Characterising the unintended consequences of antibiotic use on the microbiome and resistome: a metagenomic approach

By

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DECLARATION

I certify that this thesis:

1. does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university
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Lucy Carpenter

2nd April 2023

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All analysis shown in this thesis is my own for the purpose of my research project. General editing for spelling and grammar was performed by Ian Riley in accordance with Flinders University guidelines.

THESIS SUMMARY

Antibiotics are an essential component of contemporary healthcare but their increasing use has accelerated the rate of antibiotic resistance. Currently, data on multidrug resistant organisms (MDROs) comes from diagnostic sources such as during infection and from sources that use only culture-based techniques. However, the consequences of antibiotic use such as carriage and transmission of antibiotic resistance genes in organisms asymptomatically, as well as disruption of the protective commensal microbiome, are rarely considered. Children and elderly people who typically exhibit microbiome instability and less robust immune responses are at high risk of poor clinical outcomes when the effectiveness of antibiotic treatment is reduced. One way to better understand changes in the microbiome and resistome is the application of sequencing-based approaches. This project aimed to use such approaches in two clinical cohorts to characterise the unintended consequences of antibiotics on the gut microbiome and resistome. The first cohort, the *Cellulitis at Home or Inpatient in Children presenting to the Emergency department* (CHOICE) study, was a randomised control trial (RCT) that compared the efficacy and safety of inpatient intravenous (IV) flucloxacillin with outpatient IV ceftriaxone in children who presented to emergency with cellulitis. The second, the *Generating evidence on Resistant bacteria in the Aged Care Environment* (GRACE) study, was an observational study that investigated the impact of the residential aged care environment on the gut resistome. In both studies, sequencing-based approaches were applied to investigate how a single, controlled antibiotic exposure (CHOICE) and a multi, uncontrolled antibiotic exposure pattern (GRACE) impacted the microbiome and resistome. Responses in the gut microbiome and resistome differed substantially in each cohort. In children treated with antibiotics for cellulitis, no changes to the resistome, or carriage of genes known to be associated with broad-spectrum beta-lactam use were found. Although the microbiome was affected, this only occurred transiently and protective commensal bacteria increased in abundance shortly after treatment ceased. In a cohort of elderly, institutionalised people, the abundance of genes conferring antibiotic resistance were much higher in those who had recent and frequent antibiotic exposure compared to those who had no exposure. Microbiome richness was also much lower in these people, owing to the characteristics of the microbiome previously observed in the elderly and those in aged-care facilities. Antibiotic use has a significant impact on the gut resistome and microbiome in both young children and residents of aged-care facilities. The findings reported in this thesis indicate that the use of metagenomic sequencing should be more frequently adopted in medical decision-making, particularly in the development of antibiotic stewardship policies, where the impact of asymptomatic carriage of resistance genes and microbiome disruption is often not considered.

1. INTRODUCTION

Antimicrobial resistance is now recognised as one of the most significant threats to human health globally.[1, 2] By 2050, it is predicted that 10 million deaths per year will be attributable to antimicrobial resistant pathogens, with the most vulnerable members of the community at greatest risk.[3] With rates of resistance to first-line antibiotic treatments continuing to rise, the reliance on last line antibiotics, such as carbapenems, is becoming increasingly common.[2] In Australia, efforts to reduce inappropriate prescribing preserve the efficacy of antibiotics that are currently available, and better characterise the carriage of multidrug resistant organisms are being prioritised.[2, 4] However, these reports, together with data from culture-based screening studies, often focus on resistance in only one target pathogen, commonly after an infection has been identified, and do not consider that the gene conferring resistance may be present in other organisms, such as commensals and pathogens not causing the infection.[5] Asymptomatic carriage of resistant organisms represents a latent but significant risk to the health of those carrying them, with increasing exposure to antibiotics encouraging their selection and acquisition of resistance. Although several studies have used sequencing-based techniques to report the epidemiology of the genetics-drivers of AMR organisms in niche groups, no study has yet associated antimicrobial prescribing patterns with the resistome composition using a sequencing-based approach.[6-10] This introductory chapter aims to describe the nature of antibiotic resistance, why this occurs, how the human commensal microbiome contributes to protection against infection by antibiotic resistant pathogens, and what strategies might be adopted to address this growing risk to health. Finally, it will describe how a sequencing-based approaches, such as those used in the current project, can add to the current knowledge and contribute to a much-needed data-driven approach to antibiotic stewardship.

1.1. IMPORTANCE OF ANTIBIOTICS IN HEALTH

Following the discovery of penicillin in 1928, what has been considered the golden age of antibiotic discovery led to unrivalled advances in healthcare. In the 1940s, Australian scientist Howard Florey was at the forefront of testing and developing penicillin for safe use in humans, with sulphonamides having been introduced just a few years earlier.[11] Soon, discovery after discovery of new antibiotics were made and notifications of bacterial illnesses such as gonorrhoea and syphilis dropped significantly, alongside a marked increase in rates of recovery from septicaemia, pneumonia and meningitis.[11] Antibiotics are now one of the most common types of medication, with over 10 million Australians prescribed antibiotics each year.[2] Due to an increase in living standards and sanitation, many of the bacterial infections experienced during the early to mid-twentieth century have become increasingly rare. However, it is fortunate that infections that remain common, such as urinary tract infections (UTIs), typically respond well to antibiotic therapy.[2]

With increasing use of antibiotics came a simultaneous increase in rates of antibiotic resistance. Some of the earliest observations of antibiotic resistance were in cases of streptogramin-resistant, mutated strains of *Mycobacterium tuberculosis* during WWII.(13) Not long afterwards in the 1950s, the ability for bacteria to transfer resistance through horizontal gene transfer was observed.[12] Hundreds to thousands of β-lactamase enzymes, conferring resistance to β-lactam antibiotics (*i.e.* penicillins) have now been identified in bacteria that commonly cause infections, such as UTIs and septicaemia.[12, 13]

With antibiotic resistance leading to increased risk of antibiotic treatment failure, the consequences of reduced antibiotic efficacy started to emerge. In studies that investigated the efficacy of antibiotic treatment for septicaemia, all identified that cases of initial antibiotic treatment failure were associated with significantly higher rates of mortality compared to those that were susceptible to initial treatment.[14-16] Concerningly, the use of antibiotics deemed critically important by the World Health Organization was reported to have increased globally by 91% between 2000 and 2015.[17]

1.2. UNINTENDED CONSEQUENCES OF ANTIBIOTIC USE: ANTIBIOTIC RESISTANCE

1.2.1. HOW ANTIBIOTICS WORK

To understand how antibiotic resistance arises, it is important to first understand the mechanisms of action of antibiotics in current use. Antibiotics are grouped into different classes, each with their own mechanism of action (see Table 1.1 for examples). Cephalexin

is in the cephalosporins and amoxicillin in the penicillins. Amoxicillin-clavulanic acid is also a member of the penicillin class but includes a β-lactamase inhibitor (clavulanic acid). Both cephalosporins and penicillins have a β-lactam action, which works by inhibiting the synthesis of peptidoglycan in the bacterial cell wall, triggering autolysis and cell death (Figure 1.1).[24] Other antibiotic mechanisms of action include inhibition of protein synthesis by interfering with the bacterial ribosomal subunits, as is the case for macrolide-class antibiotics (*e.g.* roxithromycin and clarithromycin) and tetracycline-class antibiotics (*e.g.* doxycycline).[22, 26] Sulphonamide (sulphamethoxazole) and diaminopyrimidine (trimethoprim) antibiotics interfere with nucleic acid synthesis, while nitrofurantoin and metronidazole interfere with protein synthesis via disruption of the DNA.[23, 25] However, bacterial resistance has rapidly emerged across the range of mechanisms of action of all classes of antibiotics.

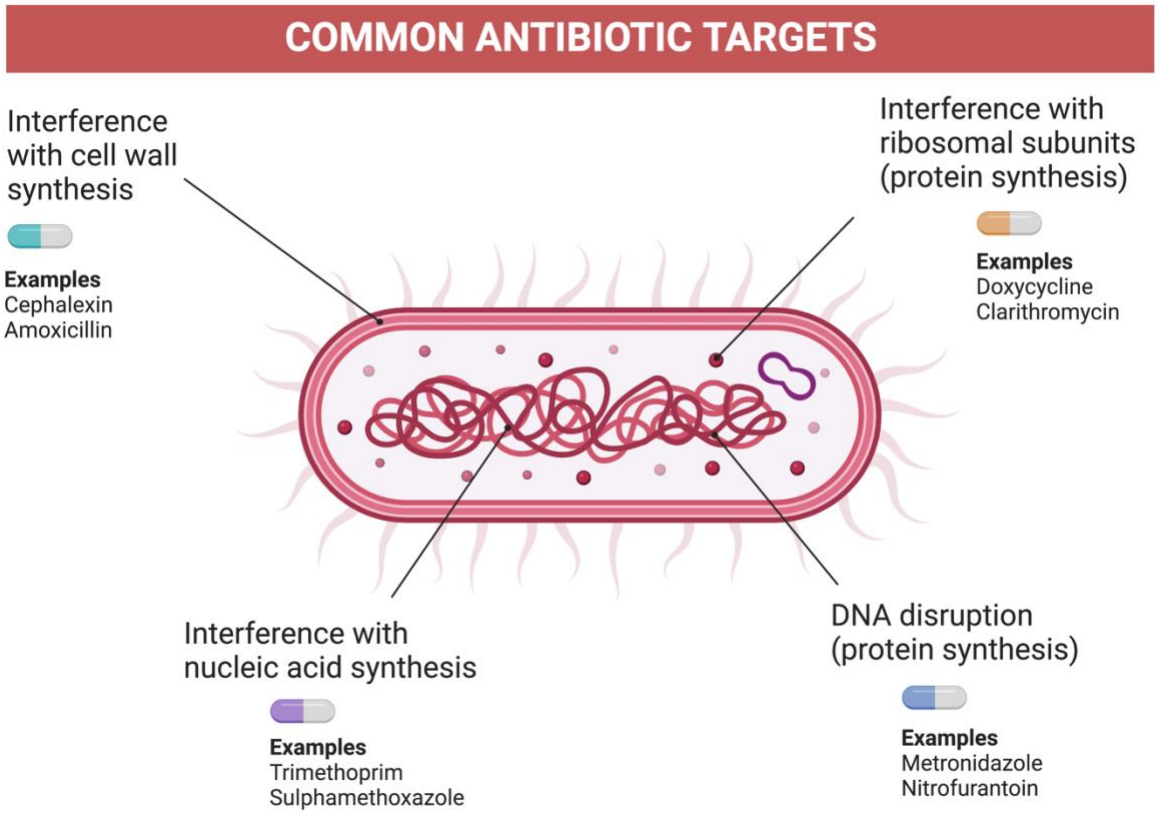

Figure 1.1 Antibiotic classes target different components of basic bacterial cell functions. This includes interferences with cell wall synthesis (*e.g.* cephalexin and amoxicillin), interference with protein synthesis via ribosomal subunits (*e.g.* doxycycline and clarithromycin) and via DNA disruption (*e.g.* metronidazole and nitrofurantoin), and interference with nucleic acid synthesis (*e.g.* trimethoprim, and sulphamethoxazole).

1.2.2. ANTIBIOTICS MOST COMMONLY USED IN AUSTRALIA

According to data from the *Antimicrobial Use and Resistance in Australia* (AURA) report, the most commonly used antibiotics in the Australian community include cephalexin, amoxicillin and amoxicillin-clavulanic acid, accounting for 21, 20 and 17% of all antibiotic prescriptions, respectively (Table 1.1).[2]

Table 1.1 Antibiotics most commonly used by the Australian public according to data from the *Antimicrobial Use and Resistance in Australia* (AURA) report, their mechanism of action and common indications for use.

Antibiotic	% of all antibiotic prescriptions	Mechanism of action	Common indications	Ref
Cephalexin	21.1	Inhibition of cell wall synthesis	UTIs, URTIs, LRTIs, skin infections	[2, 18]
Amoxicillin	20.1	Inhibition of cell wall synthesis	ENT infections, *Helicobacter pylori* eradication (in combination) LRTIs, acute bacterial sinusitis, skin infections, UTIs	[2, 19]
Amoxicillin-clavulanic acid	17.2	Inhibition of cell wall synthesis (with β-lactamase inhibitor)	Community-acquired and aspiration pneumonia, acute bacterial rhinosinusitis, UTIs, acute otitis media, skin infections	[2, 20]
Doxycycline	8.8	Inhibition of bacterial protein synthesis (30S ribosomal subunit)	Acne, malaria prophylaxis, skin infections, STIs	[2, 21]
Roxithromycin	4.3	Inhibition of bacterial protein synthesis (50S ribosomal subunit)	URTIs, LRTIs, skin infections	[2, 22]
Trimethoprim	3.3	Inhibition of nucleic acid synthesis	UTIs	[2, 23]
Flucloxacillin	2.9	Inhibition of cell wall synthesis	Staphylococcal infections (*i.e.* of the	[2, 24]

			skin) endocarditis, osteomyelitis	
Clarithromycin	2.5	Inhibition of bacterial protein synthesis (50S ribosomal subunit)	Pneumonia, sinusitis, skin infections, tonsillitis, *Helicobacter pylori* infections (in combination)	[2, 22]
Metronidazole	2.4	Disruption of DNA leading to inhibition of protein synthesis	Anaerobic bacterial infections including those causing septicaemia, intra-abdominal infections, bacterial vaginosis, LRTIs, skin infections	[2, 25]
Phenoxymethylpenicillin	2.1	Inhibition of cell wall synthesis	Strep throat, skin infections, otitis media	[2, 24]

AURA, *Antimicrobial Use and Resistance in Australia*; ENT, ear, nose and throat; LRTI, lower respiratory tract infection; URTI, upper respiratory tract infection; UTI, urinary tract infection; and STI, sexually transmitted infection

1.2.3. MECHANISMS OF RESISTANCE

Antibiotic resistance, includes both intrinsic and acquired resistance. Some bacteria are intrinsically resistant to some classes of antibiotics due to factors such as type of cell wall and genes already present in their genome that are part of their basic physiology.[27] For example, *Pseudomonas aeruginosa* cannot be treated effectively with many β-lactam antibiotics because of the low permeability of the outer membrane as well as the presence of several antibiotic efflux pumps.[28] Of most concern are species that acquire resistance and are no longer susceptible to antibiotics previously effectively used in their treatment. This can occur either through mutations in target genes in the chromosome and selection for the strains that carry resistance, or through acquisition of resistance genes from other bacteria through a process termed horizontal gene transfer. Horizontal gene transfer can occur by three mechanisms: conjugation, transformation, and transduction. Conjugation is a process whereby bacteria directly transfer genetic material encoded on plasmids. This can occur through either connection via pilus structures, in the case of gram-negative bacteria, or by chemical production, in the case of gram-positive bacteria.[27] Transformation is a process of uptake of genetic material into the cell, often from lysed bacteria that have released their cell contents into the shared environment.[27] Finally, transduction is a process of the

bacteriophage-mediated transfer of genetic material between bacteria that are infected. These genetically-mediated changes correspond to different mechanisms of antibiotic resistance including antibiotic efflux, reduction in cell wall permeability, antibiotic modification and target protection.[27]

Antibiotic efflux pumps fall into five main families: resistance-nodulation-division, small multidrug resistance, multidrug and toxic compound extrusion, ATP-binding cassette, small multidrug resistance and the major facilitator superfamily.[29, 30] Antibiotic efflux pumps can be encoded either chromosomally or on mobile genetic elements such as plasmids. However, those that confer multidrug resistance are typically chromosomally encoded, often due to having other functions in the cell in addition to transport of antibiotics.[29, 31] The first discovered antibiotic efflux resistance mechanism was a plasmid-mediated resistance protein identified in *Escherichia coli* resistant to tetracycline-class antibiotics, now known as the TetA pump.[26, 32, 33] TetA is a member of the major facilitator superfamily efflux proteins and works by exchanging a proton on the antibiotic molecule.[26, 34] These efflux pumps are found across multiple genera and now dozens of Tet proteins conferring efflux-mediated resistance to tetracyclines have been identified, with most encoded on plasmids/mobile genetic elements.[26, 35]

As mentioned above, some bacteria can be innately resistant to antibiotics simply due to the composition and low permeability of their cell wall, as in the case of *P. aeruginosa* and β-lactams.[28, 36] However, previously susceptible organisms can also prevent the antibiotic from entering the cell via a reduction in cell wall permeability. Outer membrane proteins, known as porins, are found in gram-negative bacteria and allow the transport of hydrophilic compounds in and out of the cell.[37, 38] An example of this mechanism occurs in porins found in members of the Enterobacteriaceae family including *E. coli* (*e.g.* OmpC, OmpF), and *Klebsiella pneumoniae* (*e.g.* OmpK), as well as *Acinetobacter baumannii* (*e.g.* OmpA).[39-42] Disruption to the genes that encode porins has been shown to reduce cell permeability to antibiotics including chloramphenicol, cefepime and carbapenems.[40, 42]

A well-known example of antibiotic modification/inactivation-type resistance is through the production of β-lactamases.[43] β-lactamases are one of the most common resistance mechanisms in gram-negative bacteria and work by hydrolysing the β-lactam ring found in carbapenem-, cephalosporin-, monobactam- and penicillin-class antibiotics, preventing the antibiotic from interfering with cell wall synthesis.[13] There are now thousands of classified β-lactamase enzymes that can be found in the chromosome and on plasmids, with clinically-significant carbapenemases and extended-spectrum β-lactamases (ESBLs) becoming increasingly common.[2, 13] Enzymes that inactivate aminoglycosides are another example

of the antibiotic inactivation resistance mechanism.[44] This can occur by the action of three classes of enzymes, acetyltransferases, nucleotidyltransferases and phosphotransferases, often encoded on plasmids and easily transferred between species.[44]

Target protection occurs when the protection protein binds or chemically alters the target protein such that the antibiotic is no longer biologically active.[45] One of the most well-characterised examples of this is in resistance to tetracycline through ribosomal protection proteins Tet(O) and Tet(M). These work by binding to the 30S ribosomal subunit, protecting it from tetracycline-mediated inactivation.[45-47] Importantly, these are plasmid-encoded and Tet(M) in particular is the most common cause of tetracycline resistance in several pathogens.[45] Reduced susceptibility to fluoroquinolones in Enterobacteriaceae is also mediated by target protection proteins by the *qnr* gene family also found on plasmids, further reducing the efficacy of antibiotic treatment of members of this family of bacteria.[45, 48]

1.2.4. RATES OF RESISTANCE IN COMMON PATHOGENS

Rates of antibiotic resistance in pathogens responsible for the most frequent and high-risk infections are routinely collected in Australia. One example of these programs is AURA with biannual reports that detail the use of antibiotics in the Australian population and includes rates of resistance for specific pathogens. These pathogens include Enterobacteriaceae, *Enterococcus faecalis* and *E. faecium*, *P. aeruginosa*, and *Staphylococcus aureus*.

Enterobacteriaceae are an family of bacteria that is frequently associated with UTIs and septicaemia and includes *E. coli*, *K. pneumoniae* and *Enterobacter cloacae*.[2] Importantly, resistance is easily transferred between members of this family of bacteria and is most commonly β-lactam resistance, which imparts resistance to penicillins, cephalosporins, monobactams and in some uncommon cases, carbapenems.[2, 49] According to the most recent AURA data, *E. coli* strains were most frequently resistant to ampicillin, cefazolin (first-generation cephalosporin), trimethoprim-sulphamethoxazole and trimethoprim alone (Figure 1.3A).[2] Resistance to cefazolin, fluoroquinolones (ciprofloxacin and norfloxacin), gentamicin and trimethoprim was highest in aged-care facilities whereas resistance to ampicillin, amoxicillin clavulanate, third-generation cephalosporins (ceftriaxone/cefotaxime) piperacillin-tazobactam and trimethoprim-sulphamethoxazole was highest in hospitals.[2] Meropenem resistance, a reserved carbapenem-class antibiotic, was not observed in hospitals or in aged-care facilities but was observed in <0.1% of strains from the wider community.[2] In *K. pneumoniae* strains from aged-care facilities, resistance was highest to trimethoprim, piperacillin-tazobactam and fluoroquinolones, whereas in hospitals and the wider community resistance was highest to trimethoprim, trimethoprim-sulphamethoxazole and cefazolin

(Figure 1.3B).[2] Meropenem resistance was not observed in aged-care facilities but was observed in 0.5% of *K. pneumoniae* strains from hospitals and <0.6% in the community. Finally, *E. cloacae* strains were most resistant to third-generation cephalosporins and piperacillin-tazobactam, with 0.2% of strains from hospitals identified as resistant to cefepime, a fourth-generation cephalosporin (Figure 1.3C).[1] Additionally, a large proportion of *E. cloacae* strains were resistant to trimethoprim and trimethoprim-sulphamethoxazole across the different locations.[2] Meropenem resistance was observed in 0.1% of strains from the community and 2% of strains from hospital, but this data was not available for aged-care facilities.[2]

[1] Data on cefepime resistance was only available for hospitals.

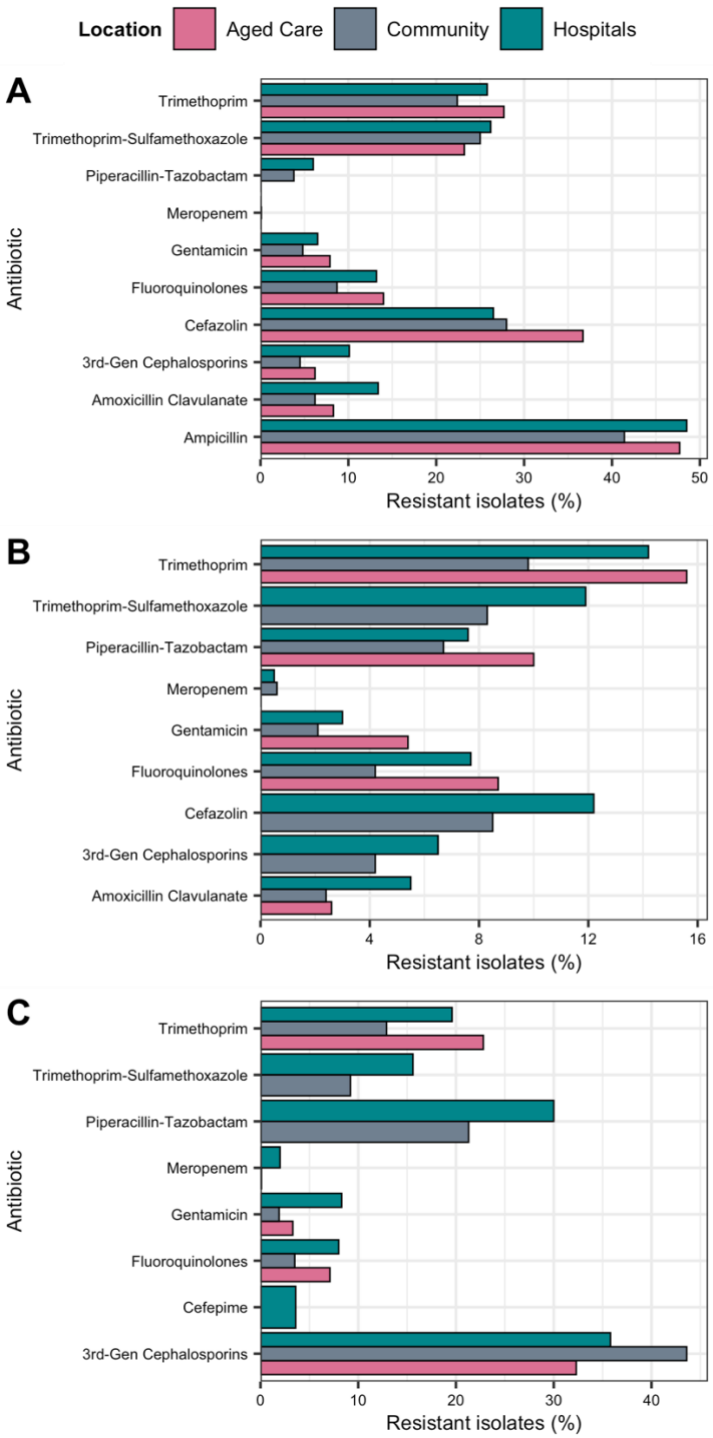

Figure 1.3 Data adapted from the *Fourth Australian report on Antimicrobial Use and Resistance in Human Health* showing the resistance rates to antibiotics used to treat A) *Escherichia coli*, B) *K Klebsiella pneumoniae* and C) *Escherichia cloacae* in aged-care facilities, community and hospitals in 2018-2019. Data was missing for *K. pneumoniae* resistance to cefazolin, third-generation cephalosporins (ceftriaxone/cefotaxime) and trimethoprim-sulphamethoxazole in aged-care facilities, *E. cloacae* resistance to cefepime, meropenem, piperacillin-tazobactam and trimethoprim-sulphamethoxazole in aged-care facilities, and *E. cloacae* resistance.

The genus *Enterococcus*, which includes *E. faecalis* and *E. faecium,* can cause septicaemia, UTIs and intra-abdominal infections, most commonly in high-risk populations such as the elderly.[2, 50] Due to the nature of their cell wall, enterococci are naturally resistant to several classes of antibiotics, including macrolides and cephalosporins.[2, 50] The first line of treatment for minor *Enterococcus* infections is non-staphylococcal penicillins including amoxicillin and ampicillin. In *E. faecalis*, resistance to ampicillin remains low in Australia (0.3-0.6%, Figure 1.4A) but is extremely high in *E. faecium*, with over 90% of strains from all locations resistant (Figure 1.4B).[2] Fluoroquinolone resistance was high in both *E. faecalis* (15-25%, excluding aged-care facilities) and *E. faecium* (over 90% in hospitals and aged-care facilities and 65% in the community) whereas resistance to nitrofurantoin was much higher in *E. faecium* (over 70% for all) compared to *E. faecalis* (<1%).[2] Vancomycin is a glycopeptide-class antibiotic that is an alternative treatment to penicillins for more severe enterococcal infections.[2, 50] Resistance to vancomycin was low in *E. faecalis* (<0.5%) but over 20% of *E. faecium* strains from each location were resistant, with the numbers in public hospitals reaching nearly 40%.[2] In cases where vancomycin is ineffective, known as vancomycin-resistant enterococci (VRE), reserved treatments such as teicoplanin (for *vanB* genotypes) and linezolid or daptomycin (for *vanA* genotypes) may be necessary.[2, 50] Resistance to teicoplanin was also low in *E. faecalis* (0.1-0.7%) but ranged between 5-11% in *E. faecium* strains from hospitals.[2]

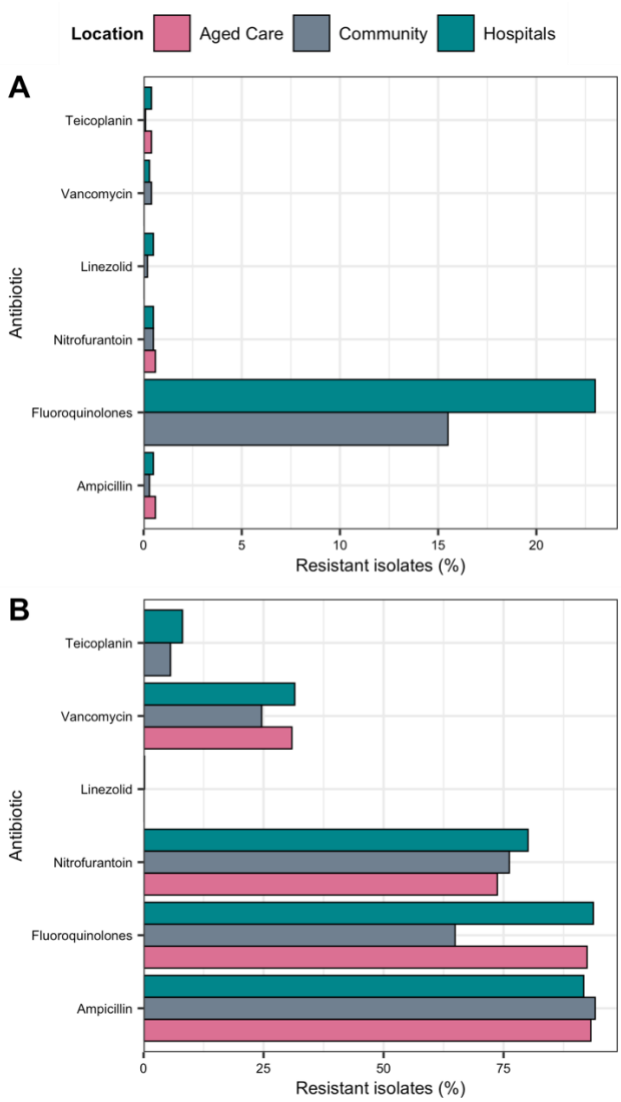

Figure 1.4 Data adapted from the *Fourth Australian report on Antimicrobial Use and Resistance in Human Health* showing the resistance rates to antibiotics used to treat A) *Enterococcus faecalis* and B) *Enterococcus faecium* in aged-care facilities, community and hospitals in 2018-2019. Data was missing for *E. faecalis* resistance to fluoroquinolones in aged-care facilities, and *E. faecium* resistance to linezolid and teicoplanin in aged-care facilities. Hospital data are an average of the data collected from both private and public hospitals.

P. aeruginosa is an opportunistic gram-negative pathogen found in hospitals and commonly associated with morbidity in cystic fibrosis patients, as well as infections in burn patients.[51] As previously mentioned, the spectrum of antibiotics available to treat *P. aeruginosa* is significantly reduced due to its innate resistance to a number of different classes.[51] It is a highly concerning pathogen due to its ability to acquire a various types of resistance, such as β-lactamase genes, and its ability to upregulate genes already present in the genome.[51] Rates of resistance in *P. aeruginosa* were highest for piperacillin-tazobactam, with 7%, 5% and 4% of strains resistant in hospitals, aged-care facilities and the community, respectively (Figure 1.5).[2] This was closely followed by ciprofloxacin (2-8%), and ceftazidime (2-6%)

resistance.[2] Meropenem resistance was highest in public hospitals (4%) and lowest in aged-care facilities (0.8%).[2]

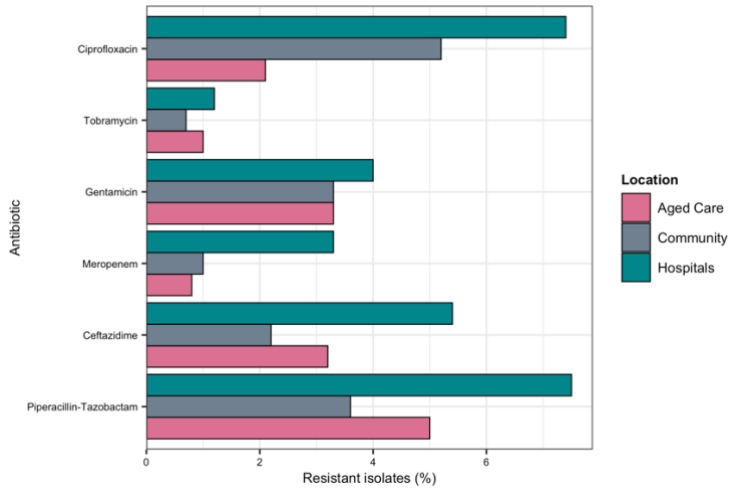

Figure 1.5 Data adapted from the *Fourth Australian report on Antimicrobial Use and Resistance in Human Health* showing the resistance rates to antibiotics used to treat *Pseudomonas aeruginosa* in aged-care facilities, community and hospitals in 2018-2019. Hospital data are an average of the data collected from both private and public hospitals.

The final example is *Staphylococcus aureus*, an organism commonly associated with skin infections but can also be present asymptomatically on the skin as well as in the nose.[52] In cases of blood infections it has a rapid and high mortality rate, with a 15% 30-day mortality rate reported in 2017.[53] Methicillin-resistant *S. aureus*, known as MRSA, is one of the most common causes of healthcare-associated infections and is resistant to first-line treatments including flucloxacillin and first-generation cephalosporins.[2, 52] Resistance to penicillin was between 80 and 90% in *S. aureus* strains from across all locations, but was highest in the community (Figure 1.6).[2] Strains from aged-care facilities had the highest rates of resistance for most antibiotics, including oxacillin/methicillin (26%), erythromycin (21%), clindamycin (19%), tetracyclines (6.3%), and trimethoprim-sulphamethoxazole (3.9%). Ciprofloxacin resistance was highest in aged-care facilities, with a resistance rate of 20%, compared to community (2.1%) and hospitals (7.8%).[2]

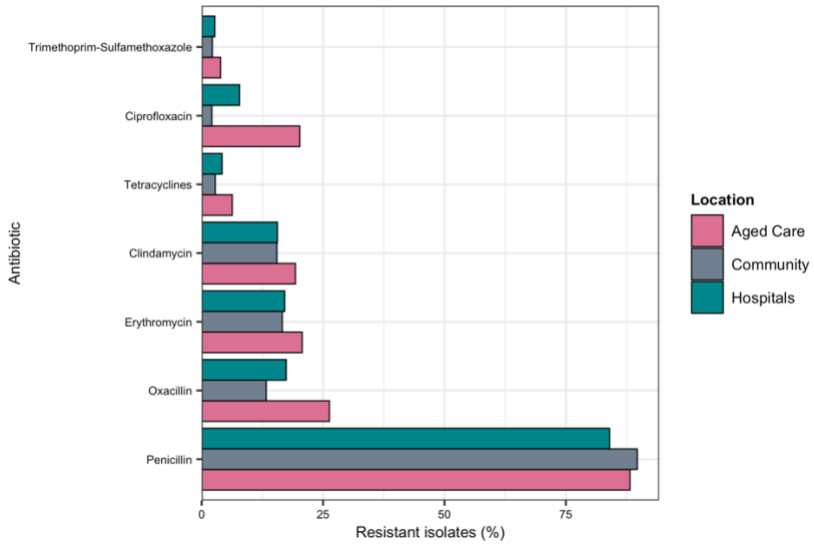

Figure 1.6 Data adapted from the *Fourth Australian report on Antimicrobial Use and Resistance in Human Health* showing the resistance rates to antibiotics used to treat *Staphylococcus aureus* in aged-care facilities, community and hospitals in 2018-2019. Hospital data are an average of the data collected from both private and public hospitals.

In addition to resistance profiles of individual species, some pathogens are classed as reportable to the federal government in Australia through the National Alert System for Critical Antimicrobial Resistances (CARAlert).[2] Included in this list of 12 resistance phenotypes is carbapenemase-producing Enterobacteriaceae (CPE) and Enterobacteriaceae that produce transferable colistin resistance, carbapenemase-producing *P. aeruginosa* (CRPA), carbapenemase-producing *A. baumannii,* vancomycin/linezolid/daptomycin-nonsusceptible *S. aureus* and linezolid-nonsusceptible *Enterococcus*. CPE was the most reported group of organisms in the CARAlert system in 2020, with roughly 650 cases making up 41% of all those reported.[2] Carbapenemase-producing *P. aeruginosa* and carbapenemase-producing *A. baumannii* were reported 44 and 25 times, respectively.[2] Linezolid-nonsusceptible *Enterococcus* was detected infrequently, with only 19 reports in 2020. Transferrable colistin resistance, a last-resort antibiotic used in extreme cases of resistance due to its high toxicity, was reported 9 times in Enterobacteriaceae in 2020.[2] In high-risk locations, such as hospitals and aged-care facilities, attempts to reduce the spread of MDROs, especially those reportable organisms such as noted here, have been addressed through implementation of infection control policies.

1.3. CURRENT EFFORTS TO PREVENT INFECTION AND PRESERVE ANTIMICROBIAL EFFICACY

Currently, infection control policies to manage multidrug-resistant organisms (MDROs) encourages contact precautions, improved antimicrobial stewardship (AMS), and has a strong focus on hand hygiene.[54] This is not unwarranted; when hand hygiene is properly implemented, rates of methicillin-resistant *S. aureus* (MRSA) infection, found commonly on the skin and in the nose, is significantly reduced.[55] Ensuring these precautions are in place will certainly continue to improve infection control outcomes. Although hand hygiene is essential for reducing the spread of resistant organisms, there are other factors involved particularly for gut pathogens. The human gut provides a unique environment which, when in a health state, can support a vast diversity of bacteria. It is, however, constantly exposed to a range of different food, foreign bacteria and medications (including antibiotics), which can significantly alter its microbial community. Consequentially, this can also allow the colonisation or expansion of pathogenic MDROs such as VRE and ESBL gram-negative bacteria, and most concerningly, CPE and CRPA. Although evidence does show that adherence to hand hygiene standards can reduce the transmission of VRE, a pathogen frequently implicated in cases of septicaemia, contact precautions are not sufficiently effective in limiting its spread.[56] In addition, transmission of ESBL-producing gram-negative bacteria, also found predominantly in the gut, is reduced by improved hand hygiene practices.[57] However, given that the incidence of infection with ESBL-producing gram-negative bacteria is also significantly associated with third-generation cephalosporin and fluoroquinolone use, AMS is equally, if not more important.[57]

One study based in a Melbourne teaching hospital showed that implementation of an electronic AMS policy significantly reduced the use of antibiotics such as later generation cephalosporins and carbapenems, and improved susceptibility in *S. aureus* and *Pseudomonas* spp. to several antibiotics.[58] Currently, all Australian health services, including aged-care facilities, dental practices and hospitals, are required to have an AMS policy in place. Although the Australian Commission into Safety and Quality in Health Care released a final version of their AMS standard in 2020, this was only a guide, and it is up to the discretion of individual healthcare providers which measures they will implement.[59]

Antimicrobial stewardship and clinical pathology are at the forefront of efforts to characterise and address the spread of AMR. It is evident that there are improvements being made to implemented policies with resistance development minimised in some of the most dangerous pathogens. Nevertheless, there is a valid argument that there are contributing factors to AMR beyond the narrow context of the target pathogen and it would be appropriate to investigate

rates of resistance in a wider range of bacteria than just those pathogens considered to be the primary cause of infectious diseases. It is now evident that antibiotic resistance genes can be transferred between bacteria in the microbiome, but clinical decisions around the use of antibiotics rarely consider this phenomenon. Essential data on how antibiotic exposure influences both the collection of antimicrobial resistance genes, termed the resistome, and the risk of resistant pathogen overgrowth is not readily available in clinical practice. Increasing evidence implicating antibiotic overuse in the development of AMR in all aspects of healthcare provides no doubt that antimicrobial stewardship will be at the forefront of its control. However, future thinking about antimicrobial stewardship must include consideration of the damage already done by unregulated use of antimicrobials in terms of development of resistance, and the contribution the commensal microbiome makes to protection from infectious pathogens

1.4. UNINTENDED CONSEQUENCES OF ANTIBIOTICS: DISRUPTING THE MICROBIOME

It is important to recognise that even in the case of narrow-spectrum antibiotic use, the impact is not limited to the bacteria causing the infection. Even for antibiotics not targeted to pathogens in the gastrointestinal tract, if they are used systemically (oral or IV), many leave an impact on the organisms there. The microbiome is the collection of microorganisms that inhabit all external niches in the host. In the human gastrointestinal tract, known less formally as the gut, this includes a highly diverse population of bacteria with a variety of functions. One such function is colonisation resistance, where commensal bacteria in the gut microbiome prevent new, potentially pathogenic bacteria from colonising by competing for space and nutrients.[60] This function also extends to pathogenic bacteria already present in the gut at asymptomatically low levels. Antibiotic use can therefore impact the microbiome and resistome through two broad mechanisms: (1) disruption and depletion of the microbiome can create opportunity for external pathogens to colonise through reduction of colonisation resistance, and (2) the disruption caused by frequent and unregulated use of antibiotics can select for bacteria carrying resistance genes and create a niche for them to proliferate and cause infection (Figure 1.7).[12, 60, 61]

Therefore, it is reasonable to postulate that a disruption of colonisation resistance could have adverse effects in terms of infection risk. In cases of *Clostridioides difficile*[2] infection, the protective effect of the commensal microbiome has been sufficiently well researched that faecal-microbiota transplantation is now an established treatment for recurrent infections.[62] Antibiotic use is a significant risk factor for developing not only infections with *C. difficile* but a

[2] An opportunistic pathogen found in the gut associated with antibiotic use.

number of AMR enteric pathogens. By eliminating commensal microbiota and competition, antibiotic use enables the expansion of resistant pathogens while simultaneously disrupting regulation of local immunity provided by the commensal microflora (Figure 1.7).[63] VRE colonisation models in mice showed that antibiotic treatment reduced the expression of RegIIIγ, a C-type lectin produced in the intestines that can kill VRE and other gram-positive species. In these models, killing of VRE was significantly reduced and could only be restored through the administration of lipopolysaccharides, produced by commensal organism *Bacteroides thetaiotaomicron* involved in the regulation of RegIIIγ.[64] Colonisation and overgrowth of ESBL-producing *K. pneumoniae* was significantly increased with the use of anti-anaerobic antibiotics such as clindamycin but not with the use of antibiotics that have minimal impact on the anaerobic gut bacteria. Hooper and colleagues demonstrated that Paneth cell secretion of Ang4 (angiogenin that has bactericidal activity in the gut) was increased in mice colonised with an adult-intestinal microbiota and with *B. thetaiotaomicron* alone.[65]

Colonisation with organisms resistant to carbapenems, including CRE and CRPA, have been associated with significantly reduced taxa richness compared to a presumed healthy microbiome.[66, 67] Importantly, the presence of a select group of commensal taxa was found to be associated with protection against colonisation by CRPA in a group of ICU patients whereas in patients in which these taxa were absent or depleted CRPA colonisation occurred.[66] Colonisation by VRE has also been frequently found to be associated with a disruption to the presumed normal microbiome composition. Antibiotic-mediated destruction of the anaerobic commensal microbiology was found to be strongly associated with colonisation by VRE in the gut and supplementation with a subgroup of selected commensal bacteria was shown to increase clearance of VRE and prevent colonisation.[68, 69] Within one aged-care cohort, reduced diversity, alongside a depletion of several anaerobic commensal taxa was found to be associated with colonisation by ESBL-producing Enterobacteriaceae even in the absence of differences in antimicrobial exposure.[70]

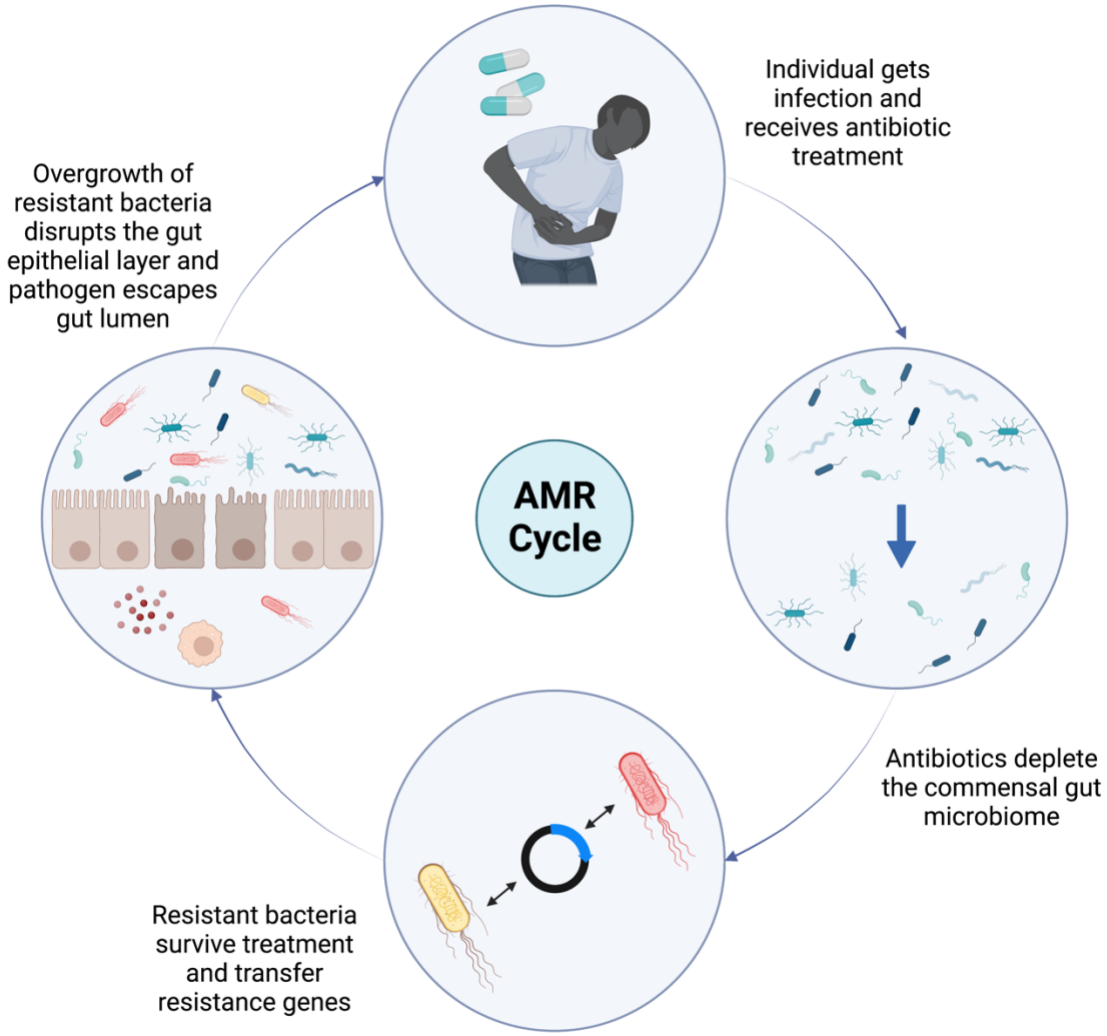

Figure 1.7 Cycle of antimicrobial resistance (AMR) and its impact in the gut microbiome. Patient presents with infection and is prescribed antibiotics (top circle). Antibiotics deplete commensal organisms that are within the spectrum and non-resistant. The reduction of commensal organisms results in reduced colonisation resistance and regulation of intestinal immunity (right circle). Bacteria that carry resistance genes survive antibiotic treatment and can also transfer mobile resistance genes through horizontal gene transfer (bottom circle). Increased resistance through horizontal gene transfer and selection for resistant bacteria provides opportunity for proliferation of the intestinal pathogen. This can trigger an immune response, disrupt the epithelial barrier and allow pathogen escape from the gut lumen (left circle). Escaped, resistant pathogens are able to cause infection which requires new antibiotics.

In the case of *C. difficile*, its overgrowth in the gut has a direct impact on gut function, producing toxins that disrupt the tight junctions of the gut epithelial layer, leading to diarrhoea, reduced absorption of nutrients and severe abdominal pain.[71] A recent review on the impact of the implementation of AMS policies for *C. difficile* infection management found that a highly restrictive stewardship policy, such a complete removal of certain medications or prior

approval requirements, was significantly associated with reduced risk of infection.[72] Wilcox and colleagues showed that the incidence of *C. difficile* diarrhoea was significantly reduced after implementation of a policy that reduced cefotaxime (broad-spectrum, third generation cephalosporin) in conjunction with preferred usage of piperacillin-tazobactam (broad-spectrum penicillin with β-lactamase inhibitor).[73] For other bacteria, such as *E. coli* and *Enterococcus* spp., an overgrowth in the gut reflects a risk of escaping and causing infection in other parts of the body, such as the cardiovascular system.[74] However, certain groups may be at higher risk of these complications than others.

1.5. WHO IS AT RISK?

Although this phenomenon poses a moderate risk to everyone using antibiotics, particular groups in the community are more vulnerable than others. Children, who have relatively naive immune systems, and whose commensal microbiology is still developing, are at a higher risk of the unintended consequences of inappropriate antibiotic use (Figure 1.8).[75] Elderly people are at a higher risk of infections due to a weakened immune system that accompanies the ageing process, termed immunosenescence (Figure 1.8).[76, 77] The mechanisms that put these groups at particular risk, and the reason for their investigation in this project, are discussed below.

Globally, infectious diseases are responsible for the most deaths in children under 5 years of age.[78] Rates of hospitalisation due to sepsis are some of the highest in infants under 1 year of age, and make up a significant proportion of all hospitalisations for this age group.[79, 80] In a European study, children under 1 year of age had the highest disease burden and rates of mortality for infections of antibiotic-resistant pathogens, particularly for pathogens in the order Enterobacteriaceae with carbapenem resistance.[81] Children also had significantly higher rates of resistant *P. aeruginosa* bloodstream infections compared to adults from the same location.[82]

Children are highly susceptible to infections and the complications of these due to their naive immune system and other factors related to birth and feeding.[75, 83] There is also evidence that the developing microbiome contributes to the shaping of the immune system. In fact, in preterm infants, inhibition of the developing microbiome has been associated with serious illness, such as sepsis.[84, 85] During this pivotal stage of life, the microbes that begin to colonise the gut have essential functions for shaping the host immune system. This phenomenon has been shown in mice, with those lacking microbiota colonisation developing significantly fewer Th17 cells, which are essential for local immunity and regulating inflammation, leading to a lower ability to resist infection by the rodent pathogen *Citrobacter*

rodentium.[86] Additionally, Bouskra and colleagues showed that commensal organisms, specifically gram-negative peptidoglycan, stimulated the genesis of intestinal lymphoid tissue through recognition by the nucleotide-binding oligomerisation domain containing 1 receptor in germ-free mice, essential for regulating local immunity.[87] It is therefore of paramount importance that the microbiome of infants and children be disrupted as little as possible during this period of development, especially given the emerging evidence of how important the commensal microbiome is in regulating immunity.[75, 88]

With AMS becoming increasingly important in medical practice, a few studies have emerged investigating the impact of a range of antibiotic treatments on health outcomes in children. For example, one such study examined the efficacy and safety of treating children who presented to the emergency department with severe cellulitis (infection of the skin), with either narrow-spectrum flucloxacillin administered in the hospital or broad-spectrum ceftriaxone administered at home.[89] Given the high-risk age group and the spectrum differences of each antibiotic, the investigators included a small sub-analysis that cultured stool samples and screened for EBSL-producing bacteria. In a study by Reyman and colleagues, the use of three antibiotic treatment regimens for suspected early-onset neonatal sepsis were investigated for their impact on the microbiome and resistome.[90] Each had a markedly different effect on the microbiome and resistome genes with no difference in treatment efficacy, suggesting that the antibiotic with the least adverse microbiological effects should be considered first for treatment.

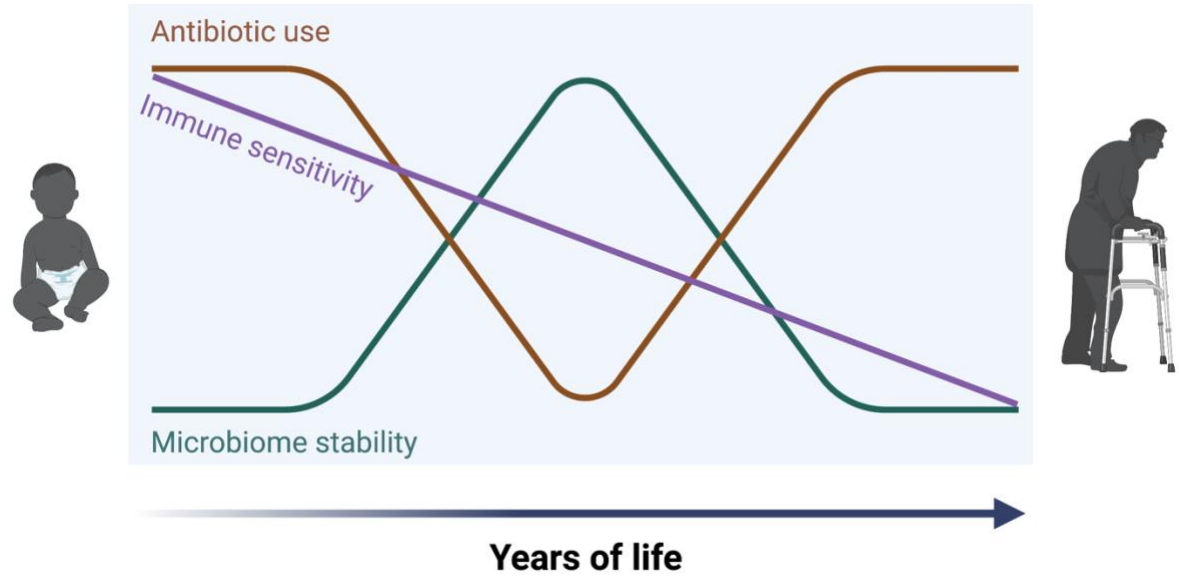

Figure 1.8 Suggested relationship between age, the microbiome and immune function. Microbiome stability is low for the first few years of life while development occurs, but is often accompanied by a high use of antimicrobials. In elderly people, a similar mechanism occurs by which they are frequently prescribed antimicrobials, with a low microbiome stability that

does not recover as quickly from perturbations. Main differences appear in the response of the immune system to the microbiome and pathogens, with a highly sensitive, naive immune system in younger people, and a depleted, weaker immune system in elderly people (immunosenescence).

In the context of residential aged-care facilities, it is not hard to understand why rates of AMR are high. Residents of aged-care facilities are in close contact with other residents, frequently visit hospital and are regularly prescribed antimicrobials.[91-93] High rates of cognitive impairment hinder management of some residents and leads to difficulty in controlling the spread of AMR. Additionally, residents of aged-care facilities have a significantly lower microbial diversity in the gut compared to elderly persons living in the community, making them highly susceptible to the effect of pathogen overgrowth.[94] There are several factors that might contribute to this. Residents of aged-care facilities are often older, frailer, and frequently suffer from malnutrition due to a reduced ability to masticate food and absorb essential nutrients.[95] Immunosenescence likely contributes to the frequency and severity of infections occurring in the elderly. Recovery from infections, such as those of the bloodstream, is significantly reduced in the elderly and is accompanied by higher risk of mortality, and incidence of urinary tract infections is twentyfold higher in elderly people than in younger people.[76, 96] Although not well understood, there is some evidence that this phenomenon may be associated with immunosenescence, involving two main components of the adaptive immune system: reduction of T cell production due to a weakened thymus, and cessation of T cell proliferation and differentiation.[77, 97] Such changes mean that the capacity of T cells to be assigned as memory cells is markedly reduced and infections with similar pathogens does not result in a more rapid and robust immune response. Additionally, NF-κB, a pro-inflammatory transcriptional factor, is upregulated with ageing, promoting local and systemic inflammation and rendering the host more vulnerable to infection.[98, 99] This effect has been linked with protection of the commensal microbiome, with the addition of clostridium-derived butyrate controlling enterocolitis through increased T_{reg} cell differentiation in a germ-free mouse model.[100] It has also been theorised that neutrophils, the first and main responders to bacterial infections, are significantly reduced in numbers and have reduced bactericidal activity, leading to a poor response to invading pathogens.[101]

Combined with the physiological effects of their advanced age, the diet provided to residents of aged-care facilities has been found to not support a diverse gut microbiome.[94] Claesson and colleagues showed that a reduced microbiome diversity was associated with a high fat/low fibre diet when compared to a low fat/high fibre diet. Importantly, around 80% of long-term aged-care facility residents were clustered in the high fat/low fibre diet group. In a study that examined the impact of providing a Mediterranean diet as an intervention on the microbiome and markers of health, a significant increase in *Faecalibacterium prausnitzii*, a bacterial species associated with reduced frailty and gut health, was observed.[102] A reduction in pro-inflammatory markers (IL-17, CRP) as well as microbes associated with inflammatory diseases such as IBD were also observed in those who adhered to the dietary intervention.[102]

Further adding to the risk of treatment failure, levels of antibiotic use are high in age care residents, with a significant proportion receiving prophylactic treatment, sometimes with no end-date indicated.[2, 4] The most commonly used antibiotics in Australia and their functions are described above, and in aged-care facilities, the usage of these antibiotics is significantly higher and less-well regulated.[4] Excluding topical antimicrobials, the most commonly used antibiotics in Australian aged-care facilities differ slightly to the general population, with cephalexin (21%), amoxicillin and clavulanic acid (7%), and trimethoprim (6%) the three most prescribed.[4] One in six antibiotic prescriptions were written as *per resident need*, and about 20% of antibiotics prescribed were for prophylaxis.[4] Of these, 47% were for UTI prophylaxis.[4] This is concerning as a large proportion of antibiotics used for urinary tract infections have been shown to impact the gut microbiota.[103-105] In fact, O'Sullivan and colleagues demonstrated that antibiotic treatment had significant and long-term effects on the gut microbiome and specific commensal members of the microbiome.[106] Challenges in enforcing hand hygiene and other infection control practices, in addition to a microbiome with a reduced capacity for controlling pathogen abundance suggests that the microbiome should be considered when managing antibiotic use in this group.

1.6. OPPORTUNITY TO APPLY SEQUENCING-BASED APPROACHES

DNA sequencing methods provide an opportunity to examine the composition of both the microbiome and the resistome. Sequencing allows a culture-free alternative to investigating the effects of various exposures on the microbiome and been used as a basis to analyse the microbial ecology of many different sample types in many different contexts (Figure 1.9).

The development of amplicon-based sequencing for microbial DNA revolutionised microbiome research and provided scientists with a way to capture the microbiome in a sample without requiring prior microbial culture.[107, 108] This was possible because all bacteria share a ubiquitous 16S rRNA gene with hypervariable regions that allow differentiation between genera. Amplicon sequencing was first applied to investigate environmental microbiomes, particularly those in the soil, but was later expanded to human, clinical samples.[109, 110] Sequencing 16S rRNA gene amplicons is an attractive option for microbiome research due to its relatively low cost, ease of sequencing and ease of data analysis post-sequencing.[111] One limitation of this is that the resolution of identification is not as high as some other methods, with the most reliable identification at genus-level rather than species-level.[111]

An alternative approach is shotgun metagenomic sequencing, a methodology that involves sequencing all DNA present in a particular sample.[108, 111] This type of sequencing provides identification that is more reliable and of higher resolution (species level), and can be used to investigate other features in the sample, such a bacterial functionality and antibiotic resistance genes.[111] However, this approach is significantly more costly than amplicon-based sequencing, so the desired research outcomes must be considered carefully when selecting the methodology to be used. [111]

Investigation of antimicrobial resistance using shotgun sequencing approaches has limitations. Given that all DNA is sequenced during this process, in cases where genes can be present in multiple bacterial species (such as those on plasmids), assignment of genes to specific bacteria is often not possible. Consequently, the resistome that is determined by this type of sequencing is defined as the collection of antimicrobial resistance genes present in the microbiome.

Whole genome sequencing (WGS) can be applied to specific strains and provides a high resolution for gene annotation. This method has been used frequently in projects investigating the genetic-drivers of infectious outbreaks.[6-10] However, this method is preferred for single-organisms, and is not as useful for assessments of the entire microbiome and resistome.

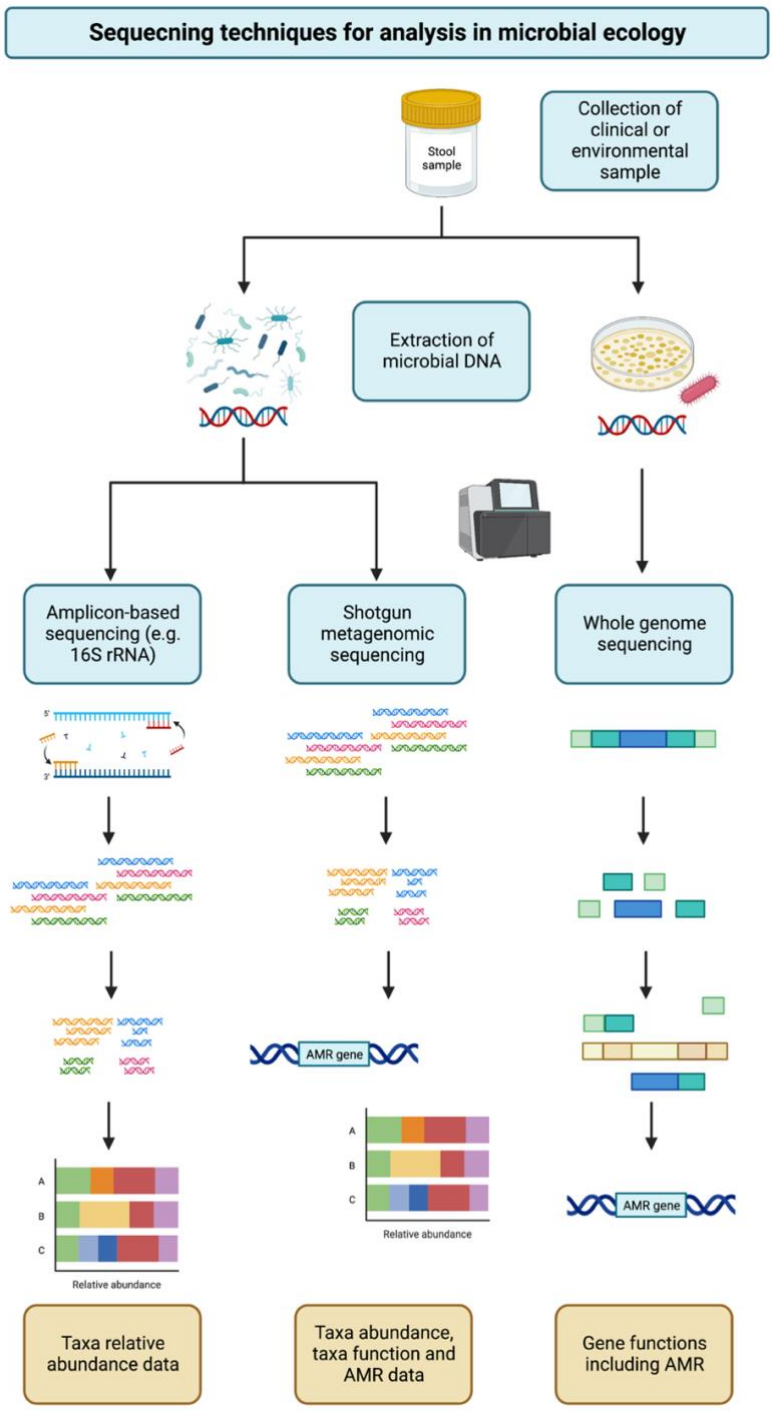

Figure 1.9. Sample collected from source (clinical or environmental) undergoes extraction of microbial DNA, and/or is isolated into pure cultures. During amplicon-based sequencing, for example 16S rRNA gene sequencing, samples undergo amplification (polymerase chain reaction) of the 16S rRNA gene followed by sequencing and bioinformatic assembly of those sequences. These are mapped to a 16S database and gives a genus-level taxa relative abundance output. During shotgun sequencing, all DNA from a sample is fragmented and sequenced. These sequences can be mapped to databases to provide information such as species-level taxa relative abundance data, bacterial functional data and antimicrobial resistance data. The isolated strains can be sequenced using whole genome sequencing whereby the fragmented genome is sequenced and matched to a reference genome in order to annotate genes present. This could include bacterial functional genes and antibiotic resistance genes.

Given that these technologies are relatively new, examples of their application to resistome analysis are limited. One applied metagenomic sequencing to arctic soil samples assumed to be free of anthropogenic impacts, and detected several genes that conferred resistance to commonly used antibiotics in clinical practice.[112] Mahnert and colleagues investigated the presence of antimicrobial resistance genes in anthropogenic-environments using metagenomics and showed a significant difference in resistome composition between controlled environments (such as ICU) and uncontrolled environments (such as private residences).[113] A study investigating the sewage resistome composition found that resistance genes detected correlated with that of *E. coli* that was currently circulating in the community.[114] In a mouse model, Xu and colleagues showed that some resistance genes in the gut microbiome are enriched with antibiotic use and that cross resistance can be found to a small number of antibiotics.[115] The human microbiome project applied a metagenomic sequencing approach to a few of their samples to investigate the microbiome composition and relatedness of different niches in the human body, but this study did not examine the resistome.[109]

For the reasons outlined above, a metagenomic-sequencing-based approach was applied in this candidature to investigate the impact of antibiotic use patterns on the microbiome and resistome, as well as a 16S rRNA gene amplicon-sequencing-based approach to investigate the microbiome in the CHOICE study component[3]. Although current, culture-based techniques are excellent for screening and observation of current carriage of resistant organisms, the ability to combine this with sequencing-based technology in clinical practice would allow a greater understanding of the pathways leading to resistance and pathogen overgrowth.

Currently, there are few reports that investigate the human resistome using metagenomic sequencing methods. To demonstrate this, a narrowly-targeted literature search was performed in PubMed to gather currently available articles that used metagenomic sequencing to investigate the effect of antibiotics on the human resistome using the search term, "(metagenomic OR shotgun) and (resistome OR resistance) and (antibiotic OR antimicrobial) and human". No language restrictions were applied and reviews/commentaries were excluded. Thirty-four articles were identified as matching to this search term and two other highly relevant articles not found using this search were added.[116, 117] Of these 34 articles, 14 were excluded for the following reasons: six used metagenomics but did not include resistome analysis, five did not use metagenomics or include resistome analysis, and one was a virus study. The remaining 22 articles were reviewed and a summary is presented in Table

[3] Details of the choice of 16S rRNA gene amplicon sequencing for the CHOICE microbiome analysis are given in Chapter 3.

1.2. Nineteen included an analysis of the gut resistome, four the respiratory resistome (sputum and throat swabs) and three single articles on skin, saliva or urine.

Table 1.2 Summary of a review of 22 selected articles that matched the PubMed search term: (metagenomic OR shotgun) and (resistome OR resistance) and (antibiotic OR antimicrobial) and human. Search was performed on 20 September 2022 for original research articles published between 1 Jan 1946 and 20 September 2022 with no language restriction and excluding reviews/commentaries.

Author	Year	Clinical context	Body site	Sample size	Ref
Bajaj *et al.*	2020	FMT in cirrhosis	Gut	20	[118]
Djamin *et al.*	2020	Macrolide maintenance treatment in chronic obstructive pulmonary disease patients	Throat	92	[119]
Doan *et al.*	2019, 2020	Azithromycin prophylaxis in preschool children from Niger, Tanzania and Malawi	Gut	30 communities	[120]
Fishbein *et al.*	2021	Oral vancomycin treatment for *C. difficile*	Gut	15	[121]
Francis *et al.*	2020	Cotrimoxazole treatment in HIV-infected children	Gut	72	[122]
Hansen *et al.*	2021	*Campylobacter* infected patients compared to healthy family members	Gut	70	[123]
Jo *et al.*	2021	Impact of systemic antibiotic exposure on skin microbiome	Skin, Throat, Gut	14	[124]
Kwak *et al.*	2020	Trial of microbiota-modifying drug	Gut	66	[125]
Langdon *et al.*	2021	Restoration of microbiota to prevent colonisation of MDROs in CDI patients	Gut	29	[126]
Leo *et al.*	2021	Shortening antibiotic treatment duration in patients with gram-negative bacteraemia	Gut	56	[127]

Li *et al.*	2021	Exposures on the infant resistome and onset of asthma	Gut	662	[116]
Millan *et al.*	2016	FMT for recurrent CDI	Gut	20	[128]
Pettigrew *et al.*	2022	Short and standard length antibiotic courses for community-acquired pneumonia in children	Throat, Gut	171	[129]
Rampelli *et al.*	2015	Hunter-gatherer vs urban microbiome and resistome	Gut	38	[130]
Rani *et al.*	2020	Multidrug resistance in urine of kidney patients and healthy controls	Urine	46	[131]
Reyman *et al.*	2022	Antibiotic treatment regimen on resistome in suspected early-onset neonatal sepsis cases	Gut	147	[90]
Taylor *et al.*	2021	Resistome of cystic fibrosis patients versus controls	Gut	35	[117]
Taylor *et al.*	2019	Azithromycin maintenance treatment in asthma patients	Sputum	61	[132]
Vaga *et al.*	2020	Microbiome and resistome of healthy gut (mucosal layer)	Gut	5	[133]
Willmann *et al.*	2019	Haematology patients receiving prophylactic antibiotic treatment	Gut	41	[134]
Zaura *et al.*	2015	Response of saliva and gut to antibiotic treatment	Gut, Saliva	66	[135]

FMT, faecal microbiota transplant; CDI, *Clostridioides difficile* infection

Metagenomic sequencing to investigate the effect of prophylactic macrolide treatment for respiratory illnesses on the resistome was performed in three studies.[117, 119, 132] Taylor and colleagues demonstrated that prophylactic use of azithromycin in patients with severe asthma was associated with a significant increase in the abundance of macrolide antibiotic resistance genes,[132] which was also observed in a study investigating the impact of azithromycin in chronic obstructive pulmonary disease patients.[119] Three studies used

metagenomic sequencing to investigate the impact of treatment of infectious diseases in children on the resistome, specifically in low income countries.[120, 122, 136] These studies recognised the importance of antibiotic treatments for preventing and controlling illnesses such as HIV-associated infections, while investigating the impact that repeated and prolonged antibiotic use had on the microbiome and resistome. The effect of different antibiotic treatments, either through antibiotic type or course duration has been investigated with a metagenomic-sequencing approach by several researchers. In a recent study by Reyman and colleagues, the type of antibiotic regimen given to infants with suspected early-onset neonatal sepsis was shown to significantly impact the resistome, with an amoxicillin and cefotaxime treatment having the strongest effect.[90] Two groups of researchers used a metagenomic-sequencing-based approach to evaluate the impact of reduced treatment lengths in different clinical contexts.[127, 129] In the bacteraemia patients, no significant changes to the gut resistome was observed with length of treatment.[127] However, in the pneumonia patients, a shorter course of antibiotics was associated with a lower abundance of antibiotic resistance genes in the throat microbiome.[129] Finally, three studies investigated various treatments for *C. difficile* infections and their impact on the gut microbiome and resistome.[121, 126, 128] In a study investigating the impact of oral vancomycin treatment for patients colonised with *C. difficile*, a significant increase in macrolide-lincosamide-streptogramin resistance genes was found in the treated group.[121] Millan and colleagues showed that a faecal-microbiota transplant recipients with recurrent *C. difficile* infections significantly reduced the number of antibiotic resistance genes in the gut.[128] This was supported by Langdon and colleagues who demonstrated that *C. difficile* infection could be prevented through a microbiome-modulating treatment.[126]

Due to the newness of this technology, there remains a gap in information on the resistome. Controlled studies, such as that of Reyman and colleagues, are a first step in merging microbiome and resistome analysis into clinical decision-making. Indeed, clinical practice would benefit from the findings of studies such as these when considering alternative treatment options.[90] However, in populations where antibiotic use is high, such as in the elderly and extremely frail, performing controlled studies is much more difficult. The impact of frequent and poorly-targeted antibiotic use on the microbiome and resistome and the possibility of using metagenomic sequencing to do this must be the first approach.

2. METHODOLOGY AND DEVELOPMENT

For this project, a mix of established methods and methods that required some further validation were utilised. Types of data collected for component of this project, as well as details of recruitment, data processing and statistical analysis are included in the component specific chapters below. This chapter presents the general methods, as well as the procedure for identifying, validating and solving issues with methods used throughout the thesis.

2.1. STANDARD METHODOLOGY

2.1.1. COLLECTION, STORAGE AND DNA EXTRACTION OF STOOL SAMPLES

CHOICE: Stool samples (whole faeces) were refrigerated and transferred to a microbiological laboratory within 12 h of collection where they were stored at -80°C.[98] DNA was extracted from stool samples by the Australian Genome Research Facility (AGRF) following their standard protocols. AGRF, an organisation offering sequencing-based research services, was used to perform all lab processing before metagenomic sequencing for the CHOICE RCT.

GRACE: Stool samples (whole faeces) were collected in 20-ml tubes with DNA stabilisation buffer (Norgen, Thorold, ON, Canada) and stored at -80°C until further processed. DNA was extracted from stool samples internally in the Microbiome Research Laboratory, using the PowerLyzer PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and as previously described.[137] Blank PBS controls were processed and quantified with each batch of extraction to maintain quality control.

2.1.2. 16S RRNA GENE SEQUENCING (CHOICE ONLY)

Sequencing of 16S rRNA genes was performed by AGRF. Hypervariable V1-V3 regions of the bacterial 16S rRNA gene were amplified using the AmpliTaq Gold 360 master mix (Life Technologies Australia Pty Ltd, Mulgrave, Vic., Australia) using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 519R (5'-GWATTACCGCGGCKGCTG-3') under the following conditions. Samples were held at 95°C for 7 min, followed by 29 cycles of 94°C for 45 s, 50°C for 1 min and 72°C for 1 min, then a final extension stage at 72°C for 7 min. A further polymerase chain reaction (PCR) for indexing amplicons was performed with TaKaRa Taq DNA Polymerase (Takara Bio USA, Inc., San Jose, CA, USA). The resulting amplicons were measured using Quant-iT PicoGreen dsDNA Assay Kits (Invitrogen, Waltham, MA, USA) and normalised before pooling. The equimolar pooled library was quantified by quantitative-PCR and sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) with 2 x 300-bp paired-end chemistry.

2.1.3. SHOTGUN METAGENOMIC SEQUENCING

Metagenomic sequencing of stool samples of sufficient DNA quality was performed by Macrogen Oceania (Bella Vista, NSW, Australia). DNA fragmentation of samples was performed with Nextera XT DNA Library Prep Kits (*GRACE*) or Truseq Nano DNA Library Prep Kits (*CHOICE*) (Illumina).[4] Minimum DNA required for Nextera XT metagenomic sequencing was 0.2 ng/μl. Minimum DNA required for TruSeq metagenomic sequencing was 1 ng/μl. Samples were sequenced to a depth of 5 Gb on an Illumina Novaseq platform with 150-bp paired-end reads. Due to the cost of metagenomic sequencing, blank controls were not submitted alongside sample DNA. However, Macrogen does internal quality checks when processing samples and provides updates to project leads when required.

2.1.4. BIOINFORMATIC PROCESSING OF SEQUENCING DATA

16S rRNA gene sequencing for microbiome output

Sequences of the 16S rRNA amplicons were processed with QIIME 2 (v2.2019.4).[138] Paired forward and reverse sequences were imported, converted to artifacts and demultiplexed with the parameters *PairedEndSequencesWithQuality*. Read quality was visualised, and truncation length was chosen based on quality of the forward and reverse reads. Sequences were truncated, denoised and merged using DADA2 (Read depth: med = 20,686, IQR = 14,21227,599) with the *denoise-paired* option.[139] Chimeric sequences were identified and removed via the consensus method in DADA2. Reads were visualised using the *feature-table summarize* function and subsampling depth was chosen according to the samples with the lowest reads (depth = 3,969). The functions *alignment mafft* and *phylogeny fasttree* were executed for alpha and beta diversity measures in the following step.[140, 141] Contaminating mitochondrial and chloroplast sequences were filtered before alpha and beta diversity estimates. Alpha and beta diversities were calculated using the input filtered table, phylogeny rooted tree and chosen subsampling depth. Once core metrics were produced, taxonomy was assigned to all sequences using a feature classifier trained with SILVA v132 97% OTU database trimmed to the V1-V3 region of the 16S rRNA gene.[142]

Bioinformatic processing of metagnomic sequencing data for microbiome and resistome output

CHOICE: Paired-end sequences were quality filtered using Trimmomatic (v0.39) and human-reads were removed using Bowtie (v2.3.5.1) using the NCBI human reference genome release GRCh38.[143, 144] Sequences were converted to fasta format using FQ2FA and microbiome composition data was extracted using MetaPhlAn (v3.0).[145] Contigs were

[4] Data used in this step was validated as described in Section 2.3.1.

assembled *de novo* using IDBA-ud (v1.1.3) and open reading frames were identified with MetaGeneMark (v1.0).[146, 147] Non-redundant genes were extracted using CD-HIT (v4.8.1) with parameters ('-c 0.95 -aS 0.9') to give genes with >95% identity and aligned length covering >90% of shorter gene, and genes <100 bp in length were removed.[148] A catalogue of 3,186,222 faecal genes were transcribed to amino acids using the European Molecular Biology Open Software Suite (EMBOSS v6.6.0).[149] Transcribed genes were mapped to antimicrobial resistance genes in the Comprehensive Antibiotic Resistance Database (CARD) using BLASTP (v2.9.0) with the parameters' -evalue 1e-10 -qcov_hsp_perc 99 -max_hsps 1 -max_target_seqs 1'.[150] Alignment of non-redundant gene catalogue with human-cleaned reads was performed with Bowtie (v2.3.5.1).[144] Gene-length normalised read count calculation was performed and antimicrobial resistance gene quantification per sample was calculated using R (v4.1.2).[151] Gene abundance is reported as reads per kb of transcript per million mapped reads (rpkm).

GRACE: Processing of metagenomic sequences for the GRACE study was performed as given above with some modifications[5]. Firstly, Prodigal was used in place of MetaGeneMark for identification of open reading frames due to compatibility with the available high-performance computing environment[6].[152] A catalogue of 12,209,321 genes were identified for GRACE. Transcribed genes were mapped to antimicrobial resistance genes in CARD using their Resistance Gene Identifier (RGI v5.2.1) tool with the BLAST alignment option.[153] Parameters were set to only include genes with a strict or perfect match in the output table. A generic version of the code generated for processing metagenomic-sequencing data in a conda environment is given in Appendix A.

2.1.5. REPORTING OF MICROBIOME AND RESISTOME DATA

Resistome data (metagonomic sequencing)

Two main measures were used to characterise the resistome. Firstly, the number of unique antibiotic resistance genes (ARGs; *i.e.* simple count of the number of unique matches) detected in each sample was determined. This metric indicates the number of ARGs that are at a detectable abundance in the resistome. Secondly, the resistome was characterised using the total abundance (sum of the abundances for each gene detected in the sample) of antibiotic resistance genes present in the sample. This reflects the selection for and abundance of bacteria carrying ARGs in the microbiome. Resistome distribution, determined

[5] Some components of the bioinformatic processing of shotgun metagenomic-sequencing data were validated as described in Section 2.2.2.
[6] Flinders University HPC DeepThought underwent significant changes during the course of this project which required movement to a conda-environment-based approach to analysis. Not all software was compatible with this new environment.

by the presence or absence of antimicrobial resistance genes within a cohort, was depicted by Sorenson transformed data on a non-metric multidimensional scaling (nMDS) plot. Coordinates were generated in Primer 7 and data were visualised using in R (v4.1.2) package "ggplot2" (v3.3.5).[154]

Microbiome data

Alpha and beta diversity calculations of the microbiome were performed on both sequencing types: 16S rRNA gene sequencing in the CHOICE study, and for metagenomic sequencing output in the GRACE study. The type of diversity metrics used was the same across both data-types and is expanded on in this section. Alpha diversity (within-sample diversity) of the microbiome was estimated in four ways: (1) Pielou's evenness (closeness in abundance of detected taxa, with a higher value indicating greater evenness), (2) taxa richness (number of unique taxa detected in the sample, with a higher number corresponding to a greater richness), (3) Shannon-Wiener diversity (combined richness and evenness of detected taxa, with a higher value indicating higher diversity), and (4) Faith's phylogenetic diversity (diversity weighted according to the phylogenetic relatedness of detected taxa, where a higher value indicates higher phylogenetic richness). Each of these measures helps to determine the risk that the particular treatment or exposure poses to the microbiome: (1) a lower evenness might indicate the overgrowth of a particular bacteria, (2) a change in Shannon-Wiener diversity indicates an overall shift in microbiome composition, and (3) a reduction in taxa richness indicates that the abundance of some bacteria have fallen below the detection threshold.

Beta diversity (between-sample diversity) was estimated using weighted UniFrac distance, which accounts for both phylogenetic relatedness and relative abundance. Here, beta diversity is visualised using nMDS plots. Microbiome dispersion, which describes the within-group variance of microbiome composition, was assessed using mean distance to centroid. Coordinates were generated in Primer 7 and data were visualised using R package "ggplot2". An additional analysis of changes in the abundance of core taxa was performed. Core taxa were defined as genera present in at least 80% of samples for each comparison. An assessment of the presence of nosocomial pathogens defined by those flagged in clinical practice and the ESKAPE list was performed at the genus-level.[2, 155]

GRACE included an additional analysis of the total sum of Proteobacteria pathogens in each sample.

2.2. VALIDATATION OF METHODOLOGY

2.2.1. METAGENOMIC SEQUENCING LIBRARY PREPARATION

There are several methods for preparing DNA samples for sequencing, with most requiring high initial DNA concentrations to yield reliable results.[156] Recent developments in sequencing technology have allowed for samples with lower initial DNA concentrations to be successfully sequenced. However, these methods, such as the Nextera XT method (Illumina), are subject to amplification biases that may impact on identifying genes later in the process.[156-158] For the CHOICE study, all samples extracted were at a sufficient concentration to be processed using the TruSeq Nano (Illumina) library preparation method, suitable for samples with a higher starting concentration. GRACE, however, contained some samples that were not of sufficient DNA concentration for this method, and therefore all samples were prepared using Nextera XT. Although the two cohorts were not compared statistically at any point during this project, it was important to ensure the quality of the data would not be impacted by the use of different methods.

Five stool samples from the GRACE study were randomly selected and sequenced using both the Truseq Nano and Nextera XT library preparation methods. Samples were sequenced to a depth of 5 Gb on an Illumina Novaseq platform with 150-bp paired-end reads. All sequences were processed as in the GRACE study. Microbiome and resistome outputs were compared for these five samples.

Total sequences before (Figure 2.1A) and after (Figure 2.1B) the trimming step did not differ significantly for samples sequenced using each method as determined using a Wilcoxon matched-pairs signed-rank test with significance set at $p < 0.05$. Neither average sequence length post-trimmomatic (Figure 2.1C) nor percentage GC content (Figure 2.1D) differed significantly between methods.

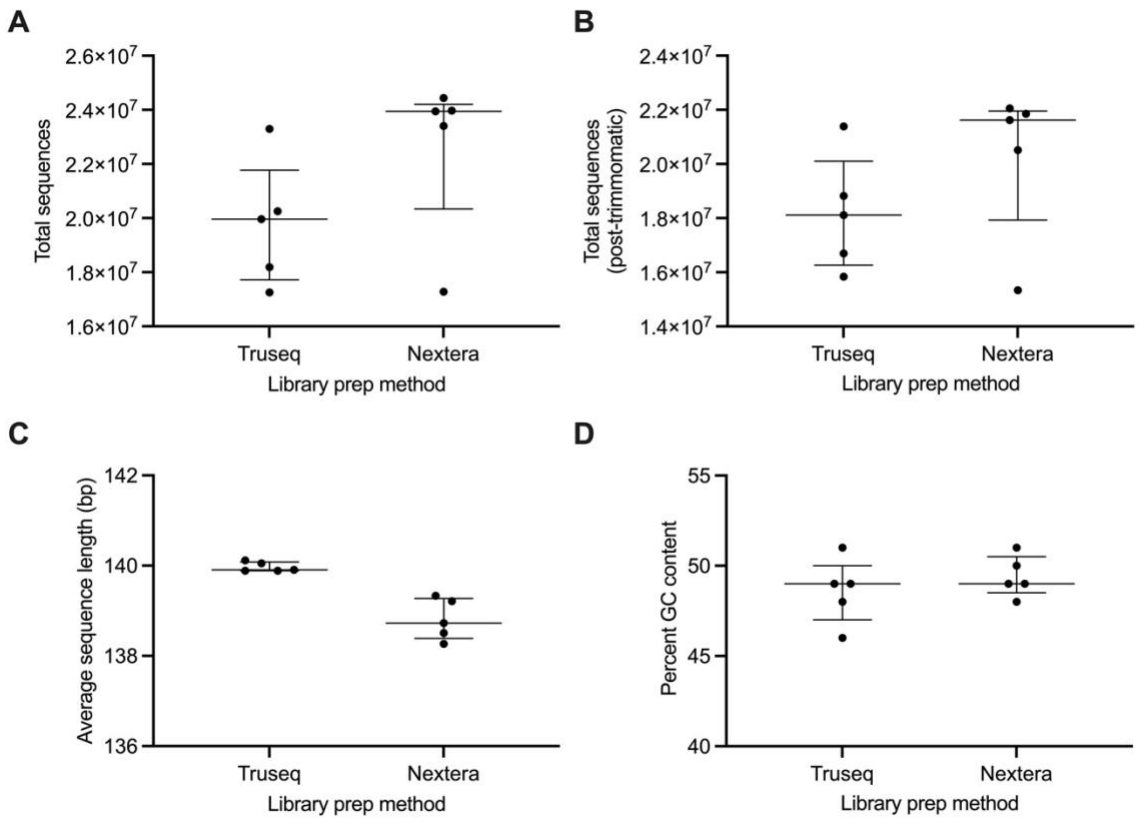

Figure 2.1 Sequencing results for the same sample processed using the TruSeq Nano library preparation method and the Nextera XT library preparation method. No difference in **(A)** total raw sequences, **(B)** total trimmed sequences, **(C)** average sequence length, or **(D)** percent GC content were found between treatment groups. Bars represent median and interquartile range.

Shannon-Wiener index, Pielou's evenness, species richness and Faith's phylogenetic diversity were compared between library methods using a Wilcoxon matched-pairs signed rank test (Figure 2.2A-D). No estimate of alpha diversity was significantly different between library preparations when treating samples as replicates.

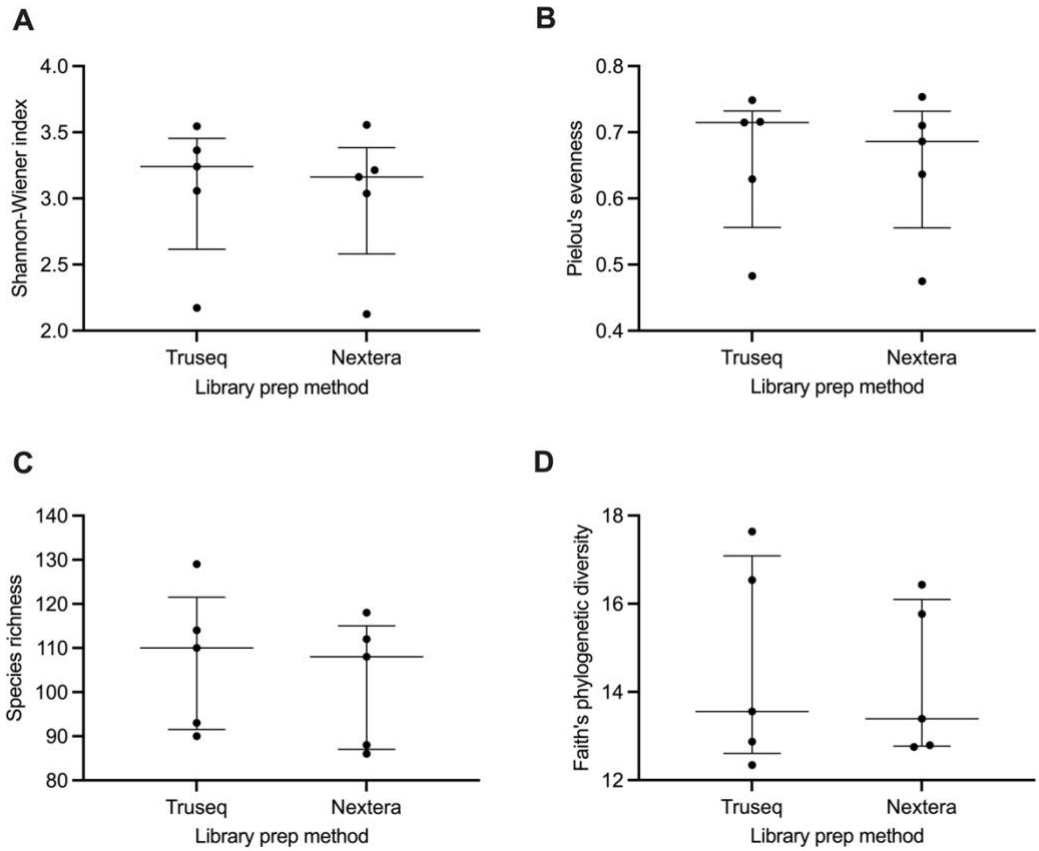

Figure 2.2 Alpha diversity as estimated by **(A)** Shannon-Wiener index, **(B)** Pielou's evenness, **(C)** species richness, and **(D)** Faith's phylogenetic diversity do not differ significantly between library preparation methods. Bars represent median and interquartile range.

Microbiome composition was also not significantly different between library preparations when controlling for sample ID (Figure 2.3).

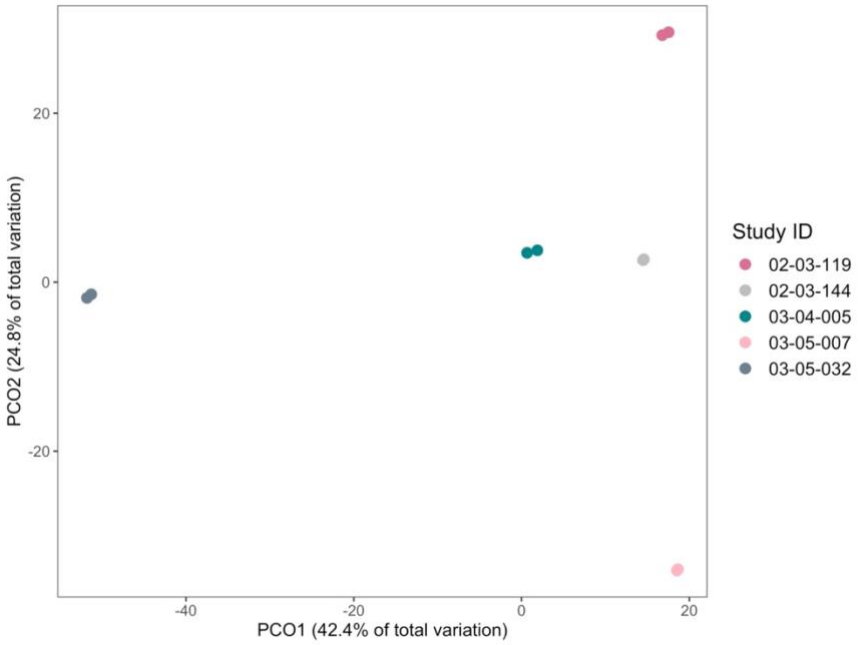

Figure 2.3 Bray-Curtis similarity matrix data displayed as a principal coordinates (PCO) biplot showing the relative location of each sample compared to others on the plot. All paired samples clustered together regardless of library preparation method.

Finally, measures of the resistome were assessed for significant differences between methods (Figure 2.4). Neither the number of genes detected, nor the total abundance of resistance genes, were found to differed significantly between library preparation methods.

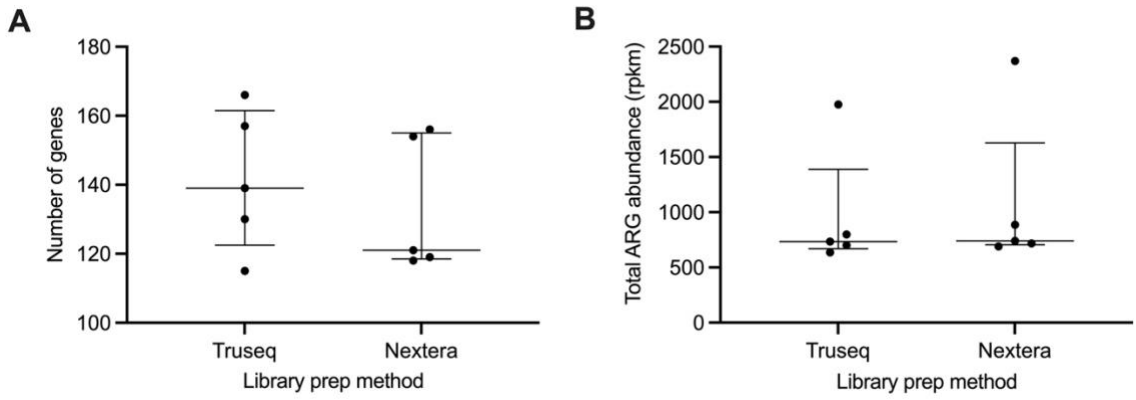

Figure 2.4 Measures of the resistome, **(A)** number of genes detected per sample and **(B)** total abundance of the genes in each sample were not significantly different between library preparation methods. Bars represent median and interquartile range.

Therefore, it was concluded that Nextera XT was an appropriate method for library preparation for metagenomic sequencing. The same approach was used for the GRACE study.

2.2.2. ALIGNMENT OF GENE CATALOGUE TO RESISTANCE GENE DATABASE

As above, one of the last steps in the bioinformatic processing of metagenomic sequences to obtain resistome data is to align the gene catalogue to CARD, the most comprehensive and updated ARG database currently available that covers all ARGs (*i.e.* not just β-lactamases). Currently, BLASTP is used to predict AMR assignments of protein sequences from the metagenomic gene catalogue.[117, 132, 159, 160] However, during the analysis of the GRACE data, some genes of clinical importance were identified by the research team as appearing at a highly unusual frequency (Table 2.1). The team from CARD created the resistance gene identifier tool (RGI) that uses BLAST or DIAMOND alignment tools to match sequences to putative AMR genes, which may have provided more reliable matches.[153]

Table 2.1 Most frequently detected, clinically important genes that were flagged as likely to have been identified incorrectly during the alignment step. Prevalence is reported as percent of the cohort where the gene was detected at least once and was generated from BLASTP alignment data.

Gene	Description	Detection frequency with BLASTP (%) n = 164
mefB	Antibiotic efflux pump conferring resistance to macrolides	100
vanB	Antibiotic target alteration gene conferring resistance to glycopeptides	99
VEB-1	Transferrable class A extended-spectrum β-lactamase conferring resistance to cephalosporins and monobactams	79
MCR-9	Mobilised colistin resistance (MCR) phosphoethanolamine transferase conferring limited resistance to colistin	70
CTX-M-52	Transferrable class A extended-spectrum β-lactamase conferring resistance to cephalosporins	63
rmtD2	Antibiotic target alteration gene conferring high resistance to all clinically available aminoglycosides	43
rmtF	Antibiotic target alteration gene conferring high resistance to all clinically available aminoglycosides	40

OXA-247	Transferrable carbapenemase-type variant of OXA-163 conferring resistance to cephalosporins and penicillins	31
qnrE2	Transferrable antibiotic target protection gene conferring resistance to quinolones	27
QnrB29	Transferrable antibiotic target protection gene conferring resistance to quinolones	26

To investigate the reliability of the matches that were considered clinically important, the hits identified by BLASTP were analysed and compared to matches from CARD's RGI method using both BLAST and DIAMOND alignment tools, as follows: (1) validity of the matches confirmed using BLASTP, (2) matches from BLASTP compared to matches for the same sequences from RGI, and (3) prevalence of problematic genes determined, if they differed between tools.

CARD has a listed bit score (reliability of matches) that it uses as a cutoff for a strict or loose match. CARD also treats a 100% sequence identity as a perfect match. BLAST output provides both percentage sequence identity and bit score, as well as other metrics (Table 2.2). Many of the genes that were identified as being present in unusually high abundance were deemed inaccurate using these metrics. For comparative purposes only, Table 2.2 also includes some examples of genes that are considered to be correctly identified.

Next, the BLASTP output was compared with the output from RGI with BLAST and RGI with DIAMOND. The following parameters were chosen for the test of RGI BLAST: '-t protein -n 8 '--alignment_tool blast --local'. The same parameters were used for the test of RGI DIAMOND with '--alignment_tool diamond' as the only modification. Other parameters which were not included as they had an undesired effect on the outcome were: '--low_quality', which allowed the prediction of partial genes; and '--exclude_nudge', which would have disallowed loose matches with a 95% or greater sequence match to be included in the output table. Table 2.3 gives gene prevalence estimated by different identification methods for some genes deemed to be either correctly or incorrectly identified.

Table 2.2 Measures to determine the reliability of gene assignment to a sequence using the BLASTP method. Bit score cutoff and match type is generated by the CARD team, all other measures were extracted from the sequencing data.

Gene	Prediction of match	Length of gene (aa)	Hits	Mean length of hit (aa)	Mean % sequence identity	Mean e value	Mean bit score	Bit score cutoff	Match type
CMY-2	Accurate	381	1	381	100	0	785	700	Perfect
DHA-1	Accurate	379	1	379	100	0	774	700	Perfect
CTX-M-15	Accurate	291	1	291	100	0	593	500	Perfect
ErmA	Accurate	243	2	243	91	6.9E-147	451	400	Strict
CMY-87	Inaccurate	381	1	381	93	0	744	700	Strict
CTX-M-52	Inaccurate	291	1	241	24	7.23E-12	60	500	Loose
MCR-4	Inaccurate	541	1	188	31	1.13E-12	61	1000	Loose
vanB	Inaccurate	341	23	265	45	2.21E-12	206	650	Loose

Table 2.3 Gene assignment for a subset of the clinically important resistance genes to demonstrate variability between methods. All methods are compared with each other in a pairwise manner, as well as an assessment to determine if the match was consistent across all three.

Gene sequence ID	BLASTP hit	RGI BLAST hit	RGI DIAMOND hit	BLASTP hit matches RGI BLAST hit	BLASTP hit matches RGI DIAMOND hit	RGI BLAST hit matches RGI DIAMOND hit	All methods match
gene1350400	CMY-2	CMY-2	CMY-2	TRUE	TRUE	TRUE	TRUE
gene5865120	CTX-M-15	CTX-M-15	CTX-M-15	TRUE	TRUE	TRUE	TRUE
gene2953700	CTX-M-24	CTX-M-24	CTX-M-24	TRUE	TRUE	TRUE	TRUE
gene1363125	DHA-1	DHA-1	DHA-1	TRUE	TRUE	TRUE	TRUE
gene8940091	ErmA	ErmA	ErmA	TRUE	TRUE	TRUE	TRUE
gene2402822	vanB	vanB	vanB	TRUE	TRUE	TRUE	TRUE
gene5303	vanB	D-Ala-D-Ala ligase	D-Ala-D-Ala ligase	FALSE	FALSE	TRUE	FALSE
gene1538836	vanB	D-Ala-D-Ala ligase	D-Ala-D-Ala ligase	FALSE	FALSE	TRUE	FALSE
gene675456	vanB	D-Ala-D-Ala ligase	vanD	FALSE	FALSE	FALSE	FALSE
gene1361110	vanB	vanE	vanE	FALSE	FALSE	TRUE	FALSE
gene1047299	vanB	D-Ala-D-Ala ligase	vanG	FALSE	FALSE	FALSE	FALSE

gene1090049	vanB	D-Ala-D-Ala ligase	vanG	FALSE	FALSE	FALSE	FALSE
gene2016841	CMY-101	CMY-137	CMY-100	FALSE	FALSE	FALSE	FALSE
gene5324124	MCR-3.12	MCR-4.4	MCR-4.5	FALSE	FALSE	FALSE	FALSE
gene7011844	rmtF	rmtF	rmtF	TRUE	TRUE	TRUE	TRUE
gene251825	VEB-1	cepA	cepA	FALSE	FALSE	TRUE	FALSE
gene8393238	VEB-1	CepA-44	cepA	FALSE	FALSE	FALSE	FALSE
gene6029391	VEB-1	CepA-49	CepA-49	FALSE	FALSE	TRUE	FALSE
gene9204102	CTX-M-52	cepA	CepA-49	FALSE	FALSE	FALSE	FALSE
gene7567187	TLA-1	cepA	cepA	FALSE	FALSE	TRUE	FALSE
gene4616126	QnrA1	QnrA6	QnrA7	FALSE	FALSE	FALSE	FALSE
gene6379546	QnrB11	QnrVC5	QnrC	FALSE	FALSE	FALSE	FALSE
gene3718078	QnrB16	QnrC	QnrA6	FALSE	FALSE	FALSE	FALSE
gene598635	rmtD2	rntD2	rmtF	TRUE	FALSE	FALSE	FALSE
gene5770673	rmtD2	rmtD2	rmtR	TRUE	FALSE	FALSE	FALSE

gene219122	MCR-4.5	MCR-4.5	MCR-4.4	TRUE	FALSE	FALSE	FALSE

Finally, an analysis of the differences in detection frequency of the clinically important, flagged genes between the alignment tools was performed (Table 2.4).

Table 2.4 Prevalence of three genes deemed incorrectly identified (vanB, rmtF, rmtD2) and one correctly identified gene (ermF) obtained by three identification methods. Genes with single hits, such as CMY-2, CTX-M-15 (correct) and CTX-M-52 (incorrect) are not included as the estimated abundances would not differ between methods. Prevalence is given as the percentage of the cohort in which the gene was detected at least once.

Gene	Prevalence (%)		
	BLASTP	RGI BLAST	RGI DIAMOND
vanB	99	42	63
rmtF	40	37	63
rmtD2	43	57	14
ermF	26	17	21

Although there was some variability between methods, RGI BLAST was considered the most appropriate for the following reasons: (1) it is regularly updated with the latest nomenclature and is a specialty tool for antibiotic resistance analysis, (2) it identified many of the clinically important extended-spectrum β-lactamases as *cepA* (or a variant of), a β-lactamase found commonly in commensal *Bacteroides* which is highly prevalent in the GRACE cohort, and (3) it uses BLAST, which is a more commonly-used tool than DIAMOND, and, despite the longer run time, stands up well[161], and both provided realistic detection frequencies for the clinically important genes. Further investigation could usefully be made to assess the reliability of DIAMOND for this data set in the future.

3. BROAD- VERSUS NARROW-SPECTRUM ANTIBIOTICS OF THE SAME ACTION DIFFERENTIALLY IMPACT THE GUT RESISTOME IN CHILDREN

3.1. CHAPTER SUMMARY

Antibiotics are frequently administered to infants and children due to their susceptibility to infection.[78-80, 161, 162] Given the significant risks associated with antibiotic resistant infections, the impact of antibiotics on the development of resistance needs to be well-characterised for this population.[81, 82] This component of the project aimed to investigate the impact of two different treatment options, a broad-spectrum antibiotic and a narrow-spectrum antibiotic, on the gut resistome and acquisition of extended-spectrum β-lactamases in children presenting with severe cellulitis to the emergency department of a Melbourne hospital.[89] Although the concept of broad and narrow spectrum is usually thought to reflect the number of possible pathogenic bacteria the antibiotic is effective against, it does not encompass the action of the antibiotic on non-target organisms. As such, this is where a metagenomic sequencing-based approach is most appropriate, and can characterise the impact of various antibiotic spectra on the whole microbiome. Here, antibiotics were deemed "broad" or "narrow" spectrum as typically classified,[89] but the analysis of their effect was not limited by these terms. In this way, a significantly higher diversity of antibiotic resistance genes in the narrow-spectrum antibiotic group than the broad-spectrum antibiotic group was observed, and the overall resistome composition clustered by treatment group. Analysis demonstrated that residual effects of systemic antibiotic use are observed in the gut resistome, and therefore should be considered in treatment selection, even for non-gastrointestinal illnesses.

3.2. INTRODUCTION TO THE CHAPTER

Antibiotics are the commonest drugs prescribed to children in developed countries with increasing use worldwide.[161, 162] By 5 years of age, up to 98% of children have received at least one course of antibiotics.[163-165] Although critical for the prevention and treatment of severe infections, overuse of antibiotics has contributed to a global crisis of antimicrobial resistance (AMR), and efforts to combat this in Australian children have been prioritised.[166-170] However, antibiotics can also contribute to adverse health outcomes through the impact on broader bacterial populations, including through the selection of AMR,[66, 171] the disruption of protective commensal microbiota,[66] and the alteration of important developmental cues linked with non-infectious disease risks.[172-174] As a consequence, narrow-spectrum antibiotic treatment options, which are effective against a limited number of species, are promoted over broad-spectrum options, when appropriate.[175]

In certain clinical contexts, this strategy can conflict with measures that aim to improve patient outcomes by reducing the need for admission to hospital. One such strategy, the use of outpatient parenteral antibiotic therapy (OPAT) in place of inpatient antibiotics, is associated with reduced rates of hospital-acquired infections and improved quality of life measures.[176-179] However, the requirement of a single daily dose in this model limits the range of suitable antibiotics. The most commonly used antibiotic for OPAT, ceftriaxone, has a broader spectrum than antibiotics administered in multiple doses per day, such as penicillins, that are often used in inpatient treatment.[176, 179]

Ibrahim, Bryant and colleagues at the Murdoch Children's Research Institute, Victoria, reported a RCT that compared clinical outcomes for children with moderate/severe cellulitis between those receiving short-course OPAT with ceftriaxone and short-course inpatient flucloxacillin.[89] Home treatment with intravenous (IV) ceftriaxone was shown to be equivalent clinically and more cost-effective than treatment in hospital with IV flucloxacillin, supporting an outpatient model becoming the standard of care for the IV treatment of moderate/severe cellulitis in children.

The aim of this component of the project was to determine whether the benefits of OPAT for children with cellulitis are offset by greater disruption of commensal microbiology or increased selection of resistance carriage, compared to standard inpatient care. This chapter focused on the stool resistome and aimed to (1) determine the impact of antibiotics on the gut resistome, and 2) investigate whether acquisition of resistant bacteria is associated with microbiome changes.

3.3. STATISTICAL ANALYSIS OF CHOICE RESISTOME

Between-group resistome analysis was performed in Statistical Analysis Software (SAS) v9.04 using a multivariate generalised linear mixed model that included adjustment for any antibiotic used prior to the intervention applied in the CHOICE study. Differences in overall resistome composition were assessed by permutational multivariate ANOVA (PERMANOVA). Correction for multiple hypothesis testing was performed using the Benjamini-Hochberg method. Summary statistics are given as mean ± standard deviation (SD) unless otherwise specified. The normality of dependent variables was assessed using the Shapiro-Wilk test. Clinical data were analysed using Fisher's exact test for categorical data and the Mann-Whitney U test for continuous data.

3.4. THE CHOICE COHORT AND INCLUSION CRITERIA FOR THIS STUDY

The CHOICE study was a single-centre, open-label, randomised, controlled, non-inferiority trial that aimed to investigate the safety and efficacy of IV ceftriaxone (50 mg/kg once per day) administered at home compared to IV flucloxacillin (50 mg/kg every 6 h) in hospital for treatment of moderate/severe cellulitis in children.[89] Both IV ceftriaxone and IV flucloxacillin treatments were followed by switch to oral cephalexin per standard practice. Recruitment ran between January 2015 and June 2017 applying inclusion and exclusion criteria as previously described.[89, 180] Briefly, the inclusion criterion was moderate/severe cellulitis requiring IV antibiotics (*e.g.* due to rapid spread) and the exclusion criteria were complicated cellulitis (*e.g.* an undrained abscess), underlying comorbidities (*e.g.* immunosuppression), clinical instability or children younger than 6 months of age. Ethical approval was obtained from the Human Research and Ethics Committee of The Royal Children's Hospital, Melbourne (approval no. HREC34254). Collection of faecal samples are described in detail in the CHOICE study.(98) Faecal samples were collected by participants at three time points: within 48 h of commencing IV antibiotics (T1), at 7-14 days (T2), and at 3 months (T3). Given that antibiotic treatments cannot normally be delayed, stool samples were mostly not available at a true zero time point, so sample collection of up to 48 h (T1) was considered to represent a baseline; a choice consistent with previous data.[181]

Samples that did not have accessible sequence data were excluded from this component of the project. Participants who had their antibiotics changed due to lack of clinical improvement were excluded. Exceptions were made for inclusion in the T1 analysis where the T1 sample had been taken before the antibiotic treatment changed.

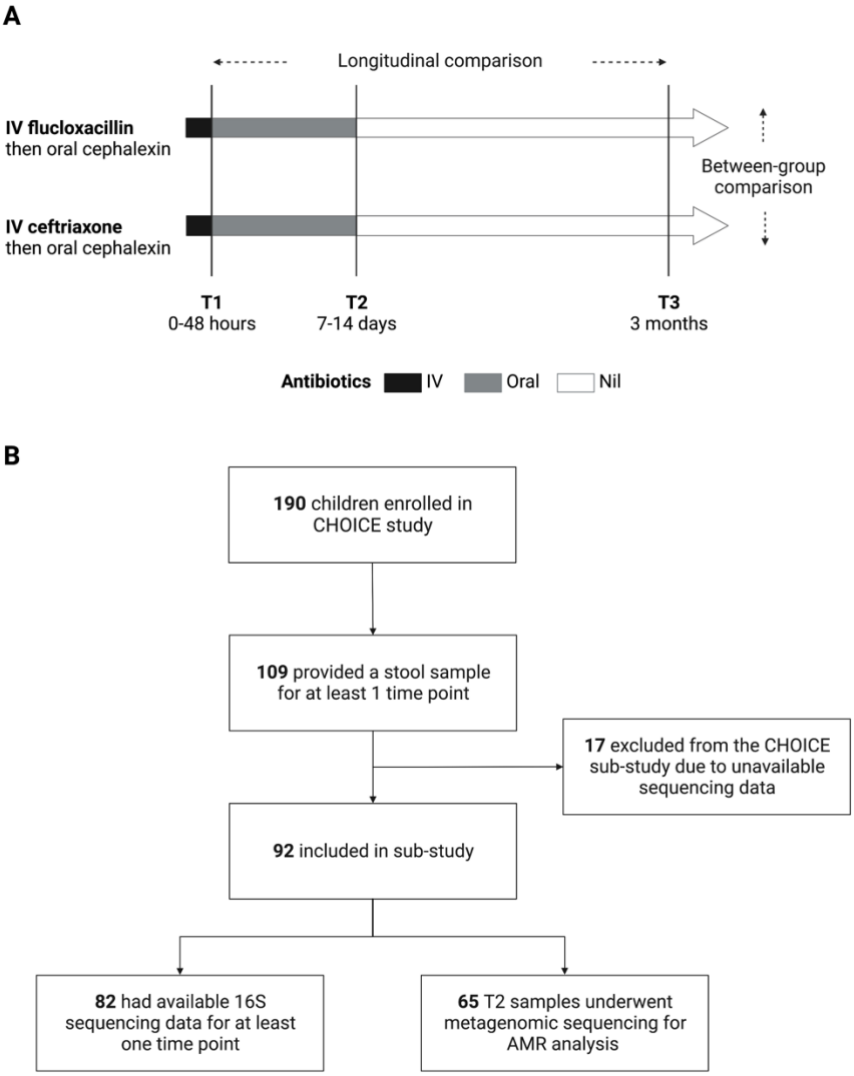

Figure 3.1. **(A)** Study design including time points where samples were collected and times at which participants were on their respective antibiotics. **(B)** Participation in the CHOICE study and subsequent gut microbiome and resistome study

Of the 188 participants in the CHOICE study, 109 provided at least one faecal sample, and for 92 sufficient material was retained for microbiome analysis: 47 at T1 (0-48 h post-antibiotics), 66 at T2 (7-14 days post-antibiotics) and 61 at T3 (3 months after antibiotics) (Figure 3.1). The characteristics of subjects that provided a faecal sample did not differ significantly from CHOICE participants who did not provide a sample (Table 3.1).

Table 3.1 Demographic and clinical characteristics of included participants and participants excluded due to unavailable sequencing data.

	Included participants (n = 92)	Excluded participants (n = 17)*	P value
Age (years), median (IQR)	4.7 (2.2-7.9)	5.6 (3.2-7.8)	0.59
Age category, 6 months to <9 years, no. (%)	71 (77.2)	16 (94.1)	0.19
Females, no. (%)	47 (51.1)	7 (41.2)	0.60
Previous oral antibiotics, no. (%)	48 (52.2)	8 (47.1)	0.79
Time between IV start and first stool collection (h), median (IQR) †	22.0 (10.2-39.2)	22.2 (17.1-40.6)	0.49
Duration of IV antibiotics (days), median (IQR)	1.7 (1.0-2.1)	1.6 (1.0-2.3)	0.85
Total duration of antibiotics (days), median (IQR)	7.9 (6.5-9.0)	6.7 (6.0-8.4)	0.24

*CHOICE participants who provided stools sample but for whom no sequencing data was available; † Excludes samples taken >48 h post IV

Ceftriaxone (n = 50) and flucloxacillin treatment groups (n = 42) also did not differ significantly in their subject characteristics (Table 3.2).

Table 3.2 Characteristics of participants with available stool data randomised to each treatment group.

	Ceftriaxone (n = 50)	Flucloxacillin (n = 42)	P value
Age (years), median (IQR)	4.4 (1.7-10.2)	5.2 (2.4-7.0)	0.78
Age category, 6 months to <9 years, no. (%)	36 (72.0)	35 (83.3)	0.22
Females, no. (%)	23 (46.0)	24 (57.1)	0.30
Previous oral antibiotics, no. (%)	30 (60.0)	18 (42.9)	0.14
Time between IV start and first stool collection (h), median (IQR) †	24.3 (13.3-38.6)	22.4 (7.4-38.2)	0.38
Duration of IV antibiotics (days), median (IQR)	1.9 (1.0-2.1)	1.5 (1.0-1.8)	0.07
Total duration of antibiotics (days), median (IQR)	7.5 (6.0-9.0)	8.0 (6.8-8.9)	0.49

3.5. PRIOR ANTIBIOTIC USE IN THE COHORT

Patients recruited to the CHOICE RCT were stratified by age and presence of periorbital cellulitis, but not prior antibiotic use.[89] As a large proportion of children received prior oral antibiotics with the potential for impact on their microbiome, the remainder of the analysis was adjusted for prior antibiotic use. Of all included participants in this microbiome study who had been exposed to antibiotics prior to enrolment (n = 48), 50% had received cephalexin, 27% flucloxacillin, 6% amoxicillin/clavulanic acid, 6% amoxicillin, 2% erythromycin, and 8% other or unknown antibiotics. Use of these prior oral antibiotics did not significantly differ between treatment groups (Table 3.3). Given the T1 stool samples representing baseline were collected up to 48 h after antibiotic initiation, a general linear regression model incorporating timing after first dose, treatment group, and prior antibiotic use was applied but it did not reveal any independent effect of baseline sample collection time on microbiome features ($p > 0.05$ for all alpha and beta diversity metrics). Consequently, a model adjusted for prior antibiotic use only was applied.

Table 3.3 Prior antibiotic use in each treatment group.

Prior antibiotic	Ceftriaxone (n = 30) % (n)	Flucloxacillin (n = 18) % (n)
Cephalexin	50.0 (15)	50.0 (9)
Flucloxacillin	26.7 (8)	27.8 (5)
Amoxicillin	3.3 (1)	11.1 (2)
Amoxicillin and clavulanic acid	6.7 (2)	5.6 (1)
Erythromycin	3.3 (1)	0 (0)
Other	6.7 (2)	5.6 (1)
Unknown	3.3 (1)	0 (0)

3.6. IMPACT OF ANTIBIOTICS ON TOTAL AND β-LACTAM RESISTANCE IN THE GUT

AMR carriage was investigated in 65 patients (38 for ceftriaxone and 27 for flucloxacillin) at T2. T2 samples were selected for metagnomic sequencing as it was the timepoint at which we expected to see a difference in resistome composition, should it occur. Samples at all timepoints were unable to undergo metagnomic sequencing due to financial and resource constraints. The number of AMR genes detected, designated AMR gene richness, was significantly higher in the flucloxacillin group compared to the ceftriaxone group (ceftriaxone 261 ± 42 vs flucloxacillin 297 ± 28; $p < 0.001$; Figure 3.2A). No significant difference in the total ARG abundance was detected between treatment groups (ceftriaxone 5,390 ± 1,100 vs flucloxacillin 5,790 ± 1,100 rpkm; $p = 0.17$; Figure 3.2B). Resistome composition (estimated by beta diversity) also differed significantly between treatment groups ($p = 0.001$; Figure 3.2C). Dispersal of the resistome was not significantly different between groups.

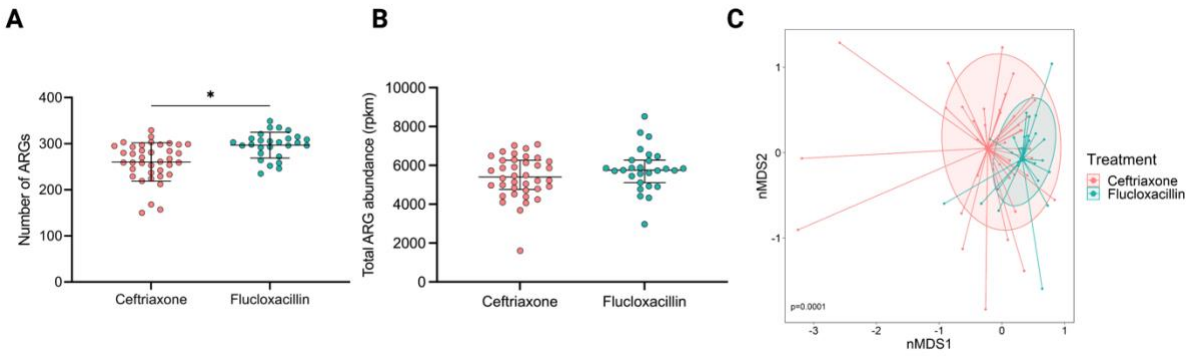

Figure 3.2 (A) Antibiotic resistance gene (ARG) richness was greater in the flucloxacillin group than the ceftriaxone group. **(B)** Total ARG abundance was not significantly different between treatment groups at T2. Bars represent mean and standard deviation. * $p < 0.05$. **(C)** Overall resistome composition, depicted by Sorenson transformed resistome data on a non-metric multidimensional scaling plot (nMDS) plot was different between treatment groups. Each point represents an individual's resistome relative to others on the plot and the circle represents 80% confidence interval.

Genes encoding ESBL and AmpC enzymes (identified using the Beta Lactamase Database) were compared between groups.[182] Of the 524 ARGs detected in the CHOICE cohort, 49 were β-lactamases and 16 were determined to be ESBL or AmpC genes (Table 3.4). Of these, eight genes (*ampC*, *ampH*, *cepA*, *cfxA2*, *cfx6*, CME-1, and VEB-6 and -7) were present in a sufficient number of samples for analysis, but none differed significantly in prevalence between treatment groups.

Table 3.4. Extended-spectrum β-lactamases and *ampC*-like genes listed in the Beta Lactamase Database that were detected in the metagenome of stool samples at T2 by treatment group.

	Ceftriaxone (n = 38)	Flucloxacillin (n = 27)	P value
Gene prevalence, No. (%)			
CMY-2	5 (13.2)	2 (7.4)	NA
ampC1	29 (76.3)	22 (81.5)	0.68
ampH	20 (52.6)	20 (74.1)	0.08
ACI-1	3 (7.9)	3 (11.1)	NA
CARB-10	1 (2.6)	0 (0)	NA
cepA	16 (42.1)	18 (66.7)	0.05
cfxA2	20 (52.6)	16 (59.3)	0.49
cfxA4	4 (10.5)	0 (0)	NA
cfxA6	26 (68.4)	21 (77.8)	0.39
CGA-1	1 (2.6)	2 (7.4)	NA
CME-1	8 (21.1)	9 (33.3)	0.25
CTX-M-15	2 (5.3)	1 (3.7)	NA
CTX-M-27	2 (5.3)	2 (7.4)	NA
OXY-1-2	2 (5.3)	5 (18.5)	NA
VEB-6	30 (79.0)	23 (85.2)	0.51
VEB-7	8 (21.1)	8 (29.6)	0.37

NA, not applicable

3.7. ACQUIRED EXTENDED-SPECTRUM β-LACTAMASE GENES IN THE GUT RESISTOME

Presence of ESBL-producing bacteria were determined with a culture-based approach as previously described.[89] An ESBL-producing strain was defined as acquired if the patient had an available prior time point sample that was negative. This included eight participants, three of whom had acquired an ESBL by T2 (3 for ceftriaxone and none for flucloxacillin) and five by T3 (2 for ceftriaxone and 3 for flucloxacillin). A comparison of the microbiome of those who had acquired an ESBL with age-, time point-, and treatment-matched controls was made to determine if there was a difference in microbiome composition. There was no significant differences in any estimates of alpha or beta diversity between ESBL-positive samples and ESBL-negative controls. (Figure 3.3A-E). There was also no significant differences in copies of *E. coli,* a bacterial species frequently associated with ESBL carriage and whose genus was frequently detected in the cohort (Figure 3.3F).

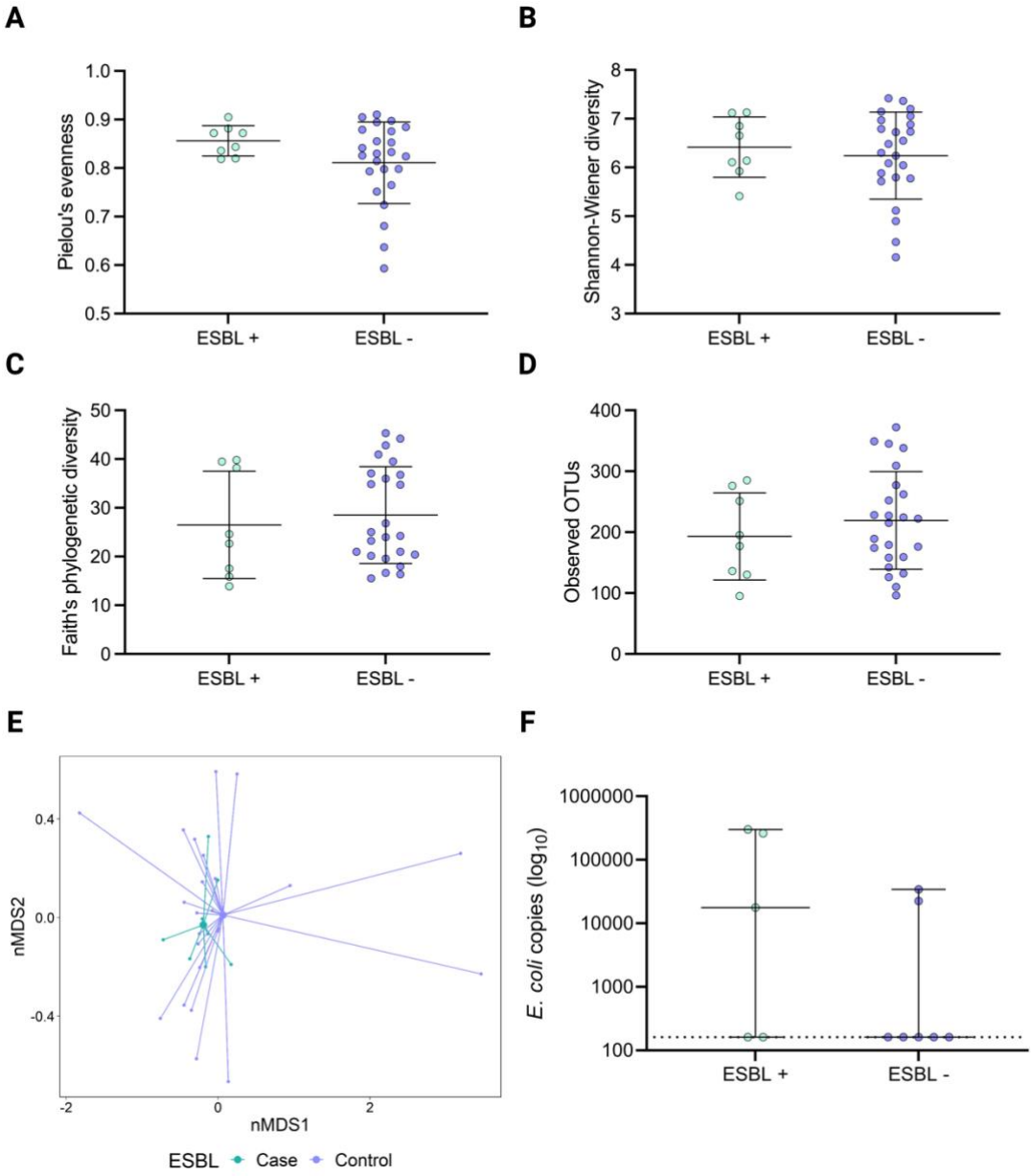

Figure 3.3 Alpha diversity as estimated by **(A)** Pielou's evenness, **(B)** Shannon-Wiener diversity, **(C)** Faith's phylogenetic diversity and **(D)** richness was not significantly different between extended-spectrum β-lactamase (ESBL) positive samples and negative age- and treatment-matched controls. Bars represent the mean and standard deviation of each group. **(E)** Microbiome composition depicted by weighted UniFrac distance of genus-level relative abundance data on a non-metric multidimensional scaling plot (nMDS) for ESBL positive case samples compared to negative controls. **(F)** Copies of *E. coli* in ESBL positive samples compared to negative controls. The dotted line represents the detection limit and bars represent the median and 95% confidence interval.

To investigate whether the microbiome characteristics of the sample preceding the acquisition of an ESBL had useful predicative value, a comparison was made of the sample prior to ESBL acquisition to age-, time point-, and treatment-matched controls where no ESBL was subsequently detected. No statistically significant differences were observed between ESBL-acquirers and controls. However, a trend towards a lower evenness (ESBL 0.74 ± 0.09 vs control 0.79 ± 0.08; p = 0.17), and Shannon-Wiener diversity (ESBL 5.3 ± 1.1 vs control 5.9

±1.1; p = 0.35) was found in the prior-samples for those who acquired an ESBL compared to those who did not (Figure 3.4A-D). Neither microbiome composition, (p = 0.86, Figure 3.4E) nor *E. coli* absolute abundance (Figure 3.4F) differed significantly between groups.

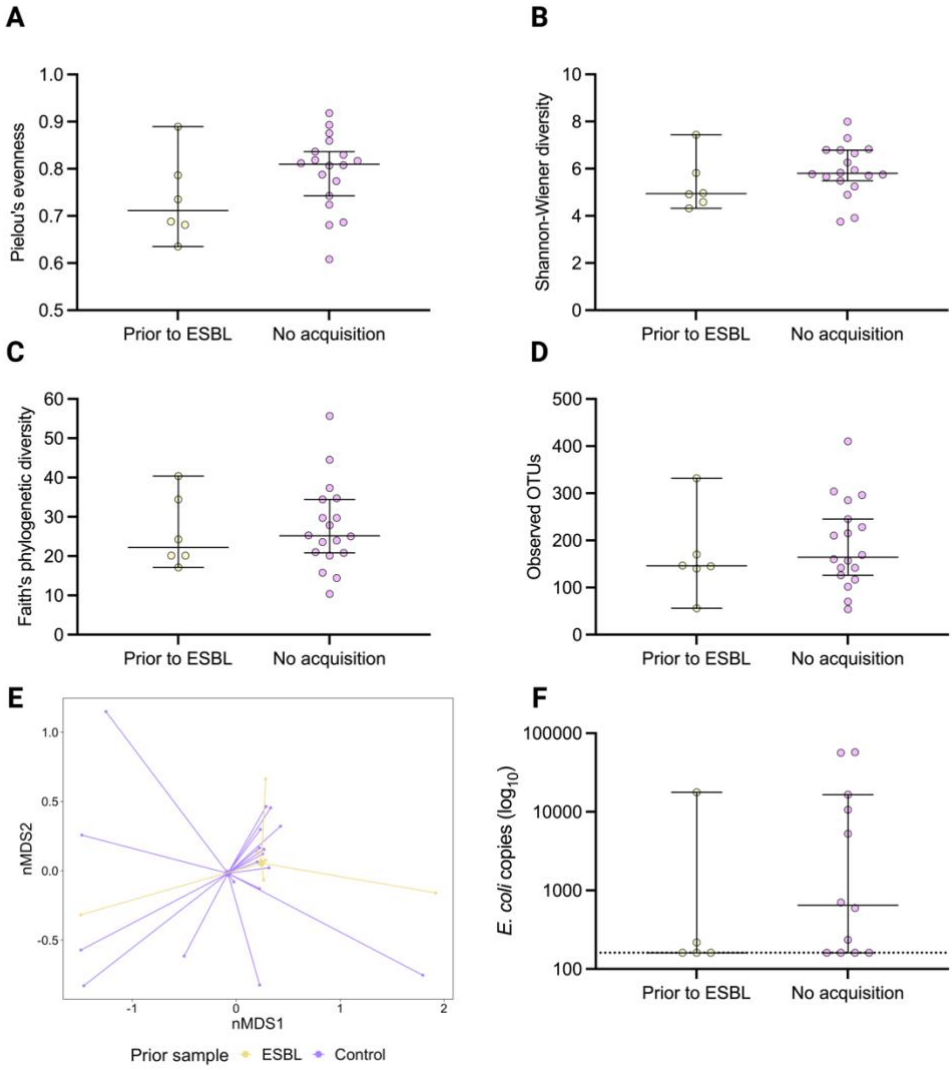

Figure 3.4. Alpha diversity as estimated by **(A)** Pielou's evenness, **(B)** Shannon-Wiener diversity, **(C)** Faith's phylogenetic diversity, and **(D)** richness was not significantly different between prior-to-extended-spectrum β-lactamase (ESBL)-acquired samples and negative age- and treatment-matched controls. Bars represent the mean and standard deviation of each group. **(E)** Microbiome composition depicted by weighted UniFrac distance of genus-level relative abundance data on a non-metric multidimensional scaling plot (nMDS) for between prior-to-ESBL-acquired samples compared to negative controls. **(F)** Copies of *Escherichia coli* in between prior-to-ESBL-acquired samples compared to negative controls. The dotted line represents the detection limit and bars represent the median and 95% confidence interval.

Microbiota characteristics of T1 samples where ESBLs were detected at T2, and T2 samples where ESBLs were detected at T3 were also compared. Although, Shannon-Wiener diversity and evenness trended lower in the time point prior to ESBL acquisition, this did not achieve statistical significance (Figure 3.5A-E). There was no significant difference in the absolute abundance of *E. coli* between the ESBL-positive samples and the sample prior (Figure 3.5F).

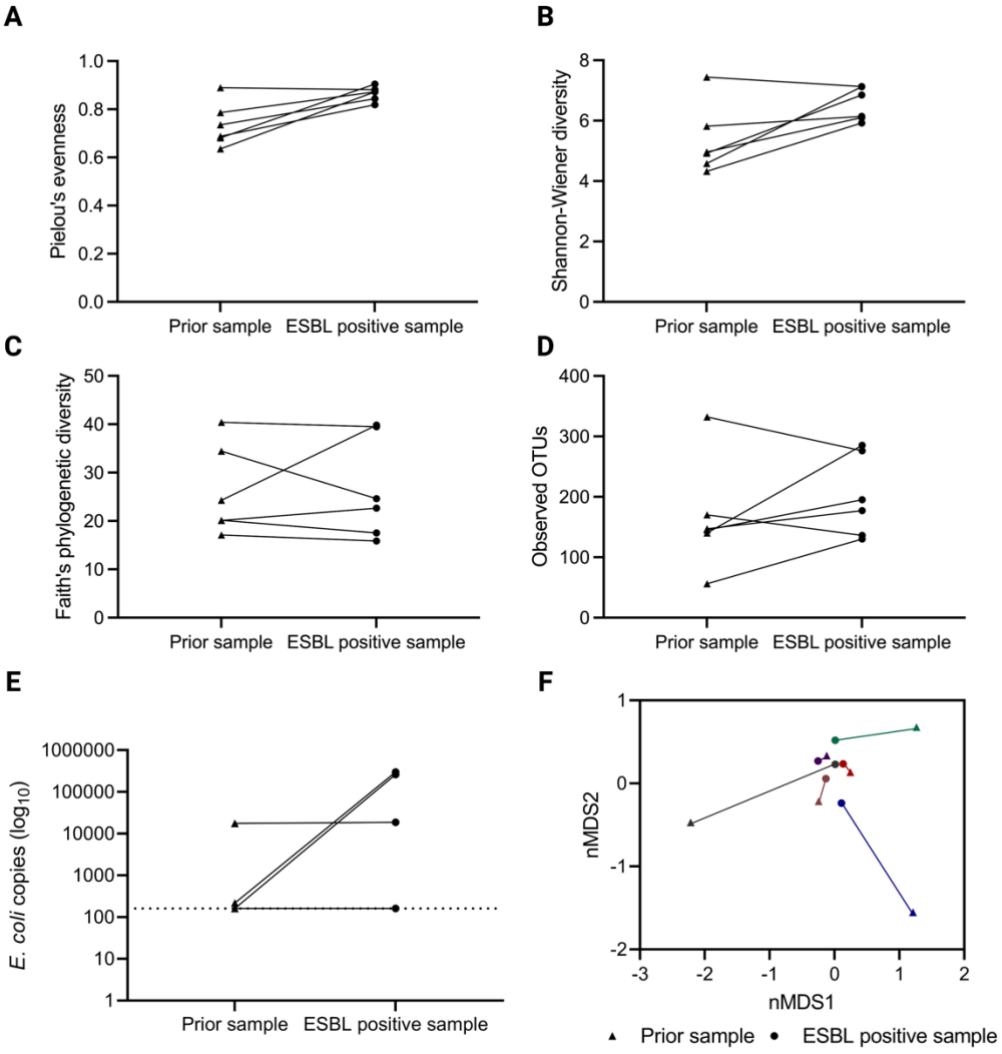

Figure 3.5 Alpha diversity as estimated by **(A)** Pielou's evenness, **(B)** Shannon-Wiener diversity, **(C)** Faith's phylogenetic diversity and **(D)** richness was not significantly different between extended-spectrum β-lactamase (ESBL) positive samples and the sample collected prior to the positive culture result. **(E)** Copies of *Escherichia coli* in the sample prior to and the sample with an ESBL-positive culture result. The dotted line represents the detection limit. **(F)** non-metric multidimensional scaling plot (nMDS) plot depicting the change in microbiome composition between the sample prior to and the sample with an ESBL-positive culture result. Paired samples are the same colour with connecting lines.

Finally, the possible genotype of the acquired ESBLs was investigated. As described above, all β-lactamase genes were screened for ESBL or AmpC candidacy (Figure 3.5).[182] Of the 21 β-lactamase genes detected in these samples, 11 were ESBL or AmpC genes and possible candidates for the phenotype detected in culture (ACI-1, AmpC1, AmpH, CepA, CIA-3, CME-1, and VEB-1, -2, -6, -7 and -9).

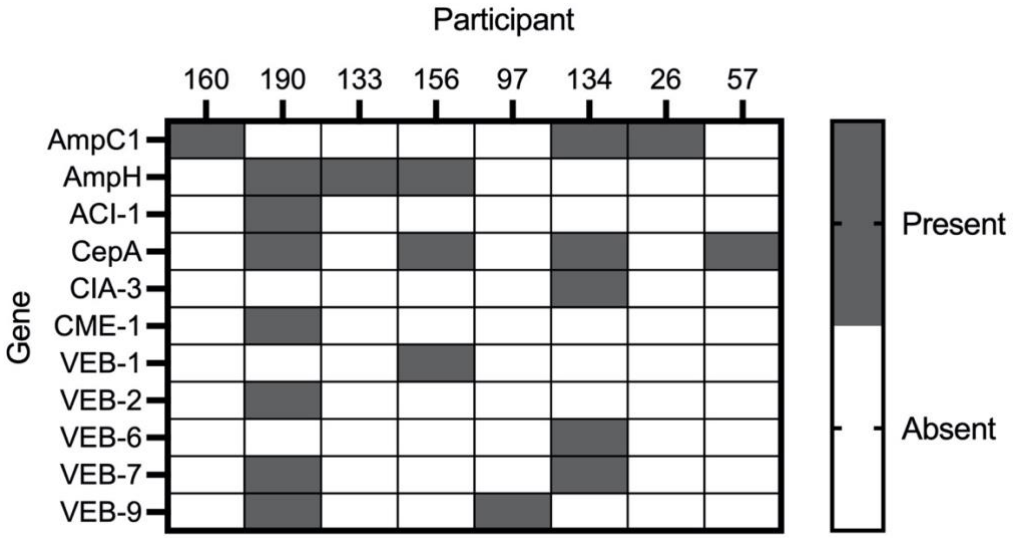

Figure 3.6 Possible genes conferring the extended-spectrum β-lactamases (ESBL) phenotype in samples that acquired an ESBL during the study period.

3.8. INTERPRETATION OF FINDINGS

In summary, overall resistome composition was significantly different between the two treatment groups, and a higher ARG richness was found in the flucloxacillin group compared to the ceftriaxone group. No statistically significant relationships between the microbiome and ESBL carriage/acquisition were detected.

Penicillin use has previously been linked with an increased gastrointestinal ARG richness.[183] However, this finding was from a study involving only a single child, and the effect observed was not compared to that of other antibiotics. In the current analysis, it is likely that the difference in resistome composition and ARG richness reflected the relative selective pressures exerted by the two antibiotics, as determined by their spectra of activity. In addition, third-generation cephalosporins have been found to be associated with the acquisition of β-lactam resistance.[61, 184] No significant intergroup difference in ESBL-producing bacteria was found in the culture component of the original RCT, nor was a greater detection of transmissible β-lactam resistance genes in the sequencing analysis of the current study. As suggested for the RCT, this finding may be due to a lack of sufficient statistical power (insufficient experimental units) in relation to what is a relatively rare event, as well as differences in this study population compared to the previous report.[89]

Additional analysis was performed to determine whether microbiome composition predicted ESBL carriage or acquisition by ESBL-producing bacteria. Although not of sufficient statistical power to draw significant conclusions, a non-significant association between ESBL-positive samples the abundance of *E. coli* was observed with positive samples having a greater abundance than negative controls. Samples from the same patient collected prior to the ESBL-positive sample did not have a statistically significant difference in microbiome composition. As shown previously, depletion of the microbiome can be associated with susceptibility to acquisition of multidrug resistant organisms.[66, 67] In this component of the project, microbiome depletion in the sample prior to culture-based detection of an ESBL in the stool might indicate an increased susceptibility to colonisation or an expansion of pathogens already present at low levels in the gut. Further investigation with a cohort large enough have sufficient statistical power is needed to confidently assess this relationship, as would be needed for comparisons with changes in the microbiome as addressed in Chapter 4.

4. GUT MICROBIOME IS TRANSIENTLY IMPACTED BY β-LACTAM ANTIBIOTICS IN CHILDREN WITH CELLULITIS

4.1. CHAPTER SUMMARY

Ensuring limited disruption to the infant and childhood microbiome has been shown to be important for a healthy immune development and disease prevention.[84, 85, 88] Therefore, in addition to determining the effect of each treatment on the selection for antimicrobial resistance, it is also important to ascertain how the microbiome composition would be affected. In Chapter 3, it was shown that two antibiotic treatments for paediatric cellulitis had differential effects on the gut resistome, which were likely reflective of changes to the microbiome. Using 16S rRNA gene amplicon sequencing, the broad-spectrum treatment was observed to have significantly reduced species richness at T2 (7-14 days post-IV antibiotic) compared to the narrow-spectrum group. Evidence of microbiome recovery in the ceftriaxone group, with several diversity estimates significantly increasing by the T3 (3 months post-antibiotic) was also shown. Levels of *E. coli* significantly changed in the flucloxacillin group over the study period, and antibiotic treatment had a significant impact on the core members of the microbiome when comparing both between and within groups. However, the observed changes to the microbiome in both groups were transient, possibly due to the plasticity of the microbiome, which has been reported for healthy adults.[103, 185]

4.2. INTRODUCTION TO THE CHAPTER

Chapter 3 outlined the rationale and the goals of the CHOICE RCT as well as the aims for the CHOICE substudy undertaken in this project, so these are not repeated here. This component of the project focused on the stool microbiome and aimed to compare the longitudinal effects of a broad-spectrum and a narrow-spectrum antibiotic on the gut microbiome in children.

4.3. STATISTICAL ANALYSIS OF CHOICE MICROBIOME

Within-group and between-group microbiome analysis was performed in SAS using a multivariate generalised linear mixed model that included adjustment for any antibiotic used prior to the study intervention. Differences in beta diversity were assessed by permutational multivariate ANOVA (PERMANOVA), which evaluates differences in the distribution of the microbiome between groups. Correction for multiple hypothesis testing was performed using the Benjamini-Hochberg method. Summary statistics are given as mean ± SD is unless otherwise specified. Statistical significance is set at $p < 0.05$.

4.4. IMPACT OF DIFFERENT ANTIBIOTICS ON THE GUT MICROBIOME OVER TIME

Analysis of the gut microbiome composition of patients randomised to receive either narrow-spectrum flucloxacillin or broad-spectrum ceftriaxone was performed at and between each time point. At T1 (24 samples for ceftriaxone and 23 for flucloxacillin), treatment group was not significantly associated with differences in alpha diversity ($p > 0.05$; Figure 4.1A-D). This was consistent with expectations because samples had been taken within the first 48 h after IV antibiotic initiation. Although beta diversity was not significantly different, dispersion (a measure of the within-group variation in microbiome composition) was significantly greater in the ceftriaxone group compared to the flucloxacillin group (ceftriaxone 1.0 ± 0.7 vs flucloxacillin 0.5 ± 0.3; $p = 0.01$, Figure 4.1E), which is likely to be indicative of an early differential impact.

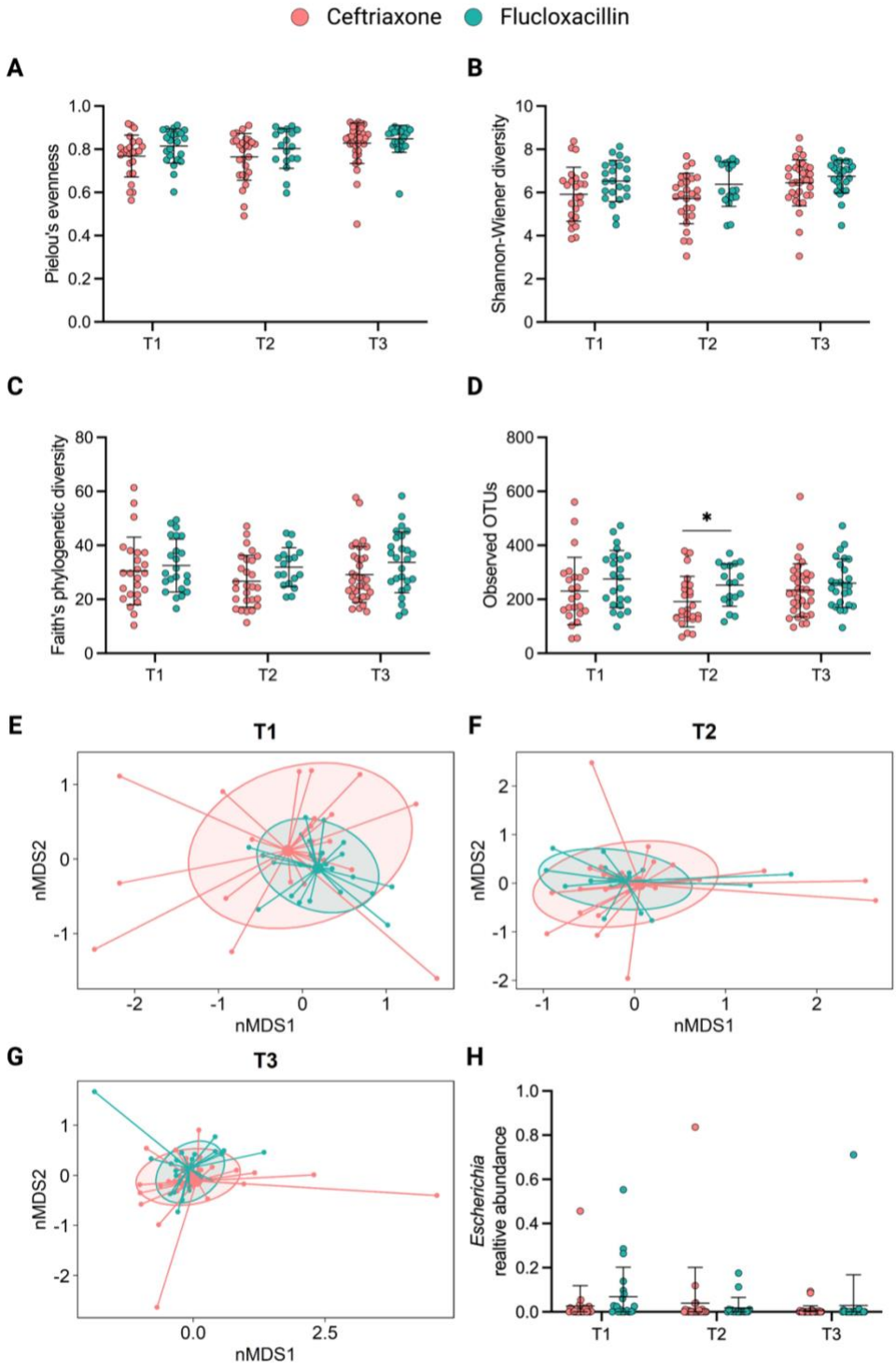

Figure 4.1 Alpha diversity between groups at each time point as estimated by **(A)** Pielou's evenness, **(B)** Shannon-Wiener diversity, **(C)** Faith's phylogenetic diversity and **(D)** richness. Overall microbiome composition between groups depicted by weighted UniFrac distance of genus-level relative abundance data on a non-metric multidimensional scaling plot (nMDS) at **(E)** T1, **(F)** T2, and **(G)** T3. Each point represents an individual's microbiome relative to others on the plot and the circle represents 80% confidence interval. **(H)** *Escherichia* relative abundance between treatment groups at each time point. Bars represent mean and SD. * $p < 0.05$.

At T2, IV treatment had ceased for both groups, and patients had received at least 5 days of oral cephalexin. Forty-five of those who provided stool samples at T2 had available 16S sequencing data for microbiome analysis (27 for ceftriaxone and 18 for flucloxacillin). Between T1 and T2, no significant changes in microbiome composition were observed in either treatment group (Figure 4.1A-D).

These findings are supported by the direct comparison between the groups at T2. There was no significant intergroup difference in evenness, Shannon-Wiener diversity or Faith's phylogenetic diversity (Figure 4.1A-C). Only richness was significantly lower in the ceftriaxone group compared to the flucloxacillin group (flucloxacillin 252 ± 78 vs ceftriaxone 191 ± 94; p = 0.046; Figure 4.1D). Beta diversity and dispersal were not significantly different between treatment groups at T2 (Figure 4.1F).

At T3, participants had completed their antibiotic course and had not received antibiotics for at least 10 weeks. Sixty participants provided stool samples at T3 (34 ceftriaxone and 26 flucloxacillin). By this time, there was no difference between treatment groups in alpha or beta diversity (Figure 4.1A-D,G). The difference in richness observed between groups at T2 was no longer present.

No significant changes to alpha or beta diversity were observed between T2 and T3 within the flucloxacillin treatment group (Figure 4.1A-D). However, within the ceftriaxone group, evenness increased significantly (T2 0.76 ± 0.11 vs T3 0.83 ± 0.1; p = 0.012), as did Shannon-Wiener diversity (T2 5.6 ± 1.2 vs T3 6.5 ± 1.1; p = 0.013) and richness (T2 183 ± 99 vs T3 232 ± 104; p = 0.041). There were no significant changes in Faith's phylogenetic diversity or beta diversity within the ceftriaxone group.

Finally, no significant differences in alpha or beta diversity between T1 and T3 were observed within the flucloxacillin group. Within the ceftriaxone group, evenness increased significantly between T1 and T3 (n = 20; T1 0.75 ± 0.09 vs T3 0.81 ± 0.1; p = 0.028), but there were no significant changes in any other estimate of alpha diversity. There were also no significant changes in beta diversity within the ceftriaxone group.

4.5. IMPACT OF DIFFERENT ANTIBIOTICS ON RELATIVE ABUNDANCE OF CORE ENTERIC GENERA OVER TIME

Core genera present at and between each time point were analysed for changes in relative abundance. At T1, seven genera met the criteria to be classed as core and were analysed for intergroup differences (*Bacteroides*, *Blautia*, *Erysipelatoclostridium*, *Faecalibacterium*, unclassified Lachnospiraceae, uncultured Peptostreptococcaceae and *Streptococcus*). There were no significant differences in relative abundance within samples between treatment groups (all p > 0.05).

At T2, six genera were identified as core (*Bacteroides*, *Blautia*, *Faecalibacterium*, unclassified Lachnospiraceae, uncultured Peptostreptococcaceae and *Streptococcus*). *Erysipelatoclostridium* did not meet the criteria at T2 and so was not analysed at this time point. Direct comparison between the treatment groups at T2 showed that only the relative abundance of unclassified Lachnospiraceae differed, being higher in the ceftriaxone group (0.05 ± 0.05) compared to the flucloxacillin group (0.04 ± 0.03). However, after correction for multiple comparisons this was not statistically significant ($p = 0.24$). Between T1 and T2, there were no significant changes in the relative abundance of core genera in either treatment group.

At T3, 11 genera were identified as being core members of the microbiome: *Anaerostipes*, *Bacteroides*, *Blautia*, *Clostridium* (*sensu stricto* 1), *Faecalibacterium*, *Fusicatenibacter*, unclassified Lachnospiraceae, uncultured Peptostreptococcaceae, *Ruminococcus torques* group, *Streptococcus* and *Subdoligranulum*. *Anaerostipes, Clostridium* (*sensu stricto* 1), *Fusicatenibacter*, *Ruminococcus torques* group and *Subdoligranulum* were included as they met the criterion for core genera at this time point. Direct comparison between the treatment groups at T3 showed no differences in genera relative abundance (all $p > 0.05$).

No changes in the relative abundance of core genera were identified between T2 and T3 for either treatment group. Additionally, no changes were observed within the flucloxacillin group between T1 and T3. However, between T1 and T3, there was a significant increase in the relative abundance of *Clostridium* (*sensu stricto* 1) (T1: 0.007 ± 0.02; T3: 0.05 ± 0.08; $p = 0.025$) within the ceftriaxone group. No other significant changes in the ceftriaxone group were observed between T1 and T3.

4.6. IMPACT OF ANTIBIOTICS ON DETECTION OF SPECIFIC ENTERIC PATHOGENS OVER TIME

To determine whether antibiotics caused reduction or enabled the expansion of pathogenic bacteria within the gut microbiota, the relative abundance of eight enteric genera. was assessed (Table 4.1). At T1, *Escherichia, Klebsiella, Citrobacter, Enterobacter, Pseudomonas, Enterococcus* and *Staphylococcus*, but not *Acinetobacter* were detected in stool samples. *Escherichia* was the only genera detected in a sufficient number of samples to be tested statistically at any time point and its relative abundance did not differ significantly between treatment groups at T1 (Figure 4.1H).

Table 4.1 Prevalence of potential pathogens throughout the study period.

Pathogen	T1 N (%)		T2 N (%)		T3 N (%)	
	Ceftriaxone n = 24	Flucloxacillin n = 23	Ceftriaxone n = 27	Flucloxacillin n = 18	Ceftriaxone n = 34	Flucloxacillin n = 26
Escherichia	17 (70.8)	17 (73.9)	12 (44.4)	12 (66.7)	20 (58.8)	11 (42.3)
Klebsiella	2 (8.3)	3 (13.0)	1 (3.7)	2 (11.1)	0 (0)	1 (3.9)
Citrobacter	0 (0)	2 (8.7)	1 (3.7)	0 (0)	1 (2.9)	0 (0)
Enterobacter	4 (16.7)	2 (8.7)	1 (3.7)	0 (0)	2 (5.9)	0 (0)
Pseudomonas	0 (0)	1 (4.4)	0 (0)	0 (0)	0 (0)	0 (0)
Enterococcus	5 (20.8)	1 (4.4)	9 (33.3)	4 (22.2)	0 (0)	0 (0)
Staphylococcus	2 (8.3)	0 (0)	0 (0)	0 (0)	1 (2.9)	0 (0)

At T2, *Escherichia, Klebsiella, Citrobacter, Enterobacter* and *Enterococcus* were detected in the stool samples, but not *Acinetobacter*, *Pseudomonas* and *Staphylococcus*. Direct comparison between the treatment groups at T2 revealed no differences in the relative abundance of *Escherichia*. Between T1 and T2, the relative abundance of *Escherichia* did not significantly change in either of the treatment groups (Figure 4.1H).

At T3, *Escherichia, Klebsiella, Citrobacter, Enterobacter* and *Staphylococcus* were detected in the stool samples, but not *Acinetobacter*, *Pseudomonas* and *Enterococcus*. Direct comparison between the treatment groups at T3 showed no differences in *Escherichia* relative abundance (Figure 4.1H).

Between T2 and T3, no significant changes in *Escherichia* were observed within either treatment group. However, within the flucloxacillin group, *Escherichia* significantly decreased in relative abundance between T1 and T3 (T1 0.11 ± 0.17 vs T3 0.002 ± 0.004; p = 0.048) (Figure 4.1H). Relative abundance of *Escherichia* did not change significantly in the ceftriaxone group between T1 and T3.

4.7. LONGITUDINAL EFFECTS OF EITHER ANTIBIOTIC ON THE STOOL MICROBIOME

To investigate the impact of *any* antibiotic treatment for moderate-severe cellulitis on the stool microbiome of children, a small longitudinal sub-analysis was performed where samples were not split into treatment groups. No estimates of alpha diversity were significantly different between samples from any treatment group at T1 and T2 (p > 0.05, n = 29, Figure 4.2A-D). Beta diversity was also not significantly different between T1 and T2 (p = 0.80). Between T2 and T3, evenness (T2 0.77 ± 0.11 vs T3 0.84 ± 0.09; p = 0.003) and Shannon diversity (T2 5.6 ± 1.2 vs T3 6.6 ± 1.0; p = 0.01) significantly increased but Faith's phylogenetic diversity and richness did not significantly change (n = 35). Beta diversity was not significantly different between T2 and T3 (p = 0.21). Evenness significantly increased between T1 and T3 (T1 0.77 ±0.09 vs T3 0.83 ± 0.09; p = 0.005), but no other estimate of alpha diversity changed significantly between these time points (n = 32). Beta diversity was not significantly different between T1 and T3 (p = 0.35).

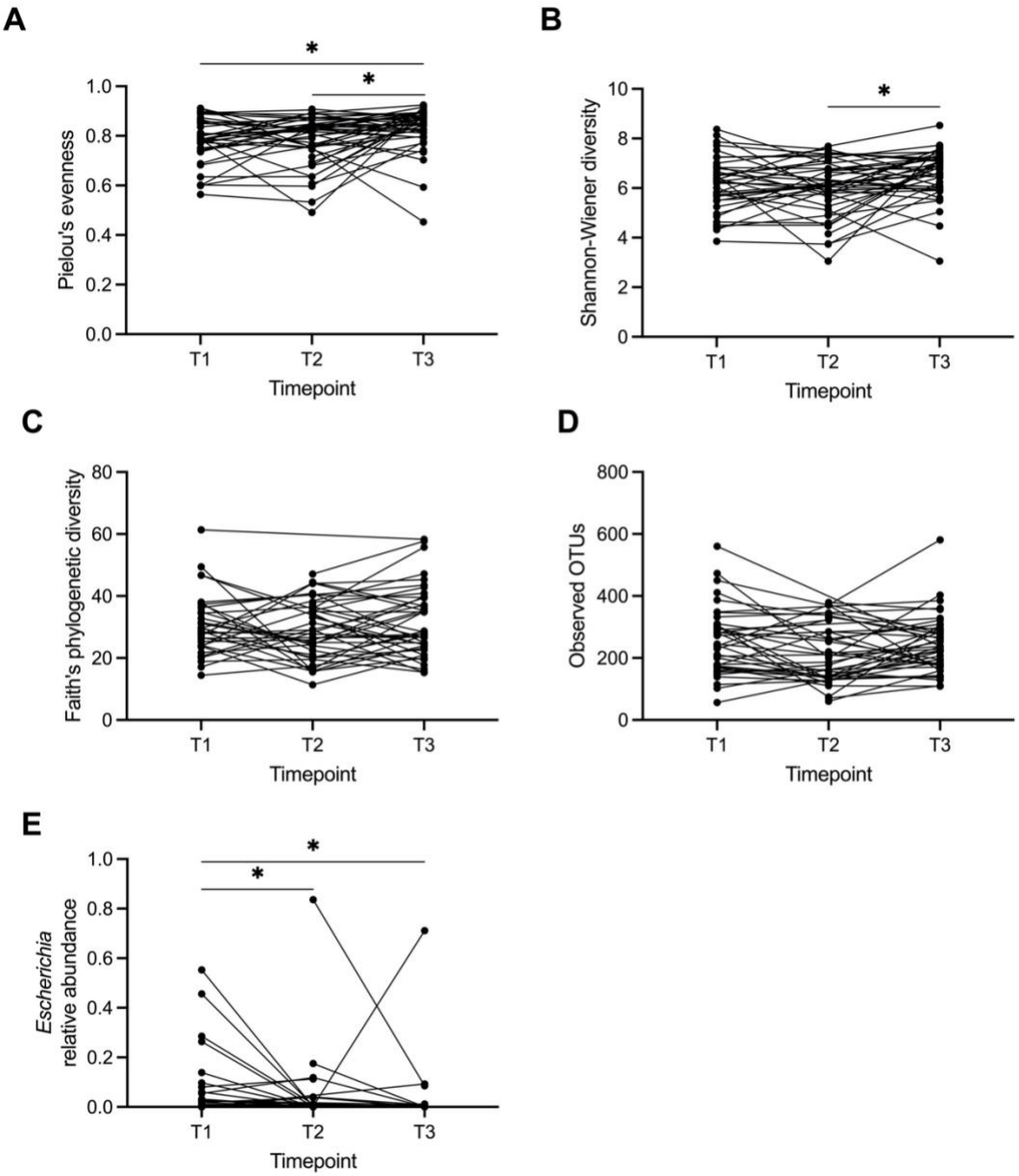

Figure 4.2 Changes in alpha diversity for all samples over the study period as estimated by **(A)** Pielou's evenness, **(B)** Shannon-Wiener diversity, **(C)** Faith's phylogenetic diversity and **(D)** richness. **(E)** Change *in Escherichia* relative abundance for all samples over the study period. * $p < 0.05$.

Core taxa present in at least 80% of all samples were assessed for changes in relative abundance over time. There was no significant change in core taxa between T1 and T2 or T2 and T3. Between T1 and T3, *Anaerostipes* (T1 0.02 ± 0.05 vs T3 0.07 ± 0.07; p = 0.002) and *Blautia* (T1 0.14 ± 0.18 vs T3 0.20 ± 0.13; p = 0.007) significantly increased in abundance. No other taxa significantly changed in relative abundance between T1 and T3. *Escherichia* significantly decreased in relative abundance between T1 and T2 (T1 0.08 ± 0.14 vs T2 0.01 ±0.03; p = 0.031) and T1 and T3 (T1 0.06 ± 0.14 vs T3 0.004 ± 0.02; p = 0.01) but did not significantly change in relative abundance between T2 and T3 (Figure 4.2E).

4.8. INTERPRETATION OF FINDINGS

Most notably, a decrease in microbiota richness was recorded 7-14 days after the initiation of IV antibiotics, and a reduction in the relative abundance of unclassified members of the Lachnospiraceae family in the ceftriaxone group. A difference in microbiome dispersal between groups at T1 was also observed, most likely due to the combination of prior antibiotics and initiation of IV treatment, although prior antibiotic use was not different between treatment groups. These intergroup differences in microbiota composition had resolved by 3 months after the commencement of the IV treatment (T3) when compared to T1 and T2 time points. Within the ceftriaxone group, there was also a clear increase in evenness, Shannon-Wiener diversity and richness between T2 and T3. An increase in the relative abundance of commensal clostridium and microbiome evenness was also seen in the ceftriaxone group between T1 and T3.

In this substudy, it was found that any antibiotic-induced changes to the microbiome composition were transient and were resolved by 3 months post treatment. Despite some early differences in composition between treatment groups, it was found that the evenness, richness, and diversity of the microbiome in the ceftriaxone group was restored between T2 and T3. This differs to the findings of a recent study in neonates, which revealed that randomised treatment with broad-spectrum antibiotics for suspected early-onset neonatal sepsis significantly impacted the microbiome even up to 12 months post treatment compared to untreated controls.[90] There are several notable differences in the current study: (1) the participants were significantly older children (≥ 6 months of age), (2) antibiotic treatments with different spectrums were compared rather than making a comparison with a untreated control group, and (3) the RCT included consideration of the benefits of at-home treatment compared to hospital treatment. However, both studies highlight the importance of antimicrobial stewardship when considering treatment options for paediatric conditions.

Some differences were identified in the relative abundance of core genera between and within treatment groups during the study period. Lachnospiraceae is a family of bacteria that contains many beneficial microbes, including *Anaerostipes, Faecalibacterium, Blautia* and *Roseburia*.[186] Many of the Lachnospiraceae that differed in abundance between treatment groups are currently unclassified, however, this family contributes to essential production of short chain fatty acids and has been shown to be important for bioavailability of nutrients and regulation of local inflammation.[186] In contrast, this family has also been associated with impaired glucose metabolism and the potential onset of prediabetes in children.[187] In this study, unclassified Lachnospiraceae were significantly greater in relative abundance in the ceftriaxone treatment group at T2 but not at T3. Given that this difference was transient, it is

not suspected to indicate a substantial risk. *Clostridium* (*sensu stricto* 1) was observed to increase in relative abundance between T1 and T3 only within the ceftriaxone group. *Clostridium* (*sensu stricto* 1) has previously been shown to increase with age and appears to be essential for modulating gut homeostasis during the entire lifespan.[188] Consequently, it was reassuring to see that the levels of this bacteria were restored by 3 months post treatment.

Use of broad-spectrum antibiotics has been found to be associated with a higher risk of acute infection,[66, 189] due to a reduction in competitive exclusion, a phenomenon whereby commensal species prevent the expansion of pathogen populations by out-competing them for resources.[60] Given the observed reduction of alpha diversity between both treatment groups, the abundance of genera that contained common pathogens (including *Escherichia, Citrobacter* and the ESKAPE pathogens) were investigated at each time point. Most pathogen-containing genera were detected at a low frequency across all samples for all time points and only *Escherichia* was detected frequently enough to be a focus for fuller investigation. However, this investigation did not reveal any significant differences in abundance of *Escherichia* between treatment groups at any time point.

Both standard and OPAT therapies were found to be associated with significant, within-group, changes in microbiome characteristics. In the ceftriaxone treatment group, evenness significantly increased between T1 and T3. Within the flucloxacillin group, levels of *Escherichia* significantly decreased between T1 and T3. Changes in the levels of *Escherichia* in the flucloxacillin group between T1 and T3 most likely resulted from natural resistance to this antibiotic. Importantly, microbiome recovery from antibiotic treatment was found at 3 months post-therapy in both groups. This finding aligns with previous studies in adults that found that the microbiome can recover from antibiotic treatment in as little as 2-6 weeks.[103, 185] Given clinical concern regarding the potential long-term impact of these antibiotics on the gut microbiome,[172-174] it is encouraging that this was also found in a cohort of children.

5. ANTIMICROBIAL USE PATTERNS IMPACT THE STOOL RESISTOME OF RESIDENTS OF AGED-CARE FACILITIES

5.1. CHAPTER SUMMARY

Both young children and elderly people have a high risk of infection and the consequences of treatment failure due to an immature or weaker immune system, microbiome instability and high frequency of antibiotic use.[75-77] After looking at the impact of antibiotic exposure in children, investigating the same effect in those at the other end of the spectrum was the next step. Specially, those in residential aged-care facilities who experience multiple exposures may be at an even higher risk of AMR and antibiotic treatment failure.

The GRACE study was set up to investigate how the residential aged-care environment influenced the risk of AMR transmission and carriage. This analysis specifically focused on how antibiotic use patterns contributed to this effect. Viable stool samples were collected from 204 participants who consented to join the GRACE study. Of these, antibiotic use data was able to be obtained from the PBS for the 12 months prior to stool sample collection for 164 participants who had provided a stool sample and for whom PBS data was available and consent had been given for access. Shotgun metagenomic sequencing was performed on all available samples and sequences were mapped to the comprehensive antibiotic resistance database (CARD) to generate a table of resistance genes.

Antibiotics had been administered at least once for 61% of the cohort, with cephalosporins and penicillins the most common (n = 53 and 55, respectively). Antibiotic use was characterised in five ways: (1) number of times exposed, (2) days since most recent antibiotic, (3) total days exposed to antibiotics, (4) number of unique antibiotic classes given, and (5) type of antibiotic received. All measures of antibiotic use significantly impacted the total abundance of ARGs, but none significantly impacted the number of unique ARGs. Doxycycline had a significant impact, not only on its own resistance profile, but also on other resistance profiles.

This component of the project demonstrated that antibiotic use has a significant impact on the resistome in permanent residents of aged-care facilities and extends beyond that of the antibiotic taken at the time.

5.2. INTRODUCTION TO THE CHAPTER

Over 190,000 people reside in aged-care facilities in Australia.[190] They are among the most frail individuals in the Australian society, share a close space, often using the same dining and bathroom facilities, frequently attend hospital and are prescribed antibiotics at a much higher rate than the rest of the country.[2] According to data from the aged-care national antimicrobial prescribing survey (acNAPs), antibiotic stewardship has scope for improvement, with one in six prescriptions of antibiotics being written for "per resident need" and 20% of prescriptions indicating they are for prophylaxis, despite antibiotics rarely being recommended for this purpose.[4] Adding to their already heightened risk of the consequences of treatment failure, this population also experiences a high incidence of skin, respiratory and urinary tract infections.[4, 76, 96] Several studies have investigated the rates of carriage for multidrug resistant organisms (MDROs), such as ESBL, MRSA, VRE and CPE, in older people, both within and outside of aged-care facilities.[5, 191-194] However, all have used culture-based approaches and many have investigated these organisms during or after an infection. Those who have applied sequencing to their analysis usually perform whole genome sequencing on a MDRO that has been involved in an outbreak.[6-10] None of these studies consider that the gene causing the resistance might be present in other organisms that are not actively involved in an infection, but still pose a high risk to the individual. The GRACE study aimed to apply a metagenomic sequencing approach to assess the risk factors for antimicrobial resistance carriage in this high-risk population. The primary outcome of the component of GRACE analysis allocated to this project was to investigate how antibiotic use patterns in a cohort of aged-care residents in a multi-exposure environment was associated with two measures of the resistome: (1) number of unique antibiotic ARGs detected in the sample, and (2) total abundance of the ARGs detected in the sample.

5.3. STATISTICAL ANALYSIS OF GRACE RESISTOME

All statistical analysis was performed in SAS. Normal distribution of both number of unique ARGs and total abundance of ARGs was tested using the SAS procedure PROC UNIVARIATE. Number of unique ARGs was categorised into quartiles using the SAS procedure PROC RANK. Total abundance of ARGs was also categorised by first assigning a value below or equal to 900 (median of the peak in the histogram) as 0 and then assigning the remaining values 1 to 3 using PROC RANK. Days since most recent administering of an antibiotic was categorised into four groups: (1) not administered, (2) administered ≥180 days prior, 3) administered <180 to >30 days prior, and 4) administered ≤30 days prior. Total days exposed to antibiotics was estimated using the anatomical therapeutic chemical code defined daily dose (ATC/DDD) toolkit provided by the WHO[195] and grouped in to four categories: (1) no antibiotic exposure, (2) ≤ 15 days exposure in the 12 months prior to stool sample collection, (3) > 15 to < 90 days exposure, and 4) ≥ 90 days exposure. Ordinal logistic regression (SAS procedure PROC LOGISTIC) was used to determine the impact of each antibiotic use variable on the two ordinal resistome variables. Multiple comparisons underwent correction using the Benjamini-Hochberg method with an FDR rate of 5%. Statistical significance was set at $p < 0.05$. The data were visualised using R (v4.1.2) package "ggplot2" (v3.3.5).

5.4. INTRODUCTION TO THE GRACE COHORT

Five aged-care facilities were included in the study with these being run by three consenting not-for-profit, aged-care providers. All current residents of these facilities were assessed for eligibility (n = 403). Of these, 344 were eligible and approached to participate, and 279 consented to join the study (Figure 5.1). A usable stool sample was provided by 204 participants and 228 participants consented to the use of their Pharmaceutical Benefits Scheme (PBS) history.[196] Full details of the variables assessed in the GRACE study and a summary of the data collected are presented in the 2021 GRACE study report (Appendix B). In addition, a comparison between the GRACE cohort and the national data from a subset of the Registry of Senior Australians was performed to demonstrate that the GRACE cohort was representative of the residential aged-care population in Australia (Appendix C).

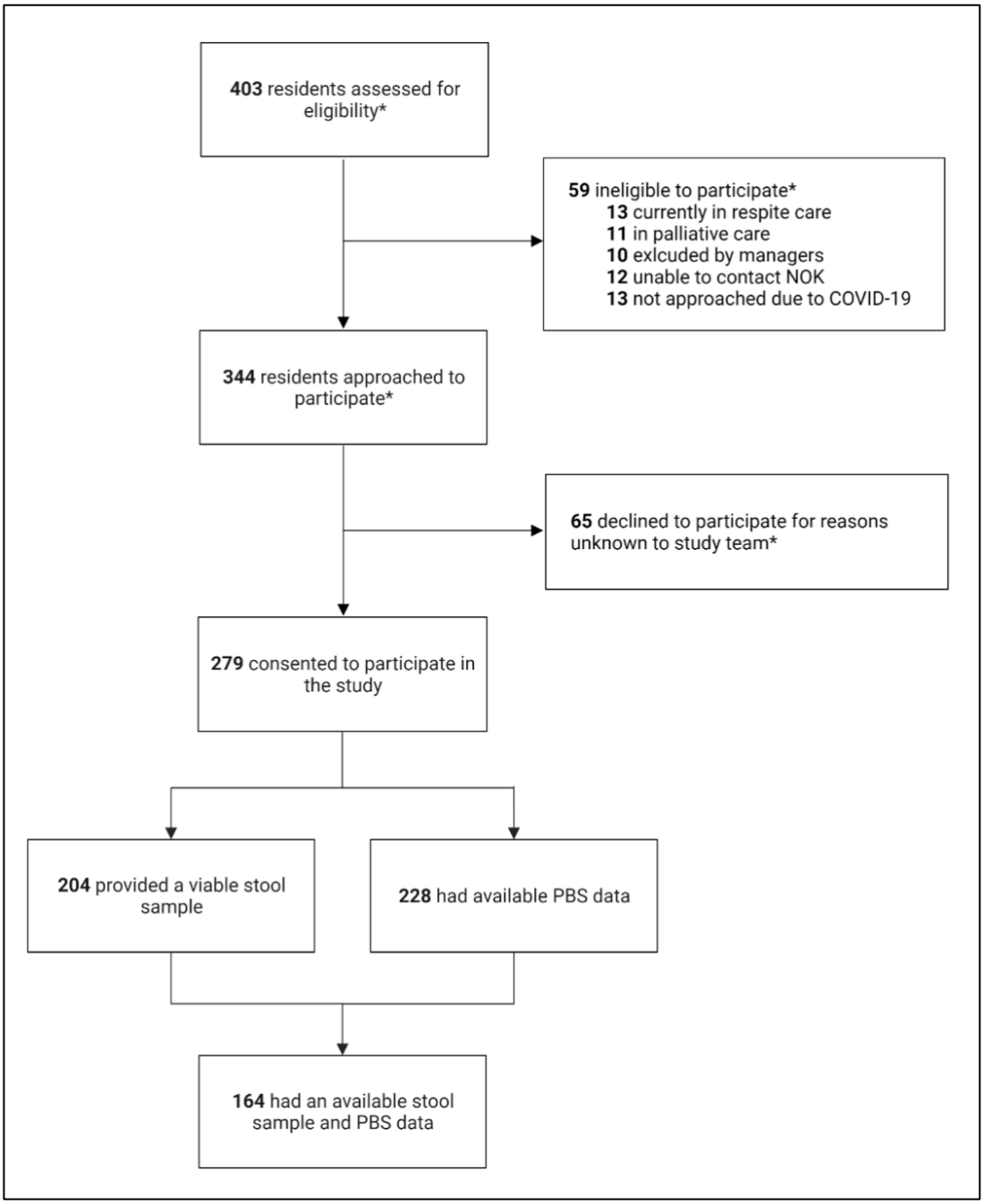

Figure 5.1 Recruitment to the GRACE study and inclusion in final analysis. *data do not include Site 1 due to a change in research nurse and recording protocols.

Analysis of 164 participants who provided stool samples and for whom PBS data was available was performed to investigate the impact of antibiotic use patterns on the resistome and microbiome of aged-care residents. A summary of their baseline characteristics is given in Table 5.1. This cohort was mostly female with a median age of 88 years. They had been living in their current facility for a median of 703 days and had a median of five comorbidities as classified by the Rx-Risk method.[197]

Table 5.1 Clinical features of the GRACE participants (n = 164) included in the final analysis.

Characteristic	Value
Age (years), median (95% CI)	88 (87-90)
Time in care (days), median (95% CI)	703 (570-861)
Sex, n (%)	
Male	46 (28)
Female	118 (72)
Site, n (%)	
1	12 (7)
2	13 (8)
3	87 (53)
4	26 (16)
5	26 (16)
Number of Rx-Risk comorbidities, median (95% CI)	5 (4-6)
Hospital admissions, median (95% CI)*	0 (0-0)

* data on hospital admissions was missing for 6% of the cohort.

5.5. ANTIBIOTIC USE PATTERNS PRIOR TO SAMPLE COLLECTION

For the purpose of this analysis, an antibiotic-use event was defined prior to analysis to represent the dispensing of an antibiotic for an unknown infectious indication. Antibiotic use was considered in five main categories: (1) number of antibiotic use events, (2) days since last antibiotic, (3) total number of days exposed to antibiotics, (4) number of unique antibiotic classes administered, and (5) the type of antibiotic used (Figure 5.2).

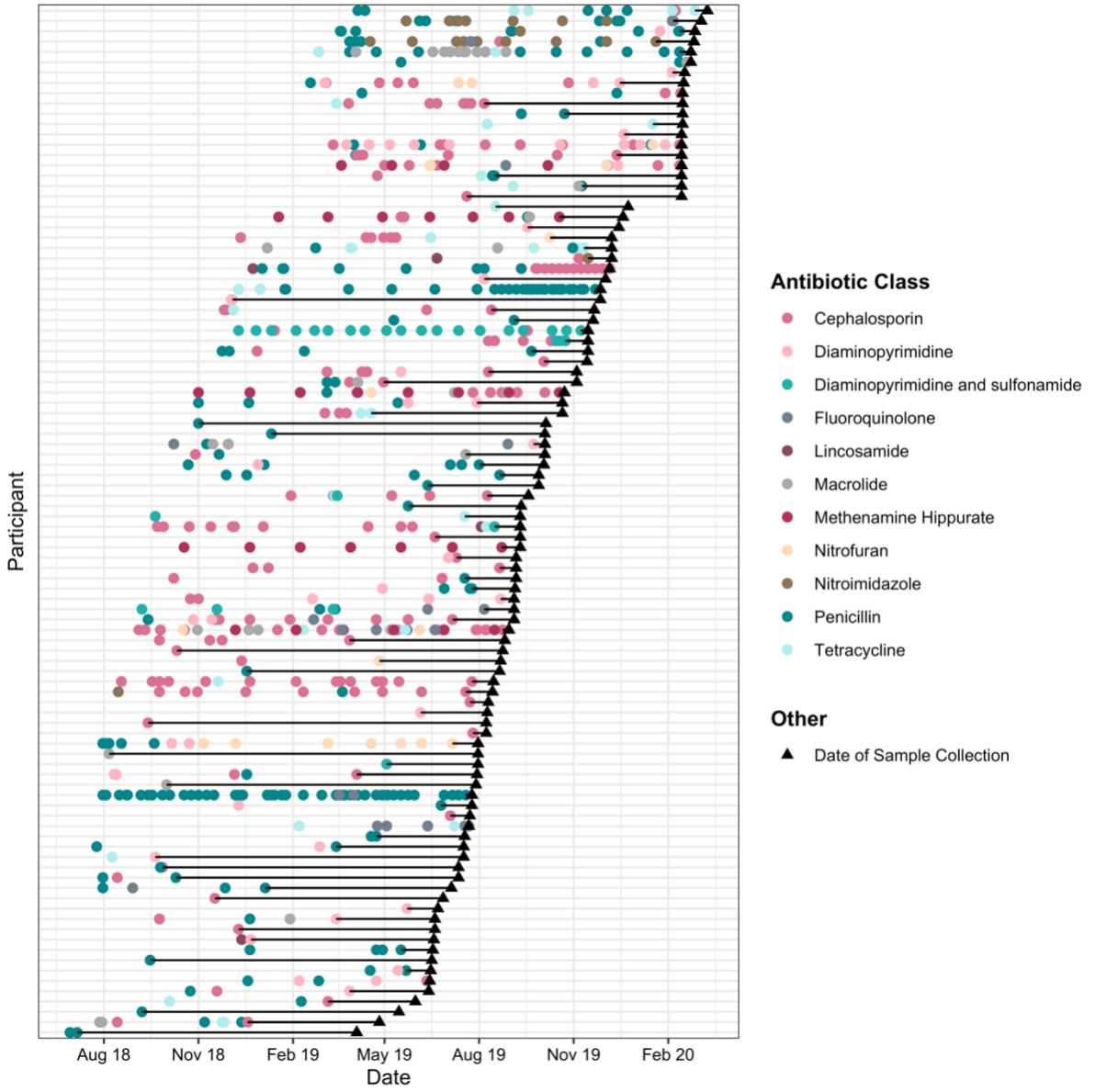

Figure 5.2 History of antibiotic use (circles) for each participant in the GRACE study up to 12 months prior to stool sample collection (triangle) (▲). Each row represents one participant. The length of the line represents the time between the most recent antibiotic use and stool sample collection.

Of the 164 included participants, 61% (n = 100) had received at least one antibiotic in the 12 months prior to collection. In total, 704 records of antibiotic dispensing were identified in this time frame, including 20 unique antibiotics. A median of four (IQR 1-8) antibiotics were administered per participant. Of those who received antibiotics, 66% (n = 66) of participants had received two or more unique antibiotics during the 12 months prior to sample collection, with the median of 2 (IQR 1-3) antibiotics. The median time between stool sample collection and the most recent antibiotic used was 62 (IQR 22-129) days. Of those who received at least one antibiotic in the 12 months prior to sample collection, the median number of days exposed to antibiotics (calculated using the ATC/DDD toolkit [196]) was 20 (IQR 6-48) days. Cephalexin

was the most commonly administered antibiotic among those who had received antibiotics, with 50% (n = 50) having at least one dose of cephalexin. This was followed by amoxicillin and clavulanic acid (40%, n = 40), amoxicillin (30%, n = 30), trimethoprim (29%, n = 29) and doxycycline (21%, n = 21). For analysis of resistome data, antibiotic use was also categorised based on their parent classes. Penicillins (55%, n = 55) and cephalosporins (53%, n = 53) were the most dispensed antibiotic classes in the cohort. The dispensed antibiotics arranged by parent classes is given Table 5.2.

Table 5.2 Classification of antibiotics used in the GRACE study into parent classes.

Parent class	Antibiotics received in the GRACE cohort
Cephalosporin	Cephalexin, Cefaclor, Ceftriaxone, Cefuroxime
Diaminopyrimidine	Trimethoprim, Trimethoprim-sulphamethoxazole
Fluoroquinolone	Ciprofloxacin, Norfloxacin
Lincosamide	Clindamycin
Macrolide	Azithromycin, Erythromycin, Clarithromycin, Roxithromycin
Nitrofuran	Nitrofurantoin
Nitroimidazole	Metronidazole
Penam (Penicillin)	Amoxicillin, Amoxicillin and clavulanic acid, Flucloxacillin
Sulphonamide	Trimethoprim-sulphamethoxazole
Tetracycline	Doxycycline

5.6. GUT RESISTOME COMPOSITION IN RESIDENTS OF AGED-CARE FACILITIES

In the stool resistome, 1,136 unique ARGs conferring resistance to 38 different antibiotic classes were detected, with a median of 274 (IQR 236-300) genes detected per participant. Genes from 254 AMR gene families were detected with the members of the OXA beta-lactamase (n = 103) and resistance-nodulation-cell division antibiotic efflux pump (n = 100) families being the most common. The median total abundance of ARGs was 890 (IQR 727-1324) rpkm. Genes conferring resistance to cephalosporins were the most common, but those conferring resistance to penicillins were the most abundant (Table 5.3).

Table 5.3 Number of times received (%) for antibiotic classes received in the GRACE study and the corresponding number of ARGs and total abundance of ARGs for each class (median and IQR).

Class	Times received	Number of ARGs	Total abundance of ARGs (rpkm)
Cephalosporin	53 (43)	81 (70-91)	245 (175-353)
Diaminopyrimidine	29 (23)	8 (6-10)	6 (2-15)
Fluoroquinolone	10 (8)	23 (19-28)	29 (17-50)
Lincosamide (clindamycin)	4 (3)	16 (13-18)	48 (31-68)
Macrolide	16 (13)	28 (24-33)	61 (43-95)
Nitrofuran (nitrofurantoin)	8 (6)	0 (0-1)	0 (0-0.3)
Nitroimidazole (metronidazole)	5 (4)	0 (0-0)	0 (0-0)
Penam/Penicillin	55 (44)	69 (60-78)	230 (160-328)
Sulfonamide (cotrimoxazole)	7 (6)	3 (2-5)	2 (0.6-5)
Tetracycline (doxycycline)	21 (17)	64 (56-71)	137 (106-196)

5.7. NUMBER OF ANTIBIOTIC EXPOSURE EVENTS IS ASSOCIATED WITH A HIGHER ABUNDANCE OF ANTIBIOTIC RESISTANCE GENES

A primary aim of this component of the project was to determine if the number of times participants were exposed to antibiotics was associated with ARG carriage. No statistically significant association between number of exposure events and number of unique ARGs was identified (Odds Ratio (OR) = 0.97, 95% CI = 0.94-1.01, p = 0.12, Figure 5.3A). However, a

statistically significant association between number of antibiotics exposure events and total ARG abundance was found (OR = 1.12, 95% CI = 1.06, 1.16, p < 0.001). Specifically, a higher number of exposure events was associated with a higher total ARG abundance (Figure 5.3B).

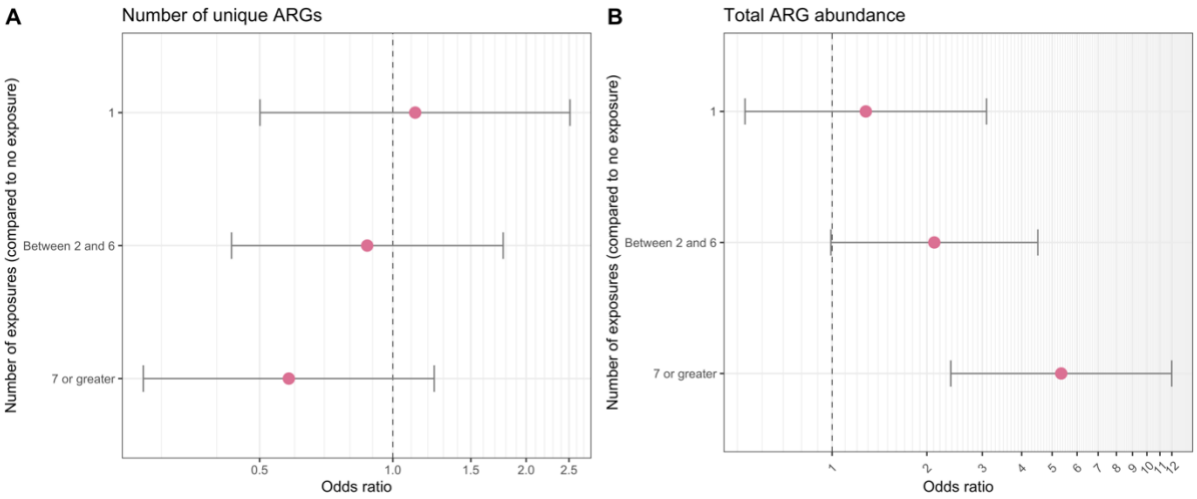

Figure 5.3 Odds of a higher **A)** number of unique ARGs and **B)** total ARG abundance with three categories of number of exposure events. Circles and bars represent the odds and 95% confidence interval of the odds. Comparisons are of each category of number of exposure events (y axis) against the no antibiotic use category.

This analysis was then repeated but adjusted for the four covariates (age, days in care, sex and site of residence) and the four most common comorbidities (depression, gastro-oesophageal reflux disease, hypertension and pain). The number of unique ARGs was not found to be significantly associated with the number of exposure events, but there was a significant association in the odds of a higher total ARG abundance and a higher number of exposure events (OR = 1.12, 95% CI= 1.06-1.18, p < 0.001).

5.8. MOST RECENT ANTIBIOTIC EXPOSURE HAS IMPACT ON TOTAL ARG ABUNDANCE

Another key aim was to investigate how the time since most recent antibiotic exposure affected the resistome. There was no significant association between number of unique ARGs and days since most recent antibiotic exposure (p = 0.39, Figure 5.4A). However, a significant difference in the odds of having a higher total abundance of ARGs was observed between those who had received an antibiotic 30 days or less prior to stool sample collection and those who had not received an antibiotic during the study period (OR = 3.66, 95% CI = 1.67-8.02, p = 0.009, Figure 5.4B). Those who had been administered an antibiotic between 30 and 180 days prior to stool sample collection (OR = 1.95, 95% CI = 0.91-4.19, p = 0.79), and those who had been administered an antibiotic ≥180 days prior (OR = 1.55, 95% CI = 0.61-4.00, p

= 0.62) did not have significantly different odds of having a higher total abundance of ARGs compared to those who were not exposed to antibiotics.

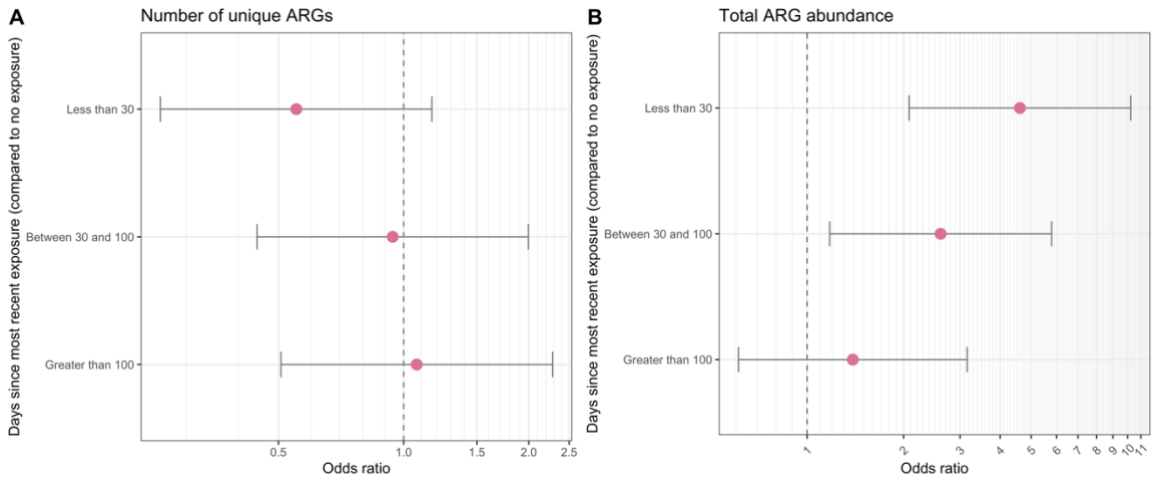

Figure 5.4 Odds of a higher **A)** number of unique ARGs and **B)** total ARG abundance with three categories of recent antibiotic exposure. Circles and bars represent the odds and 95% confidence interval of the odds. Comparisons are of each category of recent antibiotic use (y axis) against the no antibiotic use category.

Stepwise analysis adjusted for covariates revealed no significant associations between the number of unique ARGs and days since most the recent antibiotic exposure. However, total ARG abundance was found to be significantly associated with days since most recent antibiotic exposure. Specifically, the odds of a higher total ARG abundance were significantly different between those who had been administered an antibiotic within 30 days of stool sample collection and those who had no antibiotic exposure (OR = 3.58, 95% CI = 1.52-8.47, p = 0.01).

5.9. NUMBER OF EXPOSURE DAYS INCREASES ARG ABUNDANCE IN THE GUT

The total number of exposure days, as calculated using the ATC/DDD toolkit, was assessed for association with number of unique ARGs and total ARG abundance. Number of unique ARGs was not significantly associated with the number of exposure days (p = 0.33, Figure 5.5A). However, the odds of having a higher total abundance of ARGs were significantly different between those who had received 90 days or more of antibiotic exposure in the 12 months prior to stool sample collection and those who had not received an antibiotic (OR = 5.42, 95% CI = 1.89-15.6, p = 0.01, Figure 5.5B). Those who had received 15 days or less of antibiotic exposure (OR = 1.12, 95% CI = 0.51-2.48, p = 0.03) and those who had between 15 and 90 days of exposure (OR = 3.07, 95% CI = 1.46-6.48, p = 0.13) did not have significant

difference in the odds of having a higher total abundance of ARGs compared to those who had not been exposed to antibiotics.

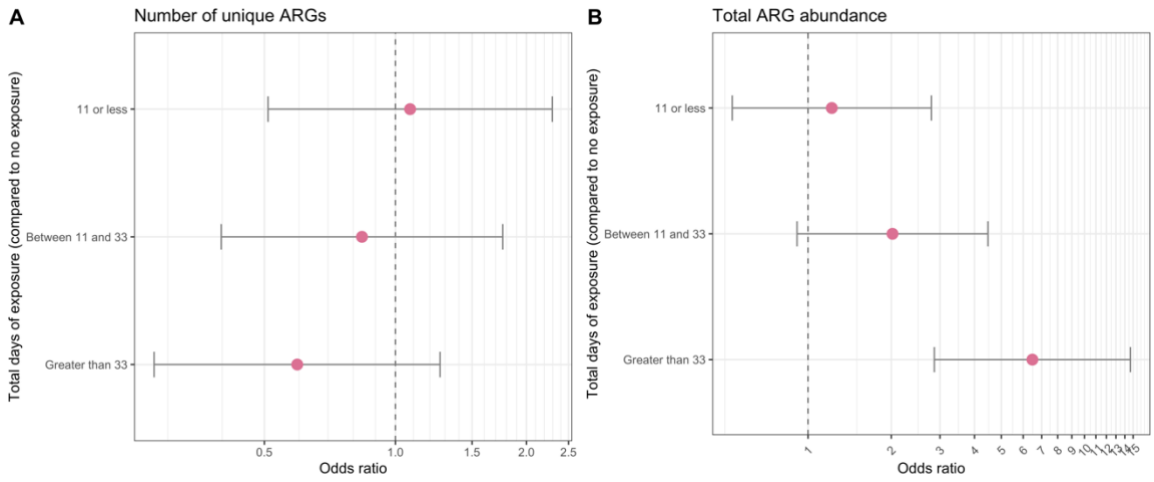

Figure 5.5 Odds of a higher **A)** number of unique ARGs and **B)** total ARG abundance with three categories of total days of antibiotic exposure in the 12 months prior to sample collection. Circles and bars represent the odds and 95% confidence interval of the odds. Comparisons are of each category of total antibiotic exposure (y axis) against the no antibiotic exposure category.

Number of unique ARGs was not significantly associated with total days exposed to an antibiotic after adjusting for covariates. However, total exposed days was associated with different odds of a higher total ARG abundance. Specifically, those who had 90 days or more of exposure had significantly higher odds of a higher ARG abundance compared to those that had not been exposed (OR = 5.61, 95% CI = 1.73-18.2, p = 0.02).

5.10. NUMBER OF UNIQUE ANTIBIOTIC CLASSES RECEIVED INFLUENCES THE RESISTOME

The next step was to investigate if the number of unique antibiotic classes given per person impacted the resistome. Number of unique ARGs was not found to be associated with number of unique antibiotic classes given (OR = 0.90, 95% CI = 0.74-1.10, p = 0.31, Figure 5.6A). Total ARG abundance, however, was significantly higher in those who had received a greater number of unique antibiotic classes in the 12 months prior to stool sample collection (OR = 1.59, 95% CI = 1.27-1.97, p < 0.001, Figure 5.6B).

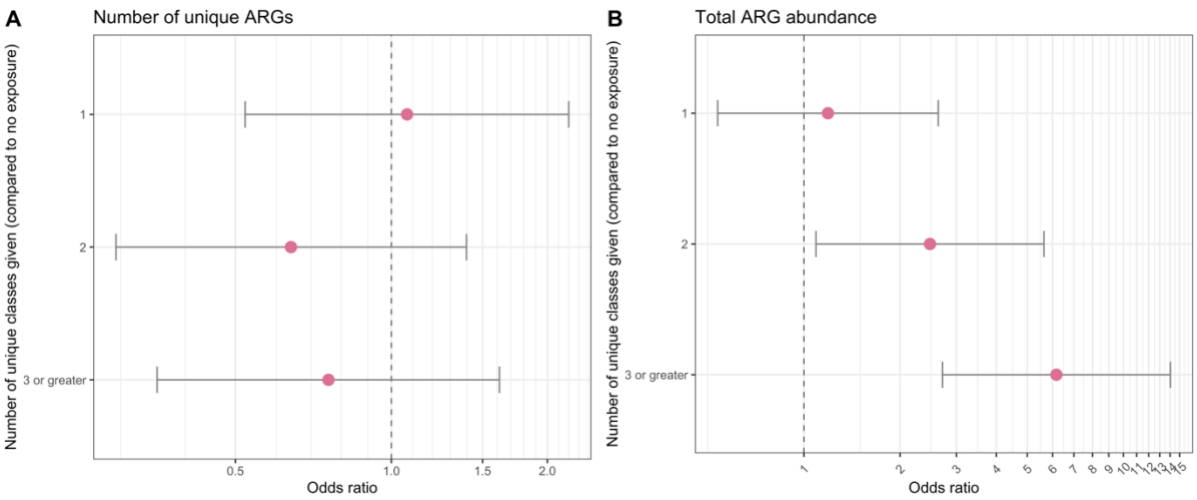

Figure 5.6 Odds of a higher **A)** number of unique ARGs and **B)** total ARG abundance with three categories of number of unique classes in the 12 months prior to sample collection. Circles and bars represent the odds and 95% confidence interval of the odds. Comparisons are of each category of number of unique classes (y axis) against the no antibiotic exposure category.

Number (a count) of unique ARGs were not significantly associated with any unique antibiotic class, even after removal of variation attributable to covariates. However, number of unique antibiotic classes were significantly associated with the odds of a higher total ARG abundance based on multivariate analysis (OR = 1.66, 95% CI = 1.30-2.12, p < 0.001).

5.11. TYPE OF ANTIBIOTIC USED IS ASSOCIATED WITH A SHIFT IN THE STOOL RESISTOME

The final possible association to be examined was that between the exposure to a class of antibiotics at least once in the study period and their effect on number of unique ARGs and total ARG abundance, both overall and for individual drug resistance profiles. Only the four most frequently used classes of antibiotics, cephalosporins, penicillins, diaminopyrimidines (trimethoprim) and tetracyclines (doxycycline), could be assessed for their impact due to small number of uses for other classes. No antibiotics were associated with a significant difference in the odds of a higher number of unique ARGs. However, cephalosporin use was associated with significantly higher odds of having a high total ARG abundance (OR = 2.4, 95% CI = 1.3-4.4, p=0.005, Figure 5.7A). Diaminopyrimidine use was significantly associated with higher odds of a high diaminopyrimidine ARG abundance (OR = 3.6 , 95% CI = 1.8-7.3, p < 0.001, Figure 5.7B). No significant association between penicillin use and ARG abundance for any class was found (Figure 5.7C) Doxycycline use at least once during the 12 months prior to stool sample collection was associated with significantly higher odds of a high total ARG abundance (OR = 9.9 , 95% CI = 3.9-24.7, p < 0.001), high cephalosporin ARG abundance (OR = 2.5 , 95% CI = 1.1-5.7; p = 0.03), high penam ARG abundance (OR = 3.0 , 95% CI =

1.3-6.9, p = 0.01) and high tetracycline ARG abundance (OR = 22.9 , 95% CI = 8.1-64.3; p < 0.001, Figure 5.7D). Adjusting for covariates did not change the significance of these outcomes.

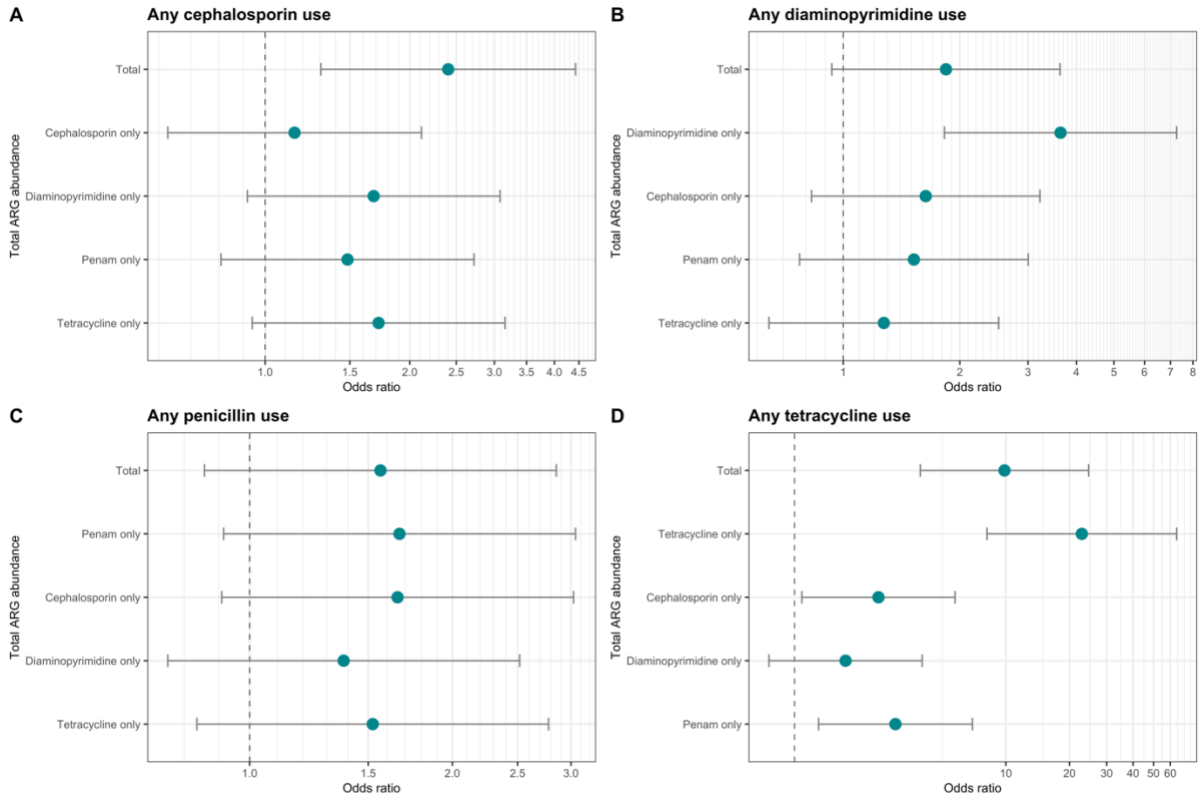

Figure 5.7 Impact of **A)** any cephalosporin use, **B)** any diaminopyrimidine use, **C)** any penicillin use or **D)** any tetracycline use at least once in the 12 months prior to sample collection on ARG abundance for all ARGs, cephalosporin ARGs, diaminopyrimidine ARGs, penam ARGs and tetracycline ARGs. Circles and bars represent the odds and 95% confidence interval of the odds. Comparisons are of each antibiotic class used against no antibiotic exposure.

5.12. INTERPRETATION OF FINDINGS

This component of the project demonstrated that antibiotic use classified by five measures significantly impacted the total abundance of ARGs but not the number of unique ARGs in a stool sample. Specifically, a higher number of exposures, a lower number of days since the most recent use of antibiotics, a higher number of total days of antibiotic use and a higher number of unique antibiotic classes used was associated with a higher total ARG abundance. Much of this is likely to be reflected in the microbiome, which is highly variable and low in diversity in older people.[94, 198] For this reason, it was unlikely to have seen a significant change in the number of unique ARGs, which reflects resistome diversity.

Given the advanced age of the GRACE cohort and limited information on antibiotic use patterns in residential aged-care,[92] analysis of the resistome was less focused on acquiring

new resistant organisms, and more focused on an increase in abundance of resistance already present in the gut microbiome. Indeed, this phenomenon was observed in the GRACE cohort, with those who had antibiotic exposure more frequently and closer to the sample collection date having a higher total ARG abundance, indicating a selection for resistance.

Use of doxycycline at least once in the 12 months prior to sample collection significantly impacted the total ARG abundance, as well as other antibiotic-specific resistance profiles, specifically penicillin and cephalosporin resistance. Tetracycline resistance is most often mediated by antibiotic efflux pumps that are also transmissible to other organisms through horizontal gene transfer.[26, 32] These efflux pumps occasionally are specific to tetracyclines, but quite often correspond to multidrug resistance, including to cephalosporins and penicillins.[199, 200] For example, certain strains of *E. coli* can have intrinsic resistance to tetracyclines due to regulation of efflux pumps such as the AcrAB-TolC complex.[199] This complex is a multidrug efflux pump shown to confer resistance to other antibiotics, such as ampicillin, cefotaxime and cefoxitin.[199, 201] Genes contributing to the functionality of this complex were identified in the gut resistome of GRACE participants. It is likely that the use of doxycycline selected for bacteria that carry these antibiotic efflux-type genes, which therefore is reflected in the total abundance of genes conferring resistance to tetracyclines, penicillins and cephalosporins. Cross resistance has been reported, with ceftriaxone use associated with VRE bloodstream infection.[202] The authors suggested that this may have been due to the lack of activity of ceftriaxone against *Enterococcus* spp., leading to a shift in the gut microbiota that allowed expansion of VRE and eventually infection.[202] It is suspected that a similar phenomenon occurred in the context of the current study, with doxycycline use selecting for *E. coli* and other resistant organisms that also carry genes encoding β-lactamases and efflux pumps. The work described in Chapter 6 aimed to confirm these assumptions through an in-depth analysis of the impact of antibiotics on the gut microbiome.

6. MICROBIOME DEPLETION FOLLOWING EXTENSIVE ANTIBIOTIC USE IN AGED-CARE RESIDENTS

6.1. CHAPTER SUMMARY

The analyses presented in Chapter 5 demonstrated that antibiotic exposure patterns are associated with significant resistome features. Based on the recognition that disruption to the microbiome creates a risk of AMR acquisition and increase in abundance,[64, 66, 67, 203] the antibiotic-mediated changes in the microbiome of aged-care residents was further investigated. The analysis again utilised a metagenomic-sequencing approach with the same measures of antibiotic use examined for their influence on the microbiome composition, specifically the presence and abundance of pathogens found in the gut. This revealed that all measures of antibiotic use significantly impacted species richness and Faith's phylogenetic diversity. In particular, a higher number of exposures, a higher number of unique antibiotic classes, a higher number of total days exposed to antibiotics and a lower number of days since the most recent antibiotic exposure was significantly associated with a lower richness and Faith's phylogenetic diversity. Beta diversity was also significantly impacted by all measures of antibiotic use. Use of any penicillin and doxycycline had a significant impact on species richness. Notably, no measures of antibiotic use significantly impacted the total abundance of Proteobacteria pathogens. The findings presented in this chapter supports the hypothesis that changes in the resistome would be reflected in the microbiome of GRACE participants and highlights the importance of maintaining a diverse commensal microbiological population in the gut.

6.2. INTRODUCTION TO THE CHAPTER

Antibiotic use has been repeatedly shown to significantly impact the commensal microbiome and a disruption of this microbiome. Elderly people, specifically those in residential aged-care facilities, have a high risk of complications due to repeated antibiotic us as discussed in Chapter 5.[2, 4, 76, 96] Importantly, research has shown that people in residential aged-care have a lower microbial diversity compared to those who live at home, and cannot recover as quickly from perturbations to their microbiome.[94, 198] Given that a robust commensal microbiome has been shown to protect against the acquisition and expansion of multidrug resistant organisms, a lack of this protective microbial community is likely to have detrimental effects.[66, 67] Previously, antibiotic use (characterised as number of exposure events, days since most recent antibiotic, total days exposed to antibiotics, number of unique antibiotic classes of antibiotics, and type of antibiotic given) was demonstrated to significantly impacted the gut resistome of GRACE participants. Specifically, these characteristics were associated

with significantly higher odds of a high total ARG abundance, which reflects the selection for resistant genotypes. Theorising that these changes were mediated by changes in the microbiome, the next step was to investigate how these same five characteristics impacted the microbiome and the abundance of potential pathogens. The aim of the work described in this chapter was to investigate the impact of the five selected antibiotic-use characteristics on: (1) alpha diversity, (2) beta diversity, and (3) the presence and abundance of potential pathogens.

6.3. STATISTICAL ANALYSIS OF GRACE MICROBIOME

All statistical analysis was performed in SAS and microbiome profiles were generated from metagenomic sequencing data. Normal distribution of Shannon-Wiener diversity, Pielou's evenness, species richness and Faith's phylogenetic diversity was tested using the SAS procedure PROC UNIVARIATE. Microbiome characterisation was performed on metaphlan data using alpha diversity and beta-diversity. Alpha diversity metrics were generated in R using the vegan[204] and phyloseq[205] packages and was measured using the following: i) Pielou's evenness, ii) taxa richness, iii) Shannon-Wiener diversity and iv) Faith's phylogenetic diversity (PD). Beta diversity was determined using a weighted UniFrac distance matrix.[206] Proteobacteria pathogen abundance was converted to ordinal categories by assigning rank 0 to less than the median and ranking remaining 1-3. Kruskal-Wallis was used for analysis of alpha diversity metrics against antibiotic exposures. Permutational analysis of variance (PERMANOVA) on a weighted UniFrac distance matrix from species-level relative abundance data was used to assess a difference between microbiome composition. Ordinal logistic regression was used for statistical analysis of Proteobacteria pathogen abundance. Multiple comparisons were adjusted for using the Benjamini-Hochberg method with an FDR of 5%. An additional logistic regression model was performed which adjusted for confounding variables. These variables included age, sex, site of residence, days living in current residence and the most common Rx-Risk comorbidities in the GRACE cohort: depression, gastro-oesophageal reflux disease, hypertension and pain.[197] Statistical significance was determined when $p < 0.05$. Data were visualised using R (v4.1.2) package "ggplot2" (v3.3.5). Mean and SD are reported as summary statistics.

6.4. NUMBER OF ANTIBIOTIC EXPOSURES INFLUENCES THE RICHNESS OF THE MICROBIOME IN AGED-CARE RESIDENTS

Four measures of alpha diversity (within-sample diversity) were assessed for their response to the number of antibiotic exposure events during the 12 months prior to stool sample collection (Figure 6.1). Number of exposure events was not significantly associated with

Shannon-Wiener diversity (p = 0.33) or Pielou's evenness (p = 0.92). Species richness (p < 0.001) and Faith's PD (p < 0.001) were significantly associated with the number of exposure events. Those who had 7 or greater exposures had significantly lower richness and Faith's PD (richness: 85 ± 21, Faith's PD: 12 ± 2.3) when compared to those who had no exposures (richness: 104 ± 16, Faith's PD: 14 ± 1.9), 1 exposure (richness: 101 ± 17, Faith's PD: 14 ± 2.0), and between 2 and 6 exposures (richness: 99 ± 19, Faith's PD: 13 ± 2.2). This analysis was adjusted for age, days in care, sex, site of residence, and the most common comorbidities, depression, gastro-oesophageal reflux disease, hypertension and pain. Adjustment did not change the statistical significance of results.

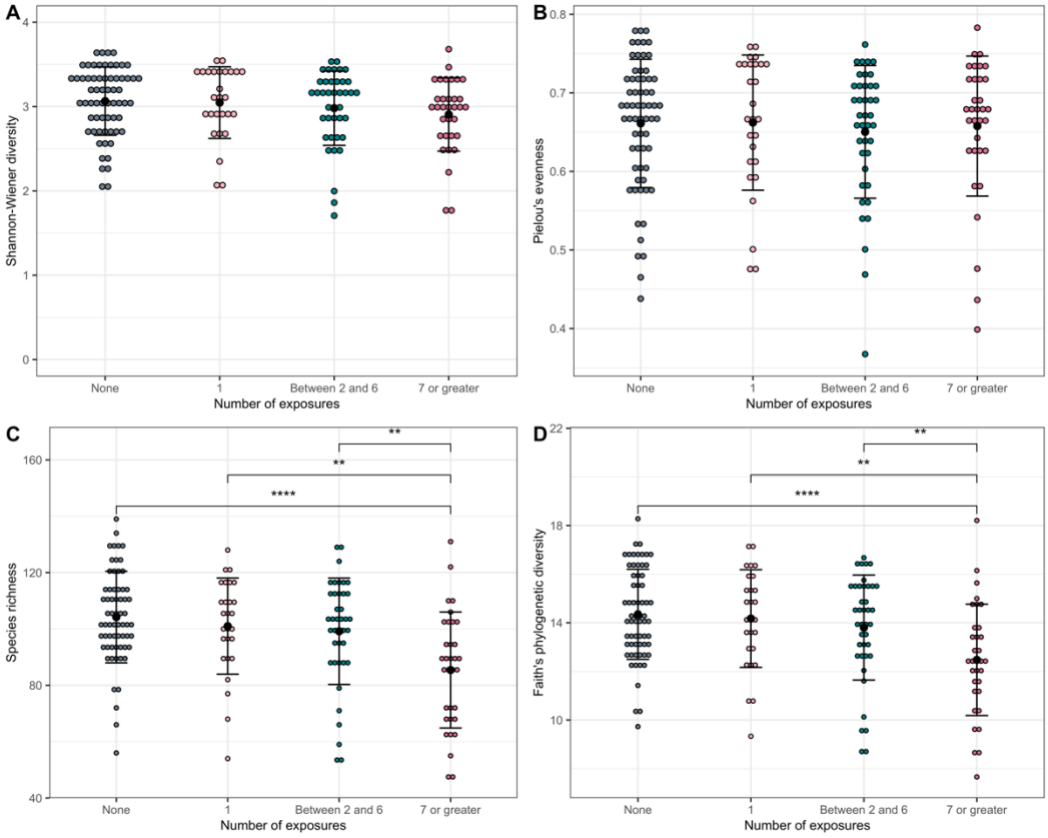

Figure 6.1 Number of antibiotic exposure events compared to **A)** Shannon-Wiener diversity, **B)** Pielou's evenness, **C)** species richness and **D)** Faith's phylogenetic diversity. Each dot represents one individual. Black dot and error bars represent mean ± SD. ** p < 0.01, **** p < 0.0001.

Number of exposure events was also assessed for its impact on beta diversity as measured by weighted UniFrac distance (Figure 6.2). Microbiome composition was significantly different between those who had no exposure and 7 or greater exposures (p < 0.001) and between 2 and 6 exposures and 7 or greater exposures (p = 0.03).

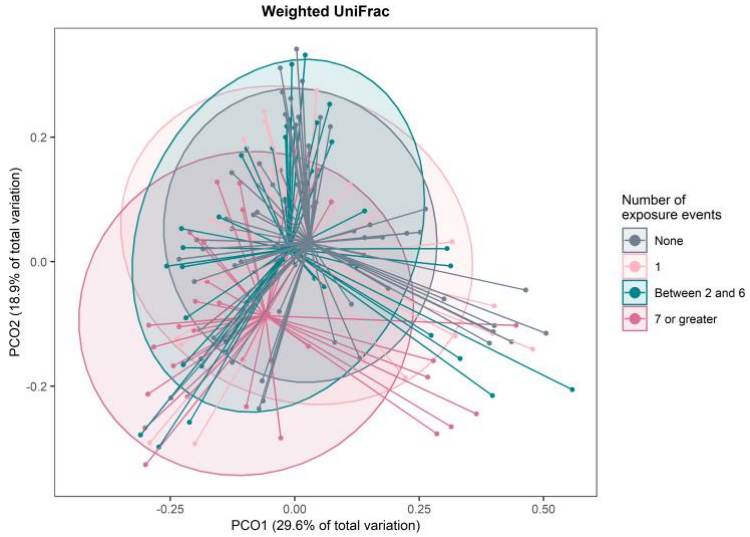

Figure 6.2 Number of antibiotic exposure events and beta diversity measured by weighted Unifrac distance and displayed on a PCO plot. Each dot represents an individual. Intergroup differences in weighted UniFrac distance determined with PERMANOVA. Distance to centroid (PERMDISP) was not significantly different between groups ($p > 0.05$).

6.5. RECENT ANTIBIOTIC USE SIGNIFICANTLY DEPLETES SPECIES RICHNESS

The next step was to investigate the time in days since most recent antibiotic use for its effect on the same four measures of alpha diversity. As previously, there was no significant impact on Shannon-Wiener diversity ($p = 0.42$) or Pielou's evenness ($p = 0.60$) (Figure 6.3). Species richness ($p < 0.001$) and Faith's PD ($p = 0.001$) were significantly different between categories of days since most recent antibiotic exposure. Those who had no exposure had significantly higher species richness and Faith's PD (richness: 104 ± 16, Faith's PD: 14 ± 1.9) compared to those who had been exposed to antibiotics less than 30 days since sample collection (richness: 89 ± 22, Faith's PD: 12 ± 2.4) and those who had been exposed between 30 and 100 days since sample collection (richness: 94 ± 18, Faith's PD: 13 ± 2.0). No change to significance was observed after adjusting for covariates.

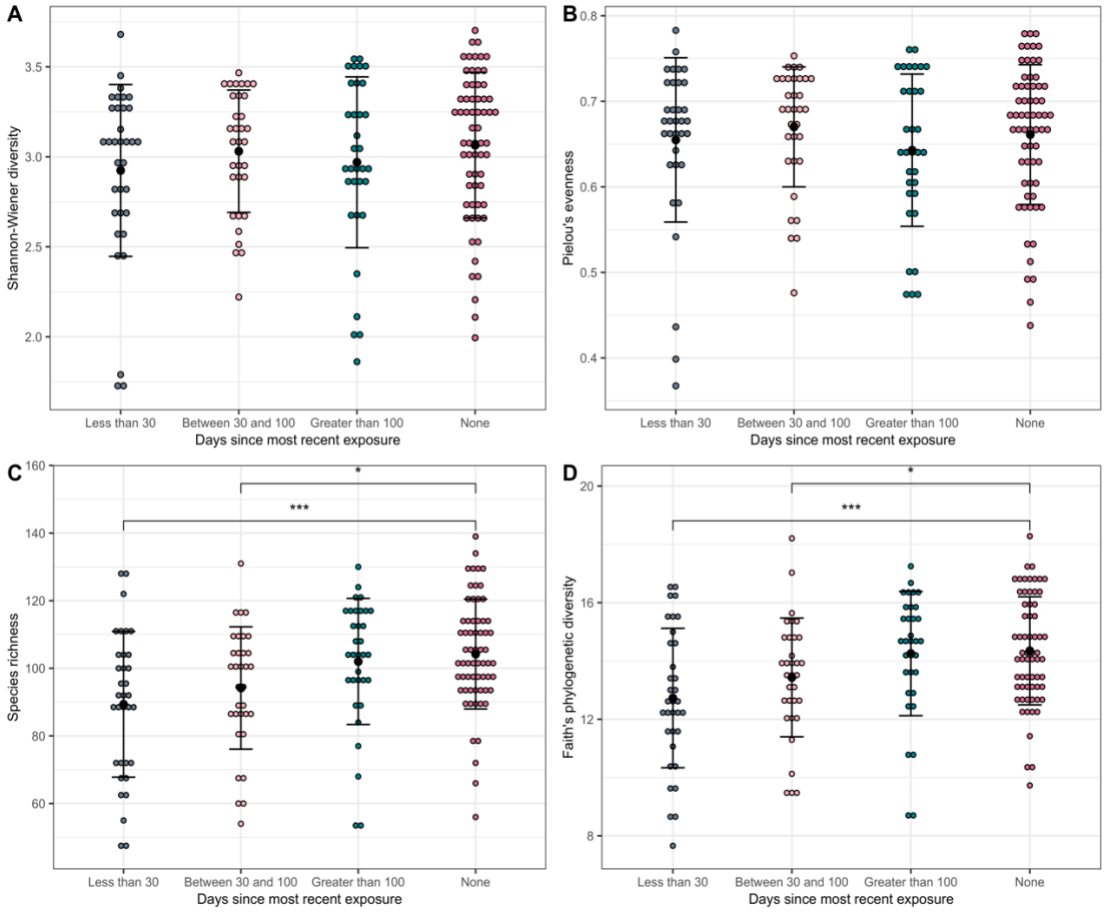

Figure 6.3 Days since most recent antibiotic exposure compared to **A)** Shannon-Wiener diversity, **B)** Pielou's evenness, **C)** species richness and **D)** Faith's phylogenetic diversity. Each dot represents one individual. Black dot and error bars represent mean ± SD. * $p < 0.05$, *** $p < 0.001$.

To determine whether microbiome composition clustered by the most recent antibiotic use interval, beta diversity (as measured by weighted UniFrac distance) was compared between antibiotic use groups (Figure 6.4). Microbiome composition was significantly different between those who had no exposure and those who were exposed to antibiotics less than 30 days prior to sample collection ($p = 0.002$) and those who were exposed between 30 and 100 days prior to sample collection ($p = 0.05$[7]).

[7] Actual p value is less than 0.05 ($p = 0.0478$).

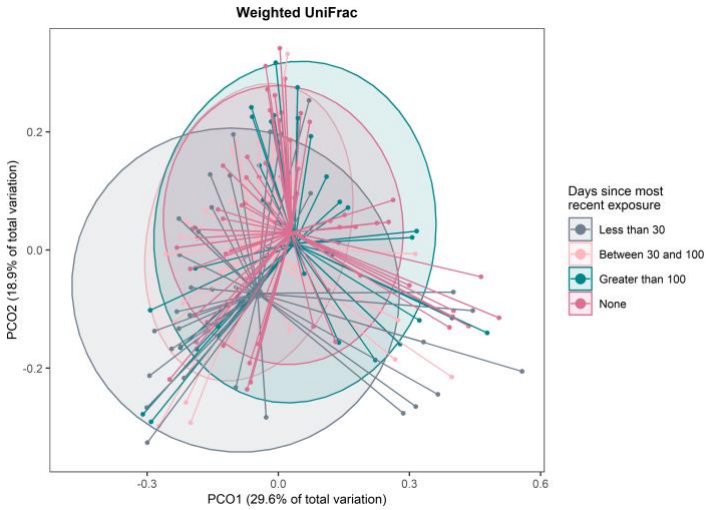

Figure 6.4 Days since most recent antibiotic exposure and beta diversity measured by weighted Unifrac distance and displayed on a PCO plot. Each dot represents an individual. Intergroup differences in weighted UniFrac distance determined with PERMANOVA. Distance to centroid (PERMDISP) was not significantly different between groups. ($p > 0.05$).

6.6. TOTAL TIME EXPOSED TO ANTIBIOTICS IS ASSOCIATED WITH STOOL MICROBIOME COMPOSITION

When assessing the effect of estimated total number of days exposed to antibiotics on alpha diversity, no significant impact was found for Shannon-Wiener diversity ($p = 0.15$) or Pielou's evenness ($p = 0.46$) (Figure 6.5). Species richness ($p < 0.001$) and Faith's PD ($p < 0.001$) were significantly different between categories of total exposed days. Those with greater than 33 days of exposure had significantly lower richness and Faith's PD (richness: 85 ± 23, Faith's PD: 12 ± 2.5) compared to those with no exposure (richness: 104 ± 16 , Faith's PD: 14 ± 1.9), 11 days or less of exposure (richness: 101 ± 17, Faith's PD: 14 ± 1.9) and between 11 and 33 days of exposure (richness: 99 ± 17 , Faith's PD: 14 ± 2.1). No change to significance was observed after adjusting for covariates.

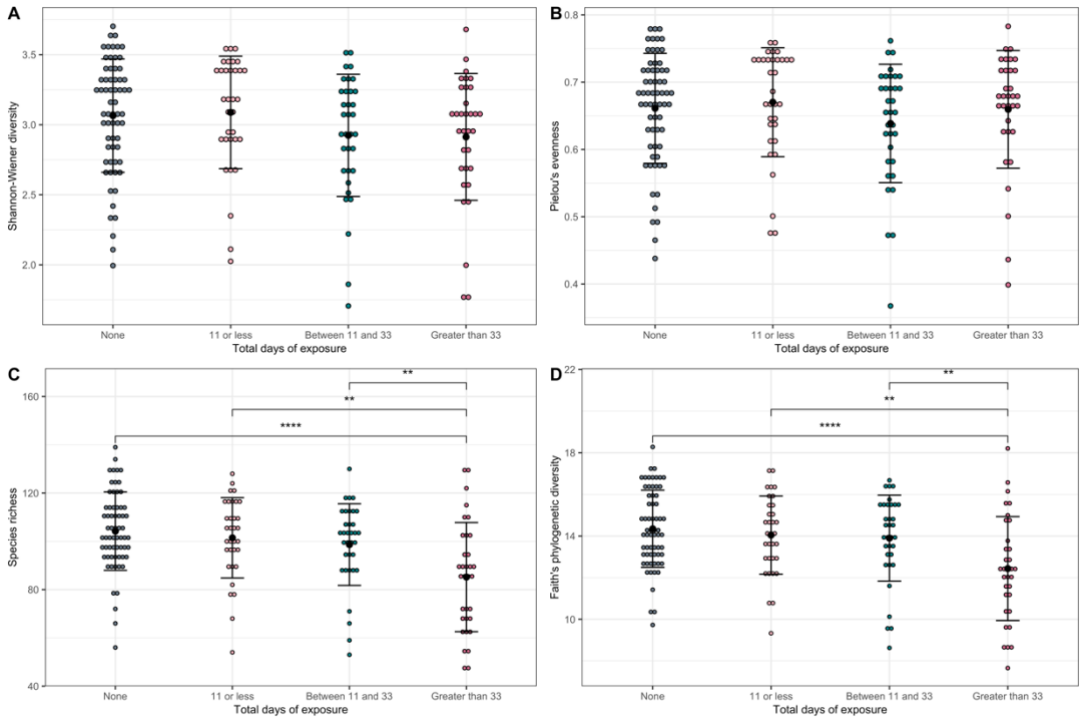

Figure 6.5 Total days of antibiotic exposure compared to **A)** Shannon-Wiener diversity, **B)** Pielou's evenness, **C)** species richness and **D)** Faith's phylogenetic diversity. Each dot represents one individual. Black dot and error bars represent mean ± SD. ** $p < 0.01$, **** $p < 0.0001$.

Beta diversity was also significantly different for categories of total days of use. Microbiome composition measured by weighted UniFrac distance was significant different between those who had greater than 33 days of exposure and those who had no exposure ($p < 0.001$), 11 days or less of exposure ($p = 0.04$) and between 11 and 33 days of exposure ($p = 0.002$).

6.7. HIGHER NUMBER OF UNIQUE ANTIBIOTIC CLASSES RECEIVED CORRESPONDS TO LOW MICROBIOME RICHNESS

Number of unique antibiotic classes was also assessed for its impact on each of the four measures for alpha diversity (Figure 6.6). Neither Shannon-Wiener diversity (p = 0.12) or Pielou's evenness (p = 0.57) were significantly impacted by the number of unique antibiotic classes received during the 12 months prior to stool sample collection. Species richness (p = 0.002) and Faith's PD (p = 0.007) were significantly impacted by the number of unique antibiotic classes exposed to. Species richness and Faith's PD were significantly lower in those who had been exposed to 3 or greater classes of antibiotics (richness: 89 ± 24, Faith's PD: 13 ± 2.7) compared to those with no exposure (richness: 104 ± 16, Faith's PD: 14 ± 1.9) No change to significance was observed after adjusting for covariates.

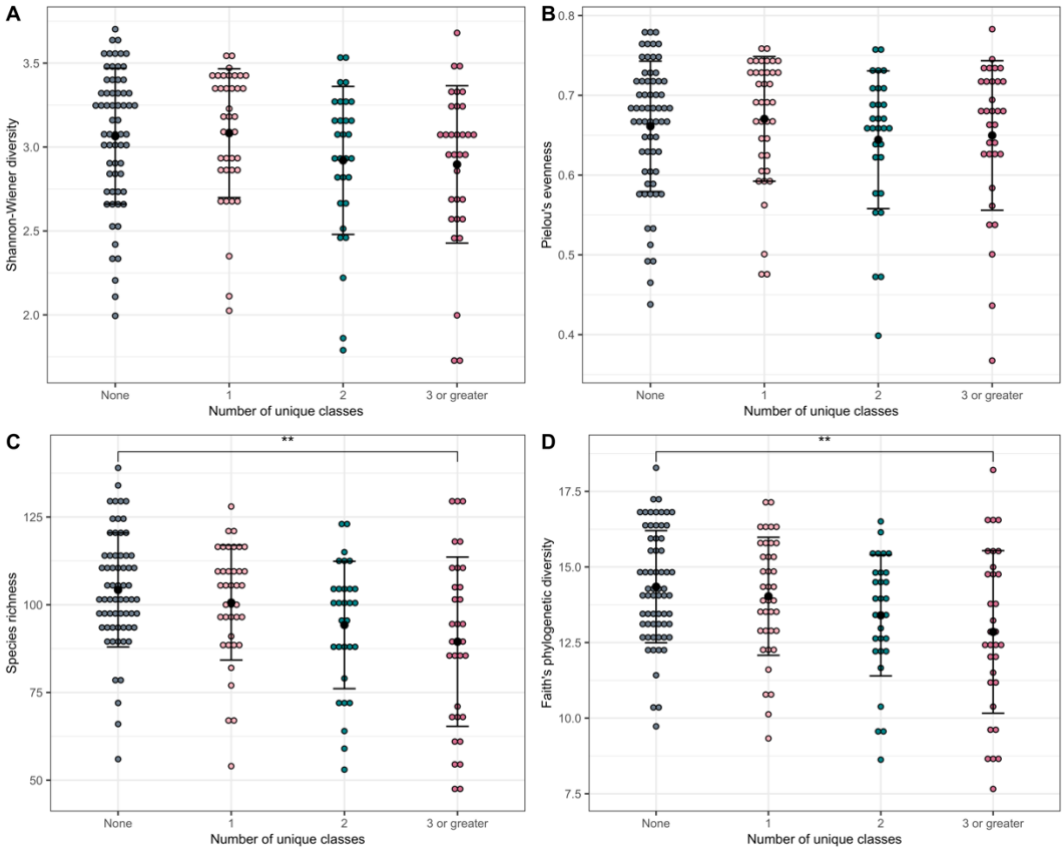

Figure 6.6 Number of unique antibiotic classes compared to **A)** Shannon-Wiener diversity, **B)** Pielou's evenness, **C)** species richness and **D)** Faith's phylogenetic diversity. Each dot represents one individual. Black dot and error bars represent mean ± SD. ** p < 0.01, **** p < 0.0001.

Number of unique antibiotic classes was also assessed for an association with beta diversity. Microbiome composition was significantly different between those who had been exposed to 3 or greater classes of antibiotics and those with no exposure (0.002) and exposure to 2 classes of antibiotics (p = 0.01).

6.8. TYPE OF ANTIBIOTIC GIVEN IS WEAKLY ASSOCIATED WITH MICROBIOME COMPOSITION

The four most frequently used antibiotics were assessed for their impact on each estimate of alpha diversity (Figure 6.7). Exposure to any diaminopyrimidine at least once in the 12 months prior to sample collection was associated with significantly lower Faith's PD (13 ± 2.2) compared to no use (14 ± 2.1, p = 0.04). Exposure to any penam antibiotic was associated with significantly lower species richness (93 ± 22, p = 0.005) and Faith's PD (13 ± 2.5, p = 0.01) compared to those with no exposure to a penam (richness: 101 ± 17, Faith's PD: 14 ± 1.9). Exposure to any tetracycline was associated with significantly lower species richness (91 ± 16) compared to those with no exposure to a tetracycline (100 ± 19, p = 0.04). No significant impact on Shannon-Wiener diversity or Pielou's evenness was found for any antibiotic exposure.

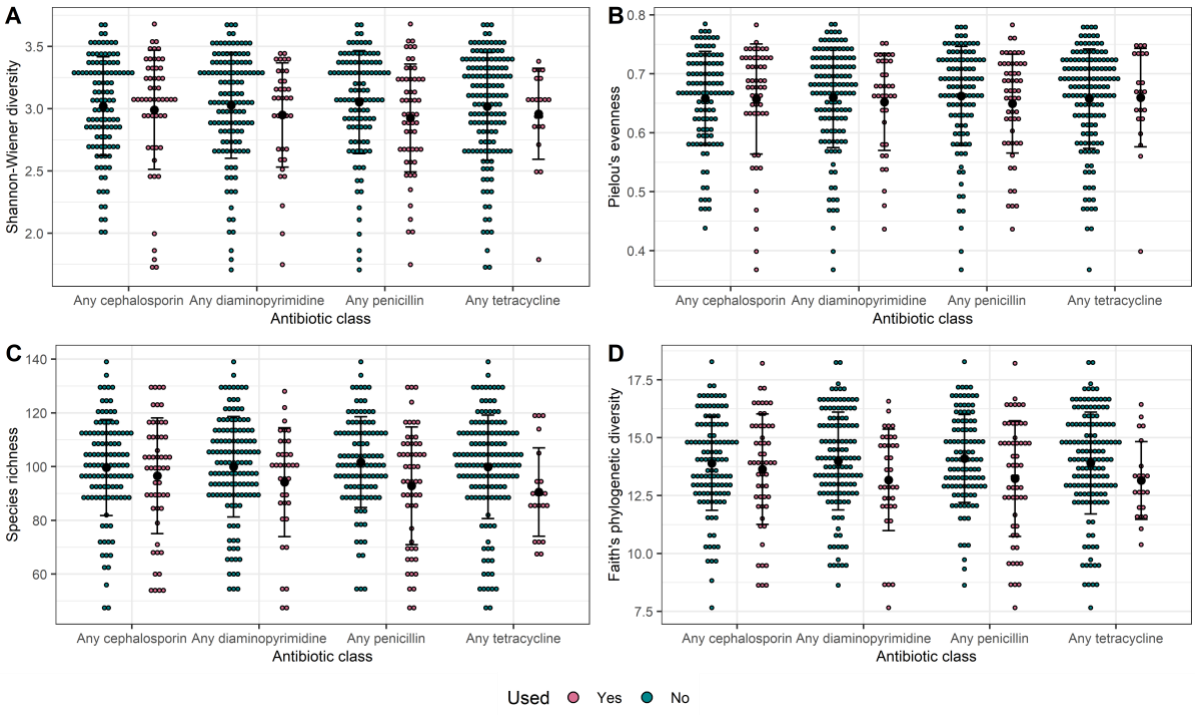

Figure 6.7 A) Shannon-Wiener diversity, **B)** Pielou's evenness, **C)** species richness, and **D)** Faith's phylogenetic diversity with the use of trimethoprim, doxycycline, any penicillin and any cephalosporin in the 12 months prior to sample collection. Each dot represents one individual. Black dot and error bars represent mean ± SD

Beta diversity as measured by weighted UniFrac distance was significantly different between those who had been exposed to any penam in the 12 months prior to enrolment compared to those who had not been exposed to a penam (p = 0.01).

6.9. ANTIBIOTIC USE AND PATHOGEN ABUNDANCE

In clinical practice, gut pathogens from the Proteobacteria phylum pose the greatest risk to the health of individuals. These pathogens (n = 32) are listed in Table 6.1 below.

Table 6.1 Pathogens from the Proteobacteria phylum summed for assessment of total abundance against antibiotic exposure categories.

Acinetobacter johnsonii	*Klebsiella aerogenes*
Pseudomonas aeruginosa group	*Klebsiella michiganensis*
Stenotrophomonas acidaminiphila	*Klebsiella oxytoca*
Citrobacter amalonaticus	*Klebsiella pneumoniae*
Citrobacter braakii	*Klebsiella quasipneumoniae*
Citrobacter europaeus	*Klebsiella variicola*
Citrobacter farmeri	*Kluyvera ascorbata*
Citrobacter freundii	*Raoultella planticola*
Citrobacter koseri	*Raoultella terrigena*
Citrobacter pasteurii	*Hafnia alvei*
Citrobacter werkmanii	*Morganella morganii*
Citrobacter youngae	*Proteus mirabilis*
Enterobacter cloacae complex	*Aeromonas caviae*
Enterobacter mori	*Aeromonas media*
Escherichia coli	*Aeromonas veronii*
Escherichia marmotae	*Achromobacter insolitus*

To evaluate the risk of antibiotic exposure, pathogens from the Proteobacteria phylum (see abundance of listed pathogens in Figure 6.8 below) were summed for the total relative abundance per person (med = 0.28, range = 0-44) and this was assessed against number of exposure events, days since most recent antibiotic exposure, total days of exposure and number of unique classes exposed to. Proteobacteria pathogen abundance was not significantly impacted by any measure of antibiotic exposure.

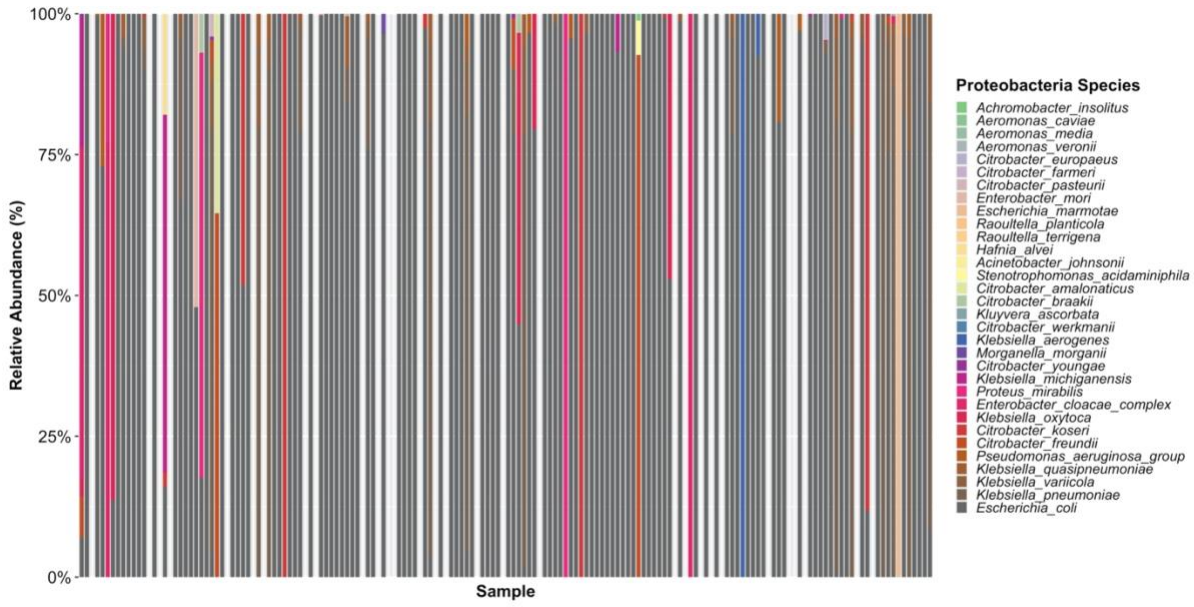

Figure 6.8 Taxa bar plot for 164 GRACE stool samples depicting the relative abundance of 32 Proteobacteria species known to be pathogenic in clinical practice.

6.10. INTERPRETATION OF FINDINGS

All five measures of antibiotic exposure significantly impacted the microbiome composition as measured by species richness and Faith's phylogenetic diversity. A higher number of exposure events was significantly associated with lower species richness and lower Faith's phylogenetic diversity. Consistent with what was observed in the resistome, both those who had been administered antibiotics within 30 days of sample collection and those who had received antibiotics between 30 and 100 days of sample collection had a significant impact on the microbiome. In younger adults, perturbations to the microbiome are short-lived, recovering within a few weeks.[103, 185, 207] In older people, the microbiome is already depleted due to lifestyle factors and physiological changes and appear to be more significantly impacted by antibiotic treatment.[94, 106, 198] It is therefore unsurprising that the effects of antibiotics were still observed in those who had received antibiotics within 30 to 100 days of sample collection, as well as those who had been exposed to one within 30 days. It is this effect that likely explains that 33 days or greater of equivalent antibiotic use was significantly associated with lower richness and Faith's phylogenetic diversity when compared to no antibiotic use. Finally, number of unique antibiotic classes was significantly associated with species richness, Faith's phylogenetic diversity and beta diversity. Unsurprisingly, the greatest difference was between those who had been exposed to 3 or greater classes of antibiotics and those who had not been exposed. This is most likely due to the increase in spectrum of activity covered by the variety of antibiotics used in the cohort, depleting more taxa.

Use of any diaminopyrimidine-class, penam-class, or tetracycline-class antibiotic was significantly associated with a change in microbiome composition. Use of any penam-class antibiotic was significantly associated with lower richness, Faith's phylogenetic diversity and a difference in beta-diversity as measured by weighted UniFrac distance. In GRACE, the two most commonly used penam-class antibiotics were amoxicillin and amoxicillin-clavulanic acid. Although the effect of amoxicillin on the gut microbiome is extremely variable across the literature, many researchers report an increase in Enterobacteriaceae.[135, 185, 208, 209] In studies that investigated the impact of amoxicillin-clavulanic acid, Enterobacteriaceae have also been reported to increase in abundance, alongside a decrease in commensal organisms such as *Bifidobacterium*, although this also varies.[210, 211] Given the extreme variability among studies investigating the effects of amoxicillin and amoxicillin-clavulanic acid, it is difficult to determine whether the findings in the GRACE study are consistent with those of previous reports. However, no significant change in the abundance of Proteobacteria pathogens (which includes the family Enterobacteriaceae) was identified with the use of penam-class antibiotics in this study. Combined with the findings presented in Chapter 5, this analysis concludes that antibiotic use patterns in residents of aged-care facilities significantly impact both the resistome and the microbiome.

7. DISCUSSION AND FUTURE DIRECTIONS

7.1. PROJECT RATIONALE

Antibiotics are life-saving medications, but in recent times overuse and inappropriate use has led to a dramatic acceleration in antibiotic resistance. This is a serious risk to the efficacy of antibiotic treatment and treatment failure has already been associated with an increase in mortality and morbidity.[14-16] Currently, information on antibiotic resistance is captured primarily at the infection-stage, with several reports investigating rates of multidrug-resistant pathogens such as ESBLs, CPE and VRE.[2, 5, 8] This is essential for targeted stewardship policies to be placed in those communities that need them most, including hospitals and aged-care facilities. However, a new theory has emerged that involves the commensal microbiome as a protective barrier against the acquisition of new resistant organisms from the external environment, as well as the proliferation of those already present in an individual's microbiome.[212] Developments in sequencing technology has provided an opportunity to investigate the microbial response to exposures, such as antibiotic use, beyond but in conjunction with that of culture-based methods.[108, 111] Metagenomic sequencing is one such technique that sequences all DNA in a sample, and can be used to thoroughly examine the microbiome and collection of antibiotic resistance genes that are present, known as the resistome. This technology had not been used widely in clinical microbiome and resistome research until recently.[90, 117, 129, 133]

This project involved applying a metagenomic sequencing technique to investigate the microbiome and resistome in two high-risk, antibiotic-exposed, clinical cohorts: the first from an RCT in children, and the other from an observational study in permanent residents of aged-care facilities. These age groups were chosen because various factors put them at a high risk of infection and complication of treatment failure. In children, interference with the developing microbiome has been shown to lead to significant adverse events including sepsis and long-term allergy and illness.[84, 85, 172-174] In older people, physiological factors such as immunosenescence, the gradual weakening of the immune system with age, have been associated with increased risk of infection.[77, 97] Permanent residents of aged-care facilities are thought to be at an even higher risk of this due to advanced age, frequent antibiotic use and increased frailty.[91, 92] This section briefly recaps the findings presented in each chapter and discusses their importance, strengths and limitations.

7.2. DISCUSSION OF FINDINGS

7.2.1. THE CHOICE STUDY

The component of the project presented in Chapter 3 investigated the impact of two antibiotic treatment regimens on the gut resistome as an extension of the CHOICE RCT. This trial compared the efficacy and safety of IV flucloxacillin in hospital compared to IV ceftriaxone administered at home in children who presented to the emergency department with severe cellulitis.[89] It also included a small culture-based component investigating presence of ESBLs in the gut. In this substudy, a significantly higher diversity of antibiotic resistance genes in the narrow-spectrum antibiotic group compared to the broad-spectrum antibiotic group was observed. This is likely because the narrow-spectrum of flucloxacillin was less deleterious in the microbiome, which was reflected in ARG richness, a phenomenon reported previously.[183] Composition of the resistome also clustered by randomised treatment group when measured by Sorenson distance, likely due to the same effect. No significant association was found between carriage of an ESBL and microbiome features in the sample collected prior to ESBL acquisition. In a previous study, the authors showed that a disruption to the microbiome increased the selection and expansion of pathogens already present in the gut at low levels but there were too few available samples to properly investigate this in the CHOICE study.[66]

Chapter 4 presents an analysis of the differences in the microbiome both between groups and within groups longitudinally. Few intergroup differences were observed across the time points, with most changes occurring within groups over the study period. Microbiome shifts within the ceftriaxone group were most prominent, with a significant reduction in alpha diversity in the treated time points (T1 and T2) compared to the long-term follow up point (T3). This differs from a single earlier report that demonstrated an impact of broad-spectrum antibiotics, including third generation cephalosporins remains even up to 12 months post treatment.[90] The factors contributing to the differences in findings between that study and the current study are more fully discussed in Chapter 4 but these are mostly likely to be due to the age of the cohorts studied, with Reyman and colleagues studying neonates versus children of at least 6 months of age in the CHOICE study.

Investigating the impact of antibiotic use on the microbiome and resistome in a paediatric clinical cohort had a number of important benefits. Firstly, study antibiotics were randomised, thereby removing the confounding effect of variation in treatment exposures. This study population provides a real-world representation of the clinical use of the antibiotics investigated. These factors, in addition to the prospective collection of data and samples, strongly supports the unbiased and clinically relevant nature of the findings. By employing a

metagenomic strategy, the current study was able to define the impact of antibiotics, not only on target taxa, but on microbiota and resistome characteristics as a whole. This is important, given that assessments of the impact of antibiotics typically focus on changes in target pathogens and their development of resistance, and fail to consider the impact of antibiotics on non-target microbial populations, despite their potential clinical significance.

The CHOICE study also had limitations that warrant consideration. First, around half of the study participants had some antibiotic exposure prior to enrolment. While this was accounted for in the analysis, it is likely to have contributed to greater variance in microbiome and resistome characteristics, and potentially reduced the ability to identify the effects of the study antibiotics. Second, the small number of children who acquired ESBL, while reassuring in relation to outpatient IV ceftriaxone use, prompts caution in interpretation of the findings from this sub-analysis, with a larger study required for this. Third, the collection of an initial faecal sample occurred after the initiation of IV antibiotics, as it would be unacceptable to delay the start of therapy. While appropriate randomisation was performed, and there were no differences in treatment group characteristics, analysis of a baseline faecal sample was not possible.

7.2.2. THE GRACE STUDY

While a cohort of children experienced a mild impact on their resistome and a transient impact on the microbiome following controlled, short-term antibiotic treatment, differences in the physiology and lifestyle of older people were likely to have a more pronounced effect. As detailed in Chapter 5, when assessing the impact of (1) number of antibiotic exposures, (2) most recent antibiotic use, (3) total days of antibiotic use, (4) number of unique antibiotic classes, and (5) type of antibiotic given, all these measures had a significant association with changes in the resistome. Specifically, all were associated with increased total abundance of resistance genes present in the sample, indicating a selection for resistant bacteria was likely to have been the underlying cause. This was likely due to the high frequency of antibiotic use in this population.[4] In this case, participants would have most probably been carrying resistant organisms asymptomatically in their gut microbiome, their expansion triggered by further antibiotic use. Doxycycline use was also associated with a higher abundance of tetracycline-resistance genes, as well as cephalosporin-resistance genes and penicillin-resistance genes. As discussed in detail in Chapter 3, this is likely to be due to the multidrug spectrum of resistance conferred by some tetracycline-resistance genes, in particular multidrug efflux pumps that can expel tetracyclines, cephalosporins and penicillins from the bacterial cell.[199] It was then hypothesised that these shifts in the resistome would also be observed in the microbiome.

Indeed, antibiotic use as measured by the five characteristics listed above was significantly associated with reduced species richness and Faith's phylogenetic diversity, as well as shifting the microbiome composition as measured by weighted UniFrac distance. This was likely to reflect a depletion of non-resistant species, therefore providing availability for resistant organisms to expand, as observed in the resistome analysis. Additionally, the use of any penicillin in the 12 months prior to sample collection was associated with a significant reduction in species richness and Faith's phylogenetic diversity . While the reports of penicillin use on the microbiome vary significantly, the reduction in richness observed in this study was likely driven by those who used amoxicillin and clavulanic acid, which also has a broad spectrum of activity.[210, 211]

GRACE, being a large-scale, observational study, had a number of important strengths. Firstly, due to a highly-competent and dedicated research nurse who spent significant time on recruiting participants to the study, the recruitment rate was 75%, providing an excellent sample size despite having to cease the study early due to the COVID-19 pandemic. Secondly, the GRACE study captured a large amount of data beyond that of antibiotic use history. This allowed robust analysis and the inclusion of models to adjust for any potential confounding effects. Thirdly, antibiotic use records from the PBS were validated using resident medication charts, considered the gold-standard for determining medication usage in aged-care facilities. Although PBS data has been regularly used and validated by collaborators in the past, it was reassuring to see this reflected within the GRACE study data set.[92, 213, 214]

The GRACE study also came with a few clear limitations. While the high recruitment rate was a strength of the study, early cessation of recruitment was a major limitation. As a result of this, some data could not be recovered from participating aged-care facilities and the study could not reach its target recruitment number. GRACE participants were also all recruited from not-for-profit aged-care facilities in metropolitan Adelaide. It is possible that this led to an unavoidable bias in the results of the study, and that inclusion of regional/remote facilities and/or government run/for-profit facilities may have been a better representation of aged care in Australia. In particular, the inclusion of only well-regarded, not-for-profit, organisations may have influenced the representation of the microbiome in aged-care residents due to factors such as better or more staffing, and better food quality. Finally, although the GRACE study did validate the PBS data against the medication chart data to a limited degree, this did not cover the full 12-month period of data capture. It is therefore possible that records of prescribed antibiotics in the PBS did not all correspond with usage of the medication by the intended resident.

7.3. PROJECT LIMITATIONS

In children, short-term, controlled antibiotic treatment had a limited impact on the resistome and a transient impact on the microbiome. In a cohort of aged-care residents this was not the case, and uncontrolled, repeated use of antibiotics was significantly associated with changes in both the resistome and the microbiome. Despite the differences in results between the cohorts, they shared some common limitations.

Firstly, neither study had a clinical outcome to quantify the clinical risk of antibiotic-mediated changes to the microbiome and resistome. Although CHOICE was a RCT, the primary outcomes were the efficacy and safety of the assigned IV treatment group, measured by initial treatment failure and adverse events.[89] Despite stool samples being collected at 3 months, long-term factors such as representation to hospital or reinfection within a set time post treatment were not recorded. In other studies, use of antibiotics in childhood has been shown to lead to adverse long-term health outcomes, such as diabetes, asthma and IBS.[172-174] In the short term, inhibition of the developing microbiome has been associated with risk of sepsis.[84, 85] Infection in particular, whether cellulitis or otherwise, would have been useful data to capture as it directly corresponds to the risk of antibiotic resistance and the risk of disrupting the commensal microbiome. For GRACE it was not as simple. Data on comorbidities, hospitalisations and other healthcare factors were available for analysis, however, the general decline in health of those in residential aged-care facilities made it nearly impossible to determine directionality between antibiotic use and illness. For example, it was not possible to confirm if antibiotic use directly related to later illness through modification of the microbiome and immune function, or if antibiotics were used to treat pre-existing illness. In actuality, it is likely to be some of both, but this is an ongoing challenge for research in older populations.

Secondly, neither study had a sufficient sample size to thoroughly investigate changes in pathogen abundance, nor to observe any potential overgrowth events if they occurred. Modification to the commensal microbiome, such as through antibiotic use, has been previously associated with the expansion of antibiotic resistant pathogens.[61, 66-68] However, these studies involved extremely high-risk participants such as those in the intensive care unit, as well as populations of people with known MDROs already present in the gut. It was anticipated that certain resistant pathogens may already be present, but the main aim was to investigate changes to the resistome as a whole in a group of individuals receiving their regular healthcare routines and behaving as they would normally. Particularly in the GRACE study, the variability in the composition of the microbiome was large, making it even more difficult to observe a specific change, such as an increase or decrease in pathogen

abundance. Nevertheless, a significant increase in the total ARG abundance in the resistome was observed, and even if this could not be attributed to a specific pathogen, it supports the hypothesis that the presence of resistance genes in organisms asymptomatically may be a subsequent risk to treatment efficacy.

Thirdly, this project was significantly impacted by frequent changes to the Flinders University high-performance computing (HPC) environment. Known as DeepThought, the Flinders University HPC is a relatively young system that was still undergoing frequent changes and optimisation at the time of project completion. After completing one component of the GRACE analysis and all of the CHOICE analysis, a substantive change in the operation of the HPC was made requiring it to be restarted and for data to be fully reprocessed. As mentioned in Chapter 2, this also required some changes to the programs that could be used in this new environment. One main aim of this project was to show how metagenomic sequencing data could be used in a clinical context to support medical decisions. Consequentially, a limitation to this is that resistome analysis is a relatively new technology and still requires more development to ensure it can be used in a streamlined and consistent manner needed and expected in medical practice.

Next, while the use of metagenomic sequencing in a clinical context is an emerging concept, the technology itself does have some limitations and alternative approaches are worth discussing. Culture-based approaches were not used in this thesis directly (although they were used in the CHOICE RCT). Culture-based microbiome analysis does have its benefits; it is relatively cheap to perform, can isolate microorganisms of clinical interest and can determine phenotypic characteristics such as antibiotic resistance profiles. The limitations of this method is that it is time-consuming to process and does not provide a snapshot of the microbiome and resistome as a whole within a clinical sample. Using a 16S rRNA gene sequencing approach does provide a picture of the microbiome at a moment in time relative to the research interest. It is low-cost compared to metagenomic sequencing and does not require significant computational resources to run. However, it is much lower resolution that metagenomic sequencing (only reliable up to the genus-level) and does not provide an output for investigating ARGs. Metagenomic sequencing provides a higher resolution than 16S rRNA gene sequencing (species-level) as well as allowing for analysis of antibiotic resistance analysis and functional analysis. However, the limitations of this approach are that it is more expensive to run and requires significantly more computational-resources to processs and analyse the output. In the context of this candidature, the use of metagenomic sequencing meant that I was unable to identify which bacteria a particular antibiotic resistance gene was from. In combination with the fact that this project did not use clinical isolates, a decrease in the accuracy and clinical relevance of the analysis shown may have occurred. One final

alternative would have been to perform whole genome sequencing on isolates grown from the clinical samples. However, while this approach does allow for assigning antibiotic resistance genes to bacteria present in the clinical samples, it is expensive and time-consuming to run. It also did not align with the research interests of this work, specifically the goal was to show how a robust, simple but informative microbiome/resistome analysis could be applied to clinical research and the benefits of doing so. The use of WGS is complex and does not provide an overall picture of the microbiome and resistome in a clinical sample.

Finally, the impact of COVID-19 had a subtle but significant impact on the progress of the two main components of this project. Given the collaborators on the CHOICE study were paediatricians and infectious disease specialists, their time was significantly taken up by the various waves of COVID-19 that occurred in Melbourne. In GRACE, the impact occurred earlier on the study, with significant delays on DNA extraction kits which prevented sequencing analysis from occurring until much later in this project. While these issues affected the length of this project, they did not impact the quality of analysis.

7.4. HOW THIS WORK CONTRIBUTES TO THE FIELD

As indicated in Section 7.3, a logical next step is to investigate the association between the shifts in the microbiome and resistome with some clinical outcomes. Within our research group this work is currently in progress, specifically within the GRACE study team. One of these projects involves investigating the gut microbiome composition as a contributor to recurrent UTIs. This was performed on a subgroup of the GRACE cohort that had available pathology data and therefore clinical evidence of a UTI or bacteriuria, as well as an available stool sample and antibiotic use data. As previously mentioned, the main limitation of a cohort of older people is assuming the directionality of the relationship between antibiotic use and illness. In this case, both directions will be considered and assessed using subgroups.

While including an infectious or clinical outcome to measure the effect of microbiome and resistome shifts is essential in the short term, this work has a much broader potential impact. As mentioned, one of the main goals of this project was to demonstrate the importance of including sequence analysis to support general medical decision-making. Until recently, sequencing analysis, particularly of the microbiome, has been a foreign concept to the medical community. Although whole genome sequencing is applied in some clinical contexts and in research contexts to assess the genetic-drivers of infectious outbreaks, metagenomic sequencing is not often included.[6-10] CHOICE was the perfect example of how metagenomic and microbiome data can be used to help inform medical decisions. Much like the Reyman and Leo studies, CHOICE applied a metagenomic sequencing-based approach

to investigate how a change in antibiotic treatment impacted the collection of resistance genes more broadly, in a cohort of people who were experiencing the relevant ailment at the time of the study.[90, 127] In the RCT, no significant impact on the efficacy or safety of either treatment was observed, but there was some evidence of ESBL acquisition in culture.[89] Subsequent analysis performed in this project demonstrated shifts in the microbiome within each group that ultimately resolved but may have allowed for the expansion of resistant organisms during the window of disruption. This type of data is paramount in considering both the immediate and later-life impact of antibiotic treatment, longevity and resistance in high-risk individuals. One major hurdle in the integration of sequencing/microbiome data into medical decision-making is the complexity of not only the technology itself, but also the interpretation of the data that emerges. This project has made an excellent start and includes some suggestions for how this data might be displayed to be impactful to the medical community, but further work and discussion between those studying the microbiome and those in medical practice is essential.

Overall, the main goal of this project was to advise how the efficacy of current antibiotics could and should be preserved and regulated. In older populations, such as those in aged-care, antibiotic stewardship has a long way to go.[4] Previous work by collaborators of our research group showed a significant increase in antibiotic use over time in the population of aged-care residents, which presents an unacceptable risk to future efficacy of treatment.[92, 214] GRACE in particular demonstrated a significant effect on the microbiome and resistome with antibiotic use as a whole, especially in those who had frequent and varied antibiotic treatment. The impact of doxycycline on the resistome was quite pronounced, impacting not only the resistance profile for its own antibiotic class, but also for other antibiotic classes. This is the first example of a metagenomic-sequencing-based approach to investigate changes in the resistome in permanent residents of aged-care facilities. Both major components of this project found that antibiotic use has consequences beyond the target infectious agent, and while there is no suggestion that antibiotics should not continue to be used, the evidence certainly reveals that overuse is having extensive, unintended consequences. Ultimately, this work described here is a guide as to how sequencing and microbiome analysis can be applied to medical decision-making and serves to support and educate others working to progress the wider application of metagenomic approaches.

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APPENDIX A: SAMPLE CODE FOR PROCESSING OF METAGENOMIC SEQUENCING DATA

Contents: Sample code of metagenomic sequencing data processing in the conda environment on Flinders HPC to generate microbiome and resistome output.

Metagenomics pipeline on DeepThought using conda environment: February 2022 (modified June 2022)

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##1 Transfer files to directory

#Files can now be transferred from the R drive to your directory on the HPC

e.g. /RDrive/CMPH-SAHMRI/USER/DIRECTORY

#Files can also be imported using the wget command if only a small number were sequenced

wget [insert link to zip file]

##2 (optional but encouraged) Match md5sum key with one generated on HPC to confirm correct file download

for i in `ls *.gz | cut -f 1 -d "." | sort -u`; do md5sum "$i".fastq.gz > /scratch/user/username/project/md5/"$i"_md5.txt; done

cat *_md5.txt > all_md5.txt

#Confirm this key matches the one provided by the institution who sequenced the samples

##3 Set up your conda environment (see 'conda_instructions' in R:\CMPH-SAHMRI\Protocols\Downstream processing\HPC information)

#Create a conda environment called SAHMRI_MHH (or whatever you would like to call it) to store all of your packages

module load Miniconda3/4.9.2

conda config --add channels defaults

conda config --add channels bioconda

conda config --add channels conda-forge

conda create --name=SAHMRI_MHH python=3.9

conda activate SAHMRI_MHH

#For up to metaphlan:

conda install fastqc

conda install multiqc

conda install trimmomatic

conda install bowtie

conda install metaphlan

#For resistome pipeline also add these:

conda install cd-hit

conda install idba

conda install bbmap

conda install prodigal

conda install emboss

conda install blast

conda install -c r r-essentials

conda install --channel conda-forge --channel bioconda --channel defaults rgi=5.2.1 #see instructions for installing this

#When asked ensure you type y to confirm

#On the headnode activate your environment using:

conda activate SAHMRI_MHH

#In a subscript activate your environment using:

source activate SAHMRI_MHH

#Deactivate your environment using:

conda deactivate

#Check your environments using:

conda env list

#For help, first activate your environment on the headnode, then use:

[progam] --help #e.g. fastqc --help

#Remeber to always add the line to load miniconda in subscripts that need a conda environment

#Scripts should look like this (noting that parameters can be different depending on the job):

#!/bin/bash

Job name

#SBATCH --job-name=[jobname]

Set email type for job

Accepted options: NONE, BEGIN, END, FAIL, ALL

#SBATCH --mail-type=NONE

email address for user

#SBATCH --mail-user=[FAN]@flinders.edu.au

Request nodes

#SBATCH --ntasks=1

#SBATCH --mem=50gb

#SBATCH --time=100:00:00

module load Miniconda3/4.9.2

Run the executable

source activate SAHMRI_MHH

[code]

conda deactivate

##4 Perform QC checks on raw files using fastqc (fastqc.sub)

mkdir fastqc

fastqc -o /scratch/user/FAN/project/raw_files/fastqc -f fastq -t 16 /scratch/user/FAN/project/raw_files/*.fastq.gz

##5 Combine QC outputs using multiqc (multiqc.sub)

cd fastqc

multiqc . -o multiqc_outdir

#What to look at?

#A Per base sequence quality- want to trim so that anything in the red section (below a phred score of 20) is removed

#B Tile quality- warm colours are bad, if there is lots of red you might want to lose that sample or get it re-sequenced

#C Per sequence quality score- above 20 acceptable

#D Per base sequence content- bases should even out and run parallel

#E Adapter content- tells you what to get rid of

##GC content and sequence duplication not important for metagen but important for WGS##

##6 Trim raw reads (trimmomatic_array.sub)

java -jar /home/FAN/.conda/envs/SAHMRI_MHH/share/trimmomatic-0.39-2/trimmomatic.jar PE -threads 4 -phred33 $r1 $r2 -baseout trimmed_fastq/$name.fastq.gz ILLUMINACLIP:adaptersPE.fa:3:30:10 SLIDINGWINDOW:4:20 MINLEN:50 HEADCROP:10

#ILLUMINACLIP removes adapter sequences

#HEADCROP removes a selected number of bases from the start of all sequences

#SLIDING WINDOW scans X number of bases and trims whenever the quality drops below Y (SLIDINGWINDOW:X:Y)

#MINLEN drops reads below a certain length from the cleaned output reads

##7 Repeat QC checks on trimmed reads (fastqc_trimmed.sub + mutliqc_trimmed.sub)

mkdir trimmed_fastq/fastqc_trimmed

fastqc -o /scratch/user/FAN/project/raw_files/trimmed_fastq/fastqc_trimmed -f fastq -t 16 /scratch/user/FAN/project/raw_files/trimmed_fastq/*.fastq.gz

cd /scratch/user/FAN/project/raw_files/trimmed_fastq/fastqc_trimmed

multiqc . -o multiqc_outdir

##8 Remove human reads from samples using bowtie (bowtie_human_array.sub)

#First move the human reference genome to your scratch folder (only do this once)

cp -R /RDrive/CMPH-GenomicsDB/human_reference_genome_grch38_for_bowtie /scratch/user/FAN

#Steps are as follows

#A Align paired reads to the human reference genome and keep those that DO NOT align

#B Rename files to be consistent with our naming conventions

#C Remove SAM files as they are not needed but take up a lot of space

#D Align unpaired forward reads to human reference genome and keep those that DO NOT align

#E As per D for unpaired reverse reads

#F As per C for all unpaired reads

bowtie2 -p 8 -x /scratch/user/FAN/human_reference_genome_grch38_for_bowtie/GRCh38_noalt_as -1 $r1 -2 $r2 --local --un-conc-gz $PWD/bowtie_human/"$name"_P_HC > $PWD/bowtie_human/$name.sam

mv $PWD/bowtie_human/"$name"_P_HC.1 $PWD/bowtie_human/"$name"_1P_HC.fq.gz

mv $PWD/bowtie_human/"$name"_P_HC.2 $PWD/bowtie_human/"$name"_2P_HC.fq.gz

rm $PWD/bowtie_human/$name.sam

bowtie2 -p 8 -x /scratch/user/FAN/human_reference_genome_grch38_for_bowtie/GRCh38_noalt_as -U $r3 --local --un-gz $PWD/bowtie_human/"$name"_1U_HC.fq.gz > $PWD/bowtie_human/"$name"_1U.sam

bowtie2 -p 8 -x /scratch/user/FAN/human_reference_genome_grch38_for_bowtie/GRCh38_noalt_as -U $r4 --local --un-gz $PWD/bowtie_human/"$name"_2U_HC.fq.gz > $PWD/bowtie_human/"$name"_2U.sam

rm $PWD/bowtie_human/"$name"_1U.sam

rm $PWD/bowtie_human/"$name"_2U.sam

##9 Convert fastq reads to fasta format (fq2fa_array.sub)

#Steps are as follows

#A Unzip paired reads, interleave forward and reverse paired reads, and convert to fasta format

#B Unzip unpaired forward reads and convert to fasta format

#C As per B for unpaired reverse reads

fq2fa --merge --filter <(gunzip -c $r1) <(gunzip -c $r2) /scratch/user/FAN/project/raw_files/trimmed_fastq/bowtie_human/fq2fa/"$name".fa

fq2fa --filter <(gunzip -c $r3) /scratch/user/FAN/project/raw_files/trimmed_fastq/bowtie_human/fq2fa/"$name"_1U.fa

fq2fa --filter <(gunzip -c $r4) /scratch/user/FAN/project/raw_files/trimmed_fastq/bowtie_human/fq2fa/"$name"_2U.fa

#Move unpaired reads to separate folder

mkdir fq2fa/unpaired

mv *U.fa unpaired

##10 Use metaphlan to generate table with taxa in each sample (metaphlan3_array.sub)

#On the headnode (only need to do this once)

rsync -av /RDrive/CMPH-GenomicsDB/chocophlan_v30 /home/FAN #Here you are moving the metaphlan database to your local folder

#In the metaphlan3_array subscript

metaphlan $r1 --input_type fasta --bowtie2db /home/FAN/chocophlan_v30 --nproc 16 -o metaphlan_out/"$name".out --bowtie2out metaphlan_bowtie/"$name".bowtieout #In array subscript

#Back on the headnode

conda activate SAHMRI_MHH

merge_metaphlan_tables.py *.out > project_metaphlan_table.txt #Here you are merging the outputs from all samples into one table

conda deactivate

###########STOP HERE IF YOU ONLY WANT METAPHLAN TAXA DATA###########

##############CONTINUE WITH BELOW FOR RESISTOME ANALYSIS ##############

##11 Build contigs using IDBA (idba_array.sub)

idba_ud -r $r1 -o idba_test/$name --num_threads 20 --pre_correction

##12 Combine all contig.fa files into one (combine_contigs.sub)

mkdir contigs

find . -type f -name "contig.fa" -exec bash -c ' DIR=$(dirname "{}"); cp "{}" "$DIR"/"${DIR##*/}"_contig.fa' \;

find . -type f -name '*_contig.fa' -exec cp {} /scratch/user/FAN/project/raw_files/trimmed_fastq/bowtie_human/fq2fa/idba/contigs \;

awk 1 contigs/*contig.fa > contigs/project_contigs.fna

cp contigs/project_contigs.fna .

##13 Use prodigal to identify protein coding regions (prodigal.sub)

prodigal -i
/scratch/user/FAN/project/raw_files/trimmed_fastq/bowtie_human/fq2fa/idba/project_contigs.fna -o
/scratch/user/FAN/project/raw_files/trimmed_fastq/bowtie_human/fq2fa/idba/project_orf.gff -d
/scratch/user/FAN/project/raw_files/trimmed_fastq/bowtie_human/fq2fa/idba/project_orf.fna -p meta

##14 Use cd-hit to identify non-redundant genes (cdhit.sub)

cd-hit-est -i
/scratch/user/FAN/project/raw_files/trimmed_fastq/bowtie_human/fq2fa/idba/project_orf.fna -o
/scratch/user/FAN/project/raw_files/trimmed_fastq/bowtie_human/fq2fa/idba/project_orf_nr.fna -c 0.95 -G 0 -aS 0.9 -g 1 -r 1 -T 16 -M 100000

##15 Remove genes less than 100bp in length (rmsmls.sub)

#Ensure removesmalls.pl is accessible

perl /scratch/user/FAN/removesmalls.pl 100
/scratch/user/FAN/project/raw_files/trimmed_fastq/bowtie_human/fq2fa/idba/project_orf_nr.fna >
/scratch/user/FAN/project/raw_files/trimmed_fastq/bowtie_human/fq2fa/idba/project_orf_nr_n100.fna

##16 Rename previously generated file (headnode)

awk '/^>/{print ">gene" ++i; next}{print}' < project_orf_nr_n100.fna >
project_orf_nr_n100_awk.fna

##17 Translate nucleic acid sequence into amino acid sequence (transeq.sub)

transeq -sequence
/scratch/user/FAN/project/raw_files/trimmed_fastq/bowtie_human/fq2fa/idba/project_orf_nr_n100_awk.fna -outseq
/scratch/user/FAN/project/raw_files/trimmed_fastq/bowtie_human/fq2fa/idba/project_orf_nr_n100_awk.faa

##18 Blast sequences against CARD database using RGI (rgi.sub)

#Make a copy of the gene catalogue and remove asterisk from the end of each line in the copy (on headnode)

cp project_orf_nr_n100_awk.faa project_orf_nr_n100_awk_nostar.faa

sed -i 's/*//g' project_orf_nr_n100_awk_nostar.faa

#Download and open database (only need to do this step once on headnode)

wget https://card.mcmaster.ca/latest/data

tar -xvf data ./card.json

rgi load --card_json /scratch/user/FAN/card.json --local

#In rgi.sub

rgi main -i project_orf_nr_n100_awk_nostar.faa -o project_rgi.txt -t protein -n 8 --local

##19 Build database for gene catalogue (bowtie_build.sub)

bowtie2-build /scratch/user/FAN/project/raw_files/trimmed_fastq/bowtie_human/fq2fa/idba/project_orf_nr_n100_awk.fna /scratch/user/FAN/project/raw_files/trimmed_fastq/bowtie_human/fq2fa/idba/project_orf_nr_n100_awk_bowtie

##20 Align interleaved paired forward and reverse reads to the database (bowtie_array_interleaved.sub)

cd /scratch/user/carp0054/GRACE/GRACE_TruSeq/raw_files/trimmed_fastq/bowtie_human/fq2fa

mkdir bowtie

bowtie2 -x /scratch/user/FAN/project/raw_files/trimmed_fastq/bowtie_human/fq2fa/idba/project_orf_nr_n100_awk_bowtie -p 4 -f --interleaved $r1 --local -S /scratch/user/FAN/project/raw_files/trimmed_fastq/bowtie_human/fq2fa/bowtie/"$name".sam

##21 Covert SAM output to RPKM counts (pileup.sub)

mkdir rpkm

for i in `ls *.sam| cut -f 1 -d\.`; do -e "pileup.sh in="$i".sam ref=/scratch/user/FAN/project/raw_files/trimmed_fastq/bowtie_human/fq2fa/idba/project_orf_nr_n100_awk.fna rpkm=rpkm/"$i".rpkm"; done

##22 Tidy output files (headnode)

mkdir combine

for i in `ls *.rpkm| cut -f 1 -d\.`; do sed '1,4d' "$i".rpkm > combine/"$i".tidyrpkm; done

##23 Merge all RPKM outputs and tidy merged file (combine_alt.sub)

#Copy "transpose.awk" to working file

cd combine

find . -name "*.tidyrpkm" -exec awk 'NR>1 {print FILENAME,$1,$6}' {} \; | sed 's/.\//"/' > raw_merge.txt

awk -v OFS='/t' -f transpose.awk raw_merge.txt > Merged_Data_bowtie.txt

sed -i 's/ \+ /\t/g' Merged_Data_bowtie.txt

sed -i 's/\t0.0000/\t0/g' Merged_Data_bowtie.txt

sed -i 's/.tidyrpkm//g' Merged_Data_bowtie.txt

sed -i 's/target_id/gene/g' Merged_Data_bowtie.txt

##24 Use R to generate final merged resistome table using rgi and bowtie outputs (left_join.sub)

#On headnode

mkdir left_join

cp Merged_Data_bowtie.txt left_join

cp /scratch/user/FAN/project/raw_files/trimmed_fastq/bowtie_human/fq2fa/idba/project_rgi.txt left_join

#Move left_join_for_rgi_bowtie.R into left_join folder and edit as instructed

#In left_join.sub

Rscript left_join_for_rgi_bowtie.R

############################## END OF PIPELINE ##############################

APPENDIX B: GRACE STUDY STAGE 2 REPORT

Contents: GRACE stage 2 descriptive report published November 2021

GRACE

Generating evidence on Resistant bacteria in the Aged Care Environment

GRACE Investigative Study Team

2021 Report

Flinders UNIVERSITY

SAHMRI South Australian Health & Medical Research Institute

FHMRI FLINDERS HEALTH & MEDICAL RESEARCH INSTITUTE

This report was written and prepared by Lucy Carpenter, Andrew Shoubridge and Erin Flynn. Cover page was designed by Bradley Rankin. We would like to acknowledge the GRACE investigative team who designed the study, contributed to recruitment and sample processing, and consulted on the analysis:

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Generating evidence on Resistant Bacteria in the Aged Care Environment (GRACE) 2021 Report

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Abbreviations

ACAP	**Aged Care Assessment Program**
ACFI	Aged Care Funding Instrument
ADL	Activities of Daily Living
AMR	Antimicrobial Resistance
ARG	Antibiotic Resistance Genes
CARD	The Comprehensive Antibiotic Resistance Database
CHC	Complex Health Care
DHS	Department of Human Services
HEP	High Energy High Protein (Diet)
IPC	Infection Prevention and Control
MBS	Medicare Benefits Schedule
MDRO	Multidrug Resistant Organism
OP	Oropharyngeal
PAS-CIS	Psychogeriatric Assessment Scale-Cognitive Impairment Scale
PBS	Pharmaceutical Benefits Scheme
PEG	Percutaneous Endoscopic Gastrostomy
RACF	Residential Aged-Care Facility
RPKM	Reads Per Kilobase of transcript, per Million mapped reads

Introduction

In keeping with trends globally, Australia is experiencing significant ageing of its population.[1] By 2031, 21% of Australians will be over 65 years of age.[2] Of these, 6% are expected to live in residential aged care facilities (RACFs), rising to 30% for individuals over 85 years.[2,3]

Residential aged care is recognised globally as a critical setting for monitoring antibiotic use and antimicrobial-resistant bacteria (AMR). High antibiotic prescribing rates,[4] individual susceptibility to infections, and high care needs likely provide an ideal environment for AMR transmission between residents and dissemination into the wider community. Despite this, the prevalence of AMR in asymptomatic individuals and dispersal of these within the RACF environment, is largely uncharacterised. Limiting the development of effective measures to prevent the spread and impact of AMR in residential aged care.[5,6]

The Generating evidence on Resistant bacteria in the Aged Care Environment (GRACE) study aimed to address five questions that are fundamental to developing strategies to reduce AMR carriage in RACF residents:

1) What factors determine the types and levels of AMR carried by RACF residents?
2) To what extent is there evidence of AMR transmission between RACF residents?
3) Is interaction with the RACF built environment likely to facilitate AMR transmission?
4) Do hospital visits for acute care significantly influence types and levels of AMR carriage?
5) To what extent do ageing-associated changes in gut microbiology influence AMR carriage?

GRACE was a cross-sectional study supported by a Medical Research Future Fund (MRFF) grant (GNT1152268) involving five aged care facilities in metropolitan Adelaide, Australia. Participants were invited to provide stool and oropharyngeal samples for metagenomic analysis to determine microbiome and resistome characteristics. Environmental samples were collected from sites within each facility to determine the role of the environment in AMR transmission. The study also accessed Pharmaceutical Benefits Scheme and Medicare Benefits Schedule data for consenting participants. Data on clinical care, facility management practices, including cleaning, provision of care, and staffing, were obtained directly from RACF providers.

This report provides an overview of participant demographics, health status and comorbidities, medication and health system utilisation, facility characteristics, and a preliminary analysis of faecal and oropharyngeal microbiota composition and resistome. Analysis of environmental samples collected from participating sites is not included. Study data are presented prior to integrative analysis to address the five study aims.

Recruitment and Sample Collection

Three residential aged care providers and five facilities participated in the GRACE study. Within these facilities, 403 residents met the study eligibility criteria and 344 were approached to participate. A total of 279 residents consented to the study, a final recruitment rate of 75% (excluding Site 1 as this data were not available) (Fig. 1, Fig. 2A). Eleven couples across four sites enrolled in the study.

Of those who consented, 111 (39.8%) provided self-consent and 168 (60.2%) provided third-party consent. Stool samples were collected from 213 participants, and 204 were of sufficient quality for sequencing. OP swabs were collected from 252 participants, of which 237 were of appropriate quality for sequencing. Primary reasons for being unable to collect a stool sample included cognitive impairment but self-toileting (n=23), staff unable to collect (n=20), refusal (n=15) and cessation of the study due to COVID-19 (n=8). Reasons for being unable to collect an OP swab included cessation of the study due to COVID-19 (n=6) and refusal (physical and verbal) (n=16) Both sample types were collected from 194 participants.

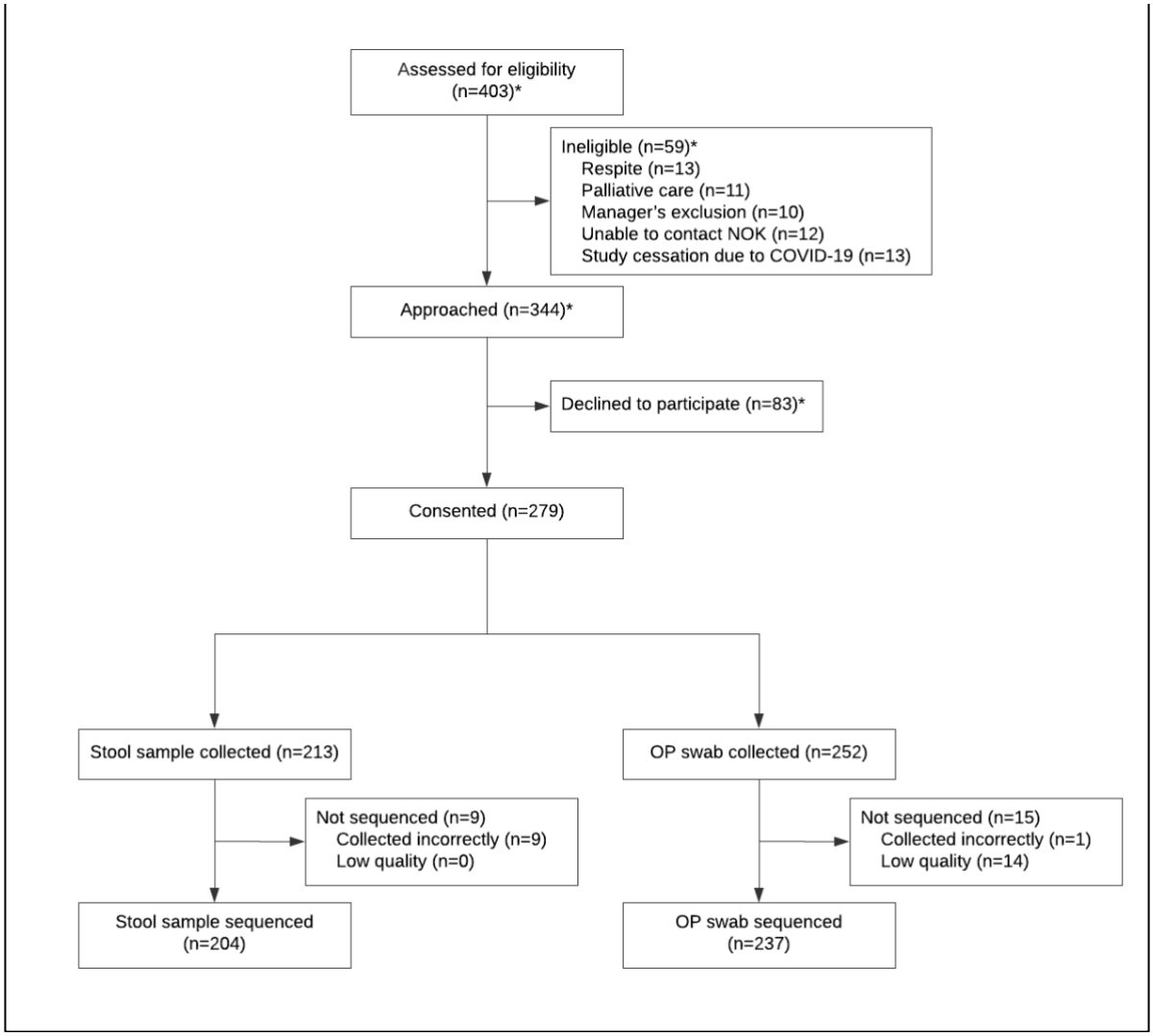


Figure 0.1. *GRACE study recruitment and sample collection. * Indicates data does not include Site 1. n=194 participants gave both sample types.*

Two-hundred and seventy-three residents provided consent to access Medicare benefits schedule (MBS) and pharmaceutical benefits scheme (PBS) data via the Department of Human Services (DHS) (Appendix A/B). DHS data was not accessible for those who provided incorrect supporting documentation (n=14) or completed the consent form incorrectly (n=11 for PBS and n=8 MBS). Finally, DHS could not provide PBS history for 20 participants and MBS history for 8 participants for reasons unknown to the study team. In total, 228 residents had accessible PBS data and 243 had accessible MBS data for analysis (Appendix B).

Site 3 was the largest site with 148 consenting residents, followed by site 5 (n=47) and site 4 (n=46) (Fig. 2B). Site 2 was the smallest site with 27 beds and 18 consenting participants. Site 1 was a pilot site with 20 residents recruited out of 110 occupied beds at the time of recruitment. Data on eligibility and consent was not adequately recorded. Study recruitment ceased in March 2020 due to the COVID-19 pandemic.

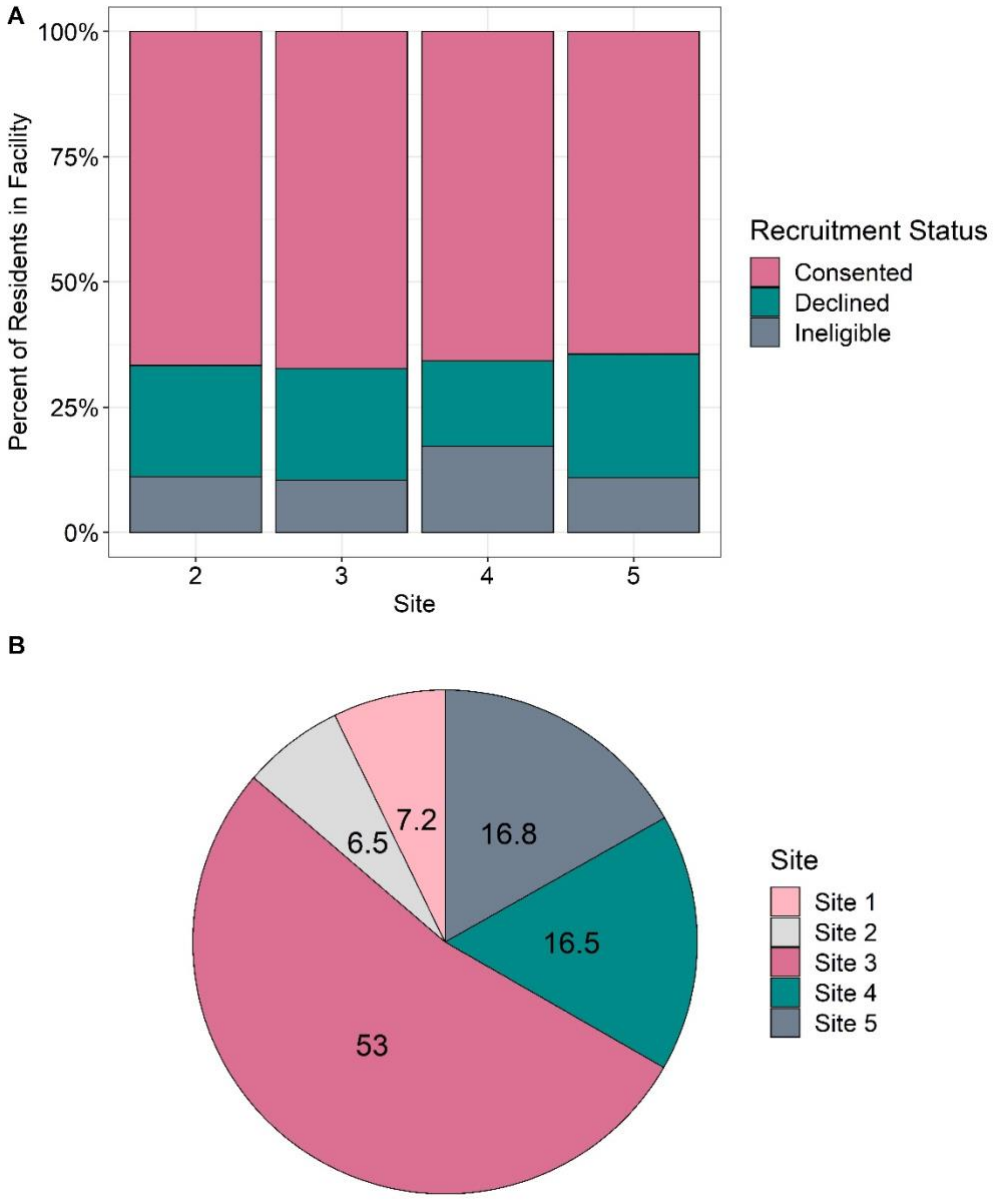


Figure 0.2. *Recruitment per site's total number of occupied beds for the GRACE study. Site 1 is not shown as this data was not collected (A). Percentage of total number of participants from each site (n=279) (B).*

Chapter 1: Facility Characteristics

Facility demographics

All three aged care providers were not-for-profit organisations and sites were located in South Australian metropolitan areas. Site 1 was run by provider A, sites 2 and 3 were from provider B, and sites 4 and 5 were managed by provider C.

Facility data was collected from all sites except for site 5 due to sudden cessation of the study from the COVID-19 pandemic, and therefore has limited variables available to report (Table 1). Site 4 was the oldest site, opened in 1963, and site 2 was the youngest site, opened in 2017. This was reflected in residents' average length of stay, with site 2, having an average of 283 days and site 4 an average of 949 days. Of the five sites, three had a memory support unit (sites 1,3 and 5). All sites had shared or public toilets, with only site 4 reporting shared bath facilities. Cooking and laundry of personal clothing were done in-house for all sites, while laundry for linen was outsourced.

Table 2. *Characteristics of facilities that participated in the GRACE study.*

	Site 1	Site 2	Site 3	Site 4	Site 5
Provider	A	B	B	C	C
Year opened	2012	2017	2009	1963	*NA*
Total beds (No.)	110	27	225	70	87
Occupied beds at time of recruitment (No.)	*NA*	27	220	70	86
Single rooms (No.)	110	27	225	70	*NA*
Shared rooms (No.)	0	0	0	0	*NA*
Average length of stay (days)					
Past 12 months	*NA*	283	624	949	*NA*
Past 3 years	*NA*	357	662	1022	*NA*
Memory support unit (Y/N)	Yes	No	Yes	No	Yes
Shared bath facilities (Y/N)	No	No	No	Yes	*NA*
Shared/public toilets (Y/N)	Yes	Yes	Yes	Yes	*NA*
Animals/pets onsite (Y/N)	Yes	Yes	Yes	No	*NA*
Food cooked fresh onsite (Y/N)	Yes	Yes	Yes	Yes	*NA*
Internal laundry (Y/N)					
Personal laundry	Yes	Yes	Yes	Yes	*NA*
Linen	No	No	No	No	*NA*

NA = not available

1.2 Facility healthcare management and infection control

Site 1 was the only site to report not having a hospital avoidance policy in place, and site 4 was the only site to report having an antimicrobial stewardship policy in place at the time of recruitment. Two out of the four sites had a polypharmacy review policy (sites 1 and 3), and all participated in the aged-care national antimicrobial prescribing survey (acNAPS).

No facility had hand sanitiser available inside resident rooms, and only site 1 reported having hand sanitiser available directly outside of resident rooms. Handwashing stations outside resident rooms ranged between 0.1 and 0.22 stations per room. All sites provided staff with formal hand hygiene training but only sites 2 and 3 reported having a dedicated infection prevention and control (IPC) nurse. As a result of COVID-19, all facilities in Australia must now appoint a nurse as the IPC site lead.

All sites except Site 4 reported having an infectious outbreak in the past 12 months, with sites 1 and 2 reporting a respiratory virus outbreak and sites 1 and 3 reporting a gastrointestinal virus outbreak.

1.3 Facility cleaning

Room cleans were performed weekly in all sites and high touch-point cleans performed daily in all sites except site 1, which reported daily to weekly touch-point cleans. All sites reported cleaning light switches, door handles, toilet seats, toilet flushes, and resident overways (Fig. 3). Bed remotes and call bells were cleaned only in sites 1 and 2, and TV remotes were cleaned in all sites but site 4. Sink taps were cleaned in all sites but site 4, and walking frames were cleaned only in sites 1 and 3.

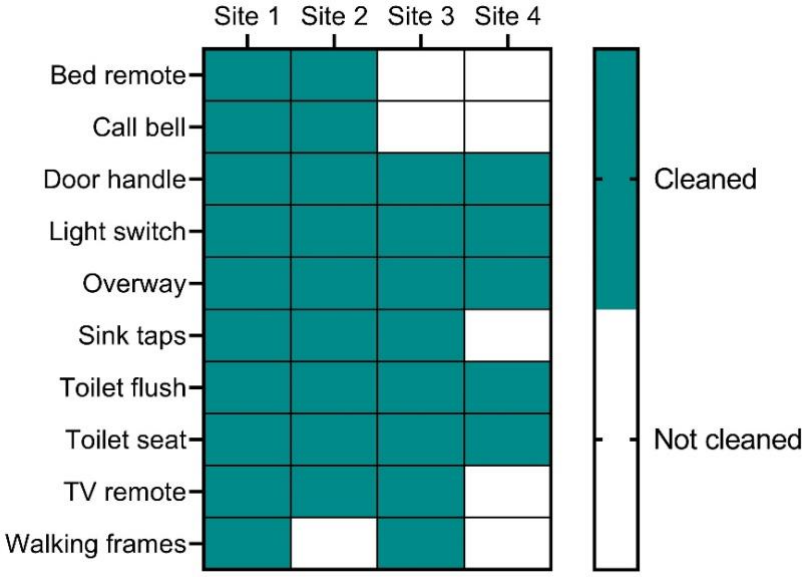


Figure 0.3. *Binary heatmap of surfaces reported as cleaned during high touch-point cleaning in each facility. Site 5 is not shown as this data was not available.*

Chapter 2: Participant Characteristics

2.1 Demographics

Participants of the GRACE study were a median of 88.6 years old (IQR: 11.3, Fig. 4A) with participants at site 4 the youngest (med=85.4, IQR=16.3) and participants at site 2 the oldest (med=90.3, IQR=6.8) (Fig. 4B).

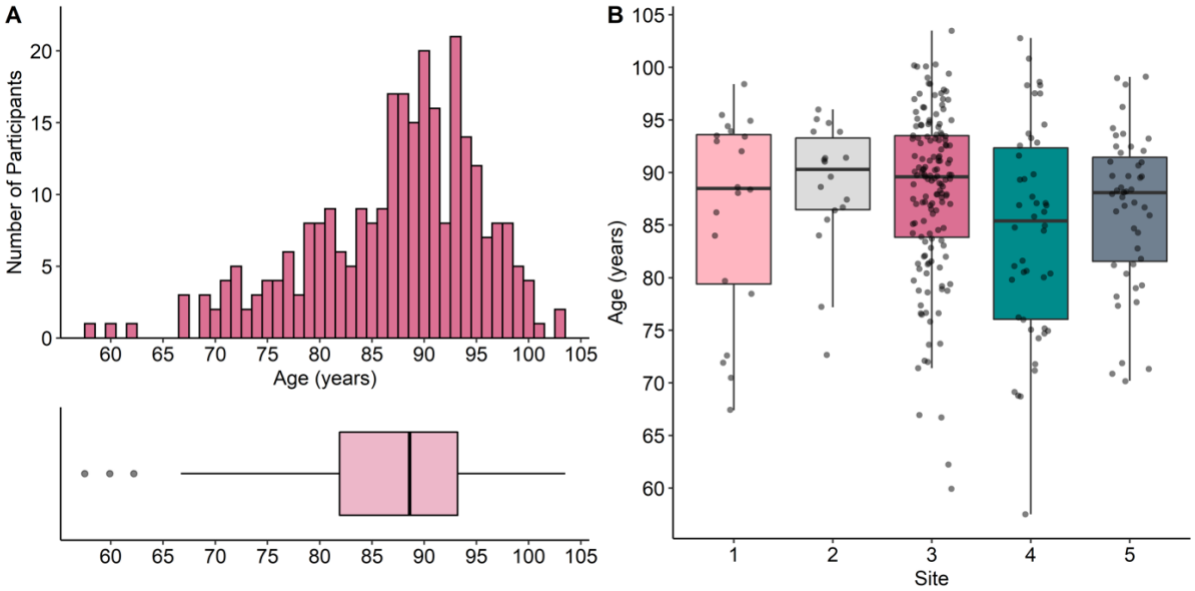


Figure 0.4. *Distribution of age and boxplot showing median, IQR and range of age within the entire GRACE population (A) and per site (B), with each dot representing an individual.*

Of the entire study cohort, 71.7% were female and 28.3% were male (Table 2). Ratio of males to females was consistent across enrolled participants in each site (Fig. 5A). Females enrolled in the GRACE study were generally older than males (females: med=89.4 years; males: med=85.8 years; Fig. 5B).

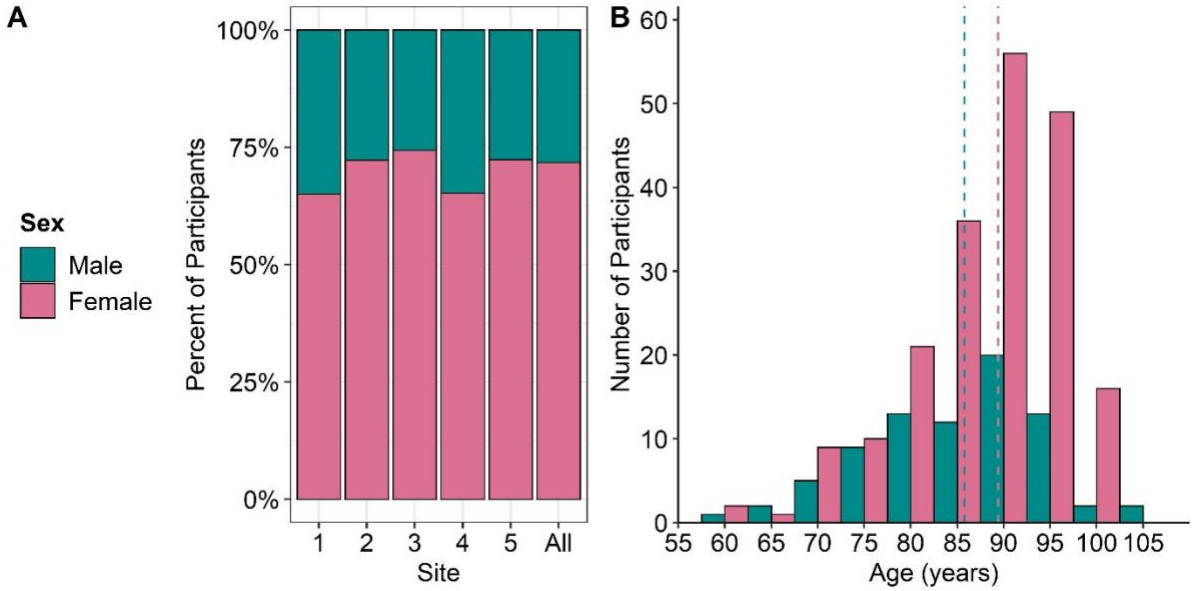


Figure 0.5. *Proportion of enrolled males and females per site (A) and age distribution of each sex (B). Dashed line represents the median age in years for each sex.*

Table 3. *GRACE participant characteristics.*

	Site 1 N (%)	Site 2 N (%)	Site 3 N (%)	Site 4 N (%)	Site 5 N (%)	Total N (%)
Total	20 (7.2)	18 (6.5)	148 (53.0)	46 (16.5)	47 (16.8)	279 (100)
Sex						
Female	13 (65.0)	13 (72.2)	110 (74.3)	30 (65.2)	34 (72.3)	200 (71.7)
Male	7 (35.0)	5 (27.8)	38 (25.7)	16 (34.8)	13 (27.7)	79 (28.3)
Age (years)						
<70	1 (5.0)	0 (0)	4 (2.7)	4 (8.7)	0 (0)	9 (3.2)
70-74	3 (15.0)	1 (5.6)	5 (3.4)	5 (10.9)	4 (8.5)	18 (6.5)
75-79	2 (10.0)	1 (5.6)	13 (8.8)	5 (10.9)	5 (10.6)	26 (9.3)
80-84	1 (5.0)	1 (5.6)	20 (13.5)	8 (17.4)	7 (14.9)	37 (13.2)
85-89	4 (20.0)	6 (33.3)	38 (25.7)	11 (23.9)	16 (34.0)	75 (26.9)
90-94	7 (35.0)	7 (38.9)	42 (28.4)	6 (13.0)	11 (23.4)	73 (26.2)
95-99	2 (10.0)	2 (11.1)	21 (14.2)	5 (10.9)	4 (8.5)	34 (12.2)
>100	0 (0)	0 (0)	5 (3.4)	2 (4.3)	0 (0)	7 (2.5)
Memory support room						
Yes	6 (30.0)	0 (0)	29 (19.6)	0 (0)	1 (2.1)	36 (12.9)
No	14 (70.0)	18 (100)	119 (80.4)	46 (100)	46 (97.9)	243 (87.1)
Shared room						
Yes	0 (0)	0 (0)	0 (0)	0 (0)	6 (12.8)	6 (2.2)
No	20 (100)	18 (100)	148 (100)	46 (100)	41 (87.2)	273 (97.8)

	Site 1 N (%)	Site 2 N (%)	Site 3 N (%)	Site 4 N (%)	Site 5 N (%)	Total N (%)
Time spent in care (days)						
<50	1 (5.0)	1 (5.6)	14 (9.5)	2 (4.3)	3 (6.4)	21 (7.5)
50-99	1 (5.0)	0 (0)	11 (7.4)	3 (6.5)	2 (4.3)	17 (6.1)
100-499	6 (30.0)	10 (55.6)	18 (12.2)	18 (39.1)	13 (27.7)	65 (23.3)
500-999	2 (10.0)	7 (38.9)	55 (37.2)	6 (13.0)	14 (29.8)	84 (30.1)
1000-1499	3 (15.0)	0 (0)	24 (16.2)	4 (8.7)	8 (17.0)	39 (14.0)
1500-1999	6 (30.0)	0 (0)	11 (7.4)	4 (8.7)	1 (2.1)	22 (7.9)
2000-2499	1 (5.0)	0 (0)	6 (4.1)	4 (8.7)	4 (8.5)	15 (5.8)
2500-2999	0 (0)	0 (0)	5 (3.4)	1 (2.2)	1 (2.1)	7 (2.5)
>3000	0 (0)	0 (0)	4 (2.7)	4 (8.7)	1 (2.1)	9 (3.2)
Urinary catheter *in situ*						
Yes	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
No	20 (100)	18 (100)	148 (100)	46 (100)	47 (100)	279 (100)
Urostomy						
Yes	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
No	20 (100)	18 (100)	148 (100)	46 (100)	47 (100)	279 (100)
Vascular catheter *in situ*						
Yes	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
No	20 (100)	18 (100)	148 (100)	46 (100)	47 (100)	279 (100)
Tracheostomy						

	Site 1 N (%)	Site 2 N (%)	Site 3 N (%)	Site 4 N (%)	Site 5 N (%)	Total N (%)
Yes	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
No	20 (100)	18 (100)	148 (100)	46 (100)	47 (100)	279 (100)
Colostomy/Ileostomy						
Yes	0 (0)	0 (0)	7 (4.7)	0 (0)	0 (0)	7 (2.5)
No	20 (100)	18 (100)	141 (95.3)	46 (100)	47 (100)	272 (97.5)
Receiving wound care^			**n=147**	**n=45**		**n=277**
None	15 (75.0)	16 (88.9)	112 (75.7)	26 (56.5)	36 (76.6)	206 (73.8)
Multiple	0 (0)	0 (0)	5 (3.4)	3 (6.5)	1 (2.1)	9 (3.2)
Skin tear	0 (0)	0 (0)	7 (4.7)	6 (13.0)	2 (4.3)	15 (5.4)
Pressure ulcer (grade 1-2)	0 (0)	1(5.6)	11 (7.4)	4 (8.7)	2 (4.3)	17 (6.1)
Pressure ulcer (grade 3-4)	0 (0)	0 (0)	0 (0)	2 (4.3)	0 (0)	2 (0.7)
Leg ulcer	2 (10.0)	1(5.6)	1 (0.7)	0 (0)	1 (2.1)	5 (1.8)
Burn/scald	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Abrasion/graze	1 (5.0)	0 (0)	3 (2.0)	0 (0)	2 (4.3)	6 (2.2)
Surgical	0 (0)	0 (0)	2 (1.4)	0 (0)	0 (0)	2 (0.7)
Lesion	0 (0)	0 (0)	0 (0)	2 (4.3)	0 (0)	2 (0.7)
Unspecified	2 (10.0)	0 (0)	6 (4.1)	2 (4.3)	3 (6.4)	13 (4.7)
Known carriage of MDRO						
Yes	4 (20.0)	2 (11.1)	8 (5.4)	2 (4.3)	0 (0)	16 (5.7)
No	16 (80.0)	16 (88.9)	140 (94.6)	44 (95.7)	47 (100)	263 (94.3)

	Site 1 N (%)	Site 2 N (%)	Site 3 N (%)	Site 4 N (%)	Site 5 N (%)	Total N (%)
Diet type^	**n=19**					**n=278**
Normal	17 (85.0)	18 (100)	139 (93.9)	44 (95.7)	44 (93.6)	262 (93.9)
Vegetarian	0 (0)	0 (0)	1 (0.7)	0 (0)	0 (0)	1 (0.4)
Lactose free	1 (5.0)	0 (0)	7 (4.7)	1 (2.2)	2 (4.3)	11 (3.9)
Gluten free	0 (0)	0 (0)	1 (0.7)	0 (0)	1 (2.1)	2 (0.7)
Halal (no pork)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Hindu (no beef)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Lactose and gluten free diet	1 (5.0)	0 (0)	0 (0)	1 (2.2)	0 (0)	2 (0.7)
Prescribed meal texture						
Regular	16 (80.0)	15 (83.3)	101 (68.2)	34 (73.9)	37 (78.7)	203 (72.8)
Finger food	1 (5.0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.4)
Soft	2 (10.0)	3 (16.7)	15 (10.1)	9 (19.6)	7 (14.9)	36 (12.9)
Minced and moist	0 (0)	0 (0)	18 (12.2)	2 (4.3)	2 (4.3)	22 (7.9)
Pureed	1 (5.0)	0 (0)	14 (9.5)	1 (2.2)	1 (2.1)	17 (6.1)
Liquidised	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Prescribed liquid texture						
Normal/Thin	19 (95.0)	17 (94.4)	132 (89.2)	43 (93.5)	44 (93.6)	255 (91.4)
Slightly thick	0 (0)	0 (0)	1 (0.7)	1 (2.2)	2 (4.3)	4 (1.4)
Mildly thick	0 (0)	1 (5.6)	10 (6.8)	2 (4.3)	1 (2.1)	14 (5.0)
Moderately thick	1 (5.0)	0 (0)	3 (2.0)	0 (0)	0 (0)	4 (1.4)
Extremely thick	0 (0)	0 (0)	2 (1.4)	0 (0)	0 (0)	2 (0.7)

	Site 1 N (%)	Site 2 N (%)	Site 3 N (%)	Site 4 N (%)	Site 5 N (%)	Total N (%)
Prescribed nutritional supplement^	**n=12**		**n=147**			**n=270**
Standard (fortified diet)	3 (15.0)	8 (44.4)	93 (62.8)	24 (52.2)	29 (61.7)	157 (56.3)
High energy & high protein (HEP)	6 (30.0)	10 (55.6)	54 (36.5)	22 (47.8)	18 (38.3)	110 (39.4)
Oral nutrition supplement	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
PEG nutrition supplement	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
HEP and oral nutritional supplements	3 (15.0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (1.1)

^ missing data: receiving wound care, 0.7%; diet type, 0.4%; prescribed nutritional supplement, 3.2%.

At the time of enrolment, study participants had resided in their facility for a median of 681 days (IQR=878; Fig. 6A). Participants in site 1 had the longest length of stay (med=872, IQR=1454.5), and site 2 the shortest (med=457, IQR=146; Fig. 6B). This difference is likely affected by a facility's age, with site 2 opening in 2017.

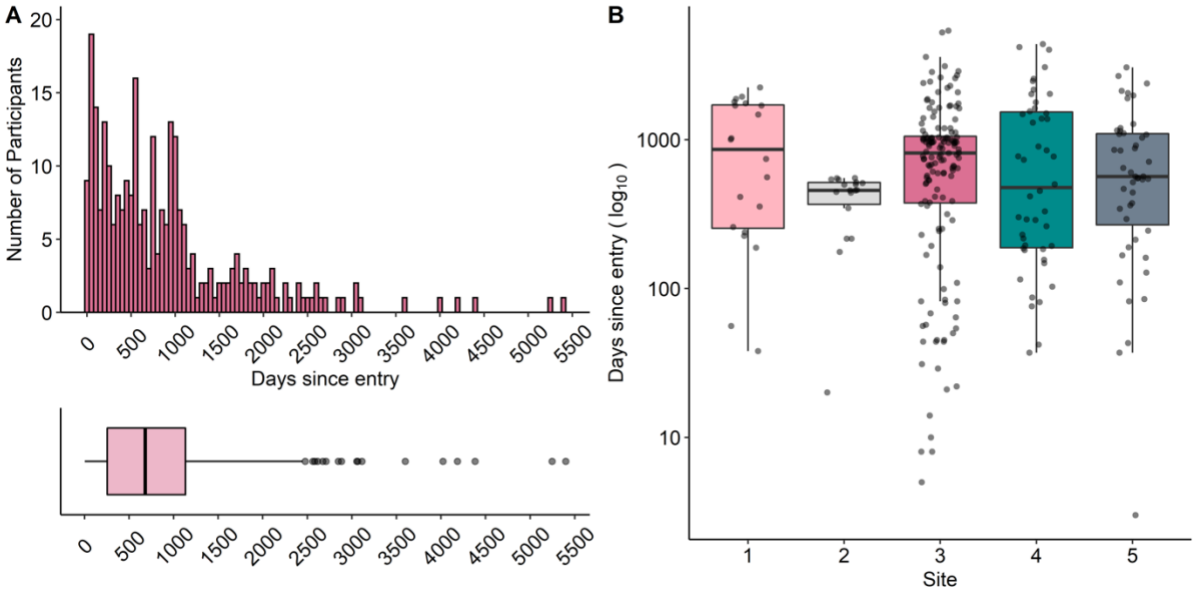


Figure 0.6. *Distribution and boxplot of length of time spent living at the participant's current facility for the entire GRACE cohort with the distribution overlayed (A) and per site (B).*

2.2 Diet type and supplementation

There was no difference in food preparation and supply between sites, with all reporting that food is prepared and cooked fresh on-site (Table 1). Most participants did not have any specific dietary requirements (n=262, 93.9%; Table 2/Fig. 7A), and this was consistent across sites. Of those that did, lactose-free was the most common (n=11, 3.9%). Most participants were able to consume their meals with a regular texture (n=203, 72.8%), however soft (n=36, 12.9%), minced and moist (n=22, 7.9%) and pureed (n=17, 6.1%) were also frequent (Fig. 7B). No participant had liquidised meals. Liquid texture was consistent across sites, with most participants consuming normal/thin textured liquid (n=255, 91.4%), followed by mildly thick (n=14, 5.0%; Fig. 7C). No participants were prescribed percutaneous endoscopic gastrostomy (PEG) supplementation or oral supplementation alone. Most participants receive a standard fortified diet (n=157, 56.3%), with a large proportion also on a high energy high protein (HEP) diet (n=110, 39.4%; Fig. 7D).

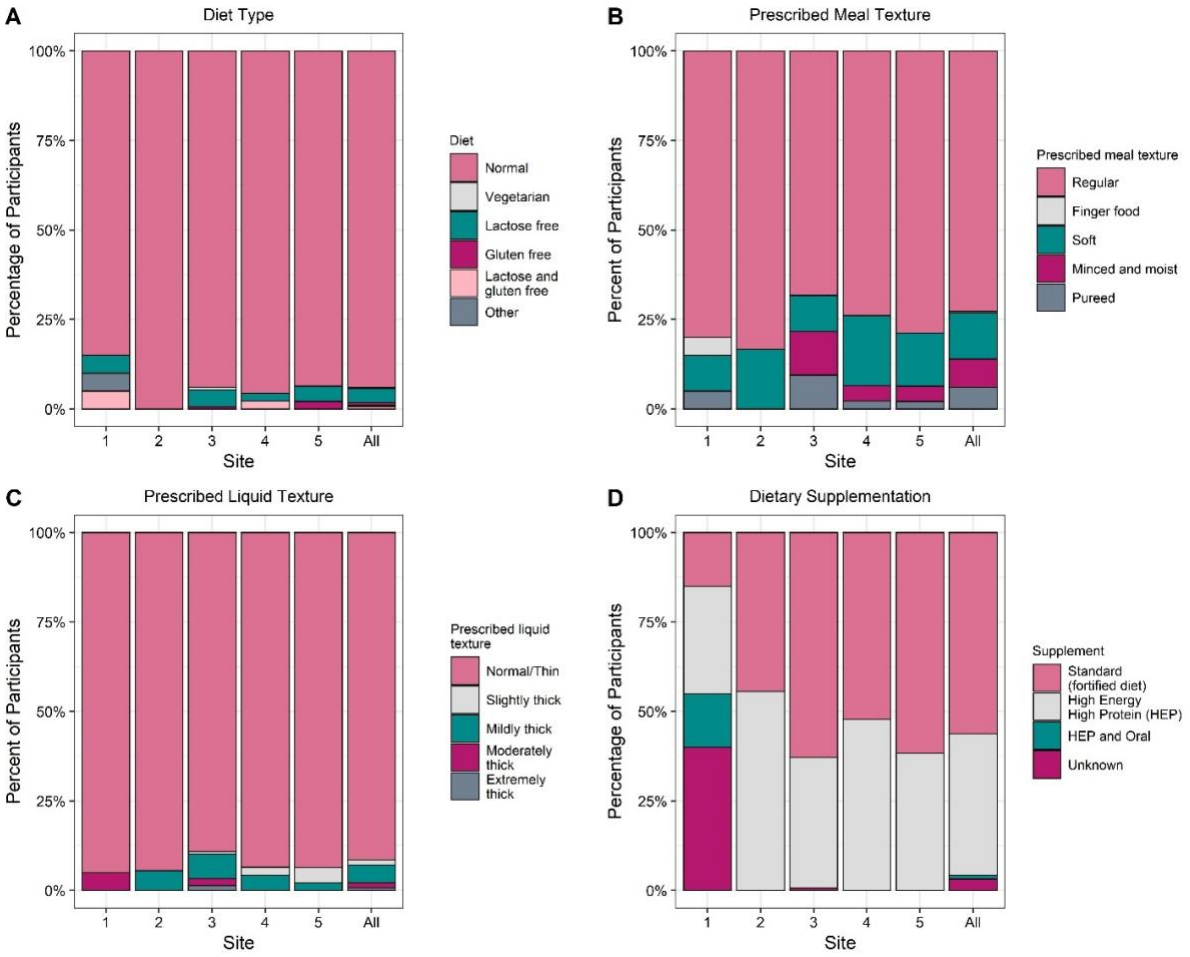


Figure 0.7. *Dietary requirements of GRACE study participants per site for diet type (A), meal texture (B), liquid texture (C) and dietary supplementation method (D).*

2.3 Care requirements

No study participants had a urinary catheter, vascular catheter, tracheostomy, or urostomy at the time of enrolment. Seven participants (2.5%), all from site 3, had a colostomy or ileostomy and 71 (25.6%) were receiving wound care (Table 2). Of those receiving wound care, most were for a grade 1-2 pressure ulcer (n=17, 23.9%) followed by a skin tear (n=15, 21.1%). Nine participants (12.7%) were receiving care for more than one wound. Site 4 had the highest percentage of participants receiving wound care (n=18, 39.1%) and site 2 had the lowest (n=2, 11.1%). Sixteen participants (5.7%) had carriage of an MDRO listed on their medical record. Of these, four were from site 1 (20% of enrolled participants from this site), two from site 2 (11.1%), eight from site 3 (5.4%), two from site 4 (4.3%) and none from site 5.

The Aged Care Funding Instrument (ACFI) assessment details the required levels of care for each of the three domains: Activities of Daily Living (ADL), Behaviour, and Complex Health Care (CHC) (Table 3, Appendix A). Most participants at each site were diagnosed with higher care needs for variables in the ADL category (classifications of C and D). Total ADL scores of high (C) were the most prevalent across the sites, (Fig. 8A) with a minimum 55.6% of participants at site 2, and maximum 72.3% at site 3. Collectively, the most diagnosed care level for each ADL variable for the entire cohort was C for Nutrition (77.4%), D for Mobility (67.4%), D for Personal Hygiene (88.9%), D for Toileting (74.6%), D for Continence, (83.5%), and C for Total ADL (66.0%).

Participants at each site were diagnosed with a range of care needs for Behaviour, which was reflected in the Total Behavioural category (Fig. 8B). High care needs (C) were the most common in sites 1 (65.0%), 3 (50.0%) and 5 (46.8%). Most frequently, participants at site 2 had moderate (B) care needs (55.6%). Site 4 was equally divided with 41.3% of participants classified as requiring moderate or high levels of behavioural care. Collectively, the most diagnosed care level for each Behavioural measure was C for Cognitive Skills (39.8%), A for Wandering (85.3%), D for Verbal (58.1%), A for Physical (49.8%), A for Depression (56.1%), and C for Total Behavioural (47.0%). The mean PAS-CIS score for the cohort was 9.6, with the lowest score at site 2 (8.1) and highest at site 1 (11.6). However, 48.8% of PAS-CIS scores for the cohort were recorded missing, likely due to cognitive impairment levels that were too high for the assessment to be done.

Within the CHC domain, participants at each site were assessed for the level of assistance they required for Medication and Complex Health Care procedures, which was reflected in the Total CHC category (Fig. 8C). A minimum of 55.0% of participants required high care at site 1, 61.1% at site 2, 65.5% at site 3, 65.2% at site 4, and 66.0% at site 5. Collectively, the most diagnosed care level for each factor of the Complex Health Care domain for the entire cohort was B for Medication (81.7%), D for Complex Health Care (63.4%), and C for Total CHC (64.5%).

Table 4. *GRACE participant ACFI assessment for each facility, and the combined cohort.*

	Site 1 N (%)				Site 2 N (%)				Site 3 N (%)			
Total Participants^	20 (7.2)				18 (6.5)				148 (53.0)			
Activities of Daily Living (ADL) Domain	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
Nutrition	2 (10.0)	2 (10.0)	11 (55.0)	4 (20.0)	0 (0)	2 (11.1)	15 (83.3)	1 (5.6)	1 (0.7)	6 (4.1)	121 (81.8)	19 (12.8)
Mobility	0 (0)	1 (5.0)	8 (40.0)	10 (50.0)	0 (0)	0 (0)	5 (27.8)	13 (72.2)	2 (1.4)	3 (2.0)	33 (22.3)	109 (73.7)
Personal Hygiene	0 (0)	3 (15.0)	1 (5.0)	15 (75.0)	0 (0)	0 (0)	0 (0)	18 (100)	0 (0)	3 (2.0)	4 (2.7)	140 (94.6)
Toileting	0 (0)	3 (15.0)	5 (25.0)	11 (55.0)	0 (0)	0 (0.)	3 (16.7)	15 (83.3)	0 (0)	5 (3.4)	20 (13.5)	122 (82.4)
Continence	3 (15.0)	2 (10.0)	0 (0.0)	14 (70.0)	2 (11.1)	3 (16.7)	1 (5.6)	12 (66.7)	10 (6.8)	2 (1.4)	4 (2.7)	131 (88.5)
Total ADL	4 (20.0)	4 (20.0)	12 (60.0)	-	1 (5.6)	7 (38.9)	10 (55.6)	-	7 (4.7)	33 (22.3)	107 (72.3)	-
Behaviour Domain	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
Cognitive Skills	0 (0)	7 (35.0)	4 (20.0)	8 (40.0)	0 (0)	10 (55.6)	6 (33.3)	2 (11.1)	3 (2.0)	28 (18.9)	61 (41.2)	55 (37.2)
Wandering	14 (70.0)	0 (0)	1 (5.0)	4 (20.0)	14 (77.8)	4 (22.2)	0 (0)	0 (0)	126 (85.1)	6 (4.1)	4 (2.7)	11 (7.4)
Verbal Behaviour	2 (10.0)	2 (10.0)	1 (5.0)	14 (70.0)	1 (5.6)	3 (16.7)	8 (44.4)	6 (33.3)	13 (8.8)	23 (15.5)	30 (20.3)	81 (54.7)
Physical Behaviour	7 (35.0)	1 (5.0)	2 (10.0)	9 (45.0)	14 (77.8)	1 (5.6)	1 (5.6)	2 (11.1)	62 (41.9)	19 (12.8)	34 (23.0)	32 (21.6)
Depression	10 (50.0)	5 (25.0)	1 (5.0)	3 (15.0)	2 (11.1)	11 (61.1)	4 (22.2)	1 (5.6)	83 (56.1)	30 (20.3)	17 (11.5)	17 (11.5)
Behavioural PAS CIS (mean (SD))	11.6 (5.4)				8.1 (3.6)				9.3 (4.5)			
Total Behavioural	3 (15.0)	4 (20.0)	13 (65.0)	-	5 (27.8)	10 (55.6)	3 (16.7)	-	22 (14.9)	49 (33.1)	74 (50.0)	-
Complex Health Care (CHC) Domain	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
Medication*	1 (5.0)	14 (70.0)	4 (20.0)	-	1 (5.6)	15 (83.3)	2 (11.1)	-	1 (0.7)	122 (82.4)	14 (9.5)	10 (6.8)
Complex Health Care	1 (5.0)	0 (0)	8 (40.0)	10 (50.0)	1 (5.6)	1 (5.6)	2 (11.1)	14 (77.8)	0 (0)	11 (7.4)	44 (29.7)	92 (62.2)
Total CHC	2 (10.0)	7 (35.0)	11 (55.0)	-	3 (16.7)	4 (22.2)	11 (61.1)	-	9 (6.1)	41 (27.7)	97 (65.5)	-

	Site 4 N (%)				Site 5 N (%)				Total N (%)			
Total Participants	46 (16.5)				47 (16.8)				279 (100)			
Activities of Daily Living (ADL) Domain	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
Nutrition	0 (0)	5 (10.9)	37 (80.4)	4 (8.7)	0 (0)	4 (8.5)	32 (68.1)	8 (17.0)	3 (1.2)	19 (6.8)	216 (77.4)	36 (12.9)
Mobility	0 (0)	0 (0.)	18 (39.1)	28 (60.9)	0 (0)	1 (2.1)	15 (31.9)	28 (59.6)	2 (0.7)	5 (1.8)	79 (28.3)	188 (67.4)
Personal hygiene	1 (2.2)	2 (4.4)	6 (13.0)	37 (80.4)	0 (0)	1 (2.1)	5 (10.6)	38 (80.9)	1 (0.4)	9 (3.2)	16 (5.7)	248 (88.9)
Toileting	1 (2.2)	2 (4.4)	14 (30.4)	29 (63.0)	0 (0)	3 (6.4)	10 (21.3)	31 (66.0)	1 (0.4)	13 (4.7)	52 (18.6)	208 (74.6)
Continence	2 (4.4)	2 (4.4)	4 (8.7)	38 (82.6)	4 (8.5)	0 (0)	1 (2.1)	38 (80.9)	21 (7.5)	9 (3.2)	10 (3.6)	233 (83.5)
Total ADL	3 (6.5)	16 (34.8)	27 (58.7)	-	3 (6.4)	14 (29.8)	28 (59.6)	-	18 (6.5)	74 (26.5)	184 (66.0)	-
Behaviour Domain	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
Cognitive skills	3 (6.5)	16 (34.8)	23 (50.0)	4 (8.7)	2 (4.3)	16 (34.0)	17 (36.2)	9 (19.2)	8 (2.9)	77 (27.6)	111 (39.8)	78 (28.0)
Wandering	42 (91.3)	1 (2.2)	0 (0)	3 (6.5)	42 (89.4)	0 (0)	0 (0)	2 (4.3)	238 (85.3)	11 (3.9)	5 (1.8)	20 (7.2)
Verbal Behaviour	6 (13.0)	2 (4.4)	4 (8.7)	34 (73.9)	10 (21.3)	3 (6.4)	4 (8.5)	27 (57.5)	32 (11.5)	33 (11.8)	47 (16.9)	162 (58.1)
Physical Behaviour	32 (69.6)	4 (8.7)	2 (4.4)	8 (17.4)	24 (51.1)	4 (8.5)	4 (8.5)	12 (25.5)	139 (49.8)	29 (10.4)	43 (15.4)	63 (22.6)
Depression	7 (15.2)	20 (43.5)	12 (26.1)	7 (15.2)	5 (10.6)	15 (31.9)	15 (31.9)	9 (19.2)	107 (38.4)	81 (29.0)	49 (17.6)	37 (13.3)
Behavioural PAS CIS (mean (SD))	9.1 (4.0)				10.3 (4.3)				9.6 (4.4)			
Total Behavioural	7 (15.2)	19 (41.3)	19 (41.3)	-	12 (25.5)	10 (21.3)	22 (46.8)	-	49 (17.6)	92 (33.0)	131 (47.0)	-
Complex Health Care Domain (CHC)	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
Medication*	0 (0)	40 (87.0)	5 (10.9)	1 (2.2)	1 (2.1)	37 (78.7)	6 (12.8)	0 (0)	4 (1.4)	228 (81.7)	31 (11.1)	11 (3.9)
Complex healthcare	0 (0)	3 (6.5)	13 (28.3)	30 (65.2)	0 (0)	2 (4.3)	11 (23.4)	31 (66.0)	2 (0.7)	17 (6.1)	78 (28.0)	177 (63.4)
Total CHC	1 (2.2)	15 (32.6)	30 (65.2)	-	2 (4.3)	12 (25.5)	31 (66.0)	-	17 (6.1)	79 (28.3)	180 (64.5)	-

^ Missing data: nutrition, 1.8%; mobility, 1.8%; personal hygiene, 1.8%; toileting, 1.8%; continence, 2.2%; total ADL, 1.1%; cognitive skills, 1.8%; wandering, 1.8%; verbal behaviour, 1.8%; physical behaviour, 1.8%; depression, 1.8%; behavioural PAS CIS, 48.8%; total behavioural, 2.5%; medication, 1.8%; complex health care, 1.8%; total CHC, 1.1%.
* Indicates score of D only applicable for assessment prior to 2017

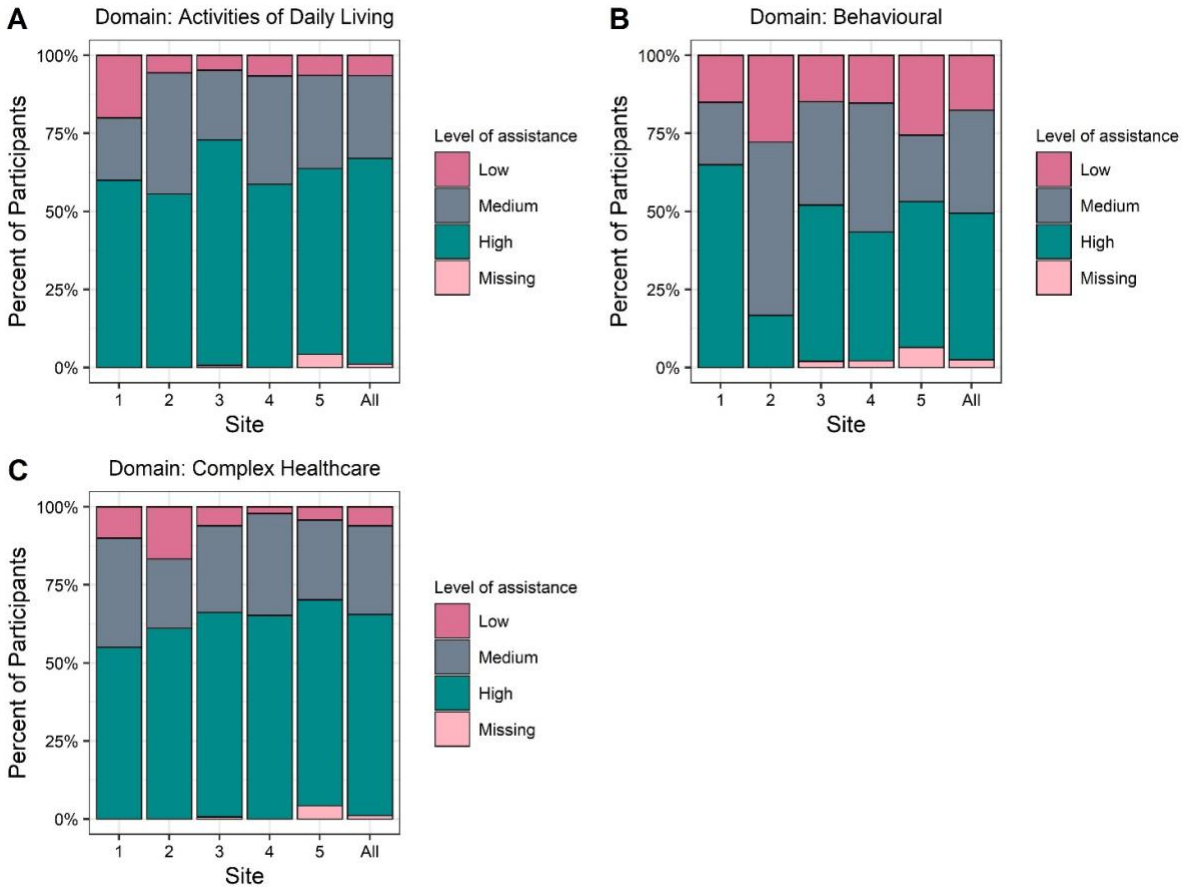


Figure 0.8. *ACFI care needs for participants per site and in total. Assessments for the three domains of aged care subsidised by the ACFI are summarised as total proportions of participants per site. The domains are categorised as activities of daily living (A), behaviour (B), and complex healthcare (C).*

2.4 Mental and behavioural diagnoses

Participants cognitive function and mental health was captured via ACFI Mental and Behavioural Diagnosis records (Table 4).

Table 5. *GRACE participant Aged Care Funding Instrument (ACFI) mental diagnoses for each facility and the combined cohort.*

ACFI Diagnosis	Site 1 N (%)	Site 2 N (%)	Site 3 N (%)	Site 4 N (%)	Site 5 N (%)	Total N (%)	Dementia type (%)
Dementia*	11 (55.0)	4 (22.2)	99 (66.9)	17 (37.0)	21 (44.7)	152 (54.5)	
Alzheimer's Disease	8 (40.0)	2 (11.1)	71 (48.0)	15 (32.6)	15 (31.9)	111 (39.8)	(73.0)
Vascular dementia	0 (0.00)	2 (11.1)	23 (15.5)	1 (2.2)	5 (10.6)	31 (11.1)	(20.4)
Dementia in other diseases (eg Parkinson's)	1 (5.0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.4)	(0.7)
Multiple dementias	0 (0)	0 (0)	2 (1.4)	0 (0)	1 (2.1)	3 (1.1)	(2.0)
Other dementias (eg Lewy body)	2 (10.0)	0 (0)	7 (4.7)	1 (2.2)	2 (4.3)	12 (4.3)	(7.9)
Delirium	0 (0)	0 (0)	9 (6.1)	1 (2.2)	6 (12.8)	16 (5.7)	N/A
Depression	11 (55.0)	10 (55.6)	69 (46.6)	32 (69.6)	36 (76.6)	158 (56.6)	N/A
Psychoses	0 (0)	0 (0)	7 (4.7)	1 (2.2)	3 (6.4)	11 (3.9)	N/A
Neurotic disorders	3 (15.0)	4 (22.2)	52 (35.1)	14 (30.4)	12 (25.5)	85 (30.5)	N/A
Intellectual/developmental disorders	0 (0)	0 (0)	5 (3.4)	0 (0)	0 (0)	5 (1.8)	N/A
Other disorders	1 (5.0)	0 (0)	0 (0)	0 (0)	3 (6.4)	4 (1.4)	N/A
Unknown	1 (5.0)	0 (0)	1 (0.7)	0 (0)	0 (0)	2 (0.7)	N/A
Cognitive impairment Category	PAS-CIS	PAS-CIS	PAS-CIS	PAS-CIS	PAS-CIS	PAS-CIS	N/A
Cognitive Impairment Score mean (SD)	11.56 (5.4)	8.12 (3.6)	9.32 (4.5)	9.12 (4.0)	10.33 (4.3)	9.56 (4.4)	N/A
Impairment level	**n=19**		**n=147**		**n=44**	**n=274**	
No or minimal impairment	0 (0)	0 (0)	3 (2.0)	3 (6.5)	2 (4.3)	8 (2.9)	N/A
Mild impairment	7 (35.0)	10 (55.6)	28 (18.9)	16 (34.8)	16 (34.0)	77 (27.6)	N/A
Moderate impairment	4 (20.0)	6 (33.3)	61 (41.2)	23 (50.0)	17 (36.2)	111 (39.8)	N/A
Severe impairment	8 (40.0)	2 (11.1)	55 (37.2)	4 (8.7)	9 (19.2)	78 (28.0)	N/A

^ Missing data: Impairment level, <1.8%.

* Three participants were diagnosed with multiple types of dementia. Multiple dementia types was not a specific ACFI diagnosis; thus the total percentage exceeds 100%.

Depression was diagnosed in 56.6% of GRACE cohort participants (n=158; Fig. 9A), ranging from 46.6% in site 2 participants to 76.6% for site 5 participants. Over half of the total participants had a dementia diagnosis (n=152, 54.5%) (Fig. 9B). The highest prevalence of dementia was among participants from site 3 (66.9%) and the lowest at site 2 (22.2%). Of the different classifications of dementia, Alzheimer's disease was the most prevalent, accounting for 73.0% (n=111) of all dementia diagnoses. Vascular dementia was the next most prevalent (20.4% of dementia diagnoses, n=31 participants), followed by other dementias, such as Lewy body dementia (7.9% of dementia diagnoses, n=12 participants).

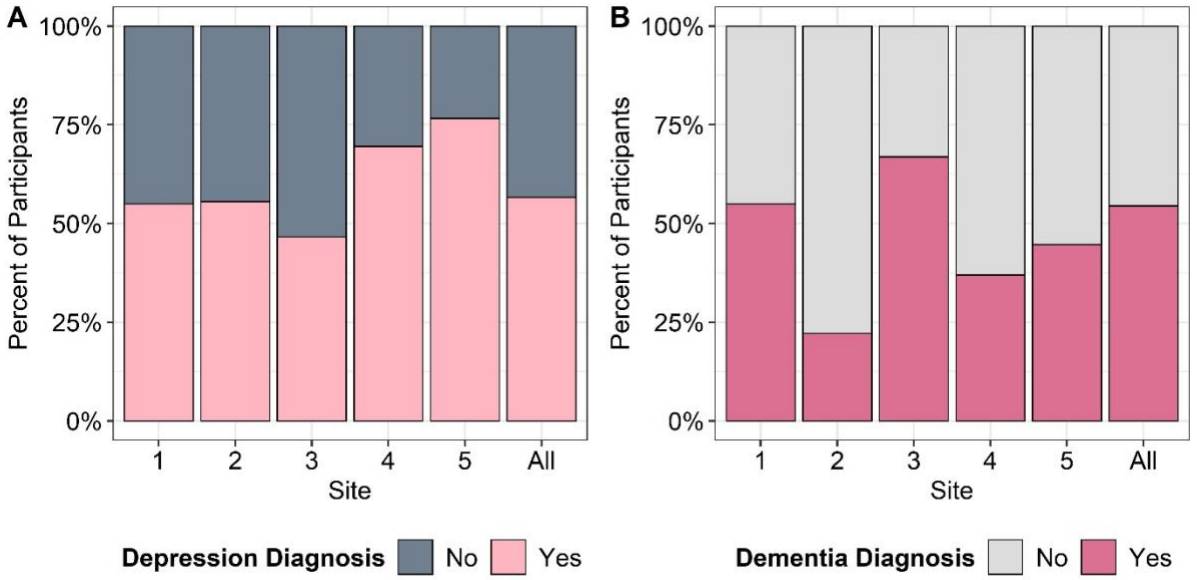


Figure 0.9. *Diagnosis of depression (A) and dementia (B) in the GRACE cohort.*

Of those with a known dementia diagnosis and PBS data available (n=123), 18 (14.6%) were supplied anti-dementia medication. Unusually, one participant was receiving anti-dementia medication but did not have a diagnosis of dementia reported. At least one antipsychotic medication was supplied to 18.7% (n=23) of participants diagnosed with dementia in the 12 months prior to study enrolment. At least one anxiolytic was supplied to 30.9% (n=38) of participants with a dementia diagnosis in this period, and at least one hypnotic/sedative was supplied to 8.9% (n=11) of participants.

Both depression and dementia were diagnosed in 29.4% (n=82) of participants and 18.2% were not diagnosed with either (n=51). Antipsychotics were supplied to 30 participants (13.6%) and antidepressants to 99 (43.4%) participants within 12 months before enrolment, regardless of a mental and behavioural diagnosis. Anxiolytics were supplied at least once to 57 (25.0%) participants, and hypnotics/sedatives were supplied at least once to 30 (13.6%) participants. No participants were supplied medications from all classes. Most frequently, participants were supplied both antidepressants and anxiolytics (n=13; Fig. 10A), and this was the same for participants with a dementia diagnosis (n=7; Fig. 10B).

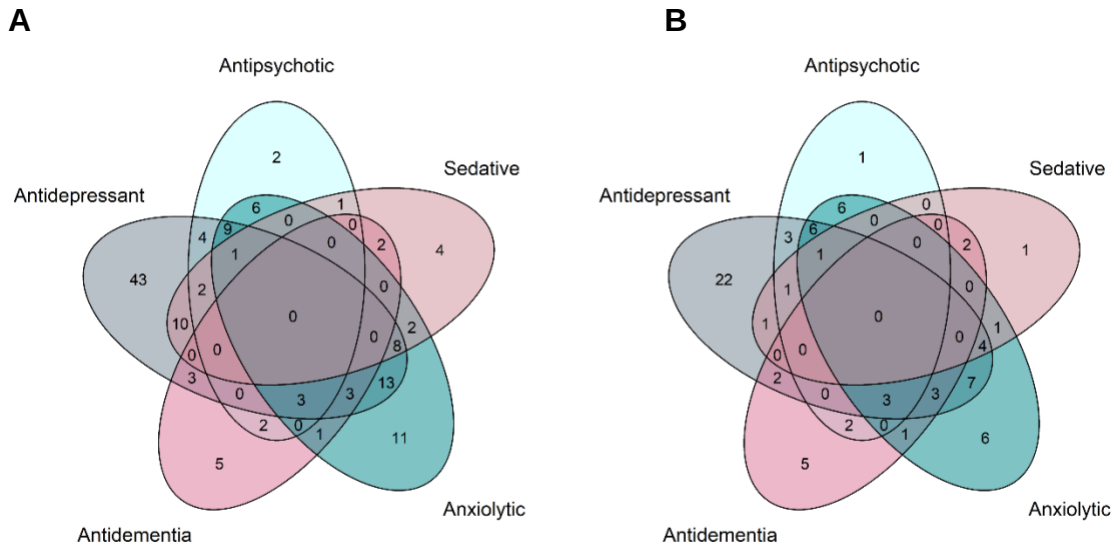


Figure 0.10. *Overlap between supply of antipsychotic, antidepressant, antidementia, anxiolytic and sedative medication for all GRACE participants who received at least 1 during the 12 months prior to enrolment (A) and all GRACE participants with these parameters and a dementia diagnosis (B).*

Cognitive impairment was inferred from a participant's cognitive assessment included in the ACFI. Moderate cognitive impairment was the highest assessment for 39.8% (n=111) of the entire GRACE cohort (Fig. 11). Severe impairment was classified for 28.0% (n=78) of the cohort, followed by mild (27.6%; n=77) and no or minimal impairment (2.9%; n=8). Recorded levels of cognitive impairment were most severe in sites 1, 3, and 5, and minimal to mild in sites 2 and 4.

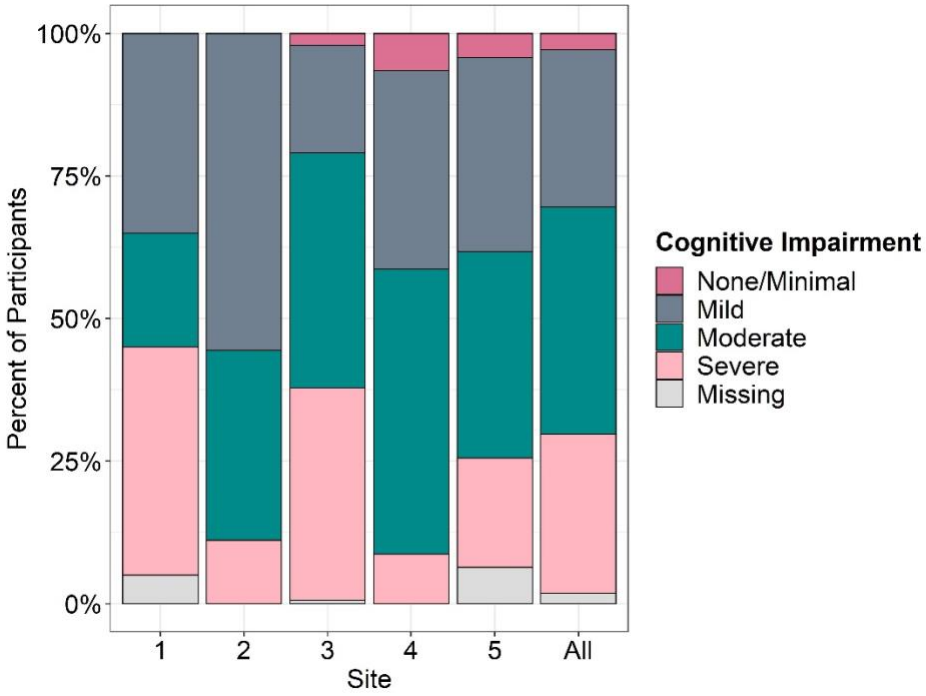


Figure 0.11. *Cognitive impairment levels per site and in total.*

2.5 Comorbidities

Participants of the GRACE study had their comorbidities inferred using the ACFI Aged Care Assessment Program (ACAP) diagnosis codes. Data on comorbidities was not available for participants in site 1 and was missing for one participant in site 3 (n=258). GRACE participants had a median of 11 medical conditions (excluding mental and behavioural conditions) per person (range = [2, 20]). Of all the medical conditions recorded for participants (excluding mental and behavioural diagnoses), the 5 most common included arthritis and related disorders (n=213, 82.6%), stress/urinary incontinence (n=180, 69.8%), hypertension (n=174, 67.4%), diseases of the intestine (n=103, 39.9%), and other diseases of the digestive system not elsewhere classified (n=101, 39.1%; Table 5). The full list of conditions recorded for the GRACE cohort can be found in Appendix C.

Table 6. *Most prevalent medical conditions present in total GRACE study participants as determined from their ACFI data.*

Medical Condition	Site 2 N (%)	Site 3 N (%)	Site 4 N (%)	Site 5 N (%)	Total N (%)
	n=18	n=147	n=46	n=47	n=258
Other arthritis and related disorders	13 (72.2)	128 (87.1)	33 (71.7)	39 (83.0)	213 (82.6)
Stress/urinary incontinence	9 (50.0)	106 (72.1)	31 (67.4)	37 (78.7)	180 (69.8)
Hypertension	10 (55.6)	102 (69.4)	30 (65.2)	32 (68.1)	174 (67.4)
Diseases of the intestine	10 (55.6)	65 (44.2)	11 (23.9)	17 (36.2)	103 (39.9)
Other diseases of the digestive system	5 (27.8)	55 (37.4)	20 (43.5)	21 (44.7)	101 (39.1)
Osteoporosis	7 (38.9)	54 (36.7)	12 (26.1)	15 (31.9)	88 (34.1)
High cholesterol	6 (33.3)	58 (39.5)	7 (15.2)	15 (31.9)	86 (33.3)
Other health condition not elsewhere specified	5 (27.8)	47 (32.0)	19 (41.3)	11 (23.4)	82 (31.8)
Deafness/hearing loss	6 (33.3)	43 (29.3)	6 (13.0)	14 (29.8)	69 (26.7)
Other diseases of the nervous system	3 (16.7)	33 (22.4)	11 (23.9)	20 (42.6)	67 (26.0)
Heart disease	4 (22.2)	43 (29.3)	8 (17.4)	5 (10.6)	60 (23.3)
Kidney and urinary system (bladder) disorders	5 (27.8)	29 (19.7)	11 (23.9)	14 (29.8)	59 (22.9)
Chronic lower respiratory diseases	6 (33.3)	24 (16.3)	11 (23.9)	15 (31.9)	56 (21.7)
Other heart diseases	8 (44.4)	24 (16.3)	10 (21.7)	14 (29.8)	56 (21.7)

Diabetes mellitus-type 2 (NIDDM)	2 (11.1)	26 (17.7)	10 (21.7)	14 (29.8)	52 (20.2)

Of all signs and symptoms, the median recorded per person was 3 (range = [0, 10]). The 5 most common signs and symptoms recorded for participants included falls (n=119, 46.1%), pain (n=118, 45.7%), oedema (n=107; 41.5%), bowel/faecal incontinence (n=98; 38.0%) and abnormalities of gait and mobility (n=53, 20.5%; Table 6).

Table 7. *Most prevalent symptoms and signs present in total GRACE study participants as determined from their ACFI data.*

Symptom/Sign	Site 2 N (%)	Site 3 N (%)	Site 4 N (%)	Site 5 N (%)	Total N (%)
	n=18	n=147	n=46	n=47	n=258
Falls (frequent with unknown aetiology)	12 (66.7)	73 (49.7)	15 (32.6)	19 (40.4)	119 (46.1)
Pain	5 (27.8)	65 (44.2)	16 (34.8)	32 (68.1)	118 (45.7)
Oedema (not specified)	3 (16.7)	66 (44.9)	10 (21.7)	28 (59.6)	107 (41.5)
Bowel/faecal incontinence	3 (16.7)	63 (42.9)	13 (28.3)	19 (40.4)	98 (38.0)
Abnormalities of gait and mobility	3 (16.7)	43 (29.3)	2 (4.3)	5 (10.6)	53 (20.5)

2.6 Medication usage

Participants with PBS data (n= 228) had been supplied with 311 different medications across the study period. Two-hundred and twenty-three participants had PBS data recorded in the 12 months prior to enrolment. Polypharmacy is most commonly defined as the daily usage of 5 or more medications.[7] In the context of the GRACE study, we have defined polypharmacy as the supply of 5 or more medications in the month prior to enrolment, as we are unable to determine daily usage. Of the participants with accessible PBS data, 45.6% (n=104) were taking 5 or more medications during this time and 7.0% (n=16) were taking 10 or more. The median number of medications supplied during this period was 5 and ranged from 0 to 17.

Medications used most frequently by GRACE participants included macrogol (n=82, 36.0%), furosemide (n=76, 33.3%), pantoprazole (n=69, 30.3%), and cephalexin (n=64, 28.1%). Appendix D contains a list of the top 10 most commonly used medications in GRACE participants.

2.7 Antibiotic use and microbiological pathology services

Systemic antibiotics were supplied 867 times in the 12 months prior to enrolment relative to each participant. Around 61% (n=139) of participants had been supplied at least one antibiotic in the 12 months prior to enrolment; 43% (n=98) had been supplied at least two antibiotics,

and 36% (n=82) had been supplied three or more. The antibiotics supplied to the most participants in the GRACE cohort included cephalexin (n=64; 28.1%), amoxicillin and clavulanic acid (n=20; 21.9%), trimethoprim (n=44; 19.3%), amoxicillin (n=36; 15.8%), and doxycycline (n=30; 13.2%; Fig. 12). Pathology services for microbiological testing were accessed at least once by 195 residents (80.3%) in the 12 months prior to enrolment. Of all residents who accessed a pathology service for microbiology (n=195), the most common reason was for a urine examination (n=122 residents, 62.6%), followed by detection of a virus or microbial antigen or microbial nucleic acid (3 or more tests; n=50 residents, 25.6%) and microscopy and culture to detect pathogenic micro-organisms from skin or other superficial sites (n=41 residents, 21.0%).

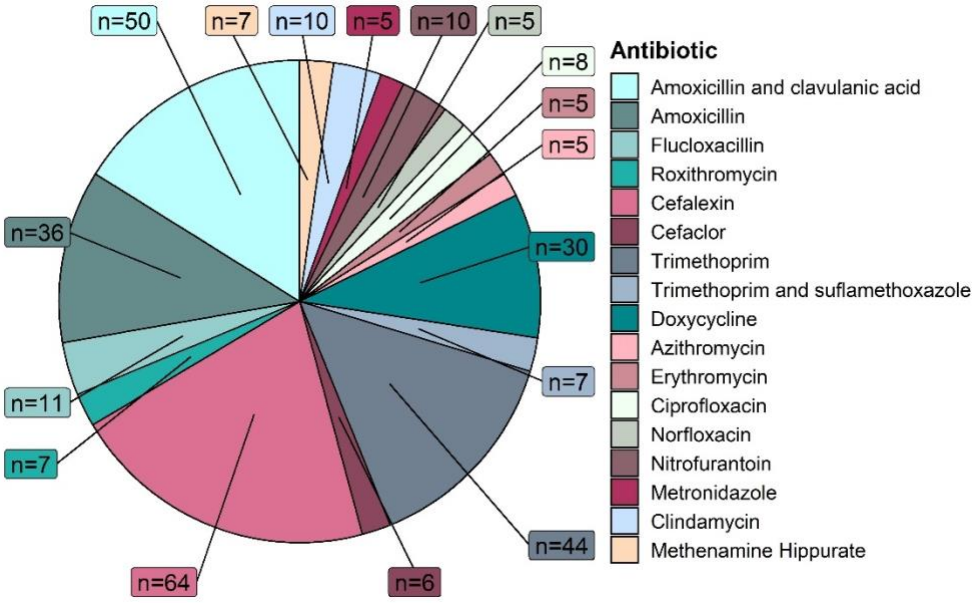


Figure 0.12. *Most frequently supplied antibiotics in the GRACE cohort up to 12 months prior to their enrolment. 'n=' refers to the number of residents who received this antibiotic at least once in this period.*

2.8 Access of healthcare services

Eighty-eight participants (31.5%) had a known hospital separation (either from an emergency, elective admission or other) at least once in the 12 months prior to enrolment. Eighty-four participants had the full 12-month period of data available. Of these, the median number of hospital visits in the 12 months prior to enrolment per person was 1 (range = [1, 5]). Of all the hospital events recorded (n=123 events), 92 of them were emergency visits (74.8%), 17 were elective admissions (13.8%) and 14 were unknown (11.4%). In cases where the number of days spent in hospital was recorded (n=89 events), the median number of days was 4 (range = [1, 63]). Most frequently, the indication for an emergency visit was an infection (including urinary tract infections (UTI), pneumonia and cellulitis; n=22 events; 23.9% of emergency visits), followed by falls (n=18 events; 19.6%) and heart complications (including heart failure and myocardial infarction; n=12 events; 13.0%). Antibiotics were supplied for 44.6% (n=41) of emergency events and was unknown for 15.2% (n=14) of cases, reflective of the high proportion of attendances that were for infections. Elective admissions were most commonly for surgical procedures (n=7 events; 41.2% of elective admissions), stroke rehabilitation (n=3 events; 17.6%) and falls (n=3 events; 17.6%). Antibiotics were prescribed in 6 (35.3%) cases, most likely prophylactically, and antibiotic use was unknown for 6 (35.3%) cases. Of all hospital events where antibiotics were prescribed (n=50), the most frequently given were amoxicillin with clavulanic acid (n=14 events; 28.0%) and ceftriaxone (n=10 events; 20.0%).

Of the 279 consenting participants in the GRACE study, MBS records could be accessed for 243 (87.1%) participants in the 12 months prior to enrolment (Appendix B). Items were identified in patients with at least one instance recorded, and according to the Australian Government Department of Health Medicare Benefits Schedule Book. For professional attendance items, general practitioner attendance after-hours was the most frequent, occurring for 85.2% (n=207) of participants. Diagnostic imaging services applied to 51.4% (n=125) of participants. Of these services, diagnostic radiology was the most frequent, required by 40.7% (n=99) of participants, followed by ultrasound to 29.2% (n=71) of participants. Pathology services were the most frequently recorded service to participants, applying to 93.4% (n=227) of participants. Within the listed pathological services, patient episode initiations were the most frequent, occurring in 93% (n=226) of participants. Details of services accessed by participants can be found in Appendix E.

Chapter 3: Microbiome and Resistome Characteristics

3.1 Microbiome composition of the stool

The human microbiome is defined as the community of microorganisms that inhabit the various surfaces of our bodies. Metagenomic assessment of the stool microbiome was performed for 204 (95.8%) available samples. Across all stool samples, 11 phyla were detected, consisting of 187 genera, or 586 species. A median of 101 (range = [39, 157]) species were detected per person. Of these, four were detected in 98.5% samples and were considered the dominant phyla. These included Firmicutes (med = 48.3%, range = [5.5, 97.4]), Bacteroidetes (med = 17.8%, range = [0, 63.0]), Actinobacteria (med = 14.6%, range = [0, 89.3]), and Proteobacteria (med = 0.94%, range = [0, 44.0]). Of the 586 species, 29 were present in at least 60% of individuals and at a relative abundance of at least 0.1% and were considered core (Fig. 13A, Appendix F). Species that were the most abundant included *Bacteroides uniformis* (med = 2.1%), *Collinsella aerofaciens* (med = 1.3%), and *Anaerostipes hadrus* (med = 1.2%). Species that were detected the most frequently among participants included *Ruthenibacterium lactatiformans* (prevalence = 99.5%), *Gordonibacter pamelaeae* (prevalence = 98.5%) and *Eggerthella lenta* (prevalence = 97.1%). Despite a core microbiome among participants, overall, microbiome compositions were highly dispersed (Fig. 13B), with a median distance to centroid of 0.13 (range = [0.002, 0.58]). Both core and non-core species contributed to this, with the relative abundance of core species ranging from 7.7 to 87.9%, (Fig. 13C).

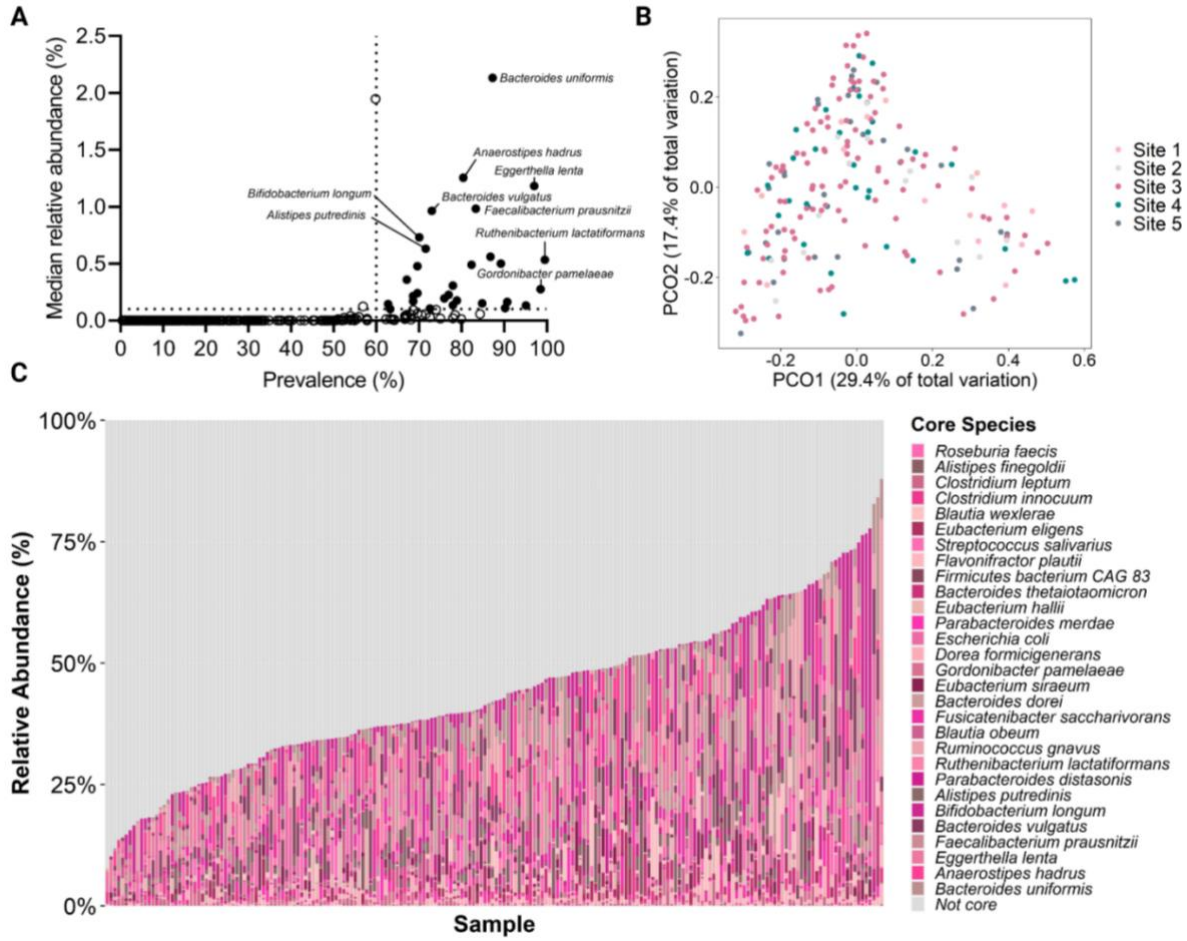


Figure 0.13. *Frequency of species detected in the stool microbiome compared to their relative abundances (A). Principle Coordinates Analysis (PCoA) plot showing dispersion of the stool microbiome among participants, where each dot represents an individual's microbiome relative to others (B). Taxa bar plot showing the distribution of 29 core species compared to non-core species in the stool microbiome of participants (C).*

3.2 Microbiome composition of the oropharynx

Metagenomic assessment of the oropharyngeal (OP) microbiome was available for 237 (94.0%) samples collected from the GRACE study. Across the OP sample, 10 unique phyla were detected, consisting of 90 genera or 333 different species. The median number of species detected per person was 49 (range = [3, 154]). Of the phyla detected in the OP microbiome for the cohort, 4 were present in 77.6% of samples and were considered dominant. Firmicutes were the most abundant (med = 61.7%, range = [2.6, 100]), followed by Actinobacteria (med = 18.4%, range = [0, 79.2]), Bacteroidetes (med = 7.5%, range = [0, 53.7]), and Proteobacteria (med = 0.6%, range = [0, 56.2]). Sixteen core genera, defined as those present in at least 60% of individuals and at a relative abundance of at least 0.1%, were identified in the OP microbiome of GRACE participants (Fig. 14A, Appendix F). Like the stool microbiome, overall, OP microbiome compositions were highly dispersed (Fig. 14B), with a median distance to centroid of 0.15 (range = [0.009, 0.45]). As per the stool samples, the relative abundance of all core species in the OP microbiome varied greatly and ranged from 0 to 99.5% (Fig. 14C). Species that were the most abundant in the OP microbiome included *Streptococcus salivarius* (med = 10.5%)*, Streptococcus parasanguinis* (med = 8.0%)*, and Veillonella atypica* (med= 2.3%). Species that were detected the most frequently among participants included *Streptococcus parasanguinis* (prevalence = 97.0%)*, Rothia mucilaginosa* (prevalence = 94.1%)*, and Streptococcus salivarius* (prevalence = 91.6%).

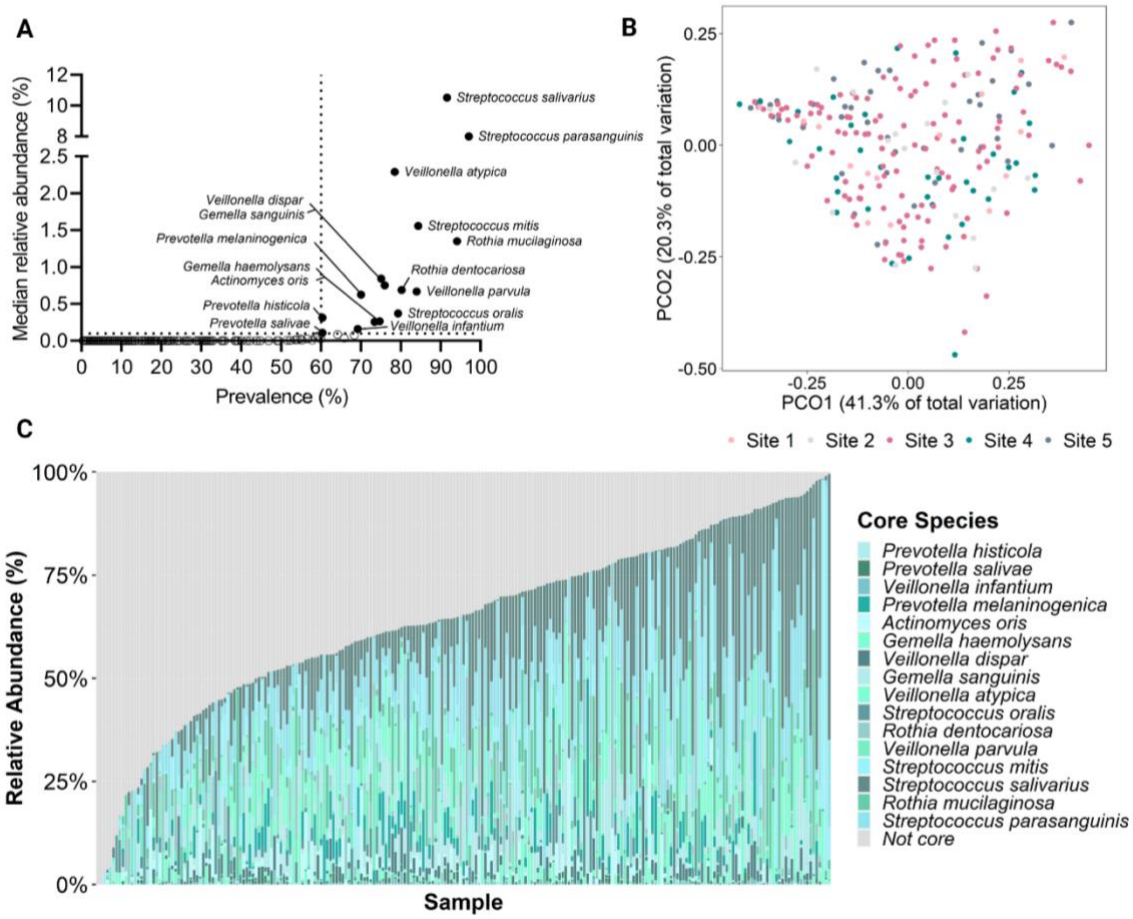


Figure 0.14. *Frequency of species detected in the OP microbiome compared to their relative abundances (A). Principle Coordinates Analysis (PCoA) plot showing dispersion of the OP microbiome among participants, where each dot represents an individual's microbiome relative to others (B). Taxa bar plot showing the distribution of 16 core species compared to non-core species in the OP microbiome of participants (C).*

3.3 Resistome composition of the stool

The resistome is the collection of ARGs that are carried in our microbiome. ARG are complex, for example they can confer resistance via more than one mechanism and to more than one drug class. Presence of ARG does not necessarily indicate that the bacteria carrying it will be an MDRO. This report describes the resistome as a whole; clinical assessment of resistance will be addressed in later analysis.

A normalised count (rpkm) of stool metagenomic reads that aligned to the Comprehensive Antibiotic Resistance Database (CARD) was used to characterise the GRACE stool resistome. In total, 690 ARG were detected across 204 participants, which conferred resistance to 37 different classes of antibiotics (Fig. 15A). Most frequently, ARGs conferring resistance to macrolides (n=137 genes), tetracyclines (n=134 genes), cephalosporins (n=125 genes) and penicillins (n=120 genes) were identified in the stool resistome of GRACE participants. ARGs conferring resistance to macrolides (med = 1659.5 rpkm) and peptides (med = 1530.4 rpkm), however, were the most abundant in the resistome (Fig. 15A). The median number of ARGs carried per person was 375 (range = [291, 437]) (Fig. 15B). Genes carried in the stool microbiome of the GRACE cohort conferred resistance to antibiotics via 6 different mechanisms, including antibiotic inactivation (n=235, 33.8%) and antibiotic efflux (n=219, 31.5%; Fig. 15C).

These genes could be classified into 108 ARG families, which describe the function of each ARG. Families that made up the largest proportion of all genes detected included resistance-nodulation-cell division (RND) antibiotic efflux pumps (n=105, 15.2%), major facilitator superfamily (MFS) antibiotic efflux pumps (n=83, 12.0%), chloramphenicol acetyltransferases (n=29, 4.2%) and OXA beta-lactamases (n=27, 3.9%). The most abundant and frequently detected gene families were ATP-binding cassette (ABC) antibiotic efflux pumps (med = 1611.0 rpkm), RND antibiotic efflux pumps (med = 946.2 rpkm), and *vanR* genes (med = 743.0 rpkm; Fig. 15D).

3.4 Resistome composition of the oropharynx

Across 237 viable OP samples, a total of 424 ARGs were detected that conferred resistance to 38 different antibiotic drug classes. Genes conferring resistance to macrolides (n=118 genes), tetracyclines (n=107 genes), peptide antibiotics (n=97 genes), and phenicol antibiotics (n=85 genes) were the most common. Genes conferring resistance to macrolides (med = 1290.5 rpkm) and tetracyclines (med = 1033.4 rpkm) were the most abundant in the OP resistome (Fig. 16A). ARG richness per person was a median of 212 genes (range = [69, 315]) (Fig. 16B). Antibiotic efflux (n=189 genes) and target alteration (n=121 genes) were the most frequent of the 6 resistance mechanisms detected (Fig. 16C).

ARGs in the OP resistome could be classified into 68 different ARG families (Fig. 16D). RND antibiotic efflux pumps (n=84, 19.8%) and major facilitator superfamily (MFS) antibiotic efflux pumps (n=74, 17.5%) made up the greatest proportion of all genes detected in the OP resistome. ABC antibiotic efflux pump (median rpkm = 1647.6) and ABC ribosomal protection protein (median rpkm = 355.6) families were the most abundant.

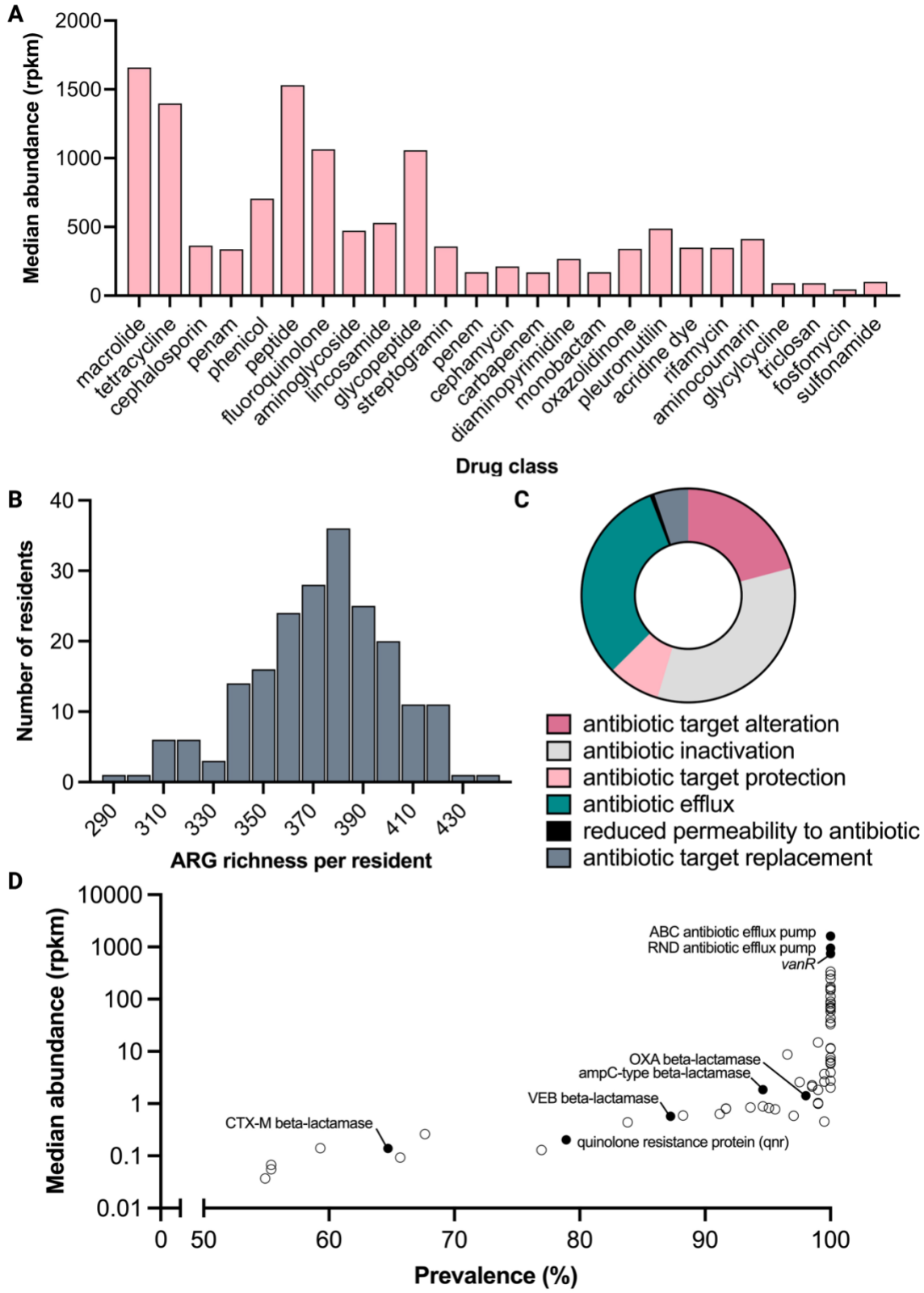


Figure 0.15. *Median abundance (rpkm) of ARG that confer resistance to antibiotic drug classes in the stool resistome (n=12 antibiotic classes not shown as they are extremely rare) (A). Distribution of stool ARG richness per person (B). Proportion of antibiotic resistance mechanisms observed in the stool resistome (C). Prevalence and abundance of ARG families in the stool resistome with most abundant and clinically important gene families labelled (n=47 families not shown as median abundance = 0 rpkm) (D). *ABC = ATP-binding cassette; RND= resistance-nodulation-cell division.*

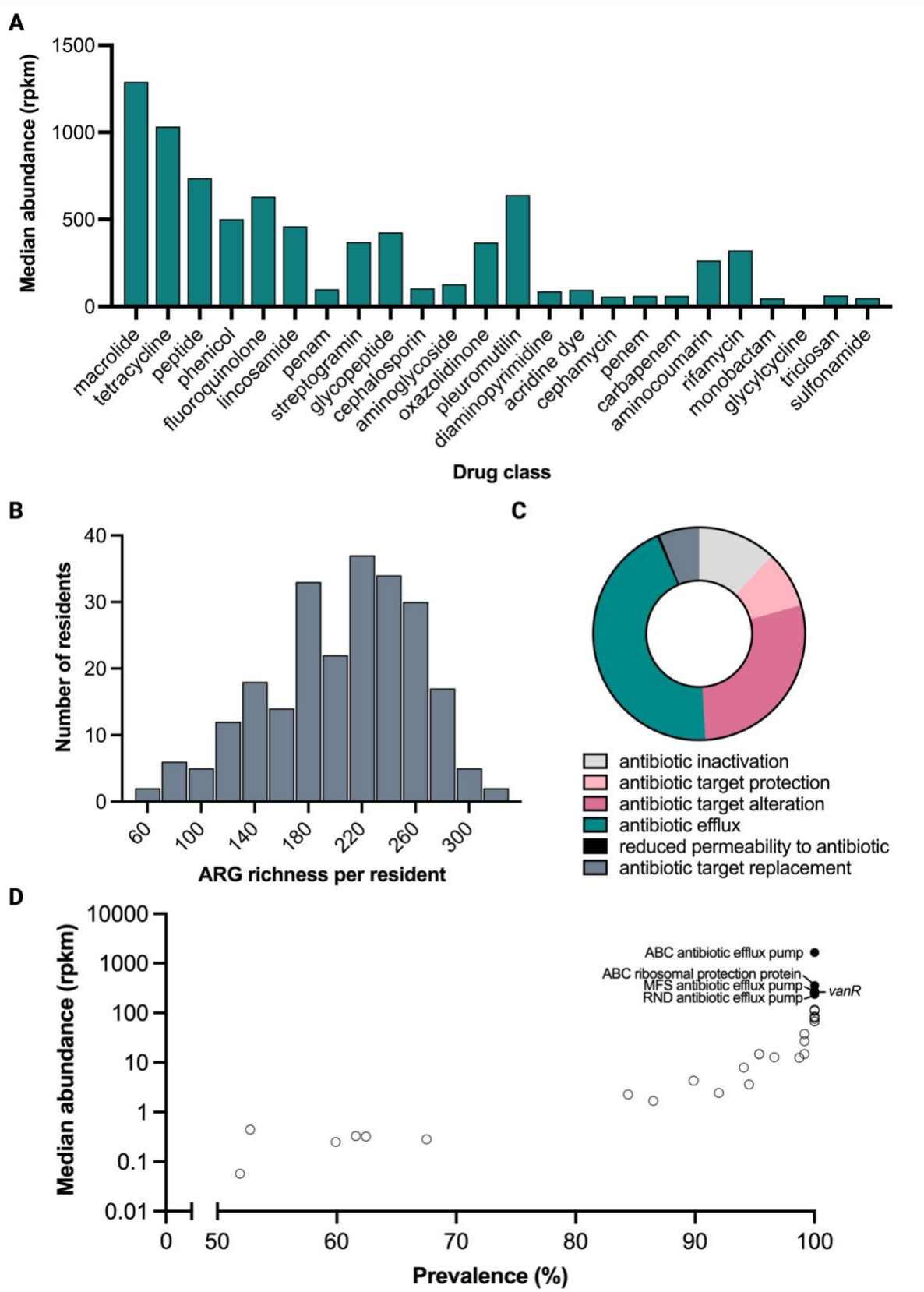


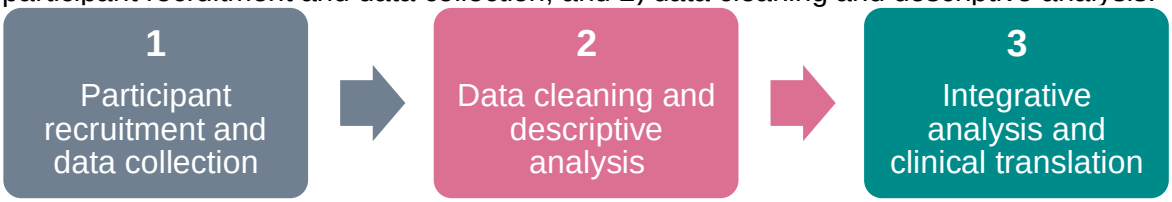
Figure 0.16. *Median abundance (rpkm) of ARG that confer resistance to antibiotic drug classes in the OP resistome (n=13 antibiotic classes not shown as they are extremely rare) (A). Distribution of OP ARG richness per person (B). Proportion of antibiotic resistance mechanisms observed in the OP resistome (C). Prevalence and abundance of ARG families in the OP resistome with most abundant gene families labelled (n=36 families not shown as median abundance = 0 rpkm) (D). *ABC= ATP-binding cassette; RND= resistance-nodulation-cell division; MFS= major facilitator superfamily.*

Conclusions

Described in this report are the clinical, environmental, and microbiological characteristics of participants of the GRACE study. Our cohort consists of permanent residents of south Australian, not-for-profit, metropolitan aged-care facilities. Participants were aged between 58 and 104 years, were mostly female and had been living in their current facility for between 3 and 5399 days. Most participants had a normal diet with standard supplementation. Over half of our cohort had a diagnosis of dementia and this was the same for a diagnosis of depression. Antibiotics were frequently used in this cohort, making up 3 of the 10 most commonly used medications. Around a third of all participants had a known hospital visit during the captured period. Large inter-individual variation in the microbiome and resistome compositions were observed and genes conferring resistance to macrolides and tetracyclines were common. As shown in this report, participants of the GRACE study were subjected to a number of exposures which we propose has a significant impact on the microbiome and resistome, and therefore risk of AMR carriage, transmission, and poor health outcomes.

Future Developments

In this report, we have presented the results from the first 2 stages of the GRACE study: 1) participant recruitment and data collection, and 2) data cleaning and descriptive analysis.



Going forward we will begin the third and final stage: 3) integrative analysis and clinical translation. We aim to use the data presented to here to determine how various exposures in the residential aged-care environment contribute to the acquisition and dispersal of AMR and answer the 5 research questions listed at the beginning of this document. We also aim to demonstrate that our study cohort is representative of those living in Australian aged-care facilities by publishing a cohort profile comparing our data to national averages. As a multidisciplinary team, we have the capacity to ensure that our findings are accessible and informative to other researchers, clinicians, as well as aged-care providers and policy makers.

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Appendices

Appendix B.A: Methods

Ethics

Ethical approval for the study was obtained from the Southern Adelaide Clinical Human Research Ethics Committee (HREC/18/SAC/244). Participants provided written informed consent themselves or where third-party consent was required, a legal guardian or family member with power of attorney provided consent on their behalf.

Setting

Three aged-care providers agreed to participate for a total of 5 sites included in the study. Recruitment started at site 1 in March 2019 and was ceased during recruitment at site 5 due to the COVID-19 pandemic in March 2020. Data was collected at the end of recruitment for each site by the study team.

Recruitment of participants

All residents living in participating aged-care facilities at the time of recruitment were invited to join in the study. Participants were not eligible to consent if: 1) they were in respite care at the time of recruitment, 2) they were receiving palliative/end-of-life care, 3) it was recommended by management that they not be approached, and 4) we were unable to contact next of kin where third-party consent was required. In addition, some participants were unable to be approached due to the COVID-19 pandemic, which caused the study recruitment at the last site to cease early. Participants who required third-party consent were identified by the participating facility and communicated to the study team.

Data sources/measurements

Facility data and participant demographical data were obtained from the facility records. Details of other data collected is explained below.

<u>Aged Care Funding Instrument (ACFI) assessment.</u>

The ACFI constitutes a series of questions and data collection instruments which determine the level of care a person requires upon admission to a residential aged care facility, and therefore how much funding that facility requires to facilitate the required level of care. The ACFI focuses on care needs related to day-to-day and high frequency needs for care. Three domains of residential care are subsidised by the ACFI: activities of daily living, behaviour, and complex health care. The metrics of assessment range from A to D, with A requiring the lowest level of care, to D requiring the most. The individual assessments within these domains are assigned specific ratings and are detailed below.

Nutrition score: The level of assistance (independent OR supervision OR physical assistance) for tasks concerning readiness to eat (using utensils and cutting up/mixing food) and eating (putting food in mouth). A = No assistance, B = Supervision in one/both tasks or physical assistance in readiness to eat task, C = Supervision in one task and physical assistance in one task, D = Physical assistance with both tasks.
Mobility score: The level of assistance (independent OR supervision OR physical assistance) for tasks concerning transfer of position (wheelchair usage, moving from chairs to wheelchairs to beds, etc.), or locomotion (walking, pushing a wheelchair, attachment or passing of mobility aids such as prosthetic limbs or braces). A = No assistance, B = Supervision or physical assistance in one task, C = Supervision in one task and physical assistance in one task, D = Physical assistance with both tasks.

Personal hygiene score: The level of assistance (independent OR supervision OR physical assistance) for tasks concerning dressing and undressing, washing and drying, and grooming. A = No assistance, B = Supervision in one task, C = Physical assistance in one task, D = Physical assistance in all tasks.
Toileting score: The level of assistance (independent OR supervision OR physical assistance) for tasks concerning the use of a toilet (setting up to use the toilet), and toilet completion (the ability to appropriately manage the toileting activity). A = No assistance, B = Supervision in one task, C = Physical assistance in one task, D = Physical assistance in all tasks.
Continence score: The presence and/or frequency of urinary and faecal incontinence. A = No episodes of incontinence or self-manages continence devices, B = Incontinent of urine less than or equal to once per day, or faeces once or twice per week, C = 2-3 daily episodes of urinary incontinence/passing of urine during scheduled toileting, or 3-4 weekly episodes of faecal incontinence/passing faeces during scheduled toileting, D = More than 3 daily episodes of urinary incontinence/passing of urine during scheduled toileting, or more than 4 weekly episodes of faecal incontinence/passing of faeces during scheduled toileting.
Total domain activities of daily living score: The summarised assistance level required across all activities of daily living. A = Low, B = Medium, C = High.

Cognitive skills score: The level of impairment determined from the Psychogeriatric Assessment Scale – Cognitive Impairment Scale (PAS-CIS). A = No or minimal impairment, B = Mild impairment, C = Moderate impairment, D = Severe impairment.
Wandering score: Assessment of occurrence/frequency of problem wandering (repeated attempts to leave the service, or where presence is unwelcome or inappropriate). A = Problem wandering occurs less than 2 days per week, B = Problem wandering occurs at least 2 days per week, C = Problem wandering occurs at least 6 days in a week, D = Problem wandering occurs twice a day or more, at least 6 days in a week.
Verbal behaviour score: Assessment of verbal refusal of care, verbal disruption (not related to an unmet need), paranoid ideation that disturbs others, or verbal sexually inappropriate advances directed at another person. A = Verbal behaviour occurs less than 2 days per week, B = Verbal behaviour occurs at least 2 days per week, C = Verbal behaviour occurs at least 6 days in a week, D = Verbal behaviour occurs twice a day or more, at least 6 days in a week.
Physical behaviour score: Assessment of physical conduct by a resident that is threatening and has the potential to physically harm another person, socially inappropriate behaviour that impacts on other residents, and being constantly physically agitated. A = Physical behaviour occurs less than 2 days per week, B = Physical behaviour occurs at least 2 days per week, C = Physical behaviour occurs at least 6 days in a week, D = Physical behaviour occurs twice a day or more, at least 6 days in a week.
Depression score: Utilises the Cornell Scale for Depression (CSD) to evaluate symptoms associated with depression and dysthymia (chronic mood disturbance), and how these symptoms interfere with daily life. A = CSD score 0-8 and minimal or no symptoms, B = CSD score 9-13 and symptoms cause mild interference with daily function, C = CSD score = 14-18 and symptoms cause moderate interference with daily function, D = Diagnosed depression, CSD score 19-38, and symptoms majorly impact daily function.
Domain behavioural PAS CIS score: The level of impairment determined from the Psychogeriatric Assessment Scale – Cognitive Impairment Scale (PAS-CIS). Scale ranges from 0-21 (nil to severe impairment).
Total domain behavioural score: The summarised impact level of resident behaviour across all forms of physical and non-physical behaviour to determine total dependency on care. A = Low, B = Medium, C = High.

Medication score: The level of assistance required to take medication administered on a regular basis (including patches, oral administration, subcutaneous, intramuscular, and intravenous). A = No medication or self-manages medication, B = Application of patches at least weekly, or needs assistance with daily medication, C = Needs daily administration of a subcutaneous, intramuscular, or intravenous drug. Assessment prior to 2017 included an

option D for medication score. For these residents, scores A and B were the same, however there are some differences to the assignment of scores C and D. Specifically, C = Needs daily assistance with medications for between 6 and 11 minutes, and D = Needs greater than 11 minutes of daily assistance and/or administration of a subcutaneous, intramuscular, or intravenous drug.
Complex healthcare score: The assessed need for ongoing complex health care procedures and activities, with ratings relating to the technical complexity and frequency of the procedures. A = Score of 0 (no procedures), B = Score of 1-4 (assistance required with a low number of complex procedures), C = Score of 5-9 (assistance required with a moderate number of complex procedures), D = Score of 10+ (assistance required with a high number of complex procedures).
Total domain complex health care score: The summarised assistance level required across all forms of complex health care to determine total dependency on care. A = Low, B = Medium, C = High.

Mental and Behavioural Diagnoses
To support the ACFI assessment, residents are also evaluated for diagnosis of a neurological impairment which may influence their care requirements, as determined by the ACFI. These diagnoses include dementia, mood disorders, psychiatric and neurotic disorders, and evaluations of cognitive impairment. Counts and proportions of residents were calculated from ACFI data.

Comorbidities
Physical comorbidities and signs and symptoms were determined using the ACAP code system which is part of the ACFI assessment as described above. Any medical conditions listed in the ACFI are required to be supported by evidence from a medical professional.

PBS/MBS
De-identified DHS-linked data was received in May 2021. PBS data was checked against the information available in resident medication charts for 41 participants. Of 134 medication entries for the available period, expectations were met in 81.3% of instances. PBS and MBS data were cleaned to ensure 12 months of pre-enrolment data was available for each participant. Drug classes were grouped and classified according to their ATC code and health services were classified based on their assigned category.

Sample collection
Stool samples and oropharyngeal (OP) swabs were collected from consenting residents, as well as environmental swabs of participant rooms and communal areas. Swabs taken in participant rooms included bed remotes, overways, door handles and toilet flushes. Swabs taken in communal areas include staff room door handles, staff phones, staff computer keyboards, medication trolleys, dining tables, servery benches, public toilet seats, public toilet flushes, public toilet door handles, wheelchairs and mechanical lifters. Stool samples were collected in 20ml tubes with DNA stabilisation buffer (Norgen, ON, Canada) and swabs were collected and placed in a 2ml screw-cap tube contained 400µl of Tris-EDTA (TE) buffer (Invitrogen). All samples were stored at -80°C until processing.

Stool DNA extraction
DNA was extracted from stool samples using the Qiagen PowerLyzer PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. Stool samples containing buffer were vortexed vigorously and 1 ml was transferred to a clean 2 ml tube. Samples were centrifuged for 20 min at 13,000 xg at 4°C and the supernatant was transferred

to a clean 2 ml screw-cap tube for storage. Faecal pellet was combined with 750 µl of bead solution and transferred to a glass bead tube. After adding 60 µl of cell lysis buffer, samples were incubated at 65°C for 10 min. Samples underwent bead-beating in a FastPrep®-24 Homogenizer (MP Biomedicals, Santa Ana, CA, USA) for 2 pulses of 1 min at 6.5m/s and were centrifuged at 10,000 xg for 3 min at room temperature. Supernatant was discarded and 250 µl of Inhibitor Removal Technology® (IRT) was added and vortexed for 5 s. Samples were incubated at 4°C for 10 min, centrifuged for 3 min at 10,000 xg at room temperature, and 600 µl of supernatant was transferred to a clean 2 ml tube. Precipitation reagent was combined with the supernatant and the sample was centrifuged for 3 min at 10,000 xg at room temperature again before transfer of 750 µl supernatant to another clean 2 ml tube. High concentration salt solution was added to the supernatant and vortexed for 5 s. Then 675 µl of supernatant was added to a Spin Filter and centrifuged for 10,000 xg for 1 min at room temperature. Flow through was discarded and this step was repeated 2 more times. 500 µl of ethanol-based wash solution was added to the spin column and centrifuged at 10,000 xg for 1 min at room temperature. Flow through was discarded and residual ethanol wash solution was removed from the spin column by a second centrifuge at 10,000 xg for 1 min. Spin columns were transferred to a clean 2 ml tube, 50 µl of UltraPure RNAse DNAse-free water was added and centrifuged for 1 min 10,000 xg at room temperature and repeated 2 more times to collect all DNA. Eluted DNA was stored at -80°C until further processing.

Swab DNA extraction

DNA from swabs was extracted using the ZymoBIOMICS miniprep kit (Zymo Research, Irvine, CA, USA). Swabs were spun down at 3374 x g for 5 min to collect all biological material and the resultant solution was added to a bead-beating tube containing 750 µl of lysis buffer. Samples underwent bead-beating for 1 min 5 times at a speed of 6.5m/s in a FastPrep®-24 Homogenizer (MP Biomedicals) for a total of 5 min with 5 min rest in between each run. Samples were centrifuged at 10,000 x g for 2 min then 700 µl was added to the III-F filter in a clean tube and centrifuged at 8,000 x g for 1 min. Filtered solution was transferred to a clean tube and 2100 µl of DNA binding buffer was added. Samples were vortexed vigorously then 800 µl of solution was added to a IICR filter and centrifuged at 10,000 x g for 1 min. Flow through was discarded and this process was repeated until all solution had been passed through the filter. After transferring the filter to a new tube, 400 µl of the first wash buffer was added to the filter and centrifuged at 10,000 x g for 1 min. Flow through was discarded then 700 µl of a second wash buffer was added to the filter, centrifuged at 10,000 x g for 1 min and a final 200 µl of wash buffer was added to ensure all wash buffer had passed through. Filters were transferred to a new clean tube and 100 µl of dH_2O at 60°C was added and incubated for 5 min. After centrifuging at 10,000 x g for 1 min, samples were added to a final spin column for purification and centrifuged at 16,000 x g for 3 min. For environmental swabs, the elution process was repeated 2 more times, then samples were concentrated and re-eluted in 75 µl of dH_2O. DNA was stored at -80°C until further processing.

Metagenomic sequencing

Stool samples and OP swabs of sufficient DNA quality underwent metagenomic sequencing. DNA fragmentation of samples was performed with Nextera XT DNA Library Prep Kit (Illumina). Samples were sequenced at a depth of 5Gb on an Illumina Novaseq platform with 150-bp paired-end reads.

Bioinformatic processing

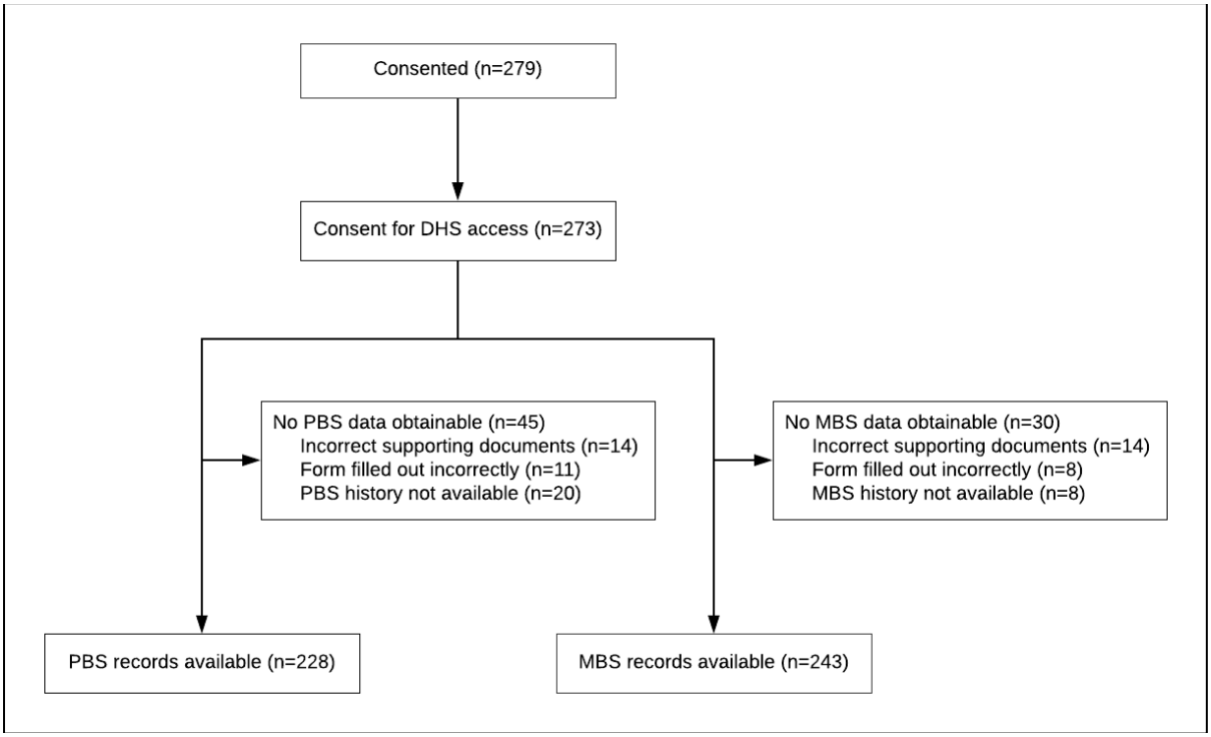
Paired-end sequences were quality filtered using Trimmomatic (version 0.39) and human reads were removed using Bowtie (version 2.3.5.1) using the NCBI human reference genome release GRCh38.[8,9] Contigs were assembled de novo using IDBA-ud (version 1.1.3) and open reading frames were identified with MetaGeneMark (version 1.0).[10,11] Non-redundant genes were extracted using CD-HIT (version 4.8.1) with parameters '-c 0.95 -aS 0.9' (genes with >95% identity and aligned length covering >90% of shorter gene) and genes less than 100 bp in length were removed.[12] A catalogue of 12,209,321 faecal genes and 2,334,932 OP genes were transcribed to amino acids using the European Molecular Biology Open Software Suite (EMBOSS v 6.6.0).[13] Transcribed genes were mapped to antimicrobial resistance genes in the Comprehensive Antibiotic Resistance Database (CARD) using BLASTP (version 2.9.0) with the following parameters: '-evalue 1e-10 -qcov_hsp_perc 99 -max_hsps 1 -max_target_seqs 1'.[14] Alignment of non-redundant gene catalogue with human-cleaned reads was performed with Bowtie (version 2.3.5.1).[9] Gene-length normalised read count calculation was performed and antimicrobial resistance gene quantification per sample was calculated in R (v4.0.2). Gene counts are reported as reads per kb of transcript, per million mapped reads (rpkm). Microbiome composition data was extracted from human-cleaned reads using MetaPhlAn (v3.0).[15]

Statistical methods

Raw data was cleaned and merged in Statistical Analysis Software (SAS) University Edition (v9.4) and exported for further processing. R (v4.0.2) and Prism (v9) was used for descriptive statistics and visualisation of data. Data was checked for normality and the appropriate metrics were reported depending on the outcome.

Appendix B.B: DHS data access

Availability of PBS and MBS data for GRACE participants and reasons where access was not possible.



Appendix B.C: Comorbidities

Full list of physical medical conditions and signs and symptoms with prevalence (number of residents) that affected the GRACE study cohort (classified by their ACAP categories).

Condition	ACAP code	N	%
Certain infectious and parasitic diseases			
Tuberculosis	0101	2	0.8
Poliomyelitis	0102	1	0.4
Diarrhoea and gastroenteritis of presumed infectious origin	0104	1	0.4
Unspecified/Unclassified infectious or parasitic disease	0199	5	1.9
Neoplasms (tumours/ cancers)			
Colorectal (bowel) cancer	0203	18	7.0
Lung cancer	0204	6	2.3
Skin cancer	0205	21	8.1
Breast cancer	0206	14	5.4
Prostate cancer	0207	17	6.6
Brain cancer	0208	2	0.8
Non-Hodgkin's lymphoma	0209	2	0.8
Leukaemia	0210	2	0.8
Other malignant tumours	0211	15	5.8
Other neoplasms	0299	12	4.7
Diseases of the blood and blood forming organs and immune mechanism			
Anaemia	0301	34	13.2
Immunodeficiency disorder (excluding AIDS)	0303	1	0.4
Other diseases of blood and blood forming organs and immune mechanism	0399	15	5.8
Endocrine, nutritional and metabolic disorders			
Disorders of the thyroid gland	0401	43	16.7
Diabetes mellitus–type 1 (IDDM)	0402	8	3.1
Diabetes mellitus–type 2 (NIDDM)	0403	52	20.2
Diabetes mellitus–other specified/unspecified/unable to be specified	0404	4	1.6
Malnutrition	0405	7	2.7
Nutritional deficiencies	0406	44	17.1
Obesity	0407	9	3.5

Condition	ACAP code	N	%
High cholesterol	0408	86	33.3
Other endocrine, nutritional and metabolic disorders	0499	22	8.5
Diseases of the nervous system			
Meningitis and encephalitis (excluding 'viral')	0601	1	0.4
Motor neurone disease	0603	2	0.8
Parkinson's disease	0604	24	9.3
Transient cerebral ischaemic attacks	0605	27	10.5
Brain disease/ disorders	0606	3	1.2
Multiple sclerosis	0607	3	1.2
Epilepsy	0608	7	2.7
Cerebral palsy	0610	2	0.8
Paralysis-non-traumatic	0611	8	3.1
Other diseases of the nervous system	0699	67	26.0
Diseases of the eye and adnexa			
Cataracts	0701	32	12.4
Glaucoma	0702	38	14.7
Blindness	0703	27	10.5
Poor vision	0704	41	15.9
Other diseases of the eye and adnexa	0799	31	12.0
Disease of the ear and mastoid process			
Ménière's disease	0801	11	4.3
Deafness/hearing loss	0802	69	26.7
Other diseases of the ear and mastoid process	0899	11	4.3
Diseases of the circulatory system			
Heart disease	0900	60	23.3
Angina	0903	6	2.3
Myocardial infarction	0904	18	7.0
Acute and chronic ischaemic heart disease	0905	41	15.9
Congestive heart failure	0906	45	17.4
Other heart diseases	0907	56	21.7
Cerebrovascular disease	0910	6	2.3
Subarachnoid haemorrhage	0911	2	0.8
Intracerebral haemorrhage	0912	2	0.8

Condition	ACAP code	N	%
Other intracranial haemorrhage	0913	5	1.9
Cerebral infarction	0914	5	1.9
Stroke (CVA)	0915	45	17.4
Other cerebrovascular diseases	0916	11	4.3
Other diseases of the circulatory system	0920	9	3.5
Hypertension	0921	174	67.4
Hypotension	0922	18	7.0
Abdominal aortic aneurysm	0923	5	1.9
Other arterial or aortic aneurysms	0924	5	1.9
Atherosclerosis	0925	3	1.2
Other diseases of the circulatory system n.e.s	0999	35	13.6
Diseases of the respiratory system			
Influenza and pneumonia	1002	15	5.8
Acute lower respiratory infections	1003	8	3.1
Other diseases of the respiratory system	1004	14	5.4
Chronic lower respiratory diseases	1005	56	21.7
Other diseases of upper respiratory tract	1099	7	2.7
Diseases of the digestive system			
Diseases of the intestine	1101	103	39.9
Diseases of the peritoneum	1102	2	0.8
Diseases of the liver	1103	7	2.7
Other diseases of the digestive system	1199	101	39.1
Diseases of the skin and subcutaneous tissue			
Skin and subcutaneous tissue infections	1201	18	7.0
Skin allergies	1202	31	12.0
Other diseases of the skin and subcutaneous tissue	1299	34	13.2
Diseases of the musculoskeletal system and connective tissue			
Rheumatoid arthritis	1301	8	3.1
Other arthritis and related disorders	1302	213	82.6
Deformities of joints/ limbs–acquired	1303	9	3.5
Back problems–dorsopathies	1304	32	12.4
Other soft tissue/ muscle disorders	1305	17	6.6
Osteoporosis	1306	88	34.1

Condition	ACAP code	N	%
Other disorders of the musculoskeletal system and connective tissue	1399	39	15.1
Diseases of the genitourinary system			
Kidney and urinary system (bladder) disorders	1401	59	22.9
Urinary tract infection	1402	46	17.8
Stress/urinary incontinence	1403	180	69.8
Other diseases of the genitourinary system	1499	34	13.2
Congenital malformations, deformations and chromosomal abnormalities			
Down's syndrome	1503	1	0.4
Other chromosomal abnormalities	1504	1	0.4
Other congenital malformations and deformations	1599	1	0.4
Injury, poisoning and certain other consequences of external causes			
Injuries to the head	1601	5	1.9
Injuries to arm/hand/shoulder	1602	20	7.8
Injuries to leg/knee/foot/ankle/ hip	1603	23	8.9
Amputation of the finger/thumb/hand/arm/shoulder–traumatic	1604	2	0.8
Amputation of toe/ankle/foot/leg–traumatic	1605	9	3.5
Fracture of neck	1606	6	2.3
Fracture of rib(s), sternum and thoracic spine	1607	18	7.0
Fracture of lumbar spine and pelvis	1608	26	10.1
Fracture of shoulder, upper arm and forearm	1609	15	5.8
Fracture at wrist and hand level	1610	7	2.7
Fracture of femur	1611	33	12.8
Fracture of lower leg and foot	1612	5	1.9
Other injury, poisoning and consequences of external causes	1699	8	3.1
Symptoms and signs n.o.s or n.e.s			
Breathing difficulties/ shortness of breath	1703	17	6.6
Pain	1704	118	45.7
Nausea and vomiting	1705	5	1.9
Dysphagia	1706	29	11.2
Bowel/faecal incontinence	1707	98	38.0
Unspecified urinary incontinence	1708	26	10.1

Condition	ACAP code	N	%
Retention of urine	1709	1	0.4
Jaundice (unspecified)	1710	1	0.4
Disturbances of skin sensation	1711	2	0.8
Rash and other nonspecific skin eruption	1712	3	1.2
Abnormal involuntary movements	1713	3	1.2
Abnormalities of gait and mobility	1714	53	20.5
Falls (frequent with unknown aetiology)	1715	119	46.1
Confusion	1716	31	12.0
Amnesia	1717	22	8.5
Dizziness and giddiness	1718	15	5.8
Restlessness and agitation	1719	4	1.6
Irritability and anger	1721	4	1.6
Speech and voice disturbances	1725	10	3.9
Headache	1726	4	1.6
Malaise and fatigue	1727	32	12.4
Blackouts, fainting, convulsions	1728	3	1.2
Oedema (not specified)	1729	107	41.5
Symptoms and signs concerning food and fluid intake	1730	29	11.2
Other symptoms and signs	1799	1	0.4
Other health condition not elsewhere specified	1899	82	31.8

n.e.s. = not elsewhere specified; n.o.c. = not otherwise classified

Appendix B.D: Most prevalent medications

Top 10 most prevalent medications used by the GRACE cohort in the 12 months prior to enrolment in the study. N refers to the number of residents who were supplied each medication at least once.

Medication	ATC Code	N (%)
Macrogol	A06AD15	82 (36.0)
Furosemide	C03CA01	76 (33.3)
Pantoprazole	A02BC02	69 (30.3)
Cephalexin	J01DB01	64 (28.1)
Hypromellose and carboxymethylcellulose (eye drops/gel)	S01XA20	57 (25.0)
Amoxicillin and clavulanic acid	J01CR02	50 (21.9)
Paracetamol	N02BE01	50 (21.9)
Denosumab	M05BX04	46 (20.2)
Trimethoprim	J01EA01	44 (19.3)
Oxycodone	N02AA05	42 (18.4)

Appendix B.E: MBS codes and usage

List of healthcare services accessed by the GRACE cohort in the 12 month prior to enrolment (classified MBS category descriptions). N refers to the number of residents who accessed the service at least once.

MBS category description	MBS category code	N (%)
Professional attendances		
General practitioner attendances to which no other item applies	A1	94 (38.7)
Specialist attendances to which no other item applies	A3	77 (31.7)
Consultant psychiatrist attendances to which no other item applies	A8	6 (2.5)
Urgent attendance after hours	A11	150 (61.7)
Health assessments by general practitioners	A14	157 (64.6)
General practitioner management plans, team care arrangements, multidisciplinary care plans	A15	197 (81.1)
Domiciliary and residential management reviews	A17	150 (61.7)
Attendances by medical practitioners who are emergency physicians (private only)	A21	9 (3.7)
General practitioner after-hours attendances to which no other item applies	A22	207 (85.2)
Attendance by specialist in geriatric medicine	A28	26 (10.7)
Diagnostic imaging services		
Ultrasound	I1	71 (29.2)
Computerised tomography	I2	59 (24.3)
Diagnostic radiology	I3	99 (40.7)
Nuclear medicine	I4	6 (2.5)
Magnetic resonance imaging	I5	13 (5.3)
Pathology services		
Haematology services	P1	167 (68.7)
Chemical services	P2	204 (84.0)
Microbiology services	P3	195 (80.3)
Immunology services	P4	27 (9.9)
Tissue pathology	P5	33 (13.6)
Cytology services	P6	6 (2.5)
Genetic tests	P7	3 (1.2)
Simple basic pathology tests	P9	2 (0.8)

Patient episode initiation	P10	226 (93.0)
Specimen referred testing	P11	3 (1.2)
Therapeutic services		
Surgical operative services	T8	61 (25.1)
Miscellaneous services		
Allied health services	M3	155 (63.8)

Appendix B.F: Core stool and oropharyngeal taxa

Prevalence and relative abundance of species identified as core in the stool and oropharyngeal microbiome of GRACE participants.

Species name	Prevalence %	Median abundance (range), %
Stool		
Roseburia faecis	63.2	0.10 (0, 30.9)
Alistipes finegoldii	72.5	0.10 (0, 17.5)
Clostridium leptum	90.2	0.12 (0, 4.7)
Clostridium innocuum	95.1	0.13 (0, 13.3)
Blautia wexlerae	77.9	0.13 (0, 16.6)
Eubacterium eligens	62.7	0.14 (0, 13.6)
Streptococcus salivarius	84.8	0.15 (0, 13.9)
Flavonifractor plautii	90.7	0.16 (0, 6.4)
Firmicutes bacterium CAG 83	68.6	0.17 (0, 13.5)
Bacteroides thetaiotaomicron	78.9	0.18 (0, 12.2)
Eubacterium hallii	76.0	0.20 (0, 12.5)
Parabacteroides merdae	68.6	0.22 (0, 10.5)
Escherichia coli	77.0	0.23 (0, 43.6)
Dorea formicigenerans	69.6	0.24 (0, 6.3)
Gordonibacter pamelaeae	98.5	0.28 (0, 5.4)
Eubacterium siraeum	77.9	0.31 (0, 10.3)
Bacteroides dorei	67.2	0.36 (0, 22.6)
Fusicatenibacter saccharivorans	69.6	0.48 (0, 12.3)
Blautia obeum	82.4	0.49 (0, 8.4)
Ruminococcus gnavus	89.2	0.50 (0, 57.4)
Ruthenibacterium lactatiformans	99.5	0.53 (0, 15.0)
Parabacteroides distasonis	86.8	0.56 (0, 13.4)
Alistipes putredinis	71.6	0.63 (0, 7.4)
Bifidobacterium longum	70.1	0.73 (0, 60.2)
Bacteroides vulgatus	73.0	0.96 (0, 31.2)
Faecalibacterium prausnitzii	83.3	0.98 (0, 19.0)
Eggerthella lenta	97.1	1.2 (0, 18.9)
Anaerostipes hadrus	80.4	1.3 (0, 34.4)
Bacteroides uniformis	87.3	2.1 (0, 28.2)

Oropharyngeal		
Prevotella salivae	60.3	0.11 (0, 4.1)
Veillonella infantium	69.2	0.16 (0, 3.4)
Actinomyces oris	73.4	0.26 (0, 29.9)
Gemella haemolysans	74.7	0.27 (0, 40.5)
Prevotella histicola	60.3	0.31 (0, 31.1)
Streptococcus oralis	79.3	0.37 (0, 54.7)
Prevotella melaninogenica	70.0	0.63 (0, 33.4)
Veillonella parvula	84.0	0.67 (0, 42.0)
Rothia dentocariosa	80.2	0.69 (0, 64.0)
Gemella sanguinis	75.9	0.75 (0, 17.2)
Veillonella dispar	75.1	0.84 (0, 21.9)
Rothia mucilaginosa	94.1	1.4 (0, 57.8)
Streptococcus mitis	84.4	1.6 (0, 93.4)
Veillonella atypica	78.5	2.3 (0, 32.0)
Streptococcus parasanguinis	97.0	8.0 (0, 45.9)
Streptococcus salivarius	91.6	10.5 (0, 70.0)

Appendix B.G: Data completeness

Data completeness in the GRACE dataset varies by data source and individual variables. We have presented the availability of data for variables reported throughout the text in the below table.

Data Item	Availability % (N)
Demographics	
Age	100 (279)
Sex	100 (279)
Memory support room	100 (279)
Single or shared room	100 (279)
Medical needs	
Urinary catheter *in situ*	100 (279)
Urostomy	100 (279)
Vascular catheter *in situ*	100 (279)
Tracheostomy	100 (279)
Colostomy/ileostomy	100 (279)
Wound care (type)	99.3 (277)
Carriage of MDRO	100 (279)
Diet	
Diet type	99.6 (278)
Meal texture	100 (279)
Liquid texture	100 (279)
Prescribed supplements	96.8 (270)
ACFI: ADL	
Nutrition	98.2 (274)
Mobility	98.2 (274)
Personal hygiene	98.2 (274)
Toileting	98.2 (274)
Continence	97.8 (273)
Total ADL score	98.9 (276)
ACFI: Behaviour	
Cognitive skills	98.2 (274)
Wandering	98.2 (274)

Verbal behaviour	98.2 (274)
Physical behaviour	98.2 (274)
Depression	98.2 (274)
PAS-CIS score	51.3 (143)
Total behaviour score	97.5 (272)
ACFI: CHC	
Medication	98.2 (274)
Complex healthcare	98.2 (274)
Total CHC score	98.9 (276)
ACFI: Mental and behavioural diagnoses	
Mental and behavioural diagnoses	100 (279)
Impairment level	98.2 (274)
ACFI: Comorbidities	
ACAP diagnosis codes	92.5 (258)
DHS-linked data	
PBS	81.7 (228)
MBS	87.1 (243)
Sample availability for microbiome and resistome composition	
Oropharyngeal swab	84.9 (237)
Stool sample	73.1 (204)
Both	69.5 (194)

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APPENDIX C: GRACE COHORT PROFILE MANUSCRIPT

Contents: Manuscript of comparative analysis between GRACE participants and the ROSA historical cohort to assess representativeness currently available on medrxiv (https://doi.org/10.1101/2022.10.26.22281199).

Cohort Profile: The Australian Generating evidence on antimicrobial resistance in the aged care environment (GRACE) study; alignment with national population characteristics

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ABSTRACT

Purpose:

The emergence of antibiotic-resistant bacteria represents a considerable threat to human health, particularly for vulnerable populations such as those living in residential aged care. However, antimicrobial resistance (AMR) carriage and modes of transmission remain incompletely understood. The Generating evidence on antimicrobial Resistance in the Aged Care Environment (GRACE) study was established to determine principal risk factors of AMR carriage and transmission in residential aged care facilities (RACF).

Participants:

Between March 2019 and March 2020, 279 participants were recruited from five South Australian RACFs. The median age was 88.6 years, the median period in residence was 681 days, and 71.7% were female. A dementia diagnosis was recorded in 54.5% and more than two thirds had moderate to severe cognitive impairment (68.8%). Sixty-one percent had received at least one course of antibiotics in the 12 months prior to enrolment.

Findings to date:

To investigate the representation of the GRACE cohort to Australians in residential aged care, its characteristics were compared to a subset of the historical cohort of the Registry of Senior Australians (ROSA). This included 142,923 individuals who were permanent residents of RACFs on June 30th, 2017. GRACE and ROSA cohorts were similar in age, sex, and duration of residential care, prevalence of health conditions, and recorded dementia diagnoses. Differences were observed in care requirements and antibiotic exposure (both higher for GRACE participants). GRACE participants had fewer hospital visits compared to the ROSA cohort, and a smaller proportion were prescribed psycholeptic medications.

Future plans:

Participant and built environment metagenomes will be used to determine microbiome and resistome characteristics. Individual and facility risk exposures will be aligned with metagenomic data to identify principal determinants for AMR carriage. Ultimately, this analysis will inform measures aimed at reducing the emergence and spread of antibiotic resistant pathogens in this high-risk population.

KEYWORDS

Infection control; geriatric medicine; microbiology

Abbreviations

GRACE	*Generating evidence on antimicrobial Resistance in the Aged Care Environment*
ROSA	*Registry Of Senior Australians*
RACF	*Residential Aged Care Facility*
AMR	*Antimicrobial Resistance*
MDRO	*Multi-Drug Resistant Organism*
PBS	*Pharmaceutical Benefits Scheme*
MBS	*Medicare Benefits Schedule*
DHS	*Department of Human Services*
ACFI	*Aged Care Funding Instrument*
PAS-CIS	*Psychogeriatric Assessment Scales – Cognitive Impairment Scale*
GP	*General Practitioner*

Strengths and limitations of this study

- The GRACE study captured a diverse array of data; demographics, medications, personal and medical care, RACF management practices, as well as oropharyngeal, intestinal, and environmental metagenomic data, allowing detailed analysis of exposure-resistome relationships.
- A high rate of participant recruitment (75% of eligible residents) was achieved, representing the spectrum of resident characteristics and care needs. This included a representative proportion of individuals with moderate or severe cognitive impairment.
- The main limitation of this cohort resulted from the early cessation of recruitment, due to stringent facility access regulations resulting from the COVID-19 pandemic. While a high recruitment rate partially compensated in terms of cohort size, we were unable to complete recruitment at our fifth site or begin recruitment at two further sites.
- Ethnic and linguistic data was not captured and so could not be compared between cohorts.

INTRODUCTION

In keeping with trends globally, Australia is experiencing significant ageing of its population.[1] By 2031, 21% of Australians will be aged over 65 years.[2] Of Australians over 65, 6% currently live in residential aged care facilities (RACFs), and of those 85 years and over, 30% do.[2,3] The threat of increasing rates of infection caused by multidrug-resistant organisms (MDRO) is particularly serious in RACFs. High rates of antibiotic prescription, poor antimicrobial stewardship, and the potential for microbial transmission between residents, all contribute to growing rates of multidrug-resistant clinical isolates.[4-6] However, the prevalence of antimicrobial resistance (AMR) in asymptomatic individuals (carried either by pathogens or commensal microbes), or the dispersal of MDRO within the RACF environment, is largely uncharacterised. Despite serious concerns about a growing inability to readily treat common infections, and the potential for RACF populations to contribute to AMR carriage within the wider community, sufficiently detailed data to support the development of effective measures to limit the spread of MDRO in aged care simply do not exist.

The Generating evidence on Resistant bacteria in the Aged Care Environment (GRACE) study enrolled residents from five RACF located in metropolitan Adelaide, South Australia and aims to address five questions that are fundamental to developing strategies to reduce AMR carriage in RACF residents: 1) What factors determine the types and levels of AMR determinants carried by RACF residents? 2) To what extent is there evidence of AMR transmission between RACF residents? 3) Is interaction with the RACF built environment likely to facilitate AMR transmission? 4) Do hospital visits for acute care significantly influence types and levels of AMR carriage? 5) To what extent do ageing-associated changes in gut microbiology influence AMR carriage?

To address these research questions, participants were invited to provide stool and oropharyngeal samples for metagenomic analysis to determine microbiome and resistome

characteristics. Environmental samples were also collected from areas within each facility. Metagenomic data will be related to a range of factors, including facility variables, resident demographics, morbidity, and polypharmacy data, to identify influences on AMR carriage and potential transmission.

Prior to these analyses, we compared GRACE cohort characteristics with those of aged care residents within the national historical cohort of the Registry of Senior Australians (ROSA) which contains data for more than 2.8 million Australians aged over 65 who accessed government-subsidised aged services from 1997 to 2017.[7] Our comparison assessed whether the GRACE cohort was representative of the wider Australian aged care population, and its validity as a basis to provide further insight.

COHORT DESCRIPTION

Study design and population

GRACE is a prospective, cohort study of permanent residents of RACFs recruited between March 2019 and March 2020. All eligible residents living in participating facilities at the time of recruitment and/or their next of kin were approached by a research nurse to provide informed consent. In addition to the consent form, study information was made available in the form of a video, a two-page brochure and on a website. Consent could be provided for one or all study procedures, including the collection of stool and/or oropharyngeal samples, collection of facility-level medical records and access to data held by the Medicare Benefits Schedule (MBS) and Pharmaceutical Benefits Scheme (PBS). Participants were not eligible if: 1) they were in respite care, 2) they were receiving palliative/end-of-life care, 3) it was recommended by management that they not be approached, and 4) we were unable to contact next of kin where third-party consent was required. Participants who required third-party consent, such as those with cognitive impairment, were identified by the participating facility and communicated to the study team. GRACE aimed to recruit 400 residents across 10 RACFs. However, due to the COVID-19 pandemic, and the imposition of strict facility entry restrictions, recruitment was ceased, resulting in a sample size of 279 residents from five facilities, with a mean recruitment rate of 75%. Site 1 was excluded from this mean as data on eligibility and participants who declined was not recorded.

Of 403 residents assessed for eligibility, 344 were approached to join the study and 279 consented to participate (Figure 1). Fifty-nine residents were ineligible and 65 declined to participate (excluding site 1). Of those who consented, 111 (39.8%) provided consent themselves, and 168 (60.2%) provided third-party consent. Two-hundred and seventy-three residents (97.8%) provided consent for Department of Human Services (DHS) data access, with MBS and PBS data available for 243 and 228 residents, respectively.

Data collection

Participant and facility data were collected at the close of recruitment at each site and included facility medical records. Information held by the PBS and MBS from the DHS was requested after all recruitment was complete. Demographical data (including age and sex), as well as data on participant living arrangements (time spent in current facility, room type, room security) were collected from facilities. In addition, data on care requirements was collected from facilities via the Aged Care Funding Instrument (ACFI), a tool used on entry to an RACF to determine the funding needed for a person's care.[8] This includes three domains representing different areas of care needs: Activities of Daily Living, Cognition and Behaviour, and Complex Healthcare. Activities of Daily Living, includes details of care required for eating, showering, toileting and general mobility. Cognition and Behaviour domain measures the cognitive skills, verbal and physical behaviour, and mental health of individuals. Complex Healthcare considers the support residents need to manage their medications and health conditions. The ACFI also includes data on cognitive and behavioural conditions, which we have used to determine the presence of dementia in our cohort. Cognitive impairment scores pre-calculated using the Psychogeriatric Assessment Scales – Cognitive Impairment Scale (PAS-CIS) method were also obtained from the ACFI data.[9] Details of hospitalisations in the 12 months prior to enrolment, diet type and texture, and medical care data (wound care, medical devices) were collected from the facility records.

Data collected from the PBS included medications prescribed during the 12 months prior to study enrolment for each participant. Specifically, data were obtained relating to medications that might directly or indirectly influence the microbiome and care needs (antibiotics, antivirals, antimycotics, medicines for constipation and acid-related disorders, insulin, antidiabetics, opioids, anti-inflammatories, corticosteroids, immunosuppressants, hormones,

lipid-modifying and beta-blocking agents, antidementia medication, antidepressants, and psycholeptics (includes antipsychotics, anxiolytics, sedatives/hypnotics)). Data collected from the MBS included general practitioner (GP) attendances, specialist attendances, allied health services, surgery, diagnostic imaging services, health assessments, and access of pathology services during the 12 months prior to study enrolment for each participant. Definitions and coding of these variables can be found in Supplementary Table 1.

RxRisk is an established tool to determine a person's actively managed health conditions using their medication data and was used to compare health conditions between GRACE and ROSA.[10] In GRACE, RxRisk health conditions were able to be assessed for 228 participants as this relied on PBS data availability. Dementia is reported using both the RxRisk method and the ACFI diagnosis as per previously reported.[7]

Comparison with the national aged care data

Data from the National Historical Cohort of the Registry of Senior Australians (ROSA) was used to evaluate the extent to which the study cohort was representative of the national residential aged care population.[7] ROSA includes Australians aged 65 years and over who accessed government-subsidised aged care services between 1997 and 2017. ROSA has integrated information from the aged care sector with various health care data sources. Datasets within ROSA include: Australian Institute of Health and Welfare's National Aged Care Data Clearinghouse datasets, Australian Government Medicare Benefits Schedule (MBS) and Pharmaceutical Benefits Scheme (PBS), state health authorities' hospitalisations (QLD, NSW, VIC, SA), and ambulance datasets (NSW, SA). All data were de-identified and integrated by approved agencies (Australian Institute of Health and Welfare, Centre for Health Record Linkage, Centre for Victorian Data Linkage, SA NT DataLink and Queensland Health's Statistical Services Branch). Details of ROSA datasets, variables, definitions, and limitations

have been published previously.[7] The June 30th, 2017 (latest available data at the time of the study), non-Indigenous national cohort of permanent residents of RACFs (n=142,923) was obtained from ROSA for comparison to the GRACE cohort. Analysis focusing on MBS subsidized health care services only included individuals without Department of Veterans' Affairs cards (n=123,555), and analysis focusing on hospitalization records only included individuals living in NSW, VIC, SA, and QLD (n=125,351).

Statistical Analysis

Descriptive statistics were used to summarise characteristics of both GRACE and ROSA derived populations. For continuous data, median (IQR) was reported. For categorical data, percent and number of participants was reported. GRACE data was exported, cleaned, and analysed in Statistical Analysis Software (SAS) University Edition (SAS Studio v3.8/SAS v9.4).

FINDINGS TO DATE

Demographics

A comparison of participant clinical data between GRACE (n=279) and ROSA (n=142,923) is shown in Table 1. GRACE participants had a median age of 88.6 (IQR=81.8-93.2) years, which was similar to that of ROSA (med=87.4, IQR=81.6-91.7). GRACE and ROSA participants were mostly female (GRACE=71.7%, n=200; ROSA=68.4%, n=97,706), had a similar prevalence of dementia (GRACE=54.5%, n=152, ROSA=53.6%, n=76,594), and residents had been in their current facility for a similar period of time at recruitment/data collection (GRACE: med=681 days, IQR=252-1147; ROSA: med=689 days, IQR=283-1391). GRACE participants all lived in metropolitan facilities, run by not-for-profit organisations, whereas ROSA participants lived in a number of locations and organisation types.

Care requirements

Care requirements represented by the three ACFI domains were assessed for both datasets (Table 1). Activities of Daily Living (ADL) care requirements were greater in the GRACE cohort compared to ROSA, with 65.9% (n=184) having a high care requirement for this domain, compared to 54.3% in ROSA (n=77,552). Cognition and Behaviour care requirements for GRACE were less than those in ROSA, with 47.0% (n=131) and 60.3% (n=86,117) having a high care score, respectively. GRACE had a higher proportion of participants with a high care requirement for Complex Healthcare (64.5%, n=180) compared to the ROSA cohort (53.3%, n=76,228).

Utilisation of healthcare services subsidised by Medicare

In the 12 months prior to enrolment/data collection, the proportions of the GRACE and ROSA cohorts that had accessed an MBS-subsidised healthcare service were similar (GRACE=100%, n=243; ROSA=99.4%, n=122,875). GRACE participants utilised GP services for non-urgent out of hours care most commonly (GRACE=85.5%, n=207; ROSA=54.7%, n=67,643; Table 1). More GRACE participants accessed urgent out of hours GP services (61.7%, n=150) compared to ROSA (33.1%, n=40,904). Both cohorts accessed standard GP attendances similarly (GRACE=38.7%, n=94; ROSA=45.7%, n=56,465). GRACE participants had team care plans (in which multidisciplinary teams manage a case; GRACE=81.1%, n=197; ROSA=56.0%, n=69,213) and collaborative domiciliary and residential management reviews (in which a GP and pharmacist review ongoing medication for a resident; GRACE=61.7%, n=150; ROSA=33.7%, n=41,696) more commonly than those in the ROSA.

GRACE and ROSA cohorts had a similar level of pathology service utilisation, with patient episode initiations the most frequently accessed service for each (GRACE=93.0%, n=226; ROSA=88.9%, n=109,896; Table 1). Of all pathology services captured, access of microbiology services differed most between the datasets (GRACE=80.3%, n=195; ROSA=65.0%, n=80,366).

Hospitalisations

GRACE had a smaller proportion of participants with at least 1 hospitalisation recorded in the 12 months prior to enrolment/data collection (31.5%, n=88) compared to ROSA (48.2%, n=60,409) The median number of hospitalisations per resident was similar (GRACE: med=1, IQR=1-2; ROSA: med=2, IQR=1-3; Table 1). GRACE also had a smaller proportion of participants with at least 1 emergency department presentation in the 12 months prior to enrolment/data collection (GRACE=26.2%, n=73; ROSA=44.7%, n=56,016).

Medications

At least 1 medication had been dispensed in the 12 months prior to enrolment/data collection for 97.8% (n=223) and 99.3% (n=141,893) of the GRACE and ROSA cohorts, respectively. GRACE participants were taking less medications per person (med=5, IQR=2-6) compared to ROSA (med=13, IQR=9-18; Table 1). Antibiotics were the most commonly supplied drug class to both cohorts during the 12 months prior to enrolment/data collection, but GRACE had a fewer number of participants who were supplied antibiotics (61.0%, n=139) compared to ROSA (74.5%, n=106,427). Psycholeptics were supplied to fewer participants in GRACE (31.1%, n=71) compared to ROSA (47.2%, n=67,465).

Table 8. *Characteristics of GRACE study participants compared to the national population in ROSA.*

	GRACE (n=279)	**ROSA (n=142,923)**
Demographics		
Age, median (IQR) (years)	88.6 (81.8-93.2)	87.4 (81.6-91.7)
Sex, % (n)		
Female	71.7 (200)	68.4 (97,706)
Male	28.3 (79)	31.6 (45,217)
Facility location, % (n) †		
Major city	100 (279)	70.1 (100,140)
Outside major city	0 (0)	29.8 (42,525)
Organisation type, % (n) †		
Government	0 (0)	4.0 (5,787)
Not-for-profit	100 (279)	56.7 (80,992)
For-profit	0 (0)	39.2 (55,958)
Days lived in facility, median (IQR)	681 (252-1147)	689 (283-1391)
Dementia diagnosis, % (n)* †	54.5 (152)	53.6 (76,594)
Care requirements (ACFI)		
Activities of Daily Living, % (n)^ †		
High	65.9 (184)	54.3 (77,552)
Medium	26.5 (74)	30.9 (44,157)
Low	6.5 (18)	13.5 (19,280)
Nil	0 (0)	0.6 (874)
Cognition and behaviour, % (n)^ †		
High	47.0 (131)	60.3 (86,117)
Medium	33.0 (92)	22.8 (32,629)
Low	17.5 (49)	11.5 (16,502)
Nil	0 (0)	4.6 (6,615)
Complex healthcare, % (n)^ †		
High	64.5 (180)	53.3 (76,228)
Medium	28.3 (79)	28.6 (40,863)
Low	6.1 (17)	15.4 (22,066)
Nil	0 (0)	1.9 (2,706)

Healthcare services, % accessed at least once in 12 months prior to enrolment (n)^		
At least 1 healthcare service accessed, % (n)	100 (243)	99.4 (122,875)
GP attendance	38.7 (94)	45.7 (56,465)
GP attendance after hours	85.5 (207)	54.7 (67,643)
Specialist attendance	31.7 (77)	27.4 (33,822)
GP management plans, team care arrangements, multidisciplinary care plans	81.1 (197)	56.0 (69,213)
Collaborative domiciliary and residential management reviews	61.7 (150)	33.7 (41,696)
Diagnostic imaging (any, per resident)	51.4 (125)	44.6 (55,126)
Health assessments	64.6 (157)	44.8 (55,375)
Geriatric medicine	10.7 (26)	7.3 (9,038)
Urgent attendance after hours	61.7 (150)	33.1 (40,904)
Medical practitioner (emergency physician) attendance	3.7 (9)	2.0 (2,516)
Allied health services	63.8 (155)	42.2 (52,164)
Surgical operations	25.1 (61)	22.9 (28,312)
Psychiatrist attendance	2.5 (6)	3.4 (4,153)
Pathology services		
Patient episode initiations	93.0 (226)	88.9 (109,896)
Chemical	84.0 (204)	78.0 (96,374)
Microbiology	80.3 (195)	65.0 (80,366)
Haematology	68.7 (167)	57.9 (71,556)
Tissue	13.6 (33)	12.3 (15,249)
Immunology	9.9 (27)	6.7 (8,285)
Cytopathology	2.5 (6)	1.7 (2,146)
Genetics	1.2 (3)	0.5 (631)
Simple basic tests	0.8 (2)	0.5 (622)
Specimen referred	1.2 (3)	1.7 (2,067)
Hospitalisation in the 12 months prior to enrolment		
Emergency department presentations per resident, median (IQR)	1 (1-1)	1 (1-3)
At least 1 emergency department presentation, % (n)	26.2 (73)	44.7 (56,016)
Hospital separations per resident, median (IQR)	1 (1-2)	2 (1-3)
At least 1 hospital separation, % (n)	31.5 (88)	48.2 (60,409)
Medications prescribed in the 12 months prior to enrolment^		
Medicines supplied per person, median (IQR)	5 (2,6)	13 (9,18)

At least 1 medication dispensed, % (n)	97.8 (223)	99.3 (141,893)
At least 1 dispensed, % (n)		
Antibiotics	61.0 (139)	74.5 (106,427)
Antivirals	1.8 (4)	1.8 (2,524)
Antimycotics	0.4 (1)	0.5 (686)
Medicines for constipation	36.0 (82)	45.1 (64,434)
Medicines for acid-related disorders	49.1 (112)	51.5 (73,550)
Insulin	7.5 (17)	6.6 (9,393)
Antidiabetics	11.4 (26)	14.2 (20,262)
Opioids	44.7 (102)	48.2 (68,819)
Anti-inflammatory/antirheumatic	7.5 (17)	9.5 (13,649)
Corticosteroids	14.9 (34)	16.2 (23,114)
Other immunosuppressants	0.9 (2)	0.6 (911)
Sex hormones	4.8 (11)	3.5 (4,973)
Lipid-modifying agents	25.4 (58)	36.7 (52,409)
Beta-blocking agents	26.3 (60	28.7 (40,969)
Antidementia	8.3 (19)	10.3 (14,754)
Antidepressants	43.4 (99)	48.3 (68,982)
Psycholeptics	31.1 (71)	47.2 (67,465)

* extracted from aged care funding instrument data

^ missing data GRACE: activities of daily living care requirement, 1.1%; cognition and behaviour care requirement, 2.5%; complex healthcare care requirement, 1.1%; healthcare services, 12.9%; medications, 18.3%.

† missing data ROSA: facility location, 0.2%; organisation type, 0.1%; dementia diagnosis, 0.7%; activities of daily living care requirement, 0.7%; cognition and behaviour care requirement, 0.7%; complex healthcare care requirement, 0.7%.

Health conditions

The median number of RxRisk conditions per participant did not differ between the two cohorts (both: med=5, IQR=3-7; Table 2). In GRACE, the most common RxRisk conditions included gastro-oesophageal reflux disease (49.1%, n=112), pain (44.7%, n=102) and depression (43.0%, n=98). Gastro-oesophageal reflux disease was also the most common condition in ROSA (49.0%, n=69,977), followed by depression (45.7%, n=65,351) and hypertension (43.1%, n=61,542). Compared to ROSA, the GRACE cohort had a higher proportion of participants being treated for osteoporosis/Paget's disease (GRACE=23.7%, n=54; ROSA=15.6%, n=22,306) and hypothyroidism (GRACE=17.5%, n=40; ROSA=10.8%, n=15,450). Most conditions were similar in their prevalence between the two datasets.

Table 9. *RxRisk-V health conditions for GRACE participants compared to ROSA.*

	GRACE (n=228)	ROSA (n=142,923)
RxRisk-V condition, % (n)^		
Gastro-oesophageal reflux disease	49.1 (112)	49.0 (69,977)
Hyperlipidaemia	25.4 (58)	32.1 (45,927)
Hypertension	40.4 (92)	43.1 (61,542)
Ischaemic heart disease: hypertension	32.9 (75)	35.6 (50,863)
Antiplatelets	19.3 (44)	19.1 (27,258)
Depression	43.0 (98)	45.7 (65,351)
Pain	44.7 (102)	41.2 (58,921)
Anticoagulants	21.9 (50)	17.4 (24,838)
Chronic airways disease	24.1 (55)	22.0 (31,493)
Congestive heart failure	18.4 (42)	17.0 (24,362)
Osteoporosis/Paget	23.7 (54)	15.6 (22,306)
Psychotic illness	13.2 (30)	15.9 (22,680)
Diabetes	15.0 (34)	15.4 (22,025)
Steroid responsive disease	14.0 (32)	10.2 (14,525)
Arrythmia	10.5 (24)	10.1 (14,389)
Anxiety	17.1 (39)	14.5 (20,742)
Glaucoma	11.0 (25)	11.3 (16,120)
Ischaemic heart disease: angina	11.0 (25)	9.7 (13,812)
Dementia	8.3 (19)	16.5 (23,520)
Hypothyroidism	17.5 (40)	10.8 (15,450)
Inflammation/pain	7.5 (17)	5.8 (8,247)
Gout	7.0 (16)	5.9 (8,403)
Parkinson's disease	11.8 (27)	7.3 (10,435)
Epilepsy	7.0 (16)	9.2 (13,167)
Liver failure	0 (0)	0.1 (81)
Incontinence	3.5 (8)	2.8 (4,063)
Benign prostatic hyperplasia	3.1 (7)	2.8 (4,064)
Malignancies	1.3 (3)	1.5 (2,174)
Renal disease	2.6 (6)	1.1 (1,630)
Hyperthyroidism	0 (0)	0.9 (1,304)

	Allergies	0 (0)	0.6 (842)
	Migraine	0 (0)	0.6 (810)
	Irritable bowel syndrome	0.4 (1)	0.6 (831)
	Smoking cessation	0.4 (1)	0.4 (581)
	Pancreatic insufficiency	0 (0)	0.4 (547)
	Psoriasis	1.3 (3)	0.5 (655)
	Bipolar disorder	0 (0)	0.5 (675)
	Transplant	0 (0)	0.1 (145)
	Alcohol dependency	0 (0)	<0.1 (39)
	Pulmonary hypertension	0 (0)	<0.1 (37)
	Hepatitis B	0 (0)	<0.1 (19)
	HIV	0 (0)	<0.1 (38)
	Hyperkalaemia	0 (0)	<0.1 (20)
	Malnutrition	0 (0)	0 (0)
	Tuberculosis	0 (0)	<0.1 (<5)
	Hepatitis C	0 (0)	<0.1 (10)
Number of conditions per person, median (IQR)		5 (3,7)	5 (3,7)

^ missing data GRACE, 18.3%.

Additional GRACE datapoints

Some characteristics could not be compared between the GRACE and ROSA cohorts and these are summarised in Supplementary Table 2. Most GRACE participants were staying in their own rooms (97.8%; n=273), with a small proportion also living in memory support areas (12.9%; n=36). Diet type and texture was highly conserved among participants, with 93.9% (n=262) reporting a normal diet, 72.8% reporting normal meal texture (n=203) and 91.4% (n=255) reporting a normal liquid texture. Most participants were receiving a standard fortified diet (56.3%; n=157), but a large proportion were receiving a high energy high protein supplemented diet (39.4%; n=110). Seven (2.5%) participants reported a colostomy/ileostomy. No GRACE participants reported a urinary catheter *in situ*, vascular catheter *in situ*, urostomy,

or a tracheostomy hence they are not included in Supplementary Table 2. Seventy-two (26.0%) participants were receiving wound care at the time of enrolment, with grade 1-2 pressure ulcers the most common wound being treated (6.5%; n=18). Using the pre-calculated PAS-CIS method, GRACE participants were most commonly assigned a moderate impairment score (39.8%; n=111), followed by severe (28.0%; n=78) and mild impairment (27.6%, n=77). Very few were assigned no to minimal impairment (2.9%; n=8).

STRENGTHS AND LIMITATIONS

The primary strength of the GRACE study is the combination of comprehensive demographic, health care, health status, medical, pharmaceutical, and facility variables with intestinal and oropharyngeal microbiome and resistome data for permanent residents of RACFs. The research design developed to establish this cohort has enabled powerful opportunities for novel and extensive investigations currently underway into relationships between risk factors in aged care, current practices in aged care, intestinal health, and disease state outcomes. For example, future work will include investigations assessing associations between cognitive and behavioural diagnoses and the composition of the microbiota to measure the impact of variables in aged care on the increasing burden of cognitive decline.

Another key strength from GRACE was the high rate of recruitment (75%) from residents and families of residents in RACFs. This was most likely attributable to the availability of a research nurse in the study team who personally and extensively communicated with residents and their families. For enhanced rates of recruitment, future studies may also benefit from dedicating significant resources towards communication strategies, particularly when involving elderly populations.

A limitation of the GRACE study resulted from the impact of the SARS-CoV-2 (COVID-19) pandemic. However, whilst this affected the final sample size, the severity of the effect was dampened by the high recruitment rate of participants. In addition, the location of participating RACFs should be considered. These were exclusively in metropolitan areas and may therefore differ in their characteristics from those located in rural or more remote areas. Similarly, only not-for-profit aged care providers participated in the study, who may have had higher staffing capabilities and different approaches to food provisions compared to other types of facilities. Subsequent studies would benefit from a diversified cross-section of RACFs in both

geographical location and funding type. Data relating to resident ethnicity was also not captured.

Specifically in relation to the emergence of antibiotic resistance, potential risk exposures, including high antibiotic use, high medication usage, a high proportion of health conditions experienced, and frequent access of after-hours GP services, were identified in the GRACE cohort, and were reflective of exposures in the wider residential aged care population.

Collaboration

The GRACE team have established a cohort with comprehensively detailed information on overall health, microbiome profiles, and medication use in RACFs. The primary aim for establishing this cohort was to investigate the existence and spread of resistant bacteria in residential aged care to help improve facility management, prevent the spread of harmful bacteria, and ultimately improve the health of aged care residents and the wider community. The authors welcome approaches from other researchers to discuss the potential for collaborative studies that utilise this valuable resource.

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Contributors

LC, EF, LP, MC, MI, and GR conceived and designed the study. LC, CL, EF, and JC acquired the data. LC and CL analysed and interpreted the data. LC, AS and EF drafted the manuscript. All authors provided intellectual input to the manuscript and critically revised the manuscript. All authors have read and approved the final version of the manuscript.

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Competing interests

CW is a board member of the aged care organisation Helping Hand.

Patient and public involvement

RACF staff provided advice on how to engage with participants, families, and other staff members, and the formulation of the participant information data capture instrument. Although residents were not involved in the design, recruitment, conduct, reporting, or dissemination of this study directly, resident committees were involved through presentations at their respective sites. Ongoing feedback is provided to aged care providers and their participating residents via printed, electronic, and in-person communication.

Patient consent for publication

Not applicable.

Ethics approval

Ethics approval for the GRACE study was obtained from the Southern Adelaide Clinical Human Research Ethics Committee (HREC/18/SAC/244). Participants provided written informed consent themselves or where third-party consent was required, a legal guardian or family member with power of attorney provided consent on their behalf. ROSA has been reviewed and approved by the following Human Research Ethics Committees (HREC): University of South Australia HREC (Ref: 200487; October 2017), Australian Institute of Health and Welfare (AIHW Ref: EO2018/1/418; February 2018), the SA Department for Health & Wellbeing HREC (Ref: HREC/18/SAH/90; November 2018), the NSW Population and Health Services Research Ethics Committee (Ref: 2019/ETH12028; September 2019) and the Aboriginal Health Research Ethics Committee (AHREC Ref 04-20-895; September 2020).

Provenance and peer review

Not commissioned.

Data availability statement

The GRACE study data are available upon reasonable request. GRACE study data described in this article are available to all interested researchers through collaboration. Please contact GR (geraint.rogers@sahmri.com). Metagenomic sequencing data will be made available via a public registry once completed. Due to data custodian restrictions related to the sharing of linked data in ROSA, these data cannot be made publicly available to other researchers.

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Figure Legends

Figure 1. GRACE study recruitment and sample collection numbers. * data does not include site 1; NOK = next of kin; OP = oropharyngeal.

SUPPLEMENTARY MATERIALS

Supplementary Table 1. *Health services, pathology, and medicines coding.*

Description	Code
Health Services	**MBS group code**
GP attendance to which no other item applies	A01
GP after hours attendances to which no other item applies	A22
Specialist attendance	A03
GP management plans, team care arrangements, multidisciplinary care plans	A15
Collaborative domiciliary and residential management reviews	A17
Diagnostic imaging^	I*
Health assessments	A14
Geriatric medicine	A28
Urgent attendance after hours	A11
Medical practitioner (emergency physician) attendance to which no other item applies	A21
Allied health services	M03
Surgical operations	T08
Psychiatrist attendance	A08
Pathology Services	**MBS group code**
Patient episode initiations	P10
Chemical	P02
Microbiology	P03
Haematology	P01
Tissue	P05
Immunology	P04
Cytopathology	P06
Genetics	P07
Simple basic tests	P09
Specimen referred	P11
Medicines	**ATC code**
Antibiotics	J01*
Antivirals	J05*
Antimycotics	J02*
Medicines for constipation	A06*
Medicines for acid-related disorders	A02*
Insulin	A10A*
Antidiabetics	A10B*
Opioids	N02A*
Anti-inflammatory/antirheumatic	M01*
Corticosteroids	H02*

	Other immunosuppressants	L04*
	Sex hormones	G03*
	Lipid-modifying agents	C10*
	Beta-blocking agents	C07*
	Antidementia	N06D*
	Antidepressants	N06A*
	Psycholeptics	N05*

^Any MBS group in the diagnostic imaging category

***Supplementary Table 2**. Additional characteristics of GRACE participants.*

	Total (n=279)
Room detail	
Room type, % (n)	
Single	97.8 (273)
Shared	2.2 (6)
Memory support room, % (n)	
Yes	12.9 (36)
No	87.1 (243)
Diet	
Diet type, % (n)^	
Normal	93.9 (262)
Vegetarian	0.4 (1)
Lactose free	3.9 (11)
Gluten free	0.7 (2)
Lactose and gluten free	0.7 (2)
Meal texture, % (n)	
Regular	72.8 (203)
Finger food	0.4 (1)
Soft	12.9 (36)
Minced and moist	7.9 (22)
Pureed	6.1 (17)
Liquidised	0 (0)
Liquid texture, % (n)	
Normal/Thin	91.4 (255)
Slightly thick	1.4 (4)
Mildly thick	5.0 (14)
Moderately thick	1.4 (4)
Extremely thick	0.7 (2)
Prescribed supplementation, % (n)^	
Standard fortified diet	56.3 (157)
High energy & high protein	39.4 (110)
Oral supplement	0 (0)
PEG supplement	0 (0)
Multiple	1.1 (3)
Medical Care	
Colostomy/ileostomy, % (n)	
Yes	2.5 (7)
No	97.5 (272)
Wound care, % (n)^	
Not receiving wound care	73 (205)

Receiving care for multiple wounds	3.2 (9)
Skin tear	5.4 (15)
Pressure ulcer (grade 1-2)	6.5 (18)
Pressure ulcer (grade 3-4)	0.7 (2)
Leg ulcer	1.8 (5)
Burn/scald	0 (0)
Abrasion/graze	2.2 (6)
Surgical wound	0.7 (2)
Lesion	0.7 (2)
Unspecified	4.7 (13)
Cognitive Impairment	
Cognitive impairment level, % (n)^	
None/Minimal	2.9 (8)
Mild	27.6 (77)
Moderate	39.8 (111)
Severe	28.0 (78)

^ missing data GRACE: diet type, 0.4%; prescribed nutritional supplement, 3.2%; receiving wound care, 0.7%; cognitive impairment level, 1.8%.