Characterising intestinal microbiome disruption in healthcare settings: towards therapeutic intervention to prevent infection

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ABSTRACT

It is increasingly recognised that microbes residing in our intestinal tract significantly influence our physiology and susceptibility to disease. Disruption of resident gut microbiota promotes pathogen colonisation and expansion thereby increasing the risk of infection. However, little is known about how medical therapies contribute to gut microbiome disruption and whether faecal microbiome transplantation (FMT) can be optimised for use as a therapeutic to restore damaged microbiomes. In order to examine these knowledge gaps this doctorate is divided into two sections: the first investigates microbiome disruption in populations at high risk of infection, and the second investigates the effects of FMT processing on bacterial viability and function.

To better understand the role of medical therapy in driving microbiome disruption two distinct cohorts of patients were studied: those receiving myelosuppressive chemotherapy and critically ill patients receiving intensive care. Faecal samples collected longitudinally underwent 16S rRNA gene amplicon sequencing to determine the within-patient changes to microbiome characteristics occurring during medical intervention. In the second part of this doctorate a propidium monoazide (PMA) based method was optimised for use in determining bacterial viability in faecal samples. This methodology was then applied to study the viability of commensal donor microbiota used in a clinical trial investigating the role of FMT in preventing recurrent urinary tract infection.

The results of the first observational study indicate that chemotherapy treatment promotes gut microbiome instability and increases the relative abundance of gram-negative commensal bacteria at the expense of gram-positive Firmicutes during periods of predicted myelosuppression. However, the microbiome disruption experienced by this cohort was minor compared to the vast shifts in microbiome diversity and composition experienced by critically ill patients. Results from the second section of this doctorate demonstrate that PMA in combination with molecular assays can be used to accurately define the viability of bacteria in donor faecal slurries and that processing FMT material in aerobic conditions significantly impairs the viability of important beneficial commensals.

Together these findings suggest medical interventions are important drivers of microbiome disruption. In both cohorts, but particularly in the critically ill, the changes observed are

likely to contribute to the risk of infection developing. Continuation of these studies will shed further light on which markers of microbiome disruption are linked to infection risk. Although FMT is a promising therapy for reconstituting disrupted microbiomes, it is critical that transplants are prepared in a manner that preserves beneficial microbes. Ultimately, further research, including randomised controlled trials such as the one developed as part of this doctorate, are required to determine the role of microbiome reconstitution as an intervention to prevent infection.

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ABBREVIATIONS

aFMT	Autologous faecal microbiota transplantation
Allo-HSCT	Allogeneic haematopoietic stem cell transplantation
AAD	Antibiotic-associated diarrhoea
ANO_2	Anaerobic conditions
ANOVA	Analysis of variance
ATCC	American type culture collection
ASV	Amplicon sequence variants
COL	Colostomy
COLSW	Colostomy swab
CDI	*Clostridioides difficile* infection
CF	Cystic fibrosis
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
Ct	Cycling threshold
CRE	Carbapenem resistant Enterobacteriaceae
CSF	Cerebrospinal fluid
ESBL	Extend-spectrum beta-lactamase
FDR	False discovery rate
FMT	Faecal microbiota transplantation

FOS	Fructo-oligosaccharide
FimH	Fibrial adhesin
FS	Faecal slurry
FT	Freeze-thaw
GI(T)	Gastro-intestinal (tract)
GVHD	Graft-vs-host disease
HAMS	High amylose maize starch
HK	Heat-killed
IBD	Inflammatory bowel disease
ICU	Intensive care unit
IMV	Invasive mechanical ventilation
IQR	Interquartile range
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
LOS	Length of stay
MAC	McConkey agar
MALDI-TOF	Matrix assisted laser desorption ionisation-time of flight mass spectrometry
MIC	Minimum inhibitory concentration
MHA	Muller-Hinton agar
MMR	Mismatch repair
MOCI	Microbiome and outcomes in critical illness study

MDRO	Multi-drug resistant organism
nMDS	Non-metric multi-dimensional scaling
NS	Normal saline
O_2	Aerobic conditions
OTU	Operational taxonomic unit
PCO	Principal coordinate
PCoA	Principal coordinates analysis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	Phylogenetic diversity
PERMANOVA	Permutational multivariate analysis of variance
PMA	Propidium monoazide
RCT	Randomised controlled trial
RS	Rectal swab
QIIME	Quantitative Insights into microbial ecology
qPCR	Quantitative polymerase chain reaction
RCT	Randomised controlled trial
ROS	Reactive oxygen species
SCFA	Short-chain fatty acid
SD	Standard deviation
SDD	Selective gut decontamination
SOD	Selective oropharyngeal decontamination

SOS	Bacterial stress response named after “save our ship” distress signal
ST	Stool
STSW	Stool swab
UC	Ulcerative colitis
UTI	Urinary tract infection
VAP	Ventilator-associated pneumonia
VRE	Vancomycin resistant Enterococci

PUBLICATIONS DURING CANDIDATURE

Manuscripts resulting from this thesis:

1. **Papanicolas LE,** Sims S, Miller SJ, Taylor S, Karapetis C, Gordon DL, Wesselingh SL, Rogers GB**.** Conventional myelosuppressive chemotherapy for non-haematological malignancy alters intestinal microbiology. BMC Cancer. 2021May 22;21(1):591. doi: 10.1186/s12885-021-08296-4.

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   Author's contributions: LP and GR wrote the review with input from DG and SW

4. **Papanicolas LE**, Wesselingh SL, Rogers GB. Do we really understand how faecal microbiota transplantation works? Authors' reply. *EBioMedicine*. 2019 Mar 19. pii: S2352-3964(19)30170-7. doi: 10.1016/j.ebiom.2019.03.030.

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5. **Papanicolas LE**, Choo JM, Wang. Y, Leong LEX, Costello SP, Gordon DL, Wesselingh SL, Rogers GB. Bacterial viability in faecal transplants: Which bacteria survive? *EBioMedicine.* 2019 Feb 19. pii: S2352-3964(19)30095-7. doi: 10.1016/j.ebiom.2019.02.023.

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6. **Papanicolas LE,** Wang Y, Choo JM, Gordon DL, Wesselingh SL, Rogers GB. Optimisation of a propidium monoazide based method to determine the viability of microbes in faecal slurries for transplantation. *Journal of Microbiological Methods.* 2019 Jan;156:40-45. doi: 10.1016/j.mimet.2018.12.001.

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7. **Papanicolas LE**, Gordon DL, Wesselingh SL, Rogers GB. Not Just Antibiotics: Is Cancer Chemotherapy Driving Antimicrobial Resistance? *Trends in Microbiology*. 2018 May;26(5):393-400. doi: 10.1016/j.tim.2017.10.009.

   Author's contributions: LP and wrote the review with input from GR, DG and SW.

Other publications during candidature:

8. Sluggett JK, Moldovan M, Lang C, Lynn DJ, **Papanicolas LE,** Crotty M, Whitehead C, Rogers GB, Wesselingh SL, Inacio MC. Contribution of facility level factors to variation in antibiotic use in long-term care facilities: a national cohort study. J Antimicrob Chemother. 2021 Feb 13:dkab007. doi: 10.1093/jac/dkab007.

9. Taylor SL, Leong LEX, Sims SK, Keating RL, **Papanicolas LE**, Richard A, Mobegi FM, Wesselingh S, Burr LD, Rogers GB. The cystic fibrosis gut as a potential source of multidrug resistant pathogens. J Cyst Fibros. 2020 Nov 26:S1569-1993(20)30912-7. doi: 10.1016/j.jcf.2020.11.009.

10. Gill M, Blacketer C, Chitti F, Telfer K, **Papanicolas L,** Dann LM, Tucker EC, Bryant RV, Costello SP. Physician and patient perceptions of fecal microbiota transplant for recurrent or refractory *Clostridioides difficile* in the first 6 years of a central stool bank. JGH Open. 2020 Aug 6;4(5):950-957. doi: 10.1002/jgh3.12396.

11. Sluggett JK, Moldovan M, Lynn DJ, **Papanicolas LE** et al National Trends in Antibiotic Use in Australian Residential Aged Care Facilities, 2005-2016 *Clin Infect Dis*. 2020;ciaa436. doi:10.1093/cid/ciaa436

12. Haifer C, Kelly CR, Paramsothy S, Andersen D, **Papanicolas LE** et al. SA1913 Consensus Guidelines for the Regulation, Production and Use of Fecal Microbiota Transplantation in Clinical Practice. *Gastroenterology* 2020;158(6):S-477-S-478
13. Haifer C, Kelly CR, Paramsothy S, Andersen D, **Papanicolas LE** et al. Australian consensus statements for the regulation, production and use of faecal microbiota transplantation in clinical practice *Gut*. 2020;gutjnl-2019-320260. doi:10.1136/gutjnl-2019-320260
14. Douglas CA, Ivey KL, **Papanicolas LE**, Best KP, Muhlhausler BS, Rogers GB. DNA extraction approaches substantially influence the assessment of the human breast milk microbiome. Sci Rep. 2020 Jan 10;10(1):123. doi: 10.1038/s41598-019-55568-y.
15. Jervis-Bardy J, Leong LEX, **Papanicolas LE**, Ivey KL, Chawla S, Woods CM, Frauenfelder C, Ooi EH, Rogers GB. Examining the evidence for an adult healthy middle ear microbiome. mSphere. 2019 Sep 4;4(5). pii: e00456-19. doi: 10.1128/mSphere.00456-19.
16. Ashokan A, **Papanicolas LE**, Leong LEX, Theodossi M, Daniel S, Wesselingh SL, Rogers GB, Gordon DL. Case report: Identification of intra-laboratory blood culture contamination with Staphylococcus aureus by whole genome sequencing. Diagn Microbiol Infect Dis. 2019 Feb 23. pii: S0732-8893(19)30069-0. doi: 10.1016/j.diagmicrobio.2019.02.016
17. Harch SAJ, Currie BJ, **Papanicolas L**, Rigas V, Baird R, Bastian I. Utility of a Rapid Lateral Flow Assay to Resolve Erroneous Identification of Burkholderia pseudomallei as Burkholderia thailandensis by Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry. J Clin Microbiol. 2018 Nov 27;56(12). pii: e01437-18. doi: 10.1128/JCM.01437-18.
18. Rogers GB, **Papanicolas LE**, Wesselingh SL. Antibiotic stewardship in aged care facilities. Lancet Infect Dis. 2018 Oct;18(10):1061-1063. doi: 10.1016/S1473-3099(18)30548-6.
19. Leong LEX, Shaw D, **Papanicolas L**, Lagana D, Bastian I, Rogers GB. Draft Genome Sequences of Two *Enterobacter cloacae* subsp. *cloacae* Strains Isolated from Australian Hematology Patients with Bacteremia. Genome Announc. 2017 Aug 17;5(33). pii: e00756-17. doi: 10.1128/genomeA.00756-17.
20. **Papanicolas LE,** Nelson R, Warner M. Influence of antimicrobial susceptibility reporting on junior doctors' decision to prescribe antimicrobials inappropriately. J Antimicrob Chemother. 2017 Apr 1;72(4):1202-1205. doi: 10.1093/jac/dkw525.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

Lito Electra Papanicolas

May, 2021

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Μαμα και Μπαμπα– τελικα ειμαστε ιδιοι– I am a *real* doctor now.

CHAPTER 1

Introduction and Literature Review

Sections of this work appear in the following publications:

Papanicolas LE, Gordon DL, Wesselingh SL, Rogers GB. Not Just Antibiotics: Is Cancer Chemotherapy Driving Antimicrobial Resistance? *Trends in Microbiology*. 2018 May;26(5):393-400. doi: 10.1016/j.tim.2017.10.009.

Papanicolas LE, Gordon DL, Wesselingh SL, Rogers GB. Improving Risk-Benefit in Faecal Transplantation through Microbiome Screening. *Trends in Microbiology*. 2020 Jan 15. pii: S0966-842X(19)30324-5. doi: 10.1016/j.tim.2019.12.009.

1.1 Introduction

Bacterial infection remains a major driver of mortality and hospitalisation around the world. The most severe manifestation of bacterial infection is sepsis, a syndrome characterised by infection-driven organ dysfunction with a mortality rate of 18-35% in developed countries (1, 2). Sepsis remains a common cause of admission to critical care units (3) and contributes to up to half of deaths in hospitalised patients (2). In Australia and New Zealand major gains in mortality from sepsis (1) are currently under threat. In many parts of the world, including in the UK and USA, the incidence and overall mortality from sepsis is increasing (2, 4). The combination of rising antimicrobial resistance in common pathogens and a patient population that is increasingly vulnerable to infection suggests that this trend will only worsen.

The first major threat to our ability to fight infection is the global spread of acquired antimicrobial resistance, resulting in infections that fail to respond to empirical antibiotic therapy. Failure to use the correct initial therapy has serious clinical consequences. Without effective empirical antibiotic therapy, the mortality of patients with hypotensive septic shock increases by 8% per hour (5). The rapid rise in resistance in common gram-negative enteric pathogens in the Enterobacteriaceae family (such as *Escherichia coli* and *Klebsiella pneumoniae*) is particularly concerning. For instance, the use of fluoroquinolone prophylaxis in cancer patients highly vulnerable to infection has been credited for reducing gram-negative bacteraemia (6, 7) and mortality (8) in this group of patients, but in many parts of the world this is now largely ineffective due to widespread fluoroquinolone resistance (9). Once multi-drug resistance is present, the in-hospital mortality of gram-negative sepsis increases threefold (10). Extend-spectrum beta-lactamase (ESBL) carriage in Enterobacteriaceae conferring resistance to almost all cephalosporin therapy, is increasing rapidly. In Australia in 2004 1.5% of clinical *E. coli* isolates were resistant to ceftriaxone (11). By 2019, resistance had risen to 10.4% in bloodstream isolates and 7.8% of urine isolates (12). Most concerning is the global spread of carbapenemase-producing Enterobacteriaceae. Although still rare in Australia, this type of resistance has become endemic in *K. pneumoniae* in parts of Asia, the Middle East and Southern Europe (13). Carbapenem resistance often comes packaged with other resistance determinants carried on highly transmissible plasmids which enable bacteria to become resistant to almost all known antibiotics (14, 15). Infection, particularly bacteraemia, with carbapenem resistant gram-negative bacteria is highly lethal, with mortality rates exceeding 50% in immunosuppressed patients (16-18).

However, it is not only acquired antibiotic resistance that results in antibiotic failure, but also infection due to pathogens with inherent antibiotic resistance- such as those caused by fungi or intrinsically resistant bacterial pathogens. In parallel, patient populations are also becoming more vulnerable to infection. Advances in other domains of medicine have resulted in an older population and the increasing use of therapies for cancer, organ transplantation and auto-immune diseases that significantly reduce host defences against infection. Many patients also require therapy in intensive care units (ICUs) where mechanical ventilation and other invasive interventions render even fit and young patients vulnerable to severe infection. The availability of effective antibiotics is a pre-requisite of many modern medical advances including critical care medicine, prosthetic device surgery, chemotherapy, bone marrow and organ transplants. The spread of resistant infection- whether due to acquired or intrinsic antimicrobial resistance, therefore threatens the ability of our health systems to successfully deliver modern medical care (19). Novel approaches to both preventing and treating infection in vulnerable patient groups is urgently required. Intervention targeting the preservation or restoration of beneficial commensal bacteria within the intestinal microbiome is one such approach.

In the literature review which follows, the critical role that the healthy human intestinal microbiome plays in preventing colonisation and infection with antibiotic resistant pathogens will be reviewed. Following this, the known effects of antibiotic treatment and non-antibiotic substances on gut microbiome composition and bacterial antibiotic resistance will be described. I will then explore how gut microbiome composition influences infection risk in vulnerable patient populations. Finally, I will review the literature that describes the use of faecal microbiota transplantation as a therapeutic intervention targeting the gut microbiome and how this could improve infectious outcomes in patients at risk of bacterial infection.

1.2 The role of the gut microbiome in promoting health and preventing infection

1.2.1. Genomic methods enable microbial communities in the gut to be defined

The human intestinal microbiome is a highly diverse microbial community composed primarily of bacteria, but also consists of viruses, fungi and parasites. Until recently, the members of the intestinal microbiome could only be identified by microbiological culture. Although in recent years there have been significant advances in our ability to culture fastidious gut bacteria (20), culture-based methods of characterising the gut microbiome are

still severely limited by the fact that most human commensal bacteria are not readily culturable. The advent of molecular microbiology enabling characterisation of micro-organisms based on the detection of their molecular material (DNA or RNA) combined with advances in sequencing technology using next generation high-throughput platforms now allows the analysis of complex microbial communities. Application of these genomic methods has revolutionised our understanding of the diversity of organisms which inhabit our gut. Of the bacterial component alone, approximately 800-1000 different bacterial species and >7000 strains have been identified (21). The genes encoded by the microbiome outnumber the entire human genome by ~150 fold. Of these, greater than 99% are of bacterial origin (22).

The most established methodology used to study the gut microbiome is 16S rRNA gene amplicon sequencing. In this process the 16S rRNA gene, a conserved region of the bacterial genome used for bacterial taxonomic identification (23), is first amplified. This yields mixed amplicons of DNA belonging to all bacteria and archaea, but not other organisms in the sample. These mixed amplicons are then sequenced using next-generation sequencing technology and resulting reads are assigned to bacterial taxa based on their homology with known bacterial DNA sequences available in databases. The results produced by 16S rRNA amplicon sequencing can be used to broadly define the bacterial composition of a community (to the genus level) and to examine the diversity of bacteria within the sample (α-diversity) or the differences between bacterial communities in different groups of samples (β-diversity) (24).

In another approach known as shot-gun metagenomics, all DNA from the sample is sequenced without prior amplification. Using this approach yields much more information than just bacterial community composition. Shot-gun metagenomics can be used to identify bacterial genes associated with function, virulence or antibiotic resistance within the sample. In addition, DNA belonging to other organisms such as viruses, fungi and eukaryotes can be detected (24).

In all these methods, the quality of the results is highly dependent on the availability of databases with accurate and complete information (25). These are constantly improving, and the advances in the ability to culture fastidious gut bacteria has also benefited those using metagenomics by adding the sequenced information of individual bacterial strains to these databases (20). However, these technologies have limitations – many based on the variability of methods used to analyse the data through bioinformatic pipelines (24). There are also inherent limitations of analysing entire communities simultaneously. For instance, it is

difficult to know which specific organisms are carrying genes found in the entire community. For these reasons our ability to interpret the information gleaned from using these methods can be substantially enhanced by supplementing with older methods such as traditional culture and nucleic acid amplification tests to precisely identify and quantify organisms or genes of interest in the sample.

1.2.2 What constitutes a healthy gut microbiome?

Broadly, the bacterial component of the human intestinal microbiome of healthy individuals is dominated by strictly anaerobic bacteria in two phyla- the Firmicutes and Bacteroidetes (26). Also frequently present in much lower numbers, typically <1% of the bacterial microbiota, are facultatively anaerobic bacteria from the phylum Proteobacteria (including *E. coli* and *Klebsiella*) and facultative anaerobes from the phylum Firmicutes such as *Enterococcus faecalis* (26, 27). Unlike strictly anaerobic commensals these bacteria are important pathogens in both community and hospitalised patients.

Nonetheless, it is nearly impossible to define what is a pathogenic or beneficial bacterium. Bacterial behaviour can be vastly variable between strains, and the surrounding microbial community and host physiology all influence the behaviour of individual bacteria (28). The case of *E. coli*, which can be both commensal and pathogen (ie a pathobiont) is illustrative. *E. coli* is a commensal present in gut microbiota of healthy individuals, and certain strains, such as the probiotic Nissle strain (which was cultured from a soldier in 1917 who remained immune from dysentery) are documented to have beneficial properties (29-31). Yet *E.coli* is also one of the most frequently encountered human pathogens, and it remains the most common cause of urinary tract infection and community-acquired sepsis (4). These strains have specific virulence factors that enhance their pathogenicity (32), such as the ability to adhere to host epithelial cells.

The difficulty in determining the beneficial or pathogenic potential of individual bacteria translates to a broader problem of defining a microbiome associated with health (eubiosis) or a microbiome associated with disease (dysbiosis), by the species present. Microbial species are highly variable between individuals, but stable over time (33). Efforts to find a core microbiome universally present in all humans have been unsuccessful, although Qin et al. found 75 species common to 50% of individuals and 57 species common to 90% in a cohort of 124 Europeans. Nonetheless, despite variations in individual species, microbiomes from healthy individuals appear to share core functional genes derived from microbes (26, 33).

Therefore, a perhaps more useful way of defining the health of a microbiome is by its functional capacity rather than by which microbes are present.

1.2.3 Colonisation resistance: The role of beneficial microbiomes in preventing infection and sepsis

A functional feature of a healthy gut microbiome is the ability to exclude competing pathogen populations. This capacity is known as colonisation resistance. This phenomenon was first observed in 1954 when it was noted that mice could not be colonised with *Salmonella*, unless their commensal gut bacteria were first disrupted using the antibiotic streptomycin (34). Some years later in 1971, Waaij et al. noted that that mice were not readily colonised by *E.coli, K. pneumoniae* or *Pseudomonas aeruginosa* without antibiotic treatment, and linked colonisation resistance to the exclusively anaerobic commensal flora of conventional mice (35). More recent experiments, again in mice, have shown that colonisation with multidrug resistant Enterobacteriaceae or vancomycin resistant enterococci (VRE) is greatly facilitated by the administration of antibiotics that disrupt the commensal anaerobic microbiota, such as metronidazole, clindamycin or vancomycin (36-39). In humans, the disruption of the commensal microbiota, and resulting reduction in colonisation resistance, is associated with the acquisition of enteric pathogens (*Salmonella, Shigella*) and higher rates of *Clostridioides difficile*, VRE, *E. coli* and *P. aeruginosa* colonisation (40-44). Unperturbed microbiota confer colonisation resistance not only to pathogenic bacteria, but also to probiotic strains. Suez et al. demonstrated that microbiota depletion through antibiotic administration was required for probiotic strains (composed of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* spp) to colonise the human gut (45).

The mechanisms by which the microbiota prevent pathogen colonisation are still being elucidated. Direct competition for resources is likely to be the primary mechanism underlying colonisation resistance, however, bacteria can inhibit each other through the production of microbially-derived proteins or through their interaction with the host's immune system (46). Non-bacterial members of the microbiota such as commensal amoebae, fungi and viruses may also play a role in colonisation resistance although their role is largely unexplored. In particular, bacteriophages, viruses that are able to infect and kill specific bacteria, seem likely to contribute to colonisation resistance (47).

In contrast, there is a growing body of research demonstrating the role of bacterial products and metabolites in colonisation resistance. For instance, certain bacterial commensals produce bacteriocins- proteinaceous compounds that are able to directly inhibit or kill competing

bacteria. Bacteria in the order Lactobacillales produce potent bacteriocins, antibiotics which inhibit a broad range of gram-positive pathogens including *Staphylococcus aureus* and *Listeria monocytogenes* (48). Similar proteins called microcins, produced by commensal Enterobacteriaceae and probiotic *E. coli*, have antimicrobial activity against closely related pathogens such as adherent-invasive *E. coli* and *Salmonella* (49).

Interaction between the microbiota and host innate and adaptive immune systems is also critical to both colonisation resistance and in regulating inflammatory responses to pathogen colonisation. In an example of immune-mediated colonisation resistance, resistance to VRE colonisation can be restored in antibiotic treated mice by stimulating the innate immune system through the systemic administration of the toll-like receptor 5 agonist flagellin (50). Rangan et al (51) demonstrated the ability of a protein derived from *Enterococcus faecium*– a commensal gut bacterium- to suppress the pathogenicity of a *Salmonella* strain in both mice and *C. elegans* worms via host innate immune pathways and by improving the host intestinal epithelial barrier. In another example, bacterial components such as Polysaccharide A of commensal *Bacteroides fragilis,* can prevent pathogen driven colitis in animal models by modulating host T cell responses.

1.2.4 The role of bacterial metabolites in pathogen inhibition

Commensal microbial metabolites play an important role in colonisation resistance by directly inhibiting the growth of pathogens in the gut but also by indirectly influencing the likelihood of infection occurring by interacting with host immune cells and by helping maintain the integrity of the luminal gut barrier.

For example, the microbial metabolism of bile acids appears to play an important role in colonisation resistance again *C. difficile*. Primary bile salts are synthesised by the liver and converted to secondary bile salts by enteric bacteria to assist with the digestion of dietary fats and oils. Under normal circumstances *C. difficile* is unable to effectively compete with the wider colonic microbiota. However, *C. difficile*'s ability to survive antibiotic exposure through spore-formation enables it to germinate and proliferate while the wider colonic microbiota is substantially depleted. Primary bile salts promote *C. difficile* proliferation by inducing endospore germination (52). However, a subset of colonic bacteria can modify primary bile acids by deconjugation or convert them into secondary bile salts. This conversion not only reduces primary bile acid levels, but secondary bile salts, such as deoxycholate, themselves inhibit *C. difficile* vegetative growth (52). For example, *C. scindens*, a commensal with the ability to produce secondary bile salts, enhances resistance to *C. difficile* infection (CDI) (53).

Commensal microbiota also induce intestinal innate immune responses that further suppress pathogen growth (46). By modifying the balance of bile salt metabolism, maintaining local immune function, and by competing directly for growth substrates, transplanted microbiota therefore substantially influence the risk of CDI recurrence (46).

However, the most important enteric bacterial metabolites yet identified that modulate colonisation resistance (and in human health more generally) are the short-chain fatty acids (SCFAs). SCFAs are major metabolic products of bacterial fermentation of fibre, and are present in high concentrations in the colon (54). Through their activation of G-protein-coupled epithelial receptors and inhibition of histone deacetylases, SCFAs contribute to the regulation of an array of host processes, including metabolism, tumour suppression, gut barrier function, innate and adaptive immune responses (55).

SCFA including acetate, propionate, butyrate, lactate and succinate differ in their ability to interact with host receptors and their effects on host physiology (55). Therefore, maintaining microbiota with the ability to biosynthesize the full range of SCFA is an important capacity of a healthy microbiome. Biosynthetic pathways for SCFA are widely distributed among bacterial groups, and likely to be found in most people, even those with disrupted microbiomes. However, only a limited group of bacteria, dominated by *Faecalibacterium prausnitzii*, *Eubacterium rectale*, *Eubacterium hallii*, *Roseburia spp* and *Anaerostipes hadrus*, are responsible for the production of butyrate (54).

SCFA play an important role in preventing infection through direct and indirect pathogen inhibition (56, 57) and by modulating the expression of pathogen virulence and invasion (58, 59). A clear example of direct anti-infective capacity is SCFA-mediated intracellular acidification and growth suppression of antibiotic-resistant *K. pneumoniae*, *E. coli*, and *Proteus mirabilis* (56). Another way by which SCFA inhibit facultative anaerobic pathogens is assisting to maintain a hypoxic gut luminal environment. Unlike the vast majority of commensal bacteria in the gut which are obligate anaerobes, species with pathogenic traits are primarily facultative anaerobes with the ability to use aerobic respiration (60). In the presence of increased luminal oxygen, facultative anaerobes gain a metabolic advantage and rapidly expand. In the process of metabolising butyrate (the main energy source of colonic epithelial cells) colonocytes consume oxygen and thereby reduce oxygen availability in the gut lumen (60). Rivera-Chavez demonstrated that expansion of *Salmonella* in the murine gut depends upon increased availability of oxygen in the gut lumen and is triggered by the loss of butyrate producing commensal bacteria following antibiotic administration (61). This process could be reversed by tributyrin treatment (61). Using a complementary mechanism mediated through the host

colonocyte receptor PPAR-γ, butyrate reduces the expansion of *E. coli* in response to increased nitrate availability (an important nutrient source for facultative anaerobes) in the gut following antibiotic treatment (62).

SCFA also influence the development of systemic infection indirectly through interactions with host immune cells. SCFA, particularly butyrate, are important regulators of host inflammatory pathways and play an important role in regulating inflammation in the gut by driving the differentiation of anti-inflammatory regulatory T cells (63, 64). By dampening gut inflammation, the presence of SCFA assist in maintaining the luminal gut epithelium which is a barrier to systemic infection with gut pathogens. Thus, the ability of a healthy microbiome to biosynthesize SCFA, particularly butyrate, is likely to be an important factor not only in limiting the expansion of pathogenic bacteria in the gut but also in the host's ability to prevent these organisms from causing disseminated infection.

1.3 Mechanisms of antimicrobial resistance development within the gut microbiota

Antimicrobial resistance in gut bacteria occurs through a variety of mechanisms. These include direct selective pressure, horizontal gene transfer within the gut microbiota, the acquisition of new resistant pathogens from the environment (facilitated by the disruption of colonisation resistance provided by commensal bacteria) and through bacterial stress responses which in turn drive *de novo* antimicrobial resistance through multiple complementary mechanisms.

1.3.1 Antibiotic use drives resistance through direct selective pressure

It is well established that direct exposure to an antibiotic creates a selective environment which promotes the survival and proliferation of bacterial populations exhibiting resistance to that type of antibiotic (65). In many cases, antibiotics intended to reach an extra-intestinal target inadvertently also affect vast numbers of gut bacteria. In a clear example of such "collateral damage" to gut microbiota, investigators from our laboratory (including myself) analysed stool samples from a cohort of patients with cystic fibrosis (CF) and showed that their stool samples showed significantly higher levels of phenotypic aminoglycoside resistance and aminoglycoside resistance gene carriage than stools from matched healthy controls (66). Interestingly, the aminoglycoside antibiotic most frequently used in this CF cohort was tobramycin administered through the inhaled route, suggesting that the inhaled

route of administration can result in antibiotic resistance in the gut, perhaps because inevitably some of the inhaled drug is swallowed

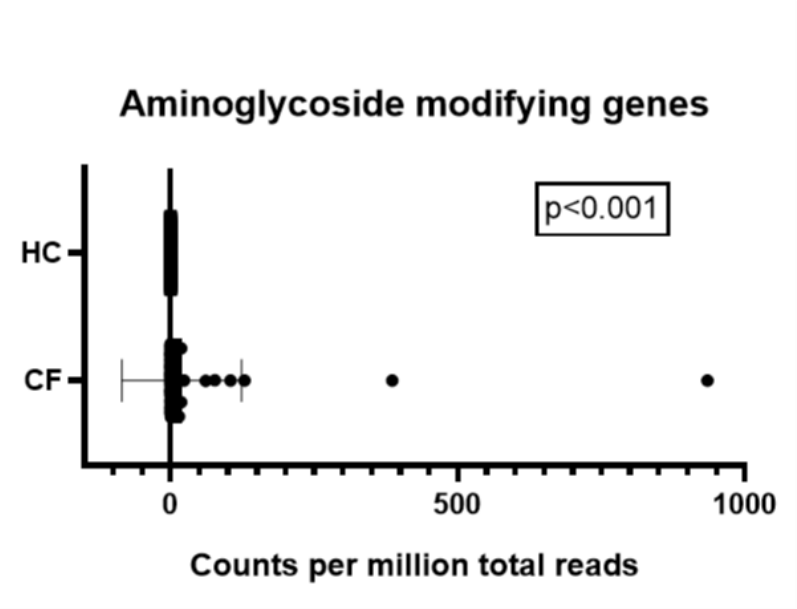


Figure 1.1 Aminoglycoside resistance genes in cystic fibrosis gut microbiome. Total reads of aminoglycoside resistance genes detected in the stool of 19 cystic fibrosis (CF) patients compared to 16 healthy controls (HC). There are significantly more reads attributed to aminoglycoside modifying genes detected in CF patients ($p < 0.001$, Wilcoxon matched-pairs signed rank test).

It has been proposed that mutants are preferentially selected during exposure to antibiotic concentrations that are above the minimum inhibitory concentration (MIC) required to inhibit the wild-type bacteria but below the MIC required to inhibit the least susceptible mutant that results from a single point mutation (67). However it has been observed that even extremely low levels of antibiotic exposure, such as found in environmental sources, can also enrich for pre-existing resistant mutants conferring high levels of resistance (68). These mutations tend to have low fitness costs to the bacteria involved- a feature which leads to the persistence of resistance (68). There are few studies which have observed the persistence of antibiotic resistances that have developed in gut microbiota in response to antibiotic treatment. However, Jernberg et al demonstrated that commensal anaerobes of the Bacteroides genera develop resistance (*erm* gene family) after a short (7-day) exposure to clindamycin- an antibiotic with broad anti-anaerobe activity (69). Furthermore, antibiotic resistance persisted for at least 2 years despite the lack of ongoing selective pressure (69).

However, the paradigm that the primary path to resistance development is through direct selective pressure exerted by an antibiotic is inadequate, particularly in the context of the gut microbiome. This paradigm does not explain how antibiotic treatment with one class can increase resistance to unrelated classes of antibiotics. For instance, high levels of plasmid mediated quinolone resistance (*qnr*) have been observed in the gut flora of children treated with non-fluoroquinolone antibiotics for respiratory illness (70). There are multiple ways that antibiotic use can promote resistance to unrelated antibiotics, with co-selection of resistance determinants spread by horizontal gene transfer being one of the most important of these mechanisms.

1.3.2 Horizontal gene transfer and antibiotic resistance in the gut microbiota

The human intestinal microbiome, which houses thousands of different bacterial species in close proximity, is an ideal environment for the transfer of antimicrobial resistance genes between different bacterial species (71). This process known as horizontal gene transfer, occurs via a variety of mechanisms such as bacterial conjugation, allowing the exchange of extra-chromosomal genes carried on plasmids, bacteriophage-mediated transformation and natural transformation allowing the movement of mobile genetic elements between bacteria (72). Mobile genetic elements, including bacterial plasmids and transposons which encode genes for antibiotic resistance, often carry multiple types of antibiotic resistance genes. For example, plasmids carrying the carbapenmase gene bla_{NDM} in Australia nearly always carry at least one type of aminoglycoside resistance gene (14). In this way selective pressure from carbapenem use can result in the acquisition of resistance not only to carbapenems but also result in co-resistance to multiple unrelated antibiotics. Resistance acquired through mobile genetic elements is therefore particularly dangerous, not only because it is able to readily spread to different types of bacteria but also because the simultaneous transfer of multiple types of resistance genes can render the receiving bacterium resistant to nearly all clinically available antibiotics.

Although it is clear that resistance genes can be passed between closely related species of bacteria within the gut (73-75) it is less clear whether genetic exchange between less closely related organisms such as human and environmental commensals plays a significant role in the emergence of resistance in bacterial pathogens. Nonetheless, there are examples where this occurs. Keen et al showed that a subset of natural lytic bateriophages coined "superspreaders" were able to release intact transformable plasmid DNA following bacterial cell lysis (76). The presence of the novel superspreader bacteriophage SUSP2 increases the

spread of plasmid-mediated kanamycin resistance between distantly related *E. coli* and *Bacillus spp* bacteria roughly 1,000-fold. The likelihood that pathogenic or commensal bacteria in the gut will subsequently transfer antimicrobial resistance genes between themselves is increased through activation of bacterial stress responses that increase horizontal gene transfer and through the disruption of commensal anaerobic gut bacteria.

1.3.3 Disruption of commensal microbiota by antibiotics

As reviewed earlier, the loss of colonisation resistance from antibiotic use is an important factor that enables antibiotic-resistant bacteria from the environment to colonise the gut of persons with disrupted microbiomes (38, 40, 42). This is facilitated when the environments where antibiotics are administered (eg hospitals), are also where antibiotic resistant pathogens are prevalent. A major factor then facilitating horizontal gene transfer between these newly acquired pathogenic bacteria is the expansion of these bacteria in the gut as a result of microbiome disruption. For instance, Stecher et al. showed in a mouse colitis model that concomitant *Salmonella* and *E. coli* blooms facilitates plasmid transfer between these different bacterial genera (77). The ability of antibiotics to disrupt commensal microbiota is therefore an important mechanism promoting antibiotic resistance acquisition and spread within the gut microbiome.

Our understanding of the disruptive effects of medical intervention on the gut microbiome is currently poor. Antibiotics are the best studied intervention in regard to microbiome disruption in humans, but the effects of non-antibiotic therapies on the gut microbiome are only just beginning to be explored (78). However, even in the case of antibiotic therapies, we are still only beginning to understand the relative impact of individual antibiotics, let alone combination therapies, adjuvants, or modes of delivery.

Much of what we do know about the antimicrobial spectrum of antibiotics has been determined by culture-based studies. However, culture-based susceptibility studies can only target a small proportion of gut anaerobes. Furthermore *in-vitro* susceptibility testing may not be the best predictor of the effects of antibiotics on *in-vivo* on microbial systems. This limits our understanding of the broader impact of antibiotics on commensal gut bacteria. The use of sequencing-based microbiome analysis can provide a more comprehensive assessment of the impact of specific antibiotics. For instance, although molecular based microbiome studies have repeatedly shown that ciprofloxacin use results in significant disruption of anaerobic commensals (83, 85), ciprofloxacin appears to lack significant anaerobic activity in culture-based studies. Microbiome based studies of antibiotic-induced microbiome disruption

(predominantly in healthy human volunteers) are consistently expanding our knowledge of how different antibiotics (or combinations of antibiotics) affect human gut microbiota (45, 79-83). Nonetheless, significant knowledge gaps persist. There are still many therapeutic substances (including antibiotics) whose effect on gut microbiota have never been documented. Even when this hurdle is passed, there will be more work required to explore the microbiome impacts of clinically relevant drug combinations and the effects on populations in clinical settings.

A first step in expanding our knowledge in this area would be for microbiome evaluation (using molecular methods) to be added as a standard assessment in therapeutic clinical trials. For instance, following landmark publications showing that microbiome composition (84, 85) and FMT (86) influence the efficacy of cancer immunotherapies there has been markedly increased interest in studying the microbiota of patients receiving these drugs. Yet despite several studies that have linked the use of antibiotics and chemotherapy that disrupt gut microbiomes with increased risk of sepsis, CDI or antimicrobial resistance carriage (40, 87-90) it is still not routine for microbiome analysis to occur in trials of antibiotic or cancer therapies.

It is important to note that antibiotics vary vastly in their ability to disrupt protective commensal microbiota. For instance, oral vancomycin administration markedly alters gut microbiota by reducing the gram-positive members of the Firmicutes phylum and in turn allowing the expansion of infection associated gram-negative species such as *E. coli* and *Klebsiella spp.* and resistant gram-positives such as Lactobacilli and Enterococci (81, 91). Reijnders et al studied changes in microbiome composition and function following the administration of either oral vancomycin or oral amoxicillin for 7 days in a randomised controlled trial. Not only were these microbiota composition changes seen, but the vancomycin-induced loss of gram-positive commensals had impacts on microbial metabolite production, including reducing SCFA production and secondary bile-acid production by the microbiota. By contrast, there was no significant impact of oral amoxicillin administration on either microbiome composition or function (81). However, when amoxicillin is combined with the beta-lactamase inhibitor clavulanic acid its anaerobic spectrum increases substantially. This combination has been shown to have significant impacts on taxonomic composition, predicted functional capacity and resistome in gut microbiota (92, 93). In another example, ciprofloxacin has been observed to alter the gut microbiota composition in multiple studies (79, 82, 94, 95), reducing the abundance of beneficial commensals

Faecalibacterium and *Ruminococcus*, while increasing the abundance of *Bacteroides* spp. This contrasts with the antibiotic nitrofurantoin, which unlike ciprofloxacin does not have significant global impacts on gut microbiota. Interestingly, nitrofurantoin was noted to increase the proportion of *Faecalibacterium* (82), a bacterium widely thought to be beneficial due to its contribution to butyrate biosynthesis in the gut (54, 96). A consistent finding across these studies (using short courses of oral antibiotics) is that although some antibiotics cause significant perturbation of gut microbiota in the short-term, gut microbial composition and diversity is largely able to return to baseline within 1-4 weeks (45, 83, 92, 93, 95). Even when very broad-spectrum intravenous antibiotics are administered for short periods (in healthy individuals) the microbiome is able to recover within 6 weeks (83). However, despite this broad recovery, there are small taxonomic changes, and acquisition of resistance determinants in the gut microbiome which can persist for months to years following antibiotic administration (79, 83, 95).

1.4 Bacterial stress responses and resistance development

Antibiotic use can also drive bacterial resistance through antibiotic-induced DNA damage that activates bacterial stress responses, including the bacterial SOS response. First described by Radman in 1975, the SOS response is triggered by single stranded DNA or double stranded DNA binding with the ubiquitous protein recA which then cleaves the regulatory protein LexA (97). One down-stream effect of activating the SOS response is that bacteria start using an error-prone DNA polymerase (Pol IV) to replicate thus increasing the number of mutants which arise (98).

Many antibiotics have been shown to activate the SOS response including penicillins and cephalosporins (99, 100). Some of the strongest inducers of the SOS response are trimethoprim, sulfamethoxazole and ciprofloxacin (99). Both trimethoprim and sulfamethoxazole interfere with bacterial synthesis of folate and prevent incorporation of thymine into bacterial DNA. Ciprofloxacin inhibits bacterial type-II topoisomerase and results in double stranded DNA breaks in affected bacteria (101). The treatment of mice with ciprofloxacin leads to the rapid appearance of resistant isolates of *E. coli*. Interestingly, when a pathogenic *E. coli* strain that encodes a non-cleavable LexA repressor is used, no ciprofloxacin-resistant mutants appear, thus showing in a murine model that development of ciprofloxacin resistance is SOS dependant (102). Thi et al went on to show that 8 different antibiotics stimulate mutagenesis in *E.coli* causing either rifampicin or fosfomycin resistance

(99). In all cases, bar one, this mutagenesis was to be shown to be dependent on SOS system activation. In these experiments trimethoprim exposure resulted in the highest mutagenicity. The authors theorised that the imbalance in the thymine nucleotide pool may act synergistically with the SOS response to increase the mutagenic response to trimethoprim. Beyond just activating error-prone polymerases (a feature that increases antibiotic resistance through point-mutation) activation of the SOS system can cause broader DNA changes by influencing chromosomal recombination. Dimpfl et al showed duplication events (which arise as a result of intrachromosomal recombination) increase greater than 10-fold during activation of the SOS system, demonstrating the broad mutagenic effect of the SOS system (103). This effect can be augmented through mutations that also affect mismatch repair mechanisms in bacteria. Antibiotic use may not only select strains that have pre-existing resistance but also strains which have higher mutation rates primarily due to deficiencies in the mismatch repair (MMR) system genes *mutS* and *mutL* (104, 105). This highly conserved system is integral to maintaining genomic stability and preventing mutation by repairing DNA base-base mismatch errors made by DNA polymerase during DNA replication (106). Both activation of the SOS system and the defects in the MMR system result in reduced editing of recombination (97, 103, 107, 108). Rayssiguier et al showed in bacterial strains with damage to the mismatch repair system due to mutations in the *mutS*, *mutL* or *mutH* genes, the requirement for DNA homology during recombination was greatly reduced (109). This allowed *E. coli* to efficiently recombine with *Salmonella typhimurium* despite the ~20% divergence in their sequence. Petit et al also investigated the combined effect of MMR system defects and SOS expression (107). Their findings were that these strains exhibited up to 30-fold increase in duplication frequency. They concluded that effects of a defective MMR system and activation of SOS system were additive but increased recombination events by different mechanisms.

Pribis et al have recently more closely examined the mechanisms behind ciprofloxacin induced mutagenesis in *E. coli* (110). In the first phase, ciprofloxacin induced DNA breaks activate the *E. coli* SOS response. However, this alone is not enough to trigger mutagenesis. A small and transient sub-population of *E. coli* cells most capable of generating reactive oxygen species (ROS) trigger a general stress response (σ^S) resulting in resistant mutants. This study showed that mutation rates to rifampicin and ampicillin increased 26- and 18-fold respectively after ciprofloxacin treatment (at low and sub-inhibitory doses). These findings expand on previous findings showing that SOS responses need to be combined with general bacterial stress response (σ^S) to optimally activate error-prone polymerases in bacteria (111)

and research showing that stimulation of ROS by sub-lethal concentrations of antibiotics increases bacterial mutagenesis (112).

In summary, antibiotic use promotes resistance formation in the gut microbiome in a variety of different, often complementary, mechanisms. Often these mechanisms promote resistance developing to antibiotics unrelated to the one being used. Every antibiotic used has a different profile in regard to the mechanisms by which it may promote antibiotic resistance formation. Some will predominantly promote resistance through direct selective pressure, others predominantly through disruption of commensal microbiota and some through strong induction of bacterial stress responses. Certain antibiotics, such as the fluoroquinolones, drive resistance through multiple mechanisms. This emphasises the need to consider the effects of antibiotic agents individually, both in regard to an antibiotic's ability to modify microbiome composition and its ability to promote antibiotic resistance.

1.5 The role of non-antibiotic substances in antibiotic resistance

The role of non-antibiotic substances in contributing to antibiotic resistance is increasingly being recognised. The mechanisms by which non-antibiotic substances can influence antibiotic resistance parallel those seen in antibiotic use; these include a capacity to disrupt commensal bacteria, co-selection of resistance mediated through horizontal gene transfer, and triggering of bacterial stress responses.

1.5.1 Metal resistance and its relationship to antibiotic resistance

The best described example of this phenomenon is the co-selection of antibiotic resistance with metal resistance in bacteria. Although this link has primarily been made in the context of bacterial communities exposed to metals in the environment (113, 114) and can be traced to the ancient origins of bacteria in environments where co-exposure to environmental metals and naturally occurring antibiotics was common (115, 116), there is emerging evidence of the relevance of this link to human pathogens and microbiota. For instance, Li et al found in a study looking at the whole genome sequence of 5,436 diverse bacteria, that genes encoding antimicrobial resistance and metal resistance were much more closely associated in human pathogens than in other types of bacteria (117).

The co-selection of antibiotic resistance and metal resistance primarily occurs through the mechanisms of cross-resistance and co-resistance (118). Cross-resistance occurs when a single mechanism confers resistance to both metals and antibiotics and is predominantly mediated

through efflux pumps. There are many examples of multidrug efflux pumps that export antibiotics in addition to metals (116). The highly conserved and chromosomal encoding of many of these efflux pumps suggests that the ability of bacteria to export both metals and antibiotics evolved long before the modern use of antibiotics as pharmacotherapy (116).

Co-resistance refers to the joint transfer of distinct resistance mechanisms due to their gene's physical proximity on mobile genetic elements. The carriage of metal-resistance frequently accompanies the carriage of antibiotic resistance determinants on mobile genetic elements, particularly bacterial plasmids and transposons (118, 119). The acquisition of such mobile genetic elements due to selective pressure from metal exposure can therefore result in co-resistance to antibiotics. In a fascinating demonstration of this phenomenon, in 1993 Summers et al. demonstrated that in primates exposed to mercury through dental fillings there was a subsequent increase in intestinal bacterial isolates with both mercury resistance and antibiotic resistance, and these resistances were able to be transferred together (120). These findings were linked to earlier observations that humans that had high levels of mercury resistance in their intestinal flora, but no recent exposure to antibiotics, were also significantly more likely to have bacterial isolates with resistance to two or more antibiotics (120, 121). Since then, co-resistance to mercury and several antibiotics has been linked to specific transposons (122).

1.5.2 Cancer chemotherapy and antimicrobial resistance

Beyond metals, there is evidence that other non-antibiotic exposures can lead to antibiotic resistance through similar mechanisms. In by far the most extensive study of its kind, Maier et al studied the in-vitro activity of >1000 marketed non-antibiotic drugs against 40 cultured bacterial isolates (21 distinct genera) representative of gut bacterial strains (78). They found that 27% of these drugs, including 24% of drugs that were not anti-infectives, exhibited anti-bacterial activity against at least one cultured commensal strain. Maier et al also identified a relationship between the expression of *tolC* in *E. coli* and other transporter families (which encode efflux pumps) and resistance to the anti-bacterial effects of non-antibiotic drugs. As a result, the investigators hypothesised that bacterial mechanisms promoting resistance to non-antibiotic drugs could result in cross-resistance to antibiotics (78).

Chemotherapeutic drugs used in cancer therapies may be particularly able to induce bacterial resistance to antibiotic drugs. As in the case of metals (notably some chemotherapy drugs such as the platinum agents contain metals), bacteria use efflux pumps to expel them, a factor that could result in cross- resistance with antibiotics. In the case of the chemotherapy drug

bleomycin, which is classed as a glycopeptide antibiotic, drug efflux occurs using the same pumps as antibiotic efflux (123). There is also evidence that chemotherapeutic agents directly inhibit some bacteria that promote colonisation resistance and encourage the expansion of pathogens. Unlike other non-antibiotic medications, chemotherapeutic drugs are also capable of strongly inducing bacterial stress responses.

In the first instance, several *in-vitro* studies have documented the ability of some chemotherapeutic agents to inhibit bacterial growth. Interestingly, cisplatin was observed to have inhibitory effects on *E. coli* even before its anti-tumour effects were discovered in 1967 (124). More recently, van Vliet et al and Maier et al have tested the antibacterial effect of chemotherapeutic agents *in vitro*. In van Vliet et al's study, daunorubicine, etoposide but not cytarabine showed a negative effect on the growth of some cultured bacteria strains (*Streptococcus mitis, C. difficile, Clostridium ramosum* and *Lactobacillus acidophilus*) (125).

In Maier et al's study, antineoplastics, hormones and nervous system agents inhibited gut bacteria more than other medications. Twenty of 37 antineoplastic drugs, including 10 of 11 antimetabolite chemotherapy drugs tested, had *in vitro* anti-commensal activity (78). Antimetabolite agents alter nucleic acid targets which are common to both human and bacterial cells thus providing a mechanism for inhibiting cell replication in both. Although these studies suggest chemotherapy in some circumstances has a significant antibacterial effect, it is difficult to extrapolate findings from *in vitro* studies to i*n vivo* conditions in the human gut in which conditions are markedly different.

Studies which look at microbiome composition changes during chemotherapy are a useful tool to document the *in vivo* effects of chemotherapy. van Vliet et al used fluorescent in situ hybridization (FISH) to quantify selected groups of anaerobic and anerobic gut bacteria before and after multi-drug chemotherapy for acute myeloid leukaemia in a paediatric population (125). This study showed up to a 10,000-fold reduction in anaerobe species and partial compensatory rises in potentially pathogenic enterococci. However, all these patients had received multiple prophylactic antibiotics which target gram-negative bacteria. Montassier et al examined the gut microbiota by 16S rRNA amplicon sequencing in 28 patients receiving myeloablative chemotherapy prior to haematopoietic stem cell transplantation (HSCT) and showed significant compositional changes day 7 post chemotherapy. These changes included a relative reduction in bacteria in the Firmicutes and Acitnobacteria phyla (ie phyla representing the majority of gram-positive strictly anaerobic

commensals) and an increase of Proteobacteria (the phylum representing facultative gram-negative bacteria including common human pathogens in the Enterobacteriaceae family) (126). Most patients examined had received prophylactic antibiotics just prior to the study commencing and these were discontinued during the study period, a factor which would have allowed Proteobacterial expansion to occur, particularly as these bacteria would presumably have been relatively suppressed by antibiotics at the first time point.

Zwielehner et al primarily used PCR based assays to assess prevalent groups of anaerobic commensals (Bacteroides, Bifidobacteria, Clostridium cluster IV and XIVa) and *C. difficile* in gut bacteria in a diverse group of patients (n=17) receiving cancer treatments. Most patients were not treatment naïve and six also received antibiotics. The most significant findings in comparison to healthy controls were reductions in total bacterial load and a non-significant reduction in Clostridium cluster XIVa. In two patients analysed by high-throughput pyrosequencing *Faecalibacterium spp.* abundances fell dramatically, whilst *E. faecium* increased.

The findings of these studies together suggest that cancer treatment has an effect on microbiota composition, favouring the relative growth of potential pathogens. However due to the small sizes of these studies, the use of multiple chemotherapeutic agents, and the recent use of antibiotics in all studies, it is difficult to disentangle the effects of particular chemotherapeutic agents themselves on the microbiota.

In addition to encouraging the relative overgrowth of pathogen populations in the gut, chemotherapy may directly cause antibiotic resistance through mutagenic effects on gut bacteria. Theoretically, many types of chemotherapy could cause *de novo* antimicrobial resistance through activation of the bacterial SOS response. platinum compounds, topoisomerase inhibitors, antimetabolite drugs and alkylating agents are known to cause DNA damage. There already exists evidence that some chemotherapeutic agents activate bacterial SOS responses and cause increased mutagenicity. Cisplatin, for instance, is able to bind to DNA covalently and as a result inhibits DNA synthesis. Razaka et al documented the ability of cisplatin to increase mutagenesis in *E. coli* three-fold (127). This effect has been attributed to the activation of the SOS system, and is dependent on the presence of the *recA* gene product (124). Mitomycin C, an alkylating agent, is a potent DNA cross-linker (128). The formation of DNA cross-links is a strong inducer of the SOS response in bacteria and mitomycin C is frequently used as a positive control in SOS responses studies (99, 129). The

SOS response can also induce transfer of mobile genetic elements directly. For example, activation of SOS response by mitomycin C increases the transcription of genes necessary to transfer the mobile genetic element that encodes resistance to multiple antibiotics in *Vibrio cholerae* (129). In addition, cell lysis following lytic bacteriophage infection can facilitate the transfer of mobile genetic elements between bacteria through transformation (121).
In summary there is evidence from a variety of sources that the use non-antibiotic substances (and perhaps chemotherapy agents in particular) may cause antibiotic resistance and microbiome disruption independently of antibiotic use.

1.6 Microbiome disruption characterises key populations at risk of sepsis and antimicrobial resistance

The effect of therapies such as antibiotics and chemotherapy on disrupting microbiome composition and encouraging the formation of antimicrobial resistance may not be particularly important to otherwise healthy individuals, although undoubtedly even small increases in antimicrobial resistance on an individual level will eventually have large environmental impacts. In healthy persons, the microbiome composition largely returns to normal after disruption (45, 93), and although small persistent changes in the resistance of commensal flora may occur (79), these changes are unlikely to be clinically important on an individual level. However, there is evidence that in vulnerable patient populations, such as those hospitalised, immunosuppressed or critically ill, that microbiome changes are likely to significantly influence clinical outcomes.

1.6.1 The influence of gut microbiota on sepsis and death in cancer patients

Cancer patients are some of the patients most vulnerable to developing infection and dying of sepsis (130). Although historically it has been noted that bacteraemias in cancer patients are frequently due to enteric pathogens (131, 132), the role that the gut microbiota play in their vulnerability to infection is only just beginning to be appreciated. Cancer patients experience myelosuppression, mucositis and invasive procedures (133), all factors that reduce host defences to infection. Neutropaenia, immunosuppression and gut inflammation as a result of chemotherapy, as well as bacterial overgrowth, are also factors known to increase the likelihood of bacterial translocation in the gut (134-137). In a study by Berg et al published in 1988 (135), translocation of gut flora to mesenteric lymph glands was noted in mice administered immunosuppressant drugs prednisolone or cyclophosphamide, a phenomenon

also later confirmed by Vetizou et al (84). In this murine model the combination of immunosuppression and pathogen overgrowth is devastating. When the mice were given prednisolone combined with clindamycin, (an anti-anaerobic antibiotic which led to the expansion of gram-negative pathogens in the gut) bacteria translocated to the peritoneum resulting in sepsis and death in all the mice studied (135). This murine experiment in the era preceding genomic technologies now available, foreshadowed the discovery of microbiome-mediated mechanisms of sepsis that are now being confirmed in immunosuppressed humans.

Of cancer patients, those receiving allogeneic hematopoietic stem cell transplantation (allo-HSCT) for haematological malignancy are arguably the most immunosuppressed and vulnerable patients to infection. The effects of gut microbiome disruption on the health outcomes of this specific population have been extensively studied by Pamer and colleagues at the Memorial Sloan Kettering Cancer Center and the literature subsequently reviewed in this section all relates to work done by this group. As a result, we now have a much deeper understanding of the important role that intestinal microbiota play in both preventing and promoting infection, graft-vs-host disease (GVHD) and death.

Firstly, it has been noted that a large proportion of patients undergoing HSCT suffer a profound disruption to their gut microbiomes (126, 138). In a cohort of 80 allo-HSCT patients only a minority (32.5%) maintain a high microbial diversity and 42.5% have very low diversity (defined as an inverse Simpson index of <2) (138). Importantly, the loss of gut microbial diversity was a significant independent predictor of death, with low diversity being associated with a mortality rate of 67% at three years compared to 36% for those with high diversity. There was a significant association between a greater abundance of Proteobacteria (especially Enterobacteriaceae) and death in this cohort, whereas the presence of anaerobic bacteria in Lachnospiraceae and Actinomycetaceae families correlated with survival.

Shono et al studied a cohort of 857 allo-HSCT patients and identified the contributing factors to gut microbiome perturbation. As also reported in Taur et al's study (138), low microbial diversity was linked to increased mortality due to an increased incidence of GVHD. The authors went on to link, using retrospective data, the use of the anaerobe-disrupting broad-spectrum antibiotics piperacillin-tazobactam and imipenem-cilastin with GVHD development. In contrast, the use of broad-spectrum antibiotics with relatively little anaerobic activity (cefepime and aztreonam) did not increase GVHD mortality. When microbiome composition was examined in a subset of patients, piperacillin-tazobactam therapy resulted in the loss of larger subsets of intestinal bacteria than cefepime or aztreonam. The link between antibiotic use driving the loss of anaerobic commensals and subsequent GVHD development was

confirmed in a murine model. These findings are clinically important because the choice of antibiotic used in treating neutropaenic fever in these patients is an easily modifiable risk factor.

The link between microbiome disruption and sepsis in this population was made first by the observation by Ubeda et al that *Enterococcus spp*. domination (>97%) in the gut microbiome of allo-HSCT patients preceded VRE bacteraemia in two patients who developed VRE bacteraemia, but not in three patients who did not (38). This effect was also demonstrated in 94 allo-HSCT patients where intestinal domination by Proteobacteria increased the risk of subsequent gram-negative bacteraemia 5-fold and Enterococcal domination increased the risk of *Enterococcus spp.* bacteraemia 9-fold (139). In this study, use of the anti-anaerobe antibiotic metronidazole was significantly associated with *Enterococcus spp* domination, whereas ciprofloxacin had a significant negative association with Proteobacterial domination. In total, 50% of patients with bacteraemia demonstrated intestinal domination with a corresponding organism, with the mean time between intestinal domination and bacteraemia a mere 7 days. These findings are supported by work from Mancini et al who showed that a faecal microbiome composition of >5% Enterobacteriaceae before allo-HSCT was significantly correlated with gram-negative bacteraemia during subsequent hospitalisation (140). Furthermore, this study reported that a pre-allo-HSCT microbiome composition of >5% Enterobacteriaceae combined with ≤10% Lachnospiraceae was associated with increased all-cause mortality.

1.6.2 Infection and its relationship to microbiome changes in critically ill patients

Patients receiving critical care therapies in ICU are another group particularly vulnerable to infection. A European point-prevalence study found that 45% of ICU patients have an infection at any given time and in 21% of cases the infection was acquired in ICU (141). The causes are multifactorial but are likely due to the use of broad-spectrum antibiotics, invasive interventions, mechanical ventilation and altered nutrition. Nosocomial infections resulting in sepsis or ventilator associated pneumonia (VAP) are major causes of morbidity and mortality in the critically ill (94, 142). Admission to an ICU results in profound disruption to these microbial communities in both the gut and respiratory tract (143-149). Although the effects of microbiome disruption on the development of infection in ICU patients have yet to be definitively established, it is very likely that disruption to these microbial communities in critically ill patients influences clinical outcomes (150). The loss of colonisation resistance mediated by healthy microbiota is a particularly important consideration in the ICU

environment, where organisms with important acquired and intrinsic antimicrobial resistance, such as *Candida glabrata*, *Pseudomonas aeruginosa* and *Acinetobacter baumanii* are frequently encountered. For example, the risk of acquisition of carbapenem-resistant *P. aeruginosa* in ICU patients has been linked to the loss of protective commensal species in patients receiving piperacillin-tazobactam (89).

The ability of selective gut decontamination (SDD) and selective oropharyngeal decontamination (SOD) to reduce rates of sepsis in critically ill patients provides strong evidence that gut microbiota composition significantly influences sepsis risk in the critically ill. Interest in SDD as an intervention to reduce sepsis has gained momentum since the publication of two randomised controlled trials (RCTs) which showed a mortality benefit from this intervention in Dutch ICUs (151, 152). A further analysis of rates of colonisation with gram-negative bacteria in the respiratory tract and rectum in patients from de Smet et al's trial showed a relationship between decolonisation from gram-negative bacteria (in both the respiratory tract and the gut) and reduced rates of gram-negative bacteraemia (153). Nonetheless, enthusiasm for SDD has been somewhat tempered by a failure to show similar results in other European ICUs, where the prevalence of multi-resistant organisms (MROs) is much higher than in the Netherlands (154).

Interestingly, although there are significant concerns about these interventions driving antimicrobial resistance, results from these studies have indicated that SDD use is not correlated with increased carriage of MROs and if anything patients randomised to SDD in clinical trials have reduced carriage of organisms with antimicrobial resistance (151, 155, 156). This is not to say that concerns about antimicrobial resistance are not valid. I personally have serious concerns about the routine use of last-line antibiotics such as colistin in SDD protocols could result in pan-resistance developing in organisms such as *P. aeruginosa* in ICU environments. However, it is worth speculating that the reason behind these seemingly paradoxical results may relate to reduced use of empirical broad-spectrum antibiotics. The antibiotics chosen as part of SDD including colistin and tobramycin are active against gram-negative bacteria, including most multi-resistant strains, but have little activity against most commensal anaerobes. The use of SDD antibiotics would likely be able to reduce the carriage of existing multi-resistant gram-negative pathogens while simultaneously keeping intact gut commensals that prevent colonisation by newly encountered drug-resistant pathogens from the environment. However, these positive effects would likely be severely attenuated in ICU settings where use of broad-spectrum anti-anaerobe antibiotics is routine.

The role of colonisation resistance in preventing infection and sepsis was clearly appreciated by the designers of SDD because as part of this intervention ICU teams are encouraged to avoid the use of antibiotics with an anti-anaerobe spectrum including broad-spectrum penicillins such as piperacillin-tazobactam (157). However, as the effects of such advice have not been tested independently of the active intervention, it is impossible to know to what degree this type of antibiotic stewardship has played in the success of both SDD and SOD interventions in the Netherlands, but not in other settings where stewardship practices are known to be different. Clearly, further research is required to establish the role of microbiomes in both the gut and respiratory tract and the efficacy of microbiome-based interventions in preventing infections in ICU patients.

1.6.3 Antibiotic use and risk of subsequent sepsis in hospitalised patients

Microbiome-mediated risk of infection is not limited to highly vulnerable patient groups but also extends to broad groups of hospitalised patients. In this population there is indirect evidence that antibiotic-driven microbiome disruption increases the risk of subsequent sepsis admission. Prescott et al used data from 43,095 hospitalisations to study the influence of microbiome perturbation on hospital re-admission rates (378). The investigators divided these admissions into categories including non-infection-related hospitalisation, infection-related hospitalisation and admission with clostridium difficile infection (CDI), based on the increasing likelihood that microbiome perturbation occurred during the admission. In their unadjusted analysis the probability of re-admission for severe sepsis in the subsequent 90 days was 3.7% for initial hospitalisation for a non-infection-related condition, 8.4% following infection-related hospitalisation and 16.8% following admission with CDI. The difference in risk remained statistically significant even after adjustment for potential confounders (by using patients as their own control subject in a self-controlled analysis and by adjusting for age). In contrast, the probability of re-admission for non-infection related reasons did not differ between the groups.

In a subsequent study by Baggs et al, hospital admission risk factors for subsequent sepsis in a cohort of nearly 13 million patients were explored further (87). Hospital use of particular antibiotics deemed high risk for microbiome disruption (third- or fourth- generation cephalosporins, fluoroquinolones, lincosamides, β-lactam/β-lactamase inhibitor combinations, oral vancomycin, and carbapenems) were linked to a statistically significant risk of re-admission with sepsis after 90 days. Use of > 4 classes of antibiotics or use for >14 days more than doubled the risk of severe sepsis occurring compared to no antibiotic use. In

contrast, the use of low-risk antibiotics had no effect. This study also confirmed Prescott et al's findings (378) that admission for CDI is a greater risk factor for subsequent sepsis than admission for other types of infection.

The evidence from these studies suggesting that sepsis risk is related to microbiome disruption is indirect but has recently been bolstered by a retrospective analysis of patients treated for CDI. In this study, Ianiro et al compared 114 patients with CDI, 57 of whom received antibiotic therapy and 57 who received faecal microbiota transplantation (FMT). Those in the FMT group were 23% less likely to develop subsequent blood stream infection. Importantly, patients who received FMT were significantly less likely to die in the subsequent 90 days, with 89% surviving post FMT compared to only 58% in the antibiotic group. Large mortality differences in favour of FMT treatment for patients with severe CDI were also found in another retrospective study published recently (158). Although these results were subject to bias from the retrospective study design, the absolute effects were very large, suggesting that prospective studies will eventually confirm these findings. These studies support the premise that sepsis risk is influenced by microbiome composition and that interventions that restore commensal microbiota such as FMT, can mitigate this risk in those with perturbed gut microbiomes.

1.7 Restoration of the intestinal microbiome as a therapy: the emergence of faecal microbiota transplantation

1.7.1 The clinical use of FMT

FMT involves the transfer of stool from a healthy donor to the GI tract of a recipient in order to achieve a therapeutic outcome. In the modern era, therapeutic FMT use in humans was first reported in 1958 for the treatment of four patients with pseudomembranous colitis (159). Over the subsequent years there have been case reports and case series describing FMT predominantly for CDI but also for treating inflammatory bowel disease, irritable bowel syndrome and constipation (160-163). In the past decade, there has been a heightened interest in use of this therapy, predominantly driven by increasing rates of recurrent CDI. During this time CDI has become more frequent, more severe, more refractory to standard treatment, and more likely to relapse (164). Standard treatment with oral metronidazole or vancomycin further alters the already abnormal gut flora of CDI patients (165), resulting in decreased microbial diversity that would usually provide colonisation resistance against *C. difficile*. For this reason, after successful initial therapy, up to 35% of patients will experience a symptomatic recurrence after ceasing antibiotics (166). A subset of patients will have multiple

recurrences and subsequent relapses occur in 45-65% of patients who have relapsed one or more times (167). For patients with recurrent CDI, FMT offers the greatest chance of cure of any therapy with success in 81- 94% (168-176). In recent years, following the publication of several RCTs, FMT has also emerged as a promising therapy for ulcerative colitis (163, 177-179). In 2020 the use of FMT as a therapy for ulcerative colitis was endorsed for the first time in consensus guidelines from Australia (180).

1.7.2 FMT mechanisms of action

FMT is the only therapy so far shown to be able to re-constitute gut microbiota to a healthy pre-perturbation state. This has been definitively shown in carefully conducted trials where patients received a stored autologous faecal microbiota transplantation (aFMT) following an intervention (usually antibiotic administration) that perturbs the gut microbiota (45, 181). These studies have shown that aFMT is highly efficacious in restoring gut microbiota to a pre-treatment composition as compared to no intervention.

In aFMT, the organisms present in the transplant are already well adapted to the recipient host. However, in allogenic donor FMT, host factors (as yet largely undefined) are thought to prevent effective engraftment of the donor microbiota in some people (182). Nonetheless, allogenic donor FMT can restore perturbed microbiomes to a state that resembles healthy donor microbiota for prolonged time periods (183-185). It is hypothesised that FMT's ability to cure CDI is related to the ability of transplanted bacteria to reconstitute the intestinal microbiota, thus restoring colonisation resistance against *C. difficile*. However, trials have not definitively linked the degree of post FMT microbe engraftment to the likelihood that FMT is clinically successful in treating CDI (186).

Since engraftment of donor gut microbiota is not always required to cure CDI, the precise mechanism by which FMT cures CDI is not known. There is no strong evidence that donor microbiome composition affects FMT's ability to cure CDI (187). Similarly, both frozen and fresh FMT appear to have similar clinically efficacy (169, 170, 188), although meta-analyses have suggested that efficacy is improved by colonoscopic delivery of FMT as compared to upper gastro-intestinal (GI) routes of administration (189, 190). However, restoration of colonisation resistance to *C. difficile* may not require reconstitution of the entire gut microbiota. Achieving this goal may only require the transplantation of a few viable bacterial species to displace *C. difficile* and inhibit its further proliferation. The successful treatment of CDI has been achieved by mixtures of bacterial cultures, bacterial spores, or lyophilised faecal material delivered orally (191-194), suggesting that in some cases, only a small

proportion of the microbiota needs to be transplanted to achieve clinical benefit. It has also been reported that even filtered stool products devoid of bacteria but still containing bacterial metabolites could be sufficient to cure CDI (195) , although this result has not been replicated. Nonetheless, to date none of these therapies have demonstrated a durable level of success sufficient to replace whole stool transplantation (196), suggesting that the complex microbial content of whole stool still remains an important determinant of clinical efficacy in CDI.

In contrast, donor-specific differences in clinical outcomes, also referred to as the super-donor effect, have been observed in the context of FMT used in inflammatory bowel disease (197, 198). In these cases, favourable donor effects were associated with bacterial richness (a measure of α-diversity) (198) and increased abundances of anaerobic bacteria in the Ruminococcaceae and Lachnospiraceae families (197), with prolonged remission linked specifically to increased butyrate producing ability after FMT (198). The mechanisms of efficacy in inflammatory bowel disease or other non-CDI indications are unlikely to be the same as in CDI, because inflammatory bowel diseases are the result of host-microbiota interactions and not simply the result of pathogen overgrowth. The ability of FMT to specifically restore microbes that produce benefit through their interactions with host cells rather than merely replace pathogenic bacteria, is likely to be clinically relevant in non-CDI contexts. It is known, for example, that many bacteria thought to confer benefit in the human host, such as the butyrate producing bacterium *F. prausnitzii*, are obligate anaerobes which cannot survive in aerobic conditions (199). At least one clinical trial has suggested that superior efficacy in the treatment of ulcerative colitis (UC) may be related to the processing of FMT under anaerobic conditions (rather than much more commonly used aerobic blending) (178). As SCFAs play an important role in colonisation resistance, the ability to preserve microbiota in faecal transplants with the ability to biosynthesize SCFA is likely to be important in FMT efficacy.

FMT material usually undergoes multiple processing steps, such as blending, filtering and freezing prior to its clinical use, often performed in ambient air. The processing methods used are highly variable and centre specific. The effects of processing on the viable microbiota composition of instilled material are not typically considered, however optimising processing methodology is important in order to preserve keystone taxa (such as those that produce SCFA) that are thought to be important in mediating colonisation resistance in gut microbiomes.

Bacterial culture is the established method for assessing bacterial viability, but use of culture to determine bacterial viability in faecal material used for FMT is limited by culture

methodology that can readily isolate only a small subset of the total gut microbiota (170). This remains true even with substantial improvements in the ability to culture fastidious bacteria from faecal samples. By contrast, molecular methods are excellent at describing the full diversity of bacteria within the gut but these methods lack the capacity to distinguish between DNA from viable cells, non-viable cells, and the extracellular environment (200). A potentially effective strategy to overcome these challenges is to combine PCR-based bacterial enumeration with propidium monoazide sample treatment (PMA-qPCR). PMA is a red fluorescent dye that is excluded from viable cells by the energised membrane of an intact cell wall. When the cell wall is compromised, PMA enters the cell and intercalates into DNA (201). The monoazide group allows PMA to covalently bind DNA upon exposure to light, thus limiting PCR amplification to DNA present within viable cells (202).

The combination of PMA treatment with PCR-based analysis has been shown to be effective in a range of contexts, including the assessment of bacterial viability in samples with mixed populations, such as in waste water or sputa (203-205). However, its efficiency may be reduced when applied to samples in which levels of non-viable bacterial DNA and extracellular DNA are high, or where sample turbidity impedes light penetration (203, 206).

The use of PMA in combination with 16S rRNA gene amplicon sequencing has been reported previously in the assessment of viable bacterial composition of FS for FMT (207). Chu and colleagues subjected undiluted simulated faecal transplant material from a single participant to various processing conditions including exposure to oxygen and freeze thaw cycles prior to treatment with PMA (207). This study showed that processing in aerobic condition or freezing had little effect on bacterial viability, except for *Faecalibacterium*- an important bacterium in the biosynthesis of the SCFA butyrate. However, a major limitation of this study was that it did not include a validation of the PMA methodology used.

1.7.3 Evidence that FMT can be used to prevent pathogen colonisation and infection

In van Nood et al's landmark randomised controlled trial of FMT for CDI it was noted that participants experienced profound drops in Proteobacterial abundances in their stool following FMT (172). This suggests that beyond its use in decolonising patients from *C. difficile*, FMT could be used to de-colonise patients from pathogenic members of the Proteobacteria such as antibiotic resistant *E. coli* or *K. pneumoniae*.

In murine models FMT can rapidly clear colonisation by both VRE and carbapenem resistant bacteria (37). In a study by Caballero et al, mice were treated with ampicillin administered via drinking water and then inoculated with either carbapenem-resistant *K. pneumoniae* or

VRE. Mice were then randomised to receive FMT from the stool of a healthy mouse or phosphate buffered saline. Following FMT treatment, *K. pneumoniae* density in faecal pellets decreased within one day and became undetectable within 7 days in all mice. VRE, on the other hand, was cleared in 60% of the mice but reduced by 3 logs in the remaining 40%.

In humans there have been several case studies that have temporally associated pathogen clearance with FMT (208-210). In a retrospective analysis of patients who received FMT for *C. difficile*, carriage of antimicrobial resistance genes was reduced following FMT (211). Similarly, FMT for *C. difficile* reduced urinary tract infection frequency in those with recurrent disease (212). Bilinski et al reported decolonisation of gut carriage of antibiotic-resistant bacteria in 75% of 20 immunocompromised cancer patients receiving FMT (213), with similar results reported by Battipaglia et al in an allo-HSCT population (214). Davido et al and Dinh et al also published a short case-series that was less encouraging, where only fewer than half of the patients were de-colonised from CRE carriage, with better results for VRE carriage (215). However, to date there has only been one published randomised controlled study of the ability of FMT, in combination with antibiotics, to eradicate resistant Enterobacteriaceae (216). While a non-statistically significant difference in the groups was observed (41% decolonisation in in the treatment arm compared with 29% decolonisation in the placebo arm), the trial included only 39 patients and was terminated early, and was therefore likely to be underpowered (216). Clinical studies of FMT for decolonisation from resistant bacteria are summarised in table 1.1

Table 1.1. Human studies of faecal microbiota transplantation for antimicrobial resistance infection or carriage

Authors	Year	Study type	Patient (sample size) and type	FMT method	AMR detection methodology	Main findings	Ref
Singh et al	2014	Case study	1 Renal transplant	Nasogastric infusion	Stool culture for ESBL	Inability to detect ESBL in stool culture for 12 weeks post FMT	(209)
Stripling et al	2015	Case study	1 Cardiac and Renal transplant	Nasogastric infusion	16S rRNA sequencing	Reversal of VRE domination in post transplant microbiome	(210)
Crum-Cianflone et al	2015	Case study	1 ICU long-stay patient	Colonoscopy	Stool culture	Clearance of CRE, ESBL and VRE colonisation 2 years following FMT, reduced number of	(208)

						infections and antibiotic use	
Lagier	2015	Case study	1	Nasogastric infusion	Stool culture	Clearance of OXA- CRE carriage	
Stalenhoef et l	2017	Case study	1 Dialysis patient with recurrent UTI	Nasoduodenal	16S rRNA sequencing and culture	Patient was decolonised from resistant *P. aeruginosa* (VIM) but not ESBL producing *E. coli*	
Millan	2016	Observational	20 recurrent CDI vs 87 healthy controls	Colonoscopy	Shotgun metagenomic DNA microarray	CDI patients had a greater number and diversity of resistance genes compared with donors and healthy controls	(198)
Bilinski et al	2017	Observational	20 patients with blood disorders and carriage of ESBL or CRE	Nasoduodenal	16S rRNA amplicon sequencing and qPCR	1 month post-FMT 60% decolonised	(147)
Davido et al	2017	Observational	8 patients, 6 CRE, 2 VRE colonised	Nasoduodenal	Culture and qPCR	1 month post-FMT, 3/8 patients were decolonised (2 CRE and 1 VRE)	(202)
Dinh et al	2019	Observational	17 patients CRE or VRE colonised	Nasoduodenal	Culture and qPCR	3 months post-FMT 4/8 decolonised from CRE and 7/8 decolonised from VRE	
Battipaglia et al	2019	Observational	10 Allo-HSCT patients CRE or VRE colonised	Nasogastric infusion or enema	16S rRNA amplicon sequencing	7/10 patients were decolonised a median of 13 months post-FMT	(217)
Saidani et al	2019	Retrospective case controlled	10 FMT 20 controls CRE colonised	Nasogastric infusion	Culture	8/10 patients were decolonised 1 month post-FMT.	
Huttner et al	2019	RCT	39 patients colonised with ESBL or CRE, 22 in intervention arm	Capsule FMT	Culture	41% decolonisation in treatment arm 29% decolonisation in the placebo arm	

1.7.4 FMT and the risk of acquired bacterial infection

Recent cases of ESBL-producing *E.coli* infections (one fatal) reported in two immunocompromised recipients following the transplant of encapsulated faecal material (218), has caused significant concern about the safety of FMT products. This has prompted a re-assessment of the risks posed by FMT and a wider recognition that screening for drug-resistance, and not just pathogen presence or absence, should be performed on FMT material. Despite these cases, FMT remains a relatively safe procedure. A recent review of FMT safety found that serious adverse events occur in 2-6% of patients, depending on the route of administration (219). The incidence of severe infections following FMT was 2.5% (27/1089), including two cases of bacteraemia with enteric pathogens (219-221). When safety checks are performed in an optimal manner, such as in stool banks who have strict donor screening requirements and also quarantine specimens by freezing while performing a range of tests for pathogen and resistance carriage, safety of the FMT is considerably greater (189).

Despite the promise of FMT, there are inherent problems with this type of therapy. Given the complexity and diversity of communities of microbes found in stool it is impossible (at least with current technology) to completely define the microbial content of a single stool sample from a single donor, let alone multiple samples from multiple donors. The intrinsic variability of these communities means that content of FMT will never be able to be precisely defined in the way that medications or laboratory produced probiotics are. The uncertainty around the microbial content of FMT links back to uncertainty around the safety of this product and creates hurdles in applying existing regulatory models to the regulation of FMT production. Therefore, the race to design microbiome-based therapies that avoid the pitfalls of FMT continues in earnest. The question is whether intervention designed by humans today can replicate the benefit provided by an incredibly complex community of microbes that have evolved in parallel with humans over thousands of years- a system which we are only just beginning to understand.

1.8 Knowledge gaps

The evidence presented in this literature review strongly suggests that gut microbiome disruption with antibiotics is a risk factor for colonisation and subsequent infection with antibiotic-resistant organisms. However, much more is unknown than known about how this process occurs and who is most at risk. Although individual antibiotics contribute differently to the subsequent risk of infection, the relative effects of individual antibiotic agents on the gut microbiome remain relatively unexplored. Furthermore, little is known about the

relationship between other forms of medical intervention, such as cancer chemotherapy or admission to a critical care unit, and microbiome-mediated risk of infection or mortality. Ultimately, it must be determined whether microbiome-based interventions can reduce the risk of recurrent bacterial infection in patients who currently have no option other than to take repeated courses of antibiotics. Since FMT is currently the best available intervention to alter microbiota, this is the most suitable intervention to test. To achieve this, FMT must be optimised as an intervention for use as a therapy to improve colonisation resistance against pathogens other than *C. difficile* and studied as an intervention to alter infection outcomes in the setting of a randomised controlled trial.

1.9 Aims of thesis

1. **To characterise the changes that occur to intestinal microbiota during cancer chemotherapy**
2. **To characterise the changes that occur to intestinal microbiota in critically ill patients during admission to an intensive care unit**
3. **To relate changes in intestinal microbiology to pathogen carriage and risk of infection**
4. **To determine the impact of FMT processing on viable faecal slurry composition**
5. **To design a clinical trial to test FMT as an intervention to reduce the frequency of recurrent bacterial infections**

CHAPTER 2

The effect of myelosuppressive chemotherapy on gut microbiome and resistome

Sections of this work may appear in the following manuscript:
Papanicolas LE, Sims S, Miller SJ, Taylor S, Karapetis C, Gordon DL, Wesselingh SL, Rogers GB**.** Conventional myelosuppressive chemotherapy for non-haematological malignancy alters intestinal microbiology. BMC Cancer. 2021May 22;21(1):591. doi: 10.1186/s12885-021-08296-4.

2.1 Abstract

Gut microbiota modulate responses to chemotherapy in both animals and humans. These same microbes influence the likelihood of adverse outcomes developing following chemotherapy including colitis and sepsis. Despite the emerging evidence that gut microbiota are likely to play an important role in the outcomes of cancer patients, very little is known about how conventional myelosuppressive chemotherapy affects gut microbiota. The aim of this study was to examine how common conventional myelosuppressive chemotherapy, independent of antibiotic use, affects gut microbiome diversity, composition, and antibiotic resistance.

Faecal samples from 19 participants with non-haematological cancers receiving conventional chemotherapy regimens (platinum agents, taxanes, antimetabolites, anthracyclines, and cyclophosphamide) for the first time were examined before chemotherapy, 7-12 days after chemotherapy, and at the end of the first cycle of treatment. Faecal samples from healthy faecal donors collected at similar time intervals were also examined. 16S amplicon sequencing and metagenomic sequencing were used to characterise the gut microbiome diversity, composition and resistome. Bacterial culture followed by agar dilution was used to determine the susceptibility of commensal *E. coli* to norfloxacin and rifampicin.

Gut microbiota changed significantly 7-12 days following chemotherapy. The overall composition of the microbiome became unstable, with much greater variability between sampling timepoints than expected ($p=0.0004$). These changes included an increase in bacterial richness ($p=0.007$) and diversity ($p=0.02$), a significant decrease in the abundance of gram-positive bacteria in the phylum Firmicutes ($p=0.003$) and a corresponding increase in the relative abundance of gram-negative bacteria in the phyla Bacteriodetes ($p=0.01$) and Proteobacteria ($p=0.021$). However, chemotherapy had no observed effect on the prevalence of microbial resistance genes or phenotypic antimicrobial resistance in commensal *E. coli*.

In conclusion, conventional myelosuppressive chemotherapy significantly alters gut microbiota even in the absence of antibiotic use. The relative increase in pathogenic gram-negative bacteria could signal an increased risk of sepsis in these patients, although these changes are also consistent with changes that drive beneficial stimulation of the host immune during chemotherapy.

2.2 Introduction

Chemotherapeutic agents have been known to inhibit bacterial growth for many decades. The inhibitory effects of cisplatin on *E. coli* preceded identification of its anti-tumour effects (124). However, in recent years, following a series of landmark publications linking gut microbial composition with the anti-tumour effects of cancer therapies (84, 143, 222, 223), there has been renewed interest in how gut bacteria affect cancer therapy. These interactions are important to understand, not only to optimise treatment efficacy, but also so that important complications of cancer treatment, such as bacterial sepsis of gut origin, can be mitigated.

Although the efficacy of conventional chemotherapies, and newer immunotherapies, have been mechanistically linked to gut microbiota changes (143, 222), there is surprisingly little known about the effects of conventional chemotherapeutic agents on human commensal gut bacteria. The antibacterial effect of certain chemotherapeutic agents, including daunorubicine and etoposide, have been demonstrated *in vitro* (125). Maier et al showed that 20 of 37 antineoplastic drugs, including 10 of 11 antimetabolite chemotherapy drugs, had the ability to inhibit the growth of some commensal gut bacteria *in vitro* (78). Several studies have also attempted to assess these effects *in vivo* (125, 126, 224). However, most of the patients assessed in these studies either received prophylactic antibiotics prior or concurrent to the study, potentially confounding assessments of chemotherapeutic impact.

In addition to disrupting commensal microbiota composition, chemotherapy may contribute directly to antibiotic resistance by promoting mutagenesis in gut bacteria through induction of stress responses. Many chemotherapeutic agents, including platinum compounds, topoisomerase inhibitors, antimetabolite drugs and alkylating agents, are known to cause DNA damage that can trigger the bacterial SOS response (105) increasing the ability of bacteria to mutate through the activation of error-prone polymerases and other mechanisms (98).

This study had two principal aims: to determine whether conventional myelosuppressive chemotherapy resulted in significant changes to gut microbiota characteristics, and to assess if chemotherapy altered phenotypic or genotypic antibiotic resistance in commensal gut bacteria.

2.3 Methods

2.3.1 Participant recruitment

Ethical approval for the study was obtained from the Southern Adelaide Local Health Network Human Research Ethics Committee (Reference number HREC/17/SAC/44). Chemotherapy-naïve patients, commencing the first cycle of conventional myelosuppressive chemotherapy for a non-haematological malignancy, were invited to participate in the study. Patients who had

received any antibiotics within four weeks of chemotherapy, a period associated with gut microbiota disruption following antibiotic exposure (95), were excluded. Patients with other potentially confounding exposures, including prior chemotherapy, immunotherapy, malignancy involving the GI lumen, or probiotics use, were also excluded (Table 2.1).

Table 2.1 Inclusion and Exclusion Criteria

Inclusion Criteria
• Aged 18-85 years • No prior chemotherapy • Commencing a new course of chemotherapy during the study period for treatment of a non-haematological malignancy
Exclusion Criteria
• Antibiotic treatment in the preceding 4 weeks • Primary or metastatic disease involving the bowel • Chronic inflammatory bowel disease or other significant gastro-intestinal disease considered to be a possible confounding factor by the investigators • Cognitive impairment • Participant wishing to take probiotics during study

2.3.2 Sample collection

Faecal samples were self-collected by participants within 7 days prior to commencement of chemotherapy (median 1 day preceding chemotherapy; IQR 2), 7-12 days after chemotherapy (median 9 days; IQR 2), and at the end of the first chemotherapy cycle (median 21 days post chemotherapy; IQR 8.5) using stool nucleic acid preservation tubes (Norgen Biotek Corp, Thorold, ON, Canada). Where possible, fresh stool samples were collected at the same time as the preserved specimens, using standard polypropylene collection pots (TechnoPlas, St Mary's SA, Australia), and transported to the laboratory on ice within 6h, to enable culture-based bacterial analysis. Fresh aliquots of stool (~500 µg) samples were stored in 1 mL 10% glycerol saline and frozen at -80°C.

2.3.3 DNA extraction

Stool DNA was weighed and then extracted using the DNeasy PowerSoil HTP 96 DNA Isolation kit (Qiagen, Chadstone VIC, Australia). The extraction protocol used is included in

Appendix 1.4. Quant-IT dsDNA Assay kit (Life Technologies, Carlsbad, CA, USA) was used to quantify DNA concentration after extraction. Extracted DNA was stored at -20°C prior to further analysis.

2.3.4 Nucleic acid amplification

Real-time quantitative polymerase chain reaction (qPCR) assay using universal primers targeting the 16S rRNA gene was used to amplify DNA from all bacteria and specific primers targeting the 16S rRNA gene to amplify DNA from *E. coli*. The primers, probes used in these assays are detailed in Appendix 2.

To determine total bacterial load 1 µL of DNA extract, 0.2 µM of each primer, 17.5 µL of 2X PowerUp SYBR Green qPCR Master Mix (ThermoFisher, Cat No. A25743, Foster City, CA, USA) and the appropriate volume of water was added to a 35 µL total reaction volume. This was then divided into triplicates of 10 µL and underwent thermal cycling (95°C for 15 sec followed by 60 °C for 1 min) for a total of 40 cycles. Real-time PCR quantitation was performed using the QuantStudio 6 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Total bacteria (as determined by 16S quantification) was quantified to a copy number per gram of stool by comparing sample Ct to a standard curve with known copy numbers.

E. coli DNA was amplified using 6 µL of DNA extract, 0.3 µM of each primer, 0.1 µM of the probe, 17.5 µL of KAPPA PROBE FAST ROX Low Master Mix reagents (Kapa Biosystems, Cape Town, South Africa) and the appropriate volume of water was added to a 35 µL total reaction volume. This was then divided into triplicates of 10 µL and underwent thermal cycling (95°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec) for a total of 40 cycles. Real-time PCR quantitation was performed using the QuantStudio 6 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Bacteria (per gram of stool) were quantified by comparing sample Ct to a standard curve using DNA extracted from a known quantity of *E. coli* (ATCC strain 36218).

2.3.5 16S rRNA gene amplicon sequencing

The bacterial composition of the preserved faecal specimens was determined by paired-end sequencing of the V4 hypervariable region of the bacterial 16S rRNA gene. Faecal microbiome characteristics were determined by 16S rRNA gene amplicon sequencing (Illumina MiSeq) as described previously (225). Paired-end reads were merged (demultiplexed) and analysed using

Quantitative Insights in to Microbial Ecology (QIIME) software (v2.2019.4) (226) using a previously described bioinformatics pipeline (225). Reads were analysed for quality and then filtered and truncated using the DADA2 algorithm (227). Sequences were assigned to amplicon sequence variants (ASVs) using an open reference approach against the SILVA 16S rRNA reference database (release 132) clustered at 97% similarity. Reads aligning with contaminants, including mitochondria, eukaryota, chloroplast and cyanobacteria were removed. Read depth median after filtering was 11,510 (IQR 7,078). Subsampling was performed on all samples to a depth of 4846 sequence reads. The taxa relative abundances and α-diversity metrics for determining taxa richness (observed species) and diversity (Shannon and Faith's PD) were computed using QIIME (v2.2019.4). Inter-sample variance (β-diversity) was determined using Bray-Curtis similarity on square root transformed taxa relative abundance using PRIMER software version 7 (PRIMER-E, Plymouth, UK).

2.3.6 Shotgun metagenomics

DNA from faecal samples were processed for shotgun metagenomic sequencing using Nextera XT DNA Library Prep Kit (Illumina Inc., CA, USA), and Nextera XT Index kit (Illumina Inc., CA, USA), as per manufacturer's instructions. Amplicon libraries were sequenced on the Illumina HiSeq 4000 platform at by Macrogen using Illumina HiSeq SBS 2 x 150bp v4 kit (Illumina Inc., CA, USA). Downstream data processing relative gene abundance from metagenomic sequencing data was determined as previously described (228) using resistance genes were categorised by function according to the Antibiotic Resistance Ontology (ARO) defined categories using CARD (Comprehensive Antibiotic Resistance Database, https://card.mcmaster.ca/).

2.3.7 *E. coli* isolation for phenotypic resistance testing Fresh faecal specimens were stored in 10% glycerol-saline at -80°C until culture-based analysis. Samples were thawed at room temperature, vortexed, and 10-fold dilution series were made in saline, producing dilutions to 10^{-6}. In order to selectively isolate *E. coli* colonies from faecal specimens, an aliquot of 100 µL of each 10-fold diluted sample was spread-plated on to solid media with ability to inhibit gram-positive cocci and differentiate *E. coli* from other coliforms based on lactose fermentation (MacConkey Agar No. 3, Oxoid Microbiology Products, Basingstoke, UK). Colonies phenotypically consistent with *E. coli* were identified on plates where single colonies were visible. Ninety-six representative colonies from each participant at each sampling time point (pre-chemotherapy, 7-12 days post chemotherapy, and at cycle completion) were picked with a plastic 1 µL loop and dispersed into 100 µL of 20% glycerol-saline solution aliquoted into

96-well plates. These plates were sealed with PCR film and stored at -80°C until further testing. Isolates were confirmed to be *E. coli* by plating onto chromogenic agar which identifies *E. coli* (CHROMagar ECC Edwards Group, New South Wales, Australia). Representative colonies from this agar that were consistent with *E. coli* phenotypically were confirmed to be *E. coli* using MALDI-TOF (Microflex LT, Bruker, Bremen, Germany).

2.3.8 *E. coli* antimicrobial resistance testing

E. coli isolates were sub-cultured onto solid media containing norfloxacin and media containing rifampicin at different concentrations. Media were prepared as described in Clinical and Laboratory Standards Institute (CLSI) guidelines (229). Mueller Hinton Agar base (MHA, Oxoid Microbiology Products, Basingstoke UK) was used with added antibiotics. Norfloxacin (Sigma Aldrich, St Louis, MO USA) containing media were prepared at the following concentrations (mg/L): 0.5, 0.25, 0.125, 0.06 and 0.03. *E. coli* was tested on MHA containing rifampicin (Sigma Aldrich, St Louis, MO USA) at the following concentrations (mg/L): 128, 64, 32, 16 and 8.

Just prior to susceptibility testing, *E. coli* isolates were sub-cultured in Luria Bertani broth for 2-4 hours until they reached an optical density (OD_{600nm}) of 0.1 (equivalent to 0.5 McFarland concentration) using a spectrophotometer (Victor 3X multimode plate reader, Perkin Elmer, Waltham, MA, United States). Following this, 2 µL of broth containing each isolate was pipetted onto antibiotic-containing agar plates (16 spots per plate). Plates were incubated overnight (18-22 hrs) at 37^{o}C in aerobic conditions and examined for colony growth the following day. Quality control of all antibiotic containing media batches was performed using the following reference strains with known MICs: *E. coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212 and *Pseudomonas aeruginosa* ATCC 27853 according to CLSI performance standards for antimicrobial susceptibility testing (229).

2.3.9 Longitudinal analysis of microbiome characteristics: analysis plan and rationale

The primary aim of this study was to assess the within-patient changes to gut microbiota occurring over time in patients receiving chemotherapy. In order to accomplish this aim, analysis compared two timepoints (Pre-chemo v Post-1) and (Pre-chemo v Post-2) using a within participant paired analysis of all sample types. Where within-participant pairing was not possible, cohorts of unpaired samples at different timepoints were compared.
The specific microbiome characteristics assessed include sample bacterial load, α-diversity, β-diversity and the relative abundance of specific taxa (at the phylum and genus level).

Alpha-diversity (within-sample microbial variance) was assessed using two measures: Observed species (richness) and Shannon's diversity index. Richness is a metric of how many different species are observed in a sample (regardless of their distribution or phylogenetic relatedness). Evenness is a measure of how equally distributed bacterial species are within an individual sample. Shannon's index is a composite index which takes into account both richness and evenness.

Beta-diversity (between-sample comparisons of microbiome composition) was determined using Bray-Curtis similarity and dissimilarity indices. These indices compare the microbial composition of samples based on the number of common species in the samples and their relative abundance. Changes in relative abundance of individual phyla and taxa (at the genus level) within participants was analysed to determine whether specific taxa were significantly likely to change after chemotherapy. Uncommon taxa, defined as being detectable in less than 20% of samples, were excluded from this analysis.

To control for natural temporal variability that occurs with repeated sampling, Bray-Curtis dissimilarity distances were also determined on faecal samples (collected as part of a separate study presented in Chapter 4) at matching time intervals from six healthy participants not exposed to chemotherapy. Apart from sampling interval, these participants were not otherwise matched to the participants receiving chemotherapy. Other microbiota characteristics (including diversity and composition of samples from healthy participants) were therefore not compared to that of the chemotherapy cohort. For this analysis each participant's own pre-chemotherapy sample served as the comparator sample. Finally, in order to fulfil the second aim of this study an analysis of resistome and phenotypic antimicrobial resistance before and after chemotherapy in subset of participants was performed.

2.3.10 Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.03 software. The Anderson-Darling and D'Agostino & Pearson tests were used to test for normal distribution. Significance (p-value <0.05) for longitudinal data linked to specific participants was determined using paired t-tests for parametric data and the Wilcoxon matched-pairs signed rank test for non-parametric data. For comparisons between unrelated cohorts unpaired t-test or 1-way ANOVA was used for parametric data and the Mann-Whitney U test for non-parametric data. All p-values were adjusted for multiple testing using the Benjamini-Hochberg correction (false discovery rate 0.05). Significance of Bray-Curtis similarity β-

diversity metrics were determined by PERMANOVA using PRIMER software version 7 (PRIMER-E, Plymouth, UK).

2.4 Results

2.4.1 Cohort characteristics

Analysis was performed on samples collected from 19 participants. The demographic profile, type of malignancy and chemotherapy used in the cohort is presented in Table 2.2. Myelosuppressive chemotherapies used included platinum agents, antimetabolites, alkylating agents, taxanes and anthracyclines. A total of 56 faecal samples were collected over the three time points for molecular analysis. Faecal samples were self-collected by participants prior to commencement of chemotherapy (median 1 day preceding chemotherapy; IQR 2), 7-12 days after chemotherapy (median 9 days; IQR 2) and at the end of the first chemotherapy cycle (median 21 days post chemotherapy; IQR 8.5) In addition, 14 fresh samples were suitable for culture (pre-chemotherapy n=5; 7-12 days post-chemotherapy n=5; end of cycle n=4). The number and types of samples collected are presented in Table 2.3.

2.4.2 Impact of chemotherapy on bacterial load and faecal microbiome α-diversity

The absolute number of bacteria present in faecal samples per gram of stool did not change with chemotherapy (pre-chemotherapy median 1.14 $X10^9$ bacterial cells/g stool [IQR 2.3 $X10^9$] vs median 1.6 $X10^9$ cells/g stool [IQR 1.6 $X10^9$] 7-12 days post-chemotherapy; p=0.76; However, compared to the pre-chemotherapy microbiota, significant increases in bacterial richness (median 117, observed species [37] vs 117 ± [38]; p=0.007, Figure 2.1A) and Shannon diversity index (median [IQR] 6.2 [0.45] vs 6.3 [0.34]; p=0.02, Figure IB), were observed 7-12 days after chemotherapy. Increased bacterial richness persisted to the end of the chemotherapy cycle (mean 125 observed species ± SD 36 p=0.02, Figure 2.1A). There was no significant change in phylogenetic diversity following chemotherapy (Faith's PD median 6.1[4.2] before chemotherapy vs median 6.5 [2.7] after chemotherapy).

Table 2.2 Cohort demographic and clinical characteristics

Study ID	Gender	Age	Malignancy type (Treatment intention)	Chemotherapy regimen included						
				Alkylating agent	Anthra-cycline	Anti-metabolite	Nucleoside analog	Platinum agent	Taxane	Topo-isomerase inhibitor
CDS3	F	48	Breast (adjuvant)	X	X				X	
CDS4	F	64	Breast (adjuvant)	X	X				X	
CDS5	F	52	Osteosarcoma		X			X		
CDS6	F	70	Cholangiocarcinoma				X			
CDS7	F	67	Endometrial (adjuvant)					X	X	
CDS8	F	80	Mesothelioma			X		X		
CDS9	F	75	Pancreatic			X		X		
CDS11	F	75	SCLC					X		X
CDS 12	M	71	Unknown primary				X	X		
CDS 13	M	57	NSCLC stage 3B					X		X
CDS 14	M	69	Urothelial (adjuvant)				X	X		
CDS 15	M	70	NSCLC stage 4				X	X		
CDS 16	M	73	NSCLC stage 4				X	X		
CDS 17	F	70	Breast (adjuvant)		X				X	
CDS 18	F	63	NSCLC stage 4				X	X		
CDS 19	F	70	Breast (adjuvant)	X	X				X	
CDS 20	M	82	Pancreatic						X	
CDS 21	F	72	Breast (adjuvant)	X	X				X	
CDS 22	M	63	Bladder (neoadjuvant)				X	X		
HC1	F	41	Healthy control							
HC2	M	30	Healthy control							
HC3	F	29	Healthy control							
HC4	F	44	Healthy control							
HC5	M	27	Healthy control							
HC6	F	30	Healthy control							

NSCLC = Non small cell lung cancer

Table 2.3 Sample types collected

Study ID	Sample type and collection interval					
	Pre-chemotherapy		Post-1		Post-2	
	NAPT*	Fresh	NAPT	Fresh	NAPT	Fresh
CDS3	X		X		X	
CDS4	X		X		X	
CDS5	X		X		X	
CDS6	X		X		X	
CDS7	X		X		X	
CDS8	X		X		X	
CDS9	X		X		X	
CDS11	X		X		X	
CDS 12	X		X		X	
CDS 13	X	X	X	X	X	X
CDS 14	X	X	X	X	X	X
CDS 15	X		X		X	
CDS 16	X		X		X	
CDS 17	X	X	X	X	X	X
CDS 18	X		X		X	
CDS 19	X	X	X	X	X	X
CDS 20	X	X	X	X		
CDS 21	X	X	X	X	X	X
CDS 22	X		X		X	

*NAPT = Nucleic Acid Preservation Tube

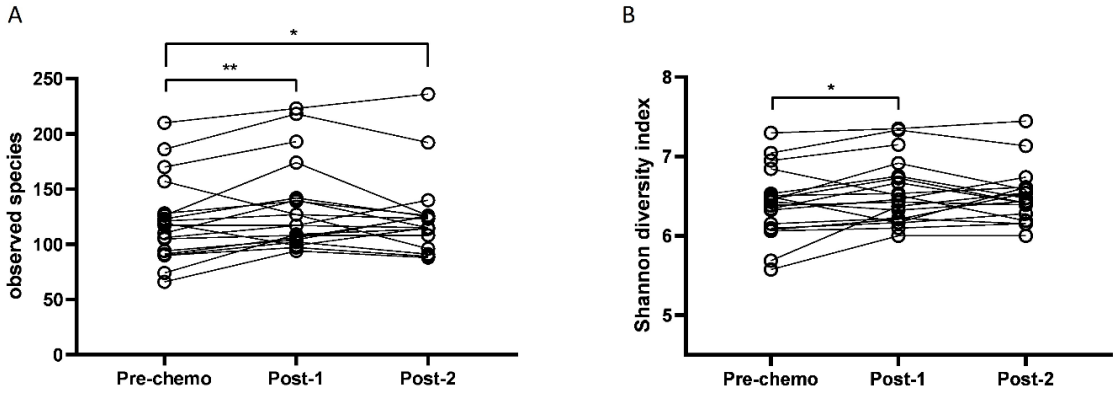


Figure 2.1. Faecal microbiome α-diversity changes during chemotherapy.

Observed species (A) and the Shannon diversity index (B) were used to measure bacterial richness and α-diversity at three time intervals: Pre-chemo (baseline samples prior to chemotherapy), Post-1 (7-12 days post start of chemotherapy) and Post-2 (at the end of one chemotherapy cycle; median 21 days after chemotherapy). Significance of comparisons were determined using the Wilcoxon matched pairs signed rank test of 19 paired subject samples (*= $p<0.05$; **= $p<0.01$).

2.4.3 Impact of chemotherapy on microbiome β-diversity

There were no significant differences in faecal microbiome distribution following chemotherapy (PERMANOVA; p=0.99, Figure 2.2) or dispersion (PERMDISP; p=0.90, Figure 2.3) In this type of analysis does not involve a within-participant analysis, instead the composition of the cohort as a whole before chemotherapy is compared to composition of the cohort as a whole following chemotherapy. For this reason, the results are influenced by large baseline differences in individual microbiomes. As demonstrated in the nMDS plot, instead of clustering by timepoint, samples from the same participant cluster together (Figure 2.2).

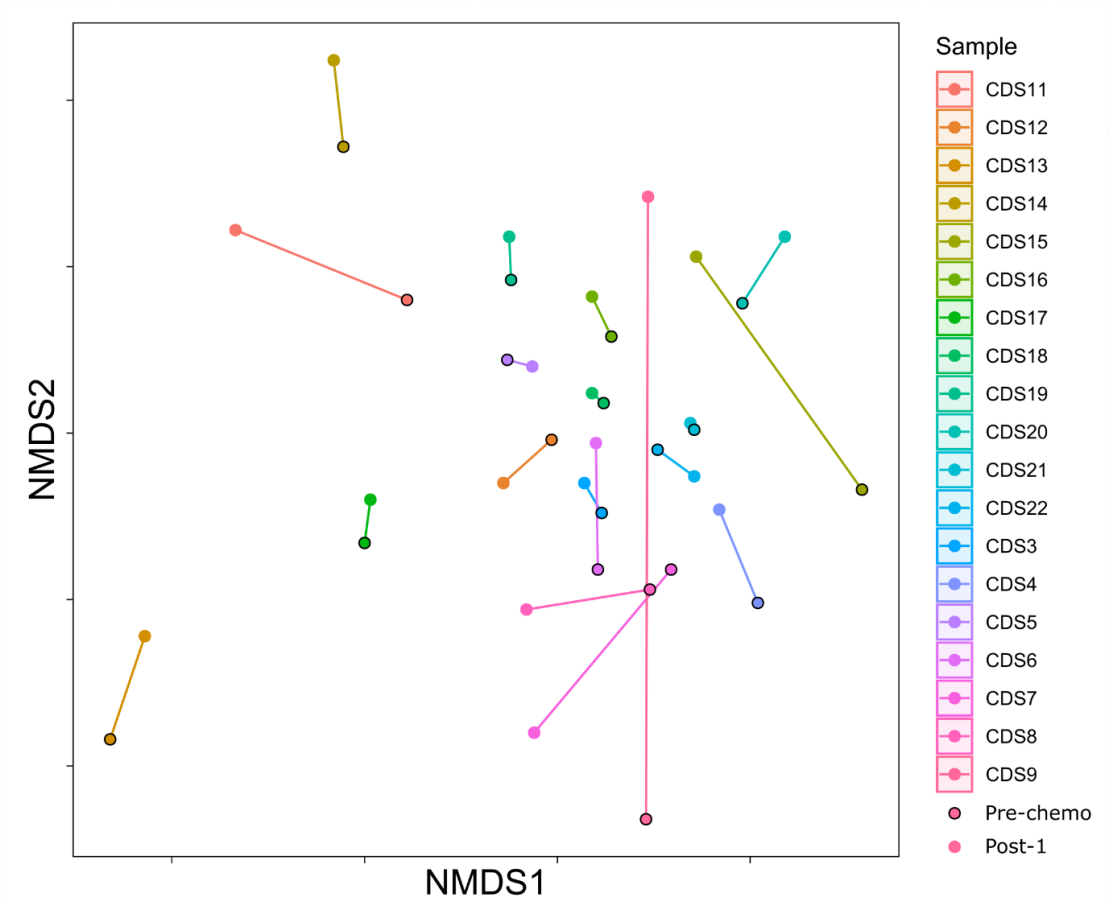


Figure 2.2 Faecal microbiome distribution in paired samples before and after chemotherapy

Non-metric multi-dimensional scaling (nMDS) plot depicts paired-sample changes to microbiota composition following 7-12 days of chemotherapy (Post-1). Each colour represents an individual participant, with the pre-chemo sample (outline) linked to the post-chemotherapy sample (no outline) by a line. Samples are shown to cluster by participant rather than by sampling time point, with no significant difference between the pre-chemo and post-1 groups PERMANOVA; p=0.99.

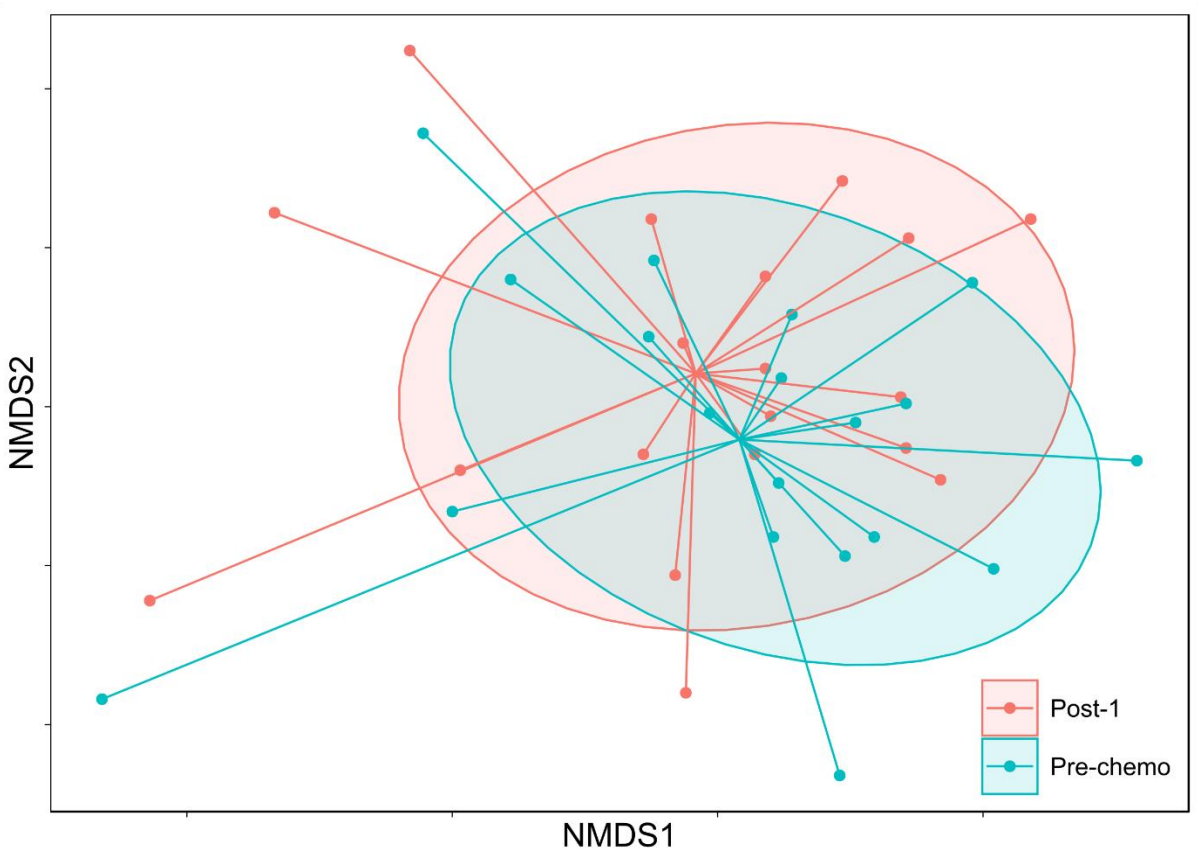


Figure 2.3. Faecal microbiome distribution and dispersal before and after chemotherapy
The nMDS plot depicts Bray-Curtis resemblance of square root transformed, genus-level, relative abundance data. Shaded ovals represent 80% confidence interval. There is no difference between the pre-chemotherapy and post-chemotherapy (post-1) faecal microbiome distribution (PERMANOVA; p=0.99) or dispersion (PERMDISP; p=0.90).

2.4.4 Impact of chemotherapy on microbiome composition

The Bray Curtis dissimilarity index (where 0 indicates sample composition is identical and 1 indicates there are no shared species) was used to evaluate the degree of difference in microbiome composition between pairs of samples. This methodology controls for an individual's baseline microbiome composition when used with paired samples from the same participant.

The Bray Curtis dissimilarity score between different participants (unmatched samples) was not significantly different before and after chemotherapy (mean Bray-Curtis dissimilarity distance 0.84±SD 0.06 pre-chemo vs mean 0.81±SD 0.06 post-1; Figure 2.4, p=0.07). As expected, paired samples from the same participant (taken before and after chemotherapy) were

more alike than samples from different participants (mean Bray-Curtis dissimilarity distance 0.58±SD 0.14 vs 0.81±SD 0.06; Figure 2.4, p <0.0001). This is expected as individual microbiomes differ substantially from each other, but typically remain stable over time (230). However, paired samples from the same participant (taken before and after chemotherapy) were significantly more dissimilar than paired samples from healthy participants who did not receive chemotherapy but whose samples were collected at similar timepoints (mean Bray-Curtis dissimilarity distance 0.58±SD 0.14 vs 0.35±SD 0.14; Figure 2.4, p <0.0001).

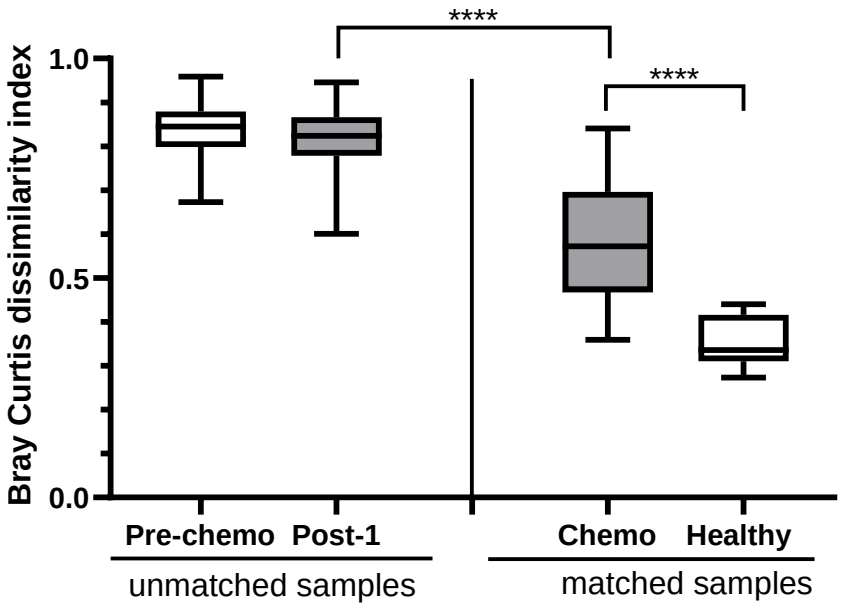


Figure 2.4. Microbiome composition similarity between paired and unpaired specimens
The box plot figure depicts the median [IQR] and range of the degree of similarity of the microbiomes in groups of sample pairs (matched participant and sample type) using the Bray Curtis dissimilarity index. The degree of similarly in unpaired samples (from different participants) before chemotherapy (pre-chemo, unpaired, n=19 participants with 171 paired comparisons) and 7-12 days following chemotherapy (post-1, unpaired, n=19 participants with 171 paired comparisons) is depicted on the left. On the right the degree of similarity between paired samples from the same participants before and 7-12 days after chemotherapy (chemo, paired, n=19) or healthy participants (healthy, paired, n=6) at matching sampling intervals are depicted. Significant comparisons are indicated by stars (****= p<0.0001; one-way ANOVA).

2.4.5 Impact of chemotherapy on the relative abundance of specific phyla

The observed changes in microbiota composition and structure resulting from chemotherapy were associated with significant shifts in the relative abundance of bacterial phyla at 7-12 days post-treatment. The four most abundant phyla (Firmicutes, Bacteriodetes, Proteobacteria and Actinobacteria) representing 99% of bacteria in the samples, were analysed. The relative abundance of gram-positive bacteria in the phylum Firmicutes was significantly reduced (pre-chemotherapy median relative abundance 0.78, IQR 0.11 vs 0.75, 0.11; p=0.003), while the relative abundances of gram-negative bacteria in the phyla Bacteroidetes and Proteobacteria were significantly increased (Bacteroidetes: median 0.16, IQR 0.13 vs 0.21, IQR 0.13; p=0.01 and Proteobacteria: 0.015, IQR 0.018 vs 0.03, IQR 0.03; p=0.02, Figure 2.5).

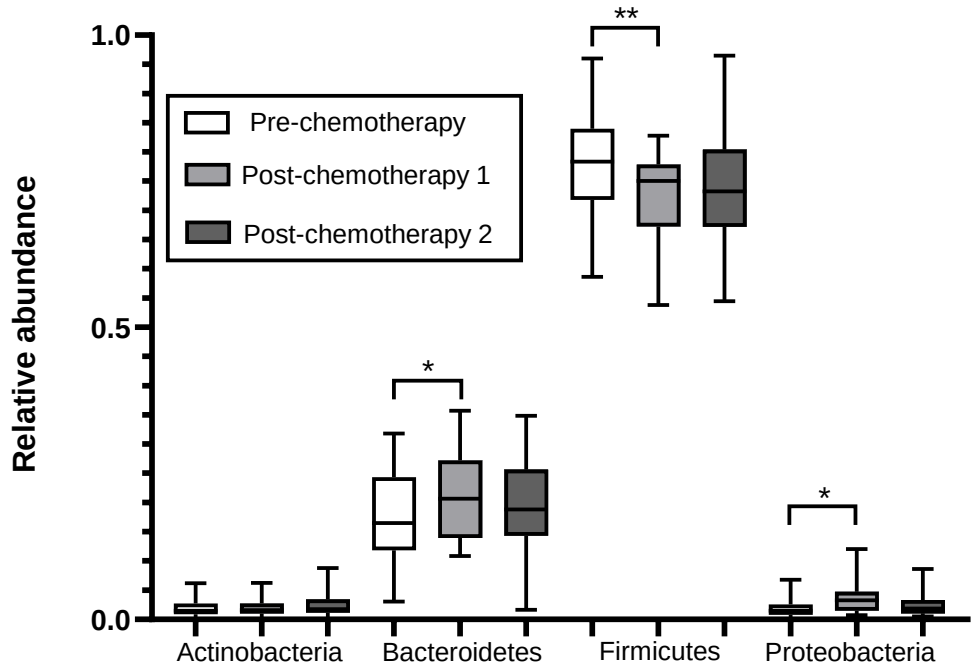


Figure 2.5. Phyla relative abundance before and after chemotherapy

Relative abundance of pre-chemotherapy samples compared to paired samples collected 7-10 days post-chemotherapy in 19 participants. Box and whiskers depict median ± interquartile range with bars representing minimum and maximum values. All significant comparisons are indicated by stars (*= p<0.05; **= p<0.01; ***= p<0.001; Wilcoxon matched-pairs signed rank test).

2.4.6 Impact of chemotherapy on the relative abundance of specific genera

In total, 259 individual taxa were identified to the genus level in the cohort's stool microbiome composition analysis. The relative abundance of individual taxa was assessed before and after chemotherapy (timepoint 1). Uncommon taxa, defined as being detectable in less than 20% of samples, were not analysed, leaving 95 taxa. Of these, 3 comparisons were statistically significant prior to correction for multiple testing. Two members of the Firmicutes phylum decreased in abundance: Ruminococcaceae UCG-014 (med 0.002, IQR 0.02 vs med 0, IQR 0.004; p=0.006) and Clostridia D_3_Clostridiales (unnamed genus) (med 0.021, IQR 0.07 vs med 0.011, IQR 0.05; p=0.025) and the genus Bacteroides of the Bacteriodetes phylum increased in abundance (med 0.123, IQR 0.11 vs med 0.153, IQR 0.11 p=0.03). However, none of these comparisons remained significant after correction for multiple testing.

2.4.7 Metagenomic analysis of antibiotic resistance gene dispersion

Ten paired samples (10 pre-chemotherapy and 10 post-chemotherapy timepoint 1) underwent shotgun metagenomic analysis. A total of 339 individual antibiotic resistance determinants were identified in the collective metagenome. Eight pairwise comparisons of specific genes pre- and post-chemotherapy were significant. The prevalence of the gene van RF (a glycopeptide resistance gene intrinsic to *Paenibacillus)* and the efflux pumps efrB, mexl, tet44, tet35 decreased following chemotherapy ($p<0.05$), whilst the genes EdeQ (instrinsic to *Brevibacillus*), MuxB (a multidrug efflux pump in *Pseudomonas*) and oqxB (an efflux pump conferring resistance to fluoroquinolones in Enterobacteriaceae) increased ($p<0.05$). However, none of these differences remained significant following correction for multiple testing.

2.4.8 Absolute abundance of *E. coli* as determined by qPCR

Before performing phenotypic antimicrobial susceptibility testing on *E. coli isolates* cultured from 5 participant stools. Total *E. coli* abundance in the entire cohort was assessed by qPCR. In one individual there was a dramatic rise in *E. coli* abundance post-chemotherapy rising from undetectable to 10^5 CFU/gram – a finding also confirmed by culture. However, when the entire cohort was evaluated there was no statistically significant change in *E. coli* absolute abundance following chemotherapy (log10 CFU/µL mean ± standard deviation: 4.1 ± 1.5 pre-chemotherapy vs 4.5 ± 1.2 post-chemotherapy timepoint 1; p=0.17).

2.4.9 Phenotypic assessment of resistance to norfloxacin and rifampicin in stool *E. coli* isolates

Ninety-six *E. coli* colonies were isolated from the stools of 5 participants who provided fresh stool samples, at three time intervals. In total, 1,440 bacterial colonies were isolated and were plated onto agar containing norfloxacin and rifampicin. The results, depicted in Figure 2.6, showed that there was no significant change in phenotypic resistance to either norfloxacin or rifampicin following chemotherapy.

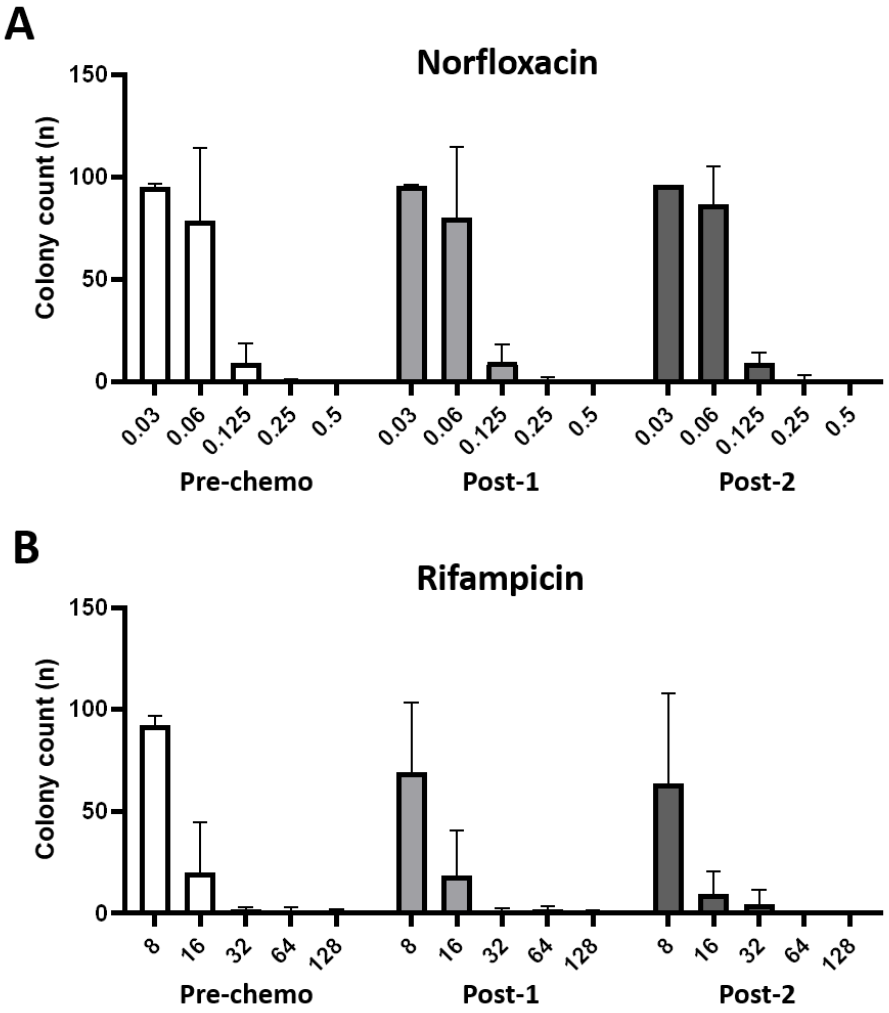


Figure 2.6 Pattern of phenotypic resistance of commensal *E. coli* isolates before and after chemotherapy

Phenotypic resistance to the antibiotics Norfloxacin (A) and Rifampicin (B). The data depicts the resistance pattern of 5 participant's stools, with 96 colonies tested for each participant. There were no significant differences before and after chemotherapy (χ^2test). The of colonies

growing on agar (mean and range) with the selected antibiotic at increasing concentration is depicted.

2.5 Discussion

The importance of assessing chemotherapy-associated changes in the absence of antibiotic exposure is highlighted by the substantial differences between our findings and those of previous studies in which patients received both chemotherapy and antibiotics (125, 224). The design of this study meant that the effects of previous antibiotic use on commensal microbiome, which can cause dynamic changes in gut microbiota for up to 4 weeks (45, 82, 83, 92, 93, 95), were minimised. Furthermore, this study also excluded patients with tumour affecting the gut lumen or other chronic gastro-intestinal disorders that could independently alter the gut microbiota during the study period. Finally, all the patients recruited were chemotherapy naïve, so that the baseline samples collected would not demonstrate changes that were the result of earlier chemotherapeutic treatments.

The results suggest that the use of conventional myelosuppressive chemotherapy drives significant shifts in gut bacterial microbiome composition. Following chemotherapy gut microbiome composition in a short timeframe becomes unstable, with much higher variability than would normally be observed. A potential limitation of this finding is that bacterial stability in our treatment cohort was compared that of unmatched healthy controls – a cohort that was significantly younger. However, significant compositional changes were also observed in this time frame and these were controlled by the use of within patient comparisons. These compositional changes include a reduction in the relative abundance of commensal gram-positive bacteria in the Firmicutes phylum, paired with a relative increase in the abundance of gram-negative bacteria in the Bacteriodetes and Proteobacteria phyla. The observed changes were not driven by extreme changes of a few bacterial species, but rather across the cohort the gut microbiome composition shifted in this pattern. Microbiota shifts were most pronounced 7-10 days following chemotherapy. Following this, just prior to the next chemotherapy cycle, the gut microbiota composition appeared to return towards the baseline composition.

The observed microbiome composition changes align with data from previous studies, with a relative decrease of Firmicutes and an increase in Proteobacteria following chemotherapy (126), and decreases in Clostridium cluster XIVa of the Firmicutes phylum following chemotherapy (224). Although the reason for this change cannot be attributed to antibiotic use,

we cannot exclude secondary effects of chemotherapy, such as loss of appetite or change in diet as being the primary driver of the change.

Unexpectedly, and in contrast to previous reports (126) of decreased bacterial diversity, we instead observed a statistically significant rise in bacterial diversity after chemotherapy. However, this diversity increase is so small in absolute terms that it may be better described as stable. The reasons for this discrepancy are not clear, although previously reported falls in diversity were reported in patients with haematological cancer and not the population studied here (126). Another crucial difference between our study's findings, and those previous studies (125, 224) whose cohorts also received antibiotics during treatment, is that we did not observe a fall in total bacterial absolute abundance during chemotherapy.

The increase in Proteobacteria and decreased Firmicutes is a change in microbiota composition that has been linked to adverse outcomes in patients with haematological cancers. In this population, a baseline composition of >5% Enterobacteriaceae (a member of the Proteobacteria) has been linked to sepsis while <10% Lachnospiraceae (from the Firmicutes Phylum) is associated with overall mortality (140). Although no participants in this cohort had a composition of <10% Lachnospiraceae, one participant's faecal microbiome consisted of >5% Enterobacteriaceae (at both time-points sampled). This was the only patient who developed sepsis and died during the study period.

All changes observed in the study were most pronounced 7-10 days following chemotherapy, a timepoint when the patients are also neutropenic, a factor which facilitates translocation of bacteria across the gut epithelial barrier and increases the risk of systemic infection (231). It is likely that gut microbiota changes at this timepoint also reflect how chemotherapy utilises commensal bacteria to stimulate host anti-tumour responses. In Viaud et al's landmark study (143), it was demonstrated following cyclophosphamide treatment in mice, that there was a disruption of the intestinal barrier and accompanying translocation of predominantly gram-positive bacteria into lymphoid organs. This bacterial translocation triggered T-cell immune responses with antitumor effects. By day 7, a decreased abundance of gram-positive bacteria in the phylum Firmicutes in the gut microbiota of the animals was reported, a finding also observed in this study. The use of vancomycin, an antibiotic with gram-positive activity significantly compromised the anti-tumour effects of cyclophosphamide. Therefore, it is possible that changes to microbiome composition found in this study reflect changes associated with chemotherapy use that correlate with efficacy. Indeed, chemotherapy may also be involved in altering the microbiome of the tumour tissue itself- a factor which could independently affect responses to therapy (232).

The second aim of the study was to determine whether conventional myelosuppressive chemotherapy altered antibiotic resistance in gut microbiota. *E. coli* was selected as a representative pathogen to test for evidence for alteration of phenotypic resistance patterns following chemotherapy. *E. coli* is a leading cause of sepsis globally (233), and unlike many other pathogens, it is an intestinal commensal and readily cultured from stool in most people. Resistance to norfloxacin and rifampicin were specifically tested because resistance to these antibiotics is known to develop in response to point mutations. This mechanism of resistance was hypothesised to be the most likely following the use of DNA damaging chemotherapy agents (as opposed to resistance mechanisms arising from the acquisition of entire genes from mobile elements).

We found no evidence altered antimicrobial resistance following chemotherapy in the resistome analysis of the gut commensal communities, nor in the phenotypic resistance testing of commensal *E. coli* bacteria isolated from participant's stools. Our negative findings could simply be a result of type 2 error, because these changes are too infrequent to be detected in a small cohort (n=5). Furthermore, these changes, if present at low levels, may only become evident if further selective pressure is applied through antibiotic use. It could also be that the mutagenic effects on bacteria occur only briefly while the drug exposure is high and then after multiple generations times the bacterial populations revert to normal. Therefore, it is possible this effect was missed due to the timing of the sampling, which occurred at least a week after the chemotherapeutic drug had been given. Alternatively, it may be that although there is strong *in-vitro* evidence that chemotherapeutic drugs increase bacterial mutagenesis, this simply is not occurring in the *in-vivo* gut environment, where the drug levels achieved and the effect of the local environment on the drug's performance has not been clearly defined.

In conclusion, when examined in the absence of concurrent antibiotic use, chemotherapy alters the human gut microbiome in unexpected ways. Within a week, the bacterial composition is substantially altered. These changes predominantly occur during a period of time when chemotherapeutic agents are also disrupting the gut epithelial barrier allowing the translocation of gut bacteria. Furthermore, a relative increase in potentially pathogenic bacteria from the phylum Proteobacteria is also observed at this time. These factors are likely to contribute to the increased the risk of systemic infection post-chemotherapy. Concurrently these changes may represent beneficial responses to chemotherapy that are crucial in driving host anti-tumour responses. Clearly, understanding a patient's gut microbiome composition during this time is critical, and requires further research. Microbiome markers should be further studied in patients receiving conventional chemotherapy to establish if there is a correlation between microbiome

stability, diversity and composition and adverse outcomes such as sepsis and chemotherapy efficacy. This knowledge could help us improve outcomes in patients receiving conventional chemotherapy treatment by tailoring antibiotic treatments to avoid damaging important commensals and by using therapies to reconstitute damaged microbiomes in selected patients.

2.6 Acknowledgements

I would like to acknowledge and thank the following people who played an important role in this study: A/Prof Chris Karapetis and Monique Swan for their assistance in collecting clinical data and recruiting participants; Steven Taylor for performing the bio-informatic processing of the metagenomic resistome data and assisting with statistical analysis; Sarah Sims and Sophie Miller for their assistance in DNA extraction and performing the phenotypic testing of *E.coli* isolates.

CHAPTER 3

Intensive care unit admission and the gut microbiome of the critically ill

3.1 Abstract

Microbiome characteristics are likely to play an important role in the outcomes of critically ill patients. Patients in intensive care units (ICU) have been shown to have profound disturbances to their gut microbiota. However, which aspects of ICU care drive this change are not yet clear. A prospective longitudinal analysis of the gut microbiota of 54 adult critically ill patients in a single ICU was performed. Rectal swab, stool swab or colostomy swab samples and were taken within 72hrs of critical illness and every 48hrs there-after for 30 days or until discharge. The bacterial microbiome was determined using 16S rRNA gene amplicon sequencing (Illumina MiSeq). Changes to individual patient's bacterial diversity and composition were assessed at baseline (t1, n=52), 48hrs after baseline (t2, n=42) and before discharge in those patients who had three or more specimens collected (tfinal, n=32). Univariate and multivariate regression was used to relate clinical factors to microbiome composition.

Microbiome characteristics changed significantly within 48hrs (between t1 and t2) and by a larger degree between t1 and tfinal. These changes included a fall in α-diversity (using both Shannon and Faith's PD metrics) and differences in composition over time (PERMANOVA t1 v t2 $p=0.015$; t1 v tfinal $p=0.0001$). Using the Bray Curtis dissimilarity metric, the within-participant change between t1 and tfinal was so large that these two samples were as dissimilar as those of two unmatched participants. A range of commensal species, mainly from Clostridium cluster XI and the Prevotellaceae almost completely disappeared between t1 and tfinal. Most participants (72%) developed intestinal domination with a single taxon during their ICU admission. Univariate regression indicated that sample type, prior hospitalisation and sepsis significantly influenced baseline microbiome composition. However, multivariate regression showed only sample type (rectal swab compared to stool) remained a significant influence on microbiome composition ($p<0.001$). For this reason, the longitudinal analysis was repeated using a matched-sample analysis controlling for sample type. This analysis confirmed the findings of the primary analysis showing again that microbiome diversity and composition change significantly over time.

In conclusion, critically ill adults experience profound changes to their gut microbiota over time in ICU. These changes are apparent within 48h but are substantially greater at later timepoints. Time in ICU appears to be the main driver of this dysbiosis rather than specific interventions such as antibiotic use. Further research is needed to link dysbiosis to outcomes in the critically ill.

3.2 Introduction

Hospital acquired infections, particularly with resistant pathogens, increase health care costs and are a leading cause of preventable mortality (234). In Australian ICUs, severe sepsis has a 30-day mortality rate of 18% (1). One of the most important ways to reduce mortality in patients with severe sepsis is the early initiation of effective antimicrobial therapy. For every hour of delay in effective antimicrobial therapy there is an 8% decrease in survival of patients with septic shock (5). However, as the prevalence of multi-drug resistant organisms (MDROs) causing infections increases (235), empirical antimicrobial therapy also becomes less effective (236, 237). The ability to identify patients at risk of sepsis prior to infection developing would improve our ability to empirically treat or prevent infections with effective targeted therapy.

An important function of healthy microbiomes is to protect the host from pathogen colonisation and expansion, a property known as colonisation resistance (44, 238). The disruption of protective commensal species results in loss of colonisation resistance and pathogen expansion. A well-known example is the development of *C. difficile* colitis following antibiotic-mediated disruption of resident gut microbiota (53). However, microbiome disruption not only increases the chance of *C. difficile* colonisation but also increases the likelihood that patients will become colonised with other opportunistic pathogens (38, 46). The presence of beneficial commensal microbiota (usually strict anaerobes) also prevents opportunistic pathogens or pathobionts from expanding through a variety of mechanisms (56, 239). This phenomenon is important because expansion of pathogens or pathobionts in gut of patients vulnerable to infection can predict bacterial infection in these patients (139, 240, 241). In patients receiving HSCT, microbiome disruption leads to the gut microbiota being dominated by pathobionts (139). This intestinal domination (defined by the authors as a single taxon alone occupying >30% of the total bacterial microbiome), substantially increased the risk of a patient developing bacteraemia with the dominating organism within the following week (139). In another study, again in this population, Enterobacteriaceae relative abundance of only >5% predicted subsequent gram-negative sepsis (241).

Admission to ICU results in profound disruption to these microbial communities in both the gut and respiratory tract (144-149, 217, 242-245). The causes are multifactorial but are likely due to the use of broad-spectrum antibiotics, invasive interventions, mechanical ventilation and altered nutrition. Disruption to microbiota resulting in the loss of protective resident microbes, may increase the risk that patients in these environments will become colonised with pathogens including MDROs such as carbapenem-resistant *P. aeruginosa* (89). The coinciding loss of

mucosal barrier integrity which occurs in many critically ill patients increases translocation of pathogenic bacteria and fungi to the bloodstream resulting in increased risk of sepsis (139). In this way the microbiome, depending on its composition, may prevent sepsis through colonisation resistance and the anti-inflammatory actions of resident microbiota (46) or drive sepsis by becoming a reservoir of pathogens (246, 247).

Significant reductions in severe sepsis mortality have already been achieved through improvements in ICU interventional care (1). Even though none of these interventions targeted the gut microbiota, there is an increasing appreciation that gut microbiota are likely to play a major role in sepsis development in the critically ill. This is highlighted by the increasing use of prophylactic antibiotics, also known as selective gut decontamination or SDD, targeting gut microbiota as an intervention to reduce sepsis rates in ICU. This type intervention has already proved efficacious in several randomised controlled clinical trials (151, 152) and is currently being trialled in Australian ICUs. However, these interventions have been instituted before we have clearly understood the role that microbiota play in driving or preventing sepsis.

In recent years there has been a rapid increase in published research documenting the gut microbiota of this population. Microbiome analysis based on high-throughput sequencing microbial DNA directly from patient samples, gives a broader picture of microbes than is possible using traditional culture (248, 249). An overview of published observational studies of gut microbiota in critically ill patients is presented in Table 1. The majority of these studies compared ICU patient's microbiota to the microbiota of unrelated healthy cohorts and found significant differences, including reduced α-diversity and altered composition. A smaller number examined within-patient changes during ICU stay (243-245, 250). In the largest prospective observational study to date, Freedberg et al concluded that the presence of the pathobionts VRE, *E. coli*, *P. aeruginosa* or *K. pneumoniae* in the rectal microbiota of patients on admission were predictive of subsequent infection with the same organisms (240). Although our understanding of the gut microbiota in this population is rapidly increasing, much more is yet to be discovered about the relationship between ICU interventions and gut microbiota and the relationship between microbiota and outcomes including nosocomial infection during ICU admission (251).

Table 3.1. Observational studies of gut microbiota in critically ill populations

Authors	Year	Sample type	Patient type (sample size)	Sampling interval	Methodology	Main findings	Ref
Zaborin et al	2014	Stool	ICU long-stay patients (n=14)	Multiple variable timepoints over 40-110 days	16S rRNA amplicon sequencing and culture	Emergence of ultra-low diversity (1-4 taxa) in 30%. Emergence of candida in culture	(147)
Ojima et al	2015	Stool	Ventilated patients in ICU (n=12)	Four timepoints:1-2, 2-4, 5-8, 7-10 days	16S rRNA amplicon sequencing	Difference in Bacteroidetes /Firmicutes ratio between survivors and patients who died.	(217)
Yeh et al	2016	Stool, tongue, skin, trachea, urine	Trauma patients in ICU (n=32) Healthy controls from pre-existing database	Single sample, mean 8.8 days	16S rRNA amplicon sequencing vs healthy control	ICU microbiota different from healthy controls (alpha and beta diversity). Intestinal domination by pathogens eg. (*Enterococcus*, *Campylobacter*)	(144)
Rogers et al	2016	Stool, tongue, skin, trachea, urine	Paediatric ICU (n=37) Healthy controls from pre-existing database	Single sample (n=23), 14 patients sampled twice weekly	16S rRNA amplicon sequencing vs healthy control	Microbiota of critically ill children differs from the microbiota of healthy children and adults.	(145)
McDonald et al	2016	Stool	ICU Patients (n=115). Healthy controls from pre-existing database	Two timepoints: within 72 h of admission, and at discharge or day 10	16S rRNA amplicon sequencing vs healthy control	Lower relative abundance of *Firmicutes* and *Bacteroidetes* and increased *Proteobacteria*.	(146)
Lankelma et al	2017	Stool	ICU patients (n=34) Healthy controls (n=15)	Single sample	16S rRNA amplicon sequencing vs healthy control	Shift in composition vs controls. Reduced diversity in 50%. In 38%, a single genus made up >50% of the gut microbiota. No associations were found between microbiota and outcomes	(242)

Howard et al	2017	Stool	Trauma ICU patients (n=12) Control patients (n=10)	Two timepoints: on admission and 24-72 hrs afterwards	16S rRNA amplicon sequencing	Controls same as patients on admission. Significant change in within patient beta-diversity after 72hrs.	(250)
Oostdijk et al	2017	Stool	10 ICU patients, Healthy controls (n=10)	30 during ICU stay, 5 post-ICU Healthly controls at 2 time points	16S rRNA amplicon sequencing qPCR for resistance genes	ICU patients had lower microbial diversity, decreased *E. coli* and anaerobic butyrate-producing bacteria of the Clostridium clusters IV and XIVa, increased abundance of Bacteroidetes and enterococci.	(252)
Livanos et al	2018	Rectal swabs	ICU patients (part of Freeberg et al's cohort) n=93	On admission and 72 hrs later	16S rRNA amplicon sequencing VRE Culture	At 72 hours following ICU admission, there was a significant decrease in the proportion of Clostridial Clusters IV/XIVa, and expansion of VRE in a within patient analysis	(243)
Freeberg et al	2018	Rectal swab	ICU patients (n=301)	Single sample (admission)	16S rRNA amplicon sequencing VRE Culture	No association of diversity with infection or death. VRE colonization and Enterococcus domination (≥30% 16S reads) were associated with death or all-cause infection.	(240)
Ravi et al	2019	Stool	ICU patients (n=24)	Daily sampling, but only two required to be in study	Shotgun metagenomics (not resistome)	Reduced microbial diversity in 67%, associated with Meropenem. Pathogen domination in 75%	(245)
Aardema et al	2020	stool	Cardio-thoracic ICU (n=97)	Three timepoints: (admission, during and after)	16S rRNA amplicon sequencing	Altered microbial composition shortly after admission. Restoration microbiota in most patients post-discharge	(244)

The primary aim of the Microbiome and outcomes in critical illness study (the MOCI study) is to document longitudinal changes in both faecal and upper respiratory tract microbiota during ICU care in a critically ill cohort. The initial MOCI study was initiated as a pilot study to form part of this doctoral thesis, and to inform the design of larger studies that can link microbiome to clinical outcomes. Since then, this project has received external funding and has expanded to include patients from two South Australian ICUs over two years and will include shot-gun metagenomic analysis in addition to 16S rRNA amplicon sequencing. However, this Chapter will focus exclusively on the analysis of gut microbiota using 16S rRNA amplicon sequencing from the initial cohort of patients recruited at the Royal Adelaide Hospital in 2019.

3.3 Methods

3.3.1 Participant recruitment

Inclusion and exclusion criteria (shown in Table 2) were designed to capture the most critically ill subset of patients in ICU who were most likely to have long stays in the unit and suffer adverse outcomes as a result. Therefore, only those patients receiving invasive mechanical ventilation (IMV) and that were deemed to be likely to be in the unit for at least 3 days were included in the study.

New admissions to the Royal Adelaide Hospital ICU were screened daily to determine eligibility to participate in the MOCI study. Patients receiving IMV were screened for eligibility by asking the intensivist responsible for the patient's medical care what the predicted length of stay in ICU was, and whether treatment withdrawal or death was deemed imminent. All patients who met eligibility criteria were enrolled in the study, with the option to opt-out of the study presented to the participant or their next of kin at the first opportunity available. The study methodology, including the use of opt-out consent, was reviewed and approved by the Royal Adelaide Hospital Human Research Ethics Committee (Reference: HREC/19/CALHN/211).

Table 3.2 Inclusion and Exclusion Criteria of the MOCI study

Inclusion Criteria
• Participant admitted to a site intensive care unit
• Aged ≥ 18
• Participant is receiving invasive mechanical ventilation (IMV)
• Participant is predicted by intensivist to have length of stay (LOS) >3 days
Exclusion Criteria
• Death is deemed to be imminent during this admission and either the attending doctor patient or substitute decision maker is not committed to active treatment
• Duration of admission is predicted to be less than 72 h.
• Unable to obtain a specimen within 72 h.

3.3.2 Sample and data collection

Once patients were enrolled, a faecal sample was collected at the earliest opportunity by the patient's nurse. In order to maximise the opportunities for sample collection, these faecal samples could be obtained by collecting passed motions, through swabbing the rectum or by collecting stool from a colostomy. Previously, microbiome data from rectal swab and stool swab samples collected at the same time have been noted to be largely concordant as are replicates of the same swab type (253).

The nurse was given a sterile stool pot and a sterile swab (COPAN FLOQSwab, Copan Italia Brescia-Italy) and was instructed to collect a stool specimen if stool had been passed at the time of the patient's hygiene care. Alternatively, a rectal swab sample was collected. In patients with a colostomy, a fresh stool specimen from the colostomy was collected. Faecal samples were subsequently collected every other day (where possible) until discharge from the unit or until the patient had completed 30 days of admission. Nurses also collected tracheal aspirates on the same days as faecal samples, but the analysis of tracheal aspirate samples is not included in this report.

Clinical data from the patient's case notes were collected onto paper data collection sheets study investigators on enrolment and on each day of sample collection. This data was subsequently entered into a web-based data management system (REDCap). The full study manual (including a detailed protocol and data collection sheets) is included in Appendix 6.

3.3.3 Sample processing and storage

Samples were kept on ice until transported to the laboratory within 6h of collection. For rectal swab samples the swab tip was stored in 1.5mL microcentrifuge tubes containing 1mL of 20% glycerol saline solution. If a stool sample was obtained, the stool specimen was swabbed in the laboratory with the same swab type as used for rectal sampling and the swab tip was stored in the same manner as the rectal swabs. Additional aliquots of stool (if there was remaining specimen) were stored separately in 1mL of 20% glycerol saline solution. All samples were stored at -80^{o}C until DNA extraction.

3.3.4 Selection of samples for downstream analysis

As the main aim of the study was to ascertain the change in gut microbiota over time in ICU, samples were selected to be analysed further if they were taken at one of the following three time intervals: timepoint 1 (t1) referring to the baseline sample taken at the time of enrolment (median 21 h [IQR 33h] after ICU admission); timepoint 2 (t2) referring to the second sampling timepoint (median 48h [IQR 9h] after enrolment) and timepoint final (tfinal) referring to the final sample collected in those patients who had a sample collected at a timepoint 3 or later (median 142h [IQR 150h] after enrolment). In addition to these samples, additional samples a single participant who developed bacteraemia during their ICU stay were analysed before and during the episode of nosocomial sepsis.

3.3.5 DNA extraction of samples

Sample DNA was extracted using the Qiagen Powerlyser Powersoil kit (Qiagen, Chadstone VIC, Australia). Stool and rectal swabs were pre-processed to remove faecal material from the swabs using centrifugation (the full protocol is included in Appendix 1.2).

3.3.6 Total bacterial load quantitation

Quantitation of total bacteria was performed using real-time PCR with universal primers targeting the bacterial 16S rRNA gene (254). 1 µL of DNA extract, 0.2 µM of each primer, 17.5 µL of 2X PowerUp SYBR Green qPCR Master Mix (ThermoFisher, Cat No. A25743, Foster City, CA, USA) and the appropriate volume of water was added to a 35 µL total reaction volume. This was then divided into triplicates of 10 µL and underwent thermal cycling (95°C for 15 sec followed by 60 °C for 1 min) for a total of 40 cycles. Real-time PCR quantitation was performed using the QuantStudio 6 Real-Time PCR system (Applied

Biosystems, Foster City, CA, USA). Total bacteria were quantified to a copy number per µL of PCR reaction by comparing sample Ct to a standard curve with known copy numbers.

3.3.7 16S rRNA gene amplicon sequencing

The bacterial composition of the preserved faecal specimens was determined by paired-end sequencing of the V4 hypervariable region of the bacterial 16S rRNA gene. Faecal microbiome characteristics were determined by 16S rRNA gene amplicon sequencing (Illumina MiSeq) as described previously (225). Paired-end reads were merged (demultiplexed) and analysed using Quantitative Insights in to Microbial Ecology (QIIME) software (v2.2019.4) (226) using a previously described bioinformatics pipeline (225). Reads were analysed for quality and then filtered and truncated using the DADA2 algorithm (227). Pre-filtering to remove poor quality sequences and non-prokaryotic sequences (eg human DNA) was performed by aligning the SILVA 16S rRNA reference database (release 132) at 80% similarity using vsearch. Sequences were then assigned to amplicon sequence variants (ASVs) using an open reference approach against the SILVA reference database clustered at 97% similarity. Contaminants reads including those from Mitochondria, Chloroplasts, Planctomycetes, Eukaryota, Alphaproteobacteria, Patescibacteria, Chloroflexi, and Cyanobacteria were excluded from the analysis. Median sequence depth was 34,870 (IQR 10,116). Subsampling was performed on all samples to a depth of 474 sequence reads. The taxa relative abundances and α-diversity metrics diversity (Shannon and Faith's PD) were computed using QIIME (v2.2019.4).

3.3.8 Longitudinal analysis of microbiome characteristics: plan and rationale

The primary aim of this study was to assess changes to gut microbiota occurring over time. In order to accomplish this aim, analysis compared two timepoints (t1 v t2) and (t1 v tfinal) using a within-participant paired analysis of all sample types. Where within-participant pairing was not possible, cohorts of unpaired samples at different timepoints were compared. The specific microbiome characteristics assessed include sample bacterial load, α-diversity, β-diversity and the relative abundance of specific taxa (at the genus level).

Total bacterial load was assessed in order to ascertain whether unexpected differences occurred at different timepoints, a factor which, if present, could affect the interpretation of the other microbiome measures.

Alpha-diversity (within-sample microbial variance) was assessed using two measures: Shannon's diversity index and Faith's phylogenetic diversity index. Shannon's index is a composite index which considers both bacterial taxa richness and evenness but is more

heavily weighted towards richness. Faith's phylogenetic diversity index measures species richness while considering the phylogenetic relatedness between the taxa present.
Beta-diversity (between sample comparisons of microbiome composition) was determined using Bray-Curtis similarity and dissimilarity indices. These indices compare the microbial composition of samples based on the number of common species in the samples and their relative abundance.
Finally, the changes in relative abundance of individual taxa within participants was measured in order to determine whether particular taxa were significantly likely to change over time in ICU (regardless of whether compositional changes were observed more broadly).
In addition to the longitudinal analysis of these standard microbiome measures, a longitudinal analysis of dominating taxa has been performed. These results are presented following the primary analysis. A detailed rationale for performing these additional analyses are discussed before these results are presented. Following this, it is explored whether other factors (beyond timepoint) could independently influence the results presented in the primary analysis. As a result, a sub-analysis by sample type was performed.

3.3.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 8.2.1) or R (R Base package). The Anderson-Darling and D'Agostino & Pearson tests were used to test for normal distribution. Significance (p-value <0.05) for longitudinal data linked to specific participants was determined using paired t-tests for parametric data and the Wilcoxon matched-pairs signed rank test or the Kruskal-Wallis test for non-parametric data. The χ^2 test was used for comparisons of categorical data. For comparisons between unrelated cohorts unpaired t-test or 1-way ANOVA was used for parametric data and the Mann-Whitney U for non-parametric data. The benjamini-hochberg correction was employed where multiple tests were used (false discovery rate 0.05). The significance of inter-sample variance (β-diversity) was determined using PERMANOVA on Bray-Curtis similarity on square root transformed taxa relative abundance using PRIMER software version 7 (PRIMER-E, Plymouth, UK). Linear regression was performed in SAS (SAS Studio release 3.8). Linear Data were visualised using graphs prepared in GraphPad Prism (version 8.2.1) nMDS and PCOA plots were created using R (ggplot2 package) or PRIMER version 7. LEfSe LDA scores and graphs were produced using the Galaxy program (https://huttenhower.sph.harvard.edu/galaxy/) using 0.5 as the Alpha value

for the Kruskal-Wallis test and 2.0 as the logarithmic LDA score cut-off for discriminative features.

3.4 Results

3.4.1 Participant and sample characteristics

Fifty-seven patients were enrolled in the MOCI study (as depicted in Figure 3.1). Of these, 54 contributed samples included in the study analysis presented here. Not all 54 patients contributed to samples at each timepoint, but all patients included in the analysis contributed a stool/rectal sample at least one sampling timepoint. The 145 samples collected from these participants were split into three timepoints for the purpose of this analysis. These include timepoint 1 (t1), timepoint 2 (t2) and timepoint final (tfinal). The sample numbers, sample collection time and the sample types analysed at each of these timepoints is presented in Table 3.3. The demographic and clinical characteristics of the cohort (n=54) are presented in Table 3.4

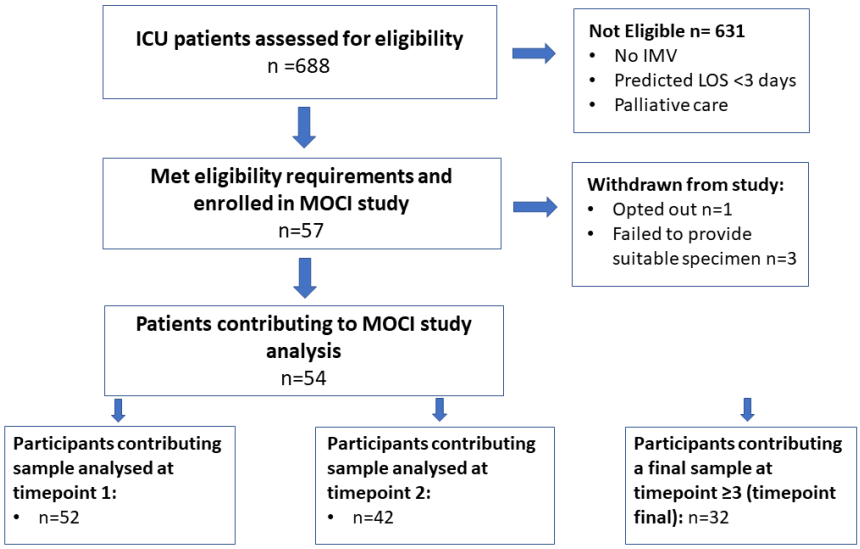


Figure 3.1 Participants screened and included in the MOCI study analysis

Table 3.3. Characteristics of samples included in MOCI analysis

	Timepoint 1 (t1)	Timepoint 2 (t2)	Timepoint final (tfinal)
Total samples analysed (N)	52	42	32

Hours from timepoint 1 Median [IQR]	0	48 [9]	142 [150]
Hours from ICU admission Median [IQR]	21 [28]	73 [28]	158 [132]
Rectal swab N (%)	43 (83%)	24 (57%)	14 (44%)
Stool swab N (%)	7 (13%)	16 (38%)	16 (50%)
Colostomy swab N (%)	2 (4%)	2 (5%)	2 (6%)
Within-participant sample matching N, (% of t1)	**t1 and t2** 39 (75%)		**t1 and tfinal** 31 (60%)

Table 3.4. MOCI study cohort demographic and clinical characteristics

Demographic and Clinical characteristics	**N, (%)**
Male	42 (77.8%)
Female	12 (22.2%)
18-49	19 (35.2%)
50-69	21 (38.9%)
≥70	14 (25.9%)
General community	32 (59.2%)
Long-term residential facility	1 (1.9%)
Hospital ward	21 (38.9%)
Respiratory disease	13 (24%)
Non-traumatic brain injury	10 (18.5 %)
Trauma/Burns	10 (18.5 %)
Cardiovascular disease	9 (16.6%)
Septic shock	6 (11.1%)
Gastro-intestinal disease	3 (5.6%)
Other diagnosis	3 (5.6%)
IMV time (median hrs [IQR])	96 [695]
Length of stay in unit (median hrs [IQR])	168 [111]
30-day mortality	13 (24%)

3.4.2 Primary longitudinal analysis

3.4.2.1 Changes to total bacterial load over time

In order to ascertain if differences in the absolute abundance of bacteria contributed to changes observed in over time, total bacterial load was assessed using 16S qPCR on DNA extracted from specimen swabs. Due to the use swabs, it was impossible to normalise the result per gram of stool. There was no relationship between total bacterial load and the sampling timepoint: median [IQR] cells/µL reaction (t1:37,486 [82,991] v t2:17,519 [15, 2474]; p=0.95; t1:37,486 [82,991] v tfinal:41,280 [214,198]; p=0.93).

3.4.2.2 Changes to bacterial microbiome α-diversity over time

Longitudinal changes to within-participant bacterial diversity were determined at three time points: timepoint 1 (t1: within 72 hrs of ICU admission, n=44), timepoint 2 (t2: collected within 48 hrs of the first sample, n=41) and the final specimen (tfinal: collected before discharge for each participant who had a further sample collected, n=33). Only participants who had an admission sample collected at t1 and at least one other sample (at t2 or tfinal of any sample type) were included in this analysis.

The within-participant paired comparisons revealed large decreases in diversity median [IQR] between the first and final sampling intervals using both Shannon and Faith's PD measures (Shannon t1 5.8 [1.1] v Shannon tfinal 5.6 [2.3] p=0.0006; Faith's t1 5.2 [2.4] v Faith's tfinal 3.0 [2.6]; p=0.0003). There were smaller but still significant decreases in diversity between t1 and t2 (Shannon t1 5.8 [1.1] v Shannon t2 5.6 [1.3]; p=0.02, Faith's t1 5.2 [2.4] v Faith's t2 4.6 [2.0]; p=0.003), and between t2 and tfinal (Shannon t2 5.6 [1.3] v Shannon tfinal 5.6 [2.3]; p=0.004, Faith's t2 4.6 [2.0] v Faith's tfinal 3.0 [2.6]; p=0.001). These results are depicted in Figure 3.2.

3.4.2.3 Changes to bacterial microbiome composition over time

There was a significant change to bacterial microbiome composition evident when all samples collected at t1 were compared to samples collected at t2 (t1 v t2 PERMANOVA; p=0.015). This change was larger and more significant when samples collected at t1 were compared to samples collected at tfinal as depicted in Figure 3 (t1 v tfinal PERMANOVA; p=0.0001). The difference in sample dispersion was also significantly different in this comparison (t1 v tfinal PERMDISP; p=0.0011). The changes to composition and dispersion between t1 and tfinal are presented in figure 3.3.

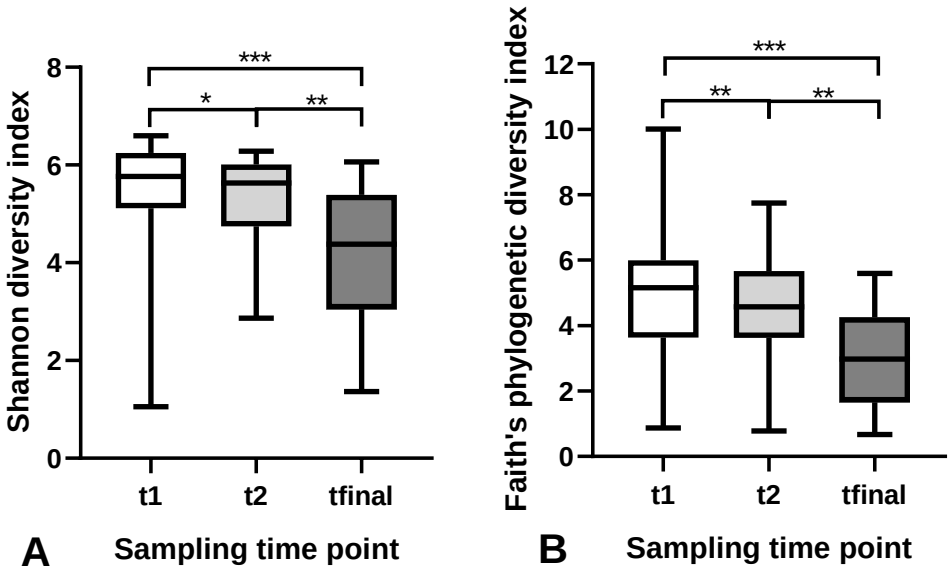


Figure 3.2 Longitudinal changes in bacterial α-diversity

This figure depicts bacterial diversity using the Shannon diversity index (plot A) and Faith's phylogenetic diversity index (plot B) at three sampling timepoints. Timepoint 1 (t1, n=44) is the sample closest to ICU admission, timepoint 2 (t2, n=41) is the sample taken 48hrs following sample t1, timepoint final (tfinal, n=33) is the sample closest to discharge in participants with three or more samples collected. The box plot depicts median ± interquartile range with bars representing minimum and maximum values. Significant comparisons are indicated by stars (*=$p<0.05$; **= $p<0.01$; ***= $p<0.001$; Wilcoxon matched-pairs signed rank test).

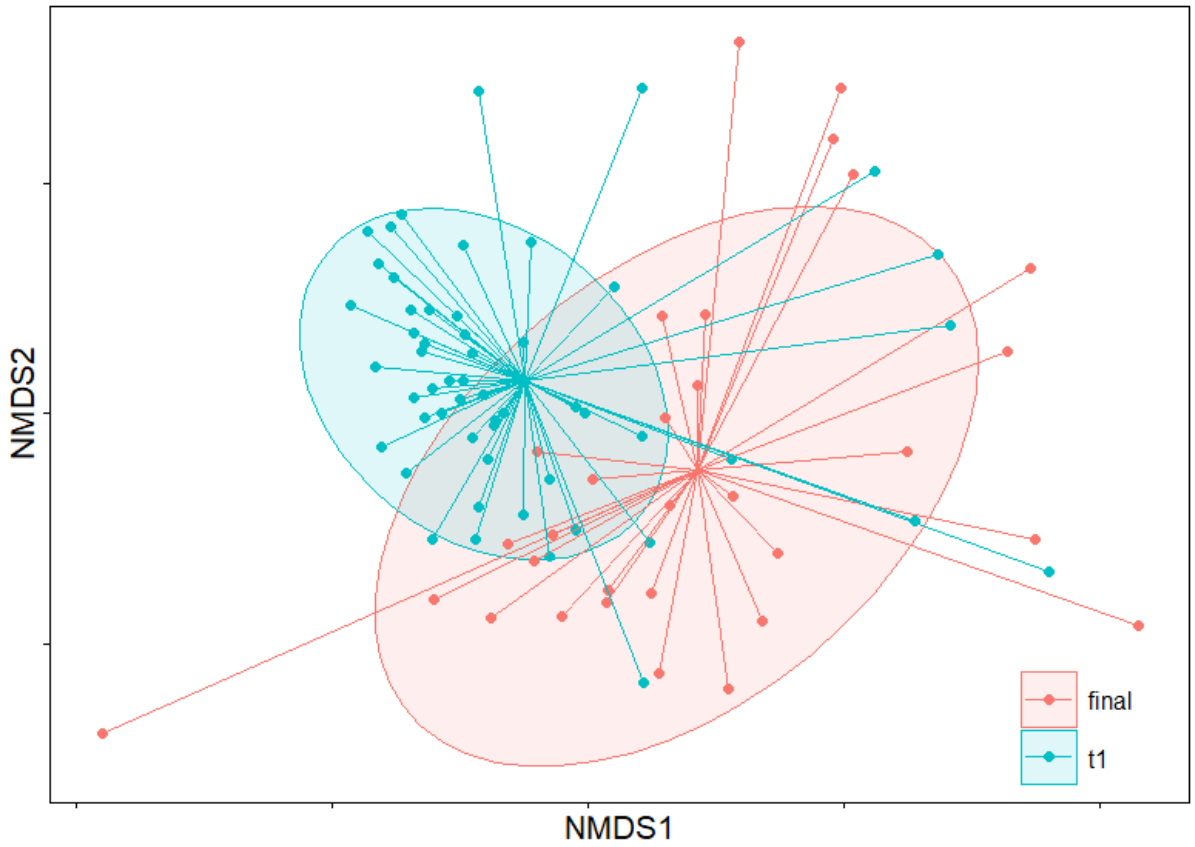


Figure 3.3 Gut microbiome composition distribution and dispersal over time

Non-metric multi-dimensional scaling (nMDS) plot showing between group comparisons of faecal microbiome distribution and dispersal. nMDS plots depicted from Bray-Curtis resemblance of square root transformed, genus-level, relative abundance data. Shaded ovals represent 80% confidence interval. There is a significant difference between the faecal microbiome distribution and dispersion of samples taken on admission to ICU (t1) compared to the final sample (PERMANOVA; p=0.0001; PERMDISP; p=0.0011).

3.4.2.4 Bray Curtis dissimilarity across time

Using the Bray Curtis dissimilarity index (where 0 indicates sample composition is identical and 1 indicates there are no shared species) it is possible to track the degree of compositional changes occurring within individual participants over time. When comparing the microbiome composition of two unrelated participants at t1, the median Bray Curtis dissimilarity index is 0.91 [0.1] (across 1, 275 paired comparisons). When participant's own baseline samples (t1) are compared to samples taken 48hrs later (t2) the median [IQR] Bray Curtis dissimilarity index is 0.73 [0.39] which is substantially lower than the dissimilarity between unrelated participants ($p<0.0001$). When participant's own baseline samples (t1) are compared with the

final sampling timepoint (tfinal) the median Bray Curtis dissimilarity index increases to 0.85 [0.30]. This is a significantly greater value than the change between t1 and t2 (p=0.002). This degree of dissimilarity is not significantly different from that seen between unrelated participants (p=0.24). These results are depicted in Figure 3.4.

When the analysis is performed

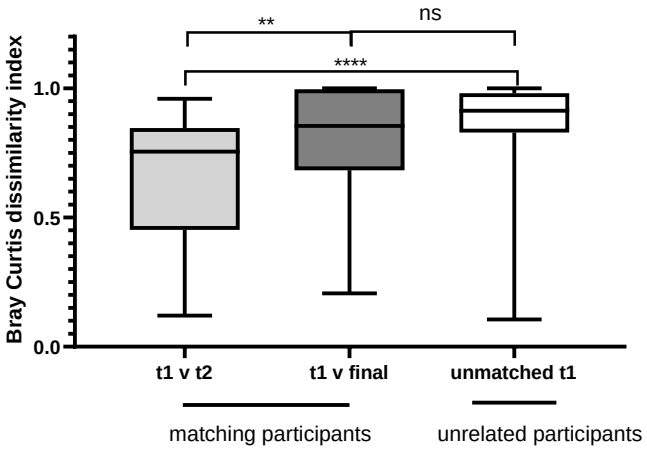


Figure 3.4 Faecal microbiome change measured by the Bray Curtis dissimilarity score in the MOCI cohort over time

The box plot figure (panel A) depicts the median, IQR and range of the degree of similarity of the microbiomes in groups of samples using the Bray Curtis dissimilarity index where 0 indicates sample composition is identical and 1 indicates there are no shared species. The degree of similarly in paired samples from unrelated participants in the cohort at timepoint 1(unmatched t1) is compared to the degree of similarity between participant's own matched samples at different timepoints (t1 v t2 and t1 v tfinal). There was no significant difference (ns) between the t1 v final Bray Curtis dissimilarity score and the dissimilarity score of unrelated participants. Significant comparisons (Mann-Whitney test) are indicated by stars (**= $p<0.01$; ****= $p<0.0001$).

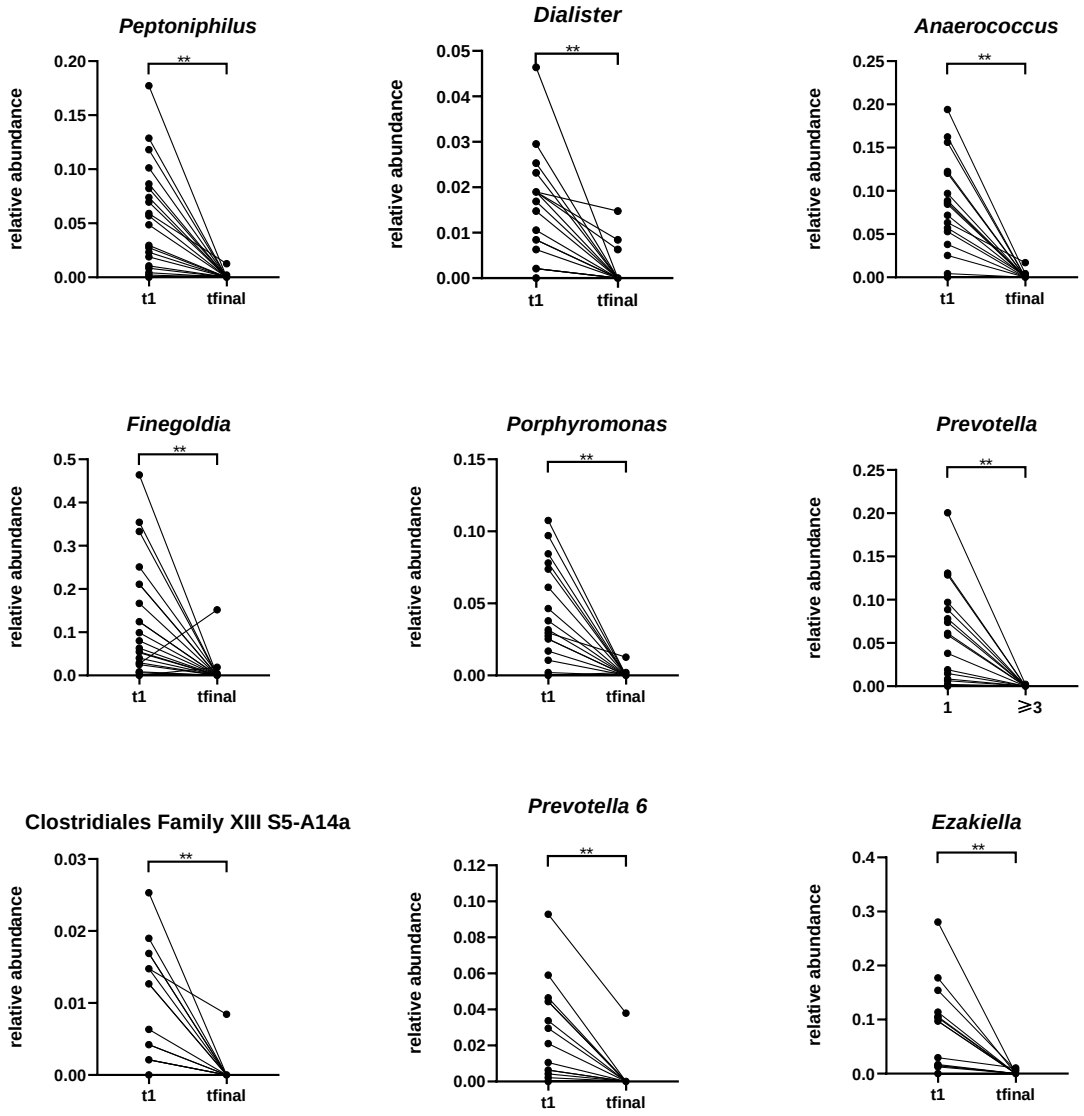


Figure 3.5. Collapse of commensal microbiota over time in ICU

This figure depicts taxa whose relative abundance significantly decreased between the first sampling timepoint (t1) and the final sampling timepoint (tfinal) in a paired within-participant analysis. Significance was determined using the wilcoxon matched-pairs signed rank test with correction for multiple testing. The level of significance is indicated by stars (*=$p<0.05$; **=$p<0.01$).

3.4.3 Predominant taxa and intestinal domination over time

The predominant taxon in each sample at each timepoint was determined and depicted in Figure 3.6. Intestinal domination by a single genus was measured in two different ways to allow correlation with previously published findings. Taur et al (139) defines the dominating taxon as a single genus comprising >30% of sequences (as long as no other taxon is more abundant) and has linked domination by pathogenic taxa (including various Proteobacteria, *Streptococcus* and *Enterococcus*) to bacteraemia with the corresponding organisms in allo-HSCT recipients. Using this definition, intestinal domination was a common phenomenon in this study, occurring in 33% of the cohort at t1 and rising to 72% by the final timepoint (p=0.001). In half of these specimens, the dominating taxon was a potential pathogen. *Enterococcus* was the most common dominant pathogen at timepoint 1 (dominant in 8%) and remained dominant in the same proportion of patients at the final timepoint.

A stricter definition of intestinal domination is when >50% of sequences in a specimen align to a single genus (242). In these patients, extreme domination is exhibited, with one genus frequently representing >90% of the bacterial sequences in the sample. As shown in Table 3.5, intestinal domination was significantly more likely to occur at the final sampling timepoint than at earlier timepoints. At t1, 10% of the cohort already exhibited extreme domination and this rose to 44% by the final timepoint (p=0.0007). Increasing levels of domination by both obligate anaerobic commensals and facultative pathobionts contributed to this phenomenon.

3.4.4 Other microbial markers correlated with infection

Beyond measuring intestinal domination, others have tied the prevalence certain of pathogenic species in the gut to the risk of future infection. Mancini et al linked a >5% relative abundance of Enterobacteriaceae in the specimen with the risk of gram-negative sepsis in haematological cancer patients (241). Additionally, Freedberg et al linked the presence of any level of *E. coli*, *C. difficile*, *P. aeruginosa* or *K. pneumoniae* at admission to ICU with subsequent infection with the same organism (240). In this study, the identification of >5% relative abundance of Enterobacteriaceae or any *Pseudomonas* did not change over time however, the proportion of samples with any *Escherichia* decreased significantly between t1 and tfinal (65% v 41% p=0.03, Table 3.6).

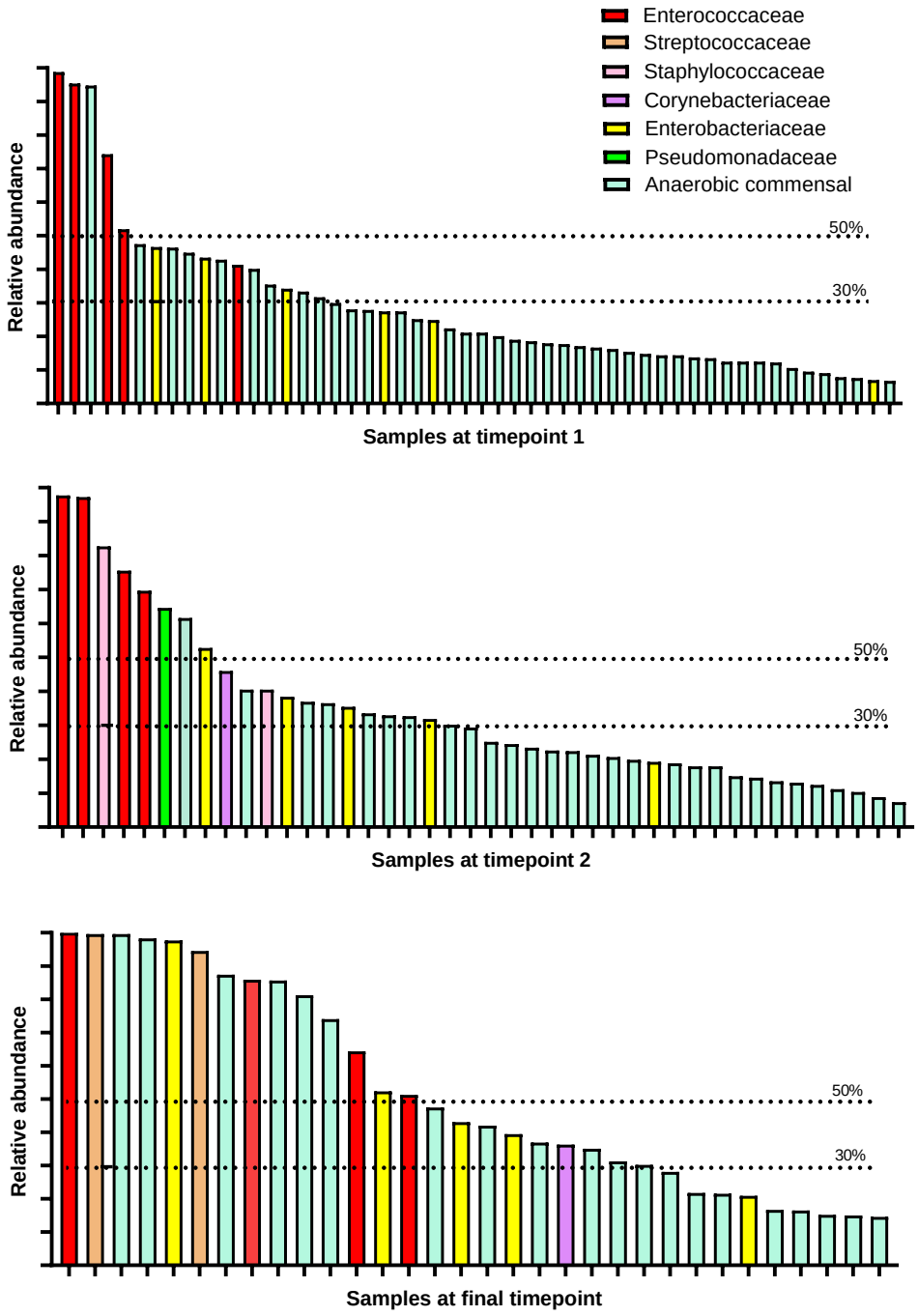


Figure 3.6. Predominant intestinal taxa over time

This figure depicts the relative abundance of the predominant taxon in each specimen analysed at timepoints 1, 2 and final. Light blue bars represent anerobic commensal species, whist the other coloured bars indicate the predominant taxon is a facultative or aerobic pathobiont. Bars taller than the 30% or 50% dotted lines indicate the predominant taxon makes up greater than 30% or 50% of the total bacterial microbiome respectively.

Table 3.5: Intestinal domination of ICU patients at different sampling timepoints

Total samples	Timepoint 1 (n=52)		Timepoint 2 (n=42)		Timepoint final (n=32)	
Dominating genus %	**>50%[1]**	**>30%[2]**	**>50%[1]**	**>30%[2]**	**>50%[1]**	**>30%[2]**
Any genus total (n, [%])	**5 (10%)**	**17 (33%)**	**7 (17%)**	**20 (48%)**	**14 (44%)**	**23 (72%)**
Number with domination different from timepoint 1? p-value (χ^2)	NA	NA	No p=0.5	No p=0.2	Yes p<0.001	Yes p=0.001
Obligate anaerobes total (n, [%])	**1 (2%)**	**9 (17%)**	**2 (5%)**	**9 (21%)**	**6 (19%)**	**12 (38%)**
Anaerococcus	0	1	0	0	0	0
Abiotrophia	0	1	0	0	0	0
Asteroplasma	0	0	0	0	0	1
Bacteroides	0	0	2	4	4	5
Bifidobacteirum	0	1	0	2	0	0
Clostridiales vadinBB60 group	0	0	0	0	0	3
Finegoldia	0	4	0	1	0	0
Lactobacillus	1	1	0	1	0	1
Parabacteroides	0	1	0	1	0	0
Ruminococcus torques group	0	0	0	0	1	1
Sphaerochaeta	0	0	0	0	1	1
Facultative/aerobic bacteria (n, [%])	**4 (7.7%)**	**8 (15%)**	**5 (12%)**	**11 (26%)**	**8 (25%)**	**11 (34%)**
*Corynebacterium**	0	0	0	1	0	1
Enterobacteriaceae (un-named)*	0	1	0	2	2	3
*Enterococcus**	3	4	2	2	3	3
Enterococcaceae (un-named)*	1	1	1	1	1	1
*Escherichia**	0	2	0	2	0	1
*Pseudomonas**	0	0	1	1	0	0
*Staphylococcus**	0	0	1	2	0	0
*Streptococcus**	0	0	0	0	2	2

1. Greater than 50% reads aligned to the dominating genus

2. Greater than 30% of reads aligned to the dominating genus (and no other genus was dominant)

* Facultative or anaerobic taxa with pathogenic potential

Table 3.6: *Pseudomonas*, *Escherichia* and Enterobacteriaceae changes over time

	Timepoint 1 (t1)	Timepoint 2 (t2)	Timepoint final (tfinal)	p-value (χ^2) (t1 v tfinal)
***Pseudomonas* (any)**	4 (7.7%)	4 (9.5%)	4 (12.5%)	**0.47**
***Escherichia* (any)**	34 (65.4%)	24 (57.2%)	13 (40.6%)	**0.03**
Enterobacteriaceae (>5%)	17 (32.7%)	14 (33.3%)	9 (28.1%)	**0.66**

3.4.5 Case study: correlation of gut microbiome with nosocomial bacteraemia

Only two patients in this cohort developed bacteraemia after a specimen was collected. In the first case the patient developed bacteraemia with *Enterococcus faecalis*. There was only a single specimen collected for this patient and there were no reads aligning to *Enterococcus* in this specimen. Therefore, there was clearly no relationship between the presence of this pathogen in the gut and this episode of bacteraemia.

The second patient developed bacteraemia with *Morganella morganii* (a member of the Enterobacteriaceae) at timepoint 9 (t9), shown in yellow in Figure 3.7 below. At baseline (t1), this patient's microbiome is characterised by a high abundance of pathobionts (5.6% *Morganella* [purlpe],10.3% other Enterobacteriaceae [grey], 2% *Staphylococcus* [red] and 41% *Enterococcus* [yellow]) and low bacterial diversity (34 observed species). Nonetheless, at t1 this microbiome also had commensals usually abundant in healthy people (*Bacteroides* 3.3%, *Faecalibacterium* 0.4% and *Bifidobacterium* 2.5%). However, this microbiome was very unstable, and by t2 the relative abundance of these three commensals had fallen to zero (and remained at this level thereafter), the richness fell to 16 observed species and microbiome became dominated by *Staphylococcus* (84%). In the samples preceding the episode of bacteraemia, *Morganella* relative abundance expanded from 2% at t7 to 15% at t9 when bacteraemia with this organism occurred. At t12 *Morganella* relative abundance increased to 30% and *Enterococcus* was the dominating organism with 51% of sequences aligning with this genus. The patient passed away a few days after this specimen was collected.

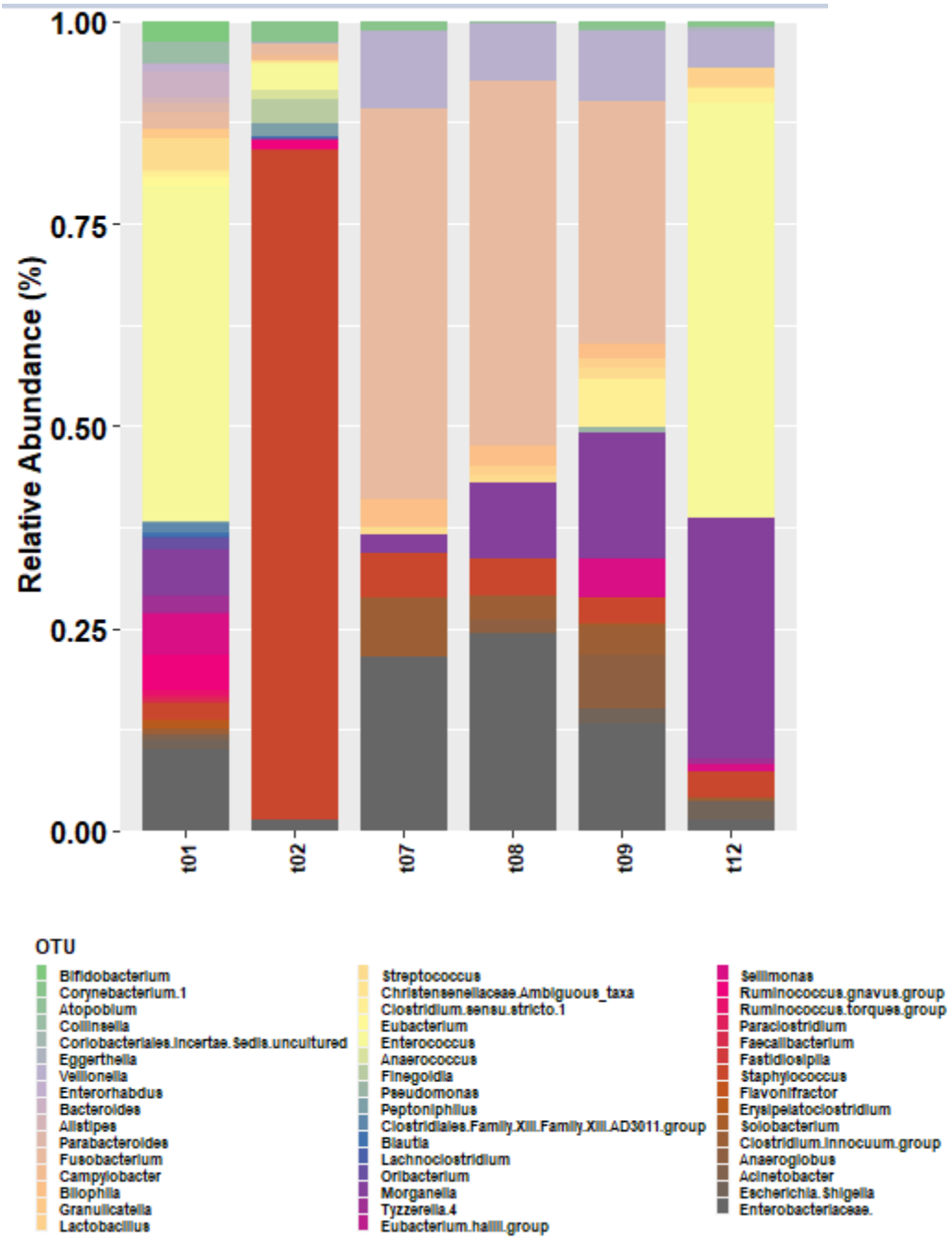


Figure 3.7. Taxa bar plot of a participant who developed bacteraemia in ICU

This bar plot shows all detected genera (n=47) and their relative abundance in a single patient who was sampled at multiple timepoints during their ICU stay. This patient developed bacteraemia with *Morganella* (purple) at timepoint 9 (t9).

3.4.6 Other microbial markers correlated with infection

At this stage it was considered whether other factors, apart from sampling timepoint, could independently influence microbiome composition. I considered which variables could plausibly influence the microbiome of the samples collected, and analysed whether sample type, age, gender, admission with sepsis, hospitalisation >48hrs prior to admission and the use of antibiotics caused significant differences between samples at baseline (t1).

3.4.6.1 Univariate and multivariate regression of variables that could affect microbiome composition

To assess the impact of each of these variables on microbiome composition, univariate and multivariate linear regression was performed. The dependant variable used to represent microbiome composition of each sample was the PCOA axis 1 coordinates (PCO1 x-axis, Figure 3.8).

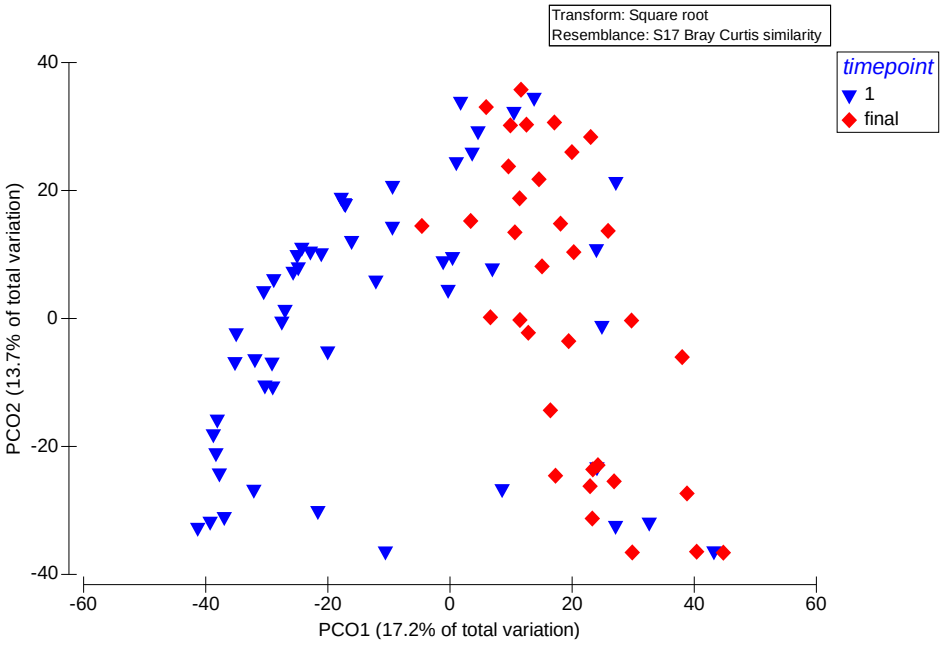


Figure 3.8. Principal coordinate analysis (PCoA) plot visualising similarity of sample composition This PCoA plot of square-root transformed Bray-Curtis similarity data at timepoint 1 (blue) and timepoint final (red) is depicted. PCO1 (x-axis) represents 17.2% of the total variation of the Bray-Curtis similarity data.

Table 3.7. Univariate and Multivariate regression of variables that could affect microbiome composition.

Univariate Model Reference category (R^2)	Variable (n=52)	Univariate Analysis		Multivariate Analysis		R^2 of each variable in multivariate analysis (R^2 of the multivariate model)
		coeff	p-value	*Coeff*	p-value	
Admission from health-care setting (0.21)	Community admission	-20.5	0.0006	2.9	0.6442	<0.01 (0.51)
No sepsis (0.13)	Has sepsis[1]	18.2	0.0036	9.5	0.1253	0.05 (0.51)
No antibiotics (0.07)	Broad[2]	13.2	0.0896	1.8	0.80	<0.01 (0.51)
	Narrow[3]	4.4	0.6658	2.2	0.78	
Male (0.02)	Female	3.6	0.3671	5.5	0.1827	0.04 (0.51)
Sample type is stool swab (0.43)	Rectal swab	-34.5	<0.0001	-29.7	0.0004	0.39 (0.51)
	Colostomy swab	14.4	0.2990	11.1	0.4303	
Age as continuous variable (<0.01)		-0.09	0.6284	-0.12	0.2811	0.02 (0.51)

[1]Sepsis as defined in the third international consensus definitions for sepsis and septic shock (Sepsis-3)
[2]Broad-spectrum antibiotics (selected due to broad anaerobic activity) include: amoxicillin-clavulanate, piperacillin-tazobactam, any carbapenem or any other antibiotic when combined with metronidazole, clindamycin or oral vancomycin.
[3]Narrow-spectrum: any antibiotic not considered broad-spectrum (as above)

As presented in Table 3.7 (above) each of the following variables were examined in 6 separate univariate models. Model 1: community admission (admission from the community within 48 h [n=30] vs admission from hospital [n=22]); Model 2: sepsis (the presence of sepsis on admission [n=19] vs no sepsis on admission [n=33]); Model 3: the use of antibiotics at timepoint 1(broad-spectrum [n=33], narrow-spectrum [n=8] vs no antibiotics [n=11, reference category]); Model 4: female (n=15) vs male (n=37); Model 5: sample type (rectal swab [n=43], colostomy swab [n=2] vs stool swab [n=7, reference category]). Model 6: age (age as a continuous variable). The univariate analysis of these 6 models showed that

community admission, the presence of sepsis, and a rectal swab sample type were all variables associated with significant changes in baseline microbiome composition (Table 3.7). These variables were assessed together in a multivariate regression (where the R-squared shows that the multivariate analysis in total was able to account for 51% of the variance of the dependent variable). Following multivariate analysis, sample-type remained the only significant variable associated with altered microbiome composition at timepoint 1 (p=0.0006) and accounted for 39% of the variance. PERMANOVA analysis of the nMDS of Bray-Curtis similarity data confirmed the significant difference between the composition of stool samples as compared to rectal swabs at both timepoint 1 and timepoint 2 (PERMANOVA; p=0.0001 rectal swabs v stool swabs at t1, PERMANOVA; p=0.001 rectal swabs v stool swabs at t2).

3.4.6.2 Specific taxa driving differences between stool samples and rectal swab samples

A LEfSe analysis (Figure 3.9, below) was performed to determine which taxa (at all phylogenetic levels) were driving the compositional changes between rectal swabs and stool swabs. It has previously been shown that facultative or aerobic skin flora which have been sampled from the rectal epithelium drive this difference (230). However, the LEfSe plot shows that only one such bacterial type (*Corynebacterium*) is more prevalent in rectal swabs, while skin flora belonging to the genus *Staphylococcus* are unexpectedly more common in the stool specimens. The remaining species are anaerobic flora that are found in stool, although many of these are species belong to Clostridium Family XI (eg *Anaerococcus, Murdochiella and Peptoniphilus*), commensals that are likely to prefer the rectal site (230). In Figure 3.9, taxa (at all phylogenic levels) that have previously been found to be more prevalent in rectal samples are indicated by an Asterix.

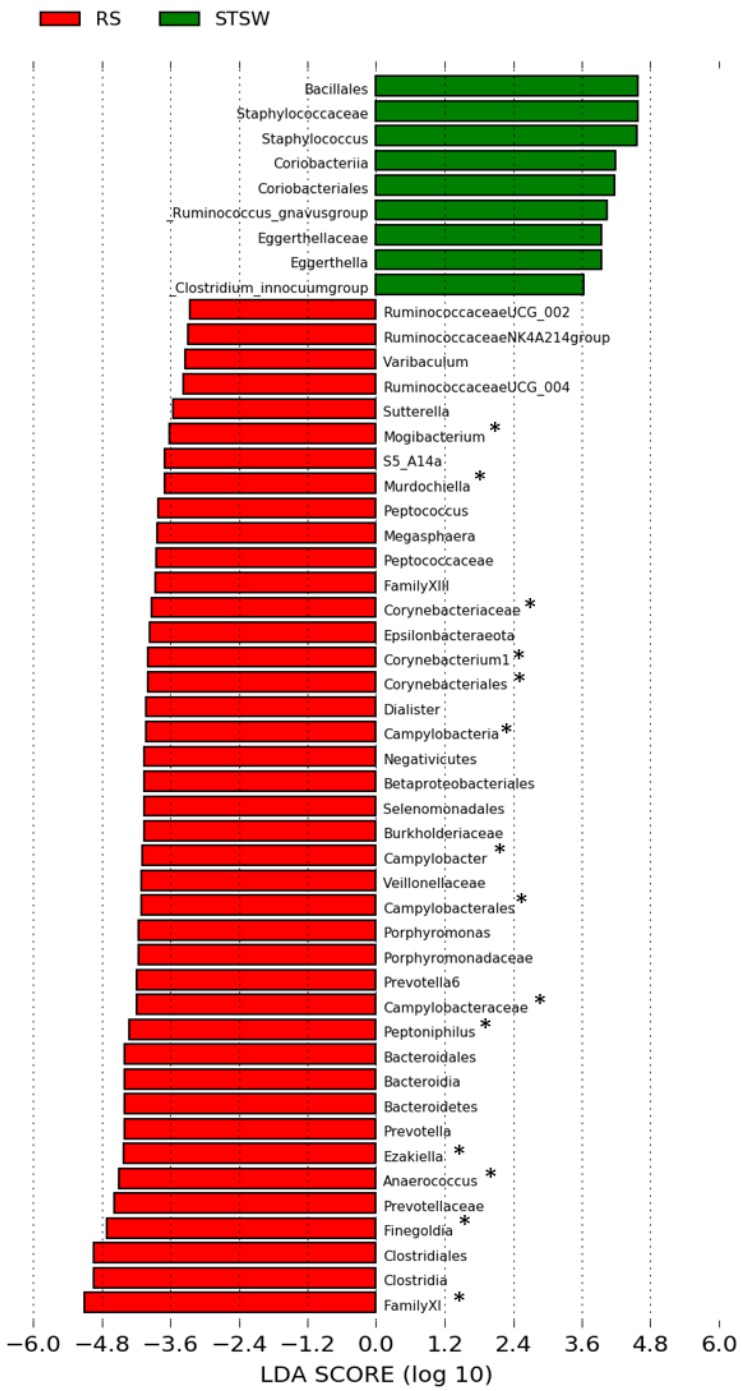


Figure 3.9. Specific taxa driving differences between stool samples and rectal swabs

A linear discriminant analysis (LDA)-based algorithm underlies the LEfSe analysis which depicts the taxa at all phylogenetic levels driving the significant difference between rectal swab (red) and stool swab samples (green). LDA scores are proportional to the relative change within the bacterial taxa depicted. The Asterix symbol indicates taxa previously shown to correlate with rectal swab sampling methodology (230).

3.4.7 Longitudinal analysis with sampling type considered

Rectal swabs were found to have significantly different composition when compared to stool swabs but were also unequally distributed between timepoints. Unlike patient-associated characteristics that remain constant over time, there was a significant difference in sample type collected at different timepoints (t1 consists of 83% rectal swabs, but by the final timepoint the proportion of the samples that are rectal swabs falls to 44%, $p<0.0001$ t1 v tfinal; Figure 10).

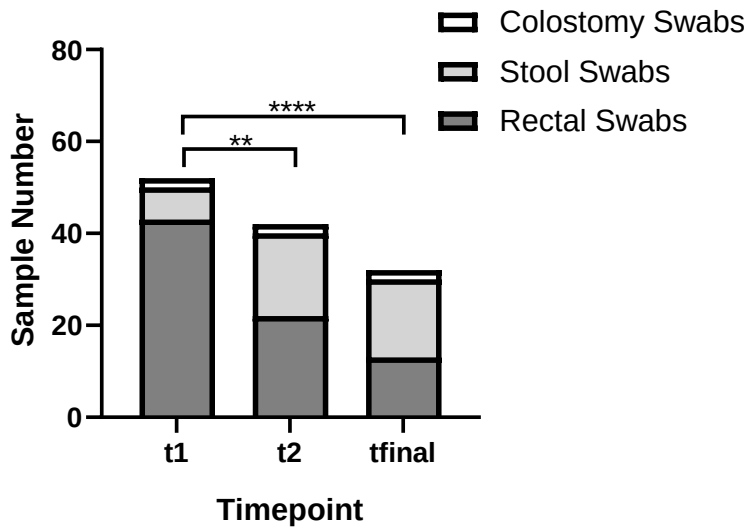


Figure 3.10. Number of samples types collected at different timepoints

The sample type collected varies significantly over time. At timepoint 1 (t1) the majority of samples (83%) are rectal swabs, by timepoint final (tfinal) the proportion falls to 44%. Significant comparisons (1-way ANOVA) are indicated by stars (**=$p<0.01$; ****= $p<0.0001$).

Given the large differences in the microbiome characteristics between rectal and stool sample types, it is possible that the longitudinal changes observed simply reflect the changing mix of sample types in the cohort. Therefore, to assess whether microbiome changes truly occur over time, the primary analysis (analysing the change in microbiome composition at three timepoints) was performed again using a matched-sample type analysis. In this analysis, when within-participant sample pairing was used, only comparisons involving the same sample type were included in the analysis. When an analysis was performed without participant pairing, the analysis was limited to rectal swabs only (since this was the most common sample type overall). The number of specimens, and the specimen types involved in the matched-specimen

type analysis (as compared to the sample numbers in the primary analysis) are presented in Table 8 below.

Table 3.8. Specimen types involved in matched sample-type analysis

	Paired timepoints	
	t1 and t2	**t1 and tfinal**
Any sample type paired (primary analysis)	39	31
Matched sample-type (sub-analysis)	21 (54%)	20 (65%)
• rectal swabs	16 (76%)	12 (60%)
• stool swabs	3 (14%)	6 (30%)
• colostomy swabs	2 (10%)	2 (10%)

3.4.7.1 Total bacterial load

To ascertain if differences in the absolute abundance of bacteria contributed to changes observed with sampling method, total bacterial load was assessed using 16S qPCR on DNA extracted from specimen swabs. Due to the use of swabs, it was impossible to normalise the result per gram of stool. There was no significant difference in bacterial load (median [IQR] cells/µL reaction) from rectal swab specimens as compared to stool swab specimens (23,432 [70,256] v 46,861 [347,993]; p=0.18).

3.4.7.2 Alpha-diversity of samples included in the matched sample-type analysis

The change over time for both indices of α-diversity using a matched sample-type analysis are depicted in Figure 3.11. There was no significant difference (median [IQR]) in the Shannon diversity index between timepoints 1 and 2 (t1 v t2: 5.4 [1.1] v 5.3 [1.3] p=0.16 and between t2 and tfinal (5.3 [1.3] v 4.9 [2.4] p=0.17) However, Faith's PD still decreased significantly during these time intervals (t1 v t2: 5.0 [2.0] v 4.6 [3.1], p=0.01 and t2 v tfinal: 4.6 [3.1] v 3.5 [2.9], p=0.03). Alpha-diversity decreased significantly and by a larger degree for both measures when t1 was compared to the final sample (Shannon 5.4 [1.1] v 4.9 [2.4] p=0.002; Faith's PD 5.0 [2.0] v 3.5 [2.9], p=0.0006).

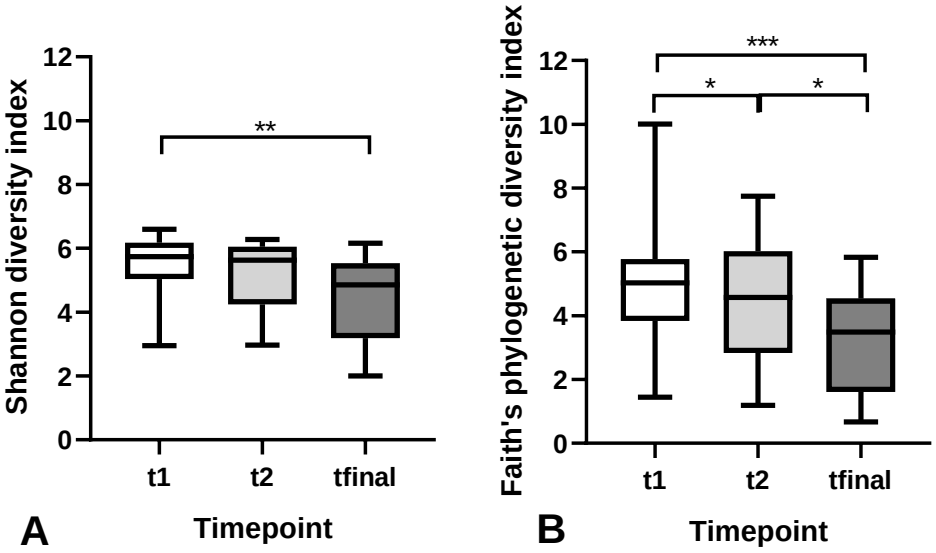
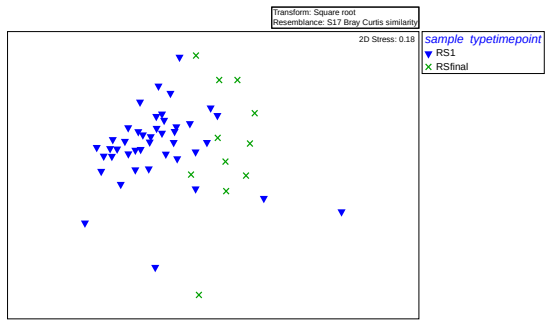


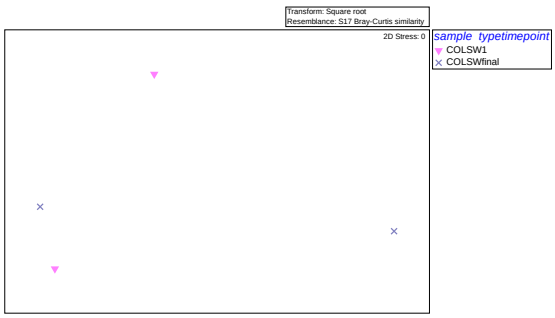
Figure 3.11. Longitudinal changes in bacterial α-diversity of matching sample types
Shannon diversity index (plot A) and Faith's phylogenetic diversity index (plot B) during ICU admission is depicted at three sampling timepoints: timepoint 1 (t1), timepoint 2 (t2), and timepoint final (tfinal). The box plot depicts median ± interquartile range with bars representing minimum and maximum values. This analysis is limited to participants with matching sample types. Significant comparisons are indicated by stars (*=p<0.05; **= p<0.01; ***= p<0.001; Wilcoxon matched-pairs signed rank test).

3.4.7.3 Beta-diversity of rectal swabs specimens over time

The changes in composition between t1 and tfinal observed in the primary analysis remained significant when controlled for sample type using an analysis of only rectal swabs (t1 v tfinal PERMANOVA p=0.0001). The data is presented in the nMDS plot below (Figure 3.12).



A



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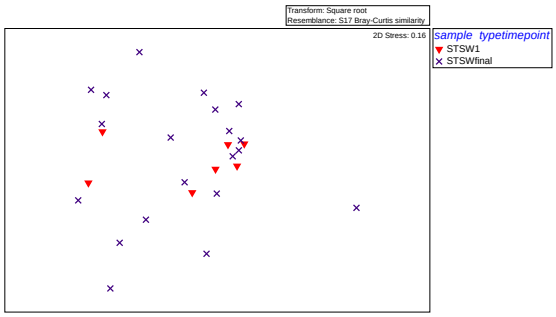

B

Figure 3.12. Non-metric multi-dimensional scaling (nMDS) plot of sample composition at t1 v tfinal by sample type.

This nMDS plot using square-root transformed Bray Curtis similarity data shows that the microbiome composition of rectal swabs taken at timepoint 1 (RS1, blue triangles) is significantly different from the composition of rectal swabs taken at the final timepoint (RSfinal, green crosses) (PERMANOVA p=0.0001).

There was no significant change over time when stool samples only (STSW1 red triangles vs STSWfinal black crosses; Panel B) were analysed (PERMANOVA p=0.7). Colostomy swab (COLSW) samples are presented in Panel C but are too few to undergo statistical analysis.

3.4.7.4 Microbiome composition change over time using a matched sample-type analysis

The Bray Curtis dissimilarity index (where 0 indicates sample composition is identical and 1 indicates there are no shared species) was used to track the degree of compositional stability occurring within individual participants over time. Results are reported as Bray Curtis dissimilarity score (median [IQR]) across groups of paired comparisons. In the analysis presented here only rectal swabs were used in the comparisons between the microbiome

composition of pairs of unrelated participants at timepoint 1 (n=1,059, 0.89 [0.15]), and between paired samples from the same participant at t1 and t2 (n=16, 0.52 [0.37]) and t1 and tfinal (n=12, 0.85 [0.12]). The results of the sample-matched analysis are presented in Figure 3.13. These results closely align with the results of the primary analysis of all samples (Figure 3.4), with significantly increased Bray-Curtis dissimilarity scores occurring over time in ICU (when t1 v t2 is compared to t1 v tfinal [p=0.002]). As in the original analysis, the degree of microbiome change occurring between t1 to tfinal is so great that the samples of matched participants are as different from each other as those of different participants (p=0.57).

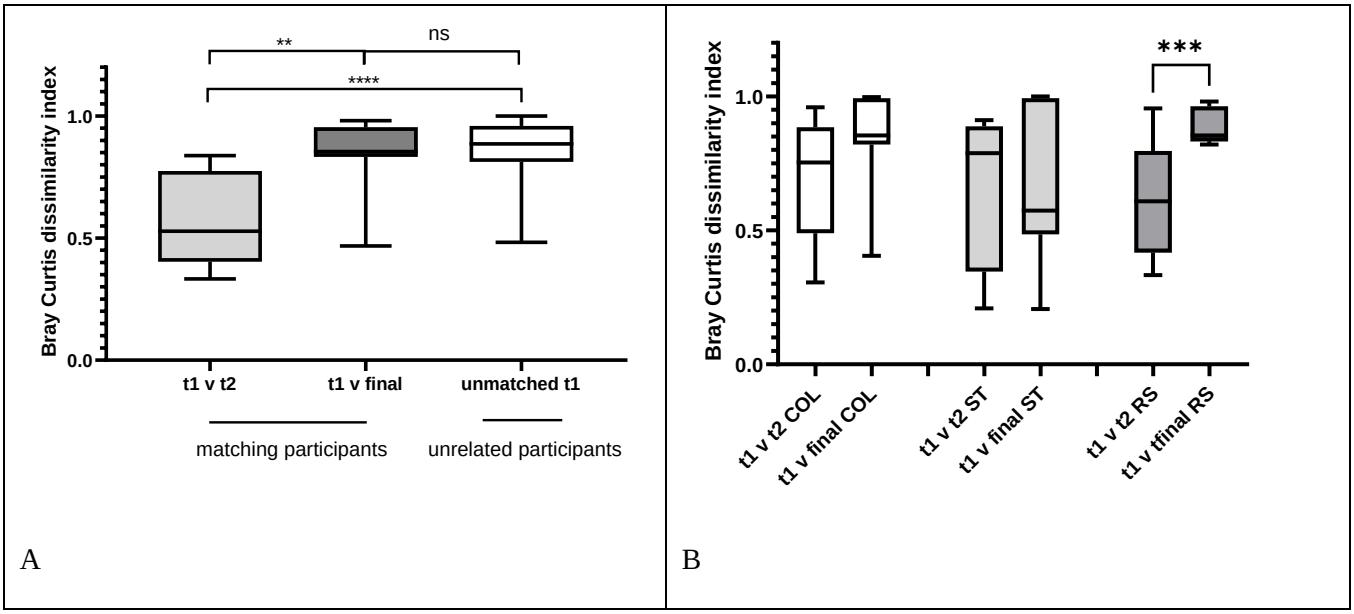

Figure 3.13. Microbiome change in rectal swabs measured by the Bray Curtis dissimilarity score in the MOCI cohort over time

The box plot figure depicts the median, IQR and range of the degree of similarity of the microbiomes in groups of samples using the Bray Curtis dissimilarity index. The degree of similarly in rectal swab samples from unrelated participants in the cohort at timepoint 1 (unmatched t1) is compared to the degree of similarity between participants with matched rectal swab samples at different timepoints (t1 v t2 and t1 v tfinal). There was no significant difference (ns) between the matched participant t1 v tfinal Bray Curtis dissimilarity scores and the dissimilarity scores between pairs of unrelated participants. Significant comparisons are indicated by stars (**= $p<0.01$; ****= $p<0.0001$).

Panel B represents the same data, broken down by sample type in matched patients who also had a matching sample type collected. There number of each sample type (colostomy: COL, stool: ST and rectal swab: RS) in each comparison is shown in table. This shows that the rectal swab sample type is the only sample type where significant change in overall microbiome composition is seen over time. Too few COL samples (n=2) were available to perform statistical analysis.

3.4.7.5 Change in individual taxa over time using a matched sample-type analysis

The results of the matched sample-type analysis analysing the changes in individual taxa over time were very similar to the primary analysis. There was strong agreement in which taxa changed between t1 and tfinal. All taxa found to change significantly in this analysis were also found to change significantly in the primary analysis. In this analysis the changes to 11 taxa were significant prior to FDR correction and of these 9 were decreases (Clostridiales Family XIII, *Peptoniphilus, Dialister, Anaerococcus, Finegoldia, Porphyromonas, Prevetolla, Prevetolla-6, Ezakiella*) from the cohort as shown in Figure 14. There was only one that increased significantly (prior to FDR correction) in relative abundance (unassigned taxa from Ruminococcaceae). Following correction for multiple testing, only the decreases in the relative abundance of *Prevotella*, *Dialister* and *Peptoniphilus* remained significant.

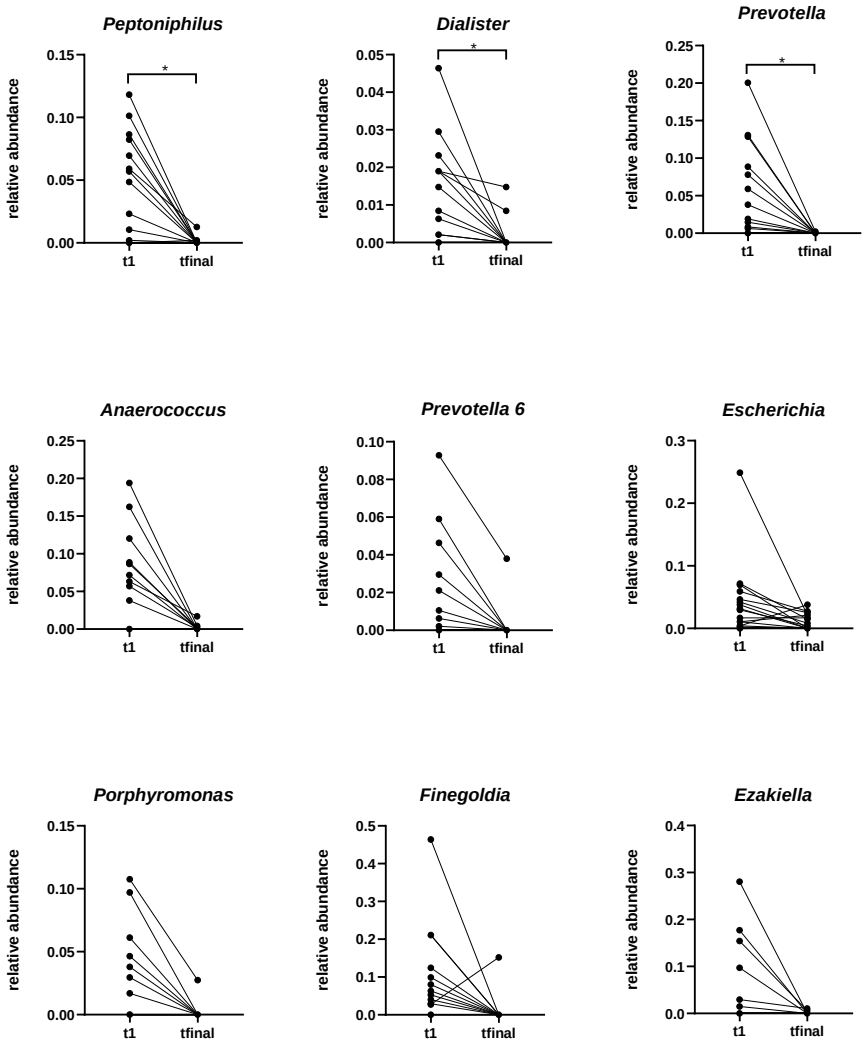

Figure 3.14. Change to specific taxa over time in ICU in a matched-sample analysis

This figure depicts all taxa whose relative abundance decreased significantly (prior to correction for multiple testing) between the first sampling timepoint (t1) and the final sampling timepoint (tfinal) in a paired within-participant matched sample-type analysis. Significance was determined using the wilcoxon matched-pairs signed rank test. For those comparisons that remained significant after correction for multiple testing, the level of significance is indicated by stars (*=$p<0.05$).

3.5. Discussion

The preliminary findings of the MOCI study presented here clearly support earlier studies (146, 147, 242) showing that gut microbiomes are profoundly disrupted during ICU care. By using a longitudinal analysis in matched individuals, we are also able to show that the strongest contributor to loss of diversity, compositional change and intestinal domination in the

microbiota of ICU patients is the sample being taken >96hrs after admission. By contrast, other factors which were hypothesised to influence the gut microbiota characteristics including the presence of sepsis, the use of broad-spectrum antibiotics, or admission from hospital appear to have relatively little impact on microbiome composition.

Interestingly, sample type was found to strongly influence microbiome composition independently of the other variables listed above. This substantial difference of microbiome composition between rectal swab samples and stool samples in ICU patients has also been recently described by Fair et al (255), who also longitudinally sampled ICU patients using either rectal swabs or stool samples. As also seen in the MOCI study, Fair et al noted that stool samples were difficult to obtain at the first sampling time interval but became the predominant sample type obtained at discharge. Fair et al concluded that the sampling method itself was the main contributor to differences in microbiome between the two sample types. However, neither this study nor the MOCI study were designed to answer this question. In order determine if analysis of rectal and stool samples result in a different microbiome composition the samples must be taken at the same time. Others (253, 256-260) have assessed microbiota from rectal swabs and stool in parallel and concluded that these samples are comparable with samples from the same person, producing a distinct individual signature that is present in both sample types. There are some notable differences in predominant taxa present in rectal swabs when compared to stool samples seen in previous studies. These differences are attributable to aerobic or facultative commensals which make up the skin flora of the rectal canal (230). Despite these differences, rectal samples still correlate with stool samples from the same individual (230, 256). When we consider the differences in the microbiota between the stool samples and rectal swabs in this study, the differences are much greater than would be expected. Therefore, it is likely that biological factors other than sampling method explain the differences between these sample types. This is supported by the observation that many of taxa which differ between these sample types are commensal anaerobic bacteria usually found in stool and not skin flora. In particular, taxa from the phylum Bacteriodetes (eg *Bacteroides* and *Prevotella*) have previously been noted to be stable regardless of sampling methodology (230, 260) or more prevalent in stool samples (255). However, in the MOCI analysis, members of the Bacteroidetes are strongly associated with rectal swabs. This finding cannot be explained by sampling methodology, but rather suggest that patients who produced stool samples at baseline had more disrupted gut microbiota. This hypothesis is supported by the fact that patients who had a stool sample collected at baseline did not have a significant change in microbiome over time unlike those who had a rectal swab at baseline (Figures 3.12 and 3.13).

Participants who produced a stool sample at timepoint 1 were hospitalised prior to ICU at much higher rates than the rectal swab cohort (100% v 31%) and used broad spectrum antibiotics at higher rates (71% v 61%). Most importantly, the median Bristol stool score of the 74 stool samples was 6 (IQR 1) indicating a "mushy stool". It is therefore likely that the ability to obtain stool as a specimen (especially at early timepoints) is a proxy for the presence of diarrhoea, itself an independent predictor of an altered microbiome (261). As a patient's bowel habit varies during admission, this is likely to reflect corresponding changes in gut microbiota. If diarrhoeal stool samples are not included in the analysis, important changes occurring to the patient's microbiota could be missed.

The ability to use both rectal swabs and stool samples is important in a longitudinal ICU study, because different types of samples are more readily obtained at different timepoints (255). At admission, when patients are less likely to pass stool but are sedated, rectal swabs are more easily obtained. However, at later timepoints it is only practical to obtain stool samples. Ethically, it is difficult to justify performing a rectal swab on a patient when a stool sample is readily available. Therefore, if the ultimate goal is to use a sampling method that is most easily available and clinically applicable, then ideally we need a measure that can be applied to faecal samples more broadly, regardless of whether that sample was obtained through sampling passed motions, colostomy output or by swabbing the rectum.

The most important finding of this study is that gut microbiota became increasingly disrupted over time in ICU. Firstly, there was a significant loss of bacterial diversity in an individual's gut microbiome over time. Using Faith's phylogenetic diversity index (but not the Shannon index) this decrease in diversity was evident after only 48hrs. By the final sampling timepoint (median 158 hrs after ICU admission), there were large and significant decreases in microbial diversity regardless of the diversity measure used or the sampling method used. It is surprising that use of broad-spectrum antibiotics at the timepoint studied did not have a significant association with loss of diversity. This may be because antibiotic exposure has cumulative effects over time that were not captured by this analysis.

Analysis of β-diversity indices also found an association between the final sampling time and highly significant changes in bacterial composition. It is remarkable that by the final sampling point an individual's microbiome had changed so much from their own baseline sample that, on average, these two samples were as dissimilar as the microbiomes of two unrelated patients.

When the specific taxa driving these changes were analysed, a clear trend emerged: most of the change was driven by the near complete collapse of a number of a number of closely related gram-positive anaerobic commensals in the order Clostridiales (Family XI: *Peptoniphilus,*

Anaerococcus, Finegoldia, Ezakiella, Mogibacterium) as well as large reductions in of typically abundant gram-negative anaerobic commensals in the order Bacteroidales (*Prevotella, Prevotella 6, Porphyomonas*). By contrast, there was no clear trend in which bacteria increased in abundance, signalling that these compensatory increases were diverse and differed between individuals.

The loss of members of the Clostridiales order, although belonging to different family clusters than observed here, has also been observed in other studies that sampled ICU patients longitudinally. Both Aardema et al (244) and Livanos et al (244) observed loss of bacteria in Clostridial clusters IV/XIVa after ICU stay (72hrs or greater) in stools and rectal swabs respectively. It should also be noted that Howard et al reported the opposite result of Clostridiales increasing after 72hrs in ICU.

Bacteria which were most likely to be lost during ICU admission, particularly members of the Clostridiales, play an important role in producing beneficial short-chain fatty acids (SCFA). In our study *Peptoniphilus* and *Anaerococcus* contribute to butyrate biosynthesis, *Prevotella* to propionate biosynthesis. However, this capacity is also present in several other abundant commensal species. Therefore, the loss of these taxa alone would be unlikely to significantly alter the capacity of the gut microbiota to produce SCFA or the functional capacity of the gut microbiota more broadly. However, the complete loss of several types of closely related bacteria could significantly contribute to loss of colonisation resistance by vacating an ecological niche, destabilising the microbiome's structure, and thereby allowing opportunistic bacteria to expand.

The abnormal expansion of opportunistic bacteria is demonstrated by the frequent observation of intestinal domination by a single taxon in the MOCI cohort. In normal populations, it is very unusual to find any one genus making up the majority (>50%) of microbiome, particularly if this bacterium is a pathobiont normally found in low abundance. In the dataset analysed in chapter 2 (consisting of 56 samples from 6 healthy faecal donors and 19 cancer patients undergoing chemotherapy) there were no samples where one taxon dominated >50% of relative abundance. By contrast, intestinal domination by one bacterial genus was present in 12% of the MOCI ICU cohort's samples at baseline, rising to 41% (>50% relative abundance) at the final timepoint. The majority of dominating organisms were facultative pathobionts in genera *Enterococcus, Streptococcus* or *Staphylococcus* or members of the Enterobacteriaceae family. These results are very consistent with those previous reported by Lankelma et al who observed intestinal domination in 38% of their ICU patient cohort using the same definition (242).

A relationship between intestinal domination (at a lower threshold of ≥ 30%) and bacteraemia has been shown previously by Taur et al (139) in patients following allogeneic stem cell transplant. In the ICU population, Freedberg et al associated Enterococcal dominance (≥ 30%) on admission with death and all cause infection (240), whilst the mere presence of certain pathobionts in the admission sample predicted subsequent infection with these organisms. Using this definition, 72% of the MOCI cohort experienced intestinal domination by the final timepoint.

At this stage our cohort is too small to assess role of the microbiome in these infrequent events, although it is possible to discuss the relative levels of the causative pathogens in the microbiomes of the two participants who experienced nosocomial bacteraemia and subsequently died within days. The first patient suffered bacteraemia with *Enterococcus faecalis*, however the microbiome from the only rectal swab sample taken during the study (and preceded this event by one day) did not show any *Enterococcus*. The second patient developed bacteraemia with *Morganella morganii* (a gram-negative member of the Enterobacteriaceae) at timepoint 10. At baseline, this participant's microbiome already was characterised by an abnormally high abundance of pathobionts including 5.6% *Morganella* and *Enterococcus* at 41%. In the samples preceding the bacteraemia, *Morganella* relative abundance expanded to reach 15% at timepoint 9 when bacteraemia with this organism occurred. Despite appropriate treatment, *Morganella* continued to expand and reached 30% relative abundance at timepoint 12 just before death. Although it is impossible to draw conclusions about the utility of using intestinal domination as a predictor of bacteraemia from only 2 cases, these cases suggest that predictive models will need to take into account multiple factors including longitudinal measures of microbiome stability, the pathogenicity of the bacterium that has expanded, and the surrounding microbial and host environment.

The main limitation of the MOCI study is that it is still underpowered to assess the effect of the microbiome on patient clinical outcomes. Expansion of the patient population studied is also required to accurately analyse the effect of specific ICU interventions such as the use of antibiotics and enteral feeding on microbiome composition. Another limitation is the lack of a control group undergoing longitudinal sampling to control for normal sample variability over time. However, because consistent trends have been observed over more than one sampling timepoint, these results are not likely to be spurious. A final important weakness of this study is that the analysis is limited to only examining bacterial composition through 16S rRNA gene amplicon sequencing. This type of analysis lacks the ability to determine whether patients also acquire clinically important antimicrobial resistance determinants during their admission, and

if such acquisition is linked to microbiome composition. Furthermore, non-bacterial pathogens, especially yeasts and other fungi, are likely to significantly expand in gut niches when bacterial populations are substantially depleted and di is required to form a complete picture of microbial dynamics in this population.

In order to address these limitations, the final MOCI study analysis will include shotgun metagenomic analysis on a subset of samples so that antimicrobial resistance genes and fungal composition can be assessed. Furthermore, the study recruitment has been expanded to include 116 patients to date. Once an interim analysis has been performed on these patients, it will be determined if further recruitment is required to adequately power the study to achieve the goal of assessing the role of gut microbiota in nosocomial sepsis and bacteraemia.

3. Acknowledgements

I would like to thank and acknowledge the RAH ICU research team, including Ben Reddi, Lee-anne Chapple, Marianne Chapman, Rhea Louis and Bethany Dunn, who assisted with the design, implementation, recruitment and clinical data collection for this study, as well as the many intensive care nurses who selflessly assisted with the sample collection for this study. I also would like to thank Sarah Sims and Levi Elms for tirelessly extracting DNA for this project and Steven Taylor for his invaluable assistance in statistical analysis, interpreting data and β-diversity analysis using PRIMER.

CHAPTER 4

Optimisation of a propidium monoazide based method to determine the viability of microbes in faecal slurries for transplantation

The contents of this chapter appear in the following publication:

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4.1 Abstract

The efficacy of faecal microbiota transplantation (FMT) as a therapeutic intervention may depend on the viability of the microorganisms in faecal slurries (FS) prepared from donor stool. However, determining the viability of these organisms is challenging. Most microorganisms in stool are refractory to culture using standard techniques, and culture-independent PCR-based methods derive signal from both viable and non-viable cells. Propidium monoazide (PMA) treatment has been shown to be effective in preventing PCR amplification of DNA from non-viable bacteria in a range of contexts. However, this methodology can be sensitive to factors such as bacterial load and sample turbidity. We describe the optimisation of a PMA treatment methodology for FS that restricts quantitative PCR-based bacterial enumeration to viable cells. When applied to concentrated FS (10-25% stool content), PMA treatment at 100 µM concentration was ineffective in preventing DNA amplification from heat-killed cells. Efficacy was not significantly improved by doubling the PMA concentration. However, PMA treatment efficacy was improved markedly following 10-fold sample dilution and was found to be optimal at 100-fold dilution. Substantial reductions in viable bacterial load could be observed following both freeze-thaw and heat-treatment of FS. This method successfully prevented DNA amplification of heat-killed *Pseudomonas* and *Staphylococcus* spiked into stool and could reliably determine the proportion of live bacteria and viable *E. coli* counts present in fresh and heat-treated stool. With appropriate sample dilution, PMA treatment excluded >97% of non-viable cells from amplification in all assays, without significantly affecting the amplification of DNA from viable cells. This method can be applied to optimise sample processing of FMT donor material, and to characterise bacterial viability within faecal samples more widely.

4.2 Introduction

Faecal microbiota transplantation (FMT) is a therapeutic intervention in which stool from one or more healthy donors is processed into a faecal slurry (FS) and delivered to the lower intestinal tract of the recipient. The ability of FMT to substantially alter gut microbiota makes it potential interventional therapy in a range of diseases (196). FMT is an established therapy for *Clostridioides difficile* colitis (172) and shows promise as a therapeutic intervention in inflammatory conditions such as ulcerative colitis (262). It is postulated that the efficacy of FMT is dependent on the ability of beneficial commensal bacteria from the donor to proliferate within the recipient (172, 263, 264). This requires those microbes to be viable at the time of

transplantation. The ability to accurately determine the viability of bacteria in donor faecal samples is critical to developing appropriate protocols for the preparation and standardisation of FMT material.

Previous efforts to assess the viability of microorganisms in faecal material used for FMT have been limited either by the use of culture methods, that can readily isolate only a small subset of the total gut microbiota (170), or molecular methods that lack the capacity to distinguish between DNA from viable cells, non-viable cells, and the extracellular environment (200). A potentially effective strategy to overcome these challenges is to combine quantitative (q)PCR-based bacterial enumeration with propidium monoazide sample treatment (PMA-qPCR). PMA is a red fluorescent dye that is excluded from viable cells by the energised membrane of an intact cell wall. When the cell wall is compromised, PMA enters the cell and intercalates into DNA (201). The monoazide group allows PMA to covalently bind DNA upon exposure to light, thus limiting PCR amplification to DNA present within viable cells (202).

The combination of PMA treatment with PCR-based analysis has been shown to be effective in a range of contexts, including the assessment of bacterial viability in samples with mixed populations, such as in waste water or sputa (203-205). However, its efficiency may be reduced when applied to samples in which levels of non-viable bacterial DNA and extracellular DNA are high, or where sample turbidity impedes light penetration (203, 206).

The use of PMA in combination with 16S rRNA gene amplicon sequencing has been reported previously in the assessment of viable bacterial composition of FS for FMT (207). Chu and colleagues subjected undiluted simulated faecal transplant material from a single participant to various processing conditions including exposure to oxygen and freeze thaw cycles prior to treatment with PMA (207). However, this study did not include a validation of the methodology.

This chapter describes the optimisation of PMA-qPCR to determine the viable bacterial content of faecal slurries for FMT. This method can reliably be applied to optimise sample processing methodologies for FMT donor material, as well as the characterisation of bacterial viability within faecal samples more widely.

4.3 Methods

4.3.1 FMT faecal slurry (FS) processing

Stool was collected with informed consent from participants being screened as stool donors for a clinical trial examining the use of FMT in patients with recurrent UTI (265). Fresh stool from

3 faecal donors were collected on separate occasions and processed immediately. Stool was collected on site and processed with 15 minutes. Stool was blended with normal saline (NS) and glycerol to produce a FS consisting of 25% (wt/vol) stool, 65% NS, and 10% glycerol, as previously described (266). Stool blending and PMA treatment were performed within an anaerobic chamber. Remaining stool was frozen at -80^{o}C in either 50 mL centrifuge tubes or 250 mL sterile pots. To assess the effects of freeze-thaw, a 50mL aliquot of FS was stored at -80^{o}C for 48 hrs and then allowed to thaw at room temperature within the anaerobic cabinet. Heat killing was performed by subjecting a 1 mL aliquot of thawed FS to 99^{o}C for 30 minutes in a heating block.

4.3.2 Dilution and PMA treatment of fresh, frozen and thawed, and heat-killed FS

Fresh, freeze-thawed and heat-treated FS was tested at four different dilutions. Neat FS (25% stool content) was serially diluted in phosphate buffered saline (PBS) 10, 100, and 1000-fold. Neat FS and each dilution were treated with PMA or control in triplicate, as described in Section 2.3.

4.3.3 PMA treatment

Stock solution was prepared by dissolving 1 mg of PMA (Biotium Inc., Fremont, CA, USA) in 1 mL of 20% dimethyl sulfoxide. For PMA treatment, 5 µL of PMA was added to 95 µL of sample to achieve 100 µM final concentration of PMA in 100 µL (203). All samples were prepared in clear RNase-free 1.5mL tubes (Ambion®, Thermo Fisher Scientific, Waltham MA, USA). Following a 30 min incubation at room temperature in the dark (267), samples were exposed to an LED light (1.5 W, Model AL329, Aqua Zonic, Singapore) at a distance of 15 cm for 20 min. A detailed protocol is included in Appendix 1.

4.3.4 Spiking of stool with heat-killed *Pseudomonas aeruginosa* and dilution of stool prior to PMA treatment

P. aeruginosa ATCC 27863 was cultured onto horse blood agar (HBA, bioMerieux, Australia) for 24 hours. Colonies were dispersed in 1 mL PBS and diluted 40-fold to give a suspension of 3.35 McFarland units (~1x10^{9} CFU/mL). Heat-killing of the neat suspension was performed by heating 1 mL aliquots to 99°C for 30 min.

To assess the effect of stool concentration on the exclusion of non-viable bacteria through PMA treatment, donor stool was spiked with heat-killed *P. aeruginosa* to produce FS consisting of 25% (vol/vol) heat-killed *P. aeruginosa*, 10% (wt/vol) stool suspended in PBS. This

suspension was further serially diluted 10-fold in PBS to produce suspensions of 1%, 0.1% and 0.01% stool. Each dilution of spiked stool was separated into six 95 µL aliquots. Three aliquots were treated with PMA (as described in section 4.3.3) and three used as untreated controls.
As the presence of stool in specimens could result in PCR inhibition or affect the performance of PMA, the performance of the *P. aeruginosa* qPCR assay in pure culture alone was compared to its performance in stool specimens. Therefore, the heat-killed *P. aeruginosa* culture was diluted to 25%, 2.5%, and 0.25% in PBS to mirror the concentration of spiked bacteria in the stool samples and treated with PMA in an identical manner.

4.3.5 Spiking of viable and non-viable *Staphylococcus aureus* into 1/100 diluted FS

Fifty µL of *S. aureus* ATCC 29213 overnight culture in cerebrospinal fluid (CSF) enrichment broth (bioMerieux, Australia) was inoculated into 3 mL CSF broth, which was grown at 37 °C with shaking for 6 hours prior to use to ensure log-phase of growth. Two 1.5 mL culture aliquots were centrifuged for 2 min at 10,000 *g*. The pellet was washed twice in NS and resuspended to provide a suspension of 4 McFarland units (~1.2 x10^9 CFU/mL).
To confirm that PMA treatment was effective in preventing PCR amplification of DNA from non-viable cells, while not affecting amplification of DNA from viable cells, dilute FS was spiked with live and heat-killed *S. aureus* cells as well as with a combination of live and heat-killed cells in a 1:1 ratio. For each, 100 µL was spiked into 900 µL of diluted (1/100) FS. The three spiked samples were treated in triplicate with PMA or PBS control. Pure bacterial cells (live, heat-killed, and combined) were similarly PMA-treated.
To determine whether a higher PMA concentration would provide greater efficacy in concentrated stool, 100 µL heat-killed *S. aureus* was spiked into 900 µL neat FS and divided into nine aliquots (three used as controls, three treated with 100 µM PMA as described above, and three treated with 200 µM PMA).

4.3.6 Assessing performance of PMA qPCR over a defined range of viable concentrations

To determine the performance of this method over a range of viable/dead concentrations, FS was prepared fresh as described in section 4.3.1, diluted 100-fold in PBS, and mixed with heat-killed FS in defined proportions. The following FS mixtures were prepared in 1mL aliquots: 100% fresh, 80% fresh/20% heat-killed, 60% fresh/40% heat-killed, 40% fresh/60% heat-killed, 20% fresh/80% heat-killed and 100% heat-killed. Each mixture was treated with and without PMA in triplicate in 100 µL aliquots as described in section 4.3.3. The proportion of total bacteria viable in each specimen was determined using PMA qPCR targeting the 16S

rRNA gene, as described in section 4.3.7. Estimated *E.coli* colony forming units (CFU/µL) were quantified using a probe based qPCR targeting the *tuf* gene as described previously (268). For each FS mixture, 100 µL of sample was plated onto three MacConkey with salt agar plates (Thermo Fisher Scientific, Therbarton SA, Australia) at three 10-fold dilutions to select for single colonies of coliforms. Plates were incubated aerobically at 37°C for 24 hr. The heat-killed aliquot was also cultured under the same conditions and demonstrated no growth. Single coliform colonies were counted and confirmed to be *E. coli* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Daltonik MALDI Biotyper, Bruker Biosciences Pty Ltd, Preston VIC, Australia).

4.3.7 DNA extraction and qPCR

DNA was extracted from samples using the PowerLyzer® PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carslbad, CA, USA) in accordance with the manufacturer's instructions and stored at 20^{o}C.

Levels of total bacteria, *P. aeruginosa*, and *S. aureus*, were determined using previously described qPCR assays (269-271) on a QuantStudio 6 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Total bacterial and *P. aeruginosa* qPCR assays were performed using sybr green fluorophore reagents (PowerUp™ SYBR™ Green Master Mix, Applied Biosystems, Foster City, CA, USA). *S. aureus* and *E. coli* specific qPCR was performed using probe-based assays (KAPA PROBE FAST ROX Low MasterMix, Kapa Biosystems, Japan). All qPCR assays were performed in triplicate and mean Ct values were converted to log10 cell/µL. Details of primers, probes and cycling conditions are available in Appendix 2.

Proportions of live cells were determined by dividing the quantity of cells amplified in the presence of PMA by the quantity of cells amplified in matching untreated controls. Statistical significance (p-value <0.05) was determined using paired t-tests for parametric data and the Wilcoxon matched-pairs single rank test for non-parametric data.

4.4 Results

4.4.1 Effect of stool concentration on PMA-qPCR efficacy in fresh, freeze-thawed, and heat-treated samples

The efficacy of PMA treatment was reduced in both neat and 10-fold diluted FS (2.5% stool content) compared to 100-fold and 1000-fold diluted FS. In neat FS, no difference in

amplification between PMA-treated and control samples was observed for fresh (p=0.496), frozen (p=0.203) or heat-treated samples (p=0.203) (Figure 4.1A). In 10-fold diluted FS (Figure 4.1B), amplification of bacterial DNA was significantly reduced in fresh (p=0.004) and heat-killed FS (p=0.004) following PMA-treatment compared to controls, but not in frozen FS (p=0.074). In 100-fold diluted FS (Figure 4.1C), amplification of bacterial DNA was significantly reduced following PMA-treatment compared to controls (p=0.004 for all three treatment conditions). While reductions in bacterial DNA amplification were also observed in 1000-fold diluted FS (p=0.004 for all three treatment conditions, Figure 4.1D), at this dilution the FS is so dilute that amplification levels are near the limit of detection of the assay even without PMA treatment. Overall, the best separation of heat-killed control and PMA treated samples is observed at the 100-fold dilution of FS (Figure 4.2, arrow)

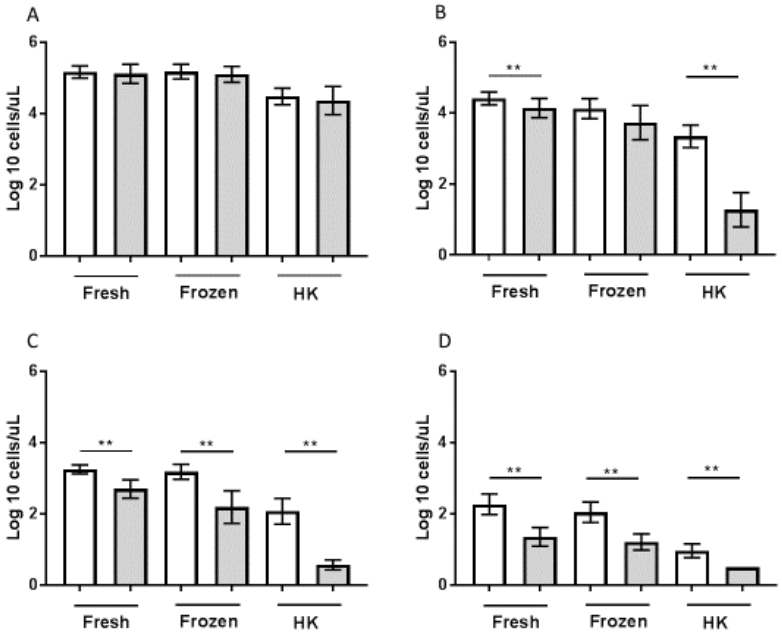

Figure 4.1. Effect of faecal slurry (FS) dilution on the ability of PMA to exclude non-viable cells from amplification in FS material processed as fresh, frozen or heat-killed (HK). **A.** Neat FS (25% stool) **B.** 1/10 diluted FS (2.5% stool) **C.** 1/100 diluted FS (0.25% stool) **D.** 1/1000 diluted FS (0.025% stool) Bars depict the mean (±SD) log cells/µL amplified by 16S qPCR from three donors, each with three replicate samples. Statistical comparisons (paired t-tests) are made between amplification from PMA treated samples (shaded bars) compared to untreated control samples (clear bars) (**=p<0.01).

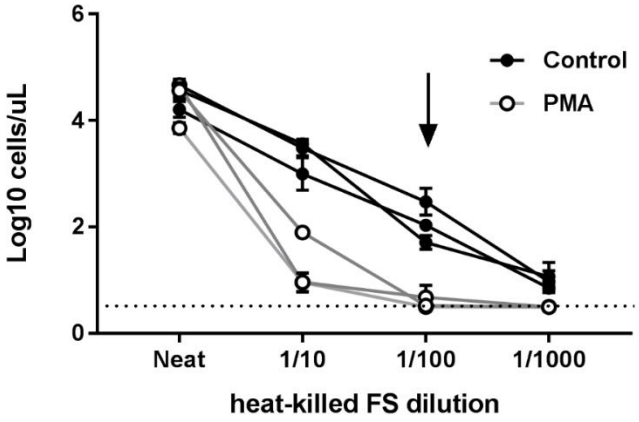

Figure 4.2. Effect of faecal slurry (FS) dilution on the ability of PMA to exclude non-viable cells from amplification in heat-killed FS material. Circle symbols depict the mean (±SD) log cells/µL amplified by 16S qPCR from three donors, each with three replicate samples. The 1/100 dilution shows the best separation between control and PMA treated specimens (arrow). The dotted line represents the assay's threshold of detection.

4.4.2 Effect of stool concentration on the ability of PMA to exclude spiked non-viable *P. aeruginosa* cells from qPCR amplification.

PMA treatment did not prevent the amplification of DNA from non-viable *P. aeruginosa* cells in the presence of 10% stool, with no significant difference observed between PMA-treated and control samples (Table 4.1). Furthermore, the level of amplification of *P. aeruginosa* DNA in spiked FS was significantly reduced compared to an equivalent level of *P. aeruginosa* DNA in pure culture (3.45 ± 0.04 vs 5.99 ± 0.05 log cell/µL $p<0.0001$, Figure 4.3), suggesting inhibition of PCR by components of stool DNA. Inhibition was also observed to a smaller degree in the 1% stool samples (4.34 ± 0.02 vs 4.81 ± 0.12; $p=0.02$), but not in the 0.1% stool sample (Figure 4.3). No *P. aeruginosa* DNA was detectable in unspiked FS.

In all samples with 1% or less stool concentration, amplification of *P. aeruginosa* DNA in the PMA-treated samples was significantly reduced compared to controls ($p <0.005$), consistent with the successful limitation of DNA amplification to viable cells (Table 4.1).

When viability was assessed as a proportion of total bacterial cells (Table 4.1), no significant difference was observed between spiked samples with ≤1% stool content. More than 97% of DNA from heat-killed cells was excluded from amplification following PMA treatment. The performance of PMA-qPCR in samples with a stool concentration of ≤1% did not differ significantly from the performance of PMA on cells from pure culture alone.

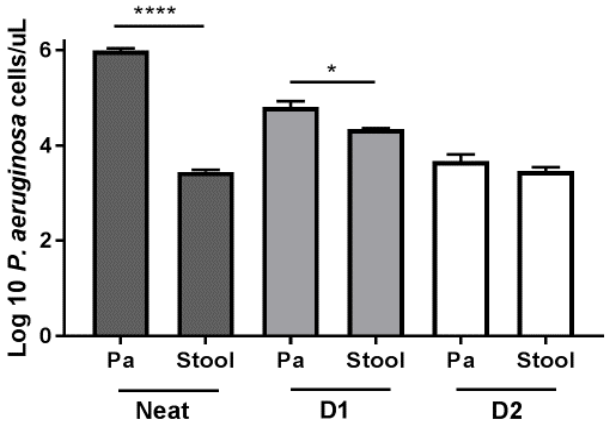

Figure 4.3. Effect of stool dilution on amplification of heat-killed *P. aeruginosa* cells

The amplification in *P. aeruginosa* cells alone (Pa) grown in pure culture is compared to amplification of the same cells at the same concentration spiked in FS without PMA treatment (paired t-tests,*=p<0.05; ***=p<0.001). The neat spiked sample consists of 10% stool, dilution 1 (D1) of 1% stool and dilution 2 (D2) of 0.1% stool.

Table 4.1. Effect of stool dilution on PMA's ability to exclude heat-killed *P. aeruginosa* DNA from amplification using *P. aeruginosa* specific qPCR

	Stool content (%)	*P. aeruginosa* cells detected[e]		Difference (p-value)	Proportion viable[f] (PMA /control)
		Control	PMA		
Neat[a]	10%	3.45 ± 0.04	3.80 ± 0.17	0.1	>100%
D1[b]	1%	4.34 ± 0.02	2.80 ± 0.17	0.005	3.0%
D2[c]	0.1%	3.46 ± 0.08	1.74 ± 0.08	0.002	2.0%
D3[d]	0.01%	2.19 ± 0.12	0.53± 0.13	0.0006	2.2%

[a]Heat-killed *P. aeruginosa* cells (5.8 log10 cells/µL) were spiked into a sample consisting of 10% stool (Neat). [b,c,d]This sample was then serially 10-fold diluted: 1/10 dilution (D1), 1/100 dilution (D2), 1/1000 dilution (D3). [e]All *P. aeruginosa* cells detected (log10 cells/µL, mean± SD of 3 replicate samples) in spiked stool samples without PMA treatment (Control) were compared to viable cells detected in corresponding PMA-treated samples (PMA). [f]The proportion of heat-killed *P. aeruginosa* cells detected as viable is determined by dividing viable cells detected in PMA treated samples by total cells detected in the control samples.

4.4.3 Efficacy of PMA-qPCR in discriminating live and dead *S. aureus* cells spiked into 100-fold diluted FS

Our initial experiment suggested that 100-fold diluted FS (0.25% stool) was optimal for PMA-qPCR determination of viable bacterial load. We sought to confirm this finding by assessing whether PMA treatment could reliably eliminate amplification from non-viable cells, while not significantly affecting the amplification of viable cells. 100-fold diluted FS was spiked with live, heat-killed, or a 1:1 ratio of live and heat-killed *S. aureus* cells, to a concentration of ~ 1.2

x10^9 CFU/mL. The use of *S. aureus* also allowed an assessment of the efficacy of PMA treatment when applied to a gram-positive organism and a different, probe-based qPCR assay. PMA treatment did not significantly affect amplification from viable cells from culture (p= 0.33, data not shown), while still providing optimal (>99%) exclusion of DNA from heat-killed cells from amplification (p= 0.004). No amplification of *S. aureus* DNA was observed with unspiked FS. PMA treatment reduced amplification of DNA from non-viable cells in FS samples spiked with 1:1 heat-killed: viable cells (p=0.003) and 100% heat-killed cells (p<0.001) when compared to FS spiked with live cells (Figure 4.4).

We also examined whether the inefficiency of PMA treatment in concentrated stool could be overcome by doubling the concentration of PMA used. However, in neat FS piked with heat-killed *S. aureus* there was no difference in amplification between PMA treated and control samples regardless of PMA concentration (control vs 100 µM PMA, p=0.47; control vs 200 µM PMA p= 0.51).

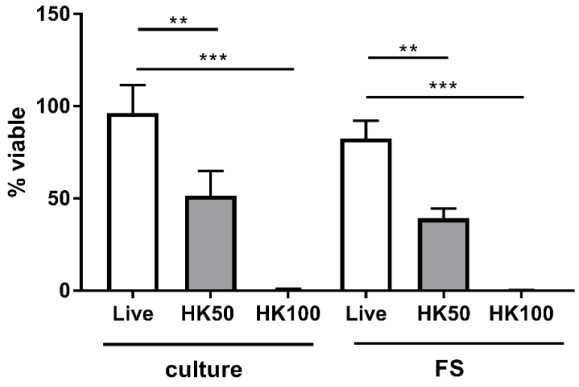

Figure 4.4. Proportion of cells determined to be viable *S. aureus* cells.

Proportion of viable cells was determined by dividing viable cells amplified in PMA-treated samples (mean ± SD of 3 replicate samples) over total number of cells amplified in non-PMA treated control samples. *S. aureus* cells consisted of live culture (Live), a mixture of 50% live culture and 50% heat-killed culture (HK50), or 100% heat-killed culture (HK100). The ability of PMA to exclude dead cells from amplification was assessed in *S. aureus* culture alone, or in *S. aureus* spiked into diluted faecal slurry consisting of 0.25% stool (FS) (paired t-tests, **=p<0.01; ***=p<0.001).

4.4.4 Performance of PMA-qPCR in varying ratios of fresh and heat-killed FS

The PMA-qPCR method performed well in predicting the proportion of live bacteria in the sample with a strong linear correlation (Figure 4.5, R^2= 0.966). As the proportion of fresh FS increased there was an increase in the variability between replicate samples, with the most variability in the 100% fresh sample (mean proportion viable 0.55 +/- SD 0.18). PMA-qPCR was used to determine *E. coli* CFU/µL and these results were compared to corresponding colony counts on selective agar. Estimates of viable bacterial load, as determined by PMA-qPCR, were closely correlated with bacterial colony counts (Figure 4.6). PMA-qPCR performed comparably to culture in identifying the proportion of live *E. coli* in the sample, with a strong linear correlation (Figure 4.6 panel A: *E. coli* culture R^2= 0.900, panel B: *E. coli* PMA qPCR R^2= 0.978).

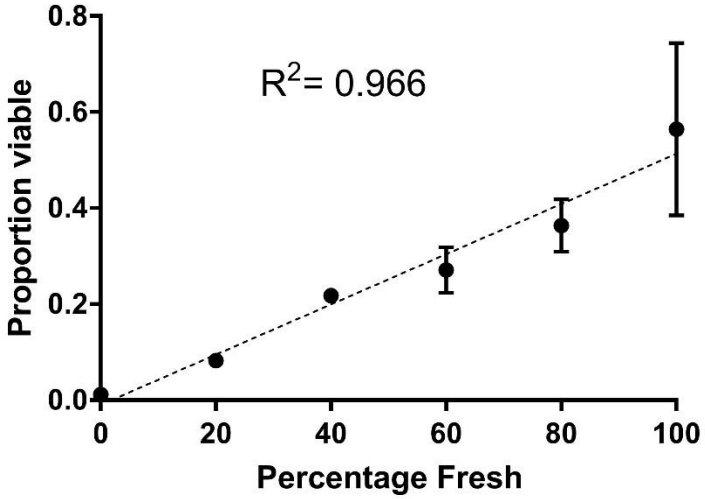

Figure 4.5 Performance of PMA-qPCR (16S) when applied to defined ratios of fresh and heat-killed FS.

Data points and error bars represent the proportion of viable bacterial cells detected in FS after 16S rRNA qPCR (mean ± SD of 3 replicate samples). Proportion viable was determined by dividing cells/ µL amplified in PMA treated samples by the amplification of the non-PMA treated in the 100% fresh sample. The strength of the linear relationship was determined by calculating the coefficient of determination, R^2.

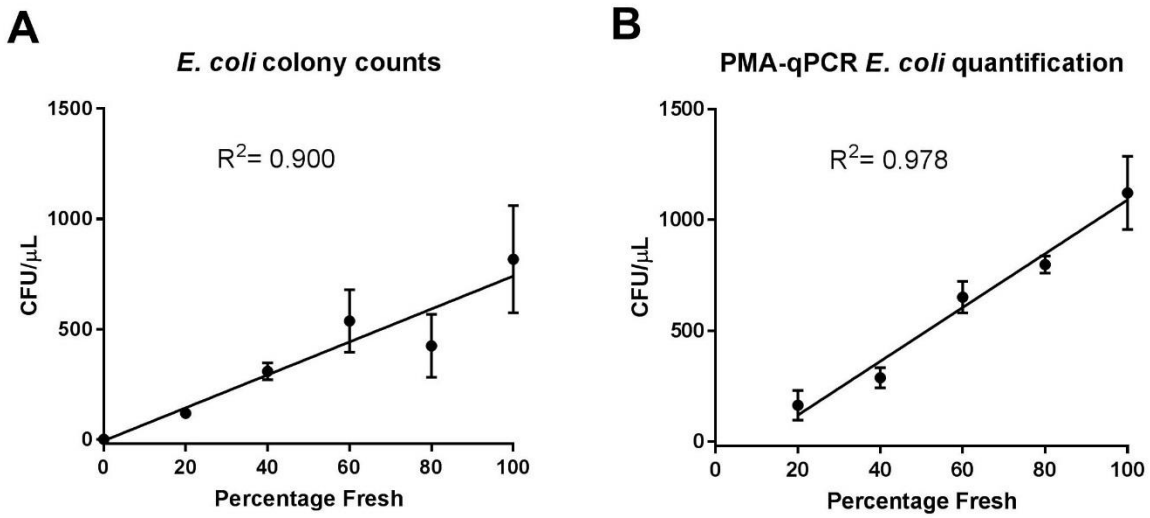

Figure 4.6. Performance of PMA-qPCR when applied to defined ratios of fresh and heat-killed *E. coli* spiked in FS.

Data points and error bars represent the CFU/µL after selective agar culture (panel A) and CFU/ µL of bacterial cells determined to be viable after *E. coli*-specific qPCR (panel B) (mean ± SD of 3 replicate samples). The strength of the linear relationship was determined by calculating the coefficient of determination, R^2.

4.5 Discussion

A methodology that can reliably determine the viability of stool bacteria, a substantial portion of which are refractory to standard culture techniques, is essential for the accurate assessment and optimisation of stool processing protocols for FMT. While the use of PMA treatment in conjunction with PCR-based bacterial enumeration has shown promise in other contexts, a failure to consider the reduced efficacy of this approach when applied to undiluted faecal slurries may have confounded previous applications in this context. We describe the optimisation of this approach and demonstrate the importance of sample dilution in achieving efficient exclusion of DNA from non-viable cells.

Stool is an inherently heterogeneous and variable material, with substantial variation in water content between samples. The degree of stool dilution incorporated into standard protocols must therefore allow effective PMA treatment on even the densest stool samples. Our results suggest that 1/100 dilution of stool or faecal slurry to ~0.25% stool content is necessary to achieve optimal results following PMA treatment. As a guide, the optical density of the FS specimens used at 0.25% stool content was 3.76 -4 McFarland units. At this dilution, PMA reliably inhibited amplification from non-viable cells in all experiments.

Although PMA treatment also performed well when stool content was further diluted, over-dilution of specimens is inadvisable, particularly when attempting to amplify targets that are already present at low concentrations (as illustrated in Figure 4.2). PMA treatment also performed well in excluding DNA from spiked non-viable cells at 1% stool content. However, at this concentration, PCR assay inhibition was observed, a phenomenon that is well-described in relation to DNA extracts from stool (272).

The inefficiency of PMA treatment at stool contents of ≥10%, could not be overcome by doubling the concentration of PMA used to 200 µM. This observation suggests that factors such as light penetration, rather than PMA concentration, limit the effectiveness of this approach in concentrated stool samples.

PMA-based methods might, under certain circumstances, be prone to underestimating numbers of non-viable bacteria (273). Therefore, our method employs a relatively high PMA concentration (100 µM), long incubation time (30 min) and long period of light exposure (20 min). These parameters are conservative, based on available evidence to optimise the elimination of non-viable cells from amplification (203, 267). By applying our method to live as well as heat-killed spiked *S. aureus* cells we confirmed that our method did not significantly affect live cells while still excluding more than 99% of DNA from non-viable cells from amplification (Figure 4.4).

The application of the optimised methodology described here demonstrates that a single freeze-thaw cycle renders the majority of bacteria in FS non-viable (Figure 4.1). These results differ from those reported by Chu et al., who did not detect a significant difference in cell viability in FS for FMT after 20 freeze-thaw cycles (207). However, in contrast to our optimised approach, Chu et al applied PMA treatment to undiluted FS (207), a methodology which is in this study is demonstrated to be ineffective. Similarly, Young and colleagues also reported applying PMA treatment to concentrated stool samples (274). In addition, they analysed samples that had already been frozen, a process which is problematic given the impact that this has on the number of viable bacteria within the sample.

Commensal intestinal bacteria are increasingly being recognised as important mediators of both human health and disease (275). Many of these organisms are only readily detectable using molecular methods. PMA-based methodologies have an important role to play in determining the viability of a wide-range of organisms in stool. Application of the optimised methodology described here will allow standardisation of appropriate preparation protocols for FMT-based therapeutics.

4.6 Acknowledgements

I would like to acknowledge Yanan Wang, Joceyln Choo and Lex Leong for their assistance with DNA extraction and PCR optimisation

CHAPTER 5

Effect of stool processing on the viability and functional potential of microbes in faecal transplant material

The contents of this chapter appear in the following publication:

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5.1 Abstract

The therapeutic potential of faecal microbiota transplantation (FMT) is under investigation for a wide range of inflammatory conditions. While mechanisms of benefit are poorly understood, most proposed models rely on the viability of transplanted microbes, either to displace disease-associated microbiota or to drive the biosynthesis of anti-inflammatory metabolites. We hypothesised that protocols commonly used in the preparation of faecal transplant material will substantially reduce both the number of viable microbes delivered through FMT and the relative abundance of beneficial commensal species.

Analysis was performed on stool from eight screened donors. Processing conditions for sample aliquots replicated commonly employed clinical protocols, including strict anaerobic conditions, homogenisation in ambient air, and freeze-thaw. Propidium monoazide (PMA) sample treatment was combined with quantitative PCR and 16S rRNA gene amplicon sequencing and short-chain fatty acid (SCFA) analysis to define the viable microbiota composition and functional potential.

Approximately 50% of the total bacterial content of stool processed immediately under strict anaerobic conditions was non-viable. Homogenisation in ambient air or freeze-thaw reduced viability to 19% and 23% respectively. Processing of samples in ambient air resulted in up to 12-fold reductions in the abundance of important commensal taxa, including the highly butyrogenic species *Faecalibacterium prausnitzii*, *Subdoligranulum variable*, and *Eubacterium hallii*. The adverse impact of atmospheric oxygen exposure on the capacity of the transplanted microbiota to support SCFA biosynthesis was demonstrated by significantly reduced butyrate and acetate production of faecal slurries processed in ambient air. In contrast, while reducing overall levels of viable bacteria, freeze-thaw did not significantly alter viable microbiota diversity or composition.

The practice of preparing material for faecal transplantation in ambient air profoundly affects their viable microbial content, disproportionately reducing the abundance of beneficial anaerobic commensals and the capacity for the biosynthesis of important anti-inflammatory metabolites.

5.2 Introduction

In recent years, following major advances in nucleic acid sequencing technology, there has been a dramatic increase in research linking gut microbiota not only to intestinal pathologies such as inflammatory bowel disease (96, 276-278) or colorectal cancer (279), but also to a

broad range of other medical conditions that include metabolic disorders and even mental health conditions (280-282). This has led to the hypothesis that modification of gut microbiota via faecal microbiota transplantation (FMT) could have a therapeutic role in a diverse range of diseases.

FMT is a therapeutic intervention in which stool from one or more healthy donors is processed into a faecal slurry (FS) and delivered into the intestinal tract of the recipient. Although FMT is best established as a therapy for recurrent *C. difficile* infection, (172, 174) there is increasing evidence for the use of FMT in inflammatory disorders, particularly ulcerative colitis (262).

Despite its increasing use, there is neither standardisation of donor screening nor standardisation of stool processing (266). This lack of standardization extends to the use of FMT in clinical trials, of which there are over 200 registered on clinicaltrials.gov, making comparison of outcomes difficult. Worryingly, FMT is also often performed in private clinics, or even by patients themselves, for unproven indications and in a completely unregulated fashion (283).

Current guidelines for processing stool for FMT are intended for the treatment of *C. difficile* colitis and are based on expert opinion in the absence of evidence (284-286). The exact mechanism by which FMT results in clearance of *C. difficile* from stool is not known, and variations in stool processing protocols appear to have little impact on the efficacy of FMT for this indication (284, 287, 288). This has resulted in protocols designed for use in *C. difficile* infection being adopted in trials using FMT for other indications, where mechanism of action is likely to be different. These protocols commonly involve the homogenisation of stool in ambient air, despite oxygen exposure being known to cause the rapid death of many obligate anaerobic bacterial commensals (289).

At present there is little evidence available to guide clinicians in selecting a stool processing methodology. Characterisation of the microbiome composition in processed FMT donor material is often not attempted, and where performed, typically involves high throughput sequencing of extracted faecal DNA (200). Such an approach will detect DNA derived from both viable and non-viable organisms and therefore have a limited capacity to indicate which bacteria are viable and capable of replicating in the recipient. Culture methods readily isolate only a small subset of the total gut microbiota (20) and are therefore unsuitable for characterising the impact of processing on many of the commensal anaerobic species present.

A strategy to overcome these challenges is combine molecular techniques, such as next-generation sequencing and targeted quantitative PCR assays, with propidium monoazide sample treatment (PMA-qPCR) (207). PMA is a red fluorescent dye which selectively enters

cells with compromised cell membranes. Upon exposure to light PMA will covalently bind to DNA in these cells, inhibiting PCR amplification (201). In this way, PMA treatment of sample material allows the selective amplification of DNA from only viable cells in the sample (201). The PMA-qPCR methodology applied in this study was specifically optimised for use in faecal slurries for transplantation and validated in comparison to culture methods (290) as presented in Chapter 4.

This study presented in this chapter documents the effects of anaerobic homogenisation, aerobic homogenisation and freeze-thaw on the viability and functional capacity of bacteria in donor stools processed for faecal microbiota transplantation.

5.3 Methods

5.3.1 FMT faecal slurry processing

Stool was collected with informed consent from healthy participants being screened as FMT donors for a clinical trial (265) with approval from the Queen Elizabeth Hospital Human Research Ethics Committee (HREC/16/TQEH/32). All donors had passed a screening questionnaire (see Appendix 5.1) used to identify potential FMT donors. Stool was collected on site and processed within 15 minutes of passage. Stool was divided into two aliquots weighing at least 30g. Each aliquot was blended (in a 240 V Waring SS515 laboratory blender at 22,000 rpm) with normal saline (NS) and glycerol to produce a FS consisting of 25% (wt/vol) stool, 65% NS, and 10% glycerol, as previously described (266). In the first aliquot (ANO_2) stool blending and PMA treatment were performed under anaerobic conditions within an anaerobic chamber. In the second aliquot (O_2), the same procedure was performed in ambient air. The resultant FS was frozen at -80^{o}C in 50 mL centrifuge tubes. To assess the effects of freeze-thaw, a 50mL aliquot of anaerobically processed FS was stored at -80^{o}C for 48 hrs and then allowed to thaw at room temperature within the anaerobic chamber. The freeze-thawed specimens were not exposed to oxygen during the processing or PMA treatment. Heat killing was performed by subjecting a 1 mL aliquot of thawed FS to 99^{o}C for 30 minutes.

5.3.2 Dilution and PMA treatment of fresh, frozen and thawed, and heat-killed FS

Immediately following the processing described above, neat FS (25% stool content) was diluted 100-fold in PBS and divided into six 95 µL aliquots in clear RNase-free 1.5mL tubes (Ambion, Thermo Fisher Scientific, Waltham MA, USA). Diluted samples were treated with and without PMA in triplicate, as described above. Samples were stored at -80^{o}C prior to

extraction. PMA-treatment was performed using a protocol specifically developed and validated for faecal slurries as described in Chapter 4. A stock PMA solution was prepared by dissolving 1 mg of PMA (Biotium Inc., Fremont, CA, USA) in 1 mL of 20% dimethyl sulfoxide. For PMA treatment, 5 µL of PMA stock was added to 95 µL of sample to achieve 100 µM final concentration of PMA. Following a 30 min incubation at room temperature in the dark, samples were exposed to an LED light (Aqua Zonic, Singapore) for 20 min. In non-PMA treated control aliquots, 5 µL of PBS was added instead of PMA. Control samples underwent identical incubation and light-exposure as the matching PMA treated samples.

5.3.3 Determination of microbiota composition

DNA was extracted from the entire (100 µL) unspun sample. DNA was extracted using the PowerLyzer PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carslbad, CA, USA) in accordance with the manufacturer's instructions (see Appendix 1.4) and stored at -20^{o}C.

The microbial composition of the faecal slurry specimens was determined by paired-end sequencing of the V4 hypervariable region of the bacterial 16S rRNA gene. Amplicon sequencing was performed on an Illumina MiSeq platform as described previously. Paired-end reads were merged, demultiplexed and analysed using Quantitative Insights in to Microbial Ecology (QIIME) software (v1.9.1) using a previously described bioinformatics pipeline (225). Sequences were assigned to operational taxonomic units (OTUs) using an open reference approach against the SILVA 16S rRNA reference database (release 128) clustered at 97% similarity. Subsampling was performed on all samples to a depth of 6188 sequence reads. The alpha diversity metrics for determining taxa richness (observed species) and phylogenetic diversity (PD whole tree) was computed using QIIME. Inter-sample variance (β-diversity) was determined using Bray-Curtis similarity on square root transformed taxa relative abundance using PRIMER software version 7 (PRIMER-E, Plymouth, UK). Sequence data was submitted to the National Center for Biotechnology Information SRA database with accession number PRJNA491383.

Taxa with zero counts were normalised to a single count across all samples. Changes in taxon relative abundance were determined by calculating the log2 values of fold change in relative abundance against the anaerobically processed matching controls across all taxa. Taxon relative abundance average fold change was computed based on the inverse logarithm of the sum of log2 fold change divided by the number of donors in which the taxa was detected. Only bacterial taxa that were present (sequence count ≥2) in at least one comparison group within each donor and were present in at least 7 of 8 donors were included in the analysis.

5.3.4 Species-specific bacterial enumeration

Levels of total bacteria, butyryl-CoA:acetate CoA-transferase gene, *Anaerostipes hadrus, Faecalibacterium prausnitzii, Eubacterium hallii, Roseburia spp/Eubacterium rectale, Bifidobacterium spp.*, *Alistipes putredinis*, and *Bacteriodes spp.* were determined using previously described qPCR assays using a QuantStudio 6 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Details of primers and probes used in these assays is available in Appendix 2. The *Subdoligranulum variable* qPCR assay was developed as part of this study (see Appendix 3). *Escherichia coli* DNA was amplified using a probe-based assay using KAPPA PROBE FAST ROX Low Master Mix reagents (Kapa Biosystems, Cape Town, South Africa). All other PCR assays were performed using SYBR green fluorophore reagents (PowerUp SYBR Green Master Mix, Applied Biosystems). Quantitative PCR assays for the butyryl-CoA:acetate CoA-transferase gene, fastidious bacteria, including *A. hadrus, E. hallii, Roseburia/E. rectale*, and *S. variable,* a 10-fold dilution series of DNA extracted from donor faecal slurry was used as the positive qPCR standard.

The proportion of bacterial cells in each sample that were viable was determined by dividing the quantity of cells amplified in the presence of PMA by the quantity of cells amplified in matching untreated controls. To determine the proportion viable in heat-killed specimens, the quantity of cells amplified in PMA-treated heat-killed samples was divided by the quantity of cells amplified in the control sample prior to heat killing. Heat-killed specimens served as negative controls, representing levels of amplification expected in non-viable specimens.

5.3.5 Assessment of metabolic functional capacity

An, alternative method of analysing the SCFA biosynthetic capacity of the faecal slurries was employed to complement the analysis of butyryl-CoA:acetate CoA-transferase gene by quantitative PCR. We assessed the capacity of differentially processed faecal slurries to biosynthesize SCFA using an *in vitro* fermentation model, as described previously (291). This method assesses the ability of microbiota in the sample to produce SCFA when incubated with a fermentation substrate, high amylose maize starch (HAMS). Briefly, stored frozen faecal slurry samples from FMT donors (n=8) that had been processed ANO_2 or O_2 as described earlier were thawed and incubated under strict anaerobic conditions with HAMS. Heat-killed faecal slurry (n=2) and HAMS only (n=2) were used as negative controls. SCFA levels were determined by gas chromatography with flame ionisation detection (Hewlett-Packard6890; Palo Alto, CA, USA). Acetate, butyrate and propionate levels were measured pre-incubation

and after 24 h of anaerobic incubation at 37ºC with shaking. Results were normalised using 4-methylvaleric acid (Sigma-Aldrich) as an internal standard.

5.3.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.03 software. Significance (p-value <0.05) was determined using paired t-tests for parametric data and the Wilcoxon matched-pairs signed rank test for non-parametric data.

5.4 Results

5.4.1 Donor characteristics

Donors median age was 28.5 years (range 21-41) and with an equal male to female ratio. An equal number of donors had South East Asian and European heritage.

5.4.2 Impact of processing methodology on bacterial viability

With immediate anaerobic processing, the mean proportion of bacteria in FS that was viable was 0.50 ± 0.24 (mean ± SD). After processing in ambient air, this proportion fell to 0.19 ± 0.07, and after anaerobic processing followed by one freeze-thaw cycle it was 0.23 ± 0.11 (Figure 5.1). A significant reduction in the proportion of bacterial cells that were viable was observed following processing in ambient air (p=0.007) and after freeze-thawing (p=0.027), as compared to anaerobic processing alone. The proportion of viable bacteria in all processing methods was greater than detected in heat-killed specimens (p >0.001).

5.4.3 Diversity of microbiota

The α-diversity of viable taxa detected in specimens processed under anaerobic conditions after PMA treatment was significantly lower than that in specimens processed using standard (without PMA) methods (Figure 5.2; taxa richness: p <0.001; PD whole tree: p=0.007). Observed viable taxon richness was significantly lower after processing in ambient air (O_2) as compared to either ANO_2 processed (p= 0.023) or FT specimens (p= 0.023). No difference in diversity was observed between ANO_2 and FT groups (Figure 5.2). Differences in PD whole tree diversity between O_2 and ANO_2 and between O_2 and FT groups were not significant.

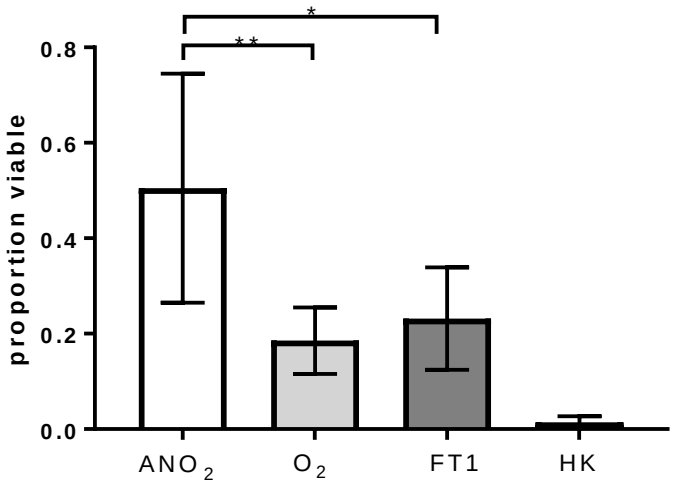


Figure 5.1. Proportion of bacteria determined to be viable using 16S rRNA gene qPCR in conjunction with PMA treatment

Proportion of viable cells was determined by dividing viable cells amplified in PMA-treated samples over total number of cells amplified in non-PMA treated control samples. Bacterial viability in faecal slurry was assessed after processing in fresh anerobic conditions (ANO_2), fresh aerobic conditions (O_2), after one cycle of freezing and thawing in anaerobically processed specimens (FT1) or after heat-killing (HK). (Bars depict mean ± SD of 8 donor faecal slurry samples *= $p<0{\cdot}05$, **=$p<0{\cdot}01$; paired t-test).

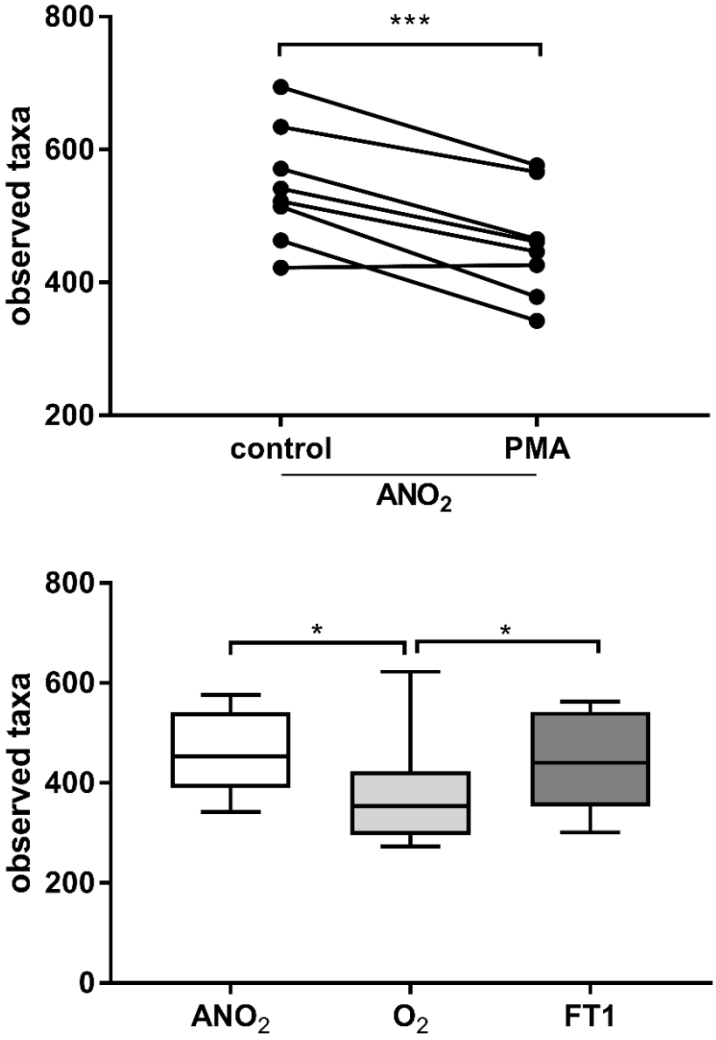


Figure 5.2. Taxa richness of FMT material from 8 donors as assessed by 16S rRNA gene amplicon sequencing with and without PMA treatment.

Viable taxa richness (PMA treated group) was significantly lower than richness observed in control specimens, even in samples processed immediately in anaerobic conditions (Figure 5.2A ***=p<0·001, paired t-test). When comparing only viable diversity between samples processed in anaerobic conditions (ANO_2), in ambient air (O_2), or after one cycle of freezing and thawing in anaerobically processed specimens (FT1) there are significantly lower observed species in specimens processed O_2, whereas freeze-thawing of specimens did not significantly reduce diversity (Figure 5.2B, box plot depicts median and IQR and error bars depict minimum to maximum values; *= p<0·05; Wilcoxon matched-pairs signed rank test).

5.4.4 Viable microbiota composition

The five most abundant taxa (OTUs) across all samples belonged to the genera *Bacteroides, Prevotella, Bifidobacterium, Faecalibacterium,* and the family *Lachnospiraceae* (0.21, 0.09, 0.05, 0.05 and 0.05 mean relative abundance, respectively). To determine which taxa were affected most by processing in ambient oxygen or freeze-thawing, taxa were ranked by relative abundance fold-change (Figure 5.4). Nineteen taxa displayed a 2.5-fold or greater drop in relative abundance after processing in ambient air, as compared to 2 taxa in the freeze-thaw group. The taxa most affected by processing in ambient air included *Faecalibacterium, Subdoligranulum, Eubacterium hallii, Eubacterium rectale, Roseburia* and *Anaerostipes,* representing major butyrate-producing taxa in healthy human gut microbiota (292). *Escherichia-Shigella* and *Alistipes* were the only taxa to show a 2.5-fold or greater increase in relative abundance processing in ambient oxygen or freeze-thawing and this was observed only following processing in ambient air.

To corroborate changes observed in taxon relative abundance, absolute levels of the taxa listed above that were substantially affected by processing were determined by qPCR. This included an assessment of all major butyrate producers that displayed a ≥2.5-fold in relative abundance, as well as those taxa displaying the greatest overall prevalence (*Bacteriodes/Prevetolla* and *Bifidobacterium spp*). Changes in relative abundance of viable bacteria (PMA-treated) in the anaerobically processed group, compared to standard analysis of the same group, is depicted in Figure 5.3.

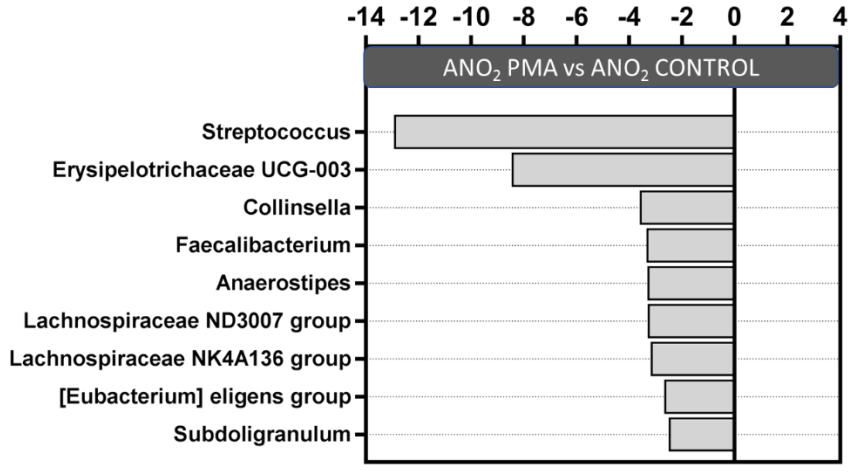


Figure 5.3. Change in the relative abundance of taxa after processing in anaerobic conditions with or without PMA

Light grey bars represent taxa with decreased relative abundance after PMA treatment. Only taxa with at least 2.5-fold change in relative abundance are depicted.

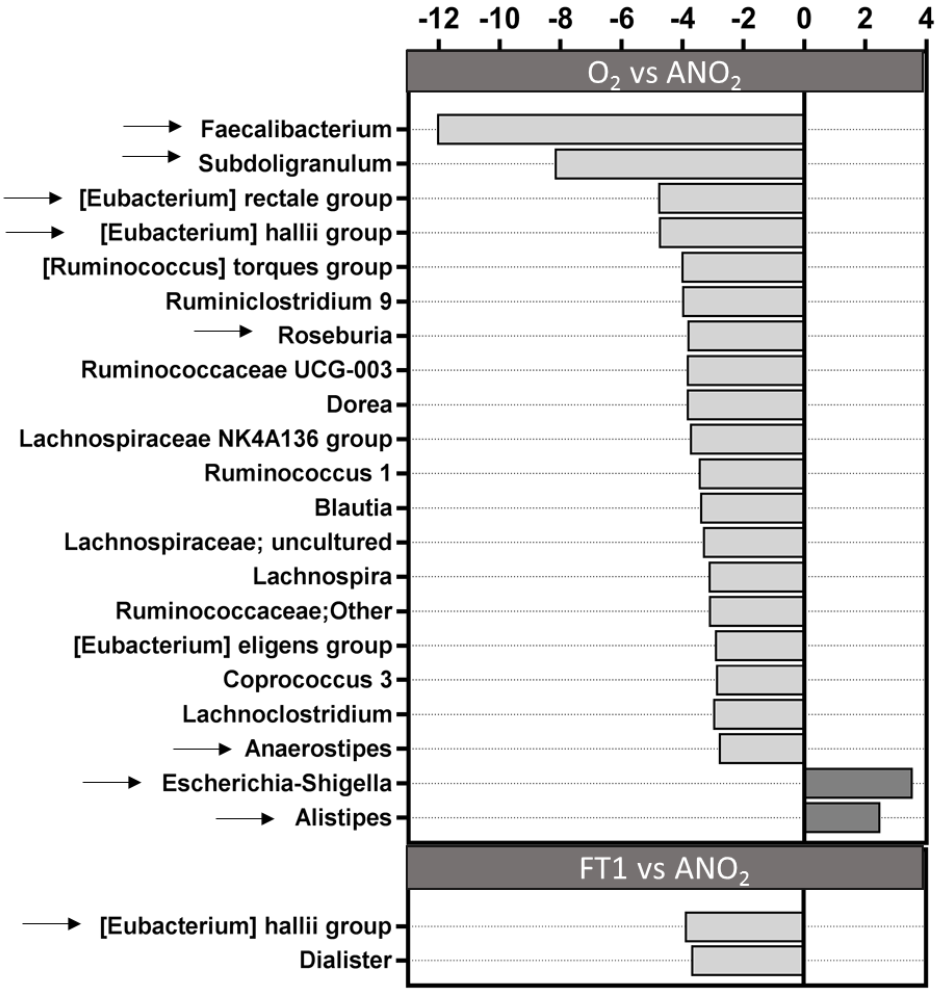


Figure 5.4. Change in the relative abundance of viable taxa after processing in ambient air (O_2 vs ANO_2) or after freeze-thawing (FT1 vs ANO_2)

Light grey bars represent decreased relative abundance and dark grey bars represent increased relative abundance. Selected bacterial taxa were further assessed by qPCR (arrows). Only taxa with at least 2.5-fold change in relative abundance are depicted.

5.4.5 Targeted amplification of viable microbial DNA

The use of targeted qPCR assays allowed the proportion of viable cells belonging to particular bacterial species to be determined (Figure 5.5). Specific assays for *F. prausnitzii*, *S. variable*, *A. hadrus*, *E. hallii*, and *Roseburia*/*E. recta*le were performed as these taxa showed >2.5 fold or greater decrease in relative abundance following processing in ambient air. Assays for *Bacteriodes/Prevotella spp*. and *Bifidobacterium spp*. were also performed as these were the genera with the highest relative abundance in our cohort. Results for individual donors for each processing condition are shown in Appendix 4: Supplemental Tables 4a-c.

Significant reductions in levels of viable bacteria following ambient air processing were observed for all taxa except the *Roseburia*/*E. rectal*e (for which no significant effects of processing were observed). In the case of *F. prausnitzii*, the proportion viable bacteria in the O_2 group was not significantly different from heat-killed aliquots, indicating likely complete loss of this species. *E. hallii* was the only species that showed a significant reduction in absolute viable levels following freeze-thaw.

An assessment of *E. coli* absolute abundance was performed as this taxon showed the greatest increase in relative abundance after processing in ambient air. The absolute amplification of *E. coli* in the donors was below the threshold of quantification in all but one individual, and there was no significant difference between the different processing methods (Appendix 4: Supplemental Tables 4a-c). *Alistipes* also showed an increase in relative abundance following processing in ambient air, but there was no increase in absolute abundance.

A semi-quantitative PCR method was used to estimate the carriage in viable bacterial cells of the gene encoding the butyryl-CoA:acetate CoA-transferase gene, the terminal enzyme in the dominant pathway of butyrate biosynthesis (this was employed as a surrogate measure of overall butyrate biosynthesis capacity) (292). When comparing amplification from viable cells processed in ambient air, levels of this enzyme are significantly lower than that detected in viable cells from anaerobically processed samples ($p=0.012$) or freeze-thawed samples ($p=0.001$). The level the butyryl-CoA:acetate CoA-transferase gene detected within viable cells processed in ambient air was equivalent to samples that had been heat-killed (Figure 5.6).

5.4.6 SCFA biosynthesis by FMT microbiota in an *in-vitro* fermentation model

In order to confirm our findings using a non-molecular method, we also measured the production of SCFA in the faecal slurries by gas chromatography before and after incubation with a fermentation substrate, high amylose maize starch (HAMS). Paired comparisons of post-fermentation SCFA levels demonstrated microbial butyrogenic and acetogenic capacity to be significantly reduced when donor stool was processed in ambient air (O_2 vs ANO_2, $p= 0.008$ and $p=0.016$ respectively, Figure 5.7). In contrast, no significant change in propionate biosynthesis was associated with oxygen exposure.

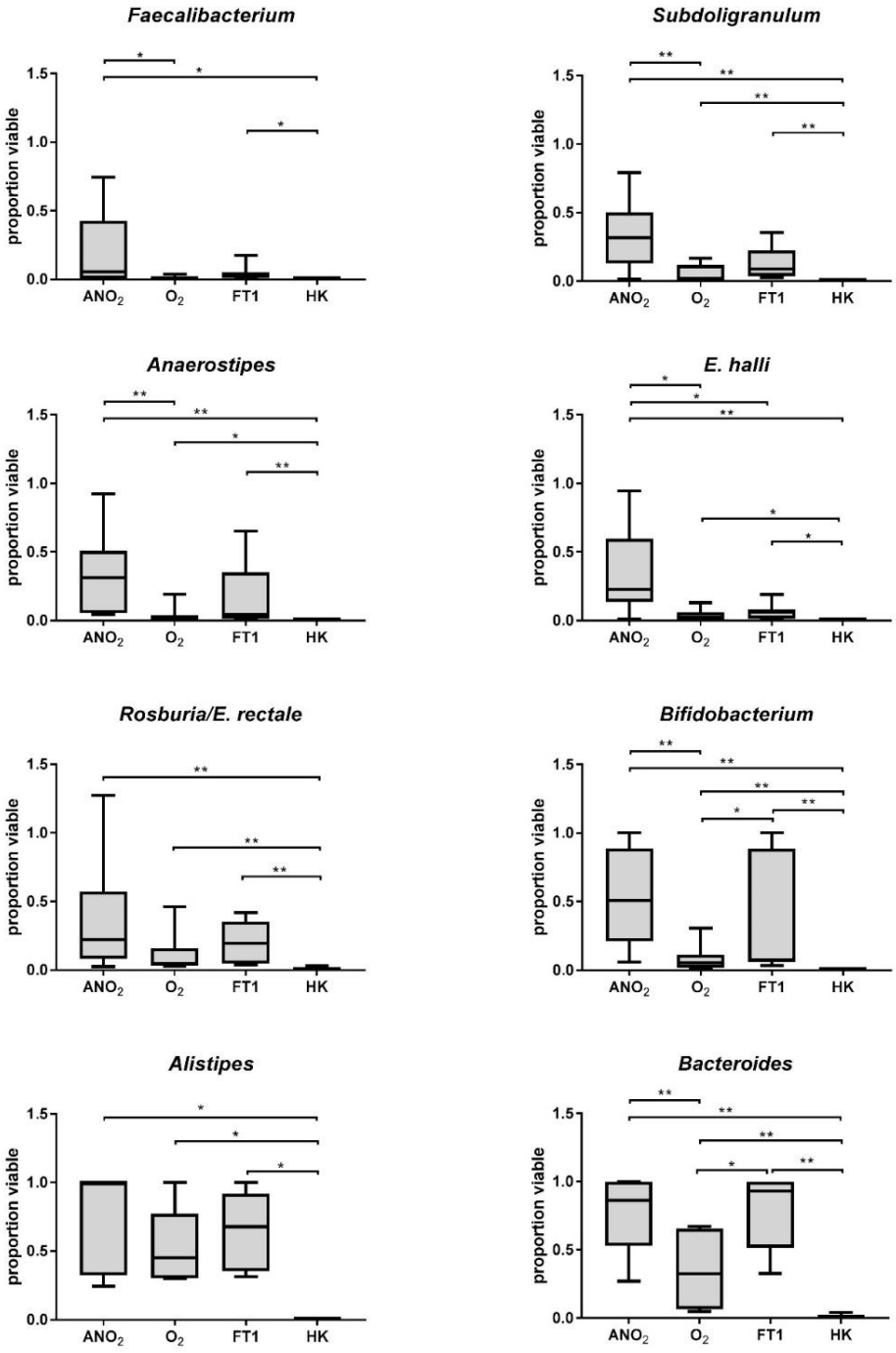


Figure 5.5 Proportion of bacteria determined to be viable by specific qPCR assays

Bacterial viability in faecal slurry was assessed after processing in fresh anaerobic conditions (ANO_2), fresh aerobic conditions (O_2), after anaerobic processing and after one cycle of freezing and thawing in anaerobically processed specimens (FT1) or after heat-killing (HK). Box plot depicts median and IQR and error bars depict minimum to maximum values of faecal

slurry samples from 8 individual donors. All significant comparisons are indicated by stars (*= p<0·05; **= p<0·01; ***= p<0·001; Wilcoxon matched-pairs signed rank test).

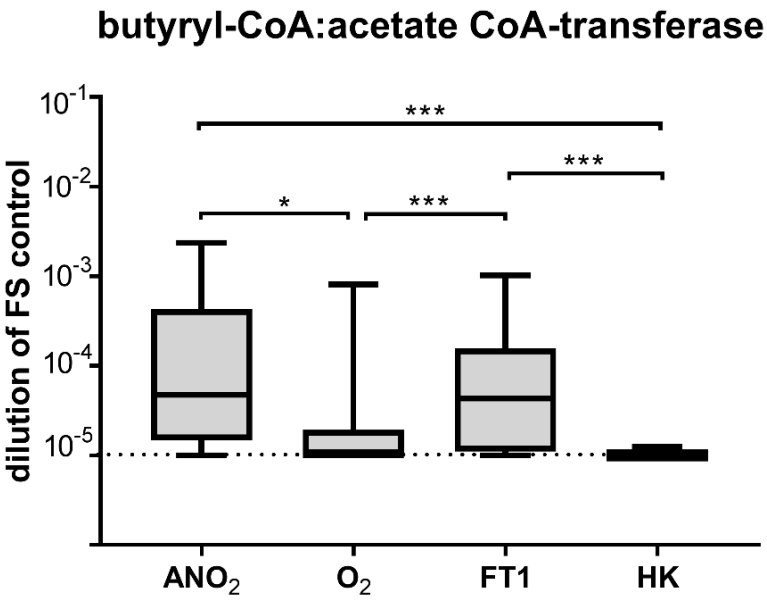


Figure 5.6 Assessment of butyrogenic capacity of FMT material by PMA-qPCR Amplification of the butyryl-CoA:acetate CoA-transferase gene, the terminal enzyme of the central butyrate synthesis pathway of human gut microbiota, in fresh anaerobic conditions (ANO_2), fresh aerobic conditions (O_2), after one cycle of freezing and thawing in anaerobically processed specimens (FT1) or after heat-killing (HK) in PMA treated samples. Butyryl-coenzyme A(CoA) CoA transferase gene levels were measured relative to amplification in a 10-fold dilution series of neat faecal slurry (FS control). The dotted line represents limit of quantification of the assay. Box plots depict median and IQR and error bars depict range from 8 individual donors. Significant comparisons are indicated by stars. (*= p<0·05; **= p<0·01; ***= p<0·001; Wilcoxon matched-pairs signed rank test).

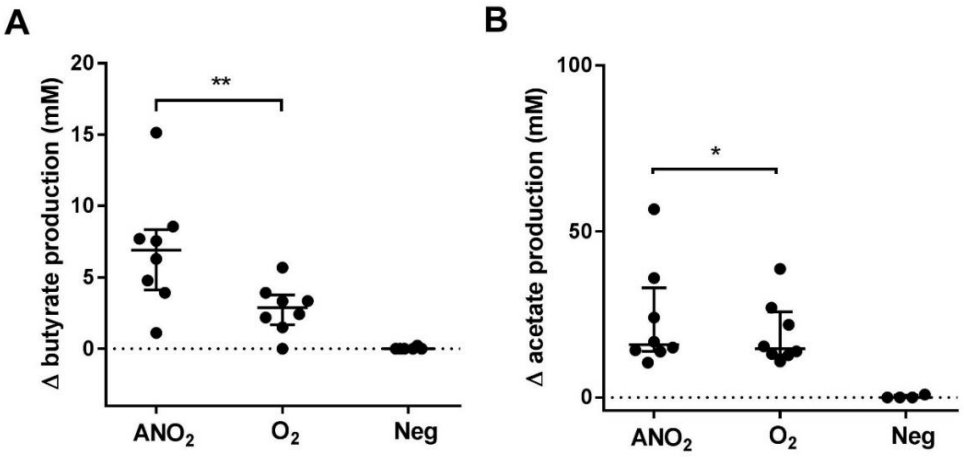


Figure 5.7. SCFA levels following *in-vitro* fermentation of faecal slurries for FMT with high-amylose maize starch.

Net production of butyrate (panel A) and acetate (panel B). Matching samples (n=8) were processed either under anaerobic conditions (ANO_2), or under aerobic conditions (O_2). Significant comparisons are indicated by stars. (*= p<0·05; **= p<0·01; Wilcoxon matched-pairs signed rank test).

5.5 Discussion

At present there is little evidence to guide clinicians using FMT on how to best ensure that the viability of donor microbiota is preserved in faecal transplant material. Analysis of microbiota in donor material is not routine, and when performed, the methods used do not assess viability. PMA based methodology can overcome many challenges in assessing the viability of the complex community of fastidious bacteria in stool. However, the main limitation of PMA methodology is that it is prone to over-estimating the number of live bacteria in a sample (201). This means the number of viable cells could be lower than found here. Furthermore, the dilution of specimens required for PMA treatment means that very rare taxa are excluded from this type of analysis.

Despite these limitations, this study shows that current methods of reporting the microbiota present in FMT material significantly overestimates the number of live bacteria transplanted. On average, only half of bacteria in faecal transplants in our study were still viable after immediate processing in strict anaerobic conditions. The use of PMA sample treatment also revealed that the diversity of bacteria in these transplants is significantly less than what would be reported using standard sequencing methods.

We observed substantial inter-donor variation in the impact of sample processing. Such differences can be explained by individual variation in microbiome composition, resulting in microbiota with different vulnerabilities to oxygen exposure and freezing, and indicate the need for viability assessments to be performed individual donor material.

This study revealed that homogenization by blending stool in ambient air has a profound impact on its viable bacterial composition. Ambient air processing is the default practice in most clinical trials and is described in American, (293) British (286) and European consensus guidelines, (284) although in many protocols stools are homogenized manually and not blended as in this study. The increased air flow produced during high-speed blending may result increased oxygen-exposure and be more detrimental to oxygen sensitive species than manual homogenization.

Obligate anaerobic gut commensal species that are most affected by oxygen exposure, including *Faecalibacterium prausnitzii, Subdoligranulum variable, Eubacterium rectale, Eubacterium hallii* and *Anaerostipes hadrus,* are major contributors to the biosynthesis of butyrate (292). Butyrate is an SCFA produced from the fermentation by the intestinal microbiota (54). Apart from being the major energy source of colonocytes, butyrate and has both anti-inflammatory and anti-carcinogenic properties (55, 294). More recently the interaction of butryrate with colonocytes has been linked to the inhibition of potentially pathogenic species such as *E. coli* (62). Reduced luminal butyrate concentration or butyrate utilisation is associated with enterocyte adenosine triphosphate depletion, loss of tight junctions, reduced mucus production and resultant colonic barrier disruption with inflammatory and immunological consequences (294).

The proportion of viable *F. prausnitzii* and *A. hadrus* in the majority of donors, as well as the levels of the butyryl-CoA:acetate CoA-transferase gene, were reduced to levels detected in heat-killed specimens when specimens were processed in the presence of oxygen. The relative reduction of butyrate producing bacteria, *F. prausnitzii* in particular, within the gut has been associated with the presence of a diverse range of chronic diseases including depression (282), obesity (281), type 2 diabetes (280) and inflammatory bowel disease (96, 276, 278). These findings suggest that processing faecal material in ambient air may negatively influence the outcome of efforts to achieve anti-inflammatory or immunomodulatory outcomes using FMT.

Bifidobacterium spp and *Bacteroides spp,* are major sources of the short-chain fatty acids, acetate and propionate respectively, and like butyrate are important energy and signalling molecules (55). Although, their beneficial effects are less well-established than those of butyrate, these metabolites have also been found to be important immune and metabolic

regulators (55). Members of these genera were also significantly reduced in abundance following oxygen exposure. However, significant residual viable populations remained, particularly in the case of *Bacteroides spp.*, suggesting the potential for re-expansion in the gut of the recipient.

Although freezing did reduce the overall viability of the transplant material (reducing overall viability to around 25%) the viable microbiota composition was not significantly different to that in fresh, anaerobically processed, specimens. Only one bacterial species, *Eubacterium hallii*, was found to be significantly reduced in freeze-thawed specimens. While *E. hallii* is a major butyrate-producer (292), this function is also performed by several other bacterial species. The potential clinical implications of the loss of this single species is uncertain.

Beyond the depletion of beneficial commensal bacteria in faecal material through processing in ambient air, the relative abundance of potentially pathogenic species, such *E. coli* and other oxygen-tolerant gram-negative bacteria, will increase proportionally. In our donor cohort levels of *E. coli* were very low in fresh stool, with no evidence of an increase in absolute abundance during processing. However, delays in sample processing that result in prolonged periods at room temperature could result in substantial increases in the abundance of opportunistic pathogens. The loss of butyrate-producing anaerobes combined with the overgrowth of oxygen-tolerant species could potentially transform faecal transplant material from healthy donors into faecal material with a microbiota profile more closely resembling those linked to inflammatory bowel disease (277, 289), type 2 diabetes (280) and colorectal cancer (279).

Processing FMT material in an anaerobic chamber achieves optimal preservation of important commensal species. In the context of *C. difficile* colitis, aerobic processing does not appear to adversely influence clinical outcomes. However optimal preservation of commensals should be attempted when FMT is being investigated for other indications where it is not yet known how the loss of commensal anaerobes could influence clinical outcomes. A limitation of this study was that the effect of delays in processing were not analyzed. Although stool specimens were processed within 15 minutes in this study, this is not achievable in most clinical settings, and a delay in processing of several hours is more typical. Such delays in processing may result in changes in viable microbiota composition. A number of previous studies employing molecular strategies, have reported delays in stool analysis to be associated with shifts in microbiota composition and viability (225, 295-297). Chu et al assessed changes in FMT material in one donor after processing delays of incremental periods up to 7 hours and saw a trend towards a change in microbiome composition over this time (207). These reports are supported by a number of culture-based studies that have demonstrated reduced recovery of

anaerobes when processing is delayed, (298) particularly at room temperature (299) and when samples were not stored in anaerobic conditions.(300) Until further research clarifies the effects or processing delays, we suggest that periods between sample collection and processing are as short as possible.

Following processing, faecal material should be frozen promptly at -80°C. The ability to freeze samples prior to instillation is important as it provides an opportunity to complete systematic testing for pathogens, including viruses and parasites (266). Freezing also allows stool to be available on demand for use in urgent clinical situations. This analysis suggests that, while freeze-thaw does impact the viable composition of stool, in general this effect is relatively limited. However, there is variability in the individual donor microbiota response to freezing. In this study, the overall bacterial viability in two donors dropped below levels seen after ambient air processing after freeze-thaw alone.

The role that stool processing plays in changing the composition of faecal transplants has been widely overlooked in the design of FMT clinical trials. Adherence to strict anaerobic stool processing protocols is likely to result in increased benefit from FMT in some clinical settings. Other factors, such as delays in stool processing and storage conditions, might have as great an impact on bacterial viability as anaerobic processing, but were not assessed in this study. It would be beneficial for future trials to assess the composition of donor transplant material with a viability assay to ensure that the microbiota composition includes a broad range of viable bacteria some of which may be crucial in mediating the therapeutic effects of FMT. A detailed analysis of the types and numbers of viable bacteria transplanted is critical to understanding of the mechanisms by which FMT produces, or fails to produce, therapeutic effects.

5.6 Acknowledgements

I would like to acknowledge Jocelyn Choo for assistance in bioinformatic processing and the interpretation of microbiome results, Yanan Wang for assistance with *in-vitro* fermentation methodology and Ge Liu from the SA Health and Medical Research Institute for performing the SCFA gas chromatography.

CHAPTER 6

Design of a randomised controlled clinical trial to investigate the effect of FMT on gut colonisation and subsequent infection with resistant gram-negative bacteria

6.1 Abstract

Gram-negative pathogens arising from the gut are the causative agents in the majority of urinary tract infections (UTIs). Antimicrobial resistance in these pathogens is increasingly prevalent resulting in increased morbidity and mortality associated with these common infections. In patients that experience recurrent infection with these pathogens, antibiotic use may contribute to increasing likelihood of future infection by disrupting beneficial gut microbiota that prevent pathogen expansion in the gut. However, more needs to be known about how gut microbiota affect the clinical course of patients with recurrent UTI and whether restoring beneficial bacteria to patient's gut microbiomes with faecal microbiota transplantation (FMT) can break the cycle of recurrent UTI.

This chapter describes the design of a randomised controlled trial to test the ability of FMT to alter outcomes in patients with recurrent resistant gram-negative UTI. The trial design, which includes monthly stool collection for microbiome analysis, will allow us to explore the relationship between gut microbiota, FMT and urinary tract infection in the participants.

6.2 Introduction

The world is facing a global epidemic of antimicrobial resistance which poses a grave threat to human health as antibiotics lose their efficacy (301). Of most concern are resistant enteric gram-negative bacteria capable of causing life-threatening infection in otherwise healthy people. These infections have emerged in the community and often manifest as urinary tract infections in otherwise healthy people (302). The most worrying multi-resistant organisms are gram-negative bacteria carrying transmissible carbapenemase genes (CRE). These organisms are intrinsically virulent, and carry resistance not only to carbapenems but in some cases to almost every other known antibiotic (303). Cases are extremely difficult to treat, and are associated with a high mortality and significant health care costs (301). CRE are already endemic in some regions of the world including Southern Europe, the Middle East and South Asia and are rapidly increasing in incidence (303). Although still relatively rare in Australia, incidence is steadily rising (12). Currently no decolonization therapy or procedure is routinely available to eliminate gut carriage of resistant organisms. Some people naturally clear these organisms over a period of months to years, however, the clearance rates are highly variable between individuals (304). It is thus imperative that innovative approaches are developed to reduce the incidence of infection with these types of bacteria.

Although some enteric gram-negative bacteria (particularly *E. coli*) are commonly present as gut commensals, these bacteria are also some of the most common human pathogens (4, 224). Organisms that have pathogenic potential but may otherwise exist as harmless members of commensal communities are also known as pathobionts. In healthy individuals these typically exist as a small fraction of the total gut bacterial community (56). When intact, the human gut microbiome has the ability to prevent the expansion of existing pathobionts and resist colonisation when exposed to extrinsic pathogens, a property known as 'colonisation resistance' (247). However, when the microbiome is disrupted by antibiotic use, subsequent colonisation with resistant pathogens is more likely to occur (40, 89, 239, 305). In murine models the reduction of microbial diversity resultant from antibiotic administration allows establishment of multi-resistant *Klebsiella pneumoniae* (37). In allogeneic hematopoietic stem cell transplant patients, the reduction of microbial diversity that occurs during treatment often results in the dominance of a single microbial taxon in the microbiome. This in turn is a predictor of subsequent bacteraemia, with a 9-fold increased risk in the case of VRE dominance, and a 5-fold increased risk in the case of Proteobacterial dominance (139). These observations form the basis of the theory that restoration of the microbiome with FMT following disruption may prevent or reduce MRO gut colonisation, and thereby prevent serious clinical infections resulting from intestinal domination with pathogenic bacteria. There is evidence that carbapenem-resistant *K. pneumoniae* colonised mice can be decolonised using FMT from healthy animals (37). The ability for FMT to reduce the abundance of gut pathobionts is also supported by data from human clinical trials that used FMT for the treatment of *C. difficile*. In the microbiome composition analysis from Van Nood et al's landmark randomised controlled trial of FMT showed that the relative abundance of gram-negative bacteria in the phylum Proteobacteria fell up to 1,000 fold post-FMT (172).

FMT used explicitly for MRO de-colonisation (including both gram-negative pathogens producing ESBLs and gram-positive MROs such as VRE) has been reported in the literature (208, 209). In a single case described by Singh et al, a man who suffered recurrent infection of his transplanted kidney allograft with ESBL-producing *E. coli* was administered FMT via nasoduodenal tube. Following FMT he remained clear of both clinical infections and faecal colonisation for 12 weeks (209). Several further case studies have been published documenting similar response to FMT; these are reviewed in Chapter 1 (Table 1.1).

Larger case series have shown more variable levels of success. Bilinski et al. reported 75% success in complete decolonisation of gut carriage of antibiotic-resistant bacteria in 20

immunocompromised cancer patients receiving FMT (213). Davido et al. also published a short case-series where only two of six patients were successfully de-colonised from CRE carriage following FMT (215). To date there has only been one published randomised controlled study of the ability of FMT, in combination with antibiotics, to eradicate resistant gram-negative pathogens (216). While a non-statistically significant difference in the groups was observed (41% vs 29% decolonisation in the treatment and placebo arms respectively), the trial included only 39 patients and was terminated early, and was therefore likely to be underpowered (216).
There is also emerging evidence that FMT may reduce the frequency of recurrent urinary tract infections. Wang et al reported a case in which FMT interrupted a 25-year history of recurrent urinary tract infection (27). In a retrospective report, Tariq et al reported that FMT significantly decreased the frequency of recurrent UTI in patients who received FMT for treatment of *C. difficile* infection (212). In support of the underlying hypothesis that the gut microbiome is an appropriate target for intervention to prevent recurrent UTI, Magruder et al (306) and Thanert et al (307) independently found clonally identical bacterial strains both in stool and in urine during symptomatic UTI in a subset of patients with recurrent UTIs. Many of these patients had sterile urine between episodes of UTI, while the uropathogen persisted in the stool (307).
Managing recurrent urinary tract infection with resistant gram-negative bacteria is an increasingly difficult clinical problem. Antibiotic therapy only provides temporary relief as infection recurs on antibiotic cessation. As multi-drug resistant bacterial infections become more prevalent in the community, antibiotic treatment options have become increasingly limited leaving only antibiotics that either have significant toxicity or must be delivered intravenously (or both). In patients in whom the reservoir of infectious pathogens is in the gut, the ability to reduce pathogen carriage at this site has the potential to produce significant clinical benefit. In order to test the hypothesis that modulation of the gut microbiota can reduce the carriage of gram-negative pathobionts in the gut and thus reduce recurrent infection by these organisms we designed a randomised controlled trial examining the ability of FMT to alter microbiota and reduce infection rates in persons with recurrent urinary tract infection.

6.3 Study Objectives

6.3.1 Primary

To determine whether FMT decreases carriage of selected gram-negative bacteria from the gastrointestinal tract, as evaluated by a significant decrease in relative abundance of these organisms in the stool microbial community

6.3.2 Secondary

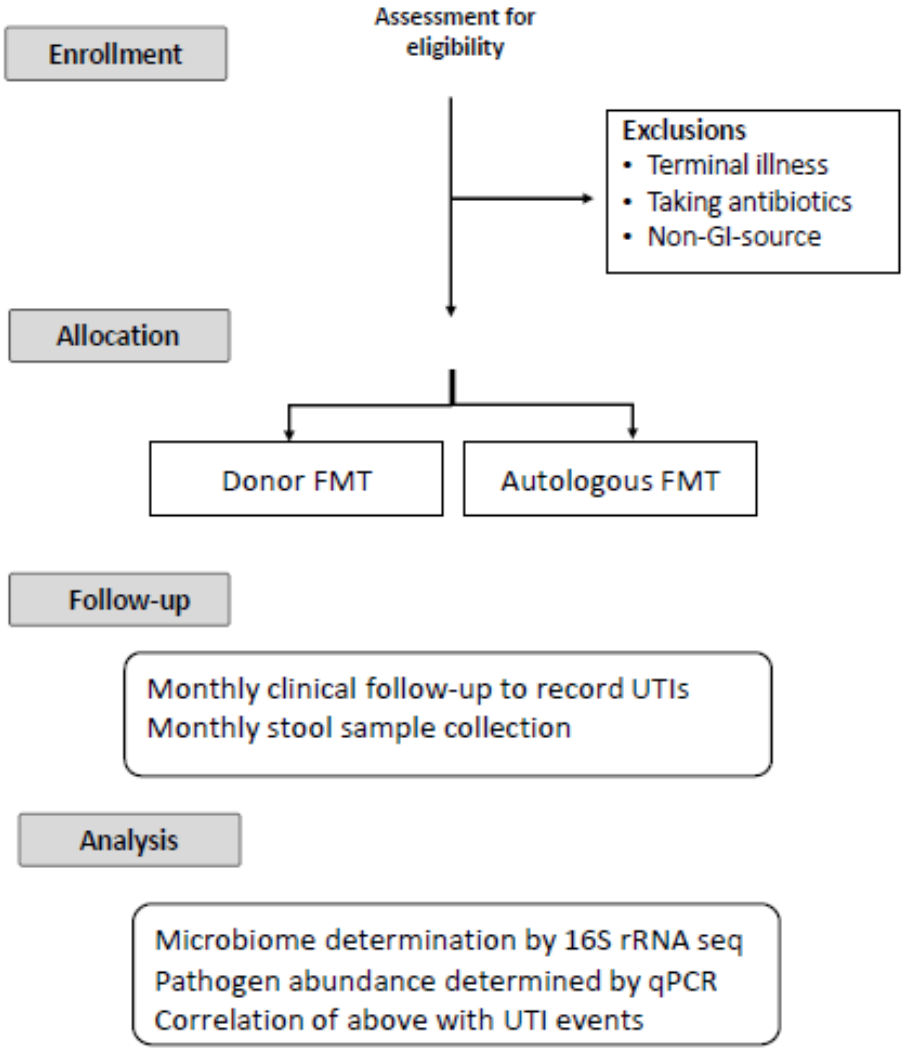
To evaluate whether FMT diminishes subsequent rates of clinical infection in persons with recurrent gram-negative bacterial infections.

6.4 Trial Design

We designed a randomised, controlled and double-blinded study as this design is the most likely to objectively test the effectiveness of a new intervention with minimal bias and is considered the gold standard design for interventional research (308). The trial has been prospectively registered on the Australian New Zealand Clinical Trials Registry (reference: ACTRN12617000561381 https://www.anzctr.org.au/). Ethical approval for this trial was granted by The Queen Elizabeth Hospital Human Research Ethics Committee (reference: HREC/16/TQEH/32).

Patients identified as having recurrent UTI with resistant gram-negative pathogens will be offered FMT via a retention enema. Following confirmation of eligibility, participants will be randomly allocated into either the donor FMT or autologous FMT (aFMT-faecal slurry prepared from the participant's own stool) group on a 1:1 basis. Participants and investigators will be blinded regarding group allocation. Due to the difficulty in delivering the total volume in one enema, the FMT is delivered in three separate intervals, one week apart. The FMT preparation and administration is detailed in section 6.4.6 below.

For 12 months following transplantation, participants will be followed up monthly to document the number of urinary tract infections that occur and participant's antibiotic use. Participants will provide a stool sample (weekly for the first 4 weeks, then monthly until study completion) for microbiome analysis using 16S rRNA amplicon sequencing to determine the relative abundance of bacterial taxa present during the monthly follow-up period. The timeline of the study intervention and follow-up is depicted in Figure 6.1.


Enrollment
Assessment for eligibility
Exclusions
• Terminal illness
• Taking antibiotics
• Non-GI-source
Allocation
Donor FMT
Autologous FMT
Follow-up
Monthly clinical follow-up to record UTIs
Monthly stool sample collection
Analysis
Microbiome determination by 16S rRNA seq
Pathogen abundance determined by qPCR
Correlation of above with UTI events

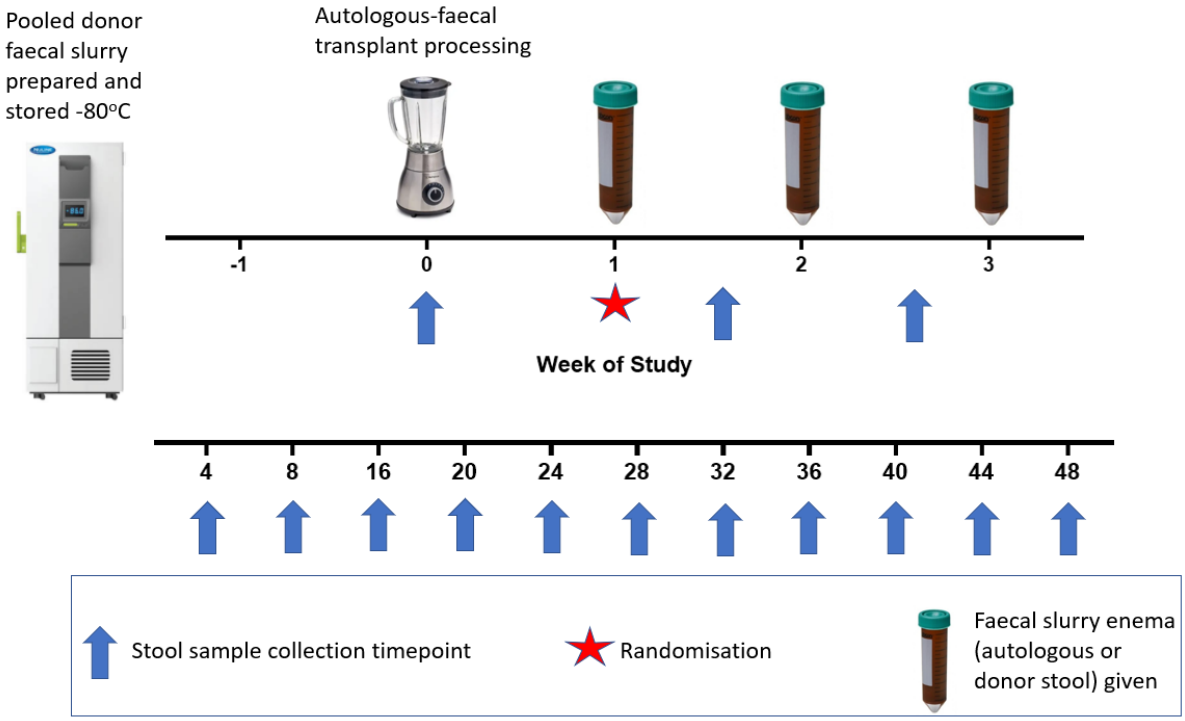


Figure 6.1. Study schema and timeline

6.4.1 Sample Size

The sample size for the study's primary outcome was calculated using a binary outcome superiority trial power calculation. From animal study data, we expect approximately 80% success in reducing (2-log or greater) Enterobacteriaceae abundance in the intervention group and 20% rate in the placebo group. Significance level (alpha) was 0.05 with a power of 90%. This yielded a sample size required per group of 10 with a total sample size of 20.

Sample size was also calculated for the secondary outcome of reduction in recurrent infection rate. For patients who have 3 or more recurrent infections in the preceding year the power required to show a 50% reduction in recurrent infection frequency is 21 (80% power at 0.05 significance).

We aim to enrol 22 patients with infection with recurrent resistant gram-negative infection. Eleven patients will be randomised to receive donor FMT and 11 will be randomised to receive aFMT. Stool samples and health data will be collected from patients weekly for 4 weeks and monthly for a further 11 months.

6.4.2 Inclusion criteria:

- Age range 18-85 years inclusive.
- Patients with refractory or recurrent infection–defined as at least 3 episodes of UTI requiring at least 3 episodes of antibiotic therapy in the preceding 12 months.
- Infection caused by a gram-negative bacterium which has acquired resistance to clinically important antibiotics routinely reported by SA Pathology. This is defined as reported resistance to one or more of the following antibiotics**:** meropenem, ceftriaxone, cefepime, ceftazidime, cefalexin, amoxicillin-clavulanate, piperacillin-tazobactam, ciprofloxacin, gentamicin or trimethoprim is this for all 3 or more episodes

6.4.3 Exclusion criteria:

- Active gastrointestinal infection
    - bacterial or viral infection causing symptoms of diarrhoea
- Pregnancy
- Current use of antibiotics*
- Cognitive impairment
- Perianal inflammation
- Life expectancy < 1 year
- Neutropaenia <0.5 $X10^9/L$
- Severe IgE mediated food allergy: urticaria or anaphylaxis
- At risk of peritonitis: including patients with ascites or peritoneal dialysis

***** The use of trimethoprim-sulfamethoxazole for pneumocystis prophylaxis in immunocompromised patients will be permitted.

6.4.4 Withdrawal criteria

Patients may withdraw from the study at any time. We will ask for their reasons for statistical purposes, however they will not be obliged to provide this. Withdrawal from the study will not affect the participant's medical care in any way.

6.4.5 Faecal donors

Donors will be anonymous volunteers. They will be recruited by poster advertising on University campuses. They will be paid $18 per hour for time spent participating in the study.

If donors are excluded from the study because of an abnormality detected on their screening test, they will be referred to their GP or other appropriate outpatient service for follow-up. Details of faecal donor screening are included in Appendix 5.

6.4.6 Faecal transplant preparation

Prior to randomisation each participant enrolled in the study will provide a stool sample of their own in order to prepare the autologous faecal transplant. The pooled FMT donor and auto-transplants will be prepared by a study investigator (Dr. Papanicolas) who will remain blinded to the participant's allocation. The stool transplants will be prepared from pooled donor stool or the participant's own stool. Faecal samples will be transported on ice on the day of passage to the laboratory and processed into FMT material immediately. FMT is prepared using stool (25%) blended with normal saline (65%) and glycerol (10%) in anaerobic conditions in an anaerobic chamber as previously described (266, 309). This faecal slurry is split into 3 aliquots of 67mL and these aliquots are immediately frozen at -80^{o}C. (266). FMT material will be placed in 250 mL capacity gamma sterilised, polystyrene containers (Techno Plas St Marys SA, Australia) in an anaerobic chamber. Following this samples will be immediately stored at -80 C until further use.

Each stool aliquot will then be numbered and recorded in the secure and confidential faecal transplant aliquot document that will list the stool donor who provided each aliquot. In this way, any possible transmission of infection or other disease could be traced. Small aliquots (1 mL) of each individual donation will be set aside and frozen individually. This will allow repeat testing and tracing of each individual donation in the future in the event of possible transmission of infection.

Before use in the study, FMT material from individual donors will be pooled under anaerobic conditions to create as uniform a specimen as possible for each participant. Stool from 5 donors will be pooled. Aliquots of 67 mL of the pooled donation will be frozen and thawed just before transplantation into the participant. The total FMT delivered will be 200 mL over 3 enemas, equivalent to 50 grams in stool content.

6.4.7 Participant allocation

Participants will be assigned a study number (consecutive 1-22) in order of recruitment to the study. Prior to the commencement of the trial block randomisation will occur so that cohort allocation (11 placebo, 11 intervention) is randomly linked to participant number using a random number generator from Random.org. Sealed envelopes prepared by a unit secretary

not otherwise involved in the study are marked with the patient's study number on the outside and contain the cohort allocation inside. On the day of the first faecal transplant procedure, the study gastroenterologist will determine the participant's pre-assigned allocation and based on this allocation will select the appropriate faecal slurry to thaw (autologous or donor). If the patient is assigned to donor FMT then their autologous transplant will be discarded.
The participants will continue to receive either donor or aFMT via enema for a further 2 weeks, for a total of three 67mL enemas given 1 week apart. The FMT is given as three smaller volume faecal transplants delivered one week apart because larger volumes are difficult to retain by participants receiving the enema.
The gastroenterologist administering the faecal enemas will be not be blinded as to which group the participant has been assigned to. This is so the proceduralist can provide a bedside check to ensure the participant does not receive an incorrect fecal transplant. Therefore, gastroenterologist will perform an unblinded safety check prior to administering the enema and then will not be involved in any aspect of participant follow-up or data analysis until the study has been completed.

6.4.8 Follow-up period

During the trial subjects will be treated as per the standard of care by their usual clinician for any medical conditions that they have. Patients will be given the telephone number of the primary investigator to contact should they have any concerns. Patients will not be taking antibiotics at enrolment, however, if the need arises for antibiotics during the study period there will be no restriction on their use.
In the first month, participants will be asked to provide specimens weekly (prior to each of the three FMT procedures). Participants are asked if they are experiencing fever or GIT disturbance or any other self-reported side-effects following FMT but will not have routine blood monitoring following the procedure. Following this, participants will be interviewed monthly (by telephone or in person) to arrange specimen collection and to determine whether they have developed any new clinical infections. Antibiotic use and/or hospitalisation for any reason will also be recorded.
Stool will be collected from study participants weekly for 3 weeks (prior to each enema administration), then monthly for one year. Where possible the stool samples will be provided fresh, these samples will be sub-aliquoted into 1.5 mL plastic tubes stored at -80^{o}C.
Participants who develop symptoms of urinary tract infection within the study follow-up period will be asked to collect a urine sample. This sample would be collected by a study

investigator to be processed separately from any urine sample testing requested by their usual doctor as part of their clinical care.

6.5 Clinical data and sample analysis plan

Clinical data collected during the trial will include documentation of every episode of symptomatic urinary tract infection that was treated with antibiotic therapy. Data collected will include the date of UTI onset, the results of diagnostic culture and all use of antibiotic therapy during the trial regardless of indication. Any admission to hospital, and the reason for this will also be recorded.

DNA from stool samples will be extracted and the V3-V4 region of the bacterial 16S rRNA gene will be amplified and sequenced (Illumina MiSeq) as previously described (309). Bio-informatic processing of sequencing data will be performed using Quantitative Insights in to Microbial Ecology (QIIME) software (226) using a previously described bioinformatics pipeline (225). QIIME outputs will be used to determine microbial α-diversity measures and the relative abundance of specific bacteria taxa. Where appropriate, specific qPCR molecular assays will be used to detect the presence, absence or absolute abundance of pathogenic bacteria species causing infections in selected participants.

6.5.1 Analysis of the primary end point

To determine whether faecal microbiota transplantation (FMT) decreases carriage of selected gram-negative bacteria from the gastrointestinal tract, the relative abundance of the phylum Proteobacteria and the specific pathogenic genus causing infection (determined individually for each participant) preceding trial enrolment will be analysed before and after the FMT intervention. Where possible, the absolute abundance of the specific pathogen species will be quantitated using qPCR.

Participants will be analysed according to their intervention allocation (either donor FMT or autologous FMT) group using paired comparisons relative abundance measures before and after intervention using three repeated tests obtained during 5 distinct time intervals (first 3 weeks following FMT, 1-3 months following FMT, 4-6 months following FMT, 7-9 months following FMT and 10-12 months following FMT). The significance of the comparison (set at $p<0.05$) will be determined using the paired Wilcoxon signed-rank test with correction for multiple testing using the Benjamini-Hochberg approach.

6.5.2 Analysis of the secondary end point

To evaluate whether FMT diminishes subsequent rates of clinical infection in persons with recurrent gram-negative bacterial infections, we will determine whether UTI occurred during the 4 distinct sample intervals (as above). The study end point will be analysed using both an intention-to-treat and a per-protocol analysis. The difference in UTI occurrence for each group in each of the 5 time intervals, and the outcome across all time point combined, will be determined using the Fisher's exact probability test.

6.6 Discussion

This trial described in this chapter is currently recruiting and has so far enrolled 9 participants. Due to the blinding involved in the trial there has not yet been an interim analysis performed, and therefore data relating to this study will not be included in this doctorate.

The purpose of this study is to investigate whether FMT reduces or eliminates carriage of gram-negative pathobionts in the gastrointestinal microbiota and thereby diminishes the incidence of clinical infection with these organisms in participants. Although FMT is not without risk, the potential benefit of this intervention significantly outweighs the risk. The global incidence of gram-negative MROs is rapidly rising and as a result there are increasingly limited therapeutic options for treating these infections (310). Many of the participants recruited to this study undergo recurrent hospital admission to receive intravenous antibiotics for their infections. Each episode of hospitalisation, particularly when associated with bacteraemia, carries a substantial risk of mortality (237, 310). A single episode of gram-negative bacteraemia has a mortality rate of nearly 20% even before resistance is taken into account (311) This contrasts to the small risk of serious adverse effects from FMT.

The main complication reported in conjunction to faecal transplantation in the literature is related to complications of anaesthetic sedation in those patients receiving FMT via endoscopy or colonoscopy (312, 313). These risks have been avoided in this study by using enema, where no sedation is used, as the mode of administration. More recently, risks of transmission of infection from FMT material have been reviewed showing that the incidence of severe infections following FMT is 2.5% (27/1089), this included two cases of bacteraemia with enteric pathogens (219-221). In addition, there has been at least two cases of viruses transmitted through FMT reported including cytomegalovirus (314) and norovirus

(315). Recent cases of the transmission of ESBL carrying *E. coli* resulting death of one patient have caused considerable concern about the safety of FMT (218). However, in these cases screening for ESBL resistance was not performed on donors. Given our aim is to use FMT to decolonise patients from these very pathogens we have taken great care to screen donors for stool carriage of multi-resistant organisms.

There are also concerns about FMT causing harm via modulation of host-microbial interactions in the gut which influence immunological and metabolic processes. The risk of long-term metabolic or immunological adverse effects following FMT are not established and remain largely theoretical. Nonetheless, given the paucity of long-term data, the possibility such long-term risks need to be considered. There have been limited reports where FMT could plausibly have resulted in such outcomes. For example, a case of weight gain has reported following FMT provided from an obese donor (175). In a follow up study of 77 patients who had received FMT for recurrent CDI, 4 patients developed new autoimmune disease during the follow up period of 3-68 months (316). This study had no control group with which to compare the follow up data and so no definite association between FMT and the development of autoimmune disease could be made. Increased insulin sensitivity has been demonstrated in obese subjects following duodenal infusion of faeces from lean donors (317). The transmission of insulin resistance via FMT is therefore a potential risk. In order to mitigate the risk of non-infectious adverse events developing in FMT recipients, the donors used in this study were screened for obesity, type 2 diabetes mellitus, hypertension, dyslipidaemia and auto-immune disease on history, examination and blood testing.

This trial employs the use of pooled donor stool from 5 individual donors processed under anaerobic conditions and then frozen at -80^{o}C prior to use. As shown in chapter 5, the use of anaerobic processing in crucial in preserving the viability of critical beneficial commensal taxa. Freezing, in contrast, has less detrimental effect on the viability of commensals, but is essential to perform to allow the screening of donor stool.

There were two main reasons for using pooled stool in this study. Firstly, pooling is likely to increase the total diversity of bacteria present in the faecal transplant material and this hypothesis is supported by at least one study (177). Secondly, the differences between individual donors can skew results when the so-called “super-donor” effect is observed. For example, in Moayyedi et al’s trial of FMT for ulcerative colitis, one of the five donors was used in 78% of the remissions observed (197). To prevent distortions occurring because of individual donor differences, pooled donations were used so that the transplant material being given represented a more uniform product in all recipients. However, pooled donations do

have the disadvantage of slightly increasing the risk of infection transmission occurring. For this reason, the practice of using pooled stool in faecal transplantations will no longer be allowed when the latest regulatory guidelines on FMT published by the Australian Therapeutic Goods Administration come into effect on July 1 2021.

To date, only 9 participants have been enrolled in this study. This includes 5 women and 4 men, 5 of whom are renal transplant recipients. A low rate of recruitment has been a persistent problem in this study. Several changes to the protocol were made in order to improve recruitment rates but also reflect the evolution of my thinking about the underlying mechanisms driving recurrent infection these patients.

When this trial was initially conceived, it was hypothesised that patients with recurrent UTIs would have consistent and easily detectable (via culture) gut colonisation with the same resistant gram-negative bacterium that was causing their urinary tract infection. It was hypothesised that donor organisms from donor FMT would be able to displace these pathogens to the point where they would no longer be detectable, thus achieving decolonisation. However, this viewpoint was challenged as the trial progressed. Firstly, the first two patients recruited to the trial could not be included the causative pathogen could not be cultured from stool despite the history of recurrent UTI. As a result, the initial protocol that stipulated that the organisms must be cultured from stool prior to faecal transplantation had to be altered and this requirement was removed. Since then, I have come to understand that gut microbiomes in a perturbed state are far more dynamic than I had initially presumed. The underlying problem in patients with disrupted gut microbiota is that they are particularly prone to instability (318). The role of intervention with FMT therefore is not to "decolonise" the patient from resistant pathogens, but rather to stabilise the recipient's microbiome to prevent repeated cycles of pathogen acquisition and expansion.

Despite it being widely accepted that uropathogens arise in the gut (319), at the time of the trial's initiation very little was known about the relationship between the gut microbiomes and urinary tract infection. Using culture and whole genome sequencing, Chen et al showed that in 4 healthy women with recurrent *E.* coli UTIs, half maintained a clonal population of the dominant uropathogenic strain in both urine and stool, whereas the other two experienced a complete shift in the strain colonising their urine and stool between UTI recurrences (320). Using similar methodology, Thanert et al related intestinal blooms of specific uropathogens to subsequent UTI with the same organism in 4 of 7 patients with recurrent gram-negative UTI (307). In late 2019 stronger evidence of a connection between gut microbiome composition and UTI emerged when Magruder et al published a large observational study

involving 16S amplicon sequencing of faecal specimens from 168 renal transplant recipients (306). This study showed an association between gut microbiome composition and subsequent UTI. Specifically, Magruder et al showed a 1% relative abundance of *Escherichia* or *Enterococcus* was an independent risk factor subsequent bacteriuria with the corresponding organism and in the case of *Escherichia* was also a risk factor for UTI (306). The findings from these studies support the premise that reducing the relative abundance of pathogens through gut microbiota modulation, rather than eliminating the carriage of these pathogens entirely, may be sufficient to alter clinical outcomes in patients with recurrent UTI. Due to low recruitment rates and the recognition that reducing the relative abundance of pathobiont bacteria in the gut (not their resistance determinants) should be the major goal of the study, some of the inclusion criteria were altered. Initially only participants with infection due to Enterobacteriaceae with ESBL or CRE type resistance were included in the study. Subsequently the inclusion criteria were broadened to include a wider range of gram-negative pathogens (such as *Pseudomonas aeruginosa*) and resistance to other classes of antibiotics. In order to be able to recruit solid-organ transplant recipients we also amended the protocol to allow the use of the antibiotic trimethoprim-sulfamethoxazole (as this is routinely used for prophylaxis from Pneumocystis infection in immunosuppressed persons). This antibiotic has a limited effect on anaerobic bacteria (321) and therefore we concluded that it's use would be unlikely to have an adverse impact on the donor stool transplant. The requirement for three infections in the year prior to enrolment has also limited recruitment, but this requirement was not altered as this would reduce the power of the study to show a change in infection frequency following the intervention. Although the rate of recruitment has gradually improved over time, the main limitation in study enrolment is that patients who are otherwise eligible for recruitment are often too unwell and unstable to be included in an outpatient clinical trial. Even though these patients are the ones with the most to gain from a successful intervention, where possible we seek to avoid recruiting participants who are likely to die during the study period as this must be treated as a serious adverse event.

In summary, conducting a clinical trial as described in this chapter will answer the important question of whether altering gut microbiomes through FMT can significantly reduce the incidence of recurrent UTIs. Given the regulatory hurdles due to be imposed by the TGA, the recruitment for this study will necessarily end in July 2021. However, difficulties in participant recruitment mean that this trial as currently implemented will not be able to achieve adequate power to answer the question we posed within that time. To move forward,

the data collected from this trial will be used to better determine the power required for a second clinical study, where the clinical outcome of reducing infection rates will be the primary endpoint. For such a study to be successful it must result from a collaboration with centres in cities and states with larger populations and receive adequate funding. Setting up this clinical trial will be a major focus of my immediate post-doctorate career.

6.7 Acknowledgements

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CHAPTER 7

Future directions: Microbiome based interventions to reduce infection and resistant pathogen carriage

Sections of this work appear in the following publications:

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Papanicolas LE, Gordon DL, Wesselingh SL, Rogers GB. Improving Risk-Benefit in Faecal Transplantation through Microbiome Screening. *Trends in Microbiology*. 2020 Jan 15. pii: S0966-842X(19)30324-5. doi: 10.1016/j.tim.2019.12.009.

7.1 Introduction

In an ideal future world, the risks of systemic infection and multi-resistant organism (MRO) carriage will be mitigated through individual faecal microbiome analysis followed by treatment with safe and effective microbiome-based precision therapeutics. However, while attempts to develop microbiome-based therapeutics proceed at a rapid pace, these efforts are hampered by substantial knowledge gaps that exist in understanding how microbiome composition influences the risk of MRO carriage and infection. While interventions to restore microbiota are awaited, efforts should be directed towards understanding how medical interventions harm commensal microbiota and focused on preventing unnecessary damage to microbial communities in our gut.

This chapter will review strategies to reduce the harm to microbiota from antibiotic use and review the progress that has been made in the rapidly expanding field of microbiome-based therapeutics. Finally, this chapter will place the findings of this thesis into the broader context of this field and will explore approaches to future research that will help to bridge important knowledge gaps.

7.2 First do no harm: strategies to reduce unnecessary damage to gut microbiomes

7.2.1 Considering the gut microbiome in antibiotic stewardship

Antibiotic stewardship involves a coordinated effort to use antimicrobials in an appropriate manner (322). Current antimicrobial stewardship efforts are mainly focused on reducing antimicrobial resistance which forms in response to direct selective pressure. For example, use of 3rd generation cephalosporins directly selects for bacteria carrying ESBLs. Consequently, antimicrobial stewardship programs that restrict the use of 3rd generation cephalosporins are used as a strategy to reduce ESBL prevalence (323). However, antimicrobial stewardship interventions often fail to consider antibiotic-related gut microbiome disruption as a factor driving the expansion and acquisition of antibiotic resistant pathogens.

Inappropriate and unnecessary use of antibiotics is common. For instance, recent audits determined that nearly one-quarter of antibiotic prescriptions Australian hospitals were inappropriate (324). Therefore, interventions that prevent the inappropriate prescription of antibiotics or that result in the cessation of antibiotics within a short time frame have high impact. Interventions that reduce inappropriate antibiotic prescriptions often involve large

education campaigns targeting both health practitioners and the public (324), although interventions based on selective pathology reporting of antimicrobial susceptibility results can also alter antibiotic prescribing behaviour (325, 326). By limiting patient exposure to any antimicrobial therapy, these interventions reduce all harmful aspects of antibiotic use including damage to gut microbiota.

However, other commonly employed stewardship interventions, such as those that involve changing the type of antibiotic used, are less impactful and could even inadvertently lead to increased damage to gut microbiota. One such intervention is encouraging early switching from intravenous (IV) antibiotics to oral antibiotics (327). This may result in a switch from an IV antibiotic which has low impact on gut microbiota due to low levels of gastrointestinal tract penetration (such as IV vancomycin) or low levels of anaerobic activity (such as IV flucloxacillin) to an oral antibiotic with significant anaerobic activity which is delivered directly to the gut microbiota through the oral route (such as clindamycin or amoxicillin-clavulanate). Even though there are other advantages to IV-to-oral switches such as reduced renal toxicity or vein thrombosis, the possible negative impacts on gut microbiota are rarely acknowledged or considered.

Another aspect of antimicrobial stewardship practice which often fails to take into account the gut microbiota is the principle of switching from broad-spectrum to a narrow-spectrum therapy where possible. Initially, "broad-spectrum" referred to the activity of an antibiotic against both gram-positive and gram-negative bacteria (328). However, anecdotally, many clinicians see this classification as reflecting the relative ability of an antibiotic to combat a wide range of pathogenic species (predominantly facultative or aerobic bacteria). However, the terms "narrow" and "broad" can be misleading and have little value in predicting effects on commensal microbiota. Indeed, antibiotics referred to as narrow-spectrum may have broad activity against commensal gut bacteria (predominantly obligate anaerobes). For instance, the narrow-spectrum antibiotic, vancomycin, has far greater adverse impact on gut microbiome composition than broad-spectrum amoxicillin when given orally (81).

There is also evidence that the use of narrow-spectrum antibiotics with broad anaerobic activity can drive antimicrobial resistance through the disruption of anaerobic commensals. For instance, Ubeda et al linked the ampicillin-mediated destruction of gut bacteria in the Bacteroidetes phylum to the dramatic expansion and domination of the gut with VRE in a murine model (38). Subsequently, Taur and colleagues demonstrated that the strongest predictor of VRE intestinal domination and VRE bacteraemia in hematopoietic stem cell transplant recipients was the use of metronidazole (139). This antibiotic is often considered

narrow-spectrum by clinicians although it is known for its activity against a broad range of anaerobes including bactericidal activity against *Bacteroides spp* (329).

Although guidelines on antibiotic use often do not explicitly take into account the impact of antibiotics on gut microbiota, the association of certain antibiotics with the development of *C. difficile* infection is usually considered. An association with *C. difficile* infection is one approach used as a surrogate indicator of that antibiotic's ability to disrupt gut microbiota (87). However, this rule of thumb is not always reliable, and can fail to identify certain antibiotics with significant microbiome-disrupting ability. Again, metronidazole fails to be recognised as a major disruptor of microbiomes because it is not considered to be associated with *C. difficile* infection (instead it is used to treat this condition).

Certain guidelines already incorporate consideration of the impact of antibiotics on commensal microbiology. For example, in patients receiving treatment for uncomplicated urinary tract infection (UTI), ciprofloxacin use results in substantially greater disruption of gut commensals than nitrofurantoin, which in contrast has minimal effects on gut microbiota (82). Such findings support current guidelines which take the impact of antibiotics on gut commensals into consideration and recommend use of nitrofurantoin as a first line agent for the treatment of UTI (330). In another example, where the oral options for treating resistant staphylococcal infection include trimethoprim-sulfamethoxazole, clindamycin or linezolid, it could be argued that trimethoprim-sulfamethoxazole should be favoured based on its minimal relative activity against commensal anaerobes (321), yet the differing effects of these antibiotics on gut microbiology are not discussed in MRSA treatment guidelines (331).

Even very broad-spectrum antibiotics that are used empirically for sepsis or febrile neutropaenia are not equivalent in regard to their microbiome-disrupting capacity. There is emerging evidence that cefepime is less disruptive to commensal microbiota than other broad-spectrum antipseudomonal antibiotics (carbapenems, piperacillin-tazobactam) and the choice of antibiotic used for febrile neutropaenia may influence the long-term survival of patients receiving haematopoietic stem cell transplantation (332). However, we currently lack prospective clinical evidence that would support using less microbiome-disrupting antibiotics, particularly when considerations such as immediate efficacy and local antibiotic susceptibility patterns clearly take precedence.

In order to successfully incorporate the concept of reducing microbiome harm into routine antimicrobial stewardship practice, clinical trials must determine the potential benefit of stewardship efforts to limit the use of antibiotics that have the greatest potential to disrupt gut microbiota, compared with current practice. These trials must be appropriately powered, multi-

centre and focus on long-term outcomes including multi-resistant organism acquisition and infection, hospital re-admission rates for sepsis and overall mortality in order to be meaningful. However, even in the absence of data from microbiome studies, the likely impact of commonly used antibiotics on commensal microbiota can at least partially be predicted based on their known antimicrobial spectrum and intestinal absorption, providing an immediate basis to refine treatment decisions. Specifically, where two antibiotic regimens are otherwise equivalent, the agent(s) with the lesser impact on commensal anaerobic bacteria should be preferred.

7.2.2 Therapeutics to block the gastrointestinal action of antibiotics

Frequently it is clinically necessary to use antibiotics that are known to disrupt the gut microbiota, even when the bacterial pathogen targeted is outside the gut. When this is the case, an innovative approach to preventing collateral damage to the gut microbiota is to use a substance to inactivate the antibiotic in the gut to protect commensal microbes from the antimicrobial effects of the antibiotic. This approach has begun to be explored by repurposing β-lactamases to protect the gut microbiota.

Beta-lactamases have long been present in gut ecosystems and are evolutionarily ancient enzymes secreted by microbes in order to protect themselves from naturally occurring β-lactam antibiotics (333). In 2008, Tarkkanen et al reported the use of a recombinant class A β-lactamase (the P1A protein) in human volunteers to successfully prevent changes to gut microbiota induced by the intravenous administration of ampicillin (334). In participants receiving ampicillin alone a significant rise in the percentage of ampicillin-resistant coliforms cultured from faeces was demonstrated (from <5% at baseline to 70-80% during treatment) whereas with the use of P1A the number of resistant isolates remained low (<10%). The drug Ribaxamase was developed after P1A, in order to achieve broader-spectrum β-lactamase activity including cephalosporins, and has reached phase II clinical trials (335). Ribaxamase degrades ceftriaxone in the human intestine without affecting the plasma concentration of ceftriaxone (335). Although Ribaxamase prevents microbiome diversity loss and the development of antibiotic resistance due to amoxicillin or amoxicillin-clavulanate in dogs, this has yet to be demonstrated in humans (336).

An alternative approach employs an activated-charcoal based drug (DAV132) to selectively adsorb drug compounds in the colon while still allowing systemic absorption in the proximal gut. The advantage is that this intervention can be used with non-β-lactam antibiotics, although its broad spectrum of activity could be problematic for those taking non-antibiotic

medications. In 2015, DAV132 was reported to be capable of protecting human participants from microbiome disruption following oral moxifloxacin administration (337).
Optimal antibiotic stewardship and the use of novel strategies to selectively inactivate antibiotics in the gut could substantially reduce collateral damage occurring to intestinal microbiota during systemic therapy. Further research is required for these therapies to become established therapeutics, but if successful, this approach will make a substantial contribution to protecting gut commensals from antibiotic disruption. However, even optimal use of approaches to reduce harm to commensal gut bacteria will not eliminate antibiotic-induced microbiome damage because many antibiotics therapies, such as those used empirically in sepsis or for abdominal infections, deliberately target a broad range of gut bacteria. Therefore, interventions to restore damaged gut microbiota to health are also required.

7.3 Interventions to restore damaged gut microbiomes

7.3.1 The role of probiotics and prebiotics in microbiome reconstitution and infection

Probiotic therapies, most commonly involving the ingestion of live bacteria associated with fermented dairy products such as *Lactobacillus spp* and *Bifidobacterium spp,* have long been thought to be health-promoting. Ingestion of these bacteria have been used as a therapy to treat antibiotic-associated diarrhoea (AAD). Although the mechanisms of AAD are likely multiple and have not been clearly established, it is likely that in a substantial proportion of patients this side-effect is related to antibiotic-mediated disruption of commensal anaerobes (338). A reduction in commensal anaerobes can lead to the reduced metabolism of carbohydrates and produce osmotic diarrhoea. This is supported by a small study showing that patients with AAD had altered SCFA levels (the main metabolic product resulting from bacterial fermentation of carbohydrates) (339).
Although certain well controlled trials have failed to show a benefit from particular probiotics (340, 341), many clinical studies of probiotic interventions suggest efficacy in reducing the incidence of AAD. A meta-analysis of 82 pro-biotic studies published in 2012 (342) found that probiotic interventions (*Lactobacillus* based therapy in the majority) showed a statistically significant association reduction of AAD with a number needed to treat of 13. Yet despite the large number of studies included in the meta-analysis only 4 proactively monitored for adverse effects and only 7 examined outcomes beyond the cessation of antibiotic use. No study examined the microbiota of the probiotic recipients, a failure which

meant that few conclusions could be drawn about mechanisms underlying the observed clinical efficacy of the intervention.

In 2018, a landmark study by Suez et al gave us new insights into how probiotics interact with antibiotic-affected microbial communities in the human adult gut (45). In this study 21 volunteers were treated with antibiotics (ciprofloxacin and metronidazole) for 1 week. This caused a significant disruption of their gut microbiota in the short term. In 7 participants that received no intervention, their microbiome composition returned to normal after 21 days. This supports the findings of numerous other studies in healthy volunteers showing that gut microbiomes are able to largely reconstitute themselves within 1-6 weeks after antibiotic use (83, 93, 343, 344). Another subset of volunteers in Suez et al's study were given a probiotic treatment consisting of 11 bacterial strains (6 species of *Lactobacillus*, 4 species of *Bifidobacterium* and *Streptococcus thermophilus*). The researchers observed that antibiotic-induced disruption of the native microbiota enhanced the ability of the administered probiotics to colonise the GI mucosa of the participants. However, this colonisation resulted in a delayed reconstitution of baseline microbiota. Further investigation by the same group in an *in-vitro* model suggested that microbial products found in *Lactobacillus* supernatants could inhibit the growth of the indigenous colonic flora (45). Importantly, the microbial species richness in the probiotic group was only half of what was observed in those who received no treatment. As reduced gut microbiome diversity has been associated with multiple chronic diseases (345, 346), this outcome could arguably be considered harmful. This study has cast significant doubt on claims that the oral administration of Lactobacilli or Bifidobacteria as probiotics is beneficial in adults. Further studies will need to be done to confirm the findings of Suez et al, but in the interim this study provides robust scientific evidence showing that administration of exogenous bacteria to a system where colonisation resistance has been disrupted by antibiotics could result in a harmful alteration of the recipient's microbiome and impede the reconstitution of indigenous microbiota that occurs naturally (45).

However, these results are not necessarily applicable to all populations. For instance, neonates have a substantially different microbiome composition than adults (347) and a diet that consists exclusively of milk. In this population, there is already strong evidence that probiotic supplementation prevents necrotizing enterocolitis (348). In a large randomised controlled trial in India, Panigrahi et al found that *Lactobacillus plantarum* plus fructo-oligosaccharide (FOS) could reduce infection related adverse outcomes in infants (349). However, it should be noted that this intervention was not a simple probiotic, but a symbiotic:

a probiotic strain combined with a prebiotic (FOS in this case) that is meant to promote the growth of the probiotic strain. Therefore, it is difficult to untangle the effects of the probiotic from the effect of the prebiotic. The prebiotic itself could be the more successful part of this intervention since a wider range of endogenous commensal bacterial could be promoted by providing a substrate for their growth, not just the probiotic strain.

A prebiotic is defined by Gibson and Roberfroid as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health." (350) However, in the settings of nutritional deprivation, including processed foods consumed in a standard western diet, the fibre consumed is completely digested in the proximal gut and therefore does not reach the distal colon where beneficial bacteria are most abundant, and their metabolites are produced. For instance, a diet rich in resistant starch found in cooked and cooled potato or rice, plantain, maize and legumes will resist digestion and reach the distal colon where resident bacteria can utilise this starch as a substrate for the biosynthesis of SCFA (351). SCFA in turn contribute to infection prevention by contributing to the colonic epithelium barrier function, dampening inflammation and maintaining and acidic and anaerobic local environment which is able to suppress the growth of disease-causing pathogens (62).

7.3.2 Autologous faecal transplantation

Thus far, the only intervention proven to reliably restore gut microbiota to a profile found in health is FMT. This has been demonstrated by a durable increase in microbial diversity found following allogenic donor faecal transplantation in multiple clinical trials investigating donor FMT for the treatment of CDI (183), ulcerative colitis (276) and metabolic syndrome (352). Although less well studied than donor FMT, microbiome reconstitution may be more effective when the transplant is made from the recipients own stool (ie autologous) rather than from an unrelated donor. Theoretically, the microbiota in autologous transplants are already ideally adapted to the host recipient, a property which should allow for optimal engraftment. Not only is autologous FMT (aFMT) likely to be highly efficacious, given that this approach has none of the safety concerns that are inherent to donor stool, this type of intervention is well positioned to become a routine therapeutic option in the future.

There is already evidence that aFMT is very effective in restoring microbiome composition following perturbation. Suez et al's landmark study that showed that probiotics delayed the

normal reconstitution of gut microbiota after antibiotic use also examined the effect of aFMT on gut microbiome reconstitution (45). A subgroup of volunteers in this study received aFMT following antibiotic use. These participants were significantly more likely to achieve rapid and sustained early reconstitution of their microbiomes, occurring as early as one day post aFMT, in contrast with the several weeks required for spontaneous recovery to occur (45). Taur et al also showed that aFMT is highly effective in reconstituting gut microbiota in patients who receive broad-spectrum antibiotics following immunosuppression for allogenic haematopoietic stem cell transplantation (allo-HSCT) (181). In contrast, Bulow et al, found no significant difference in microbiome recovery of healthy volunteers who took amoxicillin-clavulanate and either received aFMT or saline enema (92). However, the impact of amoxicillin-clavulanate appeared to be relatively mild with only few subjects experiencing major taxonomic changes at the phylum level. It appears that when antibiotic impacts are relatively mild, spontaneous microbiome reconstitution is rapid and other interventions are not required.

Autologous FMT is ideally suited to patients who have intact microbiota at baseline but will be undergoing procedures or therapies that are likely to cause significant microbiome disruption. Examples include patients undergoing allo-HSCT or other chemotherapy, patients undergoing solid-organ transplantation and those having certain elective abdominal surgical procedures. For these patients, stool could be collected, processed into FMT and stored in stool banks ahead of commencing therapy. Those receiving microbiome-disrupting therapies, such as empirical broad-spectrum antibiotics for febrile neutropaenia, could receive aFMT following completion of antibiotics. Already, aFMT is being trialled to see if it can prevent adverse outcomes, including MRO colonisation and infection in at risk populations, including in those receiving allo-HCST (ClinicalTrials.gov identifier: NCT02269150) patients with other haematological malignancy (ClinicalTrials.gov identifier: NCT02928523) or in long-term care residents (ClinicalTrials.gov identifier: NCT03061097). However, since only small numbers of patients have been recruited so far in these trials, it is likely that larger clinical trials will be required in order to assess the benefit of aFMT. Given the low risks involved with this type of intervention and the likely benefits, research efforts into this area should be increased and stool bank infrastructure that make autologous transplants feasible should be supported.

7.3.3 Donor FMT to reduce pathogen colonisation and sepsis risk

Although aFMT may be an therapeutic option in the future, it is not appropriate for those patients who have an abnormal microbiome composition at baseline or are already colonised with pathogens, and it will only be available to a minority of patients who are able to store their stool prior to undergoing microbiome-disrupting intervention. For these reasons, donor FMT will continue to be the main therapeutic option for microbiome reconstitution being investigated in patients with MRO colonisation and at high risk of sepsis. Given the scarcity of high-quality evidence that FMT is an effective intervention for this indication it is imperative that randomised controlled clinical trials, such as the one we have initiated, are performed to answer this important question. However, given the lack of standardisation of FMT donors, processing and delivery it is difficult for results from interventional studies to be compared. In this context, efforts to optimise and standardise the production of FMT for use in non-CDI conditions need to move forward.

7.3.3.1 Optimising donor selection

Donor-specific differences in clinical outcomes, also referred to as the super-donor effect, have been observed in the context of FMT used in inflammatory bowel disease (197, 198). In these cases, the donor effects were associated with bacterial richness (a measure of diversity) (198) and increased abundances of anaerobic bacteria in the Ruminococcaceae and Lachnospiraceae families (197), with prolonged remission linked to increased butyrate-producing ability after FMT (198).

However, in order to truly select the best possible donors, there must be a greater understanding of what donor characteristics are in fact most likely to be beneficial. This will require further research that examines FMT microbiome composition from every donor and correlates this with efficacy. Unfortunately, this type of analysis does not occur routinely even in the context of clinical trials. In the interim, some educated guesses can be made about characteristics of donor microbiomes that optimise efficacy in reducing pathogen colonisation.

Assessing donor microbiomes for α-diversity is an initial consideration. Reduced faecal bacterial diversity is associated with an increased risk of pathogen colonisation and systemic infection (139), suggesting this factor should be considered when assessing donor material. However, bacterial diversity is likely to be less important as an indicator of a beneficial microbiome, than as an indicator of the probability of transplanted material containing specific microbial traits. In particular, the presence of beneficial keystone taxa can have a

disproportionate influence on microbial community structure and function (353). Different short chain fatty acids (SCFA) interact with different host receptors to regulate host physiology, and therefore a microbiome's ability to biosynthesize a full range of SCFA is essential for health (55). Production pathways for propionate, acetate and lactate are widely distributed among bacterial groups and are likely to be found in all FMT donors. In contrast, a smaller subset of organisms, dominated by *Faecalibacterium prausnitzii*, *Eubacterium rectale*, *Eubacterium hallii*, *Roseburia spp* and *Anaerostipes hadrus* are responsible for the production of butyrate (54). As reviewed in Chapter 1, butyrate in particular seems to play a key role (through interactions with host epithelial cells) in maintaining intraluminal conditions that suppress pathogen expansion (62, 239). Therefore, screening donors for a high abundance of butyrate-producing taxa may be particularly important. Other key taxa important to colonisation resistance might include those with the ability to transform primary bile acids into secondary bile acids, a property associated with the inhibition of *C. difficile* (53, 354) or that produce endogenous antibacterial substances, such as lantibiotics that inhibit gram-positive pathogens including VRE (355).

However, it is important not to over-estimate the beneficial properties of individual commensal taxa. Microbes in diverse communities, such as the gut, have evolved together and developed complex interrelationships that we are only just beginning to understand (356). While we are still relatively naïve about how commensals interact with each other to provide benefit, high bacterial diversity is likely to be the best surrogate marker of normal microbiota functionality.

7.3.3.2 Considering the ratio of obligate anaerobes to facultative anaerobes

In healthy individuals, bacterial gut microbiomes are typically dominated by obligate anaerobic bacteria of the Bacteroidetes and Firmicutes phyla (26). In contrast, facultative anaerobes that can behave as commensals or human pathogens (Enterobacteriaceae, Enterococcaceae) are typically found in low abundances (<1%) (44).

Mounting evidence suggests that the high relative abundances or "blooms" of facultative anaerobes in the gut, particularly those from the phylum Proteobacteria in the Enterobacteriaceae family (such as *E. coli* or *Klebsiella sp.*) are associated with inflammatory states and sepsis (27). Disruption of the gut microbiota results in increased availability of oxygen and nitrate in the gut lumen, which facultative anaerobes are able to take advantage of to expand rapidly (61). By stimulating host immune responses, Enterobacteriaceae also contribute to changes in the gut luminal environment that further support their growth (27, 357, 358).

It is not only Enterobacteriaceae that are capable of driving inflammation and infection. Evidence from both murine models and observational human studies show that antibiotic treatment enables enterococcal blooms in the gut and that this numerical domination of the microbiome by enterococci precedes blood-stream infection (38, 359). It may therefore important to consider in FMT material whether the composition of the donor microbiota reflects the ratio of obligate anaerobes to facultative anaerobes that are observed in healthy people and avoid using donor material if the microbiome composition is dominated by facultative anaerobic bacteria with pathogenic potential.

7.3.3.3 FMT processing to preserve microbiome composition and function

FMT processing steps, particularly blending in aerobic conditions, result in profound changes in the final viable microbiota composition that are not dissimilar to that associated with exposure to antibiotics (44). As demonstrated by the work presented in this thesis (Chapter 5) and published (309), there is an overall loss of bacterial diversity, a relative enrichment for aero-tolerant Proteobacteria, and in most donors the complete loss of keystone obligate anaerobic commensals such as *Faecalibacterium prausnitzii* and *Anaerostipes hadrus* (309). Processing in aerobic conditions significantly reduces the microbiota's capacity for butyrate biosynthesis (309), an effect that is important, given the role of butyrate in reducing colonic inflammation, maintaining colonic epithelial function and preventing pathogen expansion (55, 62). In contrast, although freezing reduces overall bacterial viability, it does not affect the overall bacterial diversity or composition of the stool (309). Furthermore, the ability to freeze samples is important in providing an opportunity to perform pathogen screening and characterisation of the wider faecal microbiota. Although further evidence is required to show that processing methodologies alter the efficacy of FMT, results from a recent clinical trial suggest that clinical efficacy of FMT for ulcerative colitis could be improved by anaerobic processing to preserve butyrate-producing bacteria (178).

7.3.3.4 Modifying donor microbiota to reduce sepsis risk

The recruitment of suitable donors for FMT is a major challenge. Already stringent screening criteria mean that only 10-15% of screened donors are ultimately eligible for stool donation (360). The exclusion of donors with undesirable microbiota composition could result in a substantial further reduction in the donor pool. However, while inappropriate processing practices can modify the composition of the viable stool microbiota in a detrimental manner,

modification of donated stool to improve its microbiological characteristics might allow material to be used from a wider donor group.

Antibiotic exposure is broadly considered to be associated with changes in microbiota composition that is harmful. This is in part because certain antibiotics, for example oral vancomycin or metronidazole used in the treatment of CDI, have a significant impact on anaerobic commensals (343, 361). However, there are antibiotic options that could be employed *in vitro* to selectively deplete viable Proteobacteria from faecal transplant preparations, while increasing the relative abundance of commensal anaerobes. For example, trimethoprim, fosfomycin and nitrofurantoin, typically used to treat gram-negative UTI, have little appreciable anaerobic activity (82, 321, 362). Use of such antibiotics could leave anaerobic commensals largely intact while simultaneously reducing facultative anaerobes. In the case of nitrofurantoin, this treatment could have the added benefit of increasing the relative abundance of the beneficial commensal *F. prausnitzii* (82).

However, such an approach has several major disadvantages. Firstly, if the donor harbours a pathogen with intrinsic or acquired antibiotic resistance to the antibiotic used, this approach could inadvertently increase the risk of this strain being transmitted and causing infection by reducing competition from similar bacteria. Secondly, the use of antibiotics could increase the probability of *de-novo* antibiotic resistance arising in both commensal and pathogen populations. Finally, this approach would reduce the overall bacterial diversity, and perhaps disrupt the overall balance of microbes that were present in the donor microbiome, causing expansion of other bacteria in unpredictable ways.

An alternative to using antibiotics could be to add laboratory cultured bacterial strains or bacteriophages known to have specific antimicrobial effects to transplant material. For example, in the case of a patient receiving FMT who is known to be colonised with VRE, the addition of specific bacteria, bacteriophages or bacteriocins associated with VRE clearance could potentially reduce carriage of this pathogen and reduce the chance of future infections. In principle support for this approach comes from studies using murine models, where carriage of *Barnsiella* has been shown to be protective against VRE colonisation (363), while lantibiotic-producing commensal strains such as *Blautia producta* are able to clear this pathogen from the intestine (355, 364). This is further supported by evidence in that lantibiotic gene abundance correlates with low *Enterococcus faecium* carriage in the stool microbiota of patients at high risk of VRE colonisation (355).

The addition of growth substrates to faecal transplant slurries that provide a relative selective advantage for commensal anaerobes *in vivo* should also be considered. The availability of fibre

is an important mediator of colon mucosal health (55). In order to produce SCFA, not only must the right bacteria be present, but they must also have a suitable fibre substrate readily available (55). In a murine model, the relative absence of fibre was shown to alter microbiome composition within hours, increasing the abundance of mucin degrading bacteria that reduce colonic mucus and facilitate pathogen translocation (365). Therefore, adding fibre to the FMT product could be a strategy to improve short-term colonic epithelial barrier function. *In vitro* studies have shown, for example, that the presence of resistant starches promotes the relative abundance of important commensal taxa, as well as increasing the production of beneficial SCFAs (366).

7.3.4 Bacterial consortia to replace FMT

There is growing interest in the use of bacteriotherapies as a stool substitute. These bacteriotherapies involve using either live cultures of defined bacteria or the use of spores from defined bacteria derived from human stool and several have been used as an investigational therapy for recurrent *C. difficile* infection (CDI). One of the earliest reports of bacteriotherapy use for CDI was in 1989 when Tvede and Rask-Madsen reported the cure of 6 patients with CDI after they were administered a consortium of 10 bacterial strains (194). A follow-up case series showed a 64% response rate following this bacteriotherapy treatment (defined as no diarrhoea within 30 days of treatment) (367), but evidence from a controlled trial is lacking. In 2013 Petrof et al reported the cure of two patients with CDI following infusion of a bacterial consortium of 33 isolates (MET-1) from a healthy stool donor (368), however a clinical trial investigating this product was never completed (ClinicalTrials.gov ID: NCT01372943). Similarly, RBX2660 is a microbiota-based drug sourced from live human-derived microbes. In Phase 2 clinical trials, RBX2660 has been shown to reduce r-CDI as compared to placebo (369). Another microbiome-based therapeutic, SER-109, composed of donor-derived bacterial spores has been linked with reduced rates CDI recurrence, but only when early engraftment has occurred (370).

Only one of these bacteriotherapies has been reported to be efficacious in pathogen de-colonisation. The microbiome analysis of participants receiving RBX2660 showed that responder microbiomes came to resemble the RBX2660 product with increasing levels of Clostridia and Bacteroidetes and decreased Proteobacteria (371), a result consistent with a product able to reduce the carriage of gram-negative pathogens as well as VRE. In a published case series of participants receiving RBX2660 for recurrent CDI, of the 10 participants who tested positive for VRE during the study, 6 of 10 cleared the pathogen from

stool at the 6-month follow-up (257). However, it is very difficult to draw any firm conclusions from this report. A crucial weakness is that the study did not include a control group. Furthermore, culture based VRE testing is known to have variable sensitivity with reports of fluctuating positive tests over time making it difficult to interpret rates of clearance (372).

Compared to FMT, stool-substitute bacteriotherapy has the marked advantage of being a standardised and reproducible product with far fewer safety concerns. This type of product is far more attractive to both manufacturers and regulators than inherently complex and variable stool donations. However, although studies on live bacterial consortia have thus far suggested that they are likely to be more efficacious than placebo for CDI, overall efficacy rates have been disappointing and do not approach the impressive rates of cure achieved with whole stool faecal transplantation (196). This means that the currently available bacterial consortia will also likely fall short in achieving the more substantially more difficult aim of reducing colonisation with pathogens in the gut other than *C. difficile*.

7.3.5 Therapeutic use of microbial products

The gut microbiota produces a vast number of proteins and metabolites which could be used as or targeted by novel therapeutics. These types of therapeutics could potentially bypass the need to maintain populations of specific microbes in the gut and directly harness selected beneficial properties of microbes to treat specific conditions. Although this field of therapeutics is currently in its infancy, there are already multiple such therapeutics in development (373).

Given the evidence that SCFA, the abundant microbial by-products of fibre fermentation, are crucial in regulating multiple aspects of host physiology (55), it is not surprising that SCFAs were one of the first bacterial metabolic products considered for therapeutic use. Although the link between SCFA and pathogen suppression has been made in recent years, the direct use of SCFA in the distal gut has not been trialled in this context likely due to earlier disappointing results of using this type of therapy for ulcerative colitis.

In the 1990s there was great interest in using SCFAs in ulcerative colitis therapy, given the known links between the absence of SCFA in the colon and the development of inflammatory colitis (374). Steinhart et al trialled butyrate enemas in patients with ulcerative colitis and found no therapeutic effect (375). Two other randomised controlled trials of SCFA rectal irrigation for ulcerative colitis were similarly disappointing (376, 377), with the authors commenting that prolonged contact with rectal mucosa seemed to be required for efficacy

(376). Therefore, it could be that administration of SCFA via enema is unlikely to be beneficial because this mode of delivery cannot consistently deliver the same amount of SCFA to the colonic mucosa that resident microbiota can produce locally from the fermentation of fibre.

There are multiple other therapeutic avenues being explored as a way to selectively target pathogens or pathobionts in the gut microbiota. For instance, polysaccharide A, a bacterial capsular protein expressed on *Bacteroides fragilis,* inhibits colitis induced by the pathobiont *Helicobacter hepaticus* and is capable of supressing host pro-inflammatory pathways in a murine experimental colitis model (378). This molecule has been commercialised and is being investigated as a therapy for inflammatory bowel disease and multiple sclerosis (373). In another example, antagonists of Fibrial adhesin (FimH) are currently being investigated as a therapy to block the binding of adherent-invasive *E. coli* to epithelial receptors in the gut (ClinicalTrials.gov Identified: NCT03709628) in an attempt to limit the pathogenic role that this bacterium plays in driving inflammation in some patients with inflammatory bowel disease. Interestingly, as uropathogenic *E. coli* also use the FimH to adhere to the bladder urothelium, these same antagonists could prove to be useful in treating urinary tract infection (379). KB109 (a glycan whose purported mechanism of action is not disclosed) is another small molecule microbiome based therapeutic currently being investigated as a therapeutic to reduce pathogen carriage. It is being trialled as a therapeutic to reduce gastrointestinal carriage of multi-resistant organisms (ClinicalTrials.gov Identifier: NCT03944369).

If one thing is clear, it is that individual species of bacteria cannot be beneficial on their own, they require an entire ecosystem that supports their ability to thrive and produce benefit (356). This includes surrounding organisms that are symbionts, a tolerant host immune system and appropriate conditions for growth such as an anaerobic environment and appropriate nutrition. Our ability to produce microbiome-based therapies will be greatly improved if we are first able to have a greater understanding of how complex communities of bacteria interact with each other and their host to produce benefit. To achieve this more basic research must be done on the relationship between human microbiomes and disease states and careful dissection of how interventions that alter microbiomes – such as FMT- also alter host physiology and disease manifestations.

7.4 Addressing knowledge gaps

7.4.1 Improving our knowledge of gut commensal microbiology and microbial interactions

Despite the major advances in sequencing technology that have allowed us to identify a wide variety of previously unknown microbial species that inhabit the human GI tract, we are far from being able to reliably identify all these species. Although many types of organisms make up the human microbiome, it is mainly bacteria that are characterised in microbiome analysis (due to the expense and technological barriers involved in characterising other microbes). It is safe to say we are only just beginning to understand this immensely complex system which is unique to each of us. It is important to learn more about commensal microbes and which of their numerous gene products influence human physiology and the interactions between each other. Future research should focus not only on the role of individual microorganisms but on groups of micro-organisms working as a functional unit. By doing this we will better understand the functional redundancy of microbial groups that explains how individuals with vastly different microbiome compositions can have microbiomes that are functionally very similar (33).

Our ability to precisely characterise the bacterial composition of microbiomes is currently limited by incomplete databases containing only a few strains of difficult to culture commensal bacteria. It is now recognised that several different strains of bacterial species can co-exist in the same person (352). The ability to define the microbiome composition as precisely as possible is important since even differences at a strain level may manifest as different phenotypes that can result in functional changes to the microbiome. For instance, the phenotypes of commensal *E. coli* strains vary so greatly from one another that some strains are considered deadly pathogens while other are sold as probiotics (30). In order for optimal genomic based analysis to occur, the databases must be enhanced with the whole genome sequences of pure bacterial strains grown in culture (380). Considerable progress towards achieving this goal has been made through advances in bacterial culturing methods that have allowed many strains of bacteria previously considered unculturable to be cultured and subsequently sequenced (20).

The picture becomes even more complex when we consider that microbial metagenomes not only consists of chromosomal bacterial genes but also genes encoded by mobile genetic elements that are carried on plasmids, transposons and bacteriophages that transfer easily between bacteria and therefore must be considered separately to other genes. This is before even considering including other commensal microorganisms such as viruses, fungi and eukaryotes in microbiome analysis. However, we should expect that steady progress in this

area will mean substantially better databases will be available in the future that will markedly improve our ability to define microbial composition. In order for this progress to produce the greatest benefit, these improved databases need to be accessible to all researchers.

7.4.2 Understanding microbiome disruption caused by cancer chemotherapy and critical care intervention

In this thesis, two observational studies were preformed to further explore the role of medical intervention on the gut microbiota. In the first study presented in Chapter 2, the effect of myelosuppressive chemotherapy on gut microbiota was explored. In this study we attempted to isolate the effect of chemotherapy on the gut microbiota by excluding patients with confounding exposures from the analysis. In the second study, presented in Chapter 3, the broad impact of receiving critical care interventions in an intensive care unit was studied. These exposures, although substantially different, both resulted in temporal instability of the gut microbiota and altered microbiome composition in a way that promotes the expansion of facultative anaerobe populations within the gut. In the case of patients receiving chemotherapy in the absence of antibiotic therapy, the preservation of a high diversity of obligate anaerobic taxa in the gut microbiota of these patients will likely serve to mitigate the risk of pathogen expansion and preserve the overall functionality of the gut microbiome. Furthermore, the observed changes in microbial composition seen during chemotherapy may reflect the translocation of bacteria (as shown in Vetizou et al's murine model (130)) that play a role in driving host anti-tumour immune responses. Although, the change in bacterial composition is likely to contribute to increased risk of infection, if these changes are linked to the efficacy of chemotherapy then the changes to gut microbiology during chemotherapy may in fact prove to be on balance more beneficial than harmful.

In contrast to the chemotherapy cohort, the critically ill population experienced very substantial losses in microbial diversity, with severe dysbiosis characterised by intestinal domination by a single taxon are observed in the majority of participants. The microbiomes of individual ICU patients observed over time underwent radical changes in composition, such that their microbiomes upon discharge from ICU were as different from their own baseline sample as from a different person.

These two studies are a glimpse into how medical therapies affect microbiomes in real-life clinical contexts. They form the basis for further research which needs to answer the important question of whether these microbiome changes alter clinical outcomes.

Filling these substantial knowledge gaps will require many more studies to be conducted so that the effects of medical interventions and therapies on the commensal microbiomes of patients can be evaluated. Most importantly, theses microbiome changes must be prospectively linked with outcomes including pathogen carriage, antimicrobial resistance acquisition and the development of infection. Already, prospective examination of microbiome data in patients with haematological malignancy has led to a greater understanding of how microbiome composition relates to sepsis (139, 381) and how particular bacteria and their microbial products contribute to colonisation resistance against specific pathogens (53, 56, 355). However, relatively little is known about how microbiome changes affect outcomes in other populations.

To further contribute to knowledge in this area, the preliminary exploratory observational studies which were conducted as part of this thesis have received external funding and are being expanded. We will continue to prospectively study the gut microbiota of patients with solid organ malignancy before and after chemotherapy and link microbiome changes to outcomes including febrile neutropaenia, sepsis and bacteraemia. Similarly, the MOCI study examining the gut and tracheal aspirate microbiomes of critically ill patients has been expanded to include a second intensive care unit and will seek to link microbiomes to nosocomial sepsis and ventilator-associated pneumonia in these patients.

7.4.3 Expanding our understanding of FMT as a therapeutic intervention

Although FMT is highly efficacious in treating r-CDI, presumably by re-establishing colonisation resistance against *C. difficile*, the exact mechanisms that produce a clinical effect are not completely evident. For instance, the degree of engraftment of microbiota from the donor to the recipient does not seem to predict clinical outcomes (186) suggesting that the effects of specific bacteria may be more important. Although particular bacteria in the genus *Clostridium* with the ability to convert primary bile acids to secondary bile acids are able to inhibit *C. difficile* growth *in vitro* (354), no one has yet demonstrated that transplantation of these species alone is sufficient to cure r-CDI. The reason why whole stool FMT remains more effective than the transplantation of select bacteria, or even a large variety of spore forming bacteria remains elusive (196).

FMT remains a powerful tool in examining how altering microbiota affects clinical outcomes in a variety of disorders, including in how microbiome composition can alter our susceptibility to non-CDI infection. So far, some of the strongest evidence supporting FMT as an effective intervention in preventing future infections comes from r-CDI FMT trials

where outcomes related to antibiotic resistance carriage (211, 382) or non-CDI infectious outcomes (212, 383) have been examined retrospectively. Nonetheless, the evidence of benefit could be much stronger if these trials were specifically designed to test these outcomes.

However, it is not only further trials examining the clinical benefits of FMT in infection prevention that are required to move forward. Drawing the correct conclusions about why FMT is successful and has durable effects in some people but not in others requires much more than the mere assessment of clinical outcomes. Since FMT material is inherently highly variable due to differences in donor microbiota and processing methodologies, it is important to precisely define the microbial content of donor FMT material used in clinical trials. In depth analysis of donor FMT material should become routine as it is not only essential for understanding the mechanisms behind FMT's efficacy, but also because this type of analysis could be used to improve the safety of FMT (180).

The PMA-based methodology developed as part of this thesis (described in Chapter 4) makes an important contribution to this effort by enabling the viability of bacteria in FMT material to be determined. It allows investigators to establish if a lack of viability of bacteria in the FMT material explains why certain species that appear to be highly abundant in donors fail to engraft in recipients. This type of analysis could steer researchers away from falsely attributing non-engraftment of micro-organisms (that have been shown to be non-viable in donor material) to an incompatibility between donor microbiota and the recipient's gut environment. Once the donor material has been precisely defined, it becomes possible to better interpret microbiome changes in the recipients, and to reproduce these effects in the future. Therefore, this type of analysis of donor material should be used routinely in future clinical trials using FMT.

The other type of analysis that is essential to perform in FMT clinical trials is a longitudinal examination of the recipient's microbiota that is linked to clinical data at the same time points. Such analysis, similar to that performed by Li et al (352), can give us invaluable insights into the persistence of donor microbiota in recipients and how this relates to clinical outcomes in the trial. In the design of the clinical trial assessing FMT in people with recurrent gram-negative infections (detailed in Chapter 6), the participant's longitudinally collected samples are matched with data on urine infections occurring concurrently. This will allow us to not only gain insight into the efficacy of FMT in preventing recurrent UTI, but also into whether microbiome composition predicts such infections occurring. Clinical trials which

combine FMT intervention with rigorous analysis of the relationship between participant microbiomes and outcomes will be critical in determining the future role of FMT.

7.5 Conclusion

Our understanding of the contribution made by gut microbiota in the development of infection is rapidly expanding. This is a journey of understanding the causes of microbiome disruption, how microbiomes influence infection risk, and investigating if interventions that reconstitute microbiomes can effectively mitigate the risk of infection developing.
This thesis contributes to knowledge at several points along this journey. The first part of the thesis focuses on the effect of medical interventions on human gut microbiota. This research documents how conventional chemotherapy and critical care interventions disrupt microbiomes, setting the stage for pathogen colonisation and infection. The second part of the thesis explores the role of FMT as an intervention to mitigate the risk of bacterial infection arising from the gut. The application of PMA methodology to stool slurries improved our existing capacity to analyse the viable microbial content of FMT, thus allowing us to optimise FMT processing methods. The thesis documents the initiation of a clinical trial into the effects of FMT on recurrent bacterial infection, a crucial step towards determining if FMT will play a role in future clinical practice. Finally, in the discussion sections of this thesis, I offer an overview of how our current knowledge can be applied now to improve antibiotic stewardship and the key areas for future research required to move forward.

APPENDICES

Appendix 1: Faecal sample pre-processing and DNA Extraction

1.1 Pre-Processing: Faecal samples in Norgen tubes

1. Thaw samples and record relevant patient information
2. Weigh an empty 2 ml flip top tube.
3. Vortex sample well.
4. Using a wide bore tip, aliquot 2 ml of sample from the Norgen tube into pre-weighed tube.
    a. Usually the Norgen tube will contain buffer and it is easy to transfer. In other instances, the faecal matter is solid. If this is the case, using a sterile toothpick or tip, scoop out 0.15 g to 0.2 g of faeces into the tube and add 500 µL of PBS. Vortex well.
5. Spin down sample at 13,000 rpm for 20 minutes at 4°C.
6. Transfer supernatant to a 2 ml labelled screw cap tube. Store at -80°C
7. Weigh wet pellet and calculate the pellet weight.
8. Proceed to DNA Extraction from Faecal Sample - Qiagen PowerLyzer PowerSoil Kit Protocol (Appendix protocol 1.3)

1.2 MOCI Study stool swab and rectal swab

1. Thaw swab stored in 500µL 20% glycerol-saline solution and vortex.
2. Using sterile forceps, remove swab and transfer to 500µL of TE buffer.
3. Vortex for 10 sec.
4. Remove TE buffer solution from swab by centrifugation
    a. Take plunger out of a sterile 3mL syringe
    b. Using forceps, transfer swab to syringe barrel
    c. Paraffin wrap the bottom of syringe to the top of the 2 mL screw capped tube
    d. Put syringe with swab + tube in a 50mL eppendorf tube and screw on 50 mL cap
    e. Centrifuge 50mL tube in large centrifuge at max for 5 min at 3374G
    f. Dispose of syringe + swab and keep 2 mL tube
    g. Transfer the remaining TE buffer solution to the original 500 µL of TE solution.
5. Aliquot 250 µL into the Qiagen bead beating tube and continue with the Qiagen Powerlyser Powersoil protocol by adding 750 µL of Bead Solution.

1.3 PMA treatment pre-processing for faecal slurry specimens (Chapters 4 and 5)

Bring the following into the anaerobic chamber:

- 20mM Propidium monoazide (PMA)
- One mg of PMA dye (Biotium Inc, Hayward CA, USA) is dissolved in 1mL of 20% dimethyl sulfoxide. The reconstituted PMA is stored at 4^{o}C in a light-proof container.
- PBS or sterile Normal Saline (best kept in ANO2 chamber for days prior to use)
- LED lamp
- Vortex
- Pippettes for following tips:1000 uL wide bore tips, 200 uL tips and 20uL tips
- 1.5 uL Eppendorf tubes
- Fresh or frozen faecal slurry specimen (allow specimen to thaw in the anaerobic chamber to maintain viability of anaerobes before proceeding with dilution and PMA treatment) *Keep faecal slurry specimens anaerobic at all times*
- Heat kill a 1mL aliquot of FS by heating at 99^{o}C for 30 minutes on a heat block. Allow the specimen to cool to room temperature in the anaerobic chamber prior to using (this is your negative HK control specimen)
    - Note: If you want to use a positive control, use a freshly passed stool which is immediately processed into a FS inside the anaerobic chamber
    - Alternatively, if you are using an organism specific PCR, spike your stool with known quantities of live (from broth culture in exponential phase of growth) or dead (EtOH treated) bacteria to serve as your control.

PMA treatment of faecal slurry (FS) inside anaerobic chamber

1. For each FS (including the HK control) to be tested: Pre-label and Pre-fill 3 X 1.5 uL Eppendorf tubes with 10uL PMA in the darkened hood and cover tubes with foil, Pre-fill 3 X1.5 uL Eppendorf tubes with 10uL PBS alone and pre-fill 2 1.5 uL Eppendorf tubes with 900uL PBS for serial dilutions
2. Serial dilute FS in PBS or NS by adding 100 uL of neat FS to 900 uL PBS, vortexing to mix afterward.
3. Serially dilute twice until a 1/100 dilution is achieved. The diluted solution should be translucent.
4. Turn off lights, and work with distant low light, or with a red light (as in a dark room).
5. Briefly vortex 1/100 diluted FS
6. Aliquot 190uL of 1/100 diluted FS into each of 3 tubes pre-filled with 10uL PMA. Pipette mix each sample when adding and into each of 3 tubes pre-filled with PBS.
7. Repeat above steps (5 &6) for all additional FS samples being tested and the HK control specimen.
8. Vortex each tube briefly, cover with foil and incubate in the anaerobic chamber for a total of 30 minutes (vortex every 10 minutes during the incubation period) *Do not expose tubes to light during the incubation period*
9. Place all tubes into a clear plastic container (lid off) and place LED light (blue & white setting) ~15 cm above the samples
10. Agitate the tubes and change their position every 5 minutes during light exposure for a total of 20 minutes.
11. Tubes can now be exposed to general light and ambient air. Keep at -20C until extraction using Qiagen PowerLyzer Power Soil kit.
12. * Note: to interpret success of PMA treatment, using 16S rRNA PCR compare the amplification of PMA treated heat-killed FS (HK+PBS) to PMA-untreated heat-killed FS samples (you have prepared 3 replicates of each type of sample above). The HK+

PMA sample amplification should be at least 2 log lower than the HK (with no PMA treatment). Note that the amplification from HK+PMA is always higher than PBS negative controls but is usually within 1 log of this sample. I find that consistently fresh stool treated with PMA is within 1-log of the untreated sample translating to ~50% viability.

1.4 DNA extraction using the Qiagen* PowerLyzer PowerSoil kit

1. Add 750 µl of Bead Solution to the faecal pellet tube. Vortex suspension to mix.
2. Transfer suspension to the Glass Bead tube.
3. Check Solution C1 for precipitation.
4. Add 60 µl of Solution C1 to the mixture and vortex briefly.
5. Heat the tubes at 65ºC for 10 mins
6. Bead beat samples at a setting of 6.5 on the FastPrep for two pulses of 60 seconds
7. Centrifuge the tubes at 10,000 x g for 3 min
8. Transfer up to 500 µl of supernatant to a clean 2 ml Collection Tube.
9. Add 250 µl of Solution C2 and vortex briefly
10. Incubate at 4ºC for 10 mins
11. Centrifuge the tube at 10,000 x g for 3 min at room temperature
12. Transfer up to 600 µl of supernatant to a clean 2 ml Collection Tube
13. Add 200 µl of Solution C3 and vortex briefly.
14. Incubate at 4ºC for 10 mins
15. Centrifuge the tubes at room temperature for 3 min at 10,000 x g
16. Avoid the pellet, transfer up to 750 µl of supernatant into a clean 2 ml flip top tube
17. Shake to mix Solution C4 before use.
18. Add 1200 µl of Solution C4 to the supernatant and vortex for 5 seconds.
    a. Safe stopping point: the samples can be stored over night at 4°C
19. Load approximately 650 µl onto a Spin Filter and centrifuge at 10,000 x g for 1 min at room temperature.
20. Discard the flow through and add additional 650 µl of supernatant to the Spin Filter. Repeat centrifugation until all supernatant has been added to the Spin Filter.
21. Add 500 µl of Solution C5 and centrifuge at room temperature for 1 min at 10,000 x g
22. Discard the flow through
23. Centrifuge again at room temperature for 1 min at 10,000 x g
24. Carefully place Spin Filter in a clean 2 ml Collection Tube. Avoid splashing any of Solution C5 onto the Spin Filter
25. Add 50 µl of UltraPure RNAse DNAse water onto the filter.
26. Incubate at room temperature for 5 minutes.
27. Centrifuge at room temperature for 1 min at 10,000 x g
28. Repeat steps 25-27.
29. Store DNA at -20ºC

*previously MOBIO

Appendix 2. Primers and Probes used

Primer /Probe	Sequence (5' to 3')	Target	Annealing Temp (ºC)	Reference
qB1114F	CGGCAACGAGCGCAACCC	*Bacterial 16S rRNA gene*	60	(269)
qB1275R	CCATTGTAGCACGTGTGTAGCC			
Aput_F	TTGTACGAGGGTAAACGCAGA	*Alistipes putredenis*	65	(384)
Aput_R	CAGTATTAGAGGCACGTTCAGG			
SSC2F2	CTTTAGTAGCCAGCATATAAGG	*Anaerostipes hadrus*	60	(292)
SSC2R	TTGCTCACTCTCACGAGGCT			
Bac303-F	GAAGGTCCCCCACATTG	*Bacteroides/Prevotella*	60	(385)
Bfr-Fm-R	CGCKACTTGGCTGGTTCAG			
Bifspp-F	TCGCGTCYGGTGTGAAAG	*Bifidobacterium spp*	58	(386)
Bifspp-R	CCACATCCAGCRTCCAC			
BCoATscrF	GCIGAICATTTCACITGGAAYWSITGGCAYATG	butyryl-CoA-CoA transferase gene	53	(387)
BCoATscrF	CCTGCCTTTGCAATRTCIACRAANGC			
FPR-2F	GGAGGAAGAAGGTCTTCGG	*Faecalibacterium prausnitzii*	60	(385)
Fprau645R	AATTCCGCCTACCTCTGCACT			
EhalF	GCGTAGGTGGCAGTGCAA	*Eubacterium hallii*	60	(385)
EhalR	GCACCGRAGCCTATACGG			
E.coli-F	CATGCCGCGTGTATGAAGAA	*Escherichia coli*	60	(388)
E.coli-R	CGGGTAACGTCAATGAGCAAA			
E.coli-P	TATTAACTTTACTCCCTTCCTCCCCGCTGAA			
oprL-F	CGAGTACAACATGGCTCTGG	*Pseudomonas aeruginosa opr* gene	60	(270)
oprL-R	ACCGGACGCTCTTTACCATA			
RrecF	GCGGTRCGGCAAGTCTGA	*Roseburia spp* / *Eubacterium rectale*	63	(385)
Rrec630mR	CCTCCGACACTCTAGTMCGAC			
Nuc-F	AAATTACATAAAGAACCTGCGACA	*S. aureus nuc* gene	57	(271)
Nuc-R	GAATGTCATTGGTTGACCTTTGTA			
Nuc-P	AATTTAACCGTATCACCATCAATCGCTTT			
SubFcl_2F	TGAAGTCCTTCGGGACATCGAG	*Subdoligranulum variable*	58	Appendix 3
SubFcl_4R	TCTCGCCAGAGTCCTCTTGC			

Appendix 3: Development of a *Subdoligranulum variable* PCR assay

A *Subdoligranulum variable* quantitative PCR using specific primers targeting the 16S rRNA gene was developed and validated as part of this study. Assay primer sequences are shown in Appendix 2 above. The assay was based on sybr green fluorophore chemistry and utilised primers at 500 nM concentration and 1uL of DNA in a total volume of 35 µL. The amplification cycling conditions were 1 cycle of 3 min at 95^{o}C followed by 40 cycles of 15sec at 95^{o}C, 15 sec at 58^{o}C and 1 min at 72^{o}C with data collection at this stage. Melt curve analysis was performed at 95^{o}C, 1 min at 60^{o}C and 15 sec at 95^{o}C. The assay was run on the QuantStudio 6 Real-Time PCR system, as described in Chapter 5 methodology.

Assay specificity was assessed *in silico* using NCBI Blast. *In vitro* it was confirmed that the assay did not amplify DNA extracted from the following bacteria: *Bacteroides ovatus, Bacteroides vulgatus, Bifidobacterium bifidum, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus dysgalacticae* or the from the closest phylogenetically related bacterium, *Faecalibacterium prausnitzii.* To further validate the specificity of the assay we tested the assay on 8 adult stool where Subdoligranulum was identified as being present through 16S rRNA gene amplicon sequencing and 6 infant stools where no Subdoligranulum was detected.

A 10-fold dilution series of DNA extracted from stool from a healthy faecal donor was used to generate the standard curve for qPCR. PCR produced product with a predicted size of 130 bp and identical peaks were obtained in the on-melt curve analysis from 8 individuals in which *Subdoligranulum* was identified on 16S rRNA gene amplicon sequencing and not in 6 infant stool samples in which *Subdoligranulum* was not identified on 16S rRNA gene amplicon sequencing.

Appendix 4: Individual donor (D1-D8) PMA-qPCR results

Table 4a: **Proportion of bacteria viable on specific qPCR assay after anaerobic processing**

PCR target	D1	D2	D3	D4	D5	D6	D7	D8	Median (IQR)	p-value vs. O_2	p-value vs. FT	p-value vs. HK
All Bacteria (16S rRNA gene)	0.47	0.337	0.599	0.517	0.181	1	0.389	0.545	0.494 (0.350-0.586)	0.0068	0.027	0.0025
Alistipes putredenis	1	0.35	NA	NA	0.244	1	1	1	1.000 (0.324 - 1.00)	0.1875	0.5625	0.0313
Bacteroides spp	0.969	0.27	1	0.481	0.677	0.76	0.993	1	0.865 (0.530-0.998)	0.0078	0.8125	0.0078
Bifidobacterium spp	0.962	0.061	0.407	0.186	0.667	0.287	1	0.609	0.508 (0.211-0.888)	0.0078	0.25	0.0078
Escherichia coli	NA[1]	NA	NA	NA	0.304	NA	NA	NA	NA	NA	NA	NA
Anaerostipes hadrus	0.068	0.048	0.373	0.255	0.044	0.46	0.524	0.922	0.314 (0.053-0.508)	0.0156	0.0781	0.0078
Eubacterium hallii	0.138	0.009	0.134	0.18	0.944	0.446	0.646	0.274	0.227 (0.135-0.596)	0.0156	0.0156	0.0078
Roseburia/Eubacterium rectale	0.256	0.025	0.546	0.076	0.098	0.188	0.581	1.274	0.222 (0.082-0.572)	0.1094	0.1484	0.0078
Faecalibacterium prausnitzii	0.012	0.004	NA	0.064	0.048	0.308	0.467	0.744	0.056 (0.006 - 0.427)	0.0469	0.1563	0.0156
Subdoligranulum variable	0.314	0.014	0.247	0.092	0.319	0.509	0.479	0.792	0.317 (0.131-0.502)	0.0078	0.0781	0.0078
butyryl-CoA:acetate CoA-transferase gene	0.112	0.019	0.165	0.031	0.117	0.213	0.185	0.328	0.141 (0.051-0.206)	0.0078	0.0391	0.0078

[1]NA= no quantifiable amplification, [2] O_2=ambient air processing, [3] FT= freeze-thawing, HK= heat-killing.

Supplemental Table 4b: **Proportion of bacteria viable on specific qPCR assay after ambient air processing**

PCR target	D1	D2	D 3	D4	D5	D6	D7	D8	Median (IQR)	p-value vs. ANO_2	FT	HK
All Bacteria (16S rRNA gene)	0.26	0.15	0.187	0.186	0.257	0.236	0.049	0.158	0.187 (0.152-0.252)	0.0068	0.3621	0.0006
Alistipes putredenis	0.304	0.318	NA	NA	0.587	0.695	0.301	1	0.453 (0.303-0.772)	0.1875	0.4375	0.0313
Bacteroides spp	0.489	0.058	0.048	0.089	0.67	0.612	0.16	0.671	0.324 (0.066-0.655)	0.0078	0.0391	0.0078
Bifidobacterium spp	0.306	0.044	0.014	0.006	0.126	0.065	0.037	0.069	0.0541 (0.020-0.112)	0.0078	0.0234	0.0078
Escherichia coli	NA	NA	NA	NA	0.403	NA	NA	NA	NA	NA	NA	NA
Anaerostipes hadrus	0.047	0.016	0.014	0	0.005	0.016	0.006	0.191	0.022 (0.006-0.048)	0.0156	0.0313	0.1094
Eubacterium hallii	0.13	0.015	0.026	0.021	0.065	0.001	0	0.042	0.023 (0.005-0.060)	0.0156	0.3125	0.0156
Roseburia/Eubacterium rectale	0.46	0.029	0.17	0.031	0.063	0.036	0.035	0.129	0.050 (0.0318-0.160)	0.1094	0.1484	0.0078
Faecalibacterium prausnitzii	0.021	0.001	NA	0	0.037	0	0.003	0.014	0.002 (0.0001-0.05)	0.0469	0.0781	0.1563
Subdoligranulum variable	0.122	0.006	0.008	0.033	0.168	0.01	0.008	0.107	0.021 (0.008 - 0.118)	0.0078	0.25	0.0078
butyryl-CoA:acetate CoA-transferase gene	0.019	0.003	0.051	0.018	0.038	0.001	0.003	0.015	0.017 (0.003-0.033)	0.0078	0.1484	0.1484

[1]NA= no quantifiable amplification , [2] O_2=ambient air processing, [3] FT= freeze-thawing, HK= heat-killing

Supplemental Table 4c: **Proportion of bacteria viable on specific qPCR assay after freeze-thawing in anaerobic conditions**

PCR target	D1	D2	D3	D4	D5	D6	D7	D8	Median (IQR)	p-value vs. ANO_2	O_2	HK
All Bacteria (16S rRNA gene)	0.355	0.312	0.265	0.231	0.0878	0.088	0.171	0.344	0.248 (0.109-0.336)	0.027	0.3621	0.0031
Alistipes putredenis	0.891	1	NA	NA	0.3679	0.314	0.725	0.631	0.678 (0.354-0.918)	0.5625	0.4375	0.0313
Bacteroides spp	0.326	0.969	0.648	0.89	1	0.473	1	1	0.930 (0.517-1.00)	0.8125	0.0391	0.0078
Bifidobacterium spp	1	0.035	0.062	0.07	0.5408	0.073	0.066	1	0.0714 (0.063-0.997)	0.25	0.0234	0.0078
Escherichia coli	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Anaerostipes hadrus	0.018	0.018	0.14	0.012	0.0687	0.005	0.653	0.421	0.047 (0.008-0.351)	0.0781	0.1094	0.0391
Eubacterium hallii	0.081	0.016	0.047	0.011	0.1886	0	0.077	0.081	0.062 (0.013-0.081)	0.0156	0.3125	0.0078
Roseburia/Eubacterium rectale	0.21	0.048	0.416	0.038	0.3645	0.049	0.175	0.318	0.193 (0.048-0.352)	0.1484	0.1484	0.0078
Faecalibacterium prausnitzii	0.056	0.009	NA	0.022	0.0187	0.014	0.039	0.173	0.020 (0.01-0.05)	0.1563	0.0781	0.0156
Subdoligranulum variable	0.314	0.026	0.231	0.061	0.117	0.034	0.198	0.047	0.089 (0.037-0.223)	0.0781	0.25	0.0078
butyryl-CoA:acetate CoA-transferase gene	0.029	0.0145	0.04	0.025	0.1884	0.009	0.036	0.014	0.027 (0.014-0.390)	0.0078	0.1484	0.0156

[1]NA= no quantifiable amplification, [2] O_2=ambient air processing, [3] FT= freeze-thawing, HK= heat-killing

Appendix 5: FMT donor and recipient screening

5.1: Faecal donor screening questionnaire

Date:
Name:
Date of birth:

Have you received antibiotic therapy in the last 6 months?	Y	N
If so, when?		
Have you travelled outside of Australia in the past 6 months?	Y	N
o If yes, where?		
Do you take medication? If so, please elaborate.	Y	N
Are you a smoker?	Y	N

Do you have a history of any of these medical conditions?

• Inflammatory bowel disease	Y	N
• Irritable bowel syndrome	Y	N
• Colonic polyps	Y	N
• Malignancy or cancer	Y	N
• Any other gastrointestinal disorder	Y	N
o If yes please elaborate		
• Obesity	Y	N
• High blood pressure	Y	N
• Diabetes	Y	N
• Heart disease	Y	N
• Stroke	Y	N
• Major depression	Y	N
• Infection with Hepatitis B or C, HIV or syphilis	Y	N
• Autoimmune disease (ie rheumatoid arthritis, SLE)	Y	N
• Atopic disease	Y	N
• Chronic pain syndrome or neurological disorder	Y	N

Do you have any other medical illnesses?	Y	N
o If yes please elaborate		
Have you had unprotected sexual intercourse in the last 1 month outside of a long term monogamous relationship?	Y	N
Have you had a tattoo or body piercing within the last 6 months?	Y	N
Do any household members have infective symptoms?	Y	N
Have you used intravenous illicit drugs?	Y	N
Have you been incarcerated in prison in the past?	Y	N

Signed:

5.2 Medical interview (exclusions)

- Age: <18 or >65
- Antimicrobial therapy or probiotics in the past 3 months
- Active medical illness or symptoms
- Any medications (other than oral contraceptive pill)
- International travel in last 6 months to areas at high risk of travellers' diarrhoea
- High risk sexual activity (unprotected sex in last 1 month outside of a monogamous relationship, men who have sex with men, sex for drugs or money)
- Illicit drug use
- Tattoo or body piercing within 6 months
- Known HIV or viral hepatitis exposure in the last 12 months
- Incarceration or a history of incarceration.
- Family history of colorectal carcinoma involving 2 or more first degree relatives
- Household members with active GI infection

5.3 Medical history and Examination (exclusions)

- Any gastrointestinal disorder
- Obesity (BMI>30), hypertension, type 2 diabetes and dyslipidaemia
- Malnutrition (BMI <18)
- Autoimmune disease
- Atopic disease
- Depression
- Infection with HIV, Syphilis, Hepatitis B or C
- Malignancy
- Chronic pain syndromes, neurologic or neurodevelopmental disorders

5.4 Blood screening

Blood testing will be performed at SA Pathology using assays validated and accredited for use diagnostic use by the National Association of Testing Authorities, Australia (NATA)

- Full blood count
- Electrolytes, Urea and Creatinine (renal impairment eGFR<60)
- Liver function tests (abnormal LFTs are exclusions)

- Human T-cell lymphotropic virus 1 and 2 serology (HTLV-1 and HTLV-2)
- Epstein Barr Virus (EBV) IgM and IgG
- Cytomegalovirus (CMV) IgM and IgG
- Syphilis (Treponemal serology)
- *Strongyloides stercoralis* serology
- *Helicobacter pylori* serology
- Toxoplasma serology
- Hepatitis A virus (HAV) IgM
- Hepatitis B virus (HBV) surface antigen, core antibody
- Hepatitis C (HCV) PCR
- Human immunodeficiency virus (HIV) PCR
- Antinuclear antibody (ANA)
- Fasting lipids and blood glucose level
- C-Reactive Protein (CRP)

5.5 Stool screening

Stool testing will be performed at SA Pathology using assays validated and accredited for use diagnostic use by the National Association of Testing Authorities, Australia (NATA).

- Bacterial pathogen multiplex PCR (including Salmonella, Shigella, Campylobacter and Shiga-toxin producing *E. coli*)
- Viral pathogen multiplex PCR (Rotavirus, Norovirus and Adenovirus)
- *C. difficile* toxin PCR
- Parasitic pathogens multiplex PCR (including *Cryptosporidium spp*., *Giardia spp*., and *Entamoeba histolytica* PCR)
- Stool culture for Vancomycin-resistant enterococci (VRE), Extended-spectrum beta-lacatamse (ESBL) and Carbapenem-resistant Enterobacteriaceae (CRE).
- PCR on rectal swab testing for chlamydia, gonorrhea and herpes simplex virus DNA

5.6 Documentation and tracing of donors

Each stool donor will be recorded in the study "stool donor register" document. This will include the donor's name, date of birth, address and contact details. It will also record the result of screening history, physical examination and blood and stool tests. Each donor will be assigned a donor number.

After collection each donation will be screened for known bacterial pathogens (and each donor will also be screened as outlined in the protocol for other illnesses), then a small amount of each individual donation will be set aside, labelled with a name and donor number, and frozen individually. This will allow repeat testing for some pathogens and tracing of each individual donation in the future in the event of possible transmission of infection.
Each stool aliquot will then be numbered and recorded in a secure and confidential document designated the "faecal transplant aliquot document" (to be viewed only by the study investigators). This will list the stool donor number that contributed to each aliquot. In this way any possible transmission of infection could be traced.

5.7 FMT recipient work-up

Detailed past medical history:

- Number and type of infections due to the RGNB
- Antibiotic use history (current and prior)
- Surgical history
- Hospitalisation
- Comorbid disease

Stool infection screen for VRE, ESBL, *C. difficile* toxin, molecular tests for enteric pathogens. HIV, HBV, HCV serology test prior to FMT. CMV serostatus is noted in solid organ transplant recipients. Participants are excluded from the trial if they have tested positive for a diarrheal pathogen until the infection has resolved or been treated. If patients have tested positive to VRE or *C. difficile* (without diarrhea) these results are noted as being positive prior to FMT administration. Serology is taken just prior to FMT administration to document serostatus prior to FMT and does not change eligibility for the trial.

Appendix 6: Study Manual for the MOCI Study

Study Manual

Microbiome and Outcomes in Critical Illness (The MOCI Study)

Table of Contents

1.Study Synopsis

Title	**Microbiome and Outcomes in Critical Illness (The MOCI Study)**
Principal investigator	Dr Lito Papanicolas
Protocol registration	
Study objectives	**Primary aim:** To determine effect of residency in the ICU on microbial communities in the gut and lung. **Secondary (exploratory) outcomes:** a) To determine the effect of residency in ICU on the acquisition of antimicrobial resistance genes in gut and respiratory microbiota. b) To determine the relationship between the microbial communities and antibiotic resistance carriage with the following outcomes: a. 30-day mortality b. Length of hospital stay c. Development of sepsis in ICU d. Ventilator associated pneumonia c) To determine effect of enteral nutrition composition on microbiome communities in the gut in critically ill adults d) To determine the feasibility of longitudinal collection of faecal and respiratory samples from patients admitted to the RAH intensive care unit (ICU) for microbiome analysis in order to establish the sample sizes required for conducting larger studies.
Participant population	Inclusion criteria 1. Any patients admitted to the Royal Adelaide Hospital or Flinders Medical Centre ICU 2. Age ≥18 3. Patient is receiving invasive mechanical ventilation (IMV) 4. Patient is predicted by intensivist to have length of stay (LOS) >3 days Exclusion criteria 1. Death is deemed to be imminent during this admission and either the attending doctor, patient or substitute decision maker is not committed to active treatment 2. Duration of admission is predicted to be less than 72 hrs. 3. Patient diagnosed with COVID-19 Withdrawal criteria 1.Unable to obtain specimen within 72hrs of enrolment
Study design	MOCI is an observational cohort study.

Treatment	N/A
Study procedures	Collection of stool samples (if passed), and tracheal aspirates in intubated patients will occur three days per week starting as soon as possible after admission until the day of discharge from ICU (or until day 30 of admission). If patients are not passing stool regularly, or a stool sample cannot be collected due to the time of day that it was passed, then a rectal swab will be used as an alternative method of collecting stool. (Refer to the Schedule of Assessments)
Statistics	To determine if significant shifts in bacterial microbiome composition have occurred during ICU residency, analysis will compare the gut microbiome composition taken within 48 hrs of admission to ICU with the composition of the microbiome taken closest to the time of discharge from ICU. This will be determined using permutational analysis of variance (PERMANOVA) analysis (alpha =0.05) on weighted UniFrac distance of paired samples. Alpha diversity measures including taxa richness, Shannon and Simpson's metrics will be calculated using QIIME2 and Primer software with significance ($p<0.05$) determined using the Wilcoxon matched-pairs signed rank test. Differences in individual taxa or genes will also be determined using the Wilcoxon matched-pairs signed rank test with the Benjamini-Hochberg correction applied to correct for false discovery. Multivariate linear regression performed against alpha diversity metrics and UniFrac distance will be used to determine if there are significant differences in microbiome data in different clinical groups.
Recruitment period	RAH: 31st August – 9th October 2020 FMC: 1st of October 2020 - 1st January 2021
Participant recruitment	Recruitment for this study will occur over a defined 6-week period. The number of patients recruited per investigator per day will be limited to 6, to allow at least an hour of time required to collect data and specimens for each patient.

2.Contact Details

Study PI	Dr Lito Papanicolas	Lito.Papanicolas@sa.gov.au
Study Coordinator	Erin Flynn	Erin.flynn@sahmri.com
RAH PI	Dr Lito Papanicolas	Lito.Papanicolas@sa.gov.au
RAH Coordinator	Dr Lee-anne Chapple	Lee-anne.Chapple@sa.gov.au
FMC PI	Dr Shailesh Bihari	Shailesh.Bihari@sa.gov.au

FMC Coordinator	Tapaswi Shrestha	Tapaswi.Shrestha@sa.gov.au

3.Ethics Approvals

Committee	Approval Date	Protocol #	Reference #
Human Research Ethics Committee Central Adelaide Local Health Network Southern Adelaide Clinical Human Research Ethics Committee	30 June 2020	MOCI Protocol V6 / 19 May 2020	HREC reference: HREC/19/CALHN/211 CALHN Reference: R20190504

4.Participant Recruitment & Consent

Inclusion/exclusion criteria

Inclusion criteria	1. Any patients admitted to the Royal Adelaide Hospital or Flinders Medical Centre ICU 2. Age ≥18 3. Patient is receiving invasive mechanical ventilation (IMV) 4. Patient is predicted by intensivist to have length of stay (LOS) >3 days
Exclusion criteria	1. Death is deemed to be imminent during this admission and either the attending doctor, patient or substitute decision maker is not committed to active treatment 2. Duration of admission is predicted to be less than 72 hrs. 3. Patient diagnosed with COVID-19
Withdrawal criteria	1. Unable to obtain specimen within 72hrs of enrolment

Recruitment process

1. Screening: Daily (Monday – Friday) new ICU admissions will be identified by review of the ICU patient admission database. These patients will be screened for eligibility for inclusion in the study through review of patient case notes.
2. Enrolment: Eligible patients enrolled, and sample collection commenced

3. Withdrawal: At the first appropriate opportunity the legally acceptable representative will have the study explained to them and be given an information sheet and consent form with the opportunity to opt their relative out of the study.

Template logs available for:

- MOCI 2020 screening and sample log 'Screening Log' tab - Enter all patients who meet the inclusion criteria and select any exclusion if applicable. If no exclusions apply and patient is eligible, leave the exclusions column blank and allocate a study number to enrol the patient.
- 'Sample Log' tab – Use this log to keep track of samples collected, sample type and the date and time of collection. For ease of navigation, the sample log has been divided so that every tab contains 10 participants only.

MOCI Enrolment log – Enter enrolled patients. Can use this log to also keep track of when the consent brochure was given to next of kin and additional details.

Schedule of Assessments

Assessment / Procedure											
Study enrolment/ICU admission day	0	3	6	9	12	15	18	21	24	27	30
ALL PARTICIPANTS											
Screening & enrolment	x										
Delayed, opt-out consent		x	x								
REDCap – ICU admission form		x									
*Tracheal aspirate sample**	x	x	x	x	x	x	x	x	x	x	x
*Stool sample/rectal swab**	x	x	x	x	x	x	x	x	x	x	x
*REDCap – Sample collection form**	x	x	x	x	x	x	x	x	x	x	x
REDCap – IMV form											x
REDCap – LOS form											x

**Continue only until ICU discharge or day 30 of ICU admission, whichever comes first*

5.Sample Collection

Standard Operating Procedure (SOP)
MOCI SPECIMEN COLLECTION

1. Purpose

1.1. This SOP standardises the procedure for collecting stool samples and tracheal aspirates for the MOCI study.

2. Scope

2.1. This SOP applies to all persons involved in the collection, transport or storage of samples for the MOCI study.

3. Responsible Individuals

3.1. Principal Investigator: The Principal Investigator will assure that all staff involved with specimen collection are experienced and proficient in the collection of study specimens.

3.2. Research Coordinator: The research coordinator will assure that informed consent has been obtained prior to the collection of any specimen. The research coordinator will assure that labels and equipment needed for collection are available.

4. Materials and equipment

Stool sample	1. 20ml specimen collection container 2. Sterile dry swab 3. Specimen bag 4. 1x label per participant
Tracheal Aspirate	1. Sputum trap with collection pot 2. 1x label per participant 3. Specimen bag

5. Sample Labelling

5.1. For each participant include the following

5.2. MOCI

5.3. R2= RAH, F1=FMC

5.4. MOCI patient ID= number in order of recruitment and initials

- Eg first patient recruited Jane Doe= 001JD

5.5. Sample type

- RS= rectal swab
- ST= stool
- COL= colostomy specimen
- TA= Tracheal aspirate

5.6. Number indicating which sampling time-point you are up to eg: ST-2 (second sample collected)

5.7. Date and time to be added by nurse collecting specimen but to be confirmed by study staff (this is important to be correct on the label)

Example of final label:

MOCI R2 , 001 JD
ST-2, 20-Oct-20; 13:30

6. Sample Collection

After explaining the study to the nurse, give the ICU nurse attending to patient participant an Esky which contains ice, a stool pot, a rectal swab, 2 biohazard bags

and 2 patient specific labels. Also supply the nurse with a sputum collection set from the ward imprest system.

The ICU nurse should already be familiar with the procedures and safety precautions used to collect the specimens, as these types of specimens are routinely collected in ICU. If not, the nurse should request assistance from a nurse who is trained in how to collect the specimens.

Generally, stool specimens are collected when it is time to turn the patient as this is when routine hygiene is performed. Tracheal suctioning is also performed at regular intervals and this is when the tracheal aspirates should be collected.

6.1. Stool

a. If the patient participant has opened bowels, the nurse should collect a stool specimen using either the stool pot lid scoop or by dipping the swab into the faecal material (this option is suitable when only a small amount of faecal material is visible and is insufficient to be collected in the pot)
b. If the patient has a faecal management system (FMS), a colostomy or ileostomy request that the sample is collected from these sites instead (again either a pot or swab may be used).
c. If the patient has not opened bowels at the time of turning, the nurse should collect a rectal swab (same method used to collect VRE swabs) at that time.
d. After the specimen has been collected, the nurse should break the swab at the marked line and place the swab tip into the stool specimen pot and secure the lid
e. The stool pot (which contains either a stool specimen or the stool swab) should be labelled with a MOCI label with the patient's study number and date of collection. The nurse should add the time of collection to the label. If a nurse has used a patient label on the container this should be removed before processing.
f. Place container in the biohazard specimen bag and seal
g. Place bagged container onto ice in the Esky until transfer to SAHMRI laboratory for processing. **DO NOT** send the samples to SA Pathology via chute.

6.2. Tracheal Aspirate

a. The patient participant's nurse should collect a tracheal aspirate specimen when routine suctioning of the patient occurs.
b. The sample collection should occur using the same procedures used to collect a tracheal aspirate specimen for routine culture testing.
c. The tracheal aspirate specimen container should be labelled with a MOCI label with the patient's study number and date of collection. The nurse should add the time of collection to the label. If a nurse has used a patient hospital label on the container this should be removed before processing.
d. Place container in the biohazard specimen bag and seal

e. Place bagged container onto ice in the Esky until transfer to SAHMRI laboratory for processing. **DO NOT** send the samples to SA Pathology via chute.

7. Standard Precautions for handling specimen containers for research staff

Always wash hands before and after handling specimen containers**.** Wear gloves if handling specimen container (eg to remove or add labels)**.** Take care not to contaminate the outside of the containers/tubes

8. Transfer of specimens to SAHMRI

Samples can remain on ice for up to 24 hrs, however ideally samples will be transferred to the laboratory within a few hours of collection.

7.1.1 Contact the SAHMRI research assistant when samples are ready to be handed over.

7.1.2 RAH only: Hand over Eskys just outside the RAH on level 4 exit facing the SAHMRI building.

9. Specimen processing at SAHMRI laboratory

1. Don laboratory gown and gloves
2. Clean surface of the Class 2 biosafety cabinet with 70% Ethanol
3. Remove bagged specimens from Esky and place into biosafety cabinet
4. Open specimens only inside the biosafety cabinet
5. Clear scissors with 80% ethanol, and cut tip off stool swab. Place swab tip into a 1.5 mL tube pre-filled with 1mL 20% glycerol saline. Ensure labelled appropriately.
6. If stool remains in pot, use clean plastic 10 micron loop to place a loopful (~300 µg) stool into another 2 tubes.
7. Clean scissors using ethanol before and after every use
8. Use a new swab to swab the tracheal aspirate specimen and cut the tip off and place into 1.5 mL tube pre-filled with 1mL 20% glycerol saline. Ensure labelled appropriately.
9. If tracheal aspirate remains in pot, use clean plastic 10 micron loop to place a loopful (~300 ug) stool into another 2 tubes. Ensure labelled appropriately.
10. Store tubes in labelled boxes in appropriate -80 C freezer in SAHMRI basement freezer room.
11. Empty Esky of ice, wipe down interior with 70% Ethanol and arrange return to RAH

6.Secondary Data Collection

All data collected will be collected via the REDCap data entry program, the details of the data collection is in section 7, but the general principals are covered in this section.

The REDCap program can be accessed by approved staff members via the following website: https://redcap.sahmri.com/redcap_v9.3.1/index.php?pid=175
Data is entered into one of 6 domains on REDCap:

- ICU admission
- Sample collection
- IMV history
- Length of stay
- Microbiology
- Radiology

ICU admission form:
This form collects data related to demographic details of the patient, underlying conditions and admission diagnosis. This data is collected only once during the admission and data required should be available to investigators at the time of study enrolment.
Sample collection form:
This form collects data about the specimen collected and details about the patient's condition at the time of specimen collection. Therefore a new specimen form should be completed each day a new specimen is collected. This data is collected prospectively either from the patient's daily ICU chart and bedside case notes or EPAS/EMAR electronic systems.
As part of this form, patient SOFA scores will be recorded:

Table 1. The Sequential Organ Failure Assessment (SOFA) Score*

	SOFA Score				
Variables	**0**	**1**	**2**	**3**	**4**
Respiratory Pao_2/FIO_2, mm Hg	>400	≤400	≤300	≤200†	≤100†
Coagulation Platelets $\times 10^3/\mu L$‡	>150	≤150	≤100	≤50	≤20
Liver Bilirubin, mg/dL‡	<1.2	1.2-1.9	2.0-5.9	6.0-11.9	>12.0
Cardiovascular Hypotension	No hypotension	Mean arterial pressure <70 mm Hg	Dop ≤5 or dob (any dose)§	Dop >5, epi ≤0.1, or norepi ≤0.1§	Dop >15, epi >0.1, or norepi >0.1§
Central nervous system Glasgow Coma Score Scale	15	13-14	10-12	6-9	<6
Renal Creatinine, mg/dL or urine output, mL/d‖	<1.2	1.2-1.9	2.0-3.4	3.5-4.9 or <500	>5.0 or <200

*Norepi indicates norepinephrine; Dob, dobutamine; Dop, dopamine; Epi, epinephrine; and FIO_2, fraction of inspired oxygen.
†Values are with respiratory support.
‡To convert bilirubin from mg/dL to µmol/L, multiply by 17.1.
§Adrenergic agents administered for at least 1 hour (doses given are in µg/kg per minute).
‖To convert creatinine from mg/dL to µmol/L, multiply by 88.4.

ORGAN SYSTEM	0	1	2	3	4	9	Organ Scores
Respiration **PaO_2/FIO_2 (in mmHg)**	>400	301-400	<301	≤200 With Respiratory Support*	≤100 With Respiratory Support*	Variable not measured	
Coagulation **Platelets($x10^3/mm^3$)**	>150	101-150	51-100	21-50	≤20	Variable not measured	
Liver **Bilirubin (mg/dL)** **(μmol/L)**	<1.2 <20	1.2 - 1.9 20 - 32	2.0 - 5.9 33 - 101	6.0 - 11.9 102 - 204	>12.0 >204	Variable not measured	
Cardiovascular **Hypotension**	MAP ≥70 mmHg without vasopressors	MAP <70 mmHg without vasopressors	dopamine ≤5.0 or dobutamine (any dose) or any dose milrinone or any dose levosimendan	dopamine 5.1 – 15.0 or adr ≤0.1 or noradr ≤0.1 or any dose vasopressin or any dose metaraminol or any dose phenylephrine	Dopamine >15.0 or adr >0.1 or noradr >0.1	Variable not measured	
All drug doses are μg/kg/min							
Central Nervous System **Glasgow Coma Score^**	15	13 - 14	10 - 12	6 - 9	<6	Variable not measured	
Renal **Creatinine (mg/dL)** **(μmol/L)** **or urine output**	<1.2 <110	1.2 - 1.9 110 - 170	2.0 - 3.4 171 - 299	3.5 - 4.9 300 - 440 or <500 mL/day	>5.0 >440 or <200 mL/day	Variable not measured	

adr, adrenaline = epinephrine; noradr, noradrenaline = norepinephrine.

*Respiratory support is defined as any form of invasive or non-invasive ventilation including mask CPAP or CPAP delivered through a tracheostomy/tracheotomy or endotracheal or nasotracheal tube
PLEASE NOTE: The highest score for someone **without** respiratory support is 2.

^Collect the GCS recorded prior to administration of sedative agents and prior to intubation. Go back as far as necessary to the time at which the patient was first sedated and identify the GCS at the time of/just prior to sedation. If you cannot locate the GCS at the time of/just prior to sedation select "Variable not measured".

PLEASE NOTE: Except for GCS, score each system on data available within the previous 24 hours (from randomisation), document the value closest to randomisation (**not the most deranged value**).

Guidance on SOFA score calculation

Variables	**Instructions**
Respiratory PaO_2/FiO_2 (mm Hg)	Lowest ratio between PaO_2 and the corresponding FiO_2 values from the blood gas for the calendar day. Allocate a score between 0 – 4 accordingly as per chart. For a patient on invasive mechanical or non-invasive ventilation, they attain a score 0 – 4. With self-ventilated patients they can only score between 0 – 2. For example, if a mechanically ventilated patient had PaO_2 of 144 and FiO_2 of 0.9, then: PaO_2/FiO_2 = 144/0.9 = 160 (Allocate a score of 3).
Coagulation Platelets X 10^9/L	Lowest platelet count for the calendar day.
Liver Bilirubin, mg/dL μmol/L	Highest bilirubin levels for the calendar day.
Cardiovascular Hypotension	Lowest mean arterial pressure (MAP) on the calendar day. If patient has received any of the medications mentioned on the chart, collect the patient's weight and the maximum dose of the drug received within the calendar day, to calculate the Mcg/min/kg.
Central Nervous System Glasgow Coma score (GCS)	Lowest GCS documented prior to administration of sedatives and pre-intubation. Once patient has been extubated, find the lowest GCS.
Renal Creatinine, mg/dL μmol/L	Highest creatinine value for the calendar day.
Or urine output,	If patient is on dialysis, collect total urine output for the calendar day instead.

SOFA Score is equal to the sum of all the scores for each variable.

IMV History form:

This form prospectively collects information about ventilation including intubation, extubation and tracheostomy events, these should be updated as required during the study period.

Length of Stay form:

This form prospectively collects information about ventilation including intubation, extubation and tracheostomy events, these should be updated as required during the study period.

Microbiology and Radiology forms:

These forms will be completed retrospectively and are the responsibility of the chief PI, not site investigators.

7.REDCap

Codes

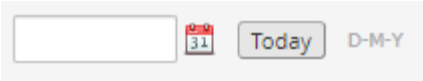
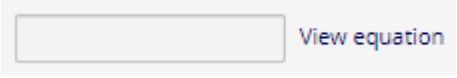
99 - Other
98 - Unknown
97 - N/A or Not Applicable
96 - Not sure / Unsure
95 - None of the above

Definitions

Current calendar day	Time starting at 00:00 (midnight) of the same day as sample collected
Preceding 48 hours	Time period 48 preceding sample collection time
Study period	Date of MOCI enrolment until day 30 of ICU admission

Data type

Multiple choice – square icons	☐ None ☐ Diabetes ☐ Cirrhosis
Single choice – circular icons	○ RAH ○ FMC
Free text	

Formatted date or date & time Calculated field (no entry required)	 or  

ICU admission

Record ID	_____
MOCI study ID:	_____ (eg. R2 - 001)
Study site:	◯ RAH ◯ FMC
Patient Initials:	_____ (eg. John Smith = JS)
Date of birth:	_____
Gender:	◯ Male ◯ Female ◯ Other
Indigenous status/residency:	◯ Aboriginal and Torres Strait Islander ◯ Non-indigenous Australian resident ◯ Not an Australian resident
Usual residence:	◯ Home ◯ Long-term care facility
Is the patient enrolled in the SuDDICU study?	◯ Yes ◯ No
What arm are they enrolled in?	◯ Washout ◯ Intervention - NG suspension & paste ◯ Intervention plus - NG suspension, paste + 4 days IVABx (Ceftriaxone, Cefotaxime or Ciprofloxacin)

Relevant Co-morbidities on admission to ICU:	☐ None ☐ Diabetes ☐ Cirrhosis ☐ Hepatic failure ☐ Haematological Malignancy ☐ Other Malignancy ☐ HIV ☐ Stem cell transplant ☐ Solid organ transplant ☐ Immunosuppressed by therapy ☐ Autoimmune disease ☐ Inflammatory bowel disease ☐ Chronic respiratory disease ☐ Current smoker ☐ Chronic cardio vascular disease ☐ Colostomy or Enterostomy ☐ Chronic renal failure ☐ Spinal cord injury ☐ Other (check all that apply)
Please describe other co-morbidity:	_____
Child-Pugh score:	_____
Which haematological malignancy?	_____
Which malignancy?	_____
CD4 count:	_____
Which transplant?	_____
Which therapy?	_____
Which autoimmune disease?	_____
Which chronic respiratory disease?	_____
NYHA score:	_____
GFR:	_____
Dialysis:	_____

Admission weight (kg):	_____
Did the patient have a MRO flag (pre admission)?	◯ yes ◯ no/unknown
Which MRO?	☐ VRE ☐ ESBL/AMP-C ☐ MRSA ☐ MRPA ☐ CRE ☐ Other
Name of other MRO:	_____
Was the patient administered antibiotics within 24 hours prior to ICU admission?	◯ yes ◯ no ◯ unknown
Which antibiotics?	☐ Penicillin/Amoxicillin ☐ Amoxicillin+clavulante ☐ Piperacillin+tazobactam (IV) ☐ Ceftriaxone (IV) ☐ Cefazolin (IV) ☐ Cephalexin (PO) ☐ Cefepime (IV) ☐ Meropenem (IV) ☐ Azithromycin ☐ Vancomycin ☐ Gentamicin ☐ Ciprofloxacin ☐ Trimethoprim/Sulfamethoxazole ☐ Metronidazole ☐ Clindamycin/Lincomycin ☐ Linezolid ☐ Other ☐ Unknown (check all that apply)
Route of penicillin/amoxicillin:	◯ IV ◯ Oral
Route of amoxicillin+clavulante:	◯ IV ◯ Oral
Route of Azithromycin:	◯ IV ◯ Oral
Route of vancomycin:	◯ IV ◯ Oral
Route of ciprofloxacin:	◯ IV ◯ Oral

Route/reason for Trimethoprim/sulfamethoxazole:	◯ IV (Prophylaxis only) ◯ Oral (Prophylaxis only) ◯ IV (Treatment) ◯ Oral (Treatment)
Route of metronidazole:	◯ IV ◯ Oral
Route of clindamycin/lincomycin:	◯ IV ◯ Oral
Route of Linezolid:	◯ IV ◯ Oral
Please state name and route of other abx:	_____
Admission diagnosis:	☐ Sepsis ☐ Skin/Soft tissue/Bone infection ☐ CNS infection ☐ Pneumonia ☐ COPD exacerbation ☐ Pulmonary embolism ☐ Chest Trauma ☐ Abdominal Trauma ☐ Brain injury or CVA ☐ GI tract haemorrhage/perforation/infarction ☐ Myocardial infarction ☐ Cardiac arrest ☐ Complications of cardio-thoracic surgery ☐ Complications of neurosurgery ☐ Complications of abdominal surgery ☐ Burns ☐ Spinal cord injury ☐ Other (check all that apply)
Please describe other admission diagnosis:	_____

Sample collection

Record ID	_____
Sample collection date and time:	_____ (Please enter for first sample collected for this instance.If time unknown please enter 12:00)
Sample number:	_____
Type of sample(s) collected:	☐ Stool ☐ Rectal swab ☐ Trachael aspirate ☐ Enterostomy output ☐ Colostomy output

SOFA Score (for current chart day)

	0	1	2	3	4
Respiratory	○	○	○	○	○
Coagulation	○	○	○	○	○
Liver	○	○	○	○	○
Hypotension	○	○	○	○	○
CNS	○	○	○	○	○
Renal	○	○	○	○	○

Lactate â‰¥2mmol/L at any point in the preceding 48 hours?	○ Yes ○ No
On renal replacement therapy within the last 3 days?	○ Yes ○ No
Type of renal replacement therapy:	○ CRRT ○ IHD ○ PD
Urine output in current calendar day (mL):	_____
Peak temperature (current calendar day):	○ â‰¥ 38.0Â°C ○ Between 35.1Â°C - 37.9Â°C ○ â‰¤35.0Â°C
Mean Arterial Pressure (lowest in current calendar day):	_____
Vasopressor required (in current calendar day)?	○ Yes ○ No

Which vasopressor:	☐ Noradrenaline ☐ Other (check all that apply)
Name of other vasopressor(s) and max hourly dose:	_____
Noradrenaline max hourly dose:	_____
GCS ICU (lowest non-sedated) total:	◯ 3 ◯ 4 ◯ 5 ◯ 6 ◯ 7 ◯ 8 ◯ 9 ◯ 10 ◯ 11 ◯ 12 ◯ 13 ◯ 14 ◯ 15 (Most recently recorded non-sedated GCS)
Tracheostomy insitu?	◯ Yes ◯ No
Ventilation type:	◯ Self ◯ NIV ◯ IMV (via ETT or tracheostomy tube) (If has tracheostomy, tick IMV if any IMV support in last 24 hrs)
Feeding type:	☐ Enteral ☐ Parenteral ☐ Oral ☐ Fasted
Tube type:	◯ NGT ◯ OGT ◯ NJT ◯ JEJ ◯ PEG
Which enteral formula?	☐ Nutrison multifibre protein plus ☐ Two CaL ☐ Other
Name of other formula:	_____
TPN Volume delivered (mL) in current calendar day:	_____

Formula volume delivered (mL) in current calendar day:	_____
Was the formula changed during the current calendar day?	◯ Yes ◯ No
Date & time of formula change:	_____
Name of new formula?	◯ Nutrison multifibre protein plus ◯ Two CaL ◯ Other
Name of other formula:	_____
Formula volume delivered in mls (current calendar day):	_____
Antibiotics administered in the current calendar day:	◯ Yes ◯ No
What antibiotics?	☐ Penicillin/Amoxicillin ☐ Amoxicillin+clavulante ☐ Piperacillin+tazobactam (IV) ☐ Ceftriaxone (IV) ☐ Cefazolin (IV) ☐ Cephalexin (PO) ☐ Cefepime (IV) ☐ Meropenem (IV) ☐ Azithromycin ☐ Vancomycin ☐ Gentamicin ☐ Ciprofloxacin ☐ Trimethoprim/Sulfamethoxazole ☐ Metronidazole ☐ Clindamycin/Lincomycin ☐ Linezolid ☐ Erythromycin ☐ Other ☐ Unknown
Drug name and route:	_____
Penicillin/Amoxicillin route:	☐ IV ☐ Oral
Amoxicillin+clavulante route:	☐ IV ☐ Oral
Azithromycin route:	☐ IV ☐ Oral
Vancomycin route:	☐ IV ☐ Oral

Ciprofloxacin route:	☐ IV ☐ Oral
Trimethoprim/Sulfamethoxazole route/reason:	☐ IV (prophylaxis only) ☐ Oral (prophylaxis only) ☐ IV (treatment) ☐ Oral (treatment)
Metronidazole route:	☐ IV ☐ Oral
Clindamycin/Lincomycin route:	☐ IV ☐ Oral
Linezolid route:	☐ IV ☐ Oral
Other medications administered in the current calendar day:	☐ Antifungal(s) ☐ Antiviral(s) ☐ Proton pump inhibitor(s) ☐ Prokinetic(s) ☐ Laxative(s) ☐ Opiate(s) ☐ Corticosteriod(s) ☐ Other immunosuppresant(s) ☐ Oral hypoglycaemic agent(s) ☐ IV/SC Insulin
Type of antifungal:	☐ Fluconazole ☐ Voriconazole/Posaconazole ☐ Amphotericin/Ambisome (IV) ☐ Antifungal mouthcare (Nystatin/Amphotericin lozenge)
Antiviral type:	☐ Aciclovir/Valaciclovir/Famciclovir ☐ Ganciclovir/Valganciclovir ☐ Oseltamivir (Tamiflu)
Route:	◯ IV ◯ Oral
Prokinetic type:	☐ Erythromycin ☐ Metoclopramide
Laxative type:	☐ Enema (G&O, Fleet) ☐ Oral (Coloxyl, Senna, MOVICOL) ☐ Lactulose ☐ Other
Name other laxative:	_____
Opiate type:	☐ Oral intermittent (oxycodone, methadone) ☐ IV/SCintermittent (fentanyl, morphine) ☐ IV continuous (fentanyl)

Corticosteroid type:	☐ Oral (prednisolone, dexamethasone) ☐ IV (methylprednisolone, hydrocortisone) ☐ Inhaled (fluticasone, beclomethasone, budesonide, mometasone)
IV/SC insulin requirement in the current calendar day:	_____
Did the patient have a procedure in theatre within preceding 48 hours of sample collection?	◯ Yes ◯ No
Type of procedure:	☐ Neurosurgery ☐ Cardiothoracic surgery ☐ Abdominal surgery ☐ Endoscopy ☐ Bronchoscopy ☐ Orthopaedic surgery ☐ Other (check all that apply)
Details of procedure:	_____ (completing this is optional)

IMV history

Record ID	_____
Total GCS at time of first intubation/start of IMV:	◯ 3 ◯ 4 ◯ 5 ◯ 6 ◯ 7 ◯ 8 ◯ 9 ◯ 10 ◯ 11 ◯ 12 ◯ 13 ◯ 14 ◯ 15 ◯ Unknown
Start (date & time) of first IMV episode:	_____ (If time unknown please enter 12:00)
End (date & time) of first IMV episode:	_____ (If time unknown please enter 12:00)
Total time (hours) of first IMV episode:	_____
Was the patient restarted on IMV for a second time?	◯ Yes ◯ No
Start (date and time) of second IMV episode:	_____
End (date & time) of second IMV episode:	_____
Total time (hours) of second IMV episode:	_____
Was the patient restarted on IMV for a third time?	◯ Yes ◯ No
Start (date & time) of third IMV episode:	_____
End (date & time) of third IMV episode:	_____
Total time (hours) of third IMV episode:	_____

Did the patient have a tracheostomy insitu at any point during their ICU admission?	◯ Yes ◯ No
Date of tracheostomy insertion?	_____
Was the patient decannulated during their ICU admission?	◯ Yes ◯ No
Date of decannulation?	_____

Length of stay

Record ID	_____
ICU admission date & time:	_____ (If time unknown please enter 00:00)
Age (in years) at time of ICU admission:	_____
Location prior to ICU admission:	◯ Ward (site or other hospital) ◯ ED (site or other hospital) ◯ Community (Medivac direct to ICU)
Hospital (site or other) admission date & time:	_____ (Please use ED admission time. If time unknown enter 00:00)
Hospitalisation time (hours) prior to ICU:	_____
Was the patient discharged from ICU during the study period?	◯ Yes ◯ No (30 days from ICU admission)
Date and time of ICU discharge:	_____
ICU discharge location:	◯ Site ward ◯ Other hospital ◯ Mortuary ◯ Other
Total time (hours) in ICU	_____
Was the patient discharged from hospital (site or other) during the study period?	◯ Yes ◯ No
Enter date and time of hospital (site or other) discharge:	_____
Hospital discharge location in known:	◯ Home ◯ Rehab ◯ Residential aged care/Hospice ◯ Mortuary ◯ Unknown
Did the patient die during the study period?	◯ Yes ◯ No
Date and time of death:	_____

REFERENCES

1. Kaukonen K-M, Bailey M, Suzuki S, Pilcher D, Bellomo R. Mortality Related to Severe Sepsis and Septic Shock Among Critically Ill Patients in Australia and New Zealand, 2000-2012. JAMA. 2014;311(13):1308-16.
2. Rhee C, Jones TM, Hamad Y, Pande A, Varon J, O'Brien C, et al. Prevalence, Underlying Causes, and Preventability of Sepsis-Associated Mortality in US Acute Care Hospitals. JAMA Network Open. 2019;2(2):e187571-e.
3. Sakr Y, Jaschinski U, Wittebole X, Szakmany T, Lipman J, Namendys-Silva SA, et al. Sepsis in Intensive Care Unit Patients: Worldwide Data From the Intensive Care over Nations Audit. Open Forum Infect Dis. 2018;5(12):ofy313.
4. Burki TK. Sharp rise in sepsis deaths in the UK. Lancet Respir Med. 2018;6(11):826.
5. Kumar A, Roberts D, Wood KE, Light B, Parrillo JE, Sharma S, et al. Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. Crit Care Med. 2006;34(6):1589-96.
6. Bucaneve G, Micozzi A, Menichetti F, Martino P, Dionisi MS, Martinelli G, et al. Levofloxacin to prevent bacterial infection in patients with cancer and neutropenia. The New England journal of medicine. 2005;353(10):977-87.
7. Cullen M, Steven N, Billingham L, Gaunt C, Hastings M, Simmonds P, et al. Antibacterial prophylaxis after chemotherapy for solid tumors and lymphomas. The New England journal of medicine. 2005;353(10):988-98.
8. Gafter-Gvili A, Fraser A, Paul M, Vidal L, Lawrie TA, van de Wetering MD, et al. Antibiotic prophylaxis for bacterial infections in afebrile neutropenic patients following chemotherapy. The Cochrane database of systematic reviews. 2012;1(1):CD004386-CD.
9. Perez F, Adachi J, Bonomo RA. Antibiotic-resistant gram-negative bacterial infections in patients with cancer. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2014;59 Suppl 5(Suppl 5):S335-S9.
10. Zilberberg MD, Shorr AF, Micek ST, Vazquez-Guillamet C, Kollef MH. Multi-drug resistance, inappropriate initial antibiotic therapy and mortality in Gram-negative severe sepsis and septic shock: a retrospective cohort study. Critical Care. 2014;18(6):596.
11. Pearson J, Turnidge J, Franklin C, Bell J, Australian Group on Antimicrobial R. Prevalence of antimicrobial resistances in common pathogenic Enterobacteriaceae in Australia, 2004: report from the Australian Group on Antimicrobial Resistance. Commun Dis Intell Q Rep. 2007;31(1):106-12.
12. Care ACoSaQiH. AURA 2019: third Australian report on antimicrobial use and resistance in human health. Sydney: ACSQHC; 2019.
13. Organization WH. Global antimicrobial resistance surveillance system (GLASS) report: early implementation 2016-2017. . Geneva: World Health Organization; 2017.
14. Wailan AM, Paterson DL, Kennedy K, Ingram PR, Bursle E, Sidjabat HE. Genomic Characteristics of NDM-Producing Enterobacteriaceae Isolates in Australia and Their bla_{NDM} Genetic Contexts. Antimicrobial Agents and Chemotherapy. 2016;60(1):136.
15. Mathers AJ, Cox HL, Kitchel B, Bonatti H, Brassinga AKC, Carroll J, et al. Molecular dissection of an outbreak of carbapenem-resistant enterobacteriaceae reveals Intergenus KPC carbapenemase transmission through a promiscuous plasmid. mBio. 2011;2(6):e00204-e211.

16. Snitkin ES, Zelazny AM, Thomas PJ, Stock F, Group NCSP, Henderson DK, et al. Tracking a hospital outbreak of carbapenem-resistant Klebsiella pneumoniae with whole-genome sequencing. Sci Transl Med. 2012;4(148):148ra16-ra16.
17. Satlin MJ, Calfee DP, Chen L, Fauntleroy KA, Wilson SJ, Jenkins SG, et al. Emergence of carbapenem-resistant Enterobacteriaceae as causes of bloodstream infections in patients with hematologic malignancies. Leuk Lymphoma. 2013;54(4):799-806.
18. Freire MP, de Oliveira Garcia D, Garcia CP, Campagnari Bueno MF, Camargo CH, Kono Magri ASG, et al. Bloodstream infection caused by extensively drug-resistant Acinetobacter baumannii in cancer patients: high mortality associated with delayed treatment rather than with the degree of neutropenia. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2016;22(4):352-8.
19. Smith R, Coast J. The true cost of antimicrobial resistance. BMJ. 2013;346:f1493.
20. Browne HP, Forster SC, Anonye BO, Kumar N, Neville BA, Stares MD, et al. Culturing of 'unculturable' human microbiota reveals novel taxa and extensive sporulation. Nature. 2016;533(7604):543-6.
21. Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. Science. 2005;307(5717):1915-20.
22. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010;464(7285):59-65.
23. Janda JM, Abbott SL. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. Journal of clinical microbiology. 2007;45(9):2761-4.
24. Jovel J, Patterson J, Wang W, Hotte N, O'Keefe S, Mitchel T, et al. Characterization of the Gut Microbiome Using 16S or Shotgun Metagenomics. Front Microbiol. 2016;7:459-.
25. Riesenfeld CS, Schloss PD, Handelsman J. Metagenomics: genomic analysis of microbial communities. Annu Rev Genet. 2004;38:525-52.
26. Human Microbiome Project C. Structure, function and diversity of the healthy human microbiome. Nature. 2012;486(7402):207-14.
27. Stecher B. The Roles of Inflammation, Nutrient Availability and the Commensal Microbiota in Enteric Pathogen Infection. Microbiol Spectr. 2015;3(3).
28. Byrd AL, Segre JA. Infectious disease. Adapting Koch's postulates. Science. 2016;351(6270):224-6.
29. Arribas B, Rodriguez-Cabezas ME, Camuesco D, Comalada M, Bailon E, Utrilla P, et al. A probiotic strain of Escherichia coli, Nissle 1917, given orally exerts local and systemic anti-inflammatory effects in lipopolysaccharide-induced sepsis in mice. Br J Pharmacol. 2009;157(6):1024-33.
30. Reissbrodt R, Hammes WP, dal Bello F, Prager R, Fruth A, Hantke K, et al. Inhibition of growth of Shiga toxin-producing Escherichia coli by nonpathogenic Escherichia coli. FEMS Microbiol Lett. 2009;290(1):62-9.
31. Maltby R, Leatham-Jensen MP, Gibson T, Cohen PS, Conway T. Nutritional basis for colonization resistance by human commensal Escherichia coli strains HS and Nissle 1917 against E. coli O157:H7 in the mouse intestine. PLoS One. 2013;8(1):e53957.
32. Gomes TAT, Elias WP, Scaletsky ICA, Guth BEC, Rodrigues JF, Piazza RMF, et al. Diarrheagenic Escherichia coli. Braz J Microbiol. 2016;47 Suppl 1(Suppl 1):3-30.
33. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. Nature. 2009;457(7228):480-4.

34. Bohnhoff M, Drake BL, Miller CP. Effect of streptomycin on susceptibility of intestinal tract to experimental Salmonella infection. Proc Soc Exp Biol Med. 1954;86(1):132-7.
35. van der Waaij D, Berghuis-de Vries JM, Lekkerkerk L-v. Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. J Hyg (Lond). 1971;69(3):405-11.
36. Hoyen CK, Pultz NJ, Paterson DL, Aron DC, Donskey CJ. Effect of parenteral antibiotic administration on establishment of intestinal colonization in mice by Klebsiella pneumoniae strains producing extended-spectrum beta-lactamases. Antimicrob Agents Chemother. 2003;47(11):3610-2.
37. Caballero S, Carter R, Ke X, Susac B, Leiner IM, Kim GJ, et al. Distinct but Spatially Overlapping Intestinal Niches for Vancomycin-Resistant Enterococcus faecium and Carbapenem-Resistant Klebsiella pneumoniae. PLoS Pathog. 2015;11(9):e1005132.
38. Ubeda C, Taur Y, Jenq RR, Equinda MJ, Son T, Samstein M, et al. Vancomycin-resistant Enterococcus domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans. J Clin Invest. 2010;120(12):4332-41.
39. Perez F, Pultz MJ, Endimiani A, Bonomo RA, Donskey CJ. Effect of antibiotic treatment on establishment and elimination of intestinal colonization by KPC-producing Klebsiella pneumoniae in mice. Antimicrob Agents Chemother. 2011;55(6):2585-9.
40. Donskey CJ. Antibiotic regimens and intestinal colonization with antibiotic-resistant gram-negative bacilli. Clin Infect Dis. 2006;43 Suppl 2:S62-9.
41. Vincent C, Manges AR. Antimicrobial Use, Human Gut Microbiota and Clostridium difficile Colonization and Infection. Antibiotics (Basel). 2015;4(3):230-53.
42. Donskey CJ, Chowdhry TK, Hecker MT, Hoyen CK, Hanrahan JA, Hujer AM, et al. Effect of antibiotic therapy on the density of vancomycin-resistant enterococci in the stool of colonized patients. N Engl J Med. 2000;343(26):1925-32.
43. Buck AC, Cooke EM. The fate of ingested Pseudomonas aeruginosa in normal persons. J Med Microbiol. 1969;2(4):521-5.
44. Vollaard EJ, Clasener HA. Colonization resistance. Antimicrob Agents Chemother. 1994;38(3):409-14.
45. Suez J, Zmora N, Zilberman-Schapira G, Mor U, Dori-Bachash M, Bashiardes S, et al. Post-Antibiotic Gut Mucosal Microbiome Reconstitution Is Impaired by Probiotics and Improved by Autologous FMT. Cell. 2018;174(6):1406-23 e16.
46. Buffie CG, Pamer EG. Microbiota-mediated colonization resistance against intestinal pathogens. Nat Rev Immunol. 2013;13(11):790-801.
47. Duerkop BA, Clements CV, Rollins D, Rodrigues JLM, Hooper LV. A composite bacteriophage alters colonization by an intestinal commensal bacterium. Proceedings of the National Academy of Sciences. 2012;109(43):17621.
48. Twomey D, Ross RP, Ryan M, Meaney B, Hill C. Lantibiotics produced by lactic acid bacteria: structure, function and applications. Antonie van Leeuwenhoek. 2002;82(1-4):165-85.
49. Sassone-Corsi M, Nuccio SP, Liu H, Hernandez D, Vu CT, Takahashi AA, et al. Microcins mediate competition among Enterobacteriaceae in the inflamed gut. Nature. 2016;540(7632):280-3.
50. Kinnebrew MA, Ubeda C, Zenewicz LA, Smith N, Flavell RA, Pamer EG. Bacterial flagellin stimulates Toll-like receptor 5-dependent defense against vancomycin-resistant Enterococcus infection. J Infect Dis. 2010;201(4):534-43.
51. Rangan KJ, Pedicord VA, Wang YC, Kim B, Lu Y, Shaham S, et al. A secreted bacterial peptidoglycan hydrolase enhances tolerance to enteric pathogens. Science. 2016;353(6306):1434-7.

52. Sorg JA, Sonenshein AL. Bile salts and glycine as cogerminants for Clostridium difficile spores. J Bacteriol. 2008;190(7):2505-12.
53. Buffie CG, Bucci V, Stein RR, McKenney PT, Ling L, Gobourne A, et al. Precision microbiome reconstitution restores bile acid mediated resistance to Clostridium difficile. Nature. 2015;517(7533):205-8.
54. Louis P, Flint HJ. Formation of propionate and butyrate by the human colonic microbiota. Environ Microbiol. 2017;19(1):29-41.
55. Koh A, De Vadder F, Kovatcheva-Datchary P, Backhed F. From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites. Cell. 2016;165(6):1332-45.
56. Sorbara MT, Dubin K, Littmann ER, Moody TU, Fontana E, Seok R, et al. Inhibiting antibiotic-resistant Enterobacteriaceae by microbiota-mediated intracellular acidification. J Exp Med. 2019;216(1):84-98.
57. Fukuda S, Toh H, Hase K, Oshima K, Nakanishi Y, Yoshimura K, et al. Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature. 2011;469(7331):543-7.
58. Lawhon SD, Maurer R, Suyemoto M, Altier C. Intestinal short-chain fatty acids alter Salmonella typhimurium invasion gene expression and virulence through BarA/SirA. Mol Microbiol. 2002;46(5):1451-64.
59. Gantois I, Ducatelle R, Pasmans F, Haesebrouck F, Hautefort I, Thompson A, et al. Butyrate specifically down-regulates salmonella pathogenicity island 1 gene expression. Appl Environ Microbiol. 2006;72(1):946-9.
60. Rivera-Chavez F, Lopez CA, Baumler AJ. Oxygen as a driver of gut dysbiosis. Free radical biology & medicine. 2017;105:93-101.
61. Rivera-Chavez F, Zhang LF, Faber F, Lopez CA, Byndloss MX, Olsan EE, et al. Depletion of Butyrate-Producing Clostridia from the Gut Microbiota Drives an Aerobic Luminal Expansion of Salmonella. Cell Host Microbe. 2016;19(4):443-54.
62. Byndloss MX, Olsan EE, Rivera-Chavez F, Tiffany CR, Cevallos SA, Lokken KL, et al. Microbiota-activated PPAR-gamma signaling inhibits dysbiotic Enterobacteriaceae expansion. Science. 2017;357(6351):570-5.
63. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature. 2013;504(7480):451-5.
64. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature. 2013;504(7480):446-50.
65. Baquero F, Negri M-C, Morosini M-I, Blázquez J. Antibiotic-Selective Environments. Clinical Infectious Diseases. 1998;27(Supplement_1):S5-S11.
66. Taylor SL, Leong LEX, Sims SK, Keating RL, Papanicolas LE, Richard A, et al. The cystic fibrosis gut as a potential source of multidrug resistant pathogens. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society. 2020.
67. Drlica K. The mutant selection window and antimicrobial resistance. Journal of Antimicrobial Chemotherapy. 2003;52(1):11-7.
68. Gullberg E, Cao S, Berg OG, Ilback C, Sandegren L, Hughes D, et al. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog. 2011;7(7):e1002158.
69. Jernberg C, Löfmark S, Edlund C, Jansson JK. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. The ISME Journal. 2007;1(1):56-66.

70. Vien le TM, Minh NN, Thuong TC, Khuong HD, Nga TV, Thompson C, et al. The co-selection of fluoroquinolone resistance genes in the gut flora of Vietnamese children. PLoS One. 2012;7(8):e42919.
71. Smillie CS, Smith MB, Friedman J, Cordero OX, David LA, Alm EJ. Ecology drives a global network of gene exchange connecting the human microbiome. Nature. 2011;480(7376):241-4.
72. Salyers AA, Amabile-Cuevas CF. Why are antibiotic resistance genes so resistant to elimination? Antimicrob Agents Chemother. 1997;41(11):2321-5.
73. Shoemaker NB, Vlamakis H, Hayes K, Salyers AA. Evidence for extensive resistance gene transfer among Bacteroides spp. and among Bacteroides and other genera in the human colon. Appl Environ Microbiol. 2001;67(2):561-8.
74. Karami N, Martner A, Enne VI, Swerkersson S, Adlerberth I, Wold AE. Transfer of an ampicillin resistance gene between two Escherichia coli strains in the bowel microbiota of an infant treated with antibiotics. Journal of Antimicrobial Chemotherapy. 2007;60(5):1142-5.
75. Papanicolas LE, Bell JM, Weldhagen GF, Bastian I. Ceftriaxone treatment failure in cephalosporin-susceptible Escherichia coli bacteraemia. International journal of antimicrobial agents. 2013;41(3):298-9.
76. Keen EC, Bliskovsky VV, Malagon F, Baker JD, Prince JS, Klaus JS, et al. Novel “Superspreader” Bacteriophages Promote Horizontal Gene Transfer by Transformation. mBio. 2017;8(1):e02115-16.
77. Stecher B, Denzler R, Maier L, Bernet F, Sanders MJ, Pickard DJ, et al. Gut inflammation can boost horizontal gene transfer between pathogenic and commensal Enterobacteriaceae. Proc Natl Acad Sci U S A. 2012;109(4):1269-74.
78. Maier L, Pruteanu M, Kuhn M, Zeller G, Telzerow A, Anderson EE, et al. Extensive impact of non-antibiotic drugs on human gut bacteria. Nature. 2018;555(7698):623-8.
79. Jernberg C, Löfmark S, Edlund C, Jansson JK. Long-term impacts of antibiotic exposure on the human intestinal microbiota. 2010;156(11):3216-23.
80. Becattini S, Taur Y, Pamer EG. Antibiotic-Induced Changes in the Intestinal Microbiota and Disease. Trends Mol Med. 2016;22(6):458-78.
81. Reijnders D, Goossens GH, Hermes GD, Neis EP, van der Beek CM, Most J, et al. Effects of Gut Microbiota Manipulation by Antibiotics on Host Metabolism in Obese Humans: A Randomized Double-Blind Placebo-Controlled Trial. Cell Metab. 2016;24(2):341.
82. Stewardson AJ, Gaia N, Francois P, Malhotra-Kumar S, Delemont C, Martinez de Tejada B, et al. Collateral damage from oral ciprofloxacin versus nitrofurantoin in outpatients with urinary tract infections: a culture-free analysis of gut microbiota. Clin Microbiol Infect. 2015;21(4):344 e1-11.
83. Palleja A, Mikkelsen KH, Forslund SK, Kashani A, Allin KH, Nielsen T, et al. Recovery of gut microbiota of healthy adults following antibiotic exposure. Nature Microbiology. 2018;3(11):1255-65.
84. Vétizou M, Pitt JM, Daillère R, Lepage P, Waldschmitt N, Flament C, et al. Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. Science (New York, NY). 2015;350(6264):1079-84.
85. Sivan A, Corrales L, Hubert N, Williams JB, Aquino-Michaels K, Earley ZM, et al. Commensal Bifidobacterium promotes antitumor immunity and facilitates anti–PD-L1 efficacy. Science. 2015;350(6264):1084.
86. Baruch EN, Youngster I, Ben-Betzalel G, Ortenberg R, Lahat A, Katz L, et al. Fecal microbiota transplant promotes response in immunotherapy-refractory melanoma patients. Science. 2021;371(6529):602-9.

87. Baggs J, Jernigan JA, Halpin AL, Epstein L, Hatfield KM, McDonald LC. Risk of Subsequent Sepsis Within 90 Days After a Hospital Stay by Type of Antibiotic Exposure. Clin Infect Dis. 2018;66(7):1004-12.
88. Prescott HC, Dickson RP, Rogers MA, Langa KM, Iwashyna TJ. Hospitalization Type and Subsequent Severe Sepsis. Am J Respir Crit Care Med. 2015;192(5):581-8.
89. Pettigrew MM, Gent JF, Kong Y, Halpin AL, Pineles L, Harris AD, et al. Gastrointestinal microbiota disruption and risk of colonization with carbapenem-resistant Pseudomonas aeruginosa in ICU patients. Clin Infect Dis. 2018.
90. Tacconelli E, De Angelis G, Cataldo MA, Mantengoli E, Spanu T, Pan A, et al. Antibiotic usage and risk of colonization and infection with antibiotic-resistant bacteria: a hospital population-based study. Antimicrob Agents Chemother. 2009;53(10):4264-9.
91. Isaac S, Scher JU, Djukovic A, Jimenez N, Littman DR, Abramson SB, et al. Short- and long-term effects of oral vancomycin on the human intestinal microbiota. J Antimicrob Chemother. 2017;72(1):128-36.
92. Bulow C, Langdon A, Hink T, Wallace M, Reske KA, Patel S, et al. Impact of Amoxicillin-Clavulanate followed by Autologous Fecal Microbiota Transplantation on Fecal Microbiome Structure and Metabolic Potential. mSphere. 2018;3(6).
93. MacPherson CW, Mathieu O, Tremblay J, Champagne J, Nantel A, Girard S-A, et al. Gut Bacterial Microbiota and its Resistome Rapidly Recover to Basal State Levels after Short-term Amoxicillin-Clavulanic Acid Treatment in Healthy Adults. Scientific Reports. 2018;8(1):11192.
94. Russo PL, Stewardson AJ, Cheng AC, Bucknall T, Mitchell BG. The prevalence of healthcare associated infections among adult inpatients at nineteen large Australian acute-care public hospitals: a point prevalence survey. Antimicrob Resist Infect Control. 2019;8:114.
95. Dethlefsen L, Huse S, Sogin ML, Relman DA. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS biology. 2008;6(11):e280.
96. Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermudez-Humaran LG, Gratadoux JJ, et al. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci U S A. 2008;105(43):16731-6.
97. Radman M. SOS repair hypothesis: phenomenology of an inducible DNA repair which is accompanied by mutagenesis. Basic Life Sci. 1975;5A:355-67.
98. Michel B. After 30 years of study, the bacterial SOS response still surprises us. PLoS biology. 2005;3(7):e255-e.
99. Thi TD, Lopez E, Rodriguez-Rojas A, Rodriguez-Beltran J, Couce A, Guelfo JR, et al. Effect of recA inactivation on mutagenesis of Escherichia coli exposed to sublethal concentrations of antimicrobials. J Antimicrob Chemother. 2011;66(3):531-8.
100. Miller C, Thomsen LE, Gaggero C, Mosseri R, Ingmer H, Cohen SN. SOS Response Induction by ß-Lactams and Bacterial Defense Against Antibiotic Lethality. Science. 2004;305(5690):1629.
101. Drlica K. Mechanism of fluoroquinolone action. Current Opinion in Microbiology. 1999;2(5):504-8.
102. Cirz RT, Chin JK, Andes DR, de Crecy-Lagard V, Craig WA, Romesberg FE. Inhibition of mutation and combating the evolution of antibiotic resistance. PLoS Biol. 2005;3(6):e176.
103. Dimpfl J, Echols H. Duplication mutation as an SOS response in Escherichia coli: enhanced duplication formation by a constitutively activated RecA. Genetics. 1989;123(2):255-60.

104. Blázquez J, Couce A, Rodríguez-Beltrán J, Rodríguez-Rojas A. Antimicrobials as promoters of genetic variation. Current opinion in microbiology. 2012;15(5):561-9.
105. Radman M, Taddei F, Matic I. Evolution-driving genes. Res Microbiol. 2000;151(2):91-5.
106. Li G-M. Mechanisms and functions of DNA mismatch repair. Cell Res. 2008;18(1):85-98.
107. Petit MA, Dimpfl J, Radman M, Echols H. Control of large chromosomal duplications in Escherichia coli by the mismatch repair system. Genetics. 1991;129(2):327-32.
108. Michel B. After 30 years of study, the bacterial SOS response still surprises us. PLoS Biol. 2005;3(7):e255.
109. Rayssiguier C, Thaler DS, Radman M. The barrier to recombination between Escherichia coli and Salmonella typhimurium is disrupted in mismatch-repair mutants. Nature. 1989;342(6248):396-401.
110. Pribis JP, García-Villada L, Zhai Y, Lewin-Epstein O, Wang AZ, Liu J, et al. Gamblers: An Antibiotic-Induced Evolvable Cell Subpopulation Differentiated by Reactive-Oxygen-Induced General Stress Response. Mol Cell. 2019;74(4):785-800.e7.
111. Layton JC, Foster PL. Error-prone DNA polymerase IV is controlled by the stress-response sigma factor, RpoS, in Escherichia coli. Molecular microbiology. 2003;50(2):549-61.
112. Kohanski MA, DePristo MA, Collins JJ. Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. Mol Cell. 2010;37(3):311-20.
113. Imran M, Das KR, Naik MM. Co-selection of multi-antibiotic resistance in bacterial pathogens in metal and microplastic contaminated environments: An emerging health threat. Chemosphere. 2019;215:846-57.
114. Seiler C, Berendonk T. Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. 2012;3(399).
115. Osborn AM, Bruce KD, Strike P, Ritchie DA. Distribution, diversity and evolution of the bacterial mercury resistance (mer) operon. FEMS Microbiol Rev. 1997;19(4):239-62.
116. Martinez JL, Sánchez MB, Martínez-Solano L, Hernandez A, Garmendia L, Fajardo A, et al. Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. FEMS Microbiol Rev. 2009;33(2):430-49.
117. Li L-G, Xia Y, Zhang T. Co-occurrence of antibiotic and metal resistance genes revealed in complete genome collection. The ISME journal. 2017;11(3):651-62.
118. Baker-Austin C, Wright MS, Stepanauskas R, McArthur JV. Co-selection of antibiotic and metal resistance. Trends Microbiol. 2006;14(4):176-82.
119. Foster TJ. Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. Microbiol Rev. 1983;47(3):361-409.
120. Summers AO, Wireman J, Vimy MJ, Lorscheider FL, Marshall B, Levy SB, et al. Mercury released from dental "silver" fillings provokes an increase in mercury- and antibiotic-resistant bacteria in oral and intestinal floras of primates. Antimicrobial agents and chemotherapy. 1993;37(4):825-34.
121. Frost LS, Leplae R, Summers AO, Toussaint A. Mobile genetic elements: the agents of open source evolution. Nat Rev Microbiol. 2005;3(9):722-32.
122. Liebert CA, Hall RM, Summers AO. Transposon Tn21, flagship of the floating genome. Microbiol Mol Biol Rev. 1999;63(3):507-22.
123. Dortet L, Girlich D, Virlouvet A-L, Poirel L, Nordmann P, Iorga BI, et al. Characterization of BRP_{MBL}, the Bleomycin Resistance Protein Associated with the Carbapenemase NDM. Antimicrobial Agents and Chemotherapy. 2017;61(3):e02413-16.

124. Johnson NP, Razaka H, Wimmer F, Defais M, Villani G. Toxicity, mutagenicity and drug resistance in Escherichia coli treated with platinum antitumor compounds. Inorganica Chimica Acta. 1987;137(1):25-9.
125. van Vliet MJ, Tissing WJE, Dun CAJ, Meessen NEL, Kamps WA, de Bont ESJM, et al. Chemotherapy treatment in pediatric patients with acute myeloid leukemia receiving antimicrobial prophylaxis leads to a relative increase of colonization with potentially pathogenic bacteria in the gut. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2009;49(2):262-70.
126. Montassier E, Gastinne T, Vangay P, Al-Ghalith GA, Bruley des Varannes S, Massart S, et al. Chemotherapy-driven dysbiosis in the intestinal microbiome. Alimentary pharmacology & therapeutics. 2015;42(5):515-28.
127. Razaka H, Villani G, Hoffman JS, Defais M, Johnson NP. Enhanced mutagenesis during post-treatment incubation of Escherichia coli treated with cis-diamminedichloroplatinum(II). Mutat Res. 1988;209(1-2):63-6.
128. Tomasz M, Lipman R, Chowdary D, Pawlak J, Verdine GL, Nakanishi K. Isolation and structure of a covalent cross-link adduct between mitomycin C and DNA. Science. 1987;235(4793):1204-8.
129. Beaber JW, Hochhut B, Waldor MK. SOS response promotes horizontal dissemination of antibiotic resistance genes. Nature. 2004;427(6969):72-4.
130. Williams MD, Braun LA, Cooper LM, Johnston J, Weiss RV, Qualy RL, et al. Hospitalized cancer patients with severe sepsis: analysis of incidence, mortality, and associated costs of care. Critical Care. 2004;8(5):R291.
131. Wingard JR, Dick J, Charache P, Saral R. Antibiotic-resistant bacteria in surveillance stool cultures of patients with prolonged neutropenia. Antimicrobial agents and chemotherapy. 1986;30(3):435-9.
132. Danai PA, Moss M, Mannino DM, Martin GS. The epidemiology of sepsis in patients with malignancy. Chest. 2006;129(6):1432-40.
133. Pagano L, Tacconelli E, Tumbarello M, Laurenti L, Ortu-La Barbera E, Antinori A, et al. Bacteremia in patients with hematological malignancies. Analysis of risk factors, etiological agents and prognostic indicators. Haematologica. 1997;82(4):415-9.
134. Tancrède CH, Andremont AO. Bacterial translocation and gram-negative bacteremia in patients with hematological malignancies. The Journal of infectious diseases. 1985;152(1):99-103.
135. Berg RD, Wommack E, Deitch EA. Immunosuppression and intestinal bacterial overgrowth synergistically promote bacterial translocation. Arch Surg. 1988;123(11):1359-64.
136. van Vliet MJ, Harmsen HJM, de Bont ESJM, Tissing WJE. The role of intestinal microbiota in the development and severity of chemotherapy-induced mucositis. PLoS pathogens. 2010;6(5):e1000879-e.
137. Blijlevens NM, Donnelly JP, De Pauw BE. Mucosal barrier injury: biology, pathology, clinical counterparts and consequences of intensive treatment for haematological malignancy: an overview. Bone Marrow Transplant. 2000;25(12):1269-78.
138. Taur Y, Jenq RR, Perales MA, Littmann ER, Morjaria S, Ling L, et al. The effects of intestinal tract bacterial diversity on mortality following allogeneic hematopoietic stem cell transplantation. Blood. 2014;124(7):1174-82.
139. Taur Y, Xavier JB, Lipuma L, Ubeda C, Goldberg J, Gobourne A, et al. Intestinal domination and the risk of bacteremia in patients undergoing allogeneic hematopoietic stem cell transplantation. Clin Infect Dis. 2012;55(7):905-14.

140. Mancini N, Carletti S, Ghidoli N, Cichero P, Burioni R, Clementi M. The era of molecular and other non-culture-based methods in diagnosis of sepsis. Clin Microbiol Rev. 2010;23(1):235-51.
141. Vincent JL, Bihari DJ, Suter PM, Bruining HA, White J, Nicolas-Chanoin MH, et al. The prevalence of nosocomial infection in intensive care units in Europe. Results of the European Prevalence of Infection in Intensive Care (EPIC) Study. EPIC International Advisory Committee. JAMA. 1995;274(8):639-44.
142. Warren DK, Shukla SJ, Olsen MA, Kollef MH, Hollenbeak CS, Cox MJ, et al. Outcome and attributable cost of ventilator-associated pneumonia among intensive care unit patients in a suburban medical center. Crit Care Med. 2003;31(5):1312-7.
143. Viaud S, Saccheri F, Mignot G, Yamazaki T, Daillère R, Hannani D, et al. The intestinal microbiota modulates the anticancer immune effects of cyclophosphamide. Science. 2013;342(6161):971-6.
144. Yeh A, Rogers MB, Firek B, Neal MD, Zuckerbraun BS, Morowitz MJ. Dysbiosis Across Multiple Body Sites in Critically Ill Adult Surgical Patients. Shock (Augusta, Ga). 2016;46(6):649-54.
145. Rogers MB, Firek B, Shi M, Yeh A, Brower-Sinning R, Aveson V, et al. Disruption of the microbiota across multiple body sites in critically ill children. Microbiome. 2016;4(1):66.
146. McDonald D, Ackermann G, Khailova L, Baird C, Heyland D, Kozar R, et al. Extreme Dysbiosis of the Microbiome in Critical Illness. mSphere. 2016;1(4).
147. Zaborin A, Smith D, Garfield K, Quensen J, Shakhsheer B, Kade M, et al. Membership and behavior of ultra-low-diversity pathogen communities present in the gut of humans during prolonged critical illness. MBio. 2014;5(5):e01361-14.
148. Zakharkina T, Martin-Loeches I, Matamoros S, Povoa P, Torres A, Kastelijn JB, et al. The dynamics of the pulmonary microbiome during mechanical ventilation in the intensive care unit and the association with occurrence of pneumonia. Thorax. 2017;72(9):803-10.
149. Dickson RP, Singer BH, Newstead MW, Falkowski NR, Erb-Downward JR, Standiford TJ, et al. Enrichment of the lung microbiome with gut bacteria in sepsis and the acute respiratory distress syndrome. Nat Microbiol. 2016;1(10):16113.
150. Kitsios GD, Morowitz MJ, Dickson RP, Huffnagle GB, McVerry BJ, Morris A. Dysbiosis in the intensive care unit: Microbiome science coming to the bedside. J Crit Care. 2017;38:84-91.
151. de Jonge E, Schultz MJ, Spanjaard L, Bossuyt PMM, Vroom MB, Dankert J, et al. Effects of selective decontamination of digestive tract on mortality and acquisition of resistant bacteria in intensive care: a randomised controlled trial. Lancet (London, England). 2003;362(9389):1011-6.
152. de Smet AMGA, Kluytmans JAJW, Cooper BS, Mascini EM, Benus RFJ, van der Werf TS, et al. Decontamination of the digestive tract and oropharynx in ICU patients. The New England journal of medicine. 2009;360(1):20-31.
153. Oostdijk EAN, de Smet AMGA, Kesecioglu J, Bonten MJM, Dutch SODSDDTG. The role of intestinal colonization with gram-negative bacteria as a source for intensive care unit-acquired bacteremia. Critical care medicine. 2011;39(5):961-6.
154. Wittekamp BH, Plantinga NL, Cooper BS, Lopez-Contreras J, Coll P, Mancebo J, et al. Decontamination Strategies and Bloodstream Infections With Antibiotic-Resistant Microorganisms in Ventilated Patients: A Randomized Clinical Trial. JAMA. 2018;320(20):2087-98.
155. Daneman N, Sarwar S, Fowler RA, Cuthbertson BH, Su DCSG. Effect of selective decontamination on antimicrobial resistance in intensive care units: a systematic review and meta-analysis. Lancet Infect Dis. 2013;13(4):328-41.

156. de Smet AMGA, Kluytmans JAJW, Blok HEM, Mascini EM, Benus RFJ, Bernards AT, et al. Selective digestive tract decontamination and selective oropharyngeal decontamination and antibiotic resistance in patients in intensive-care units: an open-label, clustered group-randomised, crossover study. Lancet Infect Dis. 2011;11(5):372-80.
157. Price R, MacLennan G, Glen J. Selective digestive or oropharyngeal decontamination and topical oropharyngeal chlorhexidine for prevention of death in general intensive care: systematic review and network meta-analysis. BMJ : British Medical Journal. 2014;348:g2197.
158. Cheng Y-W, Phelps E, Nemes S, Rogers N, Sagi S, Bohm M, et al. Fecal Microbiota Transplant Decreases Mortality in Patients with Refractory Severe or Fulminant Clostridioides difficile Infection. Clinical Gastroenterology and Hepatology. 2020.
159. Eiseman B, Silen W, Bascom GS, Kauvar AJ. Fecal enema as an adjunct in the treatment of pseudomembranous enterocolitis. Surgery. 1958;44(5):854-9.
160. Landy J, Al-Hassi HO, McLaughlin SD, Walker AW, Ciclitira PJ, Nicholls RJ, et al. Review article: faecal transplantation therapy for gastrointestinal disease. Alimentary pharmacology & therapeutics. 2011;34(4):409-15.
161. Borody TJ, Khoruts A. Fecal microbiota transplantation and emerging applications. Nature reviews Gastroenterology & hepatology. 2012;9(2):88-96.
162. Borody TJ, Warren EF, Leis S, Surace R, Ashman O. Treatment of ulcerative colitis using fecal bacteriotherapy. Journal of clinical gastroenterology. 2003;37(1):42-7.
163. Moayyedi P, Surette M, Kim P, Libertucci J, Wolfe M, Onischi C, et al. Fecal Microbiota Transplantation Induces Remission in Patients with Active Ulcerative Colitis in a Randomized Contolled Trial. Gastroenterology. 2015;149(1):102-9.
164. Kelly CP, LaMont JT. Clostridium difficile--more difficult than ever. N Engl J Med. 2008;359(18):1932-40.
165. Chang JY, Antonopoulos DA, Kalra A, Tonelli A, Khalife WT, Schmidt TM, et al. Decreased diversity of the fecal Microbiome in recurrent Clostridium difficile-associated diarrhea. The Journal of infectious diseases. 2008;197(3):435-8.
166. Pepin J. Improving the treatment of Clostridium difficile-associated disease: where should we start? Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2006;43(5):553-5.
167. McFarland LV, Elmer GW, Surawicz CM. Breaking the cycle: treatment strategies for 163 cases of recurrent Clostridium difficile disease. The American journal of gastroenterology. 2002;97(7):1769-75.
168. Gough E, Shaikh H, Manges AR. Systematic review of intestinal microbiota transplantation (fecal bacteriotherapy) for recurrent Clostridium difficile infection. Clin Infect Dis. 2011;53(10):994-1002.
169. Hamilton MJ, Weingarden AR, Sadowsky MJ, Khoruts A. Standardized frozen preparation for transplantation of fecal microbiota for recurrent Clostridium difficile infection. The American journal of gastroenterology. 2012;107(5):761-7.
170. Costello SP, Conlon MA, Vuaran MS, Roberts-Thomson IC, Andrews JM. Faecal microbiota transplant for recurrent Clostridium difficile infection using long-term frozen stool is effective: clinical efficacy and bacterial viability data. Aliment Pharmacol Ther. 2015;42(8):1011-8.
171. Kassam Z, Lee CH, Yuan Y, Hunt RH. Fecal microbiota transplantation for Clostridium difficile infection: systematic review and meta-analysis. The American journal of gastroenterology. 2013;108(4):500-8.
172. van Nood E, Vrieze A, Nieuwdorp M, Fuentes S, Zoetendal EG, de Vos WM, et al. Duodenal infusion of donor feces for recurrent Clostridium difficile. N Engl J Med. 2013;368(5):407-15.

173. Hvas CL, Dahl Jorgensen SM, Jorgensen SP, Storgaard M, Lemming L, Hansen MM, et al. Fecal Microbiota Transplantation Is Superior to Fidaxomicin for Treatment of Recurrent Clostridium difficile Infection. Gastroenterology. 2019;156(5):1324-32 e3.
174. Cammarota G, Masucci L, Ianiro G, Bibbo S, Dinoi G, Costamagna G, et al. Randomised clinical trial: faecal microbiota transplantation by colonoscopy vs. vancomycin for the treatment of recurrent Clostridium difficile infection. Aliment Pharmacol Ther. 2015;41(9):835-43.
175. Alang N, Kelly CR. Weight gain after fecal microbiota transplantation. Open Forum Infect Dis. 2015;2(1):ofv004.
176. Kelly CR, Khoruts A, Staley C, Sadowsky MJ, Abd M, Alani M, et al. Effect of Fecal Microbiota Transplantation on Recurrence in Multiply Recurrent Clostridium difficile Infection: A Randomized Trial. Ann Intern Med. 2016;165(9):609-16.
177. Paramsothy S, Kamm MA, Kaakoush NO, Walsh AJ, van den Bogaerde J, Samuel D, et al. Multidonor intensive faecal microbiota transplantation for active ulcerative colitis: a randomised placebo-controlled trial. The Lancet. 2017;389(10075):1218-28.
178. Costello SP, Hughes PA, Waters O, Bryant RV, Vincent AD, Blatchford P, et al. Effect of Fecal Microbiota Transplantation on 8-Week Remission in Patients With Ulcerative Colitis: A Randomized Clinical Trial. JAMA. 2019;321(2):156-64.
179. Rossen NG, Fuentes S, van der Spek MJ, Tijssen JG, Hartman JHA, Duflou A, et al. Findings From a Randomized Controlled Trial of Fecal Transplantation for Patients With Ulcerative Colitis. Gastroenterology. 2015;149(1):110-8.e4.
180. Haifer C, Kelly CR, Paramsothy S, Andresen D, Papanicolas LE, McKew GL, et al. Australian consensus statements for the regulation, production and use of faecal microbiota transplantation in clinical practice. Gut. 2020;69(5):801-10.
181. Taur Y, Coyte K, Schluter J, Robilotti E, Figueroa C, Gjonbalaj M, et al. Reconstitution of the gut microbiota of antibiotic-treated patients by autologous fecal microbiota transplant. Sci Transl Med. 2018;10(460):eaap9489.
182. Wilson BC, Vatanen T, Cutfield WS, O'Sullivan JM. The Super-Donor Phenomenon in Fecal Microbiota Transplantation. Front Cell Infect Microbiol. 2019;9:2.
183. Weingarden A, Gonzalez A, Vazquez-Baeza Y, Weiss S, Humphry G, Berg-Lyons D, et al. Dynamic changes in short- and long-term bacterial composition following fecal microbiota transplantation for recurrent Clostridium difficile infection. Microbiome. 2015;3:10.
184. Jalanka J, Mattila E, Jouhten H, Hartman J, de Vos WM, Arkkila P, et al. Long-term effects on luminal and mucosal microbiota and commonly acquired taxa in faecal microbiota transplantation for recurrent Clostridium difficile infection. BMC medicine. 2016;14(1):155-.
185. Fuentes S, van Nood E, Tims S, Heikamp-de Jong I, ter Braak CJF, Keller JJ, et al. Reset of a critically disturbed microbial ecosystem: faecal transplant in recurrent Clostridium difficile infection. The ISME journal. 2014;8(8):1621-33.
186. Staley C, Kelly CR, Brandt LJ, Khoruts A, Sadowsky MJ. Complete Microbiota Engraftment Is Not Essential for Recovery from Recurrent Clostridium difficile Infection following Fecal Microbiota Transplantation. mBio. 2016;7(6):e01965-16.
187. Osman M, Stoltzner Z, O'Brien K, Ling K, Koelsch E, Dubois N, et al. Donor Efficacy in Fecal Microbiota Transplantation for Recurrent Clostridium difficile: Evidence From a 1,999-Patient Cohort. Open Forum Infect Dis. 2016;3:841.
188. Youngster I, Mahabamunuge J, Systrom HK, Sauk J, Khalili H, Levin J, et al. Oral, frozen fecal microbiota transplant (FMT) capsules for recurrent Clostridium difficile infection. BMC Med. 2016;14(1):134.

189. Osman M, O'Brien K, Stoltzner Z, Ling K, Koelsch E, Dubois N, et al. Safety and Efficacy of Fecal Microbiota Transplantation for Recurrent Clostridium difficile Infection From An International Public Stool Bank: Results From a 2050-Patient Multicenter Cohort. Open Forum Infectious Diseases. 2016;3(suppl_1).
190. Ianiro G, Maida M, Burisch J, Simonelli C, Hold G, Ventimiglia M, et al. Efficacy of different faecal microbiota transplantation protocols for Clostridium difficile infection: A systematic review and meta-analysis. United European Gastroenterol J. 2018;6(8):1232-44.
191. Kao D, Roach B, Silva M, Beck P, Rioux K, Kaplan GG, et al. Effect of Oral Capsule- vs Colonoscopy-Delivered Fecal Microbiota Transplantation on Recurrent Clostridium difficile Infection: A Randomized Clinical Trial. JAMA. 2017;318(20):1985-93.
192. Jiang ZD, Jenq RR, Ajami NJ, Petrosino JF, Alexander AA, Ke S, et al. Safety and preliminary efficacy of orally administered lyophilized fecal microbiota product compared with frozen product given by enema for recurrent Clostridium difficile infection: A randomized clinical trial. PLoS One. 2018;13(11):e0205064.
193. Khanna S, Pardi DS, Kelly CR, Kraft CS, Dhere T, Henn MR, et al. A Novel Microbiome Therapeutic Increases Gut Microbial Diversity and Prevents Recurrent Clostridium difficile Infection. J Infect Dis. 2016;214(2):173-81.
194. Tvede M, Rask-Madsen J. Bacteriotherapy for chronic relapsing Clostridium difficile diarrhoea in six patients. Lancet (London, England). 1989;1(8648):1156-60.
195. Ott SJ, Waetzig GH, Rehman A, Moltzau-Anderson J, Bharti R, Grasis JA, et al. Efficacy of Sterile Fecal Filtrate Transfer for Treating Patients With Clostridium difficile Infection. Gastroenterology. 2017;152(4):799-811.e7.
196. Allegretti JR, Mullish BH, Kelly C, Fischer M. The evolution of the use of faecal microbiota transplantation and emerging therapeutic indications. Lancet. 2019;394(10196):420-31.
197. Moayyedi P, Surette MG, Kim PT, Libertucci J, Wolfe M, Onischi C, et al. Fecal Microbiota Transplantation Induces Remission in Patients With Active Ulcerative Colitis in a Randomized Controlled Trial. Gastroenterology. 2015;149(1):102-9 e6.
198. Kump P, Wurm P, Grochenig HP, Wenzl H, Petritsch W, Halwachs B, et al. The taxonomic composition of the donor intestinal microbiota is a major factor influencing the efficacy of faecal microbiota transplantation in therapy refractory ulcerative colitis. Aliment Pharmacol Ther. 2018;47(1):67-77.
199. Martín R, Bermúdez-Humarán LG, Langella P. Searching for the Bacterial Effector: The Example of the Multi-Skilled Commensal Bacterium Faecalibacterium prausnitzii. 2018;9(346).
200. Fouhy F, Deane J, Rea MC, O'Sullivan O, Ross RP, O'Callaghan G, et al. The effects of freezing on faecal microbiota as determined using MiSeq sequencing and culture-based investigations. PLoS One. 2015;10(3):e0119355.
201. Nocker A, Sossa-Fernandez P, Burr MD, Camper AK. Use of propidium monoazide for live/dead distinction in microbial ecology. Appl Environ Microbiol. 2007;73(16):5111-7.
202. Nocker A, Cheung CY, Camper AK. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J Microbiol Methods. 2006;67(2):310-20.
203. Bae S, Wuertz S. Discrimination of viable and dead fecal Bacteroidales bacteria by quantitative PCR with propidium monoazide. Appl Environ Microbiol. 2009;75(9):2940-4.
204. Rogers GB, Stressmann FA, Koller G, Daniels T, Carroll MP, Bruce KD. Assessing the diagnostic importance of nonviable bacterial cells in respiratory infections. Diagn Microbiol Infect Dis. 2008;62(2):133-41.

205. Cuthbertson L, Rogers GB, Walker AW, Oliver A, Hoffman LR, Carroll MP, et al. Implications of multiple freeze-thawing on respiratory samples for culture-independent analyses. J Cyst Fibros. 2015;14(4):464-7.
206. Varma M, Field R, Stinson M, Rukovets B, Wymer L, Haugland R. Quantitative real-time PCR analysis of total and propidium monoazide-resistant fecal indicator bacteria in wastewater. Water Res. 2009;43(19):4790-801.
207. Chu ND, Smith MB, Perrotta AR, Kassam Z, Alm EJ. Profiling Living Bacteria Informs Preparation of Fecal Microbiota Transplantations. PLoS One. 2017;12(1):e0170922.
208. Crum-Cianflone NF, Sullivan E, Ballon-Landa G. Fecal microbiota transplantation and successful resolution of multidrug-resistant-organism colonization. J Clin Microbiol. 2015;53(6):1986-9.
209. Singh R, van Nood E, Nieuwdorp M, van Dam B, ten Berge IJ, Geerlings SE, et al. Donor feces infusion for eradication of Extended Spectrum beta-Lactamase producing Escherichia coli in a patient with end stage renal disease. Clin Microbiol Infect. 2014;20(11):O977-8.
210. Stripling J, Kumar R, Baddley JW, Nellore A, Dixon P, Howard D, et al. Loss of Vancomycin-Resistant Enterococcus Fecal Dominance in an Organ Transplant Patient With Clostridium difficile Colitis After Fecal Microbiota Transplant. Open forum infectious diseases. 2015;2(2):ofv078-ofv.
211. Millan B, Park H, Hotte N, Mathieu O, Burguiere P, Tompkins TA, et al. Fecal Microbial Transplants Reduce Antibiotic-resistant Genes in Patients With Recurrent Clostridium difficile Infection. Clin Infect Dis. 2016;62(12):1479-86.
212. Tariq R, Pardi DS, Tosh PK, Walker RC, Razonable RR, Khanna S. Fecal Microbiota Transplantation for Recurrent Clostridium difficile Infection Reduces Recurrent Urinary Tract Infection Frequency. Clin Infect Dis. 2017;65(10):1745-7.
213. Bilinski J, Grzesiowski P, Sorensen N, Madry K, Muszynski J, Robak K, et al. Fecal Microbiota Transplantation in Patients With Blood Disorders Inhibits Gut Colonization With Antibiotic-Resistant Bacteria: Results of a Prospective, Single-Center Study. Clin Infect Dis. 2017;65(3):364-70.
214. Battipaglia G, Malard F, Rubio MT, Ruggeri A, Mamez AC, Brissot E, et al. Fecal microbiota transplantation before or after allogeneic hematopoietic transplantation in patients with hematologic malignancies carrying multidrug-resistance bacteria. Haematologica. 2019;104(8):1682-8.
215. Davido B, Batista R, Michelon H, Lepainteur M, Bouchand F, Lepeule R, et al. Is faecal microbiota transplantation an option to eradicate highly drug-resistant enteric bacteria carriage? J Hosp Infect. 2017;95(4):433-7.
216. Huttner BD, de Lastours V, Wassenberg M, Maharshak N, Mauris A, Galperine T, et al. A 5-day course of oral antibiotics followed by faecal transplantation to eradicate carriage of multidrug-resistant Enterobacteriaceae: a randomized clinical trial. Clin Microbiol Infect. 2019;25(7):830-8.
217. Ojima M, Motooka D, Shimizu K, Gotoh K, Shintani A, Yoshiya K, et al. Metagenomic Analysis Reveals Dynamic Changes of Whole Gut Microbiota in the Acute Phase of Intensive Care Unit Patients. Digestive diseases and sciences. 2016;61(6):1628-34.
218. DeFilipp Z, Bloom PP, Torres Soto M, Mansour MK, Sater MRA, Huntley MH, et al. Drug-Resistant E. coli Bacteremia Transmitted by Fecal Microbiota Transplant. N Engl J Med. 2019.
219. Wang S, Xu M, Wang W, Cao X, Piao M, Khan S, et al. Systematic Review: Adverse Events of Fecal Microbiota Transplantation. PLoS One. 2016;11(8):e0161174.

220. Quera R, Espinoza R, Estay C, Rivera D. Bacteremia as an adverse event of fecal microbiota transplantation in a patient with Crohn's disease and recurrent Clostridium difficile infection. J Crohns Colitis. 2014;8(3):252-3.
221. Sun W, Arunachalam A, Siddique S, Zandman D. Multi-Organism Bacteremia After Fecal Microbiota Transplantation for Relapsing Clostridium difficile Infection. Am J Gastroenterol. 2014;109:S420-S.
222. Iida N, Dzutsev A, Stewart CA, Smith L, Bouladoux N, Weingarten RA, et al. Commensal bacteria control cancer response to therapy by modulating the tumor microenvironment. Science. 2013;342(6161):967-70.
223. Sivan A, Corrales L, Hubert N, Williams JB, Aquino-Michaels K, Earley ZM, et al. Commensal Bifidobacterium promotes antitumor immunity and facilitates anti-PD-L1 efficacy. Science. 2015;350(6264):1084-9.
224. Zwielehner J, Lassl C, Hippe B, Pointner A, Switzeny OJ, Remely M, et al. Changes in human fecal microbiota due to chemotherapy analyzed by TaqMan-PCR, 454 sequencing and PCR-DGGE fingerprinting. PloS one. 2011;6(12):e28654-e.
225. Choo JM, Leong LE, Rogers GB. Sample storage conditions significantly influence faecal microbiome profiles. Sci Rep. 2015;5:16350.
226. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology. 2019;37(8):852-7.
227. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nature Methods. 2016;13(7):581-3.
228. Taylor SL, Leong LEX, Mobegi FM, Choo JM, Wesselingh S, Yang IA, et al. Long-Term Azithromycin Reduces Haemophilus influenzae and Increases Antibiotic Resistance in Severe Asthma. Am J Respir Crit Care Med. 2019;200(3):309-17.
229. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement. Methods for Dilution Antimicrobial Susceptibilty Tests for Bacteria that Grow Aerobically; Approved Standard- Ninth Edition. Wayne, PA, USA: Clinical and Laboratory Standards Institute; 2012.
230. Jones RB, Zhu X, Moan E, Murff HJ, Ness RM, Seidner DL, et al. Inter-niche and inter-individual variation in gut microbial community assessment using stool, rectal swab, and mucosal samples. Scientific Reports. 2018;8(1):4139.
231. Berg RD, Wommack E, Deitch EA. Immunosuppression and Intestinal Bacterial Overgrowth Synergistically Promote Bacterial Translocation. Archives of Surgery. 1988;123(11):1359-64.
232. Nejman D, Livyatan I, Fuks G, Gavert N, Zwang Y, Geller LT, et al. The human tumor microbiome is composed of tumor type-specific intracellular bacteria. Science. 2020;368(6494):973-80.
233. Gotts JE, Matthay MA. Sepsis: pathophysiology and clinical management. BMJ. 2016;353:i1585.
234. Dadgostar P. Antimicrobial Resistance: Implications and Costs. Infect Drug Resist. 2019;12:3903-10.
235. CARAlert annual report: 2019. Sydney Autralian Commission on Safety and Quality in Health Care; 2020.
236. Hayakawa K, Nakano R, Hase R, Shimatani M, Kato H, Hasumi J, et al. Comparison between IMP carbapenemase-producing Enterobacteriaceae and non-carbapenemase-producing Enterobacteriaceae: a multicentre prospective study of the clinical and molecular epidemiology of carbapenem-resistant Enterobacteriaceae. J Antimicrob Chemother. 2020;75(3):697-708.

237. Kang CI, Kim SH, Park WB, Lee KD, Kim HB, Kim EC, et al. Bloodstream infections caused by antibiotic-resistant gram-negative bacilli: risk factors for mortality and impact of inappropriate initial antimicrobial therapy on outcome. Antimicrob Agents Chemother. 2005;49(2):760-6.
238. Barza M, Giuliano M, Jacobus NV, Gorbach SL. Effect of broad-spectrum parenteral antibiotics on "colonization resistance" of intestinal microflora of humans. Antimicrob Agents Chemother. 1987;31(5):723-7.
239. Sorbara MT, Pamer EG. Interbacterial mechanisms of colonization resistance and the strategies pathogens use to overcome them. Mucosal Immunol. 2019;12(1):1-9.
240. Freedberg DE, Zhou MJ, Cohen ME, Annavajhala MK, Khan S, Moscoso DI, et al. Pathogen colonization of the gastrointestinal microbiome at intensive care unit admission and risk for subsequent death or infection. Intensive Care Med. 2018;44(8):1203-11.
241. Mancini N, Greco R, Pasciuta R, Barbanti MC, Pini G, Morrow OB, et al. Enteric Microbiome Markers as Early Predictors of Clinical Outcome in Allogeneic Hematopoietic Stem Cell Transplant: Results of a Prospective Study in Adult Patients. Open Forum Infect Dis. 2017;4(4):ofx215.
242. Lankelma JM, van Vught LA, Belzer C, Schultz MJ, van der Poll T, de Vos WM, et al. Critically ill patients demonstrate large interpersonal variation in intestinal microbiota dysregulation: a pilot study. Intensive Care Med. 2017;43(1):59-68.
243. Livanos AE, Snider EJ, Whittier S, Chong DH, Wang TC, Abrams JA, et al. Rapid gastrointestinal loss of Clostridial Clusters IV and XIVa in the ICU associates with an expansion of gut pathogens. PLoS One. 2018;13(8):e0200322.
244. Aardema H, Lisotto P, Kurilshikov A, Diepeveen JRJ, Friedrich AW, Sinha B, et al. Marked Changes in Gut Microbiota in Cardio-Surgical Intensive Care Patients: A Longitudinal Cohort Study. Front Cell Infect Microbiol. 2019;9:467.
245. Ravi A, Halstead FD, Bamford A, Casey A, Thomson NM, van Schaik W, et al. Loss of microbial diversity and pathogen domination of the gut microbiota in critically ill patients. Microbial genomics. 2019;5(9).
246. Donskey CJ, Ray AJ, Hoyen CK, Fuldauer PD, Aron DC, Salvator A, et al. Colonization and infection with multiple nosocomial pathogens among patients colonized with vancomycin-resistant Enterococcus. Infect Control Hosp Epidemiol. 2003;24(4):242-5.
247. Tosh PK, McDonald LC. Infection control in the multidrug-resistant era: tending the human microbiome. Clin Infect Dis. 2012;54(5):707-13.
248. Didelot X, Bowden R, Wilson DJ, Peto TEA, Crook DW. Transforming clinical microbiology with bacterial genome sequencing. Nat Rev Genet. 2012;13(9):601-12.
249. Quince C, Walker AW, Simpson JT, Loman NJ, Segata N. Shotgun metagenomics, from sampling to analysis. Nat Biotechnol. 2017;35(9):833-44.
250. Howard BM, Kornblith LZ, Christie SA, Conroy AS, Nelson MF, Campion EM, et al. Characterizing the gut microbiome in trauma: significant changes in microbial diversity occur early after severe injury. Trauma surgery & acute care open. 2017;2(1):e000108.
251. Zwielehner J, Lassl C, Hippe B, Pointner A, Switzeny OJ, Remely M, et al. Changes in human fecal microbiota due to chemotherapy analyzed by TaqMan-PCR, 454 sequencing and PCR-DGGE fingerprinting. PLoS One. 2011;6(12):e28654.
252. Buelow E, Bello González TDJ, Fuentes S, de Steenhuijsen Piters WAA, Lahti L, Bayjanov JR, et al. Comparative gut microbiota and resistome profiling of intensive care patients receiving selective digestive tract decontamination and healthy subjects. Microbiome. 2017;5(1):88-.
253. Biehl LM, Garzetti D, Farowski F, Ring D, Koeppel MB, Rohde H, et al. Usability of rectal swabs for microbiome sampling in a cohort study of hematological and oncological patients. PLOS ONE. 2019;14(4):e0215428.

254. Nadkarni MA, Martin FE, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology (Reading, England). 2002;148(Pt 1):257-66.
255. Fair K, Dunlap DG, Fitch A, Bogdanovich T, Methé B, Morris A, et al. Rectal Swabs from Critically Ill Patients Provide Discordant Representations of the Gut Microbiome Compared to Stool Samples. mSphere. 2019;4(4).
256. Bassis CM, Moore NM, Lolans K, Seekatz AM, Weinstein RA, Young VB, et al. Comparison of stool versus rectal swab samples and storage conditions on bacterial community profiles. BMC Microbiology. 2017;17(1):78.
257. Dubberke ER, Mullane KM, Gerding DN, Lee CH, Louie TJ, Guthertz H, et al. Clearance of Vancomycin-Resistant Enterococcus Concomitant With Administration of a Microbiota-Based Drug Targeted at Recurrent Clostridium difficile Infection. Open Forum Infectious Diseases. 2016;3(3).
258. Reyman M, van Houten MA, Arp K, Sanders EAM, Bogaert D. Rectal swabs are a reliable proxy for faecal samples in infant gut microbiota research based on 16S-rRNA sequencing. Scientific Reports. 2019;9(1):16072.
259. Bokulich NA, Maldonado J, Kang D-W, Krajmalnik-Brown R, Caporaso JG. Rapidly Processed Stool Swabs Approximate Stool Microbiota Profiles. mSphere. 2019;4(2):e00208-19.
260. Budding AE, Grasman ME, Eck A, Bogaards JA, Vandenbroucke-Grauls CM, van Bodegraven AA, et al. Rectal swabs for analysis of the intestinal microbiota. PLoS One. 2014;9(7):e101344.
261. Gorkiewicz G, Thallinger GG, Trajanoski S, Lackner S, Stocker G, Hinterleitner T, et al. Alterations in the colonic microbiota in response to osmotic diarrhea. PloS one. 2013;8(2):e55817-e.
262. Costello SP, Soo W, Bryant RV, Jairath V, Hart AL, Andrews JM. Systematic review with meta-analysis: faecal microbiota transplantation for the induction of remission for active ulcerative colitis. Aliment Pharmacol Ther. 2017;46(3):213-24.
263. Khoruts A, Dicksved J, Jansson JK, Sadowsky MJ. Changes in the composition of the human fecal microbiome after bacteriotherapy for recurrent Clostridium difficile-associated diarrhea. J Clin Gastroenterol. 2010;44(5):354-60.
264. Seekatz AM, Aas J, Gessert CE, Rubin TA, Saman DM, Bakken JS, et al. Recovery of the gut microbiome following fecal microbiota transplantation. MBio. 2014;5(3):e00893-14.
265. Australia New Zealand Clinical Trials Registry. Gastrointestinal eradication of multi-resistant gram negative bacteria by faecal microbiota transplantation (FMT) 2018 [Available from: https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?id=371671&isReview=true.
266. Costello SP, Tucker EC, La Brooy J, Schoeman MN, Andrews JM. Establishing a Fecal Microbiota Transplant Service for the Treatment of Clostridium difficile Infection. Clin Infect Dis. 2016;62(7):908-14.
267. Nkuipou-Kenfack E, Engel H, Fakih S, Nocker A. Improving efficiency of viability-PCR for selective detection of live cells. J Microbiol Methods. 2013;93(1):20-4.
268. Maheux AF, Picard FJ, Boissinot M, Bissonnette L, Paradis S, Bergeron MG. Analytical comparison of nine PCR primer sets designed to detect the presence of Escherichia coli/Shigella in water samples. Water Res. 2009;43(12):3019-28.
269. Denman SE, McSweeney CS. Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. Fems Microbiology Ecology. 2006;58(3):572-82.

270. Feizabadi MM, Majnooni A, Nomanpour B, Fatolahzadeh B, Raji N, Delfani S, et al. Direct detection of Pseudomonas aeruginosa from patients with healthcare associated pneumonia by real time PCR. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases. 2010;10(8):1247-51.
271. Thomas LC, Gidding HF, Ginn AN, Olma T, Iredell J. Development of a real-time Staphylococcus aureus and MRSA (SAM-) PCR for routine blood culture. Journal of microbiological methods. 2007;68(2):296-302.
272. Monteiro L, Bonnemaison D, Vekris A, Petry KG, Bonnet J, Vidal R, et al. Complex polysaccharides as PCR inhibitors in feces: Helicobacter pylori model. J Clin Microbiol. 1997;35(4):995-8.
273. Nebe-von-Caron G, Stephens PJ, Hewitt CJ, Powell JR, Badley RA. Analysis of bacterial function by multi-colour fluorescence flow cytometry and single cell sorting. J Microbiol Methods. 2000;42(1):97-114.
274. Young GR, Smith DL, Embleton ND, Berrington JE, Schwalbe EC, Cummings SP, et al. Reducing Viability Bias in Analysis of Gut Microbiota in Preterm Infants at Risk of NEC and Sepsis. Front Cell Infect Microbiol. 2017;7:237.
275. Honda K, Littman DR. The microbiota in adaptive immune homeostasis and disease. Nature. 2016;535(7610):75-84.
276. Fuentes S, Rossen NG, van der Spek MJ, Hartman JH, Huuskonen L, Korpela K, et al. Microbial shifts and signatures of long-term remission in ulcerative colitis after faecal microbiota transplantation. ISME J. 2017;11(8):1877-89.
277. Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Acad Sci U S A. 2007;104(34):13780-5.
278. Kostic AD, Xavier RJ, Gevers D. The microbiome in inflammatory bowel disease: current status and the future ahead. Gastroenterology. 2014;146(6):1489-99.
279. Wang T, Cai G, Qiu Y, Fei N, Zhang M, Pang X, et al. Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. ISME J. 2012;6(2):320-9.
280. Qin J, Li Y, Cai Z, Li S, Zhu J, Zhang F, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature. 2012;490(7418):55-60.
281. Remely M, Aumueller E, Merold C, Dworzak S, Hippe B, Zanner J, et al. Effects of short chain fatty acid producing bacteria on epigenetic regulation of FFAR3 in type 2 diabetes and obesity. Gene. 2014;537(1):85-92.
282. Jiang H, Ling Z, Zhang Y, Mao H, Ma Z, Yin Y, et al. Altered fecal microbiota composition in patients with major depressive disorder. Brain Behav Immun. 2015;48:186-94.
283. Segal JP, Abbasi F, Kanagasundaram C, Hart A. Does the Internet promote the unregulated use of fecal microbiota transplantation: a potential public health issue? Clin Exp Gastroenterol. 2018;11:179-83.
284. Cammarota G, Ianiro G, Tilg H, Rajilic-Stojanovic M, Kump P, Satokari R, et al. European consensus conference on faecal microbiota transplantation in clinical practice. Gut. 2017;66(4):569-80.
285. Trubiano JA, Cheng AC, Korman TM, Roder C, Campbell A, May ML, et al. Australasian Society of Infectious Diseases updated guidelines for the management of Clostridium difficile infection in adults and children in Australia and New Zealand. Intern Med J. 2016;46(4):479-93.
286. Mullish BH, Quraishi MN, Segal JP, McCune VL, Baxter M, Marsden GL, et al. The use of faecal microbiota transplant as treatment for recurrent or refractory Clostridium

difficile infection and other potential indications: joint British Society of Gastroenterology (BSG) and Healthcare Infection Society (HIS) guidelines. Gut. 2018;67(11):1920-41.
287. Lee CH, Steiner T, Petrof EO, Smieja M, Roscoe D, Nematallah A, et al. Frozen vs Fresh Fecal Microbiota Transplantation and Clinical Resolution of Diarrhea in Patients With Recurrent Clostridium difficile Infection: A Randomized Clinical Trial. JAMA. 2016;315(2):142-9.
288. Youngster I, Russell GH, Pindar C, Ziv-Baran T, Sauk J, Hohmann EL. Oral, capsulized, frozen fecal microbiota transplantation for relapsing Clostridium difficile infection. JAMA. 2014;312(17):1772-8.
289. Rigottier-Gois L. Dysbiosis in inflammatory bowel diseases: the oxygen hypothesis. ISME J. 2013;7(7):1256-61.
290. Papanicolas LE, Wang Y, Choo JM, Gordon DL, Wesselingh SL, Rogers GB. Optimisation of a propidium monoazide based method to determine the viability of microbes in faecal slurries for transplantation. Journal of Microbiological Methods. 2019;156:40-5.
291. Wang Y, Leong LEX, Keating RL, Kanno T, Abell GCJ, Mobegi FM, et al. Opportunistic bacteria confer the ability to ferment prebiotic starch in the adult cystic fibrosis gut. Gut Microbes. 2018:1-15.
292. Louis P, Young P, Holtrop G, Flint HJ. Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA:acetate CoA-transferase gene. Environ Microbiol. 2010;12(2):304-14.
293. IDSA. Fecal Microbiota Transplant (FMT) Protocol by Enema for Patients with Recurrent Clostridium difficile Infection. 2013.
294. McNabney SM, Henagan TM. Short Chain Fatty Acids in the Colon and Peripheral Tissues: A Focus on Butyrate, Colon Cancer, Obesity and Insulin Resistance. Nutrients. 2017;9(12).
295. Dominianni C, Wu J, Hayes RB, Ahn J. Comparison of methods for fecal microbiome biospecimen collection. BMC Microbiol. 2014;14:103.
296. Flores R, Shi J, Yu G, Ma B, Ravel J, Goedert JJ, et al. Collection media and delayed freezing effects on microbial composition of human stool. Microbiome. 2015;3:33.
297. Tedjo DI, Jonkers DM, Savelkoul PH, Masclee AA, van Best N, Pierik MJ, et al. The effect of sampling and storage on the fecal microbiota composition in healthy and diseased subjects. PLoS One. 2015;10(5):e0126685.
298. Helstad AG, Kimball JL, Maki DG. Recovery of anaerobic, facultative, and aerobic bacteria from clinical specimens in three anaerobic transport systems. J Clin Microbiol. 1977;5(6):564-9.
299. Justesen T, Jensen AM, Hoffmann S. The survival of anaerobic bacteria at 4 degrees C and 22 degrees C on swabs in three transport systems. Statistical evaluation by application of a variance component model. Acta Pathol Microbiol Immunol Scand B. 1983;91(1):17-22.
300. Hill GB. Effects of storage in an anaerobic transport system on bacteria in known polymicrobial mixtures and in clinical specimens. J Clin Microbiol. 1978;8(6):680-8.
301. Organisation WH. Antimicrobial Resistance Global Report on Surveillance. 2014.
302. Pitout JD, Laupland KB. Extended-spectrum beta-lactamase-producing Enterobacteriaceae: an emerging public-health concern. The Lancet Infectious diseases. 2008;8(3):159-66.
303. Nordmann P, Naas T, Poirel L. Global spread of Carbapenemase-producing Enterobacteriaceae. Emerging infectious diseases. 2011;17(10):1791-8.
304. A F, S E. Use of stool transplantation to clear fecal colonization with carbapenem-resistant Enterobacteriaceae (CRE): proof of concept, abstr 1805. . Abstr Soc Healthcare Epidemiol AM (SHEA) IDWeek 2014, 11 October 2014, Philadelphia, PA. 2014.

305. Kim S, Covington A, Pamer EG. The intestinal microbiota: Antibiotics, colonization resistance, and enteric pathogens. Immunol Rev. 2017;279(1):90-105.
306. Magruder M, Sholi AN, Gong C, Zhang L, Edusei E, Huang J, et al. Gut uropathogen abundance is a risk factor for development of bacteriuria and urinary tract infection. Nature communications. 2019;10(1):5521-.
307. Thänert R, Reske KA, Hink T, Wallace MA, Wang B, Schwartz DJ, et al. Comparative Genomics of Antibiotic-Resistant Uropathogens Implicates Three Routes for Recurrence of Urinary Tract Infections. mBio. 2019;10(4).
308. Concato J, Shah N, Horwitz RI. Randomized, controlled trials, observational studies, and the hierarchy of research designs. N Engl J Med. 2000;342(25):1887-92.
309. Papanicolas LE, Choo JM, Wang Y, Leong LEX, Costello SP, Gordon DL, et al. Bacterial viability in faecal transplants: Which bacteria survive? EBioMedicine. 2019;41:509-16.
310. Peleg AY, Hooper DC. Hospital-acquired infections due to gram-negative bacteria. N Engl J Med. 2010;362(19):1804-13.
311. Bryan CS, Reynolds KL, Brenner ER. Analysis of 1,186 episodes of gram-negative bacteremia in non-university hospitals: the effects of antimicrobial therapy. Reviews of infectious diseases. 1983;5(4):629-38.
312. Baxter M, Ahmad T, Colville A, Sheridan R. Fatal Aspiration Pneumonia as a Complication of Fecal Microbiota Transplant. Clin Infect Dis. 2015;61(1):136-7.
313. Kelly CR, Ihunnah C, Fischer M, Khoruts A, Surawicz C, Afzali A, et al. Fecal microbiota transplant for treatment of Clostridium difficile infection in immunocompromised patients. Am J Gastroenterol. 2014;109(7):1065-71.
314. Case 25-2014: A Man with Ulcerative Colitis and Bloody Diarrhea. New England Journal of Medicine. 2014;371(19):1848-9.
315. Schwartz M, Gluck M, Koon S. Norovirus gastroenteritis after fecal microbiota transplantation for treatment of Clostridium difficile infection despite asymptomatic donors and lack of sick contacts. Am J Gastroenterol. 2013;108(8):1367.
316. Brandt LJ, Aroniadis OC, Mellow M, Kanatzar A, Kelly C, Park T, et al. Long-term follow-up of colonoscopic fecal microbiota transplant for recurrent Clostridium difficile infection. The American journal of gastroenterology. 2012;107(7):1079-87.
317. Vrieze A, Van Nood E, Holleman F, Salojarvi J, Kootte RS, Bartelsman JF, et al. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. Gastroenterology. 2012;143(4):913-6 e7.
318. Zaneveld JR, McMinds R, Vega Thurber R. Stress and stability: applying the Anna Karenina principle to animal microbiomes. Nature Microbiology. 2017;2(9):17121.
319. Yamamoto S, Tsukamoto T, Terai A, Kurazono H, Takeda Y, Yoshida O. Genetic evidence supporting the fecal-perineal-urethral hypothesis in cystitis caused by Escherichia coli. J Urol. 1997;157(3):1127-9.
320. Chen SL, Wu M, Henderson JP, Hooton TM, Hibbing ME, Hultgren SJ, et al. Genomic diversity and fitness of E. coli strains recovered from the intestinal and urinary tracts of women with recurrent urinary tract infection. Sci Transl Med. 2013;5(184):184ra60.
321. Wust J. In vitro susceptibility of anaerobes to co-trimoxazole. Infection. 1980;8 Suppl 2:S187-9.
322. Policy statement on antimicrobial stewardship by the Society for Healthcare Epidemiology of America (SHEA), the Infectious Diseases Society of America (IDSA), and the Pediatric Infectious Diseases Society (PIDS). Infection control and hospital epidemiology. 2012;33(4):322-7.

323. Rahal JJ, Urban C, Horn D, Freeman K, Segal-Maurer S, Maurer J, et al. Class restriction of cephalosporin use to control total cephalosporin resistance in nosocomial Klebsiella. JAMA. 1998;280(14):1233-7.
324. Antimicrobial Stewardship in Australian Health Care 2018. Sydney: ACSQHC; 2018.
325. Papanicolas LE, Nelson R, Warner MS. Influence of antimicrobial susceptibility reporting on junior doctors' decision to prescribe antimicrobials inappropriately. Journal of Antimicrobial Chemotherapy. 2016;72(4):1202-5.
326. Daley P, Garcia D, Inayatullah R, Penney C, Boyd S. Modified Reporting of Positive Urine Cultures to Reduce Inappropriate Treatment of Asymptomatic Bacteriuria Among Nonpregnant, Noncatheterized Inpatients: A Randomized Controlled Trial. Infection Control & Hospital Epidemiology. 2018;39(7):814-9.
327. Khumra S, Mahony AA, Bergen PJ, Elliott RA. Evaluation of intravenous to oral antimicrobial switch at a hospital with a tightly regulated antimicrobial stewardship program. British journal of clinical pharmacology. 2021.
328. Ory EY, EM. The Use and Abuse of the Broad Spectrum Antibiotics. JAMA. 1963;185(4):273-9.
329. Tally FP, Sutter VL, Finegold SM. Metronidazole versus anaerobes. In vitro data and initial clinical observations. Calif Med. 1972;117(6):22-6.
330. Gupta K, Hooton TM, Naber KG, Wullt B, Colgan R, Miller LG, et al. International clinical practice guidelines for the treatment of acute uncomplicated cystitis and pyelonephritis in women: A 2010 update by the Infectious Diseases Society of America and the European Society for Microbiology and Infectious Diseases. Clin Infect Dis. 2011;52(5):e103-20.
331. Liu C, Bayer A, Cosgrove SE, Daum RS, Fridkin SK, Gorwitz RJ, et al. Clinical Practice Guidelines by the Infectious Diseases Society of America for the Treatment of Methicillin-Resistant Staphylococcus aureus Infections in Adults and Children. Clinical Infectious Diseases. 2011;52(3):e18-e55.
332. Shono Y, Docampo MD, Peled JU, Perobelli SM, Velardi E, Tsai JJ, et al. Increased GVHD-related mortality with broad-spectrum antibiotic use after allogeneic hematopoietic stem cell transplantation in human patients and mice. Sci Transl Med. 2016;8(339):339ra71-ra71.
333. Bush K. Past and Present Perspectives on β-Lactamases. Antimicrobial agents and chemotherapy. 2018;62(10):e01076-18.
334. Tarkkanen AM, Heinonen T, Jogi R, Mentula S, van der Rest ME, Donskey CJ, et al. P1A recombinant beta-lactamase prevents emergence of antimicrobial resistance in gut microflora of healthy subjects during intravenous administration of ampicillin. Antimicrob Agents Chemother. 2009;53(6):2455-62.
335. Kokai-Kun JF, Roberts T, Coughlin O, Sicard E, Rufiange M, Fedorak R, et al. The Oral beta-Lactamase SYN-004 (Ribaxamase) Degrades Ceftriaxone Excreted into the Intestine in Phase 2a Clinical Studies. Antimicrob Agents Chemother. 2017;61(3).
336. Connelly S, Fanelli B, Hasan NA, Colwell RR, Kaleko M. SYN-007, an Orally Administered Beta-Lactamase Enzyme, Protects the Gut Microbiome from Oral Amoxicillin/Clavulanate without Adversely Affecting Antibiotic Systemic Absorption in Dogs. Microorganisms. 2020;8(2).
337. Gunzburg JD, Ghozlane A, Ducher A, Duval X, Ruppé E, Pulse M, et al. DAV132, an Adsorbent-Based Product, Protects the Gut Microbiome and Prevents Clostridium difficile Infections During Moxifloxacin Treatments. Open Forum Infectious Diseases. 2015;2(suppl_1).
338. Bartlett JG. Clinical practice. Antibiotic-associated diarrhea. N Engl J Med. 2002;346(5):334-9.

339. Gustafsson A, Lund-Tønnesen S, Berstad A, Midtvedt T, Norin E. Faecal short-chain fatty acids in patients with antibiotic-associated diarrhoea, before and after faecal enema treatment. Scand J Gastroenterol. 1998;33(7):721-7.
340. Olek A, Woynarowski M, Ahrén IL, Kierkuś J, Socha P, Larsson N, et al. Efficacy and Safety of Lactobacillus plantarum DSM 9843 (LP299V) in the Prevention of Antibiotic-Associated Gastrointestinal Symptoms in Children-Randomized, Double-Blind, Placebo-Controlled Study. The Journal of pediatrics. 2017;186:82-6.
341. Allen SJ, Wareham K, Wang D, Bradley C, Hutchings H, Harris W, et al. Lactobacilli and bifidobacteria in the prevention of antibiotic-associated diarrhoea and Clostridium difficile diarrhoea in older inpatients (PLACIDE): a randomised, double-blind, placebo-controlled, multicentre trial. Lancet. 2013;382(9900):1249-57.
342. Hempel S, Newberry SJ, Maher AR, Wang Z, Miles JNV, Shanman R, et al. Probiotics for the Prevention and Treatment of Antibiotic-Associated Diarrhea: A Systematic Review and Meta-analysis. JAMA. 2012;307(18):1959-69.
343. Vrieze A, Out C, Fuentes S, Jonker L, Reuling I, Kootte RS, et al. Impact of oral vancomycin on gut microbiota, bile acid metabolism, and insulin sensitivity. J Hepatol. 2014;60(4):824-31.
344. Dethlefsen L, Relman DA. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. 2011;108(Supplement 1):4554-61.
345. Chua LL, Rajasuriar R, Azanan MS, Abdullah NK, Tang MS, Lee SC, et al. Reduced microbial diversity in adult survivors of childhood acute lymphoblastic leukemia and microbial associations with increased immune activation. Microbiome. 2017;5(1):35.
346. Fujimura KE, Lynch SV. Microbiota in allergy and asthma and the emerging relationship with the gut microbiome. Cell Host Microbe. 2015;17(5):592-602.
347. Milani C, Duranti S, Bottacini F, Casey E, Turroni F, Mahony J, et al. The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. Microbiology and Molecular Biology Reviews. 2017;81(4):e00036-17.
348. Robinson J. Cochrane in context: probiotics for prevention of necrotizing enterocolitis in preterm infants. Evidence-based child health : a Cochrane review journal. 2014;9(3):672-4.
349. Panigrahi P, Parida S, Nanda NC, Satpathy R, Pradhan L, Chandel DS, et al. A randomized synbiotic trial to prevent sepsis among infants in rural India. Nature. 2017;548(7668):407-12.
350. Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. The Journal of nutrition. 1995;125(6):1401-12.
351. Fuentes-Zaragoza E, Sanchez-Zapata E, Sendra E, Sayas E, Navarro C, Fernandez-Lopez J, et al. Resistant starch as prebiotic: A review. Starch-Starke. 2011;63(7):406-15.
352. Li SS, Zhu A, Benes V, Costea PI, Hercog R, Hildebrand F, et al. Durable coexistence of donor and recipient strains after fecal microbiota transplantation. Science. 2016;352(6285):586-9.
353. Banerjee S, Schlaeppi K, van der Heijden MGA. Keystone taxa as drivers of microbiome structure and functioning. Nat Rev Microbiol. 2018;16(9):567-76.
354. Kang JD, Myers CJ, Harris SC, Kakiyama G, Lee IK, Yun BS, et al. Bile Acid 7α-Dehydroxylating Gut Bacteria Secrete Antibiotics that Inhibit Clostridium difficile: Role of Secondary Bile Acids. Cell chemical biology. 2019;26(1):27-34.e4.
355. Kim SG, Becattini S, Moody TU, Shliaha PV, Littmann ER, Seok R, et al. Microbiota-derived lantibiotic restores resistance against vancomycin-resistant Enterococcus. Nature. 2019;572(7771):665-9.

356. Rakoff-Nahoum S, Foster KR, Comstock LE. The evolution of cooperation within the gut microbiota. Nature. 2016;533(7602):255-9.
357. Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC, et al. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. Cell Host Microbe. 2007;2(3):204.
358. Winter SE, Thiennimitr P, Winter MG, Butler BP, Huseby DL, Crawford RW, et al. Gut inflammation provides a respiratory electron acceptor for Salmonella. Nature. 2010;467(7314):426-9.
359. Wandro S, Osborne S, Enriquez C, Bixby C, Arrieta A, Whiteson K. The Microbiome and Metabolome of Preterm Infant Stool Are Personalized and Not Driven by Health Outcomes, Including Necrotizing Enterocolitis and Late-Onset Sepsis. mSphere. 2018;3(3).
360. Craven LJ, Nair Parvathy S, Tat-Ko J, Burton JP, Silverman MS. Extended Screening Costs Associated With Selecting Donors for Fecal Microbiota Transplantation for Treatment of Metabolic Syndrome-Associated Diseases. Open Forum Infect Dis. 2017;4(4):ofx243.
361. Brook I, Wexler HM, Goldstein EJ. Antianaerobic antimicrobials: spectrum and susceptibility testing. Clin Microbiol Rev. 2013;26(3):526-46.
362. Falagas ME, Vouloumanou EK, Samonis G, Vardakas KZ. Fosfomycin. Clin Microbiol Rev. 2016;29(2):321-47.
363. Ubeda C, Bucci V, Caballero S, Djukovic A, Toussaint NC, Equinda M, et al. Intestinal microbiota containing Barnesiella species cures vancomycin-resistant Enterococcus faecium colonization. Infect Immun. 2013;81(3):965-73.
364. Caballero S, Kim S, Carter RA, Leiner IM, Susac B, Miller L, et al. Cooperating Commensals Restore Colonization Resistance to Vancomycin-Resistant Enterococcus faecium. Cell Host Microbe. 2017;21(5):592-602 e4.
365. Desai MS, Seekatz AM, Koropatkin NM, Kamada N, Hickey CA, Wolter M, et al. A Dietary Fiber-Deprived Gut Microbiota Degrades the Colonic Mucus Barrier and Enhances Pathogen Susceptibility. Cell. 2016;167(5):1339-53 e21.
366. Wang Y, Leong LEX, Keating RL, Kanno T, Abell GCJ, Mobegi FM, et al. Opportunistic bacteria confer the ability to ferment prebiotic starch in the adult cystic fibrosis gut. Gut Microbes. 2019;10(3):367-81.
367. Tvede M, Tinggaard M, Helms M. Rectal bacteriotherapy for recurrent Clostridium difficile-associated diarrhoea: results from a case series of 55 patients in Denmark 2000-2012. Clin Microbiol Infect. 2015;21(1):48-53.
368. Petrof EO, Gloor GB, Vanner SJ, Weese SJ, Carter D, Daigneault MC, et al. Stool substitute transplant therapy for the eradication of Clostridium difficile infection: 'RePOOPulating' the gut. Microbiome. 2013;1(1):3.
369. Dubberke ER, Lee CH, Orenstein R, Khanna S, Hecht G, Gerding DN. Results From a Randomized, Placebo-Controlled Clinical Trial of a RBX2660-A Microbiota-Based Drug for the Prevention of Recurrent Clostridium difficile Infection. Clin Infect Dis. 2018;67(8):1198-204.
370. McGovern BH, Ford CB, Henn MR, Pardi DS, Khanna S, Hohmann EL, et al. SER-109, an Investigational Microbiome Drug to Reduce Recurrence after Clostridioides difficile infection: Lessons Learned from a Phase 2 Trial. Clinical Infectious Diseases. 2020.
371. Blount KF, Shannon WD, Deych E, Jones C. Restoration of Bacterial Microbiome Composition and Diversity Among Treatment Responders in a Phase 2 Trial of RBX2660: An Investigational Microbiome Restoration Therapeutic. Open Forum Infectious Diseases. 2019;6(4).
372. Baden LR, Thiemke W, Skolnik A, Chambers R, Strymish J, Gold HS, et al. Prolonged colonization with vancomycin-resistant Enterococcus faecium in long-term care patients and the significance of "clearance". Clin Infect Dis. 2001;33(10):1654-60.

373. Cully M. Microbiome therapeutics go small molecule. Nature reviews Drug discovery. 2019;18(8):569-72.
374. Parada Venegas D, De la Fuente MK, Landskron G, González MJ, Quera R, Dijkstra G, et al. Short Chain Fatty Acids (SCFAs)-Mediated Gut Epithelial and Immune Regulation and Its Relevance for Inflammatory Bowel Diseases. Front Immunol. 2019;10:277-.
375. Steinhart AH, Hiruki T, Brzezinski A, Baker JP. Treatment of left-sided ulcerative colitis with butyrate enemas: a controlled trial. Aliment Pharmacol Ther. 1996;10(5):729-36.
376. Breuer RI, Soergel KH, Lashner BA, Christ ML, Hanauer SB, Vanagunas A, et al. Short chain fatty acid rectal irrigation for left-sided ulcerative colitis: a randomised, placebo controlled trial. Gut. 1997;40(4):485-91.
377. Scheppach W. Treatment of distal ulcerative colitis with short-chain fatty acid enemas. A placebo-controlled trial. German-Austrian SCFA Study Group. Digestive diseases and sciences. 1996;41(11):2254-9.
378. Mazmanian SK, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. Nature. 2008;453(7195):620-5.
379. Mydock-McGrane LK, Hannan TJ, Janetka JW. Rational design strategies for FimH antagonists: new drugs on the horizon for urinary tract infection and Crohn's disease. Expert Opin Drug Discov. 2017;12(7):711-31.
380. Forster SC, Kumar N, Anonye BO, Almeida A, Viciani E, Stares MD, et al. A human gut bacterial genome and culture collection for improved metagenomic analyses. Nat Biotechnol. 2019;37(2):186-92.
381. Galloway-Peña JR, Shi Y, Peterson CB, Sahasrabhojane P, Gopalakrishnan V, Brumlow CE, et al. Gut Microbiome Signatures Are Predictive of Infectious Risk Following Induction Therapy for Acute Myeloid Leukemia. Clinical Infectious Diseases. 2019;71(1):63-71.
382. Jouhten H, Mattila E, Arkkila P, Satokari R. Reduction of Antibiotic Resistance Genes in Intestinal Microbiota of Patients With Recurrent Clostridium difficile Infection After Fecal Microbiota Transplantation. Clinical Infectious Diseases. 2016;63(5):710-1.
383. Ianiro G, Murri R, Sciume GD, Impagnatiello M, Masucci L, Ford AC, et al. Incidence of Bloodstream Infections, Length of Hospital Stay, and Survival in Patients With Recurrent Clostridioides difficile Infection Treated With Fecal Microbiota Transplantation or Antibiotics: A Prospective Cohort Study. Ann Intern Med. 2019.
384. Roager HM, Licht TR, Poulsen SK, Larsen TM, Bahl MI. Microbial enterotypes, inferred by the prevotella-to-bacteroides ratio, remained stable during a 6-month randomized controlled diet intervention with the new nordic diet. Appl Environ Microbiol. 2014;80(3):1142-9.
385. Ramirez-Farias C, Slezak K, Fuller Z, Duncan A, Holtrop G, Louis P. Effect of inulin on the human gut microbiota: stimulation of Bifidobacterium adolescentis and Faecalibacterium prausnitzii. Br J Nutr. 2009;101(4):541-50.
386. Rinttila T, Kassinen A, Malinen E, Krogius L, Palva A. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J Appl Microbiol. 2004;97(6):1166-77.
387. Louis P, Flint HJ. Development of a semiquantitative degenerate real-time pcr-based assay for estimation of numbers of butyryl-coenzyme A (CoA) CoA transferase genes in complex bacterial samples. Appl Environ Microbiol. 2007;73(6):2009-12.
388. Huijsdens XW, Linskens RK, Mak M, Meuwissen SG, Vandenbroucke-Grauls CM, Savelkoul PH. Quantification of bacteria adherent to gastrointestinal mucosa by real-time PCR. J Clin Microbiol. 2002;40(12):4423-7.