

Understanding the role of gut microbiota in protecting the host against intestinal colonisation by vancomycin-resistant enterococci

Olivia Grace King

A Thesis Presented in Partial Fulfilment of the Requirements for the
Degree of Doctor of Philosophy

Department of Infectious Diseases
Faculty of Medicine
Imperial College London
January 2024

Copyright Declaration

The copyright of this thesis rests with the author. Unless otherwise indicated, its contents are licensed under a Creative Commons Attribution-Non-Commercial 4.0 International Licence (CC BY-NC).

Under this licence, you may copy and redistribute the material in any medium or format. You may also create and distribute modified versions of the work. This is on the condition that: you credit the author and do not use it, or any derivative works, for a commercial purpose.

When reusing or sharing this work, ensure you make the licence terms clear to others by naming the licence and linking to the licence text. Where a work has been adapted, you should indicate that the work has been changed and describe those changes.

Please seek permission from the copyright holder for uses of this work that are not included in this licence or permitted under UK Copyright Law.

Statement of originality

I hereby declare that the research submitted in this thesis is my own, completed under the supervision of Dr Julie McDonald within the Department of Infectious Diseases at Imperial College London. Any work performed, resources contributed, or ideas shared by others have been appropriately credited, referenced, or acknowledged.

Acknowledgement

I would like to start by thanking Dr Julie McDonald for her endless enthusiasm, patience, and encouragement over the course of the last three years. The collaborative and supportive environment she has created in her lab group fosters multidisciplinary discussions, collective troubleshooting and reliable team support. She has always striven to prioritise a safe and healthy workplace which fostered creativity and success of each member of her team. Within the McDonald lab, I would like to thank my fellow PhD student, Alex Yip, for sharing weekend lab hours as well as optimising and sharing data analysis scripts and programs. Alex aided in weekend sampling for the artificial gut chemostat, methodology optimisation, undergraduate/master's student supervision and sample collection/delivery. Likewise, Dr Vicky Horrocks has been an asset for data analysis, experimental support and general degree advice. Her development of the gut commensal minimal media used in Chapter 6 was vital to the success of the commensal consortium research. Additionally, her initial profiling of monomer contamination in complex nutrients informed data analysis.

Dr Julian Marchesi's lab at the St Marys campus provided technical support for metataxonomic analysis of data in this study. Dr Marchesi provided funding for one of the three artificial gut chemostat experiment replicates included in this study in exchange for chemostat culture samples. I would like to thank Dr Nathan Danckert and Dr Jesus Miguens Blanco from the Marchesi lab who ran 16S rRNA gene sequencing with the MiSeq and Illumina platform, providing sequencing files for the faecal batch culture samples in Chapter 3. Additional thanks to Dr Marko Storch, from the London Biofoundry, who ran 16S rRNA gene sequencing with PacBio for the artificial gut chemostat model samples in Chapter 5.

Dr Tom Clarke collaborated on the design of the mouse study included in Chapter 5 and Chapter 6 and performed all the direct mouse work. The administration of antibiotics, *E. faecium*, and the commensal consortium to the mice as well as the collection of faecal samples was performed by Dr Clarke.

I would like to thank Dr Ben Mullish at the St Marys campus for helping arrange faecal sample donors for the faecal batch culture experiment. Additionally, a resounding thank you to all donors who agreed to participate in this study.

Dr Yingqi Xu assisted with the use of the ^1H NMR spectroscope by setting up the machine protocols, initiating sample reruns, troubleshooting alignment issues, and transferring raw files.

Further, I would like to thank Dr Shiranee Sriskandan and Dr Gerald Larrouy-Maumus for serving on my PhD advisory panel during my PhD. Their thoughtful questioning and insights guided my research over the last three years and I am grateful for their time and effort.

Thanks to Dr Ramesh Wigneshweraraj for providing administrative support and supervision over the course of my degree. And finally, thank you to Dr David MacIntyre and Dr James Mason for their thoughtful and thorough examination of my thesis.

Abstract

The gut is the primary reservoir for vancomycin-resistant *Enterococcus* (VRE), a multidrug-resistant pathogen which can cause severe infections, including endocarditis, bloodstream, intra-abdominal, and urinary tract infections. Broad-spectrum antibiotics kill members of the gut microbiota essential for maintaining colonisation resistance against VRE in the intestine. It was hypothesised that antibiotics disrupt microbiota-mediated colonisation resistance which can be restored through the reintroduction of key members of the gut microbiota to reestablish nutrient competition and/or metabolite inhibition against VRE. This study aimed to identify how antibiotic treatment impacted microbiota diversity and density, nutrient and microbial metabolite availability, and VRE colonisation. Faecal cultures were used to test the effects of antibiotic treatment and VRE intestinal colonisation on changes in microbiota taxa (16S rRNA gene sequencing), nutrients and microbial metabolites (¹H-NMR spectroscopy). Faecal microbiota treated with broad-spectrum antibiotics which promote VRE colonisation caused a reduction in the abundance of the *Bacteroidaceae*, *Bifidobacteriaceae*, and *Coriobacteriaceae* families. These antibiotics caused an increase in nutrient availability and a decrease in the production of microbial metabolites. This study showed that nutrients elevated following antibiotic treatment supported high levels of VRE growth. These findings were supported by longitudinal culturing of faecal microbiota using an artificial gut model, which showed persistent growth of vancomycin-resistant *E. faecium* in vancomycin-treated faecal cultures. Administration of faecal microbiota transplants to antibiotic-treated faecal cultures reestablished colonisation resistance and restricted vancomycin-resistant *E. faecium* growth. A commensal consortium designed to deplete nutrients utilised by VRE was able to successfully restrict VRE growth in both *in vitro* cultures and *in vivo* mouse model of intestinal

colonisation. These findings demonstrate that antibiotic-mediated killing of gut commensals promoted VRE intestinal colonisation by increasing the availability of nutrients that support VRE growth, and that this can be reversed by reintroducing key commensals to the microbiota that compete with VRE to use nutrients.

Table of Contents

Copyright Declaration.....	2
Statement of originality.....	2
Acknowledgement.....	3
Abstract	5
List of Figures	11
List of Tables	14
Abbreviations.....	15
Glossary	18
Chapter 1: Introduction.....	20
Antibiotic use and the rise of multidrug-resistant pathogens.....	20
Vancomycin resistant <i>Enterococcus</i>	22
General characteristics.....	22
History of the emergence of VRE as a human pathogen	23
Multidrug resistant enterococci.....	25
Antibiotic resistances and susceptibilities.....	26
Intrinsic antibiotic resistances	26
Acquired antibiotic resistance	28
Antibiotic approaches VRE treatment.....	30
Antibiotic treatment promotes VRE gut colonisation.....	32
The human gastrointestinal microbiome.....	32
Colonisation resistance	38
Indirect mechanisms of colonisation resistance	38
Direct mechanisms of colonisation resistance	42
Nutrient competition.....	42
Metabolite inhibition.....	43
Bile acid inhibition	45
Bacteriocin production	46
Emerging treatments for GI pathogen colonisation	46
Hypothesis.....	48
Aims	49
Chapter 2: Methodology.....	50
General media preparation	50
Tryptic soy media	50
Fastidious anaerobic agar	50
Columbia Agar.....	50
Reasoners 2A agar.....	50
Brilliance™ VRE agar	51
Distal gut growth medium	51
Bacterial strains	52
VRE growth conditions.....	52
Commensal growth conditions	52
Faecal donor involvement.....	53

Ethical approval.....	53
Recruitment	53
Criteria.....	54
Sample collection	54
Preparation of antibiotics known to promote VRE colonisation	54
Antibiotic minimum inhibitory concentration assays	55
Impact of antibiotics on VRE growth in pure culture	55
Faecal batch cultures	56
VRE growth in antibiotic-treated faecal batch cultures	56
Carbon utilisation capabilities of VRE.....	57
VRE minimal media	57
Individual carbon source utilisation assay	57
Nitrogen utilisation capabilities of VRE	59
VRE minimal media	59
Nitrogen utilisation “leave-one-out” experiment	59
Carbon utilisation capabilities of commensal consortium	60
Gut commensal minimal media	60
Gut commensal carbon source utilisation assay	61
Mixed nutrient utilisation assays	62
Vancomycin-resistant <i>Enterococcus</i>	62
Gut commensal consortium in coculture	63
Metabolite inhibition growth assays.....	64
Metabolite measurement from healthy human faeces	64
Mixed metabolite inhibition evaluation.....	65
Individual metabolite inhibition evaluation	65
VRE colonisation modelling in the artificial gut model	66
Artificial gut model.....	66
Vessel daily sample preparation	70
Nutrient competition assays.....	71
Gut commensal spent media assay.....	71
Faeces spent media assay	72
VRE spent media assay.....	73
Direct coculture competition assays.....	73
Mouse model of intestinal colonisation with vancomycin-resistant <i>Enterococcus</i>	74
Ethical approval.....	74
Experimental design.....	75
Preparation and administration of vancomycin.....	76
Preparation and administration of vancomycin-resistant <i>Enterococcus</i>	76
Preparation and administration of commensal consortium	77
¹H-NMR spectroscopy	78
NMR sample preparation for human faeces, human faecal culture samples, or bacterial culture samples	78
NMR sample preparation for mouse faeces	78
NMR instrument specifications.....	79
16S rRNA gene sequencing	81
DNA extraction.....	81
16S rRNA gene sequencing on the Illumina MiSeq	82
16S rRNA gene qPCR	82
PacBio genetic sequencing.....	83
Sequencing data processing with DADA2 pipeline	83

Evaluation of gene sequencing data with the mixOmics library	83
Statistical analyses	84
Chapter 3: Antibiotic treatment fundamentally reshapes the gut microbiome by reducing the abundance and diversity of the microbiota	87
Introduction	87
Results.....	88
Antibiotic treatment drives decreases of gut commensals	88
Antibiotic treatment caused an increase in nutrients and a decrease in metabolites found in the gut microbiome	91
Antibiotic-driven decreases in microbiota biomass positively correlates with increased nutrient availability and decreased metabolite production	98
Discussion.....	102
Chapter 4: Metabolic evaluation of vancomycin resistant Enterococcus within the context of the human gut.....	113
Introduction	113
Results.....	114
VRE utilise a range of nutrients which were elevated in antibiotic-treated faecal culture as carbon sources	115
VRE acts as a primary degrader for nutrients found in the distal gut	118
VRE utilise amino acids which were elevated in antibiotic-treated faecal culture as nitrogen sources ...	119
<i>E. faecium</i> and <i>E. faecalis</i> demonstrate a preference for nutrients elevated in antibiotic-treated faecal culture	124
Mixed metabolite inhibition of VRE strains is pH dependent	127
Individual metabolites do not inhibit the growth of VRE strains	128
Discussion.....	130
Chapter 5: Vancomycin-resistant <i>E. faecium</i> colonisation dynamics in response to antibiotic treatment and faecal microbiome transplant	141
Introduction	141
Results.....	143
Antibiotic treatment allows VRE exposure to become colonisation.....	145
Antibiotic treatment increases nutrient and decreases metabolite concentrations leading to <i>E. faecium</i> persistence in artificial gut chemostat models	146
Vancomycin treatment in artificial gut chemostat cultures decreases the diversity and density of the commensal microbiota	151
Vancomycin treatment in murine study promotes <i>E. faecium</i> gut colonisation	157
Faecal microbiome transplantation depletes nutrients, restores metabolite production, and restricts <i>E. faecium</i> growth	160
Discussion.....	166
Chapter 6: Harnessing specific elements of antibiotic naïve colonisation resistance for VRE treatment.....	179
Introduction	179
Results.....	180
Commensal species killed by antibiotic treatment consume the same nutrients as VRE strains	180
The commensal consortium requires longer than the VRE strains to deplete nutrients elevated by antibiotics.....	182
Nutrient depletion by a commensal consortium suppresses the growth of VRE strains.....	184

Individual commensal species outcompete VRE for limited nutrients in coculture	188
Treatment with a commensal consortium inhibited VRE colonisation in VAN-treated mice	190
Discussion.....	193
<i>Chapter 7: Conclusion</i>	<i>206</i>
Summary.....	206
Limitations and future work	211
<i>References.....</i>	<i>215</i>

List of Figures

Figure 1: History of the development of pathogenic VRE.....	24
Figure 2: Mechanisms of colonisation resistance in the gut.....	41
Figure 3: Artificial gut chemostat model used in this study.....	68
Figure 4: Artificial gut chemostat model experimental timeline.....	70
Figure 5: Mouse model of VRE colonisation experimental timeline.....	76
Figure 6: Antibiotics that promote VRE intestinal colonisation decrease the overall biomass and alter the absolute abundances of specific phyla in antibiotic-treated faecal microbiota.....	90
Figure 7: Antibiotics that promote VRE intestinal colonisation decrease the absolute abundances of several bacterial families in antibiotic-treated faecal cultures.....	91
Figure 8: Metabolic shifts drive separation of antibiotic- and antibiotic-naïve faecal cultures.....	93
Figure 9: Forced score-space separation of faecal culture shows division between antibiotic- and water-treated samples.....	94
Figure 10: Nutrient and metabolite discriminant peaks drive separation between antibiotic- and water-treated faecal culture samples.....	96
Figure 11: Antibiotic-treated faecal culture have increase nutrient and decrease metabolite abundance.....	97
Figure 12: Metabolic and taxonomic changes drive separation in antibiotic-treated versus water-treated faecal cultures.....	100
Figure 13: Bacterial families suppressed by antibiotic treatment show a positive correlation with metabolites and negative correlation with nutrients	101
Figure 14: Nutrients elevated with antibiotic treatment support the growth of VRE.....	115
Figure 15: Individual carbon sources elevated in antibiotic-treated faecal culture support the growth of VRE.....	116
Figure 16: Individual carbon sources elevated in antibiotic-treated faecal culture support the growth of VRE at high moderate or low levels.....	117
Figure 17: Complex nutrients found in faecal culture can be utilised by VRE as carbon sources.....	119

Figure 18: Eliminating key nitrogen sources decreases VRE ability to grow in media containing nutrients elevated with antibiotic-treated faecal culture.....	122
Figure 19: Individual nitrogen sources elevated in antibiotic-treated faecal culture are essential to the growth of VRE at high moderate or low levels.....	124
Figure 20: VRE isolates demonstrate a preference of nutrients when cultured with a mixture of carbon and nitrogen sources elevated in antibiotic-treated faecal culture.....	126
Figure 21: VRE inhibition by a mixture of microbial metabolites found in faecal culture is pH and concentration dependent.....	127
Figure 22: Individual microbial metabolites are ineffective at inhibiting the growth of VRE isolates.....	129
Figure 23: VRE produce microbial metabolites when cultured with a mixture of nutrients...	130
Figure 24: VRE strains can grow in the presence of broad spectrum antibiotics at concentrations measured in faeces	144
Figure 25: Antibiotic treatment in faecal culture allows for VRE isolate proliferation.....	145
Figure 26: Faecal cultures treated with vancomycin promoted high growth of vancomycin-resistant <i>E. faecium</i>	147
Figure 27: Microbial metabolites decreased in antibiotic-treated faecal culture grown in an artificial gut model.....	149
Figure 28: Nutrients increased in antibiotic-treated faecal cultures grown in an artificial gut model	150
Figure 29: Vancomycin treatment in artificial gut chemostat cultures causes a decrease in the commensal microbiota.....	153
Figure 30: Bacterial genus suppressed by antibiotic treatment show a positive correlation with metabolites and negative correlation with nutrients	154
Figure 31: Nutrients elevated and metabolites decreased with vancomycin treatment are negatively correlated with <i>E. faecium</i> growth in an artificial gut chemostat model.....	156
Figure 32: Vancomycin treatment promotes dense intestinal colonisation of vancomycin-resistant <i>E. faecium</i>	159
Figure 33: Nutrients elevated with vancomycin treatment are decreased with vancomycin-resistant <i>E. faecium</i> intestinal colonisation in murine faeces.....	159

Figure 34: Faecal microbiota transplant decreased vancomycin-resistant <i>E. faecium</i> growth in faecal culture.....	161
Figure 35: Faecal microbiota transplant treatment restores the biodiversity of the faecal microbiota in artificial gut chemostat culture following vancomycin treatment.....	162
Figure 36: Microbial metabolites associated with colonisation resistance increase in concentration following faecal microbiota transplant in an artificial gut chemostat.....	164
Figure 37: Bacterial genera reintroduced with faecal microbiota show a positive correlation with metabolites and negative correlation with nutrients	165
Figure 38: Metabolites elevated with faecal microbiota transplant are negatively correlated with <i>E. faecium</i> growth in an artificial gut chemostat model.....	166
Figure 39: Commensal strains decreased by antibiotic treatment in faecal culture consume the carbon sources necessary for VRE survival.....	182
Figure 40: The commensal consortium required longer to deplete carbon and nitrogen sources known to be elevated in antibiotic-treated faecal microbiota compared to the VRE strains.....	183
Figure 41: Commensal consortium and faeces deplete similar nutrients.....	185
Figure 42: Nutrient depletion by a 15-strain commensal consortium or faeces suppresses VRE growth.....	187
Figure 43: Individual commensal strains can suppress the growth of VRE in coculture.....	190
Figure 44: Mice treated with a commensal consortium showed suppression of VRE intestinal colonisation	191
Figure 45: Treatment with a commensal consortium decreased the nutrients available to support VRE growth in antibiotic-treated mice.....	192

List of Tables

Table 1: Commensal strains.....	53
Table 2: Concentrations of metabolites in faeces.....	64
Table 3: Donor information for artificial gut chemostat model experiments.....	69
Table 4: NMR peak assignments.....	79
Table 5: VRE strains exhibit resistance to various broad spectrum antibiotics known to promote VRE intestinal colonisation.....	143

Abbreviations

¹H-NMR	Proton-nuclear magnetic resonance
5-AVA	5-aminovalerate
<i>A. muciniphila</i>	<i>Akkermansia muciniphila</i>
AA	Acetate
ABC	ATP-binding cassette
Ala	Alanine
AMPs	Antimicrobial peptides
AMR	Antimicrobial resistance
Ara	Arabinose
Arg	Arginine
Asp	Aspartate
<i>B. adolescentis</i>	<i>Bifidobacterium adolescentis</i>
<i>B. bifidum</i>	<i>Bifidobacterium bifidum</i>
<i>B. breve</i>	<i>Bifidobacterium breve</i>
<i>B. caccae</i>	<i>Bacteroides caccae</i>
<i>B. catenulatum</i>	<i>Bifidobacterium catenulatum</i>
<i>B. dorei</i>	<i>Bacteroides dorei</i>
<i>B. fragilis</i>	<i>Bacteroides fragilis</i>
<i>B. intestinalis</i>	<i>Bacteroides intestinalis</i>
<i>B. longum</i>	<i>Bifidobacterium longum</i>
<i>B. ovatus</i>	<i>Bacteroides ovatus</i>
<i>B. pseudocatenulatum</i>	<i>Bifidobacterium pseudocatenulatum</i>
<i>B. stercoris</i>	<i>Bacteroides stercoris</i>
<i>B. thetaiotaomicron</i>	<i>Bacteroides thetaiotaomicron</i>
<i>B. thuringiensis</i>	<i>Bacillus thuringiensis</i>
<i>B. uniformis</i>	<i>Bacteroides uniformis</i>
<i>B. vulgatus</i>	<i>Bacteroides vulgatus</i>
<i>B. xylanisolvens</i>	<i>Bacteroides xylanisolvens</i>
BA	Butyrate
BCFA	Branched chain fatty acid
<i>C. aerofaciens</i>	<i>Collinsella aerofaciens</i>
<i>C. difficile</i>	<i>Clostridioides difficile</i>
<i>C. jejuni</i>	<i>Campylobacter jejuni</i>
<i>C. rodentium</i>	<i>Citrobacter rodentium</i>
<i>C. scindens</i>	<i>Clostridium scindens</i>
<i>cas</i>	CRISPR-associated
CLI	Clindamycin
CR	Colonisation resistance
CRE	Carbapenem-resistant <i>Enterobacteriaceae</i>
CRISPR	Clustered regularly interspaced short palindromic repeats
CRO	Ceftriaxone
DGGM	Distal gut growth medium
DI	Deionised
DNA	Deoxyribonucleic acid
<i>E. cloacae</i>	<i>Enterobacter cloacae</i>
<i>E. coli</i>	<i>Escherichia coli</i>

<i>E. faecalis</i>	<i>Enterococcus faecalis</i>
<i>E. faecium</i>	<i>Enterococcus faecium</i>
<i>E. histolytica</i>	<i>Entamoeba histolytica</i>
EtOH	Ethanol
<i>F. prausnitzii</i>	<i>Faecalibacterium prausnitzii</i>
FA	Formate
FAA	Fastidious anaerobic agar
FDR	False discovery rate
FMT	Faecal Microbiota transplant
Fru	Fructose
Fuc	Fucose
Gal	Galactose
Glc	Glucose
GlcNAc	N-acetylglucosamine
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
IL	Interleukin
ILC	Innate lymphoid cell
Iso	Isoleucine
Iso-BA	Isobutyrate
Iso-VA	Isovalerate
<i>K. pneumoniae</i>	<i>Klebsiella pneumoniae</i>
<i>L. monocytogenes</i>	<i>Listeria monocytogenes</i>
<i>L. murinus</i>	<i>Lactobacillus murinus</i>
<i>L. plantarum</i>	<i>Lactobacillus plantarum</i>
Leu	Leucine
Lys	Lysine
Mal	Maltose
Man	Mannose
MDR	Multidrug-resistant
Met	Methionine
MIC	Minimum inhibitory concentration
MTZ	Metronidazole
OD	Optical density
OPLS-DA	Orthogonal projections to latent structures discriminant analysis
PA	Propionate
PBP	Penicillin-binding protein
PCA	Principal component analysis
Phe	Phenylalanine
pO₂	Partial pressure of oxygen
Pro	Proline
pro-IL-1β	Pro-interleukin-1 β
qPCR	Quantitative polymerase chain reactions
R2A	Reasoner's 2A
rCCA	Regularised canonical correlation analysis
Rib	Ribose
RNA	Ribonucleic acid

<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
<i>S. ser. Typhimurium</i>	<i>Staphylococcus enterica</i> serovar Typhimurium
SCFA	Short chain fatty acid
Suc	Sucrose
TH17	T helper 17
Thr	Threonine
Tre	Trehalose
Try	Tryptophan
TSA	Tryptic soy agar
TSB	Tryptic soy broth
Tyr	Tyrosine
TZP	Piperacillin/tazobactam
Ura	Uracil
v/v	Volume/volume
VA	Vessel A
Val	Valine
VAN	Vancomycin
VB	Vessel B
VC	Vessel C
VR	Vancomycin-resistant
VRE	Vancomycin-resistant <i>Enterococcus</i>
w/v	Weight/volume
Xyl	Xylose
<i>Y. enterocolitica</i>	<i>Yersinia enterocolitica</i>
<i>Y. frederiksenii</i>	<i>Yersinia frederiksenii</i>
y.o.	Years old

Glossary

Allochthonous	Foreign to an environment, will leave the environment except under abnormal circumstances [1-3]
Antimicrobial resistance (AMR)	Lacking a response to one or more antimicrobial medications [4, 5]
Autochthonous	Native to an environment, will remain in the environment except under abnormal circumstances [1-3]
Auxotrophy	The requirement for an exogenous organic compound to facilitate growth [6]
Chemoorganotroph	A microorganism capable of obtaining energy through the oxidation of organic compounds [7]
Colonisation resistance (CR)	The ability of the microbiota to prevent the growth of exogenous microorganisms in microbiome [8, 9]
Commensal bacteria	Microorganisms which live in/on a host without causing adverse effects, maintain a mutually beneficial relationship with the host [10]
Complex nutrient	A nutrient (carbohydrate, protein etc.) which can be broken down into monomer subunits to make simple nutrients [11]
<i>Ex vivo</i>	Outside a living body [12], in this study referring to experiments which included faecal microbiota transferred from a host to a culturing vessel
Faecal Microbiota Transplant (FMT)	The process by which a faecal matter is transferred from a healthy donor to the intestinal tract of a recipient for the purpose of reestablishing microbial density and diversity [13]
Functional redundancy	Multiple members of a microbiome processing similar genes [14-16]
Homofermentative	Producing a single primary end product of fermentation (i.e. lactic acid) [17]
<i>In vitro</i>	In glass [12], experiments performed outside and without any part (including faecal microbiota) of a host
<i>In vivo</i>	In a living body (mouse, human etc.) [12]
Metabolome	The collection of metabolites and nutrients involved in the metabolism of a single organism [18-20]
Metabonome	The collection of metabolites and nutrients involved in the metabolism of a community within an environment [18, 19]
Microbial metabolite	Products of the metabolism of a microorganism [21]
Microbiome	A defined environment occupied by a microbiota with distinct physical and chemical features [22]
Microbiota	The collection of microorganisms which occupy a microbiome [23, 24]
Multidrug-resistant (MDR)	Nonsusceptible to (at least) antimicrobials from multiple antimicrobial categories [25-27]

Nutritional niche	A collection of nutrients which an organisms can successfully obtain to facilitate growth [28]
Primary degrader	Bacteria which digest complex nutrients (polysaccharides, proteins, etc.) into their monomer (simple sugars, amino acids, etc.) components [11, 29, 30]
Secondary degrader	Bacteria which consume the monomer components (simple sugars, amino acids, etc.) of complex nutrients which were digested by primary degraders [11, 29, 30]
Xenobiotic	A chemical substance foreign to the body (pesticides, heavy metals, pharmaceuticals, pollutants etc.) [31]

Chapter 1: Introduction

Antibiotic use and the rise of multidrug-resistant pathogens

Antibiotics revolutionised medicine at a cost which humanity is now beginning to understand. Since the pioneering discovery of penicillin in the early 20th century, the application and pressure for antibiotics has increased exponentially - from medical intervention and preventative care to agricultural husbandry treatments and food preservation, as the potential usage of antibiotic increases, so too does the creation of new antibiotics and combination therapies [32-34]. Antibiotics have vastly improved the success of medical treatment both as a direct agent against infections as well as providing protection prior to surgery, chemotherapy, and radiation [35]. Improvements have been made to global access to clean water, sanitation, and safe food by implementing antimicrobials into manufacture and agrarian production [35, 36].

Antibiotics are not equally used around the world. The highest level of antibiotic consumption is found in eastern Europe, the middle east, and central Asia while the lowest usage is seen in sub-Saharan Africa [32]. Access to antibiotics strongly correlates to national income [32]. Low- and middle-income countries experience a higher disease burden but a lower consumption rate of antibiotics (13.5 defined daily doses per 1,000 inhabitants per day) compared to high-income countries (25.7 defined daily doses per 1,000 inhabitants per day) [32]. This discrepancy is rapidly disappearing as access to antibiotics increases in lower income countries [37]. Between 2000 and 2018, one study found antibiotic usage increased by 46% globally [36]. It has been estimated that, if antibiotic consumption continues at the current rate without policy intervention, usage could double the 2015 estimate of 42 billion defined daily doses by 2030 [32]. Part of the escalation in antibiotic usage stems from inadequate antimicrobial stewardship [38]. Access to antibiotics as an over-the-counter medication,

misinformation regarding antibiotic application and the inability to provide confirmational testing are major causes of antibiotic abuse [38-41].

While education, regulation, and improved diagnostic testing could prove an effective strategy for stemming the influx of intentional antibiotic consumption, there is also the ever-increasing threat of secondary antibiotic intake from environmental contamination. Both human and animal usage of antibiotics leads to antibiotic residues in the environment [42]. After intake, antibiotics are excreted either through the urine or faeces, depending on the class of drug and the organ which breaks it down. Between 40-90% of the active antibiotic remains in waste matter which then passes into the environment [42-44]. The contaminated food and water can make it back into the human diet either directly or through livestock [45-49]. Further, the presence of antibiotics in the environment serves as a significant and unnatural selective pressure on environmental microbiota and contributes to the creation of multidrug-resistant (MDR) pathogens [48, 50-52].

Positive correlations have been identified between the increased global usage of antibiotics and the prevalence of MDR pathogens [5, 53]. MDR pathogens are predominantly opportunistic, pathogenic bacteria resistant to at least one class of antibiotics. These bacteria have the potential to become significant clinical obstacles by developing resistances to many available antimicrobials, including antibiotics of last resort [25, 26]. This has created a dangerous positive feedback loop where the greater the reliance on antibiotics, the greater the need for them. For many MDR pathogens, combination therapies have become the only way to treat the worst infections [54].

Unfortunately, the consequences do not end there. Antibiotics do not only target and kill pathogens, but they also inadvertently kill beneficial bacteria within the microbiome ("commensals"). The gut microbiota has many beneficial functions to the host, including

providing resistance to pathogen intestinal colonisation [55]. The “colonisation resistance” (CR) conferred by the gut microbiota can be disrupted by antibiotic usage [56-60], increasing the ease at which opportunistic pathogens can colonise the intestine and subsequently disseminate to cause invasive infections. Numerous studies have demonstrated that administration of antibiotics alters both the diversity and density of the gut microbiota [55, 57, 58]. The combination of antibiotic-disrupted gut microbiota and the intrinsic and acquired antibiotic resistance associated with multidrug-resistant pathogens can lead to difficult-to-manage infections with chronic or fatal potential.

Vancomycin resistant *Enterococcus*

One such opportunistic MDR pathogen is vancomycin-resistant *Enterococcus* (VRE). Since the mid-1980s, VRE has grown to become the second most common nosocomial infection in the world [61, 62] causing endocarditis, severe bloodstream, intra-abdominal and urinary tract infections, meningitis and wound infections [63-65]—resulting in increased hospital stays, treatment costs and death [56, 61, 63, 65-70]. The most common clinical species of VRE, *Enterococcus faecium* and *Enterococcus faecalis*, demonstrate a wide range of antibiotic resistances, including resistance to aminoglycosides, β -lactams, fluoroquinolones, glycopeptides, oxazolidinones and tetracyclines [61, 64, 65, 67, 71, 72].

General characteristics

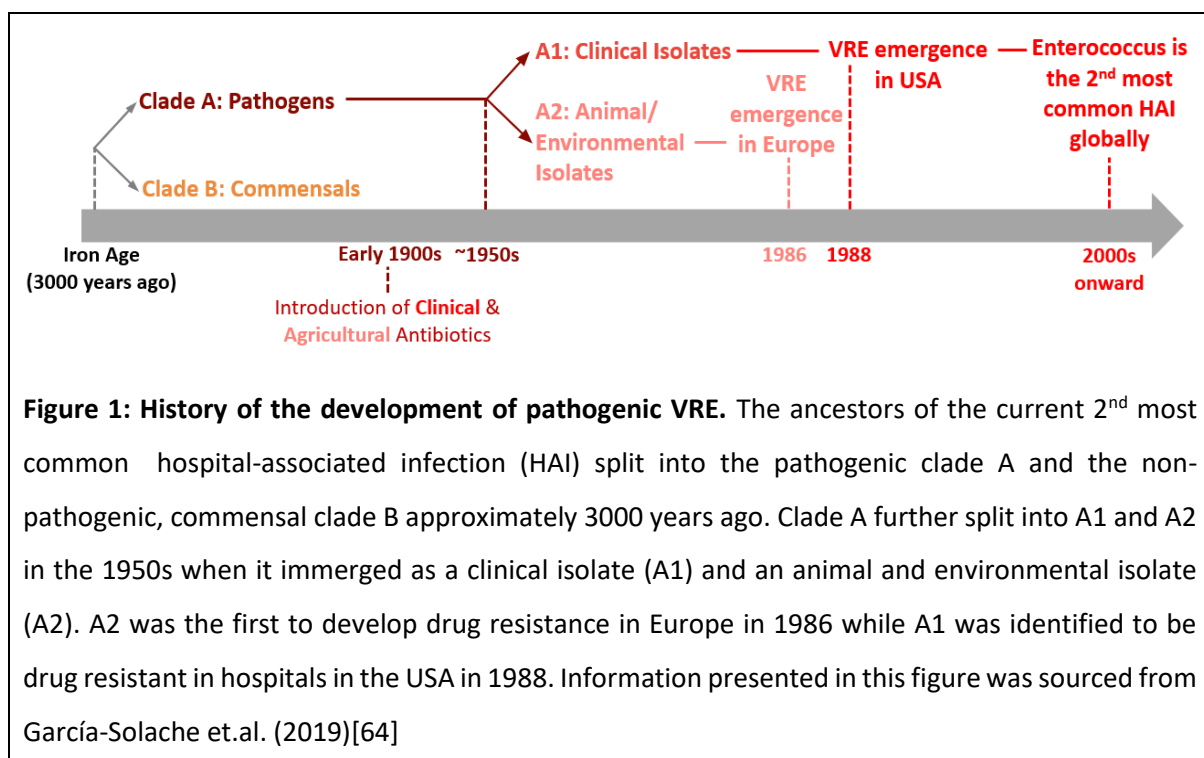
Enterococci are Gram-positive cocci which form short to medium chains [73, 74]. They belong to the phyla Bacillota (formerly Firmicutes), class Bacilli, order Lactobacillales and family *Enterococcaceae*. Due to morphological similarities to streptococci, the genus was only reclassified to enterococci in 1984 [74-76]. Enterococci have a genome size between 2.3-5.4 Mb with up to 5,107 predicted genes [64, 77, 78]. Of the (at least) 58 enterococci species, 26 have been linked to human infection [79-81]. The primary species of interest are *E. faecalis*

and *E. faecium*, and to a lesser extent, *Enterococcus avium*, *Enterococcus caccae*, *Enterococcus casseliflavus*, *Enterococcus durans*, *Enterococcus gallinarum*, *Enterococcus raffinosus*, *Enterococcus dispar*, and *Enterococcus hirae* [74, 82-84]. Both *E. faecium* and *E. faecalis* are non-motile, non-capsulated, non-spore-forming, facultative anaerobes. They are halotolerant (up to 6.5%), can grow in acidic and basic environments (pH between 4.5-10), can survive temperatures between 5-65°C, and grow between 10-45°C [64, 85-88]. Enterococci are obligate fermentative chemoorganotrophs and homofermentative, producing lactic acid following glucose and other carbohydrate fermentation [64, 73, 75, 89]. The primary reservoir for enterococci in humans is the gastrointestinal system but these bacteria can be isolated from the skin, oropharynx, and vaginal tissues. As a commensal in the human gut microbiota, enterococci are theorised to be one of the oldest symbiotic bacteria, present even in ancient evolutionary ancestors [75, 90]. Enterococcal species have been identified in the gastrointestinal (GI) tracts and faeces of mammals, reptiles, birds, and insects [91-93]

History of the emergence of VRE as a human pathogen

Approximately 3000 years ago the genetic ancestors for VRE split into two separate clades [94-96](**Figure 1**). Clade B remained commensal organisms which lack antibiotic genes and have little ability to cause infection. Clade A evolved to be pathogenic, favouring the gastrointestinal system of livestock. Different isolates of the same enterococcal species can belong in different clades; for example, *E. faecium* strains have been identified in both clade A and clade B depending on their virulence potential and origin of isolation [64, 96, 97]. The increased interaction between humans and domesticated animals has been hypothesised to have facilitated the jump of these pathogens into humans [96]. The first description of the pathogenic capabilities of enterococci was in 1899 when two groups separately identified

enterococci as a potential pathogen [98, 99]. MacCallum and Hastings reported on lethal endocarditis caused by *E. faecalis*, while Thiercelin reported that the enterococci was often found in the gut of patients with diarrhoea and could cause septicaemia through blood stream translocation. These findings led to the classification of enterococci as a commensal opportunist [75, 99]. Later, Andrewes and Horder (1906) and Orla-Jensen (1919) described *Streptococcus faecalis* and *Streptococcus faecium* respectively, further identifying pathogenic potential [100, 101]. The enterococci taxonomy was reclassified at the end of the 20th century and now consists of 58 species [74, 75].



The introduction of clinical and agricultural antibiotics in the early 20th century facilitated the development of yet another split in the lineage of pathogenic enterococci (**Figure 1**)[61, 64]. Initial antibiotic resistance in enterococcal strains emerged twice in the world, first in 1986 in Europe; the glycopeptide antibiotic, avoparcin, was commonly used as an agricultural antibiotic to promote growth of food animals [61]. Enterococci resistance was noted with little clinical impact. Members of the animal and environmental antibiotic-resistant

enterococci make up clade A2. The second emergence, in the United States in 1988, was linked with extended-spectrum cephalosporin usage against *Clostridioides difficile* infections [61, 67]. This collection of pathogenic clinically isolated enterococci form clade A1 [94-96]. Members of clade A1 have been observed to have larger genomes with greater plasticity allowing for increased antibiotic resistance and carbon utilisation [96]. These modifications help the VRE strains to survive in the host and carve out a nutritional niche in varying hostile conditions [96]. Hospitals are now a major reservoir for VRE contributing significantly to hospital-associated infections [61, 62, 102]. In addition to antibiotic resistances, VRE have been found to be able to withstand to chemical stress from alcohol and chlorine disinfectants used in hospitals [61, 103] as well as survive periods of dry starvation without sacrificing vancomycin resistance [104].

Multidrug resistant enterococci

A major contributor to the success of VRE as a pathogen is its ability to acquire antibiotic resistance genes both through horizontal gene transfer and mutation [61, 64, 69]. The abundant genetic mobility of pathogenic VRE strains allows for the integration of foreign genes and pathogenicity islands [105]. Up to 38% of the genetic material of strains in clade A1 is thought to have been incorporated from other species [106]. It is theorised that the genetic plasticity of VRE is due to its relative inability to destroy foreign nucleic acid through the CRISPR system [61, 72]. Other organisms, including archaea and bacteria [107], are able to use clustered regularly interspaced short palindromic repeats (CRISPR), and the associated systems (*cas*) and proteins, to prevent foreign plasmids and phages from making permanent changes to their DNA. The CRISPR system works like an intracellular immune system by utilising *cas*-encoded proteins to incorporate small portions of foreign DNA into the clustered

repeats. When RNA polymerase transcribes the CRISPR array containing the foreign DNA fragment, CRISPR RNA is produced which can guide the endonuclease, *cas9*, to targeted foreign material for destruction. Multiple studies have identified an inverse correlation between the presence of CRISPR elements and antibiotic resistance genes in VRE [72, 108, 109]. These studies found higher abundance of CRISPR elements in *E. faecalis* strains compared to *E. faecium* strains. The correlation is supported by data indicating that *E. faecium* strains have greater levels of antibiotic resistance than *E. faecalis* strains [64, 109-111]. The capability of VRE strains to acquire antimicrobial resistance (AMR) genes increases the vital need for the development of a non-antibiotic therapeutic against gut colonisation.

Antibiotic resistances and susceptibilities

Intrinsic antibiotic resistances

Intrinsic AMR primed enterococci species for pathogenic potential. Enterococci are naturally resistant to a wide range of antibiotics with varying antimicrobial targets. *E. faecium* has more intrinsic antibiotic resistances compared to *E. faecalis* (specifically with regards to penicillins) [64, 109-113]. Among others, many enterococcal strains are resistant to β -lactams (including penicillins, cephalosporins, and carbapenems), aminoglycosides, glycopeptides, lipoglycopeptides, and lincosamides [61, 64, 67, 69, 71, 75, 112, 114].

The β -lactams act against a bacterial cell by binding to penicillin-binding proteins (PBPs) [115]. These enzymes are essential in the final step of peptidoglycan cross-linking in cell wall formation. This class of antibiotics are active against both Gram-positive and Gram-negative bacteria. β -lactams can be broken down into penicillins, cephalosporins, carbapenems, monobactams and β -lactamase inhibitors. Enterococci are intrinsically resistant to most antistaphylococcal penicillins, cephalosporins and carbapenem as well as the monobactam,

aztreonam [73]. Compared to streptococci, the minimum inhibitory concentration (MIC) for many β -lactams is 10 to 100x higher for enterococci [116]. This can be attributed to the presentation of low-affinity PBPs on the cell surface of *E. faecalis* (PBP4) and *E. faecium* (PBP5) [117-119]. Some enterococci are also able to produce β -lactamase which, in the case of enterococci, is a membrane bound enzyme which breaks down the β -lactam ring thus deactivating the antibiotic [120]. The impact of these β -lactamases on resistance can be combatted by combination therapies including a β -lactamases inhibitor antibiotic [121].

Resistance to carbapenems is contradictory depending on the experimental environment; While some *in vitro* experiments have shown inhibition of VRE with carbapenems, there is no recommended *in vivo* monotherapy for VRE decolonisation using a carbapenem [64, 122].

Aminoglycoside antibiotics target protein synthesis in bacteria by binding to the aminoacyl site of the 16S ribosomal RNA [123, 124]. As with tetracycline antibiotics, this occurs in the 30S subunit as opposed to the 50S subunit, which is targeted by oxazolidinones, streptogramins, macrolides and lincosamides. Enterococcal resistance to aminoglycosides is theorised to be due to failure of the antibiotic to reach its target site. As ribosomes are located within the cell, the antibiotic must be able to cross the cell membrane. Aminoglycosides are theorised to be incapable of crossing the enterococcal cell envelope due to the transporter responsible being oxygen dependant while the majority of the bacteria's metabolism is anaerobic [125, 126]. For the aminoglycoside (streptomycin, gentamicin, and kanamycin), enterococcal strains produce an enzyme which can perform modifications on the aminoglycoside antibiotics, rendering them ineffective [127-131].

In *E. faecalis*, further resistance to the lincosamide, clindamycin (as well as pleuromutilins and streptogramin A antibiotics), is ascribed to a protein similar to the ATP-binding cassette (ABC)

proteins responsible for transport of molecules; in both staphylococci and enterococci, ABC transporters can export antimicrobials conferring resistances to macrolide, lincosamide and streptogramin antibiotics [132-136]. In one study, this protein was identified in 100% of the 180 *E. faecalis* strains analysed but not in the 189 other enterococcal strains [135]. Disrupting the gene encoding this protein resulted in a 40x decrease in the MIC of clindamycin, dalfopristin and quinupristin-dalfopristin combination treatment.

Enterococci are intrinsically resistant to trimethoprim-sulfamethoxazole; however this resistance is different depending on the culturing environment. Trimethoprim is a DHFR inhibitor which acts against the bacteria by reducing the dihydrofolate to the active form of folic acid, tetrahydrofolate [137]. Sulfamethoxazole is a sulphonamide which prevents the synthesis of dihydrogen folic acid from para-aminobenzoic acid [138, 139]. *In vivo*, enterococci are resistant to trimethoprim-sulfamethoxazole but the same is not seen in *in vitro* experiments [140]. This is due to the ability of enterococci to absorb folate from their environment *in vivo* thus evade the mechanisms of inhibition of the antibiotic. As such, this combination of antibiotics is not clinically used to treat infection.

Acquired antibiotic resistance

In addition to the intrinsic resistances which enable enterococci to survive in clinical and agricultural environments, new antibiotic resistance genes have been—and continue to be—acquired, thus increasing clinical pressure to successfully treat enterococcal infections. While enterococci are largely resistant to β -lactams without acquiring additional genes, there are some β -lactams which have antimicrobial impact. Aminopenicillin ampicillin, as well as the antipseudomonal-ureidopenicillins mezlocillin and piperacillin, retain medical integrity in fighting enterococcal infections [64]. The functionality of these drugs, however, is decreasing

as new resistances are acquired. Populations of highly ampicillin resistant *Enterococcus* have been identified in hospitals increasing the spread VRE to antibiotic-treated patients [62, 95].

The glycopeptide vancomycin was first released as a therapeutic in 1958. However, it was rarely prescribed until the 1980s, when it became a common antibiotic against *C. difficile* and *Staphylococcus aureus* [141, 142]. In the early 1980s, the first enterococcal strains began exhibiting resistance in Europe and the United States [143-150]. Teicoplanin, another Gram-positive targeting glycopeptide [151], was released in 1986 and also failed to eliminate resistant strains of enterococci [65]. Both vancomycin and teicoplanin prevent cell wall biosynthesis in susceptible bacteria by binding to the lipid II precursor at the d-alanyl-d-alanine moiety terminus [152-155]. Glycopeptides are not active against Gram-negative bacteria as the outer membrane prevents access to the periplasmic space [156]. Resistant strains acquired operons which replaced the d-alanine for either d-lactate or d-serine [156-163]. These substitutions reduce the binding potential of glycopeptides 1000x for d-lactate and 7x for d-serine substitution. VanA, VanB, VanD and VanM are the encoded ligases which replace the alanine in the d-alanyl-d-alanine terminus to d-lactate [158, 159, 161]. The VanC, VanE, VanG, Van L and VanM ligases replace the d-alanine with d-serine [157, 160, 162, 163]. The variants which replace the d-alanine with d-serine have a lower resistance to vancomycin compared to the d-lactate variants and remain susceptible to teicoplanin [64].

Chloramphenicol resistance, first reported in 1964 [164], is a result of the chloramphenicol acetyltransferase which works by converting the antibiotic into 3-acetyl and 1,3-diacetyl [165] [166-169]. This resistance was transferred to enterococcal strains only 15 years after the antibiotic was introduced in 1949.

Enterococci resistance to lincosamides, phenicol, pleuromutilins, oxazolidinones, and streptogramin A is linked with a methyltransferase (alters the 23S rRNA in bacteria) or an ABC transporter (exports antibiotics from the cell) [61, 170, 171]. Both resistance methods are plasmid encoded and can lead to outbreaks of resistances in hospital environments [170, 172].

Fluoroquinolones, including ciprofloxacin and levofloxacin, are potent against Gram-positive bacteria but have limited clinical application against enterococci. Ciprofloxacin and levofloxacin can still be prescribed for enterococci infections but only in the urinary tract. Mutations to the DNA gyrase enzyme and topoisomerase IV are linked with increased resistance to fluoroquinolone antibiotics [173-175]. Further, a recent study linked fluoroquinolone resistance in enterococci with efflux pumps and was able to overcome the resistance by combining ciprofloxacin antibiotics with the efflux pump inhibitor, thioridazine [176].

Antibiotic approaches VRE treatment

While treating multidrug-resistant enterococcal infections has an innate difficulty, there are still antibiotic therapies effective at eliminating the pathogen. As previously mentioned, some β -lactams can be administered against enterococcal infections, namely ampicillin, however penicillin and piperacillin can be used. Success of treatments with β -lactams is improved if done in combination with gentamicin or streptomycin. Combination of gentamicin or streptomycin to β -lactams increases clinical cure rate from 40% [209] to 70% in endocarditis [210]. This increase in inhibition when the two antibiotics are used in tandem is due to the increased ability of the aminoglycoside to enter the cell when paired with a cell wall targeting β -lactams [138]. While aminoglycosides are mostly ineffective on their own due to their

inability to cross the cell wall, the access provided by the β -lactams allows the aminoglycoside to reach the targeted ribosomes. Further, the combination of ampicillin and the third generation cephalosporin, ceftriaxone, work better as a combination therapy than either drug can achieve alone [61, 69, 177-179]. Certain combinations only work with specific species or particular infections; the streptogramin dual antibiotic, prictinamycin (combination quinupristin-dalfopristin), is active against *E. faecium* but not *E. faecalis* [180, 181]. Fluoroquinolones, an antibiotic class which targets DNA topoisomerases, has antimicrobial activity against enterococci however resistance is not uncommon in *E. faecium* [67, 182, 183]. Individual drugs, especially newer ones, have shown inhibition of enterococcal infections. Lipoglycopeptides act similarly to glycopeptides by preventing the biosynthesis of the cell wall in susceptible bacteria. This newer class of drugs includes telavancin, dalvabancin and oritavancin. Of the vancomycin-resistant enterococcal strains, only oritavancin inhibits the growth of VanA strains. All three have activity against VanB isolates [61, 184]. Tetracyclines, including lymecycline, methacycline, minocycline, and—most recently—tigecycline are intrinsically inhibitory to enterococci [61, 185, 186]. While resistance has been identified against tigecycline in *E. faecium* strains, combination therapies with gentamicin or daptomycin have shown inhibition of multidrug resistant enterococci infections [187-189]. The two oxazolidinones, linezolid and tedizolid, remain effective *in vivo* against enterococci even though there are some strains which have acquired moderate resistance [63, 64, 71, 190, 191]. Daptomycin, a lipopeptide antibiotic released to the market in 2003, acts as an antimicrobial by interfering with the cytoplasmic membrane and retains function against most enterococcal infections [61, 63, 69, 192] despite some cases of resistance identified in 2005 [193-195]. Resistance to daptomycin remains relatively rare and, in strains with higher

MICs to daptomycin, greater antimicrobial efficacy can be achieved if combined with β -lactams antibiotics [196-200].

Antibiotic treatment promotes VRE gut colonisation

Despite the current success of a limited number of antimicrobial strategies, the ability for enterococcal strains to acquire new resistance genes means many effective therapies are time limited. Further, several broad-spectrum antibiotic treatments (including but not limited to vancomycin, metronidazole, ceftriaxone, piperacillin/tazobactam and clindamycin) have been linked with increasing the risk of VRE colonisation in the gut. Studies have linked these antibiotics to promotion of VRE gut colonisation in both humans and mice [55, 61, 64, 67, 68, 70, 71, 75, 183, 201-210]. Intestinal colonisation by VRE precedes the translocation of the pathogen to the bloodstream which leads to invasive, life-threatening infections and is linked to poor outcomes in a clinical environment [63, 65, 211]. Antibiotic treatment exacerbates this by killing members of the gut microbiota responsible for maintaining the barrier integrity of intestinal epithelial cells [212, 213].

Since the primary reservoir for VRE in a host is the gut, nonantibiotic therapeutics to treat gastrointestinal colonisation are urgently needed. Therapeutics which prevent VRE from gaining a foothold in the gut, stopping it from spreading and causing invasive disease, could mitigate the risk of AMR in VRE and prevent unnecessary damage to the recipients health (through the untargeted killing of commensal bacteria).

The human gastrointestinal microbiome

Integral to the digestive system is the highly complex microbial communities that colonise the gut, collectively known as the “gut microbiota”. The gut microbiota is essential for the health of the host by contributing to metabolic functioning (including digesting food which the host

cannot) and to the immune health through both protective (such as regulating immune homeostasis) and structural (such as maintaining epithelial cell barrier integrity) means [55, 214-217]. The gut microbiota can digest nutrients found in the intestine, including non-digestible dietary fibres and host mucus [218]. The gut microbiota also plays an important role in the detoxification of xenobiotics (i.e. pesticides, pharmaceuticals, heavy metals) which could otherwise reach concentrations toxic to the host [219, 220]. The microbiota produces digestive enzymes not encoded by the human genome such as polyamines, vitamin, and short chain fatty acids (SCFAs) which not only maintain a healthy digestive system but generally improve host health [221-224]. Further, the gut microbiome has a role in the regulation of fat storage, thus relating the weight of the host to the health of the microbiome [225-227]. These commensal organisms are also essential for stimulating intestinal angiogenesis, protecting gut epithelial cells from damage, maintaining gut barrier integrity, and developing and supporting numerous immunological functions [55, 228-231]. Beyond the digestive system, dysbiosis of the gut microbiota has been linked with Alzheimer's disease [232-234], Autism spectrum disorder [235-238], depression and anxiety [239-241], chronic fatigue syndrome [242, 243], cardiovascular disease [244, 245], multiple sclerosis [246, 247], and atherosclerosis [248, 249].

The microbiota consists of primarily autochthonous microbes which remain permanently in the gut, and allochthonous microbes that are temporary inhabitants. The allochthonous microbes can be positive (probiotics), negative (pathogens) or neutral to the host. The autochthonous microbes have a significant impact on the gut homeostasis [2, 3].

The gut microbiota is composed of bacteria, viruses, fungi, archaea, and single-celled eukaryotic organisms [250]. Bacteria are the best described microorganisms in the gut

microbiota and are estimated to achieve a cell density of approximately 10^{11} to 10^{12} bacteria per millilitre in the colon [251-253]. Until relatively recently, measuring the abundances of specific members of the gut microbiota was problematic due to difficulty in culturing many gut commensal species. Culture-independent analysis techniques including 16S rRNA gene sequencing and shotgun sequencing has allowed for species identification without the need to optimise culturing strategies [254]. It is estimated that the gut microbiota contains 22 million genes, making it collectively 1000x larger than the human genome [255, 256]. The composition of the gut microbiota changes throughout the digestive system to reflect changes in oxygen levels, pH, nutrient availability, and organ structure [257]. Further, other factors such as geographical location, diet, age, gender, medication, and health of an individual massively impact the organisms found in the gut microbiota [69, 258-261]. As such, no one definition of a healthy gut microbiota exists in humans [262]. However, generally bacteria from the phyla Bacteroidota and Bacillota make up about 90% of the gut microbiota in healthy humans, with the remaining 10% of the microbiota being composed of members of the phyla Actinomycetota, Fusobacteriota, Verrucomicrobiota and Pseudomonadota [55, 57, 263-267].

The phylum Bacteroidota (formerly Bacteroidetes) contains both anaerobic and aerobic Gram-negative species [268]. These bacteria are non-spore forming rods known for having the greatest number of AMR genes of all the phyla in the gut microbiota [269]. Their ability to digest complex polysaccharides allows for the production of short-chain fatty acids as a product of metabolism [270-272]. Bacteroidota degrade complex O-glycans such as cellulose and mucin thus contributing to a mutualistic relationship with host tissue and immune cells by maintaining the mucin-rich mucus layer [273]. Due to their wide-ranging nutrient

utilisation abilities, members of the *Bacteroides* family (including *Bacteroides thetaiotaomicron*) have been shown to play a key role in nutrient utilisation and competition in the gut [274-276]. While this phylum consists of almost exclusively commensal organisms, the opportunistic pathogen *Bacteroides fragilis* can cause abdominal abscesses, bloodstream infections, and produce the *B. fragilis* toxin BFT linked to colitis and colon tumorigenesis [271, 277, 278].

The phylum Bacillota (formerly Firmicutes) consists of obligate and facultative anaerobic Gram-positive bacteria. Unlike Bacteroidota, members of the Bacillota phylum often have the ability to form spores. Spores can better survive nutrient limitation, increased oxygen concentrations, exposure to antibiotics, and extreme temperatures [56]. Bacillota contribute to promoting epithelial gut health through the inducing colonic T regulatory cells and producing the SCFA, butyrate [279]. This phylum contains the classes *Clostridia* and *Bacilli*, both which have pathogenic members including the genera enterococci and streptococci. One notable commensal of this phylum is *Faecalibacterium prausnitzii*, a commensal that is one of the main producers of butyrate in the gut, which can reduce inflammation in the intestinal mucosa [280-286]. This species is often seen in high abundance and can make up to 5% of the gut microbiota [287, 288]. The decline of *F. prausnitzii* in the gut is associated with colorectal cancer, Crohn's disease and inflammatory bowel disease—suggesting that the presence of this strain is a good indicator of gastrointestinal health [289-294].

The phylum Actinomycetota (formerly Actinobacteria) contains both aerobic and anaerobic Gram-positive bacteria. Specific strains from this phylum are often investigated for their potential use as probiotics due to their increased ability to produce SCFAs [295, 296]. Further, members of the Actinomycetota often have bile salt hydrolase and immune modulation

activity [297, 298]. Notable commensal species of this phyla include *Bifidobacterium* species, including *Bifidobacterium longum*, *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium pseudocatenulatum* and *Bifidobacterium catenulatum* [299]. Their survival in the gut is improved by their ability to adhere to the cell lining and mucosal layer [300].

The phylum Verrucomicrobiota (formerly Verrucomicrobia) are non-spore forming, Gram-negative rod or coccoid bacteria which makes up around 3-5% of the gut commensals microbiota [301]. Members of this phyla, specifically from the genus *Akkermansia*, are known for their ability to degrade mucin [301]. They are predominantly found in the mucous lining and have anti-inflammatory properties [302]. Verrucomicrobiota are not known to cause gastrointestinal diseases in humans [303]. These bacteria are negatively correlated with the risk of obesity linked with their involvement in glucose homeostasis in the gut [301, 304, 305]. A decline in *Akkermansia muciniphila* has been linked with the development of ulcerative colitis [291, 292, 306].

The phylum Pseudomonadota (formerly Proteobacteria) are Gram-negative facultative anaerobes. These bacteria make up a small percentage of a healthy gut microbiota, and an increase in abundance is often associated with disease or a perturbation to the microbiota [307]. *Escherichia coli* are members of this phylum and are often commensals in the gut microbiota, however some well-known pathogens are also part of this phylum, including pathogenic *E. coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* [308, 309].

The phylum Fusobacteriota (formerly Fusobacteria) are non-spore forming, anaerobic, Gram-negative rods. While species such as *Fusobacterium nucleatum* and *Fusobacterium necrophorum* are associated with gingival, irritable bowel disease, ulcerative colitis, and

periodontal infections, other members of this phyla are rarely associated with human gastrointestinal disease [310-315]. Other phyla, including Synergistota and Desulfobacterota (formerly Deltaproteobacteria) are found in low levels in the gut microbiota. Both Synergistota and Desulfobacterota are Gram-negative anaerobic rods [316-318]. Synergistota are commonly associated with soft tissue infections and periodontal disease [319, 320]. Desulfobacterota are sulfate-reducing bacteria associated with elevated gut inflammation and immune response [321-323].

The microbial taxa that dominate specific regions of the GI tract are heavily influenced by the environmental conditions found in that region. The intraluminal pH of the gut changes from pH 1.5-3 in the stomach to pH 6 in the duodenum, pH 7.4 in the terminal ileum; dropping to pH of 5.7 in the caecum, ranging between 5.4-5.9 in the proximal colon and 6.1-6.9 in the distal colon where the pH averages at 6.7 in the rectum [324, 325]. The microbial density of the gut increases from the stomach to the distal colon. In the stomach, there are less than 10^3 cells/g. This reaches 10^4 - 10^7 cells/g in the small intestine, and further increases in the proximal colon (10^{10} - 10^{11} cells/g), transverse colon (10^{11} - 10^{12} cells/g), and descending colon (at least 10^{12} cells/g) [251, 325]. Microbes are less abundant at the beginning of the digestive system due to the low pH, rapid transit time, and the presence of bile and pancreatic juice. The colon, by comparison, has a higher pH, slower transit time, and greater concentration of nutrients which provide more favourable growth conditions for many gut commensals [2, 326]. The increase in the gut microbiota density between the stomach and distal large intestine also correlates with the decrease in oxygen concentration. The bacteria in the colon are approximately 99% obligate anaerobes with the remaining being facultative anaerobes [264, 327, 328]. In the stomach, the partial pressure of oxygen (pO_2) is between 60-77 mmHg

[267]. This decreases to 30 mmHg in the duodenum, 10-34 mmHg in the jejunum and ileum and finally 0.5-11 mmHg in the colon. However, disruption of the gut microbiota (e.g. through antibiotic treatment) is associated with decreased butyrate production causing increased oxygen concentrations, which could favour the growth of facultative anaerobes [329-332].

To survive in the densely populated gut, each bacterial species has its own nutritional niche. The hierarchy of primary and secondary degraders allows for nutrients to be consumed by multiple species in the microbial food chain. There is a high level of functional redundancy in the genes of different microbes present in the gut microbiota, allowing for preservation of stability in the community as a whole when faced with disruption [14-16].

Colonisation resistance

The density and diversity of the gut microbiota is critical to the competitive and collaborative microbial environment that suppresses pathogenic bacteria while simultaneously supporting the health of the host [9, 333]. Colonisation resistance describes a collection of mechanisms, facilitated by members of the gut microbiota, which protects the microbiome against the incursion and expansion of exogenous, pathogenic bacteria [8]. The mechanisms implemented by the microbiota to achieve colonisation resistance can be divided by indirect and direct mechanisms.

Indirect mechanisms of colonisation resistance

Indirect mechanisms of colonisation resistance involve interaction between the gut microbiota and host, where host involvement is required to restrict pathogen growth. Some members of the gut microbiota (namely *Bifidobacterium* species) can induce mucus production through metabolite production and by inducing the expression of several host gene related to mucus glycan structure and strength (**Figure 2E**)[334-337]. The mucus

secreted by goblet cells forms a barrier between the gut microbiota and the epithelial cells thus preventing pathogen access and decreasing inflammation [338]. The mucus can also act as a nutrient source to members of the gut microbiota capable of consuming mucin [339]. During an infection, pro-interleukin-1 β (produced by gut macrophages following contact dependent stimulation of the adaptor protein MYD88) is converted to active IL-1 β by commensals to inhibit pathogens and promote the recruitment of neutrophils (**Figure 2F**)[340]. The gut microbiota signals to B cells through MYD88 to promote immunoglobulin A production which binds to microbial antigens and neutralises pathogenic activity (**Figure 2G**) [341-344]. Commensal interactions with macrophages cause the release of Interleukin (IL) 23, triggering innate lymphoid cells to produce alarmin IL22 [345]. IL22 is also produced by T helper 17 cells which are upregulated by the presence of commensal LPS and flagellin [345]. This increases the expression of antimicrobial peptides (AMPs) such as REGIII γ (**Figure 2H**) [346-349], which has been linked to the eradication of pathogenic *Citrobacter rodentium* and VRE species [348, 350-352]. Commensals can also promote the secretion of α -defensins by Paneth cells (**Figure 2I**) [353, 354].

Further, the gut commensals can interact with the host to modify the gut environment in a manner which inhibits the colonisation of pathogenic species. SCFAs protect the host from colonisation by opportunistic pathogens indirectly by decreasing the environmental pH and oxygen levels, increasing the integrity of the intestinal epithelial cells, decreasing inflammation, and acidifying the cytoplasm of the pathogens.

Many commensal organisms, including members of the Bacteroidota, Bacillota and Fusobacteriota phyla, produce butyrate as a product of metabolism [270-272, 279-286, 355-357]. Colonocytes oxidise butyrate into carbon dioxide thus decreasing the pO₂ of the

environment [358, 359](**Figure 2B**). As previously stated, antibiotic treatment and gastrointestinal disease can increase the pO_2 [359-361]. As well as potentially inhibiting many obligate anaerobic commensals, higher oxygen environments are favoured by several pathogens including *E. coli* and *Salmonella enterica* serovar Typhimurium [362-365]. Therefore, the greater the number of butyrate producing commensals in the microbiota, the greater the hypoxia of the gut. Further, within the gut, butyrate can inhibit NF- κ B transcription factor activation and interferon gamma, as well as upregulate PPAR γ , thus decreasing inflammation in the intestinal mucosa [284-286]. Propionate has been shown to inhibit *S. aureus* infection in cell culture by decreasing *S. aureus* internalisation [366, 367].

While bile acids predominantly have a direct impact on colonisation resistance, there are immune interactions which require host cell involvement. Commensals can break down bile salts to produce deoxycholic acid which can induce granulocyte monocyte progenitor immune cells in the bone marrow (**Figure 2C**) [368, 369]. Among other purposes, bile acids play a role in signalling during inflammation reactions and are a key part of the metabolism of the host and other members of the microbiota [45-47]. The commensal, *Clostridium scindens*, produces lithocholic acid which can interact with the gut epithelial cells to upregulate the transcription of the antimicrobial peptide LL-37 in in vitro cell culture [48] and was shown to inhibit *Entamoeba histolytica* [49] and the CHIKV alphavirus [50] in a mouse model.

Indirect mechanisms of colonisation resistance are vital to suppress opportunistic pathogens in immunocompetent individuals. However, many of the patients in most need of a microbiome therapeutic to eradicate gut colonisation by a nosocomial pathogen are immunosuppressed. Whether the immune system already faces challenges from other

diseases and infections, the patient is on immunosuppressant therapeutic, or antibiotics have compromised the immune system [370, 371], relying on indirect mechanisms to develop a therapeutic will fail to address the most impacted cases. As such, direct mechanisms—which do not require any host involvement—present a more realistic avenue of exploration.

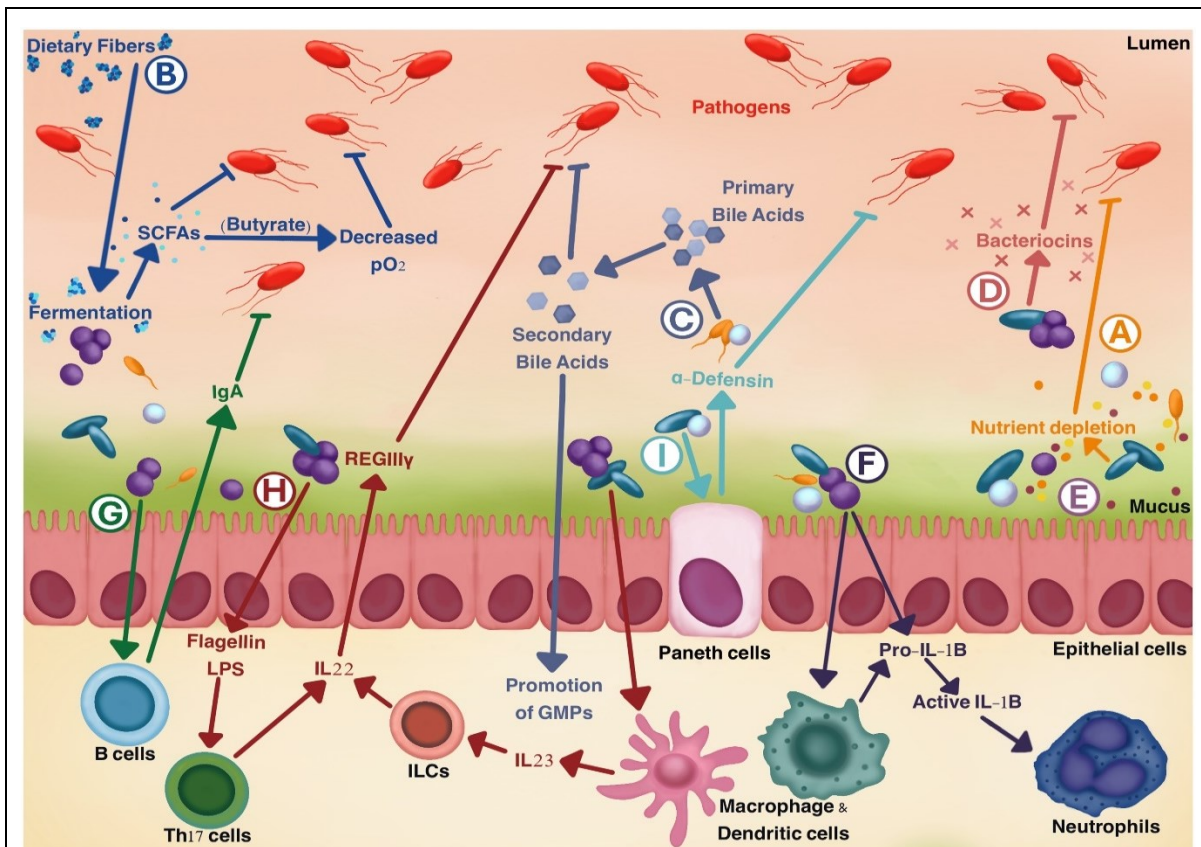


Figure 2: Mechanisms of colonisation resistance in the gut. The microbiota mediates colonisation resistance against pathogens (red cells) through direct and indirect interactions. The commensals of the gut microbiome are found on the luminal side of the epithelial intestinal wall in and around the mucus layer. **A)** The commensals of the gut compete for the limited nutrients. By depleting these nutrients, the growth of pathogens is suppressed. **B)** Dietary fibres are fermented by members of the gut microbiota producing short chain fatty acids (SCFAs) which can either directly inhibit pathogen growth by lowering the intracellular pH or indirectly (in the case of butyrate) by decreasing the partial pressure of oxygen (pO₂) in the microbiome. **C)** Primary bile acids are deconjugated by commensals into secondary bile acids which both directly inhibit the growth of pathogens as well as promote the production of granulocyte monocyte progenitor immune cells

(GMPs) in the bone marrow. **D)** Some commensals produce bacteriocins which inhibit pathogens in various ways. **E)** Some commensals increase mucus density and thickness by making SCFAs and inducing host genes associated with mucus production. **F)** Commensals stimulation of MYD88 pathway in gut macrophages causes an increase in the release of pro-IL-1 β from the immune cells. The commensals then convert the pro-IL-1 β to active IL-1 β which promotes the recruitment of neutrophils to suppress infection. **G)** The microbiota encourages the release of IgA from B cells which inhibits the growth of pathogens. **H)** Commensal flagellin and LPS encourage T helper 17 (Th17) cells to release IL22. Similarly, commensal interactions with macrophages causes an upregulation of IL23 which results in innate lymphoid cells (ILCs) producing IL22. IL22 causes an increase in REGIII γ which inhibits pathogens. **I)** The microbiota encourage Paneth cells to release the antimicrobial peptide, α -defensins, which inhibits pathogens

Direct mechanisms of colonisation resistance

Direct colonisation resistance is achieved through direct commensal-pathogen interactions, including nutrient competition, metabolite inhibition (production of SCFAs, branched-chain fatty acids (BCFAs), ethanol and secondary bile acid), bacteriocin production and bacteriophage activity [66, 334]. Direct methods of colonisation resistance are differentiated from indirect mechanisms in that they can occur independent of host cell involvement. These interactions can be modelled *in vitro* without host cell culture modelling.

Nutrient competition

Pathogens must have access to nutrients that support their growth in order to colonise the intestine. The availability of specific nutrients in the gut also significantly shapes the diversity of the gut commensal species present [56, 372-376]. The gut microbiota composition can rapidly change to reflect alterations to the host's diet [377, 378]. The gut microbiota composition varies when the host consumes plant versus animal proteins, with *Bifidobacterium* and *Lactobacillus* increased with exposure to whey and pea protein while *Bacteroides*, *Alistipes* and *Bilophila* are increased with animal protein intake [373, 379, 380]. Both monosaccharides and complex polysaccharides are consumed by the gut microbiota but

not all species can digest all of these available nutrients. Some interspecies interactions in the gut are cooperative in that one species degrades a complex nutrient and produces metabolites that other bacteria can consume. For example, sialic acid must be cleaved from mucin to become available as a nutrient source in the gut [381]. *B. thetaiotaomicron* expresses the sialidase enzyme which allows it to cleave sialic acid from mucin, thus supporting the growth of other members of the gut microbiota which require sialic acid for survival through cross feeding interactions [274, 275].

Microbial colonisation in the intestine requires the establishment of a “nutritional niche” that a specific microorganism can occupy, where this microorganism can outcompete other microbes with closely related or overlapping metabolic requirements [263, 382-384]. Microbes that can outcompete other microbes for key nutrients can restrict the growth of its competitors (**Figure 2A**). This direct mechanism of colonisation resistance has been demonstrated to successfully decolonise MDR pathogens including *E. coli* and *S. ser. Typhimurium* [385-389]. Commensal strains of *E. coli* have been shown to outcompete pathogenic *E. coli* O157:H7 for essential proline and sugars found in the intestinal mucous [385-387]. Likewise, the suppression of *S. ser. Typhimurium* by commensal *E. coli* strains was achieved due to the depletion of iron and zinc reservoirs by *E. coli* [388, 389]. As every commensal strain has a unique metabolic profile, the higher the microbial diversity in the gut, the less likely nutrients will be available to support the growth of an opportunistic pathogen.

Metabolite inhibition

In order to successfully colonise the intestine, microbes must be able to grow in the presence of metabolites that are produced by the gut microbiota. Diets rich in fibre increase the concentration of SCFAs found in the distal gut, which favours the colonisation of microbial species that are capable of withstanding lower pH and oxygen levels [390]. SCFAs are the

fermented products of commensal bacteria consumption of nondigestible, plant- or mucin-derived dietary fibres, proteins, and amino acids [11]. SCFAs are carboxylic acids with an aliphatic chain containing fewer than 6 carbons. Increasing in order by one carbon in the aliphatic tail, the SCFAs found in the gut are formate, acetate, propionate, butyrate and valerate. However, the most common SCFAs in the gut are acetate, propionate, and butyrate making up 90-95% of the SCFAs in the distal gut [66, 391]. Isobutyrate and isovalerate are branched chain fatty acids (BCFAs) that have two and three carbons respectively and are produced through protein fermentation [11, 392, 393]. Gut commensals produce polysaccharidases and glycosidases that hydrolyse the complex carbohydrates through several pathways including the pentose phosphate and Embden-Meyerhof-Parnas pathways to produce pyruvate, which is further metabolised to produce SCFAs [394]. Acetate and propionate are predominantly produced by members of the Bacteroidota phylum while butyrate is produced primarily by members of the Bacillota phylum [395].

Various pathogens have been shown to respond to colonisation resistance by metabolite inhibition. SCFAs are predominantly found in the colon where there is an inverse correlation between pH and SCFA concentration [396]. The pH affects the ionisation state of the SCFAs (pKa 4.8-4.9) [390, 397, 398] such that, at lower pHs, the metabolite inhibition by SCFAs is most effective [30, 399-401]. Within the bacterial cell cytoplasm, nonionised SCFAs dissociate and lower the intracellular pH, inhibiting microbial growth (**Figure 2B**). Acetate has also been shown to inhibit the growth of *E. coli* by preventing the synthesis of methionine, leading to the subsequent accumulation of toxic homocysteine [391, 402]. In another example, restoring valerate concentrations in an antibiotic-treated gut microbiome was shown to reduce *C. difficile* vegetative growth by 94% [403]. A combination of acetate, propionate, butyrate and

valerate was shown to inhibit the colonisation of Carbapenem-resistant *Enterobacteriaceae* (CRE) in a mouse model [30].

Since the beneficial impact of SCFAs is concentration dependent, disruptions to their production can lead to decreased metabolite inhibition in the gut [404]. Antibiotics which decrease the diversity and density of the gut microbiota result in a decrease in metabolite production, thus limiting the efficiency of colonisation resistance.

Bile acid inhibition

Some members of the gut microbiota can perform bile acid deconjugation and 7 α -dihydroxylation reactions, which convert conjugated primary bile acids into secondary bile acids [56, 405, 406]. Bile acids are produced in the liver using micelles of cholesterol and phospholipids and are excreted by the gallbladder. As the bile acids make their way along the digestive system, they improve host absorption of lipid soluble vitamins, fatty acids and triglycerides [407, 408]. While a majority of the bile acids produced are reabsorbed in the ileum, some make it to the colon. In addition to facilitating the immune reactions previously mentioned, bile acid metabolism can directly inhibit pathogens (**Figure 2C**). For example, *C. difficile* spores can be inhibited by deconjugated secondary bile acids (deoxycholate and lithocholate), while the primary bile acids taurocholate and cholate promotes spore germination and vegetative growth [409]. The aforementioned gut commensal *C. scindens* has 7 α -dehydroxylase activity and produces deoxycholate and lithocholate through metabolism of deconjugated primary bile acids. As such, this commensal strain is strongly correlated with *C. difficile* inhibition [410]. Deoxycholate can also inhibit the growth of other pathogens (such as *S. aureus*) by causing membrane disruption [411, 412].

Bacteriocin production

Bacteriocins are a direct mechanism of colonisation resistance that can restrict the growth of bacteria in the intestine (**Figure 2D**). Bacteriocins are toxic peptides that can induce cell death in bacteria by preventing RNA and DNA production, inhibition of cell wall and protein synthesis, and pore formation [66, 413-417]. Both Gram-positive and Gram-negative bacteria can produce bacteriocins, however the main species involved in bacteriocin production are *Streptococcus* species, lactic acid bacteria, and *Enterobacteriaceae* [66]. Bacteriocins can be either class I post-translationally modified peptides, class II unmodified peptides, or class III proteins [416, 418].

While some bacteriocins require other compounds to inhibit pathogens [419-423], successful growth suppression has been demonstrated for many pathogens. *Listeria monocytogenes* can be inhibited with Abp118 produced by *Lactobacillus salivarius* [383]. Colicin F_v, produced by members of *Yersiniaceae* such as *Yersinia frederiksenii*, can inhibit *Yersinia enterocolitica* [424]. Various *C. difficile* strains are inhibited by the class I peptide thuricin CD, produced by *Bacillus thuringiensis*, to a greater extent than metronidazole treatment [66, 413, 425-427]. Both VRE [428] and *S. ser. Typhimurium* [384] were suppressed by the microcin, *Escherichia coli* Nissle 1917 (EcN), produced by commensal *E. coli* Nissle 1917.

Emerging treatments for GI pathogen colonisation

Antibiotic treatment kills gut commensals and disrupts colonisation resistance in the intestine [56, 66, 75, 351, 394, 429]. Reintroducing gut commensals into the gut that were killed by antibiotic treatment may restore colonisation resistance and protect the host against MDR pathogen intestinal colonisation and the subsequent development of invasive infections. Following this logic, previous clinical trials have investigated the use of faecal microbiota transplants (FMTs) as a therapeutic to eliminate a gastrointestinal infection by reintroducing

biodiversity—and, consequently, colonisation resistance—to the gut [69, 75, 403, 430]. FMT involves transferring homogenised faeces from a healthy donor into the intestine of a patient with MDR pathogen colonisation. FMT has been very successful at treating *C. difficile* infection and has more recently been explored for decolonisation of CRE and VRE [69, 431].

However, FMTs are not without risk. Although the procedure is generally considered safe, there is the potential to transfer a pathogen from the faeces of the donor into the intestine of the recipient, which is a particular danger as the recipient patients are often immunocompromised. Fatalities following FMT treatments have been caused by the accidental transmission of MDR pathogens (in one case leading to toxic megacolon) as well as aspiration of the culture [429, 432-438]. Although donor faeces used to prepare the FMT are screened for specific pathogens, screening is not possible for all pathogens, and moreover some screening methods may not be sensitive enough to detect all pathogens. If an immunocompromised patient received an FMT with an undetected opportunistic pathogen, the resulting infection could cause severe damage, extended hospitalization, or death [436-438]. Furthermore, when a FMT successfully eliminates an infection, the exact mechanisms and/or polymicrobial interactions which led to the pathogen eradication are unknown.

A targeted, rationally-designed, and safer treatment can be designed to decolonise MDR pathogens by identifying which gut commensal strains and microbial metabolites can restrict MDR pathogen growth in the intestine [439]. A “synthetic stool” composed of 33 gut commensal species has been shown to cure two patients with recurrent *C. difficile* infection [440]. Another study demonstrated that a filtered faecal suspension which contained the microbial debris and microbial products (e.g. metabolites) from faeces was able to cure five patients with *C. difficile* infections [441]. Therefore, the potential for a defined microbial

consortium or microbial metabolites to restrict intestinal pathogen growth has been previously demonstrated and has the potential to be effective for MDR pathogens as well.

Hypothesis

This study aimed to combine taxonomic and metabolomic analysis to elucidate the interactions between VRE and the gut microbiota with the goal of developing a microbiome therapeutic which harnessed the colonisation resistance mechanisms of a healthy gut.

Limited research has been conducted regarding the potential for a treatment based on metabolite inhibition or the reestablishment of nutrient competition. VRE requires nutrients to support its colonisation of the gut but very little is known about which nutrients are pivotal to its survival and how antibiotic killing of commensals decreases the nutrient competition between the microbiota and the VRE. Metabolites are a product of the utilisation of nutrients by the gut microbiota. While other studies have shown potential application of the metabolites against pathogenic colonisation of the gut [30, 403], research regarding their impact on VRE is limited. This study was designed to determine how antibiotic treatment alters the nutrient competition and metabolite production in the gut microbiome and how VRE strains exploit these gaps in colonisation resistance.

There were two hypotheses that were tested in this thesis:

1. Antibiotic usage decreases gut microbiota-mediated colonisation resistance by killing members of the gut microbiota which:
 - a. Outcompete VRE for essential nutrients, and/or
 - b. Produce microbial metabolites capable of inhibiting VRE growth.

2. Colonisation resistance to VRE can be re-established in an antibiotic-treated gut by reintroducing key members of the gut microbiota, or specific metabolites that these key bacteria produce.

Aims

The aims of this project are:

1. To measure VRE growth, gut microbiota composition, nutrient availability, and metabolite production in an antibiotic-treated vs antibiotic-naïve gut microbiota.
2. To characterise the nutrient utilisation abilities and preferences of VRE and gut commensal bacteria.
3. To characterise the effects of microbial metabolites on VRE growth and to identify gut commensal bacteria that produce these metabolites.

Chapter 2: Methodology

Microbial metabolite concentration data presented in this chapter (derived from faecal batch cultures performed in this study) was published in Nature Communications [30] (second author).

General media preparation

Tryptic soy media

Tryptic soy broth (TSB) was purchased from Sigma and prepared based on the manufacture specifications (30 g/L) with deionised (DI) water. Tryptic soy agar (TSA) plates were used as a nonselective medium prepared by adding 4% (weight/volume, w/v) agar to TSB and autoclaving. Plates were stored at 4 °C and used within 4 weeks of preparation.

Fastidious anaerobic agar

Fastidious anaerobic agar (FAA) was purchased from SLS UK (MED 1332) and prepared based on manufacture specifications (46 g/L) with DI water and 4% (w/v) agar. After autoclaving, the media was cooled to 55 °C before adding 5% (volume/volume, v/v) defibrinated horse blood. Plates were stored at 4 °C and used within 4 weeks of preparation.

Columbia Agar

Columbia agar base was purchased from VWR, UK (84621.05) and prepared based on manufacture specifications (39 g/L) with DI water. After autoclaving, the media was cooled to 55 °C before poured into plates. Plates were stored at 4 °C and used within 4 weeks of preparation.

Reasoners 2A agar

Reasoners 2A (R2A) agar plates were used as a nonselective minimal media to limit the transfer of carbon sources to broth culture in nutrient utilisation experiments. The R2A mix contained casein hydrolysate (0.5 g/L), dextrose (0.5 g/L), soluble starch (0.5 g/L), yeast

extract (0.5 g/L), dipotassium phosphate (0.3 g/L), sodium pyruvate (0.3 g/L), casein peptone (0.25 g/L), meat peptone (0.25 g/L), magnesium sulphate (0.024 g/L) and agar (15 g/L). The mix was added to DI water and autoclaved. Plates were stored at 4 °C and used within 4 weeks of preparation.

Brilliance™ VRE agar

Brilliance™ VRE agar plates are an Oxoid product and were purchased from Thermo Fisher Scientific UK (PO1175). *Brilliance™ VRE agar* contains peptone mix (25 g/L), salt mix (12 g/L), agar (12.5 g/L), titanium dioxide (1 g/L), chromogenic mix (0.45 g/L) and an antibiotic cocktail including vancomycin (5 mL). The plates were designed as a selective and differential media for the growth of NCTC 12201 *E. faecalis* and NCTC 12202 *E. faecium* VRE strains. On these cream coloured plates, *E. faecalis* forms light blue colonies while *E. faecium* forms dark indigo colonies. The plates were stored 4 °C and used prior to the expiration date listed on the package.

Distal gut growth medium

Distal gut growth medium (DGGM) was designed to replicate the nutrients found in the human distal gut for culture of faecal microorganisms. The media, initially described by Macfarlane et. al. [442, 443], was optimised by Dr Julie McDonald for the artificial gut chemostat model used in this study [30, 403, 444]. The media contains unmodified wheat starch (5 g/L), casein (3 g/L), inulin from Dahlia tubers (1 g/L), sodium chloride (0.1 g/L), peptone water (2 g/L), yeast extract (2 g/L), sodium bicarbonate (2 g/L), pectin from citrus (2 g/L), xylan from beechwood (2 g/L), arabinogalactan (2 g/L), calcium chloride (0.01 g/L), porcine gastric type II mucin (4 g/L), potassium phosphate monobasic (0.04 g/L), potassium phosphate dibasic (0.04 g/L), magnesium sulphate heptahydrate (0.01 g/L), hemin (0.005 g/L), menadione (0.001 g/L), bile salts (0.5 g/L), and L-cysteine hydrochloride (0.5 g/L). The media

was prepared with DI water with a final pH of 8.0-8.5. For use in the artificial gut chemostat experiments, autoclaved antifoam (5 mL/L) was added. Media was stored at 4 °C for no more than 2 weeks prior to use.

Bacterial strains

VRE growth conditions

Two representative vancomycin-resistant *Enterococcus* strains were used in this study: *E. faecalis* (NCTC 12201) and *E. faecium* (NCTC 12202). Both strains were isolated in 1986 in the UK and were responsible for the first VRE infections in the country [102, 445-448]. The strains, along with 53 other VRE were isolated from 22 patients in multiple organ failure in Dulwich Public Health Laboratory [445]. Both strains were identified to be highly resistance to vancomycin and teicoplanin making them VanA resistance phenotypes [449]. These strains were purchased from Public Health England and received in February 2021. All stocks were maintained in cryogenic freezer media (120 g/L, 10 mL/L DMSO, 10 mL/L glycerol) at -80 °C. Strains were grown in 10 mL sterile TSB in sterile vials with loose lids or on R2A, TSA, or Brilliance VRE plates anaerobically or aerobically at 37 °C for 24 hours prior to use.

Commensal growth conditions

Fifteen gut commensal strains (**Table 1**) were used in this study. These strains were isolated from stool (from a healthy 30 years old (y.o.) female donor who had not been on antibiotics in six months prior to donation, with a vegetarian diet) and sequenced by the McDonald lab or purchased from LMG or DSM in September 2022. Gut commensals were all stored in cryogenic freezer media (listed above) at -80 °C. All strains were grown anaerobically at 37 °C on FAA with 5% (v/v) defibrinated horse blood for 24 hours.

Table 1: Commensal strains. 15 commensal strains were used in this study from the phyla Actinomycetota and Bacteroidota. The strains were either purchased from LMG or DSM or isolated from healthy faeces by the McDonald laboratory.

Phylum	Family	Species	Isolated from	Strain ID
Actinomycetota	Bifidobacteriaceae	<i>Bifidobacterium adolescentis</i>	Healthy human faeces	LMG 29394
Actinomycetota	Bifidobacteriaceae	<i>Bifidobacterium bifidum</i>	Healthy human faeces	LMG 29396
Actinomycetota	Bifidobacteriaceae	<i>Bifidobacterium catenulatum</i>	Human intestine or faeces	LMG 11043
Actinomycetota	Bifidobacteriaceae	<i>Bifidobacterium longum</i>	Healthy human faeces	10MRS-AN
Actinomycetota	Bifidobacteriaceae	<i>Bifidobacterium pseudocatenulatum</i>	Healthy human faeces	LMG 29397
Actinomycetota	Coriobacteriaceae	<i>Collinsella aerofaciens</i>	Healthy human faeces	7BHI-AN
Bacteroidota	Bacteroidaceae	<i>Bacteroides caccae</i>	Healthy human faeces	13TSA-AN
Bacteroidota	Bacteroidaceae	<i>Bacteroides dorei</i>	Healthy human faeces	12TSA-AN
Bacteroidota	Bacteroidaceae	<i>Bacteroides intestinalis</i>	Human faeces	DSM 108646
Bacteroidota	Bacteroidaceae	<i>Bacteroides ovatus</i>	not listed	LMG 28902
Bacteroidota	Bacteroidaceae	<i>Bacteroides stercoris</i>	Healthy human faeces	B6Y1
Bacteroidota	Bacteroidaceae	<i>Bacteroides thetaiotaomicron</i>	Healthy human faeces	9FAA-AN
Bacteroidota	Bacteroidaceae	<i>Bacteroides uniformis</i>	Healthy human faeces	29FAA-AN
Bacteroidota	Bacteroidaceae	<i>Bacteroides vulgatus</i>	Healthy human faeces	5NA-AN
Bacteroidota	Bacteroidaceae	<i>Bacteroides xylanisolvens</i>	Healthy human faeces	15FAA-AN

Faecal donor involvement

Ethical approval

Faecal samples were collected from healthy donors under the Imperial Hepatology and Gastroenterology Biobank ethics (REC Reference No. 20/SC/0389, approved by the London-Queen Square Research Ethics Committee (19/LO/0112) and the South Central-Oxford C Research Committee (16/SC/0021 and 20/SC/0389) on 12 Feb 2021).

Recruitment

All donors used in this research were recruited within the guidelines of the Biobank ethical criteria. Prior to providing a donation, donors were verbally assessed for participation before filling out case report and consent forms. Fully informed and voluntary participation in this study was confirmed through the completion of an information sheet and consent form which thoroughly detailed the objectives of the research.

Criteria

All participants were over 18 years of age (20-56 y.o.) and had not undergone antibiotic treatment in the six months prior to their involvement. Donors were excluded if they were currently experiencing any digestive irregularity or had a family history of gastrointestinal disease (including but not limited to Crohn's disease, inflammatory bowel disease, irritable bowel syndrome, or gastrointestinal cancer). This study included 12 donors of whom seven were female and five were male with an age distribution of 29.5 ± 9.5 . Nine of the participating donors consumed a western diet, one was vegetarian and two were pescatarian.

Sample collection

For each experiment requiring a faecal sample, a donation was collected immediately prior to the initiation of the experiment. All samples were collected in sterile stool collection containers and immediately placed in a sealed bag containing an anaerobic gas generating sachet (Thermo Scientific UK, AN0025A). Samples were transferred into an anaerobic chamber within one hour of donation. For each donation, three cryovials were filled with faeces and frozen at $-80\text{ }^{\circ}\text{C}$. Additionally, one aliquot was prepared for 1-hydrogen nuclear magnetic resonance ($^1\text{H-NMR}$) spectroscopy by creating a 25% (w/v) faeces diluted in HPLC-grade water, vortexing for 10 minutes, centrifuging ($17,000 \times g$) for 10 minutes at $4\text{ }^{\circ}\text{C}$, and freezing the supernatant at $-80\text{ }^{\circ}\text{C}$ [30].

Preparation of antibiotics known to promote VRE colonisation

Five antibiotics were selected based on their ability to promote the growth of VRE in the intestine [55, 61, 64, 67, 68, 70, 71, 75, 183, 201-210]. The antibiotics included a β -lactam/ β -lactam inhibitor penicillin (piperacillin/tazobactam, TZP), a cephalosporin (ceftriaxone, CRO), a glycopeptide (vancomycin, VAN), a lincosamide (clindamycin, CLI), and a nitroimidazole

(metronidazole, MTZ). The penicillins, cephalosporin and glycopeptide have effector mechanisms inhibiting cell wall synthesis, the lincosamides inhibits 50S protein synthesis and the nitroimidazole causes DNA damage [459-461, 462, 463]. Each antibiotic was tested at concentrations previously measured in human faecal samples to best assess the impact of these antibiotics on the gut microbiota and gut pathogens *ex vivo* [68, 201, 202, 205, 206, 209, 210, 450]. Antibiotic solutions were prepared by dissolving the antibiotic stock powder or liquid in water before filter sterilization using a 0.2-micron filter. Stocks were made fresh immediately prior to every experiment.

Antibiotic minimum inhibitory concentration assays

Minimum inhibitory concentration (MIC) assays were run for the five selected antibiotics for both NCTC 12201 *E. faecalis* and NCTC 12202 *E. faecium*. Per EUCAST guidelines (version 13, 2023)[451], broth microdilution MIC assays were performed in Mueller-Hinton broth in 96-well plates with the VRE inoculated in at 5×10^5 CFU/mL [30, 452]. The plates were aerobically incubated with a plate seal at 36 °C for 18 hours. The final OD ($\lambda=600$ nm) was read and the inoculated well ODs were normalised by subtracting the sterile well ODs. EUCAST guidelines only provided breakpoint values of MICs for *Enterococcus* for VAN (≤ 4 mg/L sensitive, >4 mg/L resistant); MTZ, CRO, CLI, and TZP were not provided.

Impact of antibiotics on VRE growth in pure culture

All antibiotics were tested on both *E. faecalis* and *E. faecium* in triplicate at concentrations mimicking their concentrations measured in human faeces (as described above). Test conditions included water control, VAN (1000 μ g/mL), MTZ (25 μ g/mL), CRO (152 μ g/mL), TZP (139 μ g/mL), and CLI (383 μ g/mL). Conditions were prepared in triplicate by adding antibiotics or water to TSB or DGGM and inoculating with 1.5×10^4 CFU/mL of either *E. faecalis* or *E.*

faecium (final concentration 10^3 CFU/mL). TSB cultures were incubated aerobically while DGGM cultures were incubated anaerobically, both at 37 °C for 24 hours. To quantify growth in each sample, eight serial dilutions (10^0 to 10^{-7}) were prepared from the cultures using peptone water. Five 10 µL drops of each dilution were plated on Columbia agar using the Miles and Misra plating protocol [453] before plates were incubated for 24 hours at 37 °C.

Faecal batch cultures

Faecal batch culture experiments were set up for 12 unique healthy faecal donors, where each condition was performed in triplicate. The donors for this experiment included seven females (31 ± 11.8 y.o.) and five male (27.6 ± 5.5 y.o.) with an average age of 30. Of the participating donors, nine consumed a western diet, one was vegetarian and two were pescatarian. A 20% (w/v) faecal inoculum was prepared by homogenizing freshly donated faeces and 0.9% (w/v) degassed saline in a stomacher strainer bag using a Stomacher® 80 Biomaster Lab Blender (Seward UK, 0080/000/EU) for one minute on normal speed. Faeces were inoculated into DGGM at a final concentration of 2% (w/v). Antibiotics were added to a final concentration measured in faeces (1000 µg/mL VAN, 25 µg/mL MTZ, 152 µg/mL CRO, 139 µg/mL TZP, and 383 µg/mL CLI) [68, 201, 202, 205, 206, 209, 210, 450]. Samples were incubated with loose lids anaerobically for 24 hours at 37 °C. Samples were analysed by ^1H -NMR spectrometry, 16S rRNA gene sequencing, and 16S rRNA quantitative polymerase chain reactions (qPCR) as described below.

VRE growth in antibiotic-treated faecal batch cultures

Repeat faecal batch cultures were run using 5-8 unique donors per condition, performed in triplicate. The participants included five female and three male donors, all but one of whom were consumers of a western diet while the remaining was vegetarian. The age distribution

was 26.8 ± 4.3 years (27 ± 5.1 y.o. for female, 26.3 ± 3.5 y.o. for male). Preparation of the faeces and antibiotics was identical to the previous faecal batch culture experiment described above. The culture was incubated with loose lids anaerobically for 24 hours at 37 °C. After 24 hours, *E. faecalis* or *E. faecium* were inoculated to a final concentration of 10^3 CFU/mL and the cultures were incubated for a further 24 hours anaerobically at 37 °C. Serial dilutions (10^0 to 10^{-7}) were prepared using peptone water. To quantify VRE growth five 10 µL drops of each dilution were plated on Brilliance™ VRE agar using the Miles and Misra plating protocol [453] before plates were aerobically incubated for 24 hours at 37 °C.

Carbon utilisation capabilities of VRE

VRE minimal media

To test the individual carbon utilisation ability of VRE strains, a minimal media was designed and consisted of the following: potassium phosphate monobasic (4 g/L), potassium phosphate dibasic (14 g/L), sodium citrate (1 g/L), magnesium sulphate heptahydrate (0.2 g/L), ammonium sulphate (2 g/L), adenosine (0.04 g/L), thymine (0.04 g/L), Kao and Michayluk vitamin solution [Sigma, K3129, 50x Stock](10 mL/L), and amino acid solution [Sigma, R7131, RPMI-1640, 50x](50 mL/L) was prepared in DI water. If the study was performed under anaerobic conditions, fumarate (5.2 g/L) was added to increase growth of VRE strains. Media was filter sterilised with a 0.22-µm filter and used immediately for aerobic experiments or degassed overnight for anaerobic experiments.

Individual carbon source utilisation assay

VRE strains were grown on R2A plates overnight before the cells were suspended in 0.9% (w/v) saline to an optical density (OD) of 0.38 ($\lambda=600$ nm). Then, a 10% dilution of the cell suspension was prepared using VRE minimal media to achieve a final well OD of 0.06 ($\lambda=600$

nm) greater than the sterile media OD. To measure carbon utilisation in 96 well plates, 100 μ L of the diluted cell suspension (prepared in minimal media) was added to 100 μ L of 1% (w/v) carbon source (prepared in DI water and filter sterilised). The carbon sources individually assessed were L-alanine, L-arabinose, L-arginine, L-aspartic acid, D-fructose, L-fucose, D-galactose, D-glucose, L-glutamic acid, L-glycine, L-isoleucine, L-leucine, L-lysine HCL, D-maltose, D-mannose, L-methionine, N-acetylglucosamine, L-phenylalanine, L-proline, D-ribose, succinate, sucrose, L-threonine, D-trehalose, L-tryptophan, L-tyrosine, uracil, L-valine, and D-xylose. Aerobic plates were incubated with a plate seal while anaerobic plates were not (plate seal was not compatible with the anaerobic plate reader). The aerobic plate reader was set to read the plate (λ =600 nm, 37 °C, 22 flashes per well, orbital shaking of 100 rpm for 15 seconds prior to each read) every 15 minutes for 24 hours. The anaerobic plate reader (Stratus®, Cerillo, Charlottesville, VA, USA) read the plate (λ =600 nm, 37 °C, stationary) every 15 minutes for 24 hours. The initial OD was subtracted from the final OD for each well and growth curves were generated from the data. Glucose was used as a positive control while water was used as a negative control. On each plate, uninoculated media was diluted in water at a 1:1 ratio to confirm sterility. Each carbon source was prepared in triplicate for each plate, and plates were prepared in triplicate at minimum (biological n=2-3, technical n=6-9). Each plate had inoculated culture supplemented with water instead of a carbon source as a negative control and uninoculated media for sterility testing.

Complex carbon sources were assessed with plate counts in three independent experiments following the protocol described above. VRE minimal media was supplemented with peptone, casein, pectin, mucin, starch, arabinogalactan, bile salts, inulin, xylan, yeast extract, or pyruvate. Cultures were inoculated at 1×10^8 CFU/mL (the cell density equivalent to the 0.38

OD₆₀₀ used for soluble carbon sources described above) and incubated aerobically for 24 hours. As some of the complex substrates were cloudy, VRE growth was measured using plate counts. Serial dilutions were prepared and plated on Columbia agar, and the plates were incubated aerobically and counted the following day.

Nitrogen utilisation capabilities of VRE

VRE minimal media

To test the individual nitrogen utilisation ability of VRE strains, a minimal media consisted of the following: potassium phosphate monobasic (4 g/L), potassium phosphate dibasic (14 g/L), sodium citrate (1 g/L), magnesium sulphate (0.2 g/L), ammonium sulphate (2 g/L), adenosine (0.04 g/L), thymine (0.04 g/L), Kao and Michayluk vitamin solution [Sigma, K3129, 50x Stock](5x), and glucose (1 g/L). If the study was performed under anaerobic conditions, fumarate (5.2 g/L) was added to increase growth of VRE strains. Media was filter sterilised with a 0.22-µm filter and used immediately for aerobic experiments or degassed overnight for anaerobic experiments.

Nitrogen utilisation “leave-one-out” experiment

The NCTC 12201 and 12202 had undefined amino acid auxotrophies, therefore a leave-one-out experiment was performed. VRE strains were grown on Reasoner’s 2 Agar overnight before the cells were suspended in 0.9% (w/v) saline to an OD of 0.38 ($\lambda=600$ nm). A 10% dilution of the cell suspension was prepared using VRE minimal media to achieve a final well OD of 0.06 ($\lambda=600$ nm) greater than the sterile media OD. To test nitrogen source utilisation, a nitrogen source solution was prepared to mimic the amino acid solution, containing L-alanine (2 g/L), L-arginine (2.5 g/L), L-asparagine (0.71 g/L), L-aspartate (0.25 g/L), L-cysteine (0.625 g/L), L-glutamate (0.25 g/L), L-glycine (0.125 g/L), L-histidine (0.1875 g/L), L-isoleucine

(0.625 g/L), L-leucine (0.625 g/L), L-lysine HCl (0.5 g/L), L-methionine (0.1875 g/L), L-phenylalanine (0.1875 g/L), L-proline (0.25 g/L), L-serine (0.375 g/L), L-threonine (0.25 g/L), L-tryptophan (0.0625 g/L), L-tyrosine (0.29 g/L), L-valine (0.25 g/L), N-acetylglucosamine (2 g/L), and uracil (2.5 g/L). To test the strain's ability to utilise individual nitrogen sources, separate solutions were prepared eliminating one nitrogen source each. The change in growth compared to the complete nitrogen source solution control was used to determine nitrogen utilisation. To measure nitrogen utilisation in 96 well plates, 100 μ L of the diluted cell suspension was added to 100 μ L of the nitrogen source mixture (complete or leave-one-out, filter sterilised) that had been prepared in minimal media. Aerobic plates were incubated with a plate seal while anaerobic plates were not. The aerobic plate reader was set to read the plate (λ =600 nm, 37 °C, 22 flashes per well, orbital shaking of 100 rpm for 15 seconds prior to each read) every 15 minutes for 24 hours. The anaerobic plate reader read the plate (λ =600 nm, 37 °C, stationary) every 15 minutes for 24 hours. The initial OD was subtracted from the final OD for each well and growth curves were generated from the data. The complete nitrogen source solution was used as a positive control while water was used as a negative control. Each leave-one-out nitrogen source solution was plated in three wells and plates were prepared in triplicate at minimum (biological n=2-3, technical n=6-9). Each plate had inoculated culture supplemented with water instead of the nitrogen source mix as a negative control and uninoculated media for sterility testing.

Carbon utilisation capabilities of commensal consortium

Gut commensal minimal media

A minimal media was developed by the McDonald lab which supports the growth of all 15 gut commensal strains used in this research following supplementation with an appropriate

carbon source. The media contained the following: potassium phosphate dibasic (18.8 g/L), potassium phosphate monobasic (8.4 g/L), ammonium sulphate (2.252 g/L), L-cysteine hydrochloride (2.005 g/L), yeast extract (2 g/L), sodium chloride (1.78 g/L), calcium chloride (162 mg/L), magnesium sulphate (60.6 mg/L), magnesium chloride (48 mg/L), ethylenediaminetetraacetic acid (10.1 mg/L), manganese (II) chloride (10.1 mg/L), pantetheine (10 mg/L), nicotinic acid (10 mg/L), hemin (7.5 mg/L), L-arginine (5 mg/L), L-asparagine (5 mg/L), L-aspartate (5 mg/L), L-glutamine (5 mg/L), L-glycine (5 mg/L), L-histidine (5 mg/L), L-isoleucine (5 mg/L), L-leucine (5 mg/L), L-lysine HCl (5 mg/L), L-methionine (5 mg/L), L-phenylalanine (5 mg/L), L-serine (5 mg/L), L-threonine (5 mg/L), L-tryptophan (5 mg/L), L-tyrosine (5 mg/L), L-valine (5 mg/L), iron sulphate (4 mg/L), zinc chloride (2 mg/L), adenosine (2 mg/L), thymine (2 mg/L), uracil (2 mg/L), guanosine (2 mg/L), menadione (0.75 mg/L), nickel dichloride (0.4 mg/L), cobalt (II) chloride (0.4 mg/L), copper (II) chloride (0.2 mg/L), boric acid (0.2 mg/L), Kao and Michayluk vitamin solution [Sigma, K3129, 50x Stock](30 ml/L), and tween (0.2 mL/L). The media was prepared in DI water and sterilised with a 0.2- μ m filter. The media was made one day prior to use in order to allow the media to degas in the anaerobic chamber overnight.

Gut commensal carbon source utilisation assay

Gut commensal strains were grown on FAA plates with 5% (v/v) defibrinated horse blood overnight before the cells were suspended in 0.9% (w/v) saline to an OD of 0.38 ($\lambda=600$ nm). A 10% dilution of the cell suspension was prepared using gut commensal minimal media to achieve a final well OD of 0.06 ($\lambda=600$ nm) greater than the sterile media OD. To measure carbon utilisation in 96 well plates, 100 μ L of the diluted cell suspension was added to 100 μ L of 1% (w/v) carbon source (filter sterilised) that had been prepared in minimal media. The individual carbon sources assessed were L-arabinose, D-fructose, D-galactose, D-glucose, D-

maltose, D-mannose, N-acetylglucosamine, D-ribose, sucrose, and D-trehalose. Plates were incubated anaerobically without a plate seal for 24 hours at 37 °C. An endpoint OD ($\lambda=600\text{nm}$) was measured and inoculated well ODs were normalised by subtracting the sterile media well ODs. Three biological replicates were run with 3 technical replicates in each. Each plate had inoculated culture supplemented with water instead of a carbon source as a negative control and uninoculated media for sterility testing.

Mixed nutrient utilisation assays

Vancomycin-resistant *Enterococcus*

A nutrient source solution was prepared, with each nutrient at a concentration of 0.03% (w/v): L-alanine, L-arabinose, L-arginine, L-aspartic acid, D-fructose, L-fucose, D-galactose, D-glucose, L-glutamic acid, L-glycine, L-isoleucine, L-leucine, L-lysine HCL, D-maltose, D-mannose, L-methionine, N-acetylglucosamine, L-phenylalanine, L-proline, D-ribose, succinate, sucrose, L-threonine, D-trehalose, L-tryptophan, L-tyrosine, uracil, L-valine, and D-xylose. From this point on, this solution of 29 carbon sources (0.03% (w/v) stock to be diluted to 0.015% (w/v) working concentration) will be referred to as the nutrient mixture. The VRE minimal media was prepared as follows: potassium phosphate monobasic (4 g/L), potassium phosphate dibasic (14 g/L), sodium citrate (1 g/L), magnesium sulphate (0.2 g/L), ammonium sulphate (2 g/L), adenosine (0.04 g/L), thymine (0.04 g/L), Kao and Michayluk vitamin solution [Sigma, K3129, 50x Stock] (10 mL/L). VRE cells from an overnight R2A plate were suspended in 0.9% (w/v) saline solution to achieve an OD of 0.38 ($\lambda=600\text{ nm}$). A 10% dilution was prepared with the cell suspension using the minimal media to achieve a final well OD of 0.06 ($\lambda=600\text{ nm}$) greater than the sterile media OD. The inoculated media was added in equal volume with the nutrient source solution or sterile DI water and aliquoted into sterile twist cap sample tubes and incubated at 37 °C aerobically and anaerobically. Based on pre-

conducted aerobic and anaerobic growth curves (OD, $\lambda=600\text{nm}$, one read every 15 minutes for 24 hours) of the VRE strains, 1 mL was removed from the culture tubes at the time of inoculation, during exponential phase (4.5 hours post inoculation), during stationary phase (9 hours post inoculation) and at 24 hours post inoculation. A dilution series was plated on Columbia agar for each sample time to assess VRE growth. The remaining volume was centrifuged ($4\text{ }^{\circ}\text{C}$, $17,000 \times g$) for 10 minutes before the supernatant was collected and stored at $-80\text{ }^{\circ}\text{C}$ for $^1\text{H-NMR}$ spectroscopy. The experiment was performed in triplicate.

Gut commensal consortium in coculture

The nutrient mix, described above for VRE, was also used for the gut commensal consortium nutrient utilisation. The solution was diluted to a final concentration of 0.015% in the gut commensal minimal media described above. One day prior to usage, the media was degassed in the anaerobic chamber.

All 15 commensal strains (shown in Table 1) were grown on FAA plates with 5% (v/v) defibrinated horse blood overnight. A single colony was used to inoculate each of the commensals into individual 2 mL cultures of the media described above. These cultures were incubated for 24 hours at $37\text{ }^{\circ}\text{C}$ in the anaerobic chamber. Overnight cultures yielded individual strain cell density between 1×10^8 and 6×10^9 CFU/mL. The individual cultures were mixed together in equal volumes, for a total concentration of between 1×10^9 - 2×10^9 CFU/mL. This gut commensal consortium was inoculated into degassed gut commensal minimal media with the nutrient mix to a final concentration of 1×10^7 CFU/mL. The inoculated culture was incubated anaerobically with a loose cap for 24 hours. A 1 mL sample was removed from the culture tubes at the time of inoculation, 4.5 hours post inoculation, 9 hours post inoculation and 24 hours post inoculation. The culture was centrifuged ($4\text{ }^{\circ}\text{C}$, $17,000 \times g$) for 10 minutes

before the supernatant was collected and stored at -80 °C for ¹H-NMR spectroscopy. The experiment was performed in triplicate.

Metabolite inhibition growth assays

Metabolite measurement from healthy human faeces

In order to determine the concentration of metabolites found in the distal gut of healthy individuals, ¹H-NMR spectra were generated from faecal samples of 12 healthy donors who had not been on antibiotics for six months prior to donation. Of the donors, five were female (30.9±12.1 years old) and seven males (27.8±5.3 years old). The average, minimum, and maximum concentration (**Table 2**) for each metabolite was determined using Chenomx NMR Suite (Chenomx, Canada, version 8.6).

Table 2: Concentrations of metabolites in faeces. Using ¹H-NMR spectra generated from faecal samples from 12 healthy donors, the concentrations of 10 metabolites were measured (mM). The undiluted concentrations were used to inform the selection of a minimum, average and maximum test concentration used in metabolite inhibition experiments.

Metabolite	Raw concentrations from NMR (mM)			Concentrations tested in inhibition assays (mM)		
	Minimum	Average	Maximum	Minimum	Average	Maximum
Formate	0.07	0.11	0.17	0.05	0.1	0.15
Acetate	10.54	64.08	122.73	10	65	120
Propionate	5.68	16.1	34.69	5	15	35
Butyrate	2.94	16.38	38.75	3	15	40
Valerate	0.52	3.67	12.01	0.5	3.5	12
Isobutyrate	0.33	2.2	5.28	0.5	2	5
Isovalerate	0.48	2	4.55	0.5	2	5
Lactate	0.26	0.67	1.2	0.3	0.7	1.2
5-aminovalerate	0.29	1.06	4.15	0.3	1	4
Ethanol	0.64	10.46	59.52	0.6	10	60

A Shapiro-Wilk normality test on the metabolite concentrations from the 12 donor showed normal distribution for all but valerate (p=0.0031), 5-aminovalerate (p=0.0001), and ethanol

($p < 0.0001$). Metabolites quantified were known to be produced by the gut microbiota and were found to be depleted in antibiotic-treated faecal cultures.

Mixed metabolite inhibition evaluation

Using the minimum, average and maximum concentrations found in **Table 2**, three stocks of the mixed metabolites (containing formate, acetate, propionate, butyrate, valerate, isobutyrate, isovalerate, lactate, 5-aminovalerate and ethanol) were prepared in TSB and the pH was adjusted to 6.0, 6.5 or 7.0 (to mimic the pH range of a healthy large intestine) [30] prior to being sterilised with a 0.22- μ m filter. One day before inoculation, TSB cultures of the VRE strains were incubated anaerobically at 37 °C. The VRE overnight culture was diluted in saline and inoculated into the mixed metabolite TSB cultures at 10^3 CFU/mL and incubated anaerobically for 16 hours at 37 °C before endpoint OD readings ($\lambda = 600$ nm) were measured. Each concentration of the mixed metabolites (minimum, average, maximum), at each of the three pHs (6.0, 6.5, 7.0), was tested in technical triplicates in three independent experiments (biological replicates $n=3$, technical replicates $n=9$).

Individual metabolite inhibition evaluation

The impact of individual metabolites on the growth of the VRE strains was assessed with the minimum, average and maximum concentrations (**Table 2**) found in faeces. Individual stocks of each metabolite at each concentration were prepared in TSB. The pH was adjusted to 6.0 prior to sterilization with a 0.22- μ m filter. Overnight anaerobic VRE stocks were diluted in saline and inoculated into the individual metabolite media to a final concentration of 10^3 CFU/mL. The cultures were incubated anaerobically for 16 hours at 37 °C before endpoint OD readings ($\lambda = 600$ nm) were taken. For each metabolite, a total of 24 technical replicates were run in 6 independent experiments.

VRE colonisation modelling in the artificial gut model

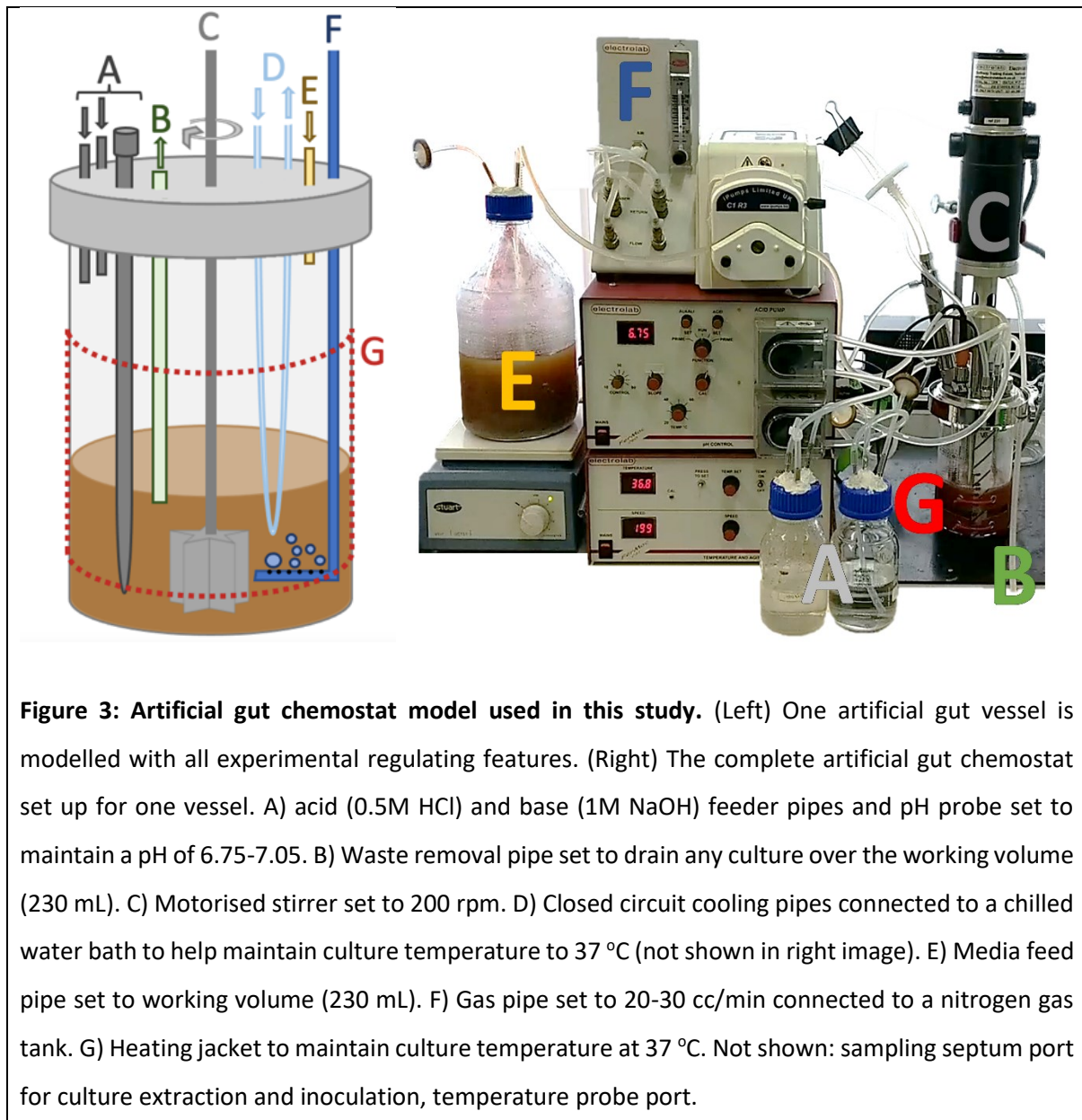
Artificial gut model

While mouse models are commonly used for MDR pathogen studies [55, 67, 75], differences in the gut microbiome of a mouse and human may provide data that cannot be easily translated into human treatments. Therefore, an artificial gut chemostat model (“aka twin vessel single-stage distal gut chemostat model”) was used to simulate the gut microbiome *ex vivo* and to investigate the complex multispecies interactions in a VRE colonised faecal microbiota. The artificial gut model, developed by McDonald et al. [444], allows for longitudinal studies of the gut microbiota when challenged with antibiotic or infection. Artificial gut models have previously been used to assess the impact of antibiotics and FMT treatments on the microbiome’s susceptibility to MDR pathogen colonisation [376, 403, 444, 454].

For this study, three identical bioreactor systems were used for each experiment (Electrolab FerMac 200 series, Electrolab Biotech Ltd). The artificial gut model experiment, using the three chemostat vessels, was performed in three independent replicates. The three vessels received the same set up, inoculation, operation, and growth media according to previously published protocols [403, 444]. The environment within the artificial gut vessel was controlled to mimic the environment found in the distal gut (**Figure 3**). The temperature, pH, anaerobic conditions, media replenishment, and waste removal can be maintained between several vessels run simultaneously. The temperature of the culture was maintained at 37 °C using a heating jacket and a cooling pipe connected to a water bath set at 14 °C. The pH was measured with a pH probe which always remained in the culture. The pH was set between 6.7-7.0 with the addition of either sterile 0.5M hydrochloric acid or 1M sodium hydroxide.

The culture level was maintained at 230 ml with a media addition rate of 10.4 ml per hour—mimicking a gut transit time of 22 hours—and waste removal through media and waste pipes, respectively. The media input was cycled between two ports every week to prevent microbial back growth. The distal gut growth media was kept at ambient temperature on a stir plate to prevent settling of growth medium components. Waste bottles were changed weekly. Media bottles not treated with VAN were changed weekly; however, during the antibiotic treatment period, when VAN was added to the media bottle as well as the vessel, the media bottles were changed daily. Within the vessel, anaerobic conditions were maintained by a gas pipe which input anaerobic gas at a rate of 20-30 cc/min. Finally, the culture was stirred by agitators set to 200 rpm. The stirring was only turned off briefly during daily sampling.

The human faecal donors for both the initial inoculum used to seed the artificial gut model cultures and faecal microbiota transplants (FMTs) were healthy individuals (**Table 3**) who had not received antibiotic treatment for more than 12 months prior to involvement in this study. All three vessels (containing 130 mL of DGGM initially) were inoculated with 100 mL of a 10% (w/v) faecal slurry (such that the final volume was 230 mL of 4.3% faecal slurry), prepared anaerobically in degassed DGGM. The faeces was homogenised in a strainer stomacher bag (normal cycle, 250 rpm, 1 minute) to prepare a liquid inoculum and to remove solid matter. For 24 hours after the faeces were inoculated, the vessels were heated, stirred, and pH controlled but the media pumps were left off and waste tubes closed. This allowed the bacteria to establish growth and prevented them being washed out by the continuous media flow. Throughout the experiment, daily sampling was performed in which 9 mL of culture was extracted from each vessel for archiving or data analysis (described below).



The vessels were first left without intervention for 21 days to allow stabilisation of the faecal culture (**Figure 4**). From days 21-40, Vessel A (VA) and vessel B (VB) received daily vancomycin treatments of a final concentration of 1000 µg/ml to mimic the concentration of vancomycin measured in human faeces [55, 201-203]. Vancomycin was prepared by dissolving the antibiotic powder in DI water and sterilizing with a 0.22-µm filter. From day 21 through day 40, media bottles were changed daily rather than weekly for all three vessels. The antibiotics were added both to the vessel for immediate dosing, and to the media bottle to ensure

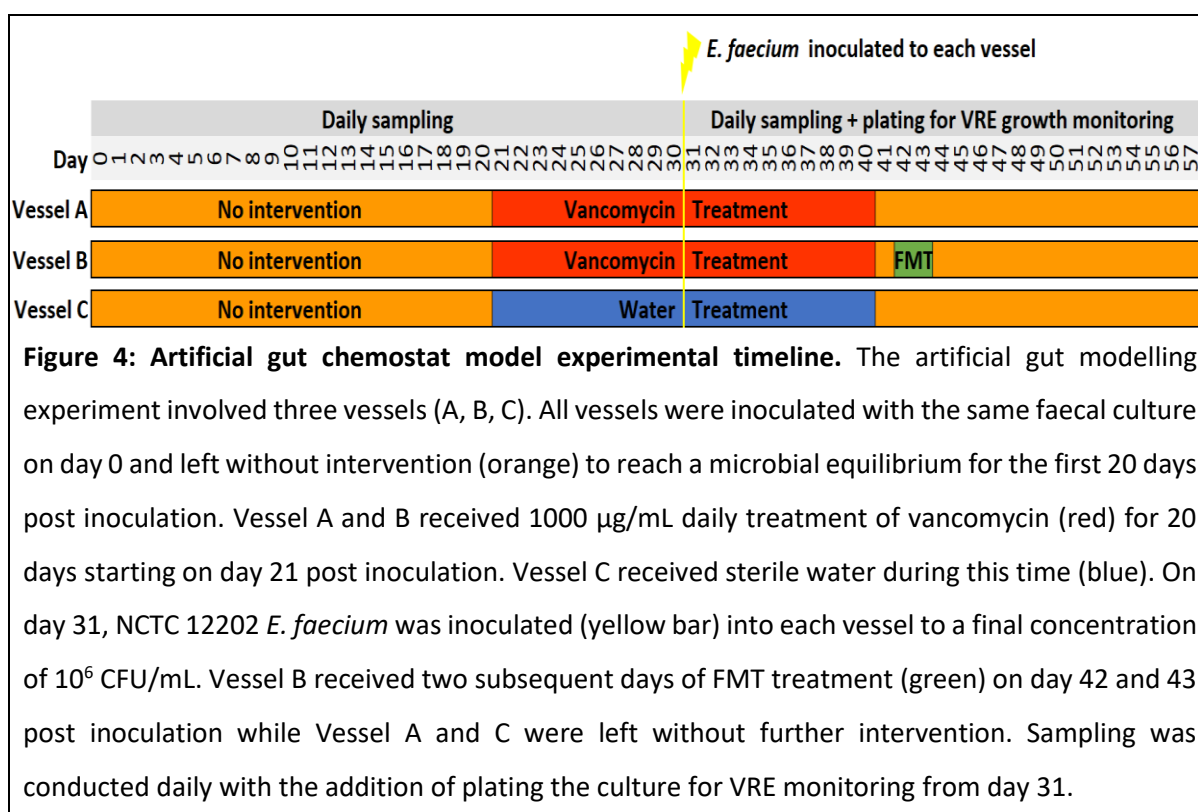
continuous administration of the vancomycin for the full 20-day treatment. During this time, vessel C (VC) received an equal volume of sterile water treatments as a control.

Table 3: Donor information for artificial gut chemostat model experiments. The table shows the donor for the initial inoculum and FMT treatments for each of the three artificial gut experiment replicates. Four healthy donors were used in total, three females (F) and one male (M), with an age distribution of 31.7 ± 1.5 y.o., three of whom consumed a western diet while one was vegetarian.

Replicate	Initial Donor	FMT Donor
1	Donor A (F, 30 y.o., Western)	Donor B (F, 33 y.o., Vegetarian)
2	Donor B (F, 33 y.o., Vegetarian)	Donor C (M, 32 y.o., Western)
3	Donor C (M, 32 y.o., Western)	Donor D (F, 25 y.o., Western)

On day 31 post inoculation, all three vessels were inoculated with NCTC 12202 *E. faecium* to a final concentration of 10^6 CFU/mL [376]. From this point on, daily sampling also involved preparing serial dilutions of the culture from each vessel and plating on *Brilliance*TM VRE agar plates for VRE growth monitoring. The plates were incubated aerobically at 37 °C for 24 hours. VA and VC were left without any further intervention till the end of the experiment. On day 42 and 43 post inoculation, VB received two subsequent FMT treatments. The FMTs were prepared anaerobically by making 50 mL of 50% (w/v) faecal slurry of healthy faeces using degassed 0.9% (w/v) saline. The faeces was homogenised in a strainer stomacher bag (normal cycle, 250 rpm, 1 minute). VB was left without further intervention until the end of the experiment on day 57.

VC, receiving no antibiotic treatment, acted as a water-treated control for antibiotic-treated VA and VB. VA, having received the same antibiotic treatment as VB but no FMT, acted as a control for the impact of the FMT given to VB.



Vessel daily sample preparation

Each day, 9 mL of culture was extracted from each vessel through the sampling port using a sterile needle and syringe. Four 2 mL aliquots were archived as is at -80 °C. The remaining 1 mL was centrifuged (17,000 x g) for 10 minutes. The supernatant was extracted and frozen at -80 °C to be used for ¹H NMR analysis. In addition to daily samples, the faeces from the initial donor sample and the two FMT donor samples were aliquoted either as is or as 25% (w/v) faecal water prepared in high-performance liquid chromatography-grade water and vortexed (3000 rpm, 10 minutes) before being centrifuged with the other samples. The prepared inoculum (10% (w/v) faecal slurry in DGGM) and the prepared FMTs (50% (w/v) faecal slurry in 0.9% (w/v) saline) were also aliquoted and centrifuged as normal.

Nutrient competition assays

Gut commensal spent media assay

This experiment was designed to evaluate the impact of the 15-strain commensal consortium on nutrient utilisation and VRE growth. All 15 commensal strains (see **Table 1**) were grown anaerobically on FAA plates with 5% (v/v) defibrinated horse blood overnight. A single colony was used to inoculate each of the commensals into individual 2 mL cultures of the gut commensal minimal medium containing the nutrient mixture. The individual cultures were incubated anaerobically for 24 hours at 37 °C. The following day, the individual cultures were mixed together in equal volumes, for a total concentration of between 1×10^9 - 2×10^9 CFU/mL. This mix was inoculated as a 1:10 dilution into fresh degassed gut commensal minimal media with the nutrient mixture, with a final cell density of the gut commensal consortium of 1×10^7 CFU/mL. This culture was incubated anaerobically for 24 hours at 37 °C.

The following day, the pH of the culture was adjusted back to 7.0, then the culture was centrifuged at 4000 x g for 10 minutes at room temperature. The supernatant was collected and sterilised with a 0.22- μ m filter. A 1 mL sample of the spent media was removed for ^1H NMR spectroscopy. This sample was centrifuged (17,000 x g, 10 minutes, 4 °C) and stored at -80 °C. The remaining sterile supernatant was then divided into four aliquots (two for each VRE strain) for reinoculation with the two VRE strains. One aliquot for each strain was supplemented with 0.5% (w/v) glucose while the other was left without further supplementation.

Using overnight cultures of VRE grown in the same minimal media, the VRE strains were diluted in saline and inoculated into the spent media at a final concentration of 1×10^6 CFU/mL. The cultures were incubated at 37 °C in the anaerobic incubator for 24 hours. The next day, 1 mL was collected from each culture and prepared as previously described for ^1H NMR

spectroscopy. Serial dilutions were plated on FAA with 5% (v/v) defibrinated horse blood to quantify VRE growth. The plates were incubated anaerobically for 24 hours before they were counted. The experiment was run in three biological replicates, with three technical replicates for every biological replicate.

Faeces spent media assay

As a positive control for the gut commensal spent media assay described above, faecal cultures were also analysed in the spent media assays. Fresh faecal samples were collected from three healthy human donors (two female and one male, average age 30 y.o.) who had not received antibiotics for six months prior to donation. The samples were prepared into a 10% (w/v) faecal slurry in 0.9% (w/v) saline. The faeces were homogenised in a strainer stomacher bag (normal cycle, 250 rpm, 1 minute). The homogenised faecal suspension was inoculated as a 1:10 dilution into degassed gut commensal minimal media with the nutrient mixture. The culture was incubated anaerobically for 24 hours at 37 °C. The next day, the pH of the culture was readjusted to a pH of 7 before being centrifuged (17,000 x g, 10 minutes, 4 °C). The filter sterilised supernatant was divided into four aliquots (two for each strain) and inoculated with VRE (at a final concentration of 1×10^6 CFU/mL). One aliquot for each strain was supplemented with 0.5% (w/v) glucose while the other was left without supplementation. The reinoculated culture was incubated at 37 °C in the anaerobic incubator for 24 hours before serial dilutions were prepared and plated on FAA with 5% (v/v) defibrinated horse blood. As with the commensal spent media assay, culture was collected and prepared for ^1H -NMR spectroscopy before and after the VRE incubation step. The 3 donors served as 3 biological replicates in this experiment and each trial was performed with 3 technical replicates.

VRE spent media assay

The faeces served as a positive control for the commensal spent media assay to show the nutrients depleted by the complete faecal microbiota compared to the selected commensal consortium. VRE served as a secondary positive control to show how the VRE strains grew in nutrient media depleted by the same strains. One day prior to the initial inoculation, VRE strains were grown on FAA with 5% (v/v) defibrinated horse blood in the anaerobic chamber. The VRE was inoculated into the degassed gut commensal minimal media (containing the nutrient mixture at a 0.015% (w/v) final concentration for each nutrient) at 1×10^7 CFU/mL final concentration. As with the other spent media assays, the culture was grown for 24 hours anaerobically, the pH was adjusted to 7.0, centrifuged ($17,000 \times g$, 10 minutes, 4°C), and filter sterilised. The spent media was divided into two aliquots (one supplemented with 0.5% (w/v) glucose and the other without supplemented nutrients), as only the strain which served as the initial inoculum was reinoculated. The corresponding VRE strain was reinoculated into the culture at 1×10^6 CFU/mL. The spent media culture was again incubated for 24 hours anaerobically. The next day serial dilutions were prepared and plated on FAA with 5% (v/v) defibrinated horse blood. Culture was collected and prepared for ^1H -NMR spectroscopy before and after the VRE incubation step. The experiment was run with 3 biological replicates, each with 3 technical replicates.

Direct coculture competition assays

Growth curves were produced for both gut commensal strains and VRE strains grown in gut commensal minimal media supplemented with glucose. First, gut commensal strains and VRE strains were grown on degassed FAA with 5% (v/v) defibrinated horse blood anaerobically at 37°C for 24 hours. The following day, each strain was individually inoculated into gut commensal minimal media containing 0.5% (w/v) glucose. The cultures were incubated

anaerobically at 37 °C for a further 24 hours. The following day, the gut commensals and VRE were individually inoculated into fresh gut commensal minimal media containing 0.5% (w/v) glucose at a final concentration of 1×10^7 CFU/mL and cultures were incubated anaerobically at 37 °C for a further 24 hours. OD₆₀₀ measurements were taken every 15 minutes for 24 hours.

The gut commensal strains had longer lag phases (5.25-14 hours) compared to the VRE strains (3 hours for *E. faecalis* and 5 hours for *E. faecium*). Therefore, for the coculture experiments the gut commensal strains were inoculated into the media first and each VRE strain was inoculated later (between 2.25-11 hours for *E. faecalis* and 0.25-9 hours for *E. faecium*)(final concentration of 1×10^6 CFU/mL), to ensure that both strains reached the exponential phase at the same time in their respective mono-culture controls. The coculture was incubated anaerobically at 37 °C for 24 hours. The following day, serial dilutions were prepared and plated on FAA with 5% (v/v) defibrinated horse blood. These plates were incubated aerobically at 37 °C for 24 hours so that the oxygen could serve as a selective mechanism and only the VRE colonies would be counted. The growth was compared to the growth of VRE from single culture conditions in the same media. This experiment was performed in biological triplicate with 3 technical replicates for each trial.

[Mouse model of intestinal colonisation with vancomycin-resistant *Enterococcus*](#)

[Ethical approval](#)

Mouse experiments were performed in collaboration with Dr Thomas Clarke from the Department of Infectious Disease at Imperial College London. Imperial College London Animal Welfare and Ethical Review Body (PF93C158E) granted ethical approval for mouse experiments. The experiment was performed under the authority of the UK Home Office Animals (Scientific Procedures) Act 1986 and followed all standards outlined by Animal

Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines. The direct handling and treatment of the mice in this experiment was performed by Dr Thomas Clarke, the experiment was designed by Dr Julie McDonald based on previous studies [455-457], and experiment preparation and mouse faecal sample processing and analysis were performed by Olivia King.

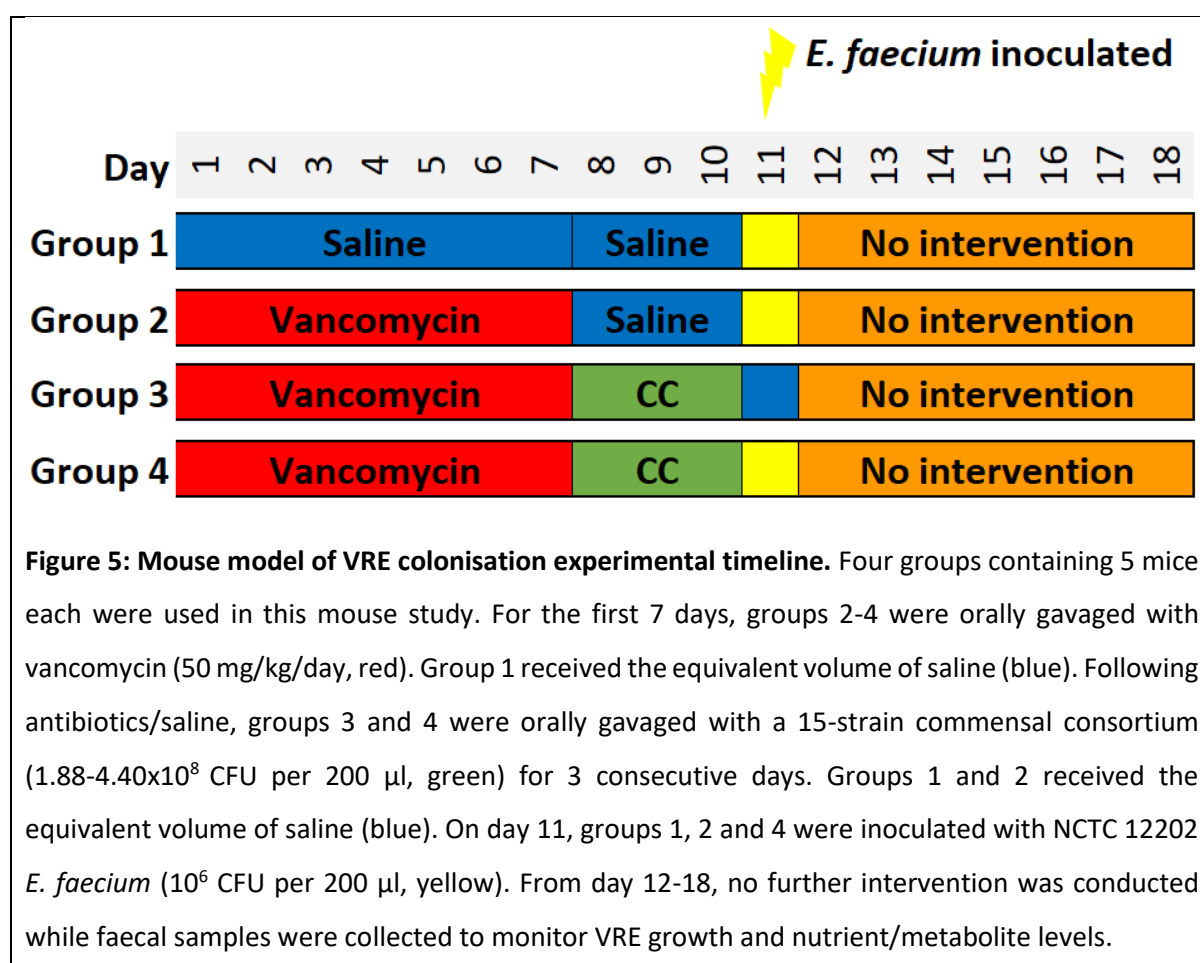
Experimental design

Twenty female C57BL/6 mice (8-10 weeks old) were purchased from Envigo (Huntington, UK). For one week prior to the start of the experiment, the mice were acclimated to their environment and were housed in individually ventilated cages of 5 mice per cage. Food (RM1, Special Diet Services, Essex, UK), water, and bedding (Aspen chip 2 bedding, NEPCO, Warrensburg, New York) were autoclaved before administration. Water was provided ad libitum and the environment was maintained at 20-22 °C with 12-hour light to dark cycles.

The experiment was designed to determine the impact of antibiotic treatment, *E. faecium* colonisation, and gut commensal colonisation on nutrient availability and metabolite production in mouse faeces (**Figure 5**). The mice were divided into 4 groups (5 mice per group). Vancomycin treatments were given as 200 µL oral gavage at a concentration of 50 mg/kg per day for 7 days (groups 2-4) or 200 µL oral gavage of 0.9% (w/v) saline (group 1) [457]. Groups 3 and 4 were orally gavaged with a mixture of the gut commensals (see **Table 1**) at a concentration of 1×10^9 CFU per 200 µL, while groups 1 and 2 were orally gavaged with 200 µL of 0.9% (w/v) saline [455]. Vancomycin-resistant *E. faecium* was orally gavaged in groups 1, 2, and 4 at a concentration of 10^6 CFU per 200 µL while groups 3 were orally gavaged with 200 µL of 0.9% (w/v) saline.

Preparation and administration of vancomycin

The antibiotics used in the mouse experiment were prepared as a 5 mg/mL stock by dissolving vancomycin into DI water and sterilizing with a 0.22-micron filter. The antibiotics were prepared fresh daily immediately prior to treatment. The treated mice were given 200 µL of vancomycin solution via oral gavage. The untreated control mice were orally gavaged with 200 µL filter sterilised saline.



Preparation and administration of vancomycin-resistant *Enterococcus*

The vancomycin resistant NCTC 12202 *E. faecium* was plated on TSA two days prior to oral gavage in the mice. The plate was incubated at 37 °C aerobically overnight. The next day, a single colony was used to inoculate a TSB culture which was incubated aerobically for 24 hours at 37 °C. On the day of treatment, the VRE was administered to the mice via oral gavage at a

final concentration of 10^6 CFU per 200 μ L. The control mice were orally gavaged with 200 μ L sterile 0.9% (w/v) saline.

Preparation and administration of commensal consortium

A commensal consortium or saline was administered to mice for three consecutive days. Fresh cell suspensions of the commensal consortium was prepared for each day of treatment. Gut commensal strains were grown on degassed FAA with 5% (v/v) defibrinated horse blood plates anaerobically at 37 °C for 24 hours. The plated cultures were used to inoculate individual broth cultures of the commensal minimal medium containing the nutrient mixture. The individual cultures were incubated anaerobically for 24 hours at 37 °C. On the treatment days, the individual commensal cultures were combined so each strain was between 1.2×10^8 and 5.8×10^9 CFU/mL, corresponding to the concentrations of each strain when grown as an overnight culture. In the case of commensal strains which did not achieve a minimum of 10^8 CFU/mL growth in the gut commensal minimal media, a 200x (*B. ovatus* and *B. bifidum*), 150x (*B. pseudocatenulatum*), or 33x (*B. adolescentis*) concentration was required to generate a commensal mix with equal volumes of each strain. When combined, each strain in the commensal consortium was present at concentrations ranging between 9.4×10^8 – 2.2×10^9 CFU/mL. The treated mice were orally gavaged with 200 μ L of the commensal consortium which averaged a total cell density of 2.96×10^8 CFU per 200 μ L (4.4×10^8 CFU per 200 μ L on day 8, 2.61×10^8 CFU per 200 μ L on day 9, 1.88×10^8 CFU per 200 μ L on day 10) over the three treatment days. The control mice were orally gavaged with 200 μ L sterile 0.9% (w/v) saline.

¹H-NMR spectroscopy

NMR sample preparation for human faeces, human faecal culture samples, or bacterial culture samples

NMR buffer was prepared using sodium phosphate monobasic (5.25 g), sodium phosphate dibasic (28.85 g), 3-(Trimethylsilyl)propionic-2,2,3,3-d₄ acid sodium salt (TSP-d₄)(1 mM), sodium azide (3 mM), and made up to 1L using deuterium oxide with a final pH of 7.4. NMR samples were prepared on the day they were collected by centrifuging for 10 minutes (17,000 x g) at 4 °C. Faecal samples were prepared for NMR by adding 300 mg of faeces to 900 µL of high-performance liquid chromatography-grade water, vortexing (3000 rpm, 10 minutes) and centrifuging (17,000 x g, 10 minutes, 4 °C). The supernatant was collected and, from this point on, the faecal samples were prepared like other faecal culture or bacterial culture samples. The prepared artificial gut sample, faecal batch culture sample supernatants, mixed nutrient utilisation assay samples, and spent media assay samples were stored at -80 °C prior to NMR sample preparation. Once thawed, samples were centrifuged for 10 minutes (17,000 x g) at 4 °C. Supernatant (400 µL) from each culture was added to 250 µL NMR buffer and vortexed to mix. 600 µL of the supernatant/buffer mixture was loaded into 5 mm NMR sample tubes.

NMR sample preparation for mouse faeces

Mouse faecal sample preparation and analysis followed a previously published protocol [30]. The buffer was prepared using sodium phosphate monobasic (20.4 g), 3-(Trimethylsilyl)propionic-2,2,3,3-d₄ acid sodium salt (TSP-d₄)(5.8 mM), sodium azide (2 mM), and made up to 100 mL using deuterium oxide and the final pH was adjusted to 7.4. The samples were prepared by vortexing 20 mg of mouse faeces in 560 µL of high-performance liquid chromatography-grade water (3000 rpm, 10 minutes) then centrifuged (17,000 x g, 10 minutes, 4°C). The 540 µL of supernatant was added to 60 µL of buffer. The

supernatant/buffer mix was loaded into 5 mm NMR tubes. Samples were maintained at 4°C while in the NMR spectrometer.

NMR instrument specifications

NMR spectra were acquired at 300 K using a Bruker AVANCE III 800 MHz NMR spectrometer (Bruker Bio-Spin, Rheinstetten, Germany) equipped with a 5mm CPTCI 1H-13C/15N/D Z-gradient cryoprobe. The NOESY pulse sequence was set as follows: 90° pulse length was ~10 µs with a recycle delay of four seconds and a mixing time of 100 ms. For each spectrum, 32 scans were collected for faeces, faecal culture, and bacterial culture samples and 128 scans were collected for mouse samples. Auto processing with Topspin (v3.2.6) was performed as follows: zero-order auto phasing (command apk0), auto baseline correction (command abs), auto referencing, water suppression, and line broadening by 0.3 Hz (command sref). Spectra were imported into MATLAB R2020b (The Mathworks, USA) where the water peak (4.6 to 5 ppm) was removed and manual alignment was conducted. Spectra were normalised to TSP with a custom script ("*JTPnormalise*") and TSP (-0.2 to 0.2) was removed. Peak selection was performed in MATLAB and Chenomx NMR Suite version 9.2 (Chenomx, Canada). Peak ranges selected for the various nutrients and metabolites assessed in this study are shown (**Table 4**). Once representative peaks were assigned, the associated peak ranges were integrated for quantification of identified substances. The signal-to-noise ratio (3:1) defined the limit of detection.

Table 4: NMR peak assignment. NMR peak integration was performed on MATLAB using the Chenomx NMR Suite (version 9.2) profiler database to help identify peaks. The untransformed peak cluster centres (ppm) for the nutrients and metabolites are listed. Some nutrients and metabolites have multiple used peaks which were selected for each experiment to reduce interference from the spectra baseline or the adjacent peaks.

	Cluster Centre	
	Used peaks	Additional peaks
Alanine	1.47	3.78
Alloisoleucine	0.95	0.92, 1.33, 1.42, 2.05, 3.73
Arginine	1.64, 3.24	1.72, 1.89, 1.92, 3.76
Asparagine	2.85, 2.94	4.00
Aspartate	2.70, 2.8	3.89
Cysteine	3.03	3.09, 3.98
Glutamate	2.12, 2.33, 2.36	2.04, 3.75
Glutamine	2.11, 2.14, 2.43,	3.77
Glycine	3.55	
Histidine	3.98	3.14, 3.24
Isoleucine	0.93, 1.00	1.25, 1.46, 3.66
Leucine	0.95	0.94, 1.67, 1.70, 1.73, 3.73
Lysine	3.02	1.43, 1.50, 1.71, 1.88, 1.91, 3.75
Methionine	2.13, 2.63	2.11, 2.19, 3.85
Phenylalanine	3.12, 7.42	3.28, 3.99, 7.32, 7.37
Proline	2.01, 3.33, 4.12	1.98, 2.06, 2.34, 3.41
Threonine	1.32, 3.58, 4.25	
Tryptophan	7.28, 7.72	3.30, 3.47, 4.05, 7.19, 7.31, 7.53
Tyrosine	6.89, 7.18	3.04, 3.19, 3.93
Valine	0.98, 1.03	2.26, 3.60
Ethanol	1.17	3.65
Arabinose	4.51, 5.29	3.50, 3.64, 3.65, 3.67, 3.68, 3.78, 3.79, 3.81, 3.83, 3.87, 3.89, 3.93, 3.98, 4.00, 4.01, 4.03, 4.04, 4.09, 4.11, 5.23, 5.24
Fructose	3.89, 4.01	3.55, 3.56, 3.59, 3.67, 3.70, 3.71, 3.79, 3.82, 3.99, 4.10, 4.11
Fucose	1.21, 1.24, 4.55	1.20, 3.44, 3.56, 3.63, 3.74, 3.76, 3.79, 3.80, 3.85, 3.86, 3.87, 3.97, 4.01, 4.03, 4.07, 4.19, 5.19, 5.22, 5.27
Galactose	4.58, 5.26	3.48, 3.64, 3.70, 3.72, 3.74, 3.77, 3.80, 3.85, 3.92, 3.98, 4.01
Glucose	3.24, 4.64, 5.23	3.40, 3.41, 3.46, 3.48, 3.53, 3.71, 3.72, 3.76, 3.82, 3.84, 3.89
Mannose	4.89, 5.17	3.37, 3.37, 3.65, 3.73, 3.76, 3.80, 3.84, 3.87, 3.90, 3.93, 3.94
Ribose	4.92, 5.38	3.52, 3.60, 3.64, 3.66, 3.68, 3.75, 3.81, 3.83, 3.85, 3.88, 3.92, 3.97, 3.99, 4.08, 4.09, 4.10, 4.12, 4.2, 4.85, 5.24
Xylose	3.22, 3.31, 4.57	3.43, 3.52, 3.60, 3.62, 3.65, 3.67, 3.69, 3.92, 5.19
Maltose	4.65, 5.40	3.27, 3.41, 3.42, 3.57, 3.58, 3.59, 3.63, 3.68, 3.69, 3.71, 3.72, 3.76, 3.77, 3.80, 3.85, 3.91, 3.93, 3.96, 5.23
Sucrose	4.04, 4.21, 5.40	3.47, 3.55, 3.67, 3.68, 3.76, 3.80, 3.81, 3.82, 3.83, 3.88
Trehalose	3.45, 5.18	3.64, 3.76, 3.81, 3.85, 3.86
N-acetylglucosamine	2.04, 5.20	3.45, 3.48, 3.53, 3.67, 3.74, 3.76, 3.79, 3.84, 3.85, 3.87, 3.90, 4.71, 8.09
Formate	8.45	
Acetate	1.91	
Propionate	2.17	1.04
Butyrate	0.88, 1.55	2.15
Valerate	0.88, 2.17	1.29, 1.51
Isobutyrate	1.05	2.38
Isovalerate	0.90	1.94, 2.04
Phenylacetate	7.29, 7.3	3.53, 7.37
5-aminovaleate	2.22	1.62, 1.66, 3.01
Lactate	1.32, 4.11	
Succinate	2.39	
Uracil	5.79, 7.53	

16S rRNA gene sequencing

DNA extraction

All samples used for 16S rRNA gene sequencing were stored at -80 °C prior to DNA extraction. Microbial DNA was isolated from faecal batch culture samples using the PowerLyzer PowerSoil DNA isolation kit (QIAGEN, Germany, Cat. No. 12855-100) and from the artificial gut model samples using QIAamp PowerFecal Pro DNA Kit (QIAGEN, Germany, Cat. No. 51804). For both kits, the corresponding protocol from the manufacturer was followed, with the modification that the microbial cells were lysed by bead beating for three 1-minute cycles using a FastPrep 24 (SKU 116004500) instrument, where each cycle was followed by a 1 minute rest between cycles. Extracted DNA was aliquoted and stored at -80 °C. DNA concentrations were quantified using a Qubit dsDNA BR Assay Kit (Cat. No. Q32850) and a Qubit fluorometer (Cat. No. Q32866). Using the results of the quantification, samples were diluted down to a concentration of 5 ng/μL in a sterile 96 well plate (or left undiluted if the concentration was already less than 5 ng/μL) before being delivered to Prof. Julian Marchesi's lab in the Department of Metabolism, Digestion and Reproduction at Imperial College London for library preparation and 16S rRNA gene sequencing of the V1-V2 variable region on an Illumina 16S metagenomic sequencing MiSeq for library preparation [458]. For PacBio sequencing (Pacific Biosciences of California, Inc), the DNA was diluted to 1 ng/μL in sterile 96 well plates (or left undiluted) before being delivered to SynbiCITE London Biofoundry (White City Campus, Imperial College London) to Dr Marko Storch for library preparation and near full length 16S sequencing using PacBio [459-461].

16S rRNA gene sequencing on the Illumina MiSeq

Faecal batch culture sample libraries were prepared based on previously described protocols [458] to amplify the V1-V2 region of the 16S rRNA gene. Sequencing was performed on an Illumina MiSeq platform (version 3) using paired end 300 bp reads (Illumina Incorporated, UK). The forward primer mix included 28F-YM, 28F-Borrellia, 28F-Chloroflex, and 28F-Bifdo primers (prepared at a ratio of 4:1:1:1), while 388R served as the reverse primer [30]. The SequalPrep Normalisation Plate Kit (Life Technologies, UK) was utilised to normalise and clean index PCR reactions while the NEBNext Library Quant Kit for Illumina (New England Biolabs, UK) was used for quantification of sample libraries.

16S rRNA gene qPCR

The total bacterial biomass in each sample (from the faecal batch culture and artificial gut chemostat experiments) was measured using 16S rRNA gene qPCR. The protocol was based on previously published work [403, 444]. For each sample, 20 μ L reactions were prepared which consisted of the DNA (5 μ L) with 2x Platinum Supermix UDG with ROX (500 nM) (Life Technologies, Cat. No. 11730-017, Carlsbad, USA), 1.8 μ M BactQUANT forward primer (5'-CCTACGGGAGGCAGCA-3'), 1.8 μ M BactQUANT reverse primer (5'-GGACTACCGGTATCTAATC-3'), 225 μ M probe ((6FAM) 5'-CAGCAGCCGCGGTA-3' (MGBNFQ)), and PCR grade water (Roche, Penzberg, Germany). The PCR run contained *E. coli* DNA (Sigma-Aldrich)(3-300,000 copies per reaction in 10x serial dilution) which served as a positive control and produced a standard curve. Additionally, a negative control was included on each plate which did not contain template DNA. Samples, standards, and controls were prepared for amplification in triplicate. DNA samples were diluted to less than 40 ng/ μ L to ensure all samples fit in the standard curve. The Applied Biosystems StepOnePlus Real-Time PCR System

(Thermo Fisher Scientific, UK) was set to follow the PCR cycle conditions 1) 50 °C for 2 minutes, 2) 95 °C for 10 minutes, 3) 40 cycles of 95 °C for 15 seconds then 60 °C for 1 minute. The generated qPCR data was used to express 16S rRNA gene sequencing data as absolute abundances as previously described [30, 403] using the following formula:

$$\text{Absolute abundance of taxa} = \text{relative abundance of taxa} \times \left(\frac{16S \text{ rRNA gene copy number in sample}}{\text{highest 16S rRNA gene copy number in sample set}} \right)$$

PacBio genetic sequencing

Artificial gut chemostat samples were prepared for PacBio [459-461] sequencing by diluting all DNA to 1 ng/μL in 20 μL Tris-Ethylenediaminetetraacetic acid buffer. The sequencing was conducted by use of a SMRT cell (PacBio, Cat. No. 101-389-001) and Sequel II sequencing kit (PacBio, Cat. No. 102-194-400) for sequencing on the Sequel II system. Further the KAPA HiFi Hot Start ReadyMix DNA polymerase (Roche) was used.

Sequencing data processing with DADA2 pipeline

The generated data from 16S rRNA gene sequencing with Illumina or PacBio was processed with the standard DADA2 pipeline (version 1.18.0) [462]. The pipeline assigned quality scores and filtered reads with a score lower than 30, then calculated sequencing errors. Sample inference ensured only biological sequences remained, the forward and reverse reads were merged for the Illumina data set only (PacBio only generates sequencing reads in one direction) and chimeric sequences were removed. The SILVA bacterial database (version 138.1)(<https://www.arb-silva.de/>) was used to classify the sequence variants [463].

Evaluation of gene sequencing data with the mixOmics library

The *mixOmics* library was used in R to correlate 16S rRNA gene sequencing data (at the family or genus levels) with ¹H NMR spectroscopy data [403, 464]. The generated regularised canonical correlation analysis (rCCA) plots show a single faecal culture sample as a point projected into the XY-variate space, which shows positively correlated points in the

correlation circle projected in the same space. Negatively correlated points are projected in opposite directions. Distance from the origin is proportional to the strength of correlation, so that the strongest correlations are shown farthest from the origin.

Statistical analyses

Software used in statistical testing include Python (version 3.11), R (version 4.2.2), Simca (version 16) and GraphPad Prism (version 10.1, La Jolla, California). With all data, a p value less than 0.05 was defined as statistically significant (n.s. ($p > 0.05$), * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), **** ($p \leq 0.0001$)).

NMR spectra were imported into MATLAB (r2019b) and processed with a custom script available in the Figshare repository (<https://doi.org/10.6084/m9.figshare.21948233>) modified from the code available at the Zenodo repository (<https://doi.org/10.5281/zenodo.3077413>). Integration values from peak assignment of ^1H -NMR spectra were analysed using either Wilcoxon signed rank testing with Benjamini & Hochberg false discovery rate (FDR) correction (Graph Pad Prism, v. 10.1), principal component analysis (PCA) and Orthogonal projections to latent structures discriminant analysis (OPLS-DA) with Hotelling T2 with a 95% confidence interval and CV-ANOVA (Simca, v. 16), MixOmics correlational analysis (previously described, R, v. 2.7.18), two-way ANOVA with pairwise Bonferroni correction, or two-tailed unpaired t-test with Benjamini & Hochberg multiple comparison test (Graph Pad Prism, v. 10.1).

The 16S rRNA gene sequencing data was weighted as absolute abundance using the qPCR data and analysed with Wilcoxon two-tailed sign ranked Benjamini & Hochberg FDR corrections (Graph Pad Prism, v. 10.1) or MixOmics correlational analysis (previously described, R, v. 2.7.18),

The growth curves generated from the carbon and nitrogen utilisation assays were assessed in the AMiGA software in Python (v. 3.6.5). In the software, subtraction of the no substrate control was used to normalise the data. The software performed Gaussian Process regression to assess the functional difference in the OD ($\lambda=600\text{nm}$) between samples with added nutrients and the negative control left without. Growth was categorised based on the functional difference (FD) as calculated by the AMiGA analytical software, between the growth with the single carbon source and growth with no carbon source. For the analysis of nitrogen utilisation, the functional difference was calculated between growth with the complete nitrogen mix and the “leave-one-out” mix. Growth was classified as negligible (0-0.5), low (0.5-2), moderate (2-3) or high (≥ 3) based on the functional difference.

The growth of VRE and commensal isolates as either plate counts and OD₆₀₀ readings was assessed in Graph Pad Prism, (v. 10.1), using Shapiro-Wilks normality test followed by either one-way ANOVA with Dunnett’s multiple comparison test, two-tailed unpaired t-tests, or Kruskal Wallis with Dunn’s multiple comparison test.

Short Asynchronous Time-series Analysis (SANTA) was used to review artificial gut chemostat data from ¹H-NMR spectroscopy. This allowed comparison of the concentration of nutrients and metabolites over a period of time between two groups. The two data sets were assessed using 4 degrees of freedom to avoid overfitting the model. The p(distBH) values generated to show statistical significance were calculated using the area between the mean group fitted curves and corrected with Benjamini-Hochberg FDR. For the comparison between VAN-treated and untreated *E. faecium* growth, day 7-11 (post VRE inoculation) plate count values were used. To determine the impact of the VAN-treatment on nutrient and metabolite concentrations, the ¹H-NMR spectroscopy peak abundances from pre-VAN treatment days

15-21 were compared to post-VAN treatment days 25-31. To determine the impact of the FMT-treatment on nutrient and metabolite concentrations, the ^1H -NMR spectroscopy peak abundances from VA and VB treatment days 45-51 were used.

Chapter 3: Antibiotic treatment fundamentally reshapes the gut microbiome by reducing the abundance and diversity of the microbiota

Introduction

Antibiotics consumed by humans, either as a direct antibiotic treatment or secondarily through food and environmental exposure, are processed by either the kidneys or the liver depending on the type of drug and method of treatment [465]. Active antibiotics which make it to the gut impact the growth of gut commensals. In addition to inhibiting the intended pathogen(s), antibiotics decrease the diversity and density of the commensal organisms which are susceptible to the drug. Further, unsusceptible bacteria can increase in abundance following antibiotic treatment as a result of reduction in competition within the gut.

As the biomass of the microbiome is altered by antibiotics, so too will the presence of the nutrients that these organisms consume and metabolites that are produced. When there are fewer bacteria in the microbiome, nutrients which these species would otherwise consume can increase in concentration. Conversely, any microbial metabolite, such as short chain fatty acids, may decrease in concentration. These shifts in metabonome of the gut decrease colonisation resistance conferred by the microbiota. Increased nutrient concentration can be exploited by fastidious opportunistic bacteria and lead to intestinal domination by pathogens. Similarly, with decreased production of SCFAs, secondary bile acids, and bacteriocins, weaknesses develop in the ability of the microbiota to restrict the proliferation of pathogens.

The experiments in this chapter aimed to identify the impact antibiotic treatment on the taxonomic composition and metabonome of the gut microbiota. Ex vivo batch cultures, inoculated with faecal samples from 12 healthy donors, were treated with water or antibiotics at physiologically relevant concentrations. The antibiotics selected in this study—vancomycin

(VAN, glycopeptide), metronidazole (MTZ, nitroimidazole), ceftriaxone (CRO, 3rd generation cephalosporin), clindamycin (CLI, lincosamide), and piperacillin/tazobactam (TZP, penicillin/ β -lactamase inhibitor combination therapeutic)—are clinically linked to the promotion of intestinal colonisation by VRE. Antibiotic-induced changes to the faecal cultures were analysed through metabonomic and taxonomic techniques to see how the treatments altered both the diversity and density of the bacterial community as well as the concentration of nutrients and inhibitory metabolites. By identifying how the treatments impact the microbiome, a better idea of how VRE might exploit an antibiotic-perturbed gut can be attained.

Results

Antibiotic treatment drives decreases of gut commensals

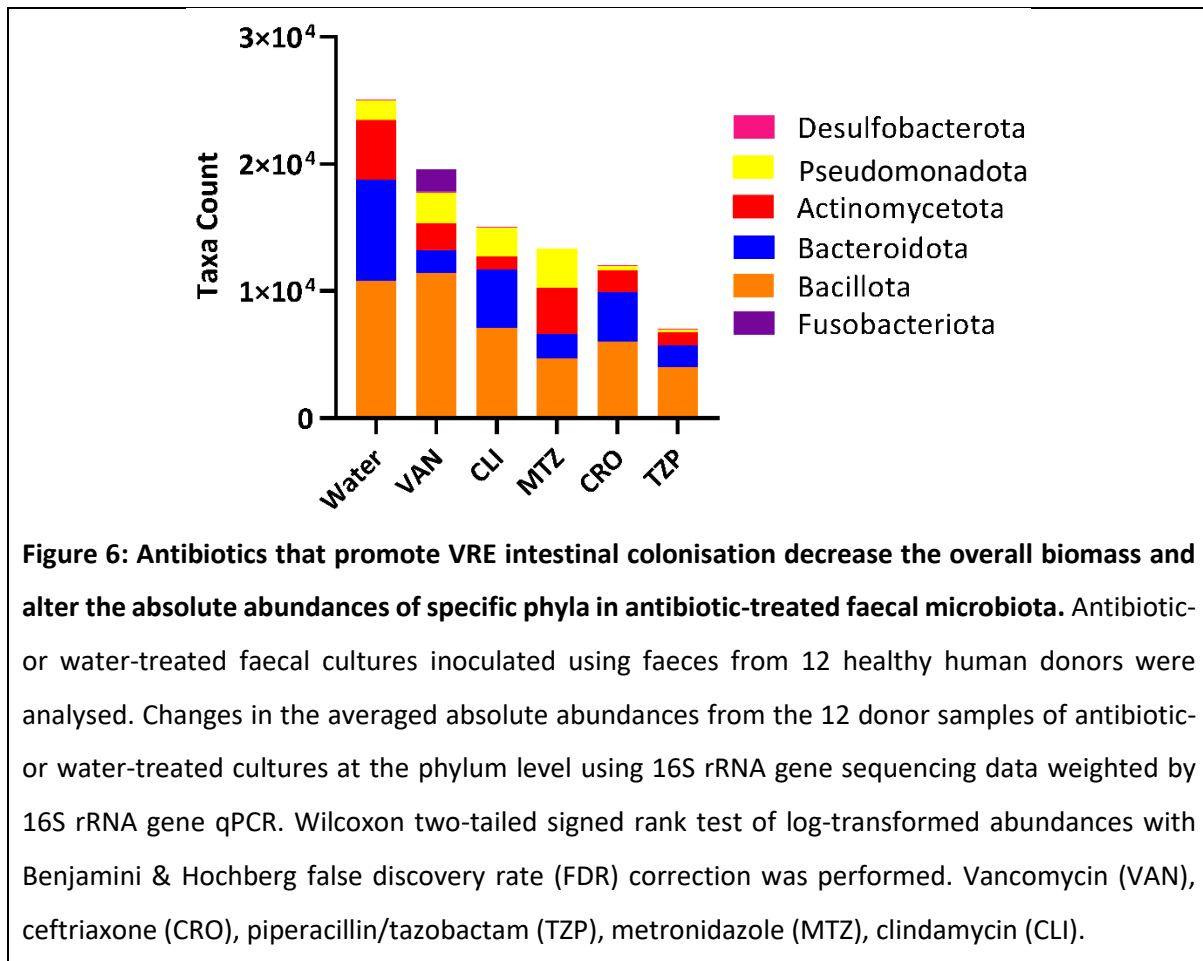
Taxonomic analysis with 16S rRNA gene sequencing weighted with 16S qPCR showed that antibiotic treatment of faecal microbiota resulted in a decrease in the absolute abundance of several bacterial phyla compared with water-treated control samples (**Figure 6**). When evaluated using a nonparametric Kruskal-Wallis multiple comparisons test, the 16S rRNA qPCR data showed a significant difference between the 16S rRNA gene counts of water-treated and CLI- ($p = 0.014$), MTZ- ($p = 0.024$), CRO- ($p = 0.0015$) and TZP-treated ($p < 0.0001$) samples. VAN-treatment did not produce a significant difference in 16S rRNA gene counts compared to the water-treated controls ($p > 0.30$).

When the absolute abundance of the 16S rRNA gene sequencing data is reviewed at the phyla level (**Figure 6**), differences were seen between the water-treated and antibiotic-treated samples when compared with nonparametric Wilcoxon matched-pairs signed rank tests. In water-treated samples, Bacillota was the most abundant phylum (43.11%), followed by

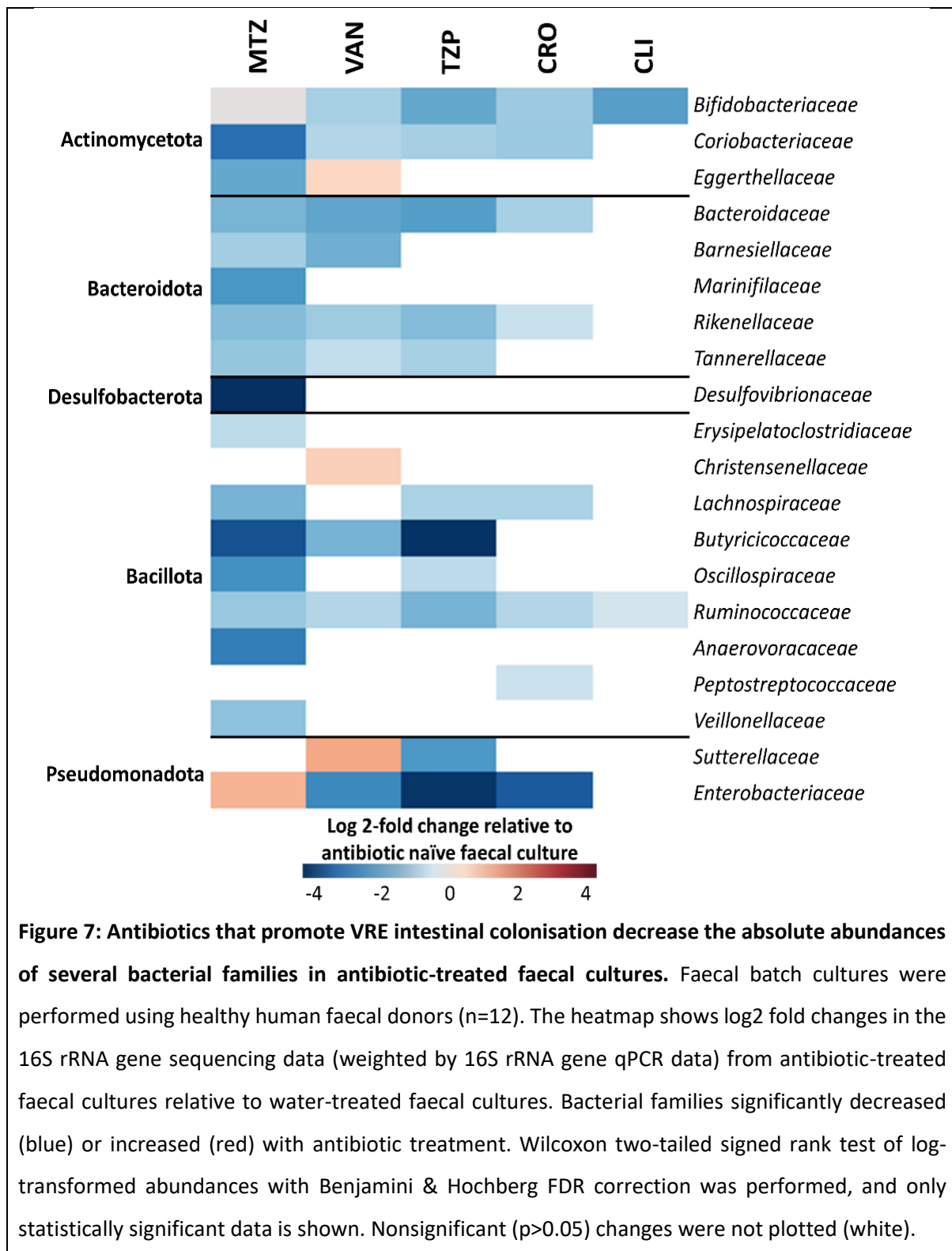
Bacteroidota (31.75%), Actinomycetota (18.57%), Pseudomonadota (6.17%), Desulfobacterota (0.2%) and Fusobacteriota (0.09%).

While the overall abundance of 16S rRNA gene counts was not significantly changed with VAN treatment, a significant decrease in the proportion of Bacteroidota (9.35%)($p = 0.0005$) and Actinomycetota (10.5%)($p = 0.0068$) was seen. There was a nonsignificant increase in Bacillota (58.01%)($p = 0.85$), Pseudomonadota (12.37%)($p = 0.11$), Desulfobacterota (0.48%)($p = 0.42$), and Fusobacteriota (8.9%)($p = 0.25$).

Much like with VAN treatment, CLI treatment resulted in a significant decrease in Bacteroidota (30.67%)($p = 0.016$) and Actinomycetota (6.93%)($p = 0.0010$). A nonsignificant increase was seen in the Bacillota (47.1%)($p = 0.064$), Pseudomonadota (14.71%)($p = 0.79$), and Desulfobacterota (0.52%)($p = 0.32$) abundance. Following MTZ treatment, the decreased abundance of Bacillota (35.18%)($p = 0.0005$), Bacteroidota (14.5%)($p = 0.0005$) and Desulfobacterota (0.01%)($p = 0.002$) was seen to be statistically significant. Pseudomonadota (23.15%)($p = 0.0093$) increased with MTZ treatment. Actinomycetota (29.96%)($p = 0.15$) and Fusobacteriota (0.07%)($p = 0.99$) did not significantly change. CRO treatment caused an increase in the abundance of Bacillota (50.11%)($p = 0.043$), Bacteroidota (32.45%)($p = 0.0093$), Fusobacteriota (0.13%)($p = 0.63$), and Desulfobacterota (0.36%)($p = 0.999$), however only the former two were significant. A significant decrease was seen for Actinomycetota (13.95%)($p = 0.0005$) and Pseudomonadota (2.93%)($p = 0.0015$) abundance. Finally, TZP treatment caused a reduction in Bacteroidota (24.94%)($p = 0.0015$), Actinomycetota (15.11%)($p = 0.001$), and Pseudomonadota (2.77%)($p = 0.0015$) abundance and an increase in the abundance of Bacillota (56.78%)($p = 0.0122$). Changes to Fusobacteriota (0.02%)($p = 0.5$) and Desulfobacterota (0.34%)($p = 0.1$) were not significant.



At the family level, the greatest number of statistically significant changes was found following MTZ treatment (**Figure 7**). Decreases were seen in several notable families including *Coriobacteriaceae*, *Bacteroidaceae*, *Barnesiellaceae*, *Butyricicoccaceae* and *Ruminococcaceae*. A small, yet significant decrease in *Bifidobacteriaceae* was seen. An increase in *Enterobacteriaceae* was seen with MTZ treatment. VAN treatment, like MTZ, caused a decrease in the same noted families with the addition of a decrease in *Enterobacteriaceae*. The greatest number of significantly increased families was seen following VAN treatment. TZP and CRO both caused decreases to key families *Bifidobacteriaceae*, *Coriobacteriaceae*, *Bacteroidaceae*, *Ruminococcaceae*, and *Enterobacteriaceae*. CLI only caused a significant decrease to *Bifidobacteriaceae* and *Ruminococcaceae*.



Antibiotic treatment caused an increase in nutrients and a decrease in metabolites found in the gut microbiome

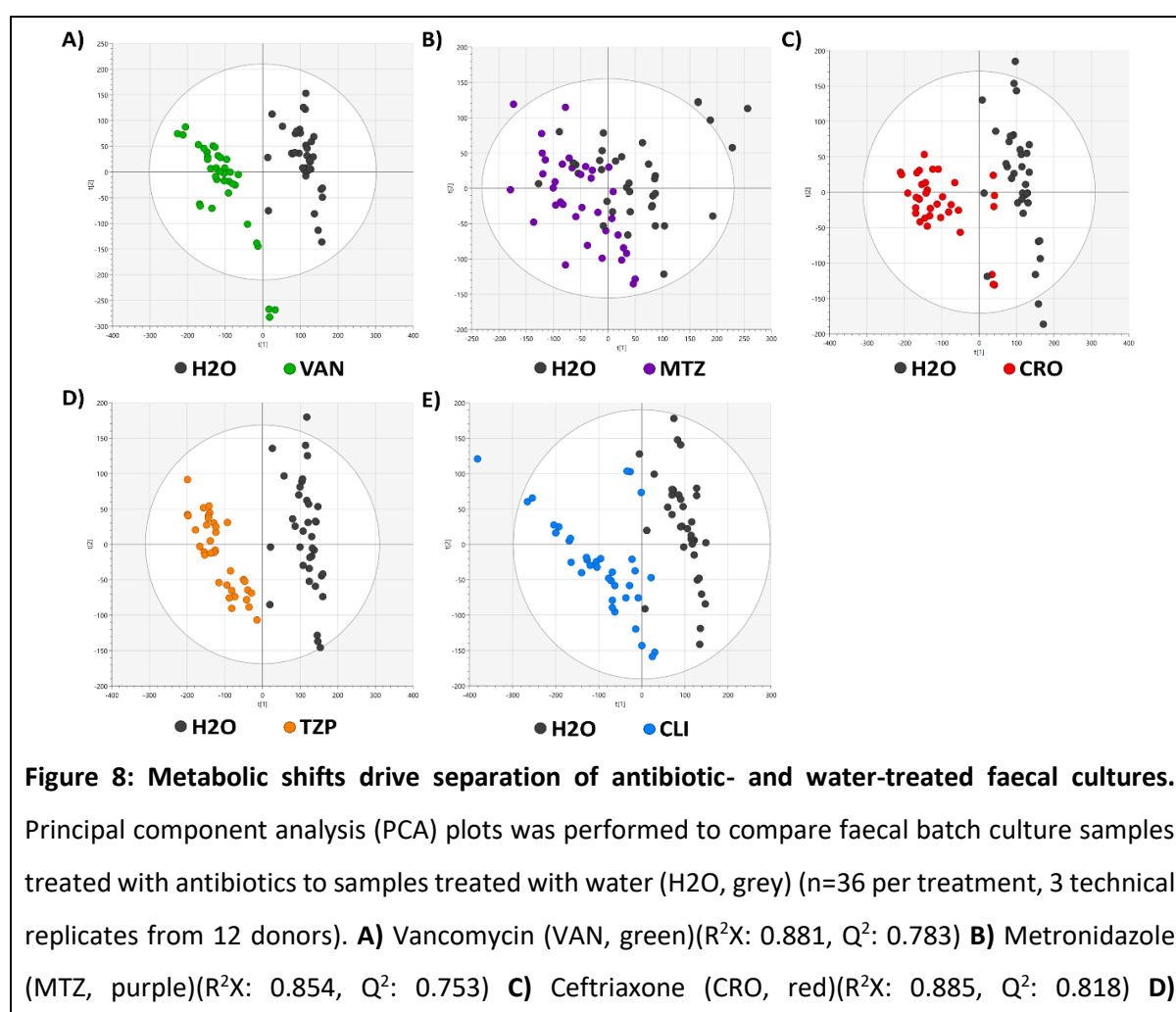
To assess the metabolic changes induced by antibiotic treatment, ¹H-NMR spectroscopy was performed on the faecal culture samples. The peak intensities from 43 amino acids, alcohols,

monosaccharides, disaccharides, amino sugars, fatty acids, organic acids, amines, and nucleotides were used to generate unsupervised principal component analysis (PCA) plots to compared antibiotic-treated and water-treated samples from all 12 donors (**Figure 8**). In generating a PCA model, the diagnostic values for the fraction of the explained variation (R^2X) and the predicted variation (Q^2X) were produced. The R^2X value denotes the significance of the number of components in the analysis in which the closer the value is to 1, the greater the significance. The Q^2X is calculated in the same way as R^2X but using observations not used to construct the PCA model. The Q^2X value increases as more components are added until the ideal number is reach, at which point Q^2X decreases.

The R^2X values were between 0.854-0.896 while the Q^2X values were between 0.753-0.828. Clear separation was seen between water-treated control cultures and cultures treated with VAN (**Figure 8A**), CRO (**Figure 8C**), TZP (**Figure 8D**), CLI (**Figure 8E**) along the first component. While a greater spread was seen with MTZ-treated samples, the paired water- versus antibiotic-treated samples for each donor were separate.

The same data was then used to produce supervised orthogonal projections to latent structures discriminant analysis (OPLS-DA) plots to assess the forced score-space separation of antibiotic- versus water-treated samples (**Figure 9**). The differences between antibiotic- and water-treated samples calculated in each component of the PCA model is compressed into a single component in the OPLS-DA model. This amplifies the separation between the groups which is shown along the X axis while the differences within the group between individual samples is shown along the Y axis. As with the PCA model, Q^2 represents the goodness of the prediction where the higher the value, the better the model parameters. Unlike the PCA model, CV-ANOVA can be performed on OPLS-DA models to better identify

significant changes. Further, the OPLS-DA model was used to detect which peaks in the ^1H NMR spectra drove the changes between groups (**Figure 10**). The PCA plots (**Figure 8**) confirmed general trends of separation between antibiotic-treated and antibiotic-naïve samples. This data was needed to validate the grouping created by the OPLS-DA plots (**Figure 9**) [466]. Significant differences were seen between antibiotic-treated samples and water-treated samples for all five antibiotics which showed Q^2 values between 0.785-0.953. Again, MTZ (**Figure 9B**) showed the least separation between antibiotic- and water-treated samples. However, all p-values produced by the OPLS-DA models were under 0.05 thus showing a significant difference between the five antibiotic-treated faecal cultures compared to water-treated faecal cultures.



Piperacillin/tazobactam (TZP, orange)(R^2X : 0.896, Q^2 : 0.828) **E** Clindamycin (CLI, blue)(R^2X : 0.877, Q^2 : 0.793). Hotelling's T², with 95% confidence interval, is represented by the ellipse. The X and Y axis show the first principal component (t[1]) and the second principal component (t[2]) respectively.

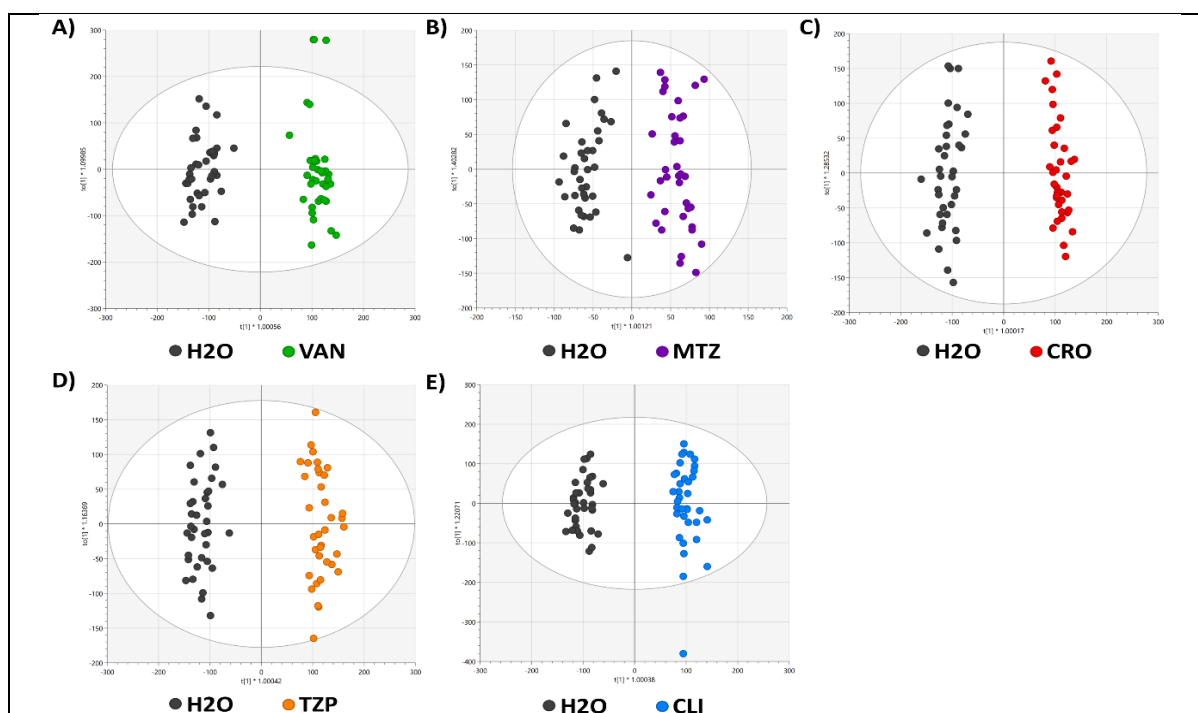


Figure 9: Forced score-space separation of faecal culture shows division between antibiotic- and water-treated samples. Orthogonal projections to latent structures discriminant analysis (OPLS-DA) plots was performed to compare batch culture samples treated with antibiotics to samples treated with water (H₂O, grey) (n=36 per treatment, 3 technical replicates from 12 donors). Hotelling's T², with 95% confidence interval, is represented by the ellipse. All five comparisons yielded a CV-ANOVA regression $p > 0.0001$. **A**) Vancomycin (VAN, green)(R^2X : 0.642, Q^2 : 0.917) **B**) Metronidazole (MTZ, purple)(R^2X : 0.76, Q^2 : 0.785) **C**) Ceftriaxone (CRO, red)(R^2X : 0.789, Q^2 : 0.942) **D**) Piperacillin/tazobactam (TZP, orange)(R^2X : 0.672, Q^2 : 0.953) **E**) Clindamycin (CLI, blue)(R^2X : 0.776, Q^2 : 0.937). The X and Y axis show the first principal component (t[1]) and the second principal component (t[2]) respectively.

From the OPLS-DA models, S-line plots were produced to highlight which NMR peaks drove the separation of antibiotic-treated samples from water-treated samples. **Figure 10** shows an example of the S-line graphs used to identify key nutrients and metabolites impacted by antibiotic treatment. Although the chemical shift in the S-line plot is identical to that of an

NMR spectrum, the S-line plot allows for the comparison between control and antibiotic spectra. Positive peaks ($p(\text{ctr})$) values correspond to an increase in the metabolite in antibiotic-treated samples compared to water-treated samples, while a negative peak indicates that the metabolite was in higher concentrations in the water-treated samples. The greater the correlation coefficient ($p(\text{corr})$) the greater the metabolite drove the separation between water and antibiotic-treated samples. In the case of VAN (**Figure 10**), the S-line plot generated by the OPLS-DA model showed that with antibiotic treatment, glucose ($p(\text{corr})$: 0.782), galactose ($p(\text{corr})$: 0.801), N-acetylglucosamine ($p(\text{corr})$: 0.815), uracil ($p(\text{corr})$: 0.870), glycine ($p(\text{corr})$: 0.888), and proline ($p(\text{corr})$: 0.538) increased in concentration. Conversely, formate ($p(\text{corr})$: 0.608), ethanol ($p(\text{corr})$: 0.706), acetate ($p(\text{corr})$: 0.946), and butyrate ($p(\text{corr})$: 0.364) decreased with antibiotic treatment compared to the water-treated samples.

The peaks in **Figure 10** were chosen as a representative selection. It was in using this analysis however, a more thorough array of discriminatory metabolites and nutrients could be identified for further analysis. A heatmap (**Figure 11**) was generated to illustrate the significant changes in specific nutrients and metabolites in antibiotic- versus water-treated samples. Sixteen amino acids (one basic, two acidic, three polar, 10 nonpolar), 13 sugars (eight monosaccharides, three disaccharides, one amino sugar, one amine sugar), six fatty acids (five SCFAs, one BCFA), and two organic acids, ethanol (alcohol), and uracil (nucleotide) were shown to have a significant shift with one of more of the antibiotic treatments.

Not all antibiotics caused metabolites and nutrients to increase or decrease uniformly; MTZ treatment, for example, resulted in a rise in trehalose and lactate while the other five antibiotics decreased (or did not affect) the concentration of the metabolites. Amino acids increased with antibiotic treatment with the exception of lysine which decreased with CRO

and VAN treatment and aspartate which decreased with VAN and TZP treatment. A similar trend is seen with sugars which increase in abundance with antibiotic treatment with the exception of 5-aminovalerate and trehalose which decreased with several of the antibiotic treatments. Fatty acids were more likely to decrease with antibiotics, specifically formate, acetate, and butyrate which showed significant drops in concentration with many of the antibiotic treatments. Of the 39 compounds evaluated, TZP and CRO treatment resulted in the greatest number of statistically significant shifts in concentration followed by CLI, VAN, and MTZ.

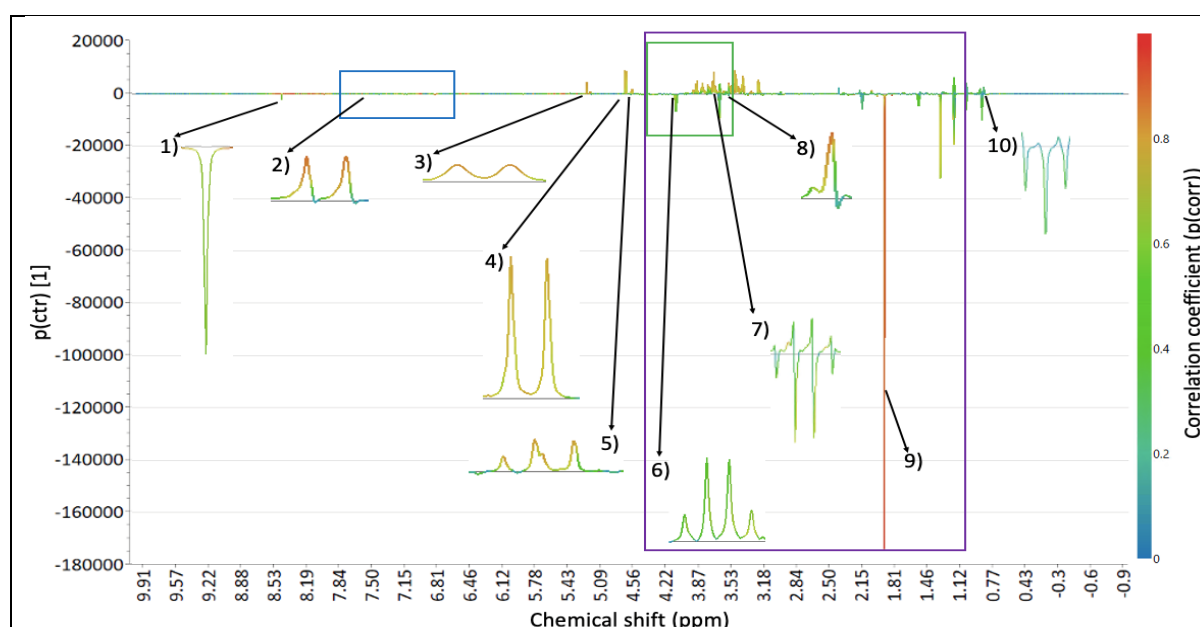
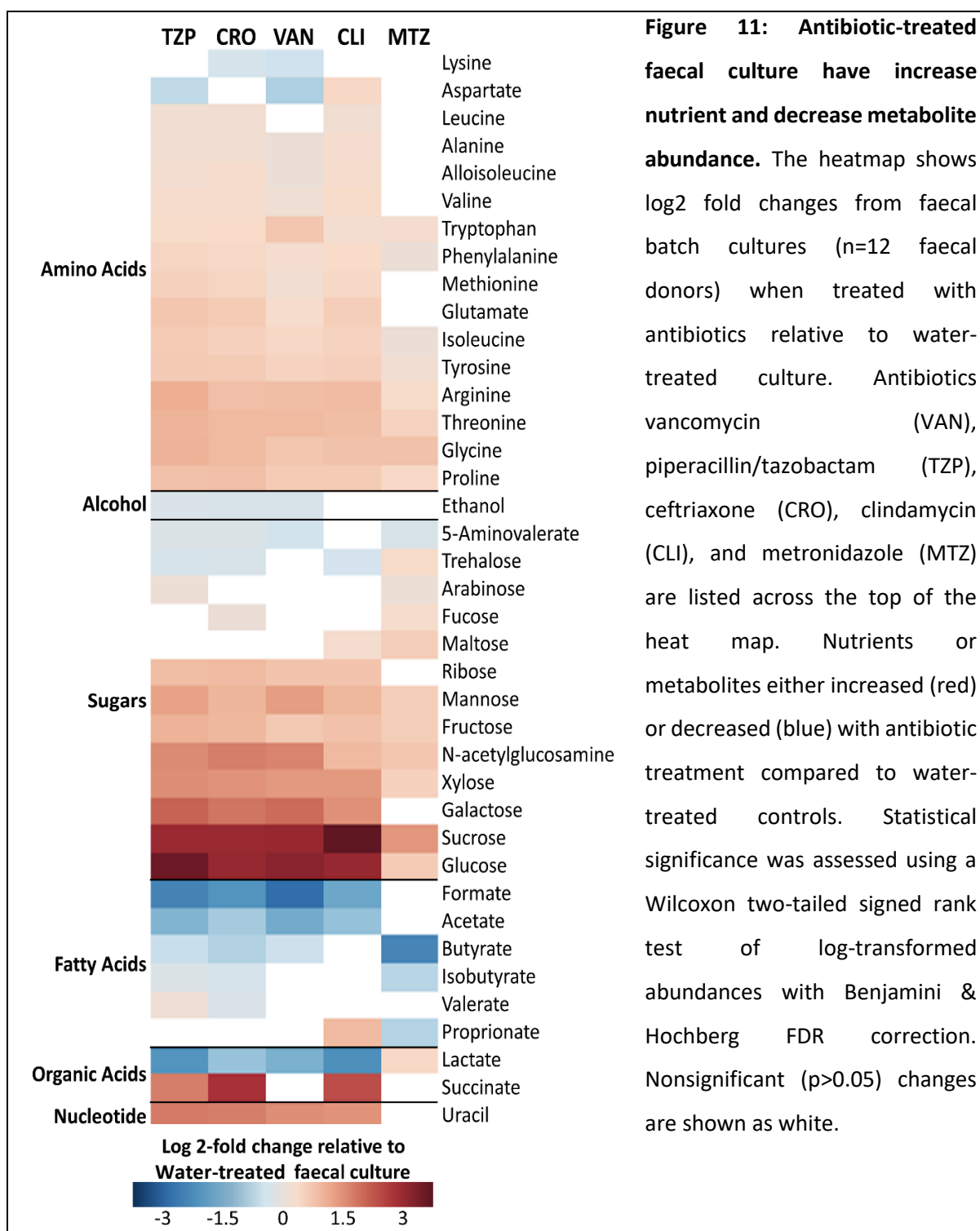


Figure 10: Nutrient and metabolite discriminant peaks drive separation between antibiotic- and water-treated faecal culture samples. An example OPLS-DA S-line plot visualizing the discriminant metabolites in the ^1H NMR spectra for VAN-treated faecal cultures versus water-treated faecal cultures ($R^2\text{X}$: 0.725, $R^2\text{Y}$: 0.929, Q^2 : 0.893, $p < 0.05$). The peaks are coloured based on the correlation coefficient of the variables. Metabolites peaks facing upward (+p(ctr)) have a higher concentration in VAN-treated samples while peaks facing downward (-p(ctr)) are in higher concentration in water-treated samples. The seven metabolites shown are examples of metabolites with high p(corr) values. 1) formate (p(corr): 0.608) 2) uracil (p(corr): 0.870) 3) N-acetylglucosamine (p(corr): 0.815) 4) glucose (p(corr): 0.782) 5) galactose (p(corr): 0.801) 6) proline (p(corr): 0.538) 7) ethanol (p(corr): 0.706) 8) glycine (p(corr): 0.888) 9) acetate (p(corr): 0.946) 10) butyrate (p(corr):

0.364). Boxes correspond with aromatic (blue) and aliphatic (purple) regions of the NMR spectra as well as the region corresponding to oligosaccharides and CH groups of amino acids (green).



Antibiotic-driven decreases in microbiota biomass positively correlates with increased nutrient availability and decreased metabolite production

To determine how the taxonomic and metabolic results related to each other, an unsupervised regularised canonical correlation analysis (rCCA) was performed. This allowed for multivariate data analysis for the trends between each antibiotic-treated culture compared to the water-treated control.

The first part of this analysis was to create a unit representation plot (**Figure 12**). While similar in appearance to the PCA (**Figure 8**) graphs, the rCCA unit analysis incorporates the 16S rRNA gene sequencing data. Each plot projects individual donor samples on the graph based on both 16S rRNA gene sequencing data (X axis) and ^1H -NMR spectroscopy data (Y axis) [467]. The separation shown along the first canonical variate demonstrates the impact of antibiotic treatment of the gut microbiota.

For each plot, the samples are listed as D1-D12 in either blue for the water-treated control samples or orange for the antibiotic-treated samples. The degree of separation between matching treated and untreated donor samples indicates the impact of the antibiotic on the taxonomic and metabolic data. Groups separated across both the first and second canonical variates was seen with VAN (**Figure 12A**), CRO (**Figure 12C**), and TZP (**Figure 12D**) treatment. CRO did not appear to drive separation for the donor 1 samples as the treated and untreated data points overlap. MTZ (**Figure 12B**) separated along the first canonical variant while CLI (**Figure 12E**) separated more along the second canonical variant. For all treatments and donors, the paired antibiotic- and water-treated samples are separated along either the first or second canonical variant.

To determine which interactions drive the separations shown in **Figure 12**, correlation circle plots were produced to show correlations between bacterial families and nutrients or

metabolites. The mixOmics processing identifies these correlations and sorts through noise to focus on key interactions [30, 468, 469]. Positive correlations in this model were shown to project in same direction from the origin, negative correlations were positioned in opposite directions from the axis, and non-significant correlations were not plotted. Strong correlations are shown farther from the origin than those close to the origin. Many of the bacterial families which decreased with antibiotic treatment were negatively correlated with the concentration of amino acids, monosaccharides, disaccharides, succinate and uracil. Conversely, a positive correlation was seen between these bacterial families and fatty acids, ethanol, and 5-aminovalerate.

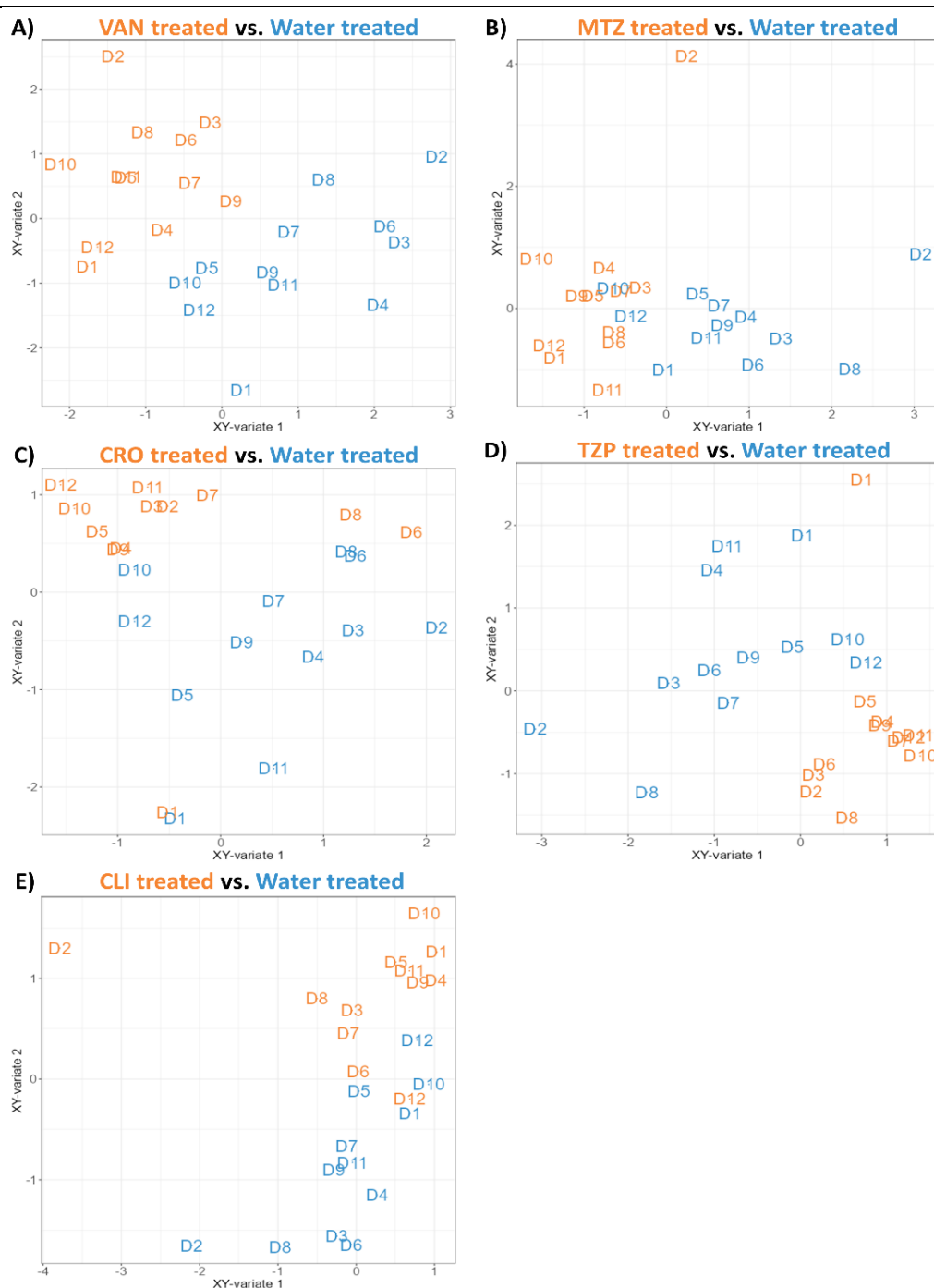
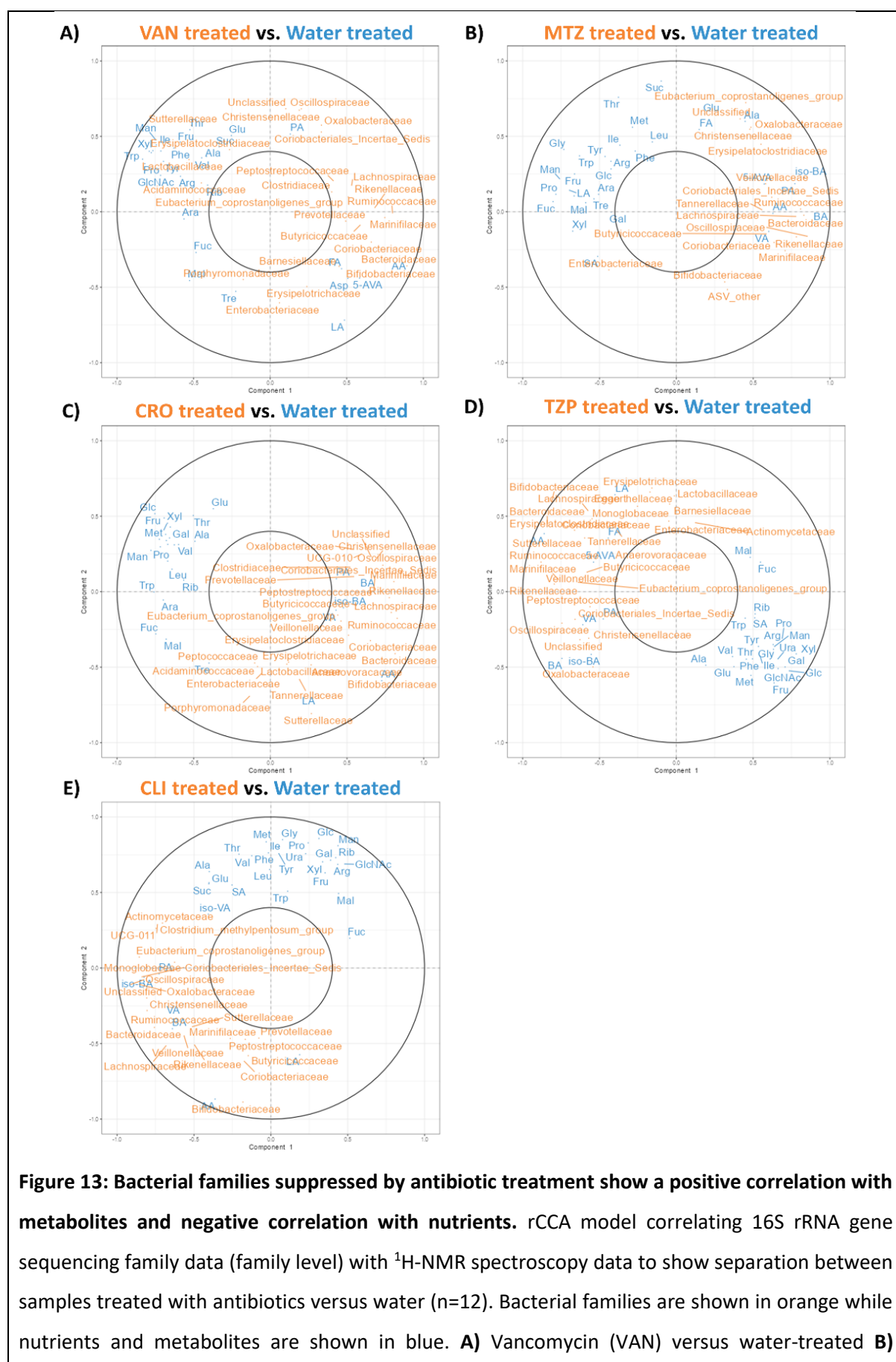


Figure 12: Metabolic and taxonomic changes drive separation in antibiotic-treated versus water-treated faecal cultures. rCCA representation unit plots correlate 16S rRNA gene sequencing family data with ^1H -NMR spectroscopy data to show separation between samples treated with antibiotics versus water ($n=12$). Data points for each donor (D1-12), both water-treated controls (blue) and antibiotic-treated samples (orange), are the first two canonical variates showing correlation. **A)** Vancomycin (VAN) versus water-treated **B)** Metronidazole (MTZ) versus water-treated **C)** Ceftriaxone (CRO) versus water-treated **D)** Piperacillin/tazobactam (TZP) versus water-treated **E)** Clindamycin (CLI) versus water-treated.



Metronidazole (MTZ) versus water-treated **C**) Ceftriaxone (CRO) versus water-treated **D**) Piperacillin/tazobactam (TZP) versus water-treated **E**) Clindamycin (CLI) versus water-treated. Nutrients and metabolites included in the data are arabinose (Ara), fructose (Fru), fucose (Fuc), galactose (Gal), glucose (Glc), mannose (Man), ribose (Rib), xylose (Xyl), N-acetylglucosamine (GlcNAc), maltose (Mal), sucrose (Suc), trehalose (Tre), alanine (Ala), arginine (Arg), aspartate (Asp), glutamate (Glu), glycine (Gly), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), threonine (Thr), tryptophan (Trp), tyrosine (Tyr), valine (Val), uracil (Ura), succinate (SA), lactate (LA), 5-aminovalerate (5-AVA), formate (FA), acetate (AA), propionate (PA), butyrate (BA), valerate (VA), isobutyrate (iso-BA), isovalerate (iso-VA), and ethanol (EtOH).

A negative correlation was seen between the grouping of bacterial families and the grouping of amino acids, sugars, succinate and uracil for antibiotic treatment with MTZ (**Figure 13B**), CRO (**Figure 13C**), TZP (**Figure 13D**), and CLI (**Figure 13E**). The only antibiotic-treated faecal cultures where positive correlation was seen between nutrients and some bacterial families was with VAN (**Figure 13A**). *Sutterellaceae*, *Erysipelatoclostridiaceae*, *Lactobacillaceae*, and *Acidaminococcaeae* positively correlated with the nutrients in this model.

Discussion

In this chapter, data showed that antibiotics that promote VRE intestinal colonisation caused a disruption to the gut microbiota that resulted in an increase in nutrients and a decrease in microbial metabolites. In an uncompromised microbiome, the abundant and diverse microbiota deplete nutrients and produce secondary metabolites. The antibiotic-driven decrease in the microbiota disrupts the natural metabonomic profile of the gut microbiome. The five antibiotics used in this study were shown to decrease the abundance of key bacterial families linked with contributing to colonisation resistance thus leading to an increase in nutrient availability and a decrease in the prevalence of microbial metabolites. These findings can help to identify what elements of an antibiotic-perturbed gut microbiome could be

exploited by VRE during colonisation. While the theoretical understanding of the impact of antibiotics on the microbiome was previously known, this study's combined approach of metabonomic and taxonomic analysis provided a uniquely thorough examination of the impact of a broad range of antibiotics on the gut.

Antibiotics were selected for this study which have been clinically linked to promoting VRE intestinal colonisation [55, 61, 64, 67, 68, 70, 71, 75, 183, 201-210]. These broad spectrum antibiotics are from different antibiotic classes and have different mechanisms of action. The glycopeptide (VAN), cephalosporin (CRO), and penicillin- β -lactamase (TZP) antibiotics disrupt cell wall formation. CRO and TZP show potent, broad-spectrum activity against both Gram-positive and Gram-negative bacteria while VAN targets Gram-positive bacteria [459-461]. The nitroimidazole (MTZ) and lincosamide (CLI) antibiotics prevent protein synthesis. MTZ targets Gram-negative bacteria while CLI has broad-spectrum activity [462, 463]. The differences in mechanism of action and spectrum of activity account for the differing impact these antibiotics had on the microbiota.

Overall, antibiotics that promote VRE intestinal colonisation decreased the absolute abundance of several important commensal bacterial families in the faecal microbiota. The contextualisation of using 16S rRNA qPCR to determine absolute abundance of the members of the microbiota identified through the 16S rRNA gene sequencing is vital to accurate evaluation of the impact of antibiotics. Relative abundance data sets, such as those collected through high-throughput sequencing, are compositional and can mask changes in the community [470]. With absolute abundance comparisons, it was clear that the antibiotic-treated faecal cultures showed a significant reshaping of the microbiota in both diversity and density. This could disrupt colonisation resistance due to the resultant accumulation of

nutrients and a depletion of microbial metabolites, together creating an environment that may promote VRE growth. Overlap in nutritional niches—a fundamental component of colonisation resistance—creates functional redundancy [15, 16, 471]. This redundancy, in which multiple commensals compete for the same nutrients and produce the same metabolites, means that if one commensal family is depleted with antibiotic treatment, the other family will fill the space left preventing pathogens from taking advantage of the loss. However, decreases in the absolute abundances across several bacterial families, in combination with overall decreases in total biomass, alters the nutrient landscape that pathogens such as VRE encounter in an antibiotic-treated gut environment.

All five antibiotics reshaped the gut microbiota in faecal cultures. Within the phyla which showed a significant change with antibiotics, most families decrease with antibiotic treatment. With the decrease in abundance of so many families in the cultures, the colonisation resistance that they conferred will likewise decrease. The nutrients which are no longer consumed by commensals can serve as carbon and nitrogen sources for opportunistic pathogens. The microbial metabolites that would otherwise have been produced by these strains to keep pathogen growth suppressed will no longer contribute to the protection of the microbiome against pathogenic colonisation.

Families including *Bifidobacteriaceae*, *Coriobacteriaceae*, *Bacteroidaceae*, *Rikenellaceae*, *Lachnospiraceae* and *Ruminococcaceae* which were shown to decrease in faecal culture with most (or all) of the antibiotic tested are linked with imbuing colonisation resistance in a healthy gut. *Bifidobacteriaceae* has been shown to protect against *E. coli* colonisation in mice [472-474]. One notable species, *B. longum*, was administered to infants to suppress virulence factor gene abundance by reducing the pH of the gut [475-477]. *Coriobacteriaceae*, another

key Actinomycetota family, are known consumers of most monosaccharides, disaccharides, and amino acids thus aiding in ensuring nutrient competition in the gut [30]. A negative correlation was found between CRE colonised patients and the presence of the strain *C. aerofaciens* [478]. Strains from the *Bacteroidaceae* family have shown to suppress growth of *S. ser. Typhimurium* [367], *C. difficile* [458], and *K. pneumoniae* [479]. *Rikenellaceae* disappearance from the microbiota has been described as a risk factor for CRE colonisation [480]. By means of nutrient competition, members of the *Lachnospiraceae* family have been shown to restrict *S. ser. Typhimurium* growth [14]. One study postulated that *Ruminococcaceae* and *Lachnospiraceae* are critical to the maintenance of colonisation resistance through the essential production of inhibitory microbial metabolites. Depletion in these families has been reported in patients with *C. difficile* [9, 481-483].

The significant remodelling of the gut microbiota by antibiotic treatment influences which nutrients are consumed and the production of the resulting microbial metabolites, some of which have been demonstrated to inhibit pathogens such as CRE [30] and *C. difficile* [9, 403, 481-483]. The metabonomic changes alone were significant enough to drive clear separation between antibiotic- and water-treated samples in both PCA and OPLS-DA plots. The unsupervised existence of separation in the PCA plots justified the forced separation seen in the OPLS-DA plots and allowed for analysis of the NMR spectra with OPLS-DA S-line plots. By observing the magnitude with which the peak intensities changed with antibiotic treatment, a better idea of overall environmental changes was formed.

Treatment by any of the five tested antibiotics resulted in an increase in the concentrations of amino acids, monosaccharides and disaccharides. Primary degraders in the gut microbiota are capable of breaking down complex nutrient sources found in the gut such as

arabinogalactan (arabinose, galactose), casein (amino acids), inulin (fructose, glucose, sucrose), mucin (amino acids, fucose, galactose, N-acetylglucosamine), pectin (arabinose, fucose, galactose, glucose, mannose, xylose), peptone (amino acids), starch (glucose, maltose, trehalose), xylan (arabinose, xylose), and yeast (amino acids, ribose, uracil) [30, 484-500]. This tiered digestion frees up simpler nutrients for secondary degraders.

Similar trends of elevation in amino acids and sugars has been shown with CRO and TZP, as well as other antibiotics associated with promoting pathogenic colonisation such as imipenem/cilastatin, meropenem, ertapenem, ciprofloxacin, ceftazidime, and cefotaxime [30]. Between the antibiotics evaluated in this study and those tested in Yip et al., a wide range of antibiotic classes have been shown to elevate nutrient levels in faecal cultures. The risk of antibiotic-associated nutrient elevations leading to a failure in colonisation resistance likely extends beyond promotion of VRE; any opportunistic pathogen which can exploit the disruption to the microbiome metabonomic profile could take advantage of antibiotic-driven dysbiosis.

When antibiotic treatment decreases bacterial diversity, the impact of the antibiotics can have secondary consequences. If species are reliant on primary degraders, such as members of the *Bacteroides* and *Akkermansia* genera [501, 502] and those primary degraders are killed by the antibiotic treatment, the down-stream effect on nutrient availability may lead to the starvation of secondary degraders. *Bifidobacterium* and *Collinsella* have been identified to consume simple sugars and amino acids in the gut microbiome [503-505]. *Bifidobacterium* has been shown to exhibit close nutritional ties with *Bacteroides* in the gut microbiome [506]. Key microbial families were seen to be negatively correlated with the concentration of amino acids and sugars, suggesting that these bacteria normally consume these nutrients in a

healthy gut microbiome. In the rCCA models for each treatment, *Bacteroidaceae*, *Bifidobacteriaceae*, *Coriobacteriaceae*, *Ruminococcaceae*, *Lachnospiraceae* and *Butyricicoccaceae* were shown to negatively correlate with nutrients when cultures were treated with all five antibiotics.

SCFAs, BCFAs, lactate (organic acid), and ethanol (alcohol) decreased with antibiotic treatment. The SCFAs (formate, acetate, butyrate, propionate and valerate) as well as isobutyrate (BCFA), 5-aminovalerate, ethanol and lactate can act as inhibitory metabolites to aid in colonisation resistance [30, 366, 391, 402, 403, 507, 508]. Isovalerate, though also an inhibitory metabolite BCFA, did not show a statistically significant change with any antibiotic treatment.

Formate, the shortest SCFA with only one carbon, is a regular end product of bacterial fermentation and is liberated when pyruvate is converted to acetyl-CoA [509-512]. While many organisms are capable of producing formate, key members of the gut microbiome associated with healthy levels of formate include *Bacteroides* species such as *B. thetaiotaomicron* and *F. prausnitzii* [283, 328, 513]. Both formate and *Bacteroidaceae* were shown to decrease with most of the antibiotic treatments. Modelling the correlation between the taxonomic and metabonomic changes, the positive correlation between *Bacteroidaceae* and formate with both VAN and TZP treatment in this study support the previous findings.

Acetate, the two carbon SCFA, helps regulate the pH of the gut [514, 515]. It is the most concentrated SCFA in the gut [2, 516, 517]. It can be produced through either conversion of pyruvate to acetyl-CoA or through the Wood-Ljungdahl pathway [357, 518, 519]. Like formate, most gut-dwelling microorganisms can produce acetate. Those associated with acetate production from the pyruvate digestion pathway include species belonging to the

genera *Ruminococcus*, *Bifidobacterium*, *Bacteroides*, *Akkermansia* and *Prevotella* [520]. The bifid shunt pathway, as the name implies, is utilised by members of the *Bifidobacteriaceae* family to produce acetate and lactate [521, 522]. The Wood-Ljungdahl pathway is used to produce acetate in members of the *Streptococcaceae*, *Clostridiaceae* and *Lachnospiraceae* families [520]. Positive correlations were seen between acetate and the *Bifidobacteriaceae*, *Bacteroidaceae*, *Ruminococcaceae*, and *Lachnospiraceae* families with most (or all) antibiotic treatments in this study indicating the importance of these commensal families in maintaining acetate concentrations.

Propionate typically makes up 20% of the SCFAs in the healthy gut [517]. It can be a secondary metabolite of the succinate, acrylate or propanediol pathway in the gut [523-525]. Production of propionate is associated with members of the *Bacteroidaceae*, *Acidaminococcaceae*, *Enterobacteriaceae*, *Veillonellaceae*, and *Ruminococcaceae* families [520, 526]. In this study, propionate was only shown to significantly decrease with MTZ treatment which caused a reduction in *Veillonellaceae* and *Ruminococcaceae* families. The rCCA plot for MTZ treatment, showed a positive correlation between propionate and these families suggesting they play a primary role in propionate production in the gut.

Butyrate is a four carbon SCFA which has been linked to reducing gastrointestinal inflammation and serves as an energy source for colonocytes [527-530]. It can be produced by the enzyme phosphotransbutyrylase and butyrate kinase or through the butyryl-CoA and acetate-CoA-transferase route in which either lactate or acetate serves as the substrate [523, 531]. The former method is associated with *Lachnospiraceae* bacteria, specifically those of the *Coprococcus* genus, while the later has been linked to member of the *Lachnospiraceae*, *Eubacteriaceae*, and *Ruminococcaceae* families. Butyrate was decreased with all antibiotic

treatments except for CLI treatment. The *Lachnospiraceae* family decreases with MTZ, CRO and TZP treatment. With MTZ, CLI and CRO treatment, butyrate concentration was seen to be highly correlated with the presence of *Ruminococcaceae* and *Lachnospiraceae*.

Valerate is a five carbon SCFA linked with restoring colonisation resistance against *C. difficile*, reducing paracellular permeability and ensuring gut barrier integrity [403, 532, 533]. Valerate can be produced through the coupling of propionate and ethanol [11, 534]. Additionally, proline can be reduced by Stickland-type fermentation to produce 5-aminovalerate which is fermented to valerate [535, 536]. In addition to identified decreases in ethanol and propionate with antibiotic treatment, 5-aminovalerate was decrease following antibiotics. These associates are reflected in the positive correlation between valerate, propionate and 5-aminovalerate in several of the antibiotic treatments in this study. Key producers of valerate are members of the *Clostridiaceae* and *Oscillospiraceae* family, the latter of which decreased with MTZ and TZP treatment. The positive correlation seen between valerate and *Oscillospiraceae* with most of the antibiotic treatments could indicate that *Oscillospiraceae* were more effected by antibiotic compared to *Clostridiaceae* and that, although a functional redundancy exists, the antibiotic treatment dysregulates the microbiome to thoroughly for valerate concentrations to be maintained.

The branched chain fatty acids (BCFAs) isobutyrate and isovalerate can also serve as inhibitory metabolites against pathogen colonisation in the gut [30, 537]. BCFAs are produced at lower levels in the gut than SCFAs and their impacts on health are less established [538]. The fermentation of branched chain amino acids such as isoleucine, leucine and valine are associated with the production of isovalerate and isobutyrate. While isovalerate was not shown to significantly change, isobutyrate decreased following several antibiotic treatment.

Isobutyrate tended to correlate positively with bacterial families including *Bacteroidaceae*, *Ruminococcaceae*, *Lachnospiraceae*, and *Oscillospiraceae*, as well as other fatty acids in the rCCA models.

Lactate, a carboxylic acid, has been linked with enhancing colonisation resistance to *S. ser. Typhimurium* and *Campylobacter jejuni* [539, 540]. Lactate can be produced via glycolysis following the reduction of pyruvate, through the pentose phosphate pathway or through the bifid shunt pathway [541]. The glycolysis pathway is the most common mechanism of producing lactate. Among others, members of the *Lactobacillaceae* family generate lactate through the pentose phosphate pathway; However, none of the antibiotics tested on the faecal cultures resulted in a significant change to *Lactobacillaceae*. The decrease in lactate concentration following antibiotic treatment was mirrored by a decrease in the concentration of *Bifidobacteriaceae* for four of the five antibiotics tested, likely related to the family's usage of the bifid shunt pathway to produce lactate and acetate. The rCCA models for antibiotic treatment showed lactate positively correlating with bacterial families (*Bifidobacteriaceae*, *Lachnospiraceae*, *Bifidobacteriaceae*) and other potentially inhibitory metabolites.

The antibiotic associated decline in diversity and density in faecal microbiota cultures and alteration to the nutritional landscape was shown to drive separation in antibiotic-treated versus water-treated cultures. A decline in the inhibitory metabolites paired with an elevation in nutrient availability, creates an ideal opportunity for pathogen colonisation in an antibiotic-perturbed gut. By identifying where treatment alters the microbiome in antibiotic-compromised gut compared to an antibiotic-naïve gut, the mechanism of VRE colonisation may be better understood.

Batch culture experiments performed in this chapter were highly reproducible, followed a standardised protocol, and allowed for the parallel comparison of multiple interventions on a single gut microbiota (including comparison to a water-treated control). This facilitated a comparison of the impact of the antibiotics under reproducible conditions between faecal donors with different gut microbiota compositions, controlling for confounding variables. Faecal donors consumed a mix of western, vegetarian, and pescetarian diets. The donors were both male and female and were a range of ages. Because each antibiotic-treated sample was compared to the water-treated sample from the same donor, the diversity of the donors served to strengthen the findings rather than introduce uncontrolled variables into the analysis.

Batch cultures, though a well-established method of short-term analysis, have the limitation in length of the study, control of environmental factors and inclusion of host immune mechanisms [542-544]. During incubation, nutrients depleted cannot be replenished, waste products cannot be removed, and the acidification of the cultures cannot be readjusted. As such, the information gleaned from the faecal batch cultures is limited to the impact of the antibiotics on the microbial communities' diversity and density as well as how shifts in the community lead to changes in what environmental materials are consumed and produced over the 24 hour incubation period.

To further investigate the potential opportunities presented by data in this chapter, a longitudinal study is necessary to confirm that the decreases in nutrients and increases in metabolite concentrations following antibiotic treatment are replicated. First, however, which of the antibiotic-induced changes VRE are capable of exploiting will need to be explored. Understanding the nutrient utilisation capabilities of VRE will inform how the influx

in nutrient concentrations following antibiotic treatment influences VRE colonisation success. Microbial metabolites have been shown to be inhibitory to other opportunistic pathogens but the susceptibility or resistance of VRE strains has yet to be established. By evaluating the optimal growth conditions for VRE in the gut, the findings on the impact of antibiotic treatment on the gut microbiota can be contextualised and prioritised by potential significance.

Chapter 4: Metabolic evaluation of vancomycin resistant *Enterococcus* within the context of the human gut

Introduction

Vancomycin-resistant *Enterococcus* is the second most common nosocomial infection worldwide with a majority of infections caused by *Enterococcus faecalis* and *Enterococcus faecium* [61, 62, 102, 545]. Enterococci are Gram-positive cocci, belonging to the phyla Bacillota (class Bacilli, order Lactobacillales, family *Enterococcaceae*), which form short to medium chains [73, 74]. Since its initial emergence as a MDR pathogen in the 1980s, VRE has been identified to cause endocarditis, severe bloodstream, intra-abdominal and urinary tract infections, meningitis and wound infections [63-65]. VRE possess intrinsic and acquired antibiotic resistances to aminoglycosides, β -lactams, fluoroquinolones, glycopeptides, oxazolidinones and tetracyclines [61, 64, 65, 67, 71, 72].

While research has gone into antimicrobial treatments for VRE, the mechanism by which it overcomes colonisation resistance in an already antibiotic-compromised gut remains unclear. In the previous chapter, the impact of antibiotics that promote VRE intestinal colonisation was measured on healthy faecal microbiota. Antibiotic treatment altered the concentration of amino acids, sugars, organic and fatty acids, alcohols and nucleotides. These nutrients and metabolites may play an important role in nutrient competition and metabolite inhibition to protect against VRE intestinal colonisation in a healthy gut microbiota. Amino acids, sugars, uracil and succinate increased following antibiotic treatment, while short and branched chain fatty acids, ethanol and lactate decreased following antibiotic treatment. To determine how antibiotic-driven alterations of the nutrient and metabolite landscapes are exploited by VRE, next determination of which nutrients can be consumed by VRE to support their growth and which microbial metabolites can restrict VRE growth was explored.

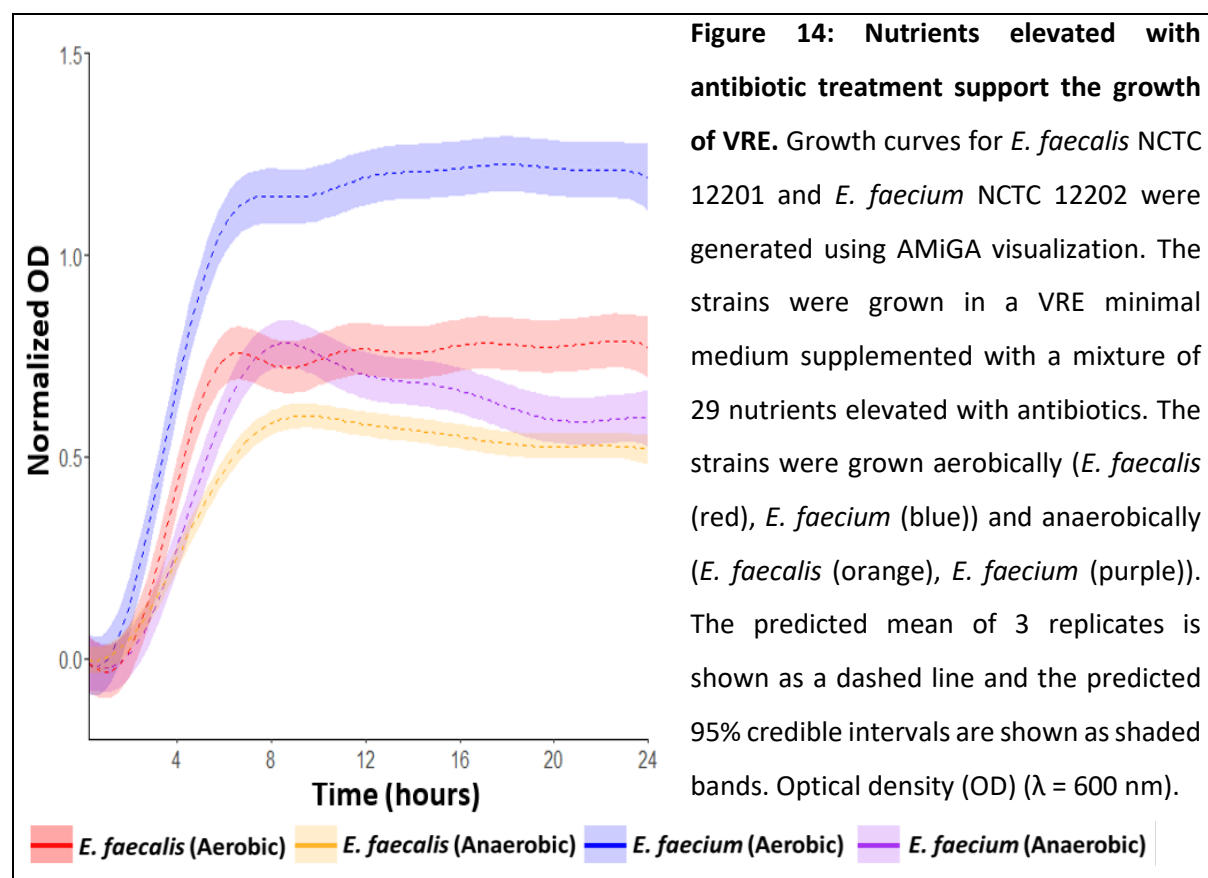
As nutrients increased following antibiotic treatment, it is critical to understand the ability of VRE to consume key nutrients as carbon and/or nitrogen sources, their preference for these nutrients, and their ability to act as primary and secondary degraders in the gut. This information will allow for the determination of whether the decrease in nutrient competition promoted the intestinal colonisation and proliferation of VRE. Secondary degraders would be capable of digesting amino acids, monosaccharides, and disaccharides [503-505]. However, disruption of primary degraders (capable of breaking down complex nutrients such as starch, mucin, arabinogalactan, inulin, casein, and xylan) may also impact the availability of these more complex nutrients [501, 502]. Therefore, it is also important to determine whether VRE strains are also capable of acting as primary fermenters to degrade these complex nutrients. Metabolites produced by the commensal microbiota, including SCFAs, BCFAs, alcohols, 5-aminovalerate and lactate, have been shown to inhibit the growth of some gut pathogens [66, 334]. Thus, the impact of these inhibitory metabolites on the VRE strains growth needed to be determined to establish if the decrease in metabolites following antibiotic treatment allows for VRE colonisation.

Evaluating how VR-*E. faecalis* and VR-*E. faecium* growth in pure culture is promoted by individual nutrients or restricted by individual metabolites will provide a better understanding of how VRE growth is controlled in the presence or absence of antibiotics in the intestine. The knowledge of how the VRE strains exploits the altered nutrient profile of an antibiotic-treated microbiome will be used to devise a target for novel microbiome-based therapeutics.

Results

Experiments in the previous chapter demonstrated that antibiotic treatment of faecal microbiota increased the concentration of 29 nutrients (**Figure 11**). Therefore, VRE isolates

were cultured in a minimal medium supplemented with a mixture of these nutrients (referred to as the “nutrient mix”) to determine whether this enriched nutrient landscape was supportive of the strains’ growth. The growth was tested under both aerobic and anaerobic conditions since, although the distal gut is an anaerobic environment, it becomes more aerobic with antibiotic treatment or disease [329-332]. Both strains grew in the nutrient mix under aerobic and anaerobic conditions, however *E. faecium* and *E. faecalis* growth was higher under aerobic conditions (**Figure 14**).



VRE utilise a range of nutrients which were elevated in antibiotic-treated faecal culture as carbon sources

After showing that the nutrient mix supported *E. faecium* and *E. faecalis* growth under both aerobic and anaerobic conditions, the nutrients were tested individually as a sole carbon source to narrow down which nutrients were capable of supporting growth. The individual nutrients were supplemented into a VRE minimal media which did not support the growth of

the strains without the addition of a carbon source. Growth was measured under both aerobic and anaerobic conditions (**Figure 15, Figure 16**).

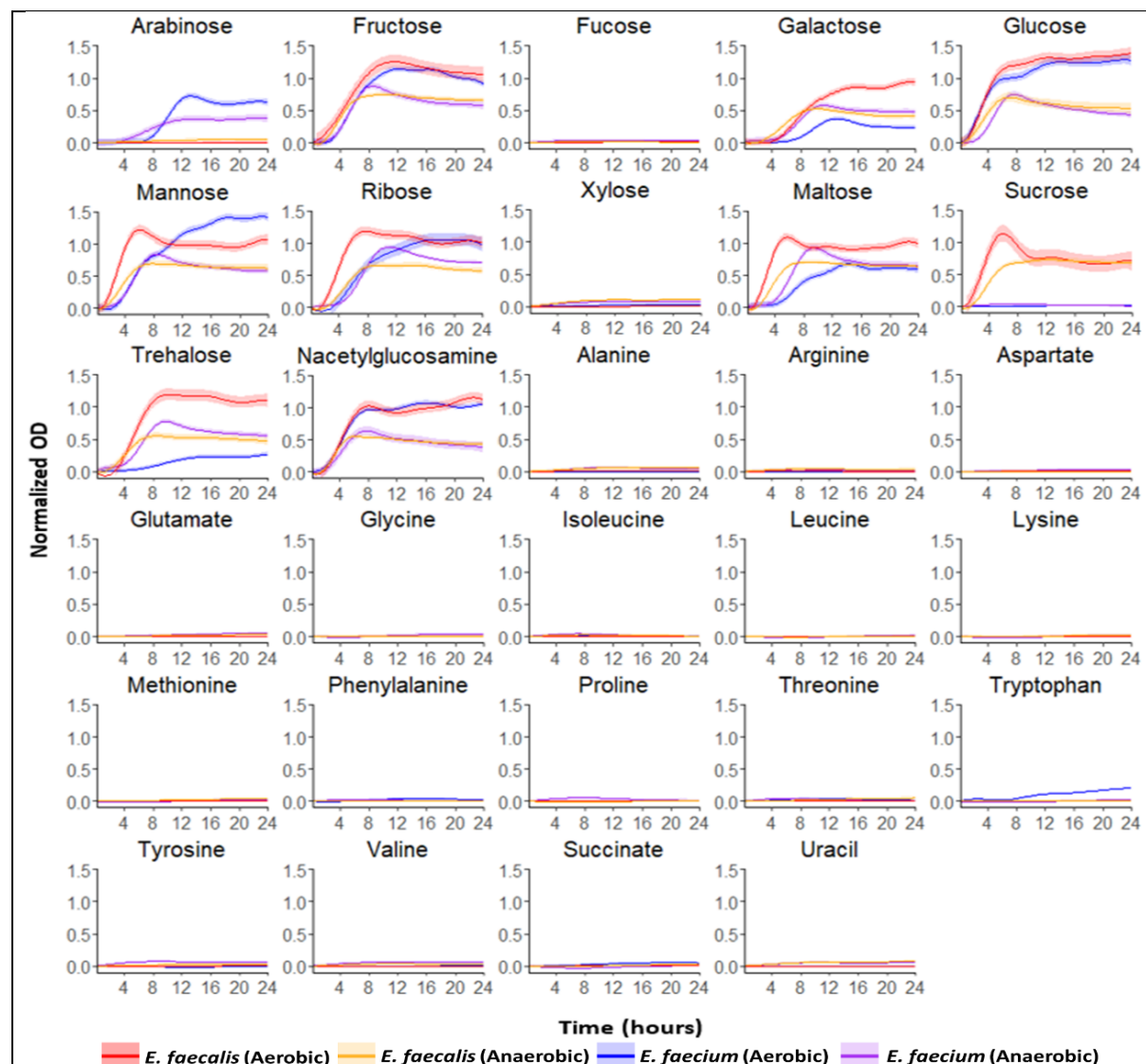
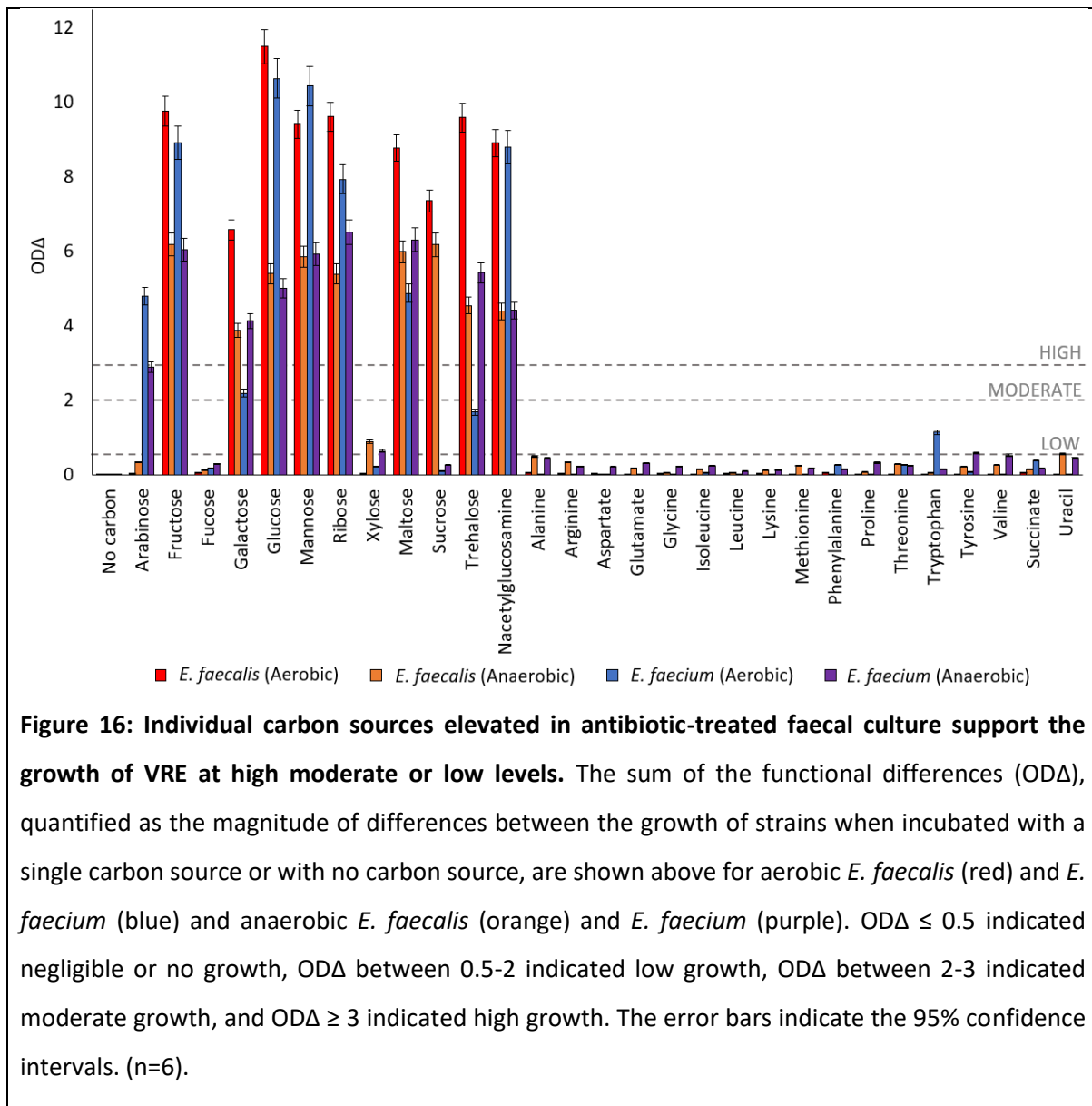


Figure 15: Individual carbon sources elevated in antibiotic-treated faecal culture support the growth of VRE. AMiGA predicted growth curves for *E. faecalis* NCTC 12201 and *E. faecium* NCTC 12202 when growth on VRE minimal medium supplemented a single carbon source (0.5%) or water as a no-carbon control. The strains were grown aerobically (*E. faecalis* (red), *E. faecium* (blue)) and anaerobically (*E. faecalis* (orange), *E. faecium* (purple)). The predicted mean of at least 6 replicates (from 2-3 independent experiments) is shown as a dashed line and the predicted 95% credible intervals are shown as shaded bands. Optical density (OD) ($\lambda = 600$ nm).



E. faecalis grew to high levels on fructose, galactose, glucose, mannose, ribose, maltose, sucrose, trehalose, and N-acetylglucosamine under both aerobic and anaerobic conditions with higher growth in aerobic conditions compared to anaerobic conditions. Under anaerobic conditions uracil and xylose supported low growth of *E. faecalis* which was not seen in aerobic conditions. The lag times of anaerobic growth were greater than in aerobic conditions for glucose, mannose, ribose, maltose, and sucrose. Glucose, mannose, ribose, maltose and sucrose supplementation resulted in stationary phase being reached on or before six hours

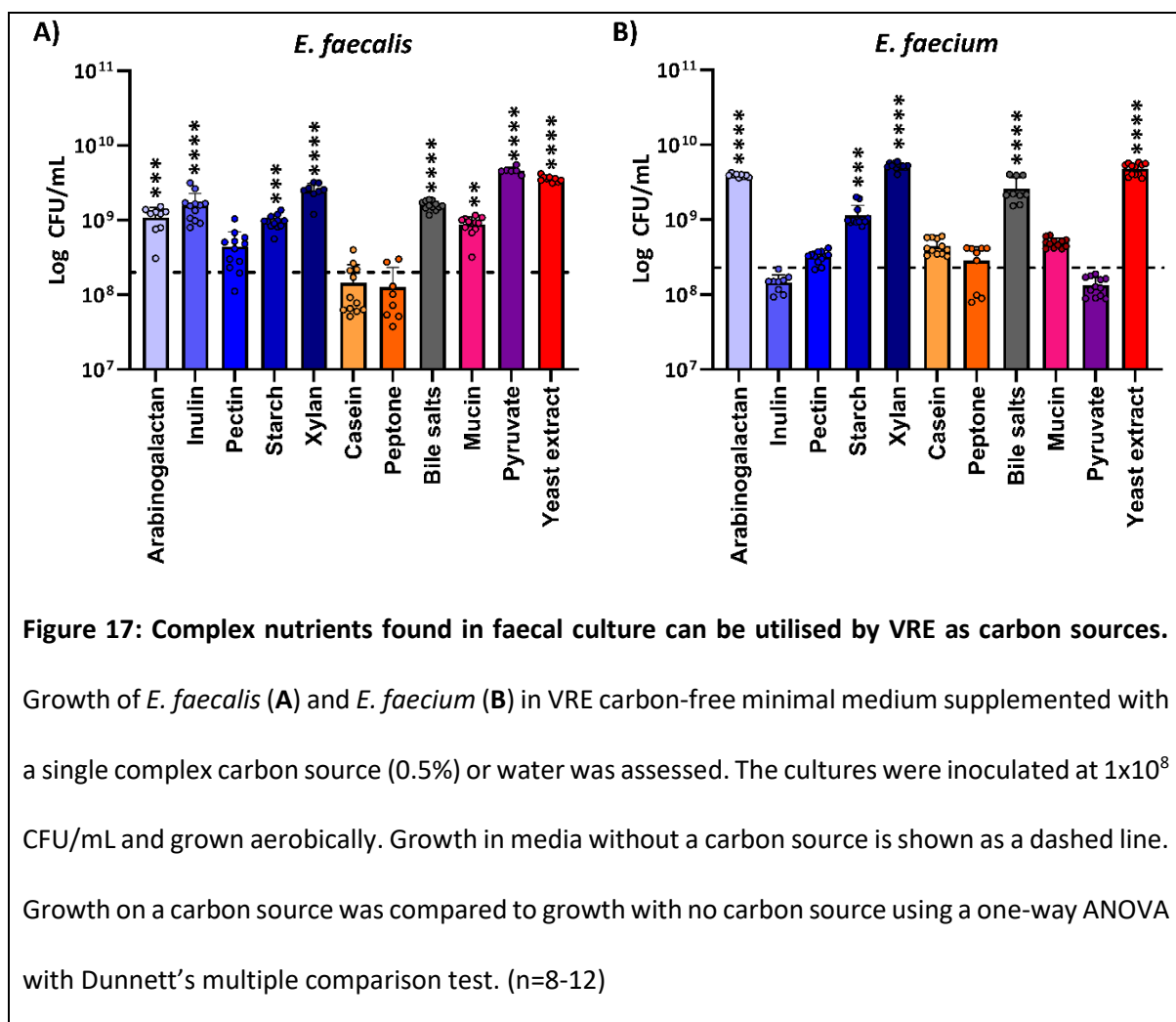
post inoculation. Stationary phase was reached on or after eight hours when *E. faecalis* was grown on fructose, galactose, trehalose, and N-acetylglucosamine.

E. faecium was seen to utilise arabinose, fructose, galactose, glucose, mannose, ribose, maltose, trehalose, and N-acetylglucosamine under both aerobic and anaerobic conditions. While growth was higher aerobically for most carbon sources tested, galactose, maltose, and trehalose supported higher growth anaerobically. *E. faecium* grew to low levels on xylose anaerobically. Tryptophan supported low growth in aerobic conditions. Log phase was reached quicker when *E. faecium* was grown on arabinose, galactose, maltose, and trehalose anaerobically but quicker on glucose in aerobic conditions. Growth on glucose and N-acetylglucosamine resulted in *E. faecium* reaching stationary phase faster than growth on arabinose, fructose, galactose, mannose, ribose, maltose, and trehalose.

VRE acts as a primary degrader for nutrients found in the distal gut

The antibiotic-treated faecal cultures from the previous chapter showed a decrease in known primary degraders such as members of the *Bacteroidaceae* and *Akkermansiaceae* families [501, 502]. While the concentrations of complex substrates were not directly measured with ¹H-NMR spectroscopy, the potential for these nutrients to have increased following antibiotic-killing of primary degraders, thus serving as a potential nutrient source for the VRE strains, had to be tested. In addition to several complex carbohydrates, pyruvate (a carbohydrate metabolism intermediate) and bile salts were assessed as potential nutrient sources for the VRE strains. As not all of the complex nutrients were fully soluble, OD₆₀₀ measurements could not be utilised. Therefore, *E. faecium* and *E. faecalis* growth was assessed using end point plate counts measured 24 hours after inoculation.

E. faecalis grew on arabinogalactan ($p=0.0002$), inulin ($p<0.0001$), starch ($p=0.0009$), xylan ($p<0.0001$), bile salts ($p<0.0001$), mucin ($p=0.004$), pyruvate ($p<0.0001$), and yeast extract ($p<0.0001$) (**Figure 17A**). *E. faecium* grew on arabinogalactan ($p<0.0001$), starch ($p=0.0005$), xylan ($p<0.0001$), bile salts ($p<0.0001$), and yeast extract ($p<0.0001$) (**Figure 17B**). Neither strain grew on pectin, casein, or peptone.



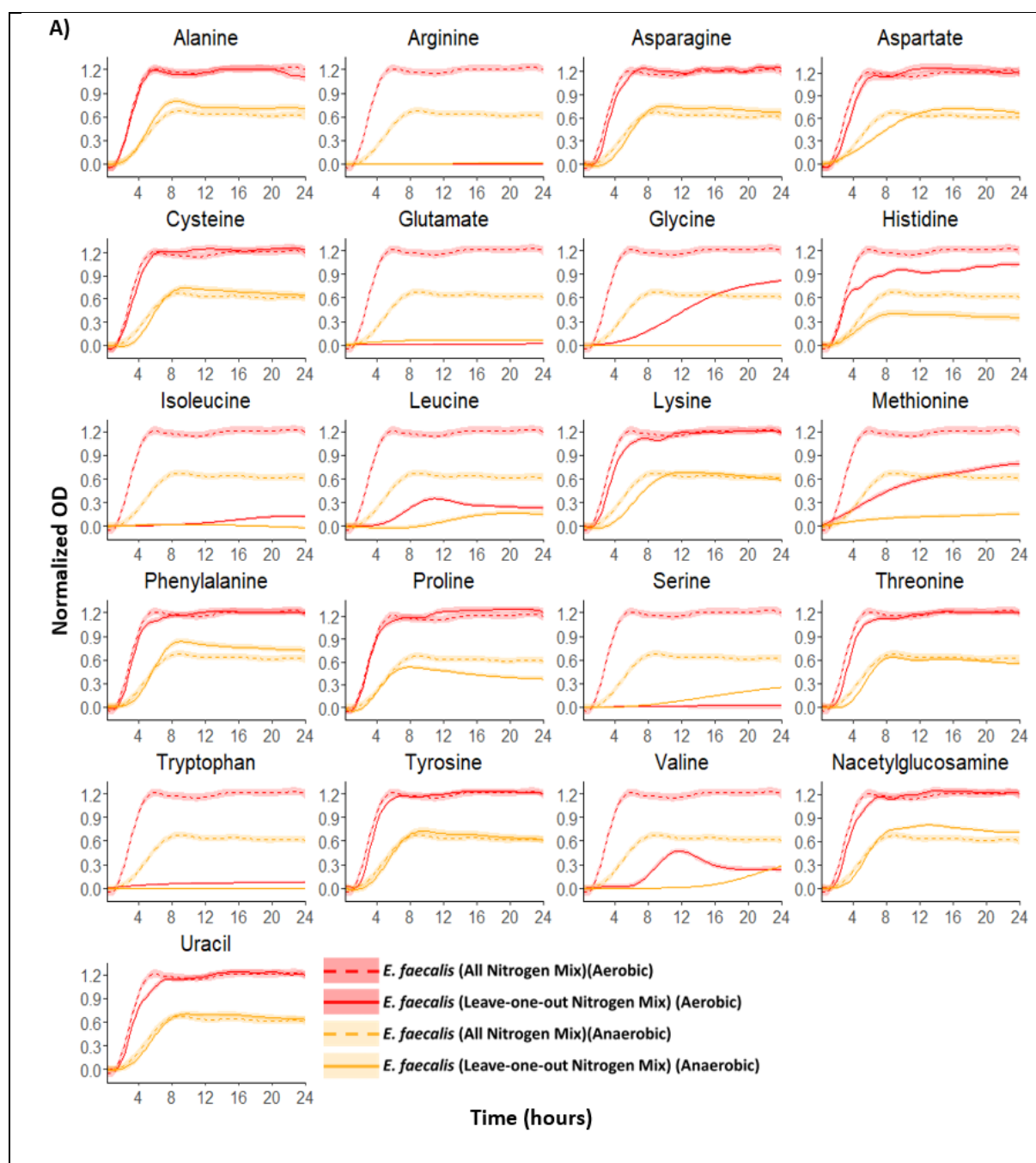
VRE utilise amino acids which were elevated in antibiotic-treated faecal culture as nitrogen sources

In addition to nutrients serving as carbon sources, some of the nutrients increased with antibiotics could also act as nitrogen sources to support *E. faecium* and *E. faecalis* growth in

an antibiotic-treated gut. The *E. faecium* and *E. faecalis* strains used in this study had undefined amino acid auxotrophies, and therefore to assess nitrogen utilisation a leave-one-out experimental design was necessary. In this design, a mixture of all the available nitrogen sources (19 amino acids, N-acetylglucosamine and uracil) was used as a positive control added to an otherwise nitrogen-free VRE minimal media supplemented with glucose as the carbon source. To assess whether the strains used the nutrient as a nitrogen source, one nitrogen source was left out at a time and the impact on growth was measured. A decrease in growth when one nutrient was left out compared to the complete mixture indicated that the source was used by the strain. No growth when a nitrogen source was left out indicated the nitrogen source was essential for the growth of the strain. The nitrogen source mix was replaced with water in the negative control.

Our experiment demonstrated that *E. faecalis* used fewer nitrogen sources than *E. faecium*. Both aerobic (**Figure 18A, Figure 19, red**) and anaerobic (**Figure 18A, Figure 19, orange**) conditions showed no or dramatically reduced growth of *E. faecalis* when arginine, glutamate, isoleucine, leucine, serine, tryptophan, and valine were left out of the media. Eliminating histidine from the nitrogen mix caused decreased growth of the strain in both aerobic and anaerobic conditions. Anaerobically, the elimination of glycine and methionine resulted in no growth of *E. faecalis* while in aerobic conditions, the growth was only decreased. Both lysine and threonine caused an increase in the lag time of *E. faecalis* when eliminated from the nitrogen mixture in both growth conditions. Leaving out proline caused a decrease in the growth of *E. faecalis* anaerobically but had no impact on aerobic growth. Asparagine, cysteine, and phenylalanine elimination resulted in slightly longer lag time but higher overall growth of *E. faecalis* in anaerobic conditions.

E. faecium growth required arginine, glutamate, serine, threonine, and tryptophan in both aerobic (**Figure 18B, Figure 19**, blue) and anaerobic (**Figure 18B, Figure 19**, purple) conditions. Cysteine, histidine, phenylalanine, and valine elimination from the nitrogen source mix negatively impacted recovery of *E. faecium*, increasing the lag time and decreasing the overall growth in both conditions. In anaerobic conditions, eliminating glycine, isoleucine, leucine, lysine, and methionine caused a greater decrease in growth of *E. faecium* compared to elimination of these nitrogen sources in aerobic conditions. Alanine and aspartate elimination increased the lag time in aerobic growth but had no impact on anaerobic growth.



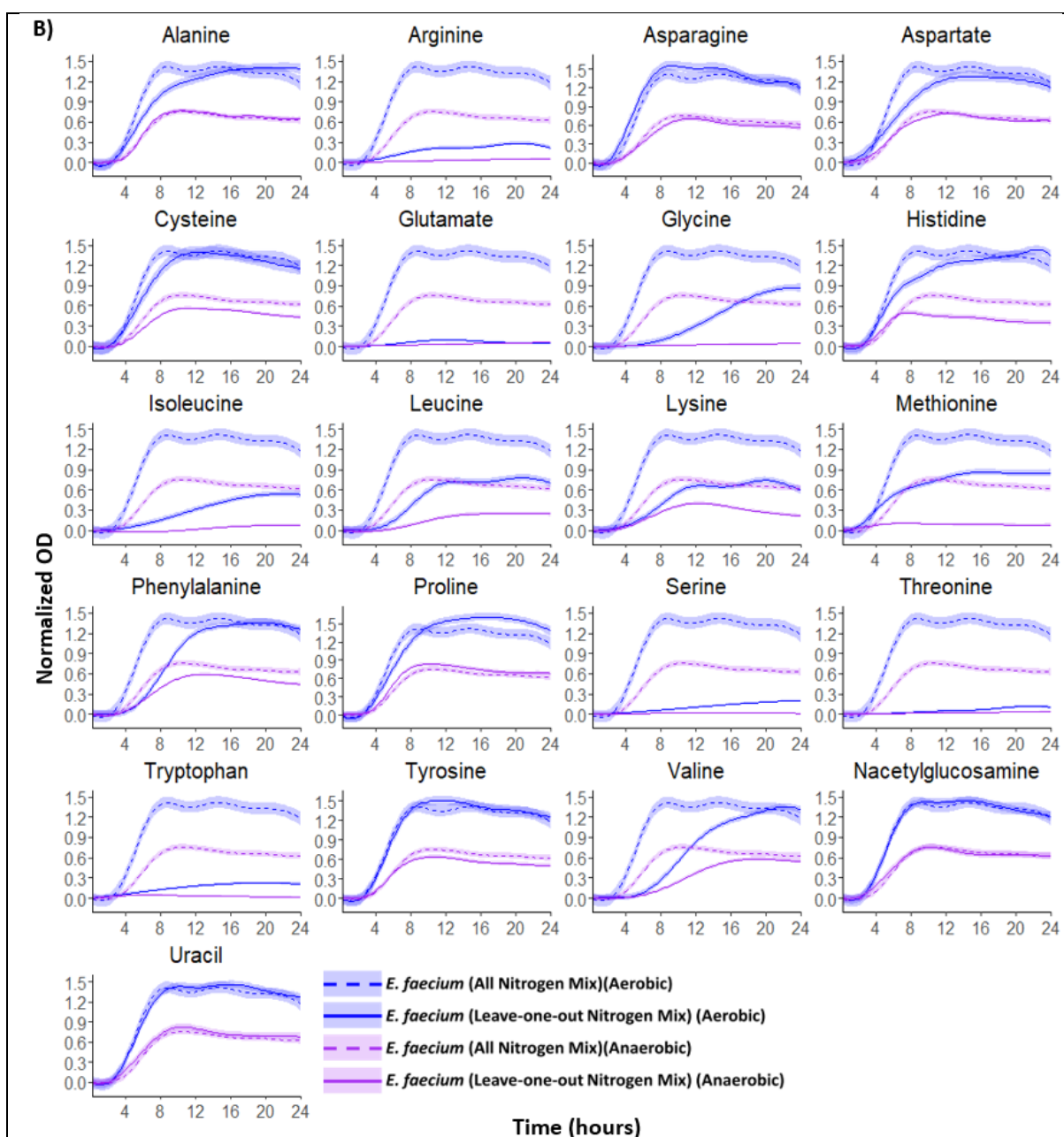
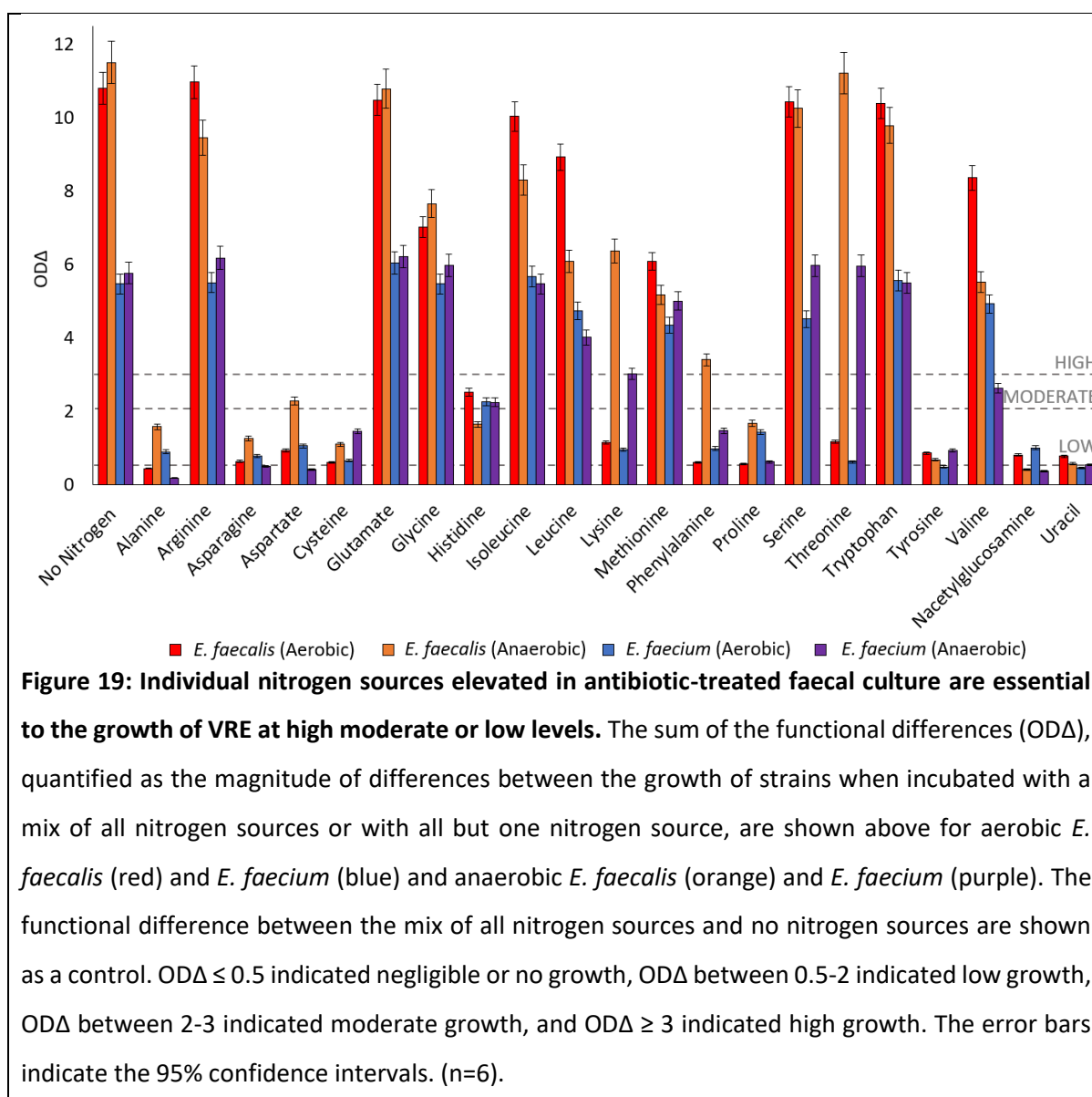


Figure 18: Eliminating key nitrogen sources decreases VRE ability to grow in media containing nutrients elevated with antibiotic-treated faecal culture. AMiGA predicted growth curves for A) *E. faecalis* NCTC 12201 and B) *E. faecium* NCTC 12202 when growth on VRE minimal medium supplemented with a leave-one-out mix of nitrogen sources (0.5%) or a complete mix of nitrogen sources as a control. The strains were grown aerobically (*E. faecalis* (red), *E. faecium* (blue)) and anaerobically (*E. faecalis* (orange), *E. faecium* (purple)). The predicted mean of at least 6 replicates (from 2-3 independent experiments) is shown as a solid line for the experimental treatment or a dashed line for the positive control. The predicted 95% credible intervals are shown as shaded bands. Optical density (OD) ($\lambda = 600$ nm).



E. faecium and *E. faecalis* demonstrate a preference for nutrients elevated in antibiotic-treated faecal culture

Although this study has demonstrated that *E. faecalis* and *E. faecium* can utilise specific nutrients elevated with antibiotics (**Figure 14, Figure 15, Figure 18**), previous studies have shown that bacteria have preferences for specific nutrients over others [30], and these preferences are currently undefined for the VRE strains. Moreover, previous studies have also demonstrated that the presence of some nutrients can influence the utilisation of other nutrients [30, 546]. The mix of nutrients elevated with antibiotic treatment was added to minimal media inoculated with *E. faecium* or *E. faecalis* and incubated under aerobic or

anaerobic conditions. Culture samples were collected at 0, 4.5, 9, and 24 hours post inoculation to determine changes in nutrient utilisation over time, during the log phase, early lag phase and late lag phase (**Figure 14**). The nutrient levels were quantified using ^1H -NMR spectroscopy and normalised to the baseline levels found in the 0-hour samples (**Figure 20**).

E. faecalis first depleted most of the supply of fructose, glucose, mannose, N-acetylglucosamine, ribose, trehalose, and sucrose. Arginine, galactose, maltose, tyrosine and—to a lesser extent—succinate, were used later in the incubation. By the final timepoint, aspartate, glutamate, glycine, isoleucine, leucine, phenylalanine, threonine, and valine had all decreased in concentration compared to the initial concentration. *E. faecalis* did not utilise arabinose or uracil as a nutrient source to a statistically significant degree.

E. faecium first depleted fructose, galactose, glucose, mannose, N-acetylglucosamine, ribose, trehalose and arginine. Arabinose, maltose, sucrose, tyrosine and succinate were used later in the incubation. *E. faecium* did not use aspartate, glycine, isoleucine, leucine, phenylalanine, threonine, or valine to a statistically significant degree.

Alanine, fucose, lysine, methionine, proline, tryptophan, and xylose did not show any significant changes during incubation with either strain in either growth condition and were thus not included in **Figure 20**.

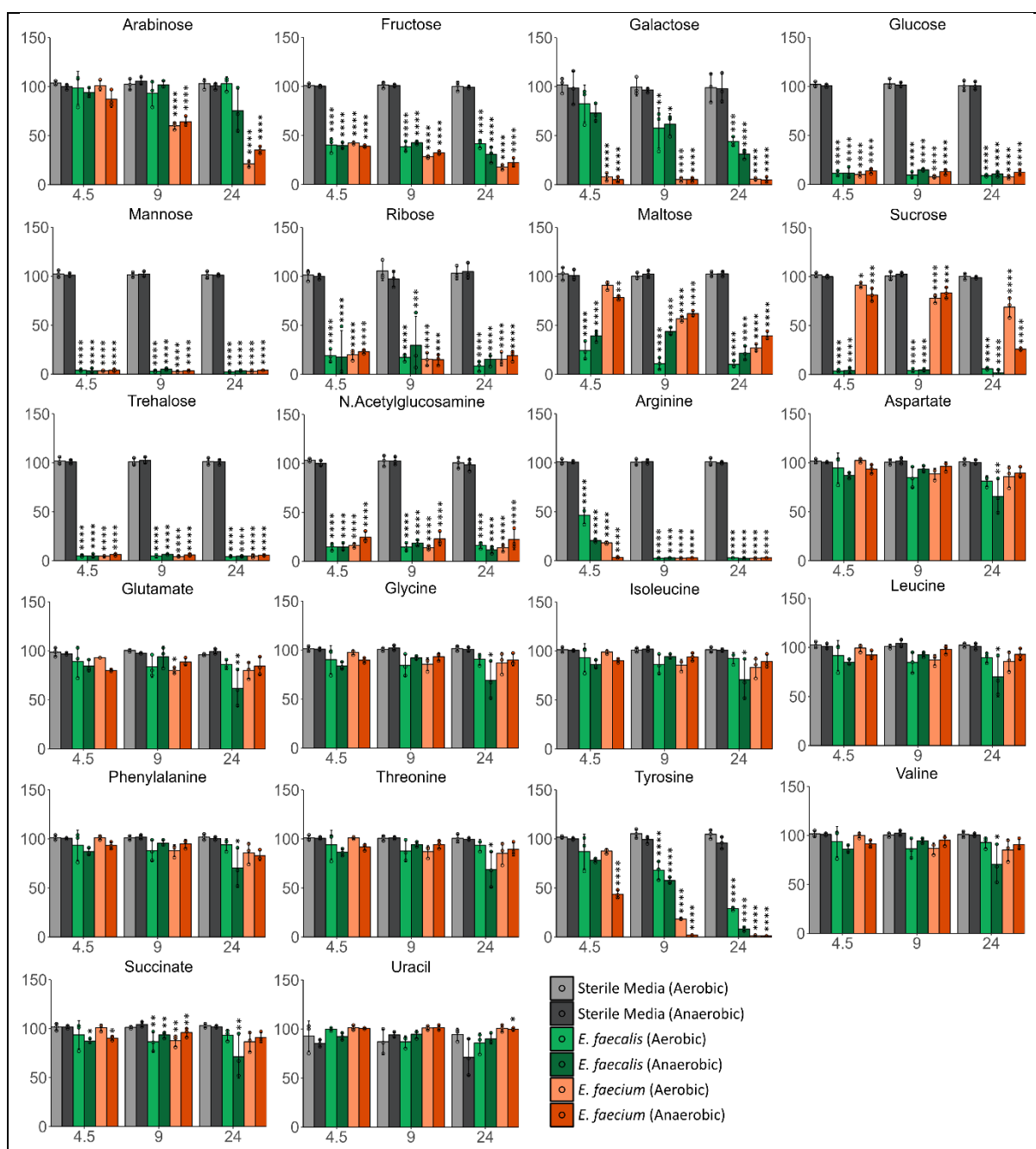
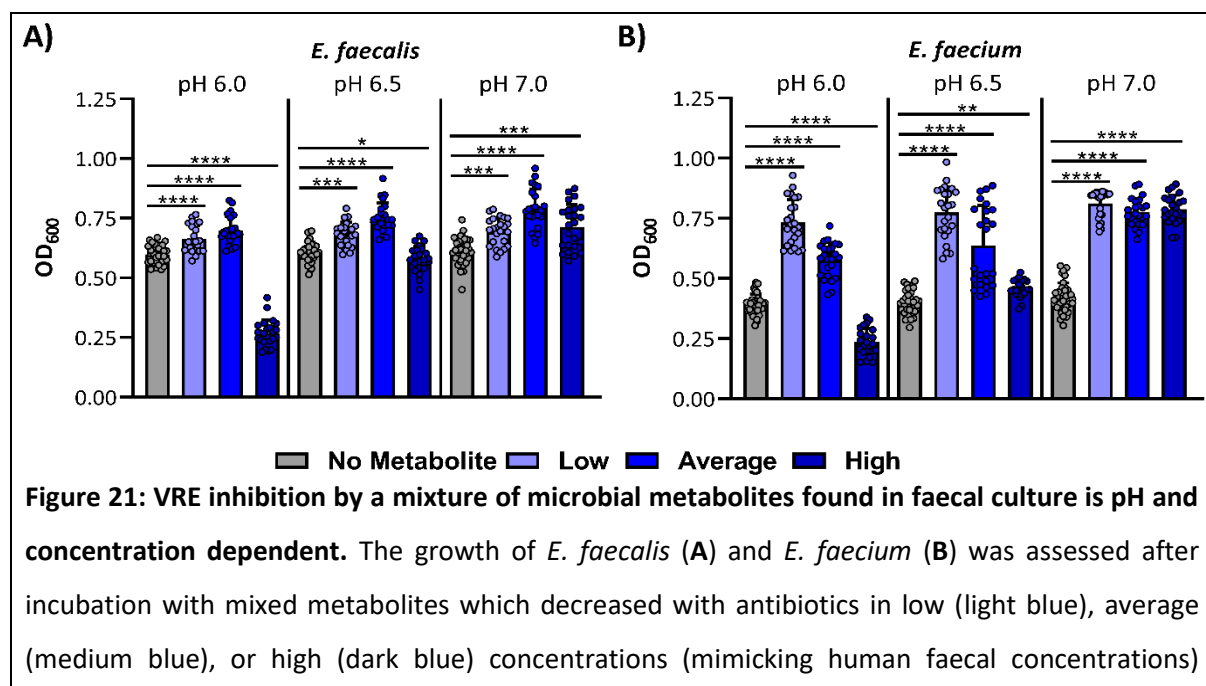


Figure 20: VRE isolates demonstrate a preference of nutrients when cultured with a mixture of carbon and nitrogen sources elevated in antibiotic-treated faecal culture. The percentage of nutrients remaining in VRE minimal media supplemented with antibiotic-increased nutrient mix and inoculated with *E. faecalis* (green) or *E. faecium* (orange) was calculated based on initial nutrient abundances measured by ^1H -NMR spectroscopy. Results from uninoculated, sterile media are shown in grey. The experiment was performed aerobically and anaerobically. Results of two-way mixed ANOVA followed by pairwise comparisons with Bonferroni correction are shown. Nonsignificant ($p > 0.05$) changes are not shown. ($n=3$).

Mixed metabolite inhibition of VRE strains is pH dependent

In addition to antibiotic treatment causing an increase in nutrient availability, it also resulted in a decrease in inhibitory metabolite production (**Figure 11**). To assess how this might influence VRE proliferation, the impact of inhibitory metabolites on the VRE strains' growth was tested in TSB media. The minimum, maximum and average concentrations of the microbial metabolites was calculated from faecal samples from 12 healthy human donors (**Table 2**).

When testing mixed metabolites, the pH was adjusted to 6.0, 6.5 or 7.0 to reflect the pH range found in a healthy human colon [30, 547, 548]. The expected inhibition of the *E. faecalis* and *E. faecium* was seen at the highest concentration at pH 6.0 for *E. faecium* and pH of 6.0 and 6.5 for *E. faecalis* (**Figure 21**). However, at pH 7.0, the metabolite mix increased *E. faecium* and *E. faecalis* growth at the low, average, and high concentrations tested. At pH 6.5 and 6.0 the metabolite mix increased *E. faecium* and *E. faecalis* growth at the low and average concentrations. *E. faecium* increased in growth at high concentrations at pH 6.5.



prepared to pH of either 6.0, 6.5 or 7.0. Positive control for each pH shown as the grey bar to the left of metabolite treated conditions. Results of unpaired two-tailed t-test the metabolite mixtures to the no metabolite control at the corresponding pH are shown. (n=6) Optical density (OD) ($\lambda = 600$ nm).

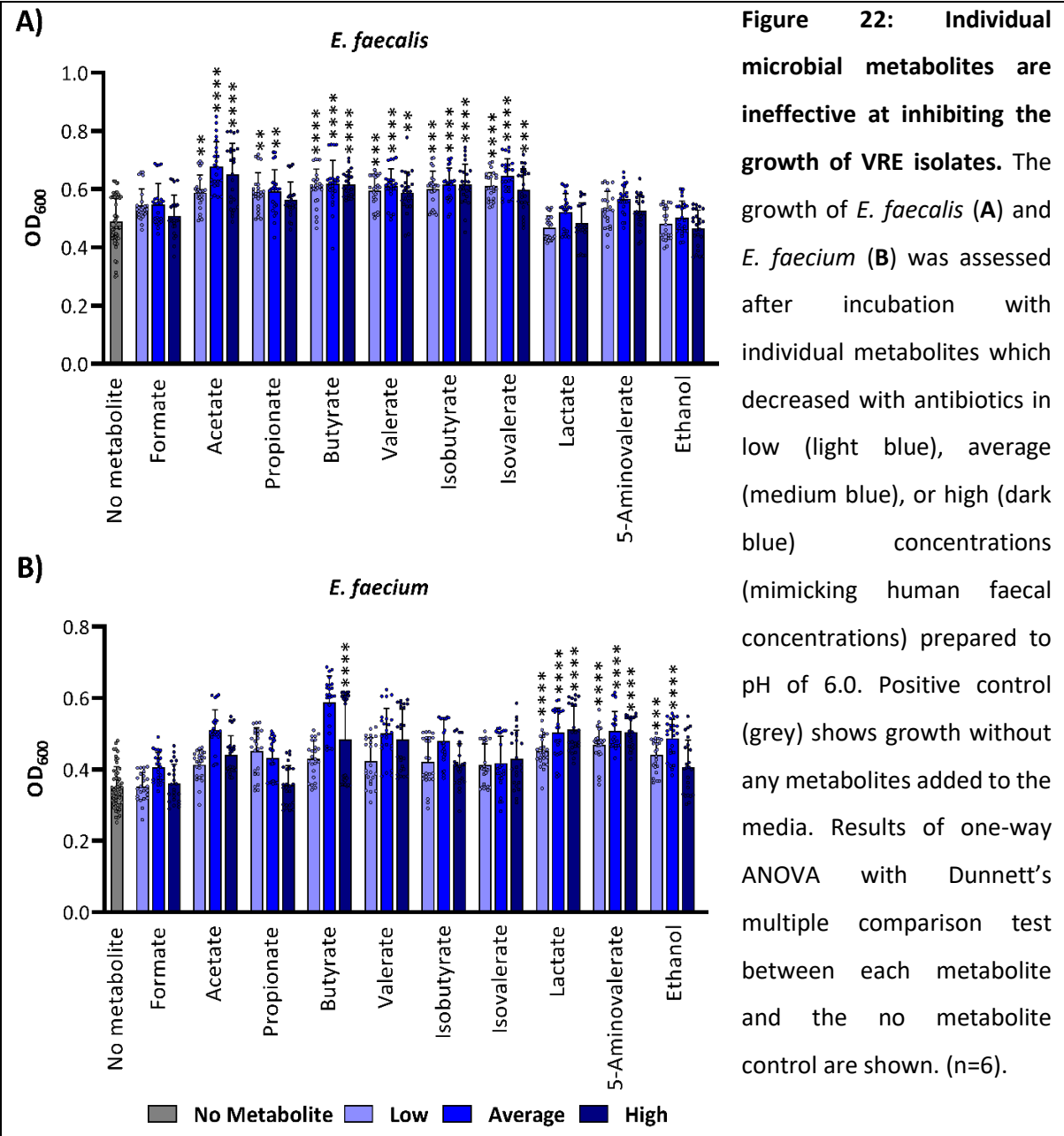
Individual metabolites do not inhibit the growth of VRE strains

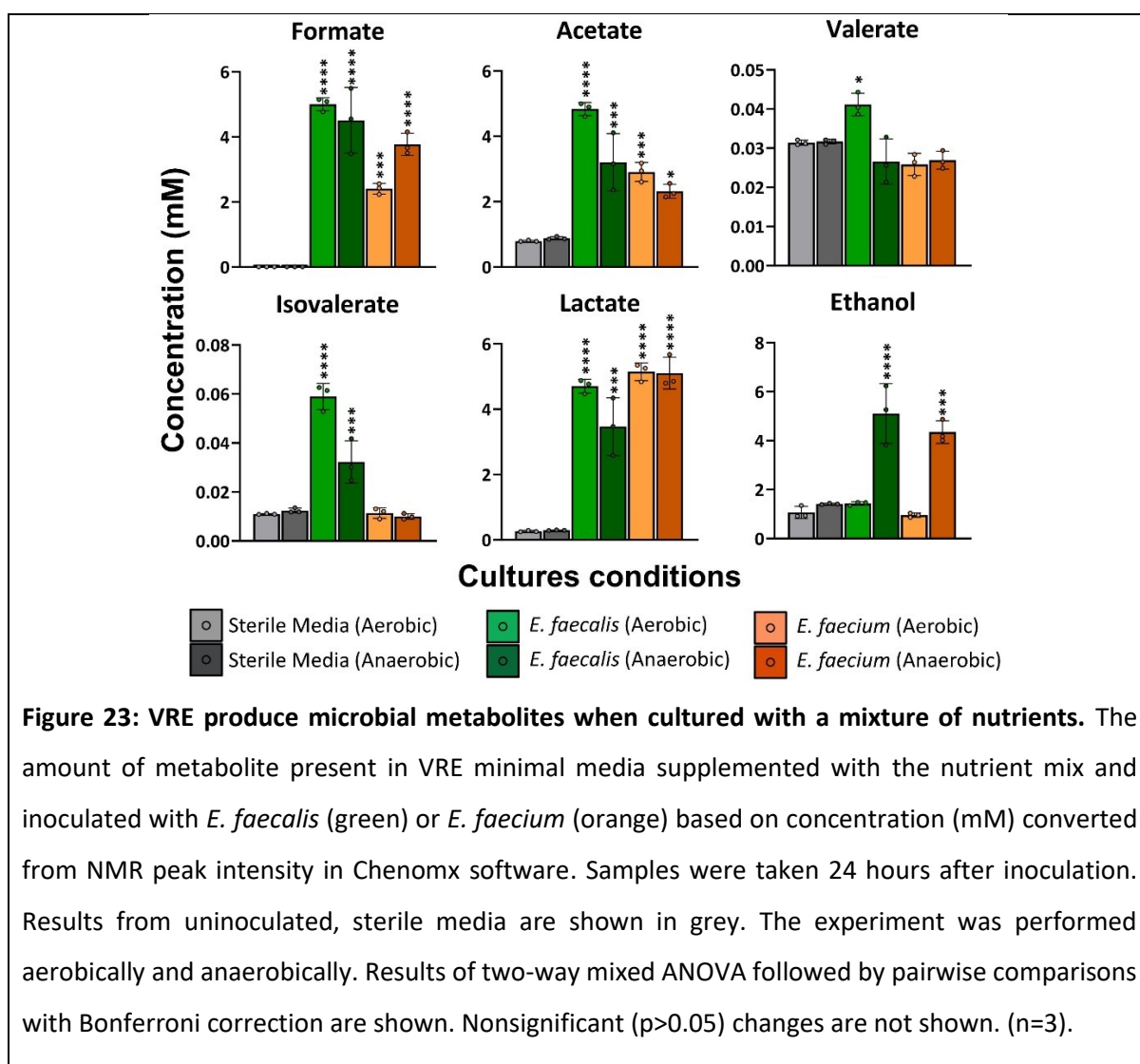
To determine which of the individual metabolites might be responsible for the changes in growth following treatment with the metabolite mix, the individual metabolites were tested at all three concentrations (low, average, and high)(**Table 2**). The pH for the individual metabolite test was adjusted to 6.0 as this was the only condition that resulted in a decrease in growth for both strains.

Individually, none of the metabolites caused a significant decrease in the growth of *E. faecium* or *E. faecalis* (**Figure 22**). Further, the concentration did not appear to consistently influence the results for either strain for any of the individual metabolites. Acetate, propionate, butyrate, valerate, isobutyrate and isovalerate increased the growth of *E. faecalis*. *E. faecium* growth was increased by lactate, 5-aminovalerate and ethanol.

To understand why these metabolites do not inhibit the VRE strains, the concentrations of the metabolites was measured when the strains were grown in the nutrient mixture. *E. faecalis* was shown to produce formate, acetate, valerate, isovalerate, lactate and ethanol (**Figure 23**). In anaerobic growth conditions, significantly more ethanol was produced compared to in aerobic growth conditions. *E. faecium* was shown to produce formate, acetate, lactate and ethanol. Like with *E. faecalis*, ethanol was produced more in anaerobic than aerobic samples. No statistically significant change was noted to valerate or isovalerate levels in *E. faecium* inoculated cultures. Isobutyrate concentrations decreased slightly in *E. faecium* and *E. faecalis* inoculated cultures compared to sterile cultures over the 24 hour

incubation. No statistically significant changes were seen for butyrate over the course of the incubation period. Propionate and 5-aminovalerate were not detected in the $^1\text{H-NMR}$ spectra for any sample.





Discussion

As demonstrated in Chapter 3, antibiotics that promote VRE intestinal colonisation depleted the diversity of key commensal members of the microbiota, resulting in increased concentrations of nutrients and decreased concentrations of microbial metabolites in a human faecal culture. Nutrient competition and metabolite inhibition are two potential mechanisms of colonisation resistance that may inhibit VRE growth. However, we need to pinpoint which nutrients are used by the two VRE strains in this study and whether the metabolites can inhibit the strains' growth to confirm the roles of nutrient competition and metabolite inhibition in restricting VRE growth in a healthy gut microbiota. Identification of

the nutrients utilised by *E. faecium* and *E. faecalis* and the metabolites that inhibit the strains will allow for the rational design of a microbiome therapeutics that can prevent or restrict VRE intestinal colonisation.

This chapter demonstrated novel insights into the roles of nutrient competition and metabolite inhibition in colonisation resistance against VRE. The data from this chapter provides a distinctively comprehensive examination of the VRE strains' role as both a primary and secondary degrader of nutrients including monosaccharides, disaccharides, polysaccharides, proteins/polypeptides, and amino acids which were shown to be elevated in the gut microbiome by antibiotic treatments (Chapter 3). New insights into the auxotrophies of VRE nitrogen requirements were shown to be a fundamental element of VRE growth. We showed that aerobic conditions generally encouraged higher growth indicating that antibiotic-associated increases in oxygen in the gut could facilitate VRE colonisation. Using an innovative growth phase based metabolomic assay, a preference between nutrients was demonstrated for both carbon and nitrogen sources for both strains. This indicates the potential for the VRE to adapt its nutrient consumption based on nutrient availability. Finally, while microbial metabolites were seen to inhibit VRE growth, the inhibition was pH dependent creating complications in exploring metabolite inhibition as a clinical avenue for VRE suppression.

To determine which nutrients support the VRE strains' growth, a minimal media was designed which lacked a growth-supporting carbon source, allowing for supplementation of each carbon source compared to a non-supplemented negative control. Several similar VRE minimal medias have been discussed in literature [549-551]. Previously developed media were modified to create a minimal media which yielded no background growth for the two strains unless supplemented with a nitrogen or carbon source.

To initially establish if the nutrients elevated by antibiotic treatment would support the two strains' growth, all 29 nutrients (amino acids, monosaccharides, disaccharides, succinate and uracil) seen to increase with antibiotic treatment in Chapter 3 were supplemented into minimal media in both aerobic and anaerobic conditions. The mix was able to support both *E. faecalis* and *E. faecium*. For both strains, the aerobic conditions resulted in higher OD₆₀₀ readings than when the cultures were grown anaerobically. This indicates that antibiotic-driven disruption of the nutrient competition is a contributing factor in the colonisation of the gut by VRE. Nutrient competition restricts pathogen growth by depleting nutrients that the pathogen requires to grow. One study identified that the depletion of fructose availability by the microbiome, suppressed the colonisation of VR-*E. faecium* in a murine model [456]. Further, due to functional redundancy in the gut microbiome, if one species of bacteria were to be eliminated from the microbiota by antibiotics, species with adjacent niches could fill the gap and suppress an opportunity for infection [14-16, 263, 382-384]. However, because broad spectrum antibiotics (like the five used in this study) kill so many types of bacteria, the built-in overlaps in colonisation resistance—namely nutrient competition—become leaky and nutrients increase in concentration. This is taken advantage of by opportunistic pathogens such as VRE.

Further, dysregulation of the biodiversity of the gut microbiota from disease or antibiotic usage leads to increases in oxygen in the microbiome [329-332]. VRE strains were shown to grow to higher levels in the presence of oxygen. Moreover, more than 99% of the gut commensals are obligate anaerobes, meaning that their growth would be suppressed by increased oxygen availability [264, 327, 328]. Therefore, an increase in oxygen availability in the gut following antibiotic treatment may further promote VRE intestinal colonisation and

growth. The favourable exposure to oxygen paired with increased nutrient availability and decreased competition with commensal organisms could prove to be the catalyst for VRE colonisation. Aerobic expansion in the gut has been shown to promote *S. ser. Typhimurium* and CRE [362, 552]. The data from this study showed that the VRE strains grew on nutrients elevated by antibiotic treatment, and were further promoted by oxygen expansion, thus supporting hypotheses regarding similar opportunistic pathogens.

Next, the individual impact of each nutrient on *E. faecium* and *E. faecalis* growth was assessed to determine which specific nutrients that were increased with antibiotics were capable of supporting VRE growth. Although *E. faecium* and *E. faecalis* grew to higher levels aerobically compared to anaerobically, both strains were able to utilise the same types of nutrients. In the few cases in which a nutrient was used in one condition but not the other, the nutrient yielded very low OD₆₀₀ readings in the condition which showed statistically significant growth. This is in contrast to CRE, where more nutrients were used aerobically compared to anaerobically [30]. For pathogens which show a dramatic increase in nutrient utilisation aerobically, there could be restriction of colonisation if oxygen is not increased in abundance by a specific antibiotic or if the oxygen returns to pre-treatment levels. The similarity in nutrients used aerobically compared to anaerobically could mean its growth is less susceptible to being impacted as oxygen levels fluctuate in the gut.

Both VRE strains used a variety of monosaccharides and disaccharides as carbon sources. In total, *E. faecalis* used nine simple carbon sources aerobically and 11 sources anaerobically. *E. faecium* used 10 simple carbon sources aerobically and anaerobically with nine in common between the growth conditions. While most simple carbon sources were used by both strains, the major differences were seen in the consumption of the monosaccharide arabinose and

the disaccharide sucrose. Similarities between the carbon usage of these two strains could indicate conserved nutrient preferences for other VRE strains. Further investigation involving more VRE strains would be needed to reach any conclusions.

Nitrogen sources were similarly assessed for utilisation by VRE strains. *E. faecium* and *E. faecalis* strains used in this study had undefined amino acid auxotrophies, therefore a leave-one-out method was used. Both *E. faecalis* and *E. faecium* used a variety of amino acids as nitrogen sources.

Several VRE minimal media—including the minimal media used in this research—required a collection of amino acids in order to facilitate growth [549-551]. The findings from this study showed that no single nitrogen source was sufficient to support the growth of the VRE strains, thus supporting previously published conclusions.

Evaluating the growth rate of the VRE strains on the different nutrients could indicate how increased availability of certain nutrients would impact the nutrient competition. Rapid acceleration of the growth rate of both strains on nutrients such as glucose and N-acetylglucosamine could allow the strains to deplete the nutrients before a commensal in a direct competition assay. Thus, in designing a microbiome therapeutic based on nutrient competition, the growth rates of both the VRE and the commensal species will have to be assessed based on the targeted nutrients.

Additional complex nutrients (which are not identifiable with $^1\text{H-NMR}$) could have had decreased utilisation during antibiotic-treatment. Therefore, complex substrates found in the human gut were evaluated as potential nutrients utilised by VRE. Complex nutrients are broken down in the gut by primary degraders (including *Bacteroides* and *Akkermansia* species) which digest the complex nutrients sources into their monomer components [501,

502]. The simple components can then be consumed by secondary degraders (such as *Bifidobacterium* and *Collinsella* species) [503-506]. The data showed that the VRE strains can act as primary degraders of complex nutrients. Thus these sources, as well as the simple sources, could support the survival of VRE in an antibiotic-treated gut microbiome.

E. faecalis was shown to consume eight complex nutrients and *E. faecium* was shown to consume five complex nutrients. The digestion of these nutrients involves breaking down the complex sources into monomer subunits; arabinogalactan (arabinose, galactose)[484, 485], starch (glucose, maltose, trehalose)[495-497], xylan (arabinose, xylose)[30], bile salts (organic acids such as deoxycholic and lithocholic acid)[368, 369], and yeast (amino acids, ribose, uracil) [30] were all shown to support the growth of both strains. The digestion of these complex nutrients further frees up many simpler carbon sources which were shown to be utilised by the VRE strains. Neither strain was able to utilise pectin (arabinose, fucose, galactose, glucose, mannose, xylose) [492, 493], casein or peptone as a carbon source. Both casein and peptone break down into polypeptides and then amino acids in the gut [486, 487, 494]. As the VRE strains are not able to utilise most amino acids as sole carbon sources, this explains why the digestion of these complex substrates would not support high (or any) growth.

The ability to utilise a nutrient when it is the sole option does not necessarily mean that the nutrient will be used by the VRE strains when an abundance of other nutrients are available; previous studies have demonstrated that the presence of some nutrients can influence the utilisation of others [30, 546, 553]. In order to determine which nutrients were preferred by the strains, an experiment was run to test the levels of the nutrients during mid-exponential, early stationary, and late stationary phase of VRE inoculated cultures. The order and degree

to which nutrients were consumed indicated nutritional preference [30]. It was seen that, by mid-exponential phase, both strains had significantly (if not entirely) depleted the levels of several monosaccharides and disaccharides. Both strains consumed all the arginine in the media by mid-exponential phase, indicating that this was the preferred nitrogen source.

As a sole carbon source, sucrose only supported the growth of *E. faecalis*. The monomers of sucrose are glucose and fructose—both of which independently support the growth of the VRE strains as a sole carbon source. It is possible, since (despite the results indicating the sucrose cannot support the growth of *E. faecium* as a sole carbon source) sucrose was seen to decrease in *E. faecium* inoculated nutrient preference samples, that the disaccharide can be broken down by *E. faecium* if other nutrients support the strains growth simultaneously. Previous research has shown similar conditional usage of nutrients for VRE (specifically concerning the consumption of fructose) and CRE [30, 456].

While neither strain showed an ability to use tyrosine as a carbon or nitrogen source, the amino acid was nearly completely depleted by the end of the incubation period for both strains. *E. faecalis* was not noted to utilise aspartate or phenylalanine as a carbon or nitrogen source to a statistically significant degree yet both nutrients were depleted by late stationary phase when the culture was incubated anaerobically. As all nitrogen source utilisation was assessed with glucose as the carbon source, it is possible that with other carbon sources available, other nitrogen sources can be used.

The utilisation and preference for certain nutrients is essential knowledge if the increased concentration of nutrients following antibiotics is exploited by VRE during colonisation. These preferred nutrients could serve as targets for the reestablishment of colonisation resistance through nutrient competition. If commensal strains can be identified which consume these

nutrients more readily than the VRE, a targeted microbiome therapeutic could be designed to reestablish nutrient competition and, thus, colonisation resistance. This method has been shown to successfully suppress the growth of other pathogens including *E. coli* [385-387] and *S. ser. Typhimurium* [388, 389] by identifying commensal organisms which outcompeted the pathogens for essential nutrients (proline and sugar in the case of *E. coli*, zinc and iron in the case of *S. ser. Typhimurium*). While VRE's diverse nutrient utilisation abilities could make this approach difficult, knowing which nutrients VRE strains use first creates a hierarchy of nutrients to target.

While antibiotic treatment of faecal culture in Chapter 3 led to an increase in nutrient availability, it caused a decrease in the concentration of microbial metabolites such as SCFAs, BCFAs, alcohols and organic acids. These metabolites have been shown to inhibit pathogens including, CRE [30], *E. coli* [391, 402], *C. difficile* [403], *S. aureus* [366] and *C. jejuni* [507]. Metabolites produced by commensal bacteria have been shown to inhibit the growth of pathogens by decreasing both the environmental pH of the gut and the intracellular pH of pathogens [30, 471].

To replicate the conditions of the distal gut, the pH of the metabolite mix was adjusted to 6.0, 6.5, or 7.0. The pH of the distal gut ranges between 6.0-7.0 in healthy individuals [30, 324, 547, 548, 554]. Variation in the pH of a healthy gut can be due to microbiota diversity and density, diet, age and medication [555]. Antibiotic treatment, gastrointestinal inflammation, colorectal cancer and infection can increase the pH of an unhealthy gut as well [556]. Testing the microbial metabolite mix at varying pHs allowed for a better idea of how the VRE strains' would react in different circumstances.

The low and average concentrations of the metabolite mix caused a statistically significant increase in growth compared to untreated cultures for both strains at all pHs indicating that, contrary to expected, these concentrations promoted rather than inhibited VRE growth. The impact of the high concentration metabolite mix was pH dependent, however, only when the mix was adjusted to a pH of 6.0 was inhibition seen for both strains. Similar pH dependent VRE suppression by SCFAs was recently reported by Odenwald et. al [557]. The study found a reduction from pH of 7.0 to 5.5 increased the susceptibility of VRE to acetate (pKa of 4.76), butyrate (pKa of 4.84) and propionate (pKa of 4.87). When individual metabolites were tested at all three concentrations, none were able to inhibit the growth of VRE strains to a significant degree. Prior research conducted for this study showed that, although VRE inhibition by faecal transplant or commensal competition has been achieved in various models [376, 558, 559], metabolite inhibition by SCFAs and BCFAs on their own has never been shown to be a viable target for VRE decolonisation. When considering the clinical viability of metabolite inhibition for restoring colonisation resistance, the pH dependence of this mechanism would prove a challenge to inhibit VRE in a gut with the antibiotic-elevated pH conditions associated with target recipients. Data showed promotion of VRE at higher pHs; If metabolites were administered as a therapeutic to an individual whose gut experienced antibiotic-elevated pH conditions, the treatment could serve to promote rather than inhibit the VRE. Thus, in order to optimise the inhibitory potential of a microbial metabolite mixture, the acidic pH of the gut would have to first be restored. Further, if a microbiome therapeutic was designed to reintroduce commensals capable of restoring nutrient competition, microbial metabolite concentrations would likewise increase since nutrient consumption and metabolite production are connected through bacteria metabolism.

In an effort to better understand why the metabolites were promoting VRE growth, the metabolites were individually supplemented into minimal media to determine if they were used as nutrient sources for the strains. No statistically significant promotion of growth was seen and thus the data from this experiment was not shown. To test if the strains produce these metabolites, ¹H-NMR spectroscopy data from the nutrient preference experiment was reviewed for changes to the metabolite concentrations. It was seen that formate, acetate, ethanol, and lactate were all produced by both of the VRE strains as products of their consumption of sugars. Valerate and isovalerate were produced by *E. faecalis* but not *E. faecium*. Previous research has shown that *E. faecalis* but not *E. faecium* was able to produce BCFAs from acetyl-CoAs due to the presence of *ptb* and *buk* genes [560, 561].

As the 10 metabolites tested in this chapter do not appear to consistently negatively impact the VRE strains' growth, it is unlikely that SCFAs or BCFAs provide colonisation resistance against VRE. It is possible that, when combined with other growth challenges (such as elevated nutrient competition) in a more physiologically relevant environment, inhibition by SCFAs, BCFAs, alcohols and organic acids is more effective. Further, other colonisation resistance mechanisms such as bile acid inhibition by lithocholic acid have been shown to inhibit VRE colonisation [562]. Evaluating the impact of other metabolic biproducts—individually or as combination therapies—could provide more promising avenues for VRE inhibition.

Based on the results of nutrient utilisation capabilities of the VRE strains, targeting the reestablishment of nutrient competition may prove an effective mechanism for decreasing VRE growth following antibiotic treatment. A collection of commensals which consume key nutrients required for VRE growth at a rate equal or greater to that of the VRE strains could

be given as a microbiome therapeutic to starve VRE strains and reestablish colonisation resistance. As the VRE strains have been shown to consume a wide range of both simple and complex nutrients, likely several commensals from different families will be needed to cover all of the nutrients utilised by VRE.

This study was limited by the number of VRE strains tested. Further research incorporating more strains associated with intestinal colonisation could validate the results of this study and contextualise the behaviour of these two strains in wider VRE-associated behaviours. The expansion of this research beyond these strains is ongoing for the McDonald lab.

Additionally, the use of ^1H -NMR spectroscopy to measure nutrient and metabolite abundance has limitations in what can be identified. The abundances of complex substrates can be unreliable. An ongoing collaboration with the National Phenome Centre aims to develop a mass spectrometry based assay to measure these polysaccharides. Initial results of polysaccharide analysis with ^1H -NMR spectroscopy showed that the complex substrates contained contamination by monosaccharides, disaccharides and amino acids. This may have impacted the growth of the VRE strains on these nutrient sources. Further evaluation of substrate purity through ^1H -NMR spectroscopy and developed mass spectrometry techniques could improve analysis.

Chapter 5: Vancomycin-resistant *E. faecium* colonisation dynamics in response to antibiotic treatment and faecal microbiome transplant

Introduction

Broad-spectrum antibiotic treatment leads to the killing of members of the healthy gut microbiota. The decrease in diversity and density of the gut commensals causes an increase in nutrient availability (due to reduced nutrient competition) and a decrease in the production of inhibitory metabolites (due to killing of gut commensals that produce these metabolites). The altered nutrient- and metabolite-defined niches that are created in the intestine following antibiotic treatment can be exploited by opportunistic pathogens like VRE to grow. Understanding how VRE can colonise an antibiotic-treated gut while failing to proliferate in an antibiotic-naïve gut can help inform the design of targeted, non-antibiotic therapeutics against VRE colonisation.

Many *in vivo* and *in vitro* models have been designed to study the impact of perturbations on the susceptibility to microbial colonisation within the gut [403, 442, 444, 563-572]. *In vivo* models show the impact of an experimental variable on colonisation within the context of the host's immune system. This, however, can be a disadvantage if specific microbe-to-microbe or microbe-to-substrate interactions are being targeted. Confounding variables, including indirect mechanisms of colonisation resistance, host related immune activity, and individual variables such as age, sex, BMI, and diet, could skew findings regarding the gut commensal microbiota's role in inhibiting pathogenic colonisation [573, 574]. Due to the lack of host interference, *in vitro* models offer clearer assessments of changes within and to the microbiota.

Evaluating VRE colonisation in a physiologically relevant manner requires a longitudinal model capable of maintaining relevant conditions over an extended time. Within the gut, the pH,

temperature, fluid shear, and oxygen levels are maintained by both host and microbiota mediated mechanisms. Further, the movement of fresh nutrients and the removal of waste products are regulated through digestion. While short term models, such as faecal batch cultures, can provide valuable insights into the immediate impact of a treatment, nutrient depletion leads to waste product build up which causes the pH of the culture to shift away from the baseline. If the culture is left to incubate for longer than the capacity of the model, the impact of the treatment can become distorted by uncontrolled variables.

Artificial gut models have been used when modelling the impact of antibiotics, colonisation, and FMT treatments on faecal microbiota cultures [376, 403, 444, 454, 575]. This model allows cultures to be maintained at physiologically relevant conditions for as long as the experimental design requires. By running the artificial gut chemostat model with multiple vessels concurrently, inoculated with the same faecal sample, control cultures are run parallel to experimental cultures. High reproducibility has been shown for cultures inoculated with the same faecal sample ensuring that variable comparisons are controlled [403]. This model has been previously used to study colonisation by VRE [376] and *C. difficile* [403, 483, 576, 577].

The experiments in this chapter aimed to link the data from the previous two chapters to determine which antibiotic-driven changes in faecal cultures are exploited by VRE during colonisation. Using both an artificial gut chemostat model and a murine model, the taxonomic and metabonomic characteristics of an antibiotic-perturbed gut leading up to and during VRE colonisation were modelled. This information could inform the development of a targeted non-antibiotic treatment for VRE through the reestablishment of colonisation resistance in the gut microbiome.

Results

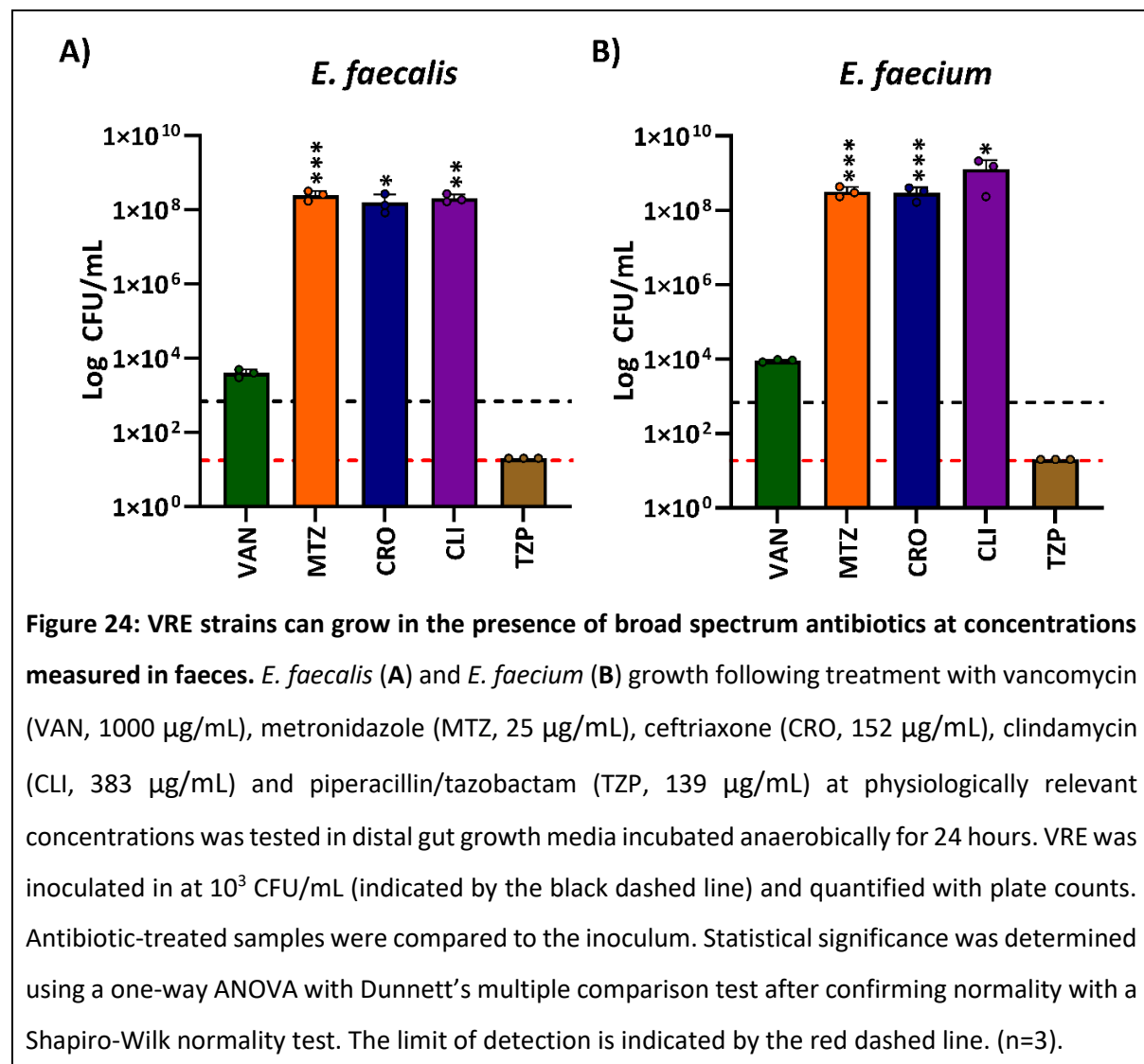
In order to select which antibiotic would be used in the longitudinal modelling of VRE colonisation in an antibiotic-perturbed gut microbiome, the susceptibility of the VRE strains to each antibiotic had to be tested. Initial MIC assays were run in accordance with EUCAST standards. The experiment, performed in triplicate, provided the resistance breakpoints for each of the five antibiotics (**Table 5**). Both strains were resistant to all five antibiotics tested though there was variability to the MICs for each treatment. Both strains were resistant to MTZ up to 64 mg/L. *E. faecalis* (NCTC 12201) demonstrated resistance to higher concentrations of VAN (128 mg/L), CRO (1024 mg/L), and CLI (>8192 mg/L) than *E. faecium* (4 mg/L for VAN and CRO, 2048 mg/L for CLI). Conversely, *E. faecium* was resistant to TZP (32 mg/L) at a concentration 8x higher than *E. faecalis* (4 mg/L).

Table 5: VRE strains exhibit resistance to various broad spectrum antibiotics known to promote VRE intestinal colonisation. Minimum inhibitory concentration assays were performed with the broth microdilution technique on the VRE strains using vancomycin (VAN), metronidazole (MTZ), ceftriaxone (CRO), piperacillin/tazobactam (TZP) and clindamycin (CLI). The MICs were conducted aerobically in Mueller-Hinton broth following EUCAST standards. The breakpoint is listed (mg/L) for each strain (n=3).

Strain	VAN mg/L	MTZ mg/L	CRO mg/L	TZP mg/L	CLI mg/L
<i>E. faecalis</i> NCTC 12201	128 (resistant)	64 (resistant)	>128 (resistant)	4 (resistant)	>128 (resistant)
<i>E. faecium</i> NCTC 12202	4 (resistant)	64 (resistant)	4 (resistant)	32 (resistant)	>128 (resistant)

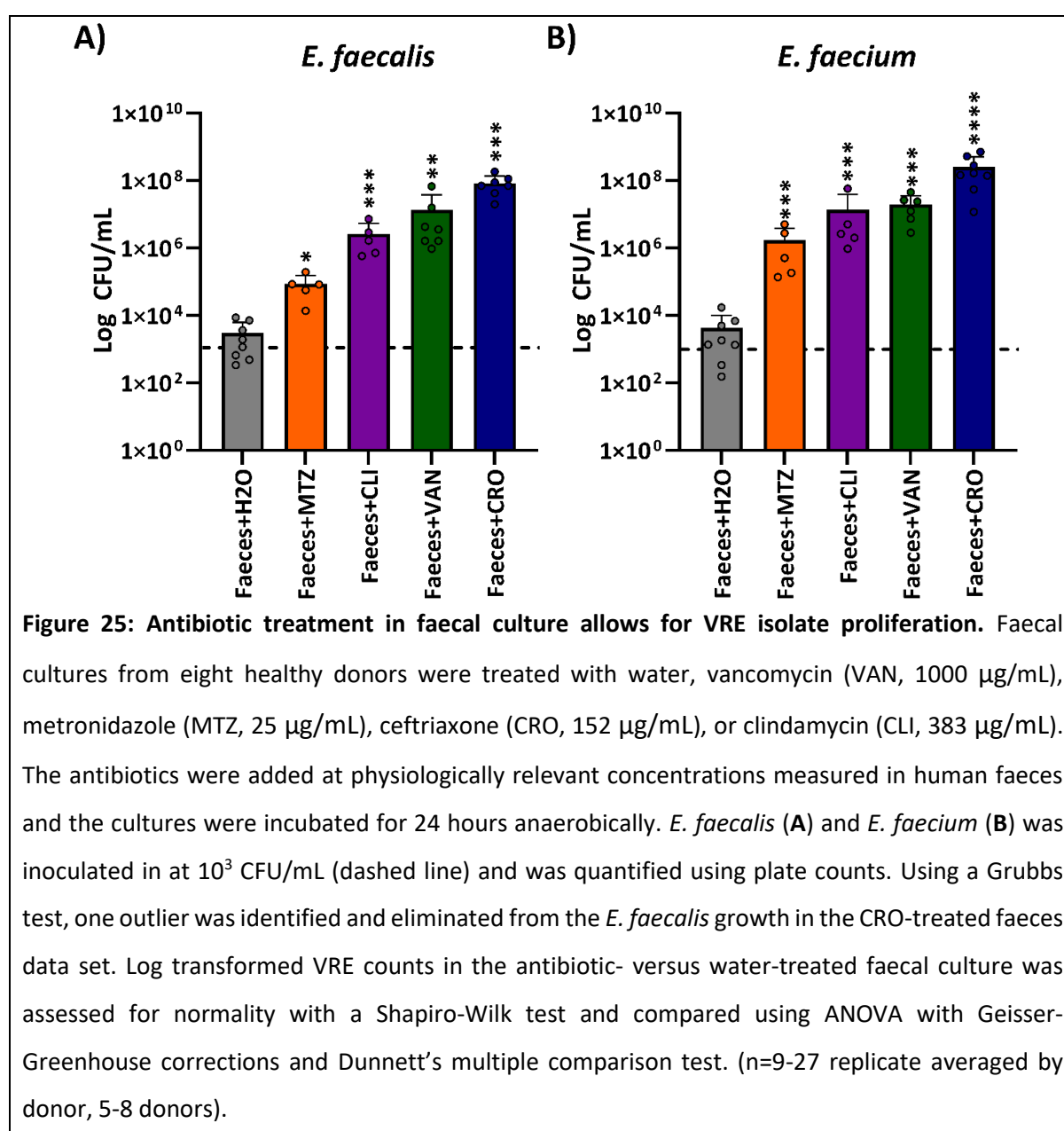
Since the impact of antibiotic treatment can vary based on the media used, a further resistance assay was performed in DGGM used in both the faecal batch cultures and the artificial gut model, under anaerobic conditions with the antibiotics concentrations set at that which are found in faeces [68, 201, 202, 205, 206, 209, 210, 450]. Plate counts were used to

quantify the growth of the strains following incubation with the antibiotics. For *E. faecalis* (Figure 24A) and *E. faecium* (Figure 24B), treatment with VAN resulted in statistically nonsignificant growth above the inoculum ($p>0.9999$). For *E. faecalis*, growth above 10^8 CFU was seen in media treated with MTZ ($p=0.0008$), CRO ($p=0.0296$), and CLI ($p=0.0047$). Likewise, statistically significant growth was seen for *E. faecium* when treated with MTZ ($p=0.0001$), CRO ($p=0.0003$), and CLI ($p=0.0107$) when compared to the inoculating concentration. TZP reduced both *E. faecalis* and *E. faecium* to below the limit of detection when the cultures were plated ($p>0.9999$).



Antibiotic treatment allows VRE exposure to become colonisation

As all five antibiotics used in this study are associated with increased risk of subsequent VRE colonisation [55, 61, 64, 67, 68, 70, 71, 75, 183, 201-210], the growth in faecal cultures treated with antibiotics was tested. The batch cultures were only performed with four of the five antibiotics as TZP completely inhibited the VRE strains. The growth of the VRE strains in antibiotic-treated faecal cultures was compared to growth in water-treated faecal cultures (Figure 25).



VRE strains grown in water-treated faecal culture were not significantly different to the inoculum when compared using an unpaired t-test indicating the antibiotic-naïve faeces suppressed VRE growth. Treatment of the faecal cultures with all four antibiotics promoted the growth of both VRE strains compared to water-treated faecal cultures. Faecal cultures treated with CRO had the highest growth of *E. faecalis* ($p<0.0001$) and *E. faecium* ($p<0.0001$). VAN-treated faecal cultures resulted in the second highest growth of *E. faecalis* ($p=0.0002$) and *E. faecium* ($p=0.0003$), followed by CLI-treated faecal cultures (*E. faecalis* $p<0.0001$, *E. faecium* $p=0.0009$). MTZ-treated faecal cultures yielded the lowest growth for both *E. faecalis* ($p=0.0091$) and *E. faecium* ($p=0.0005$).

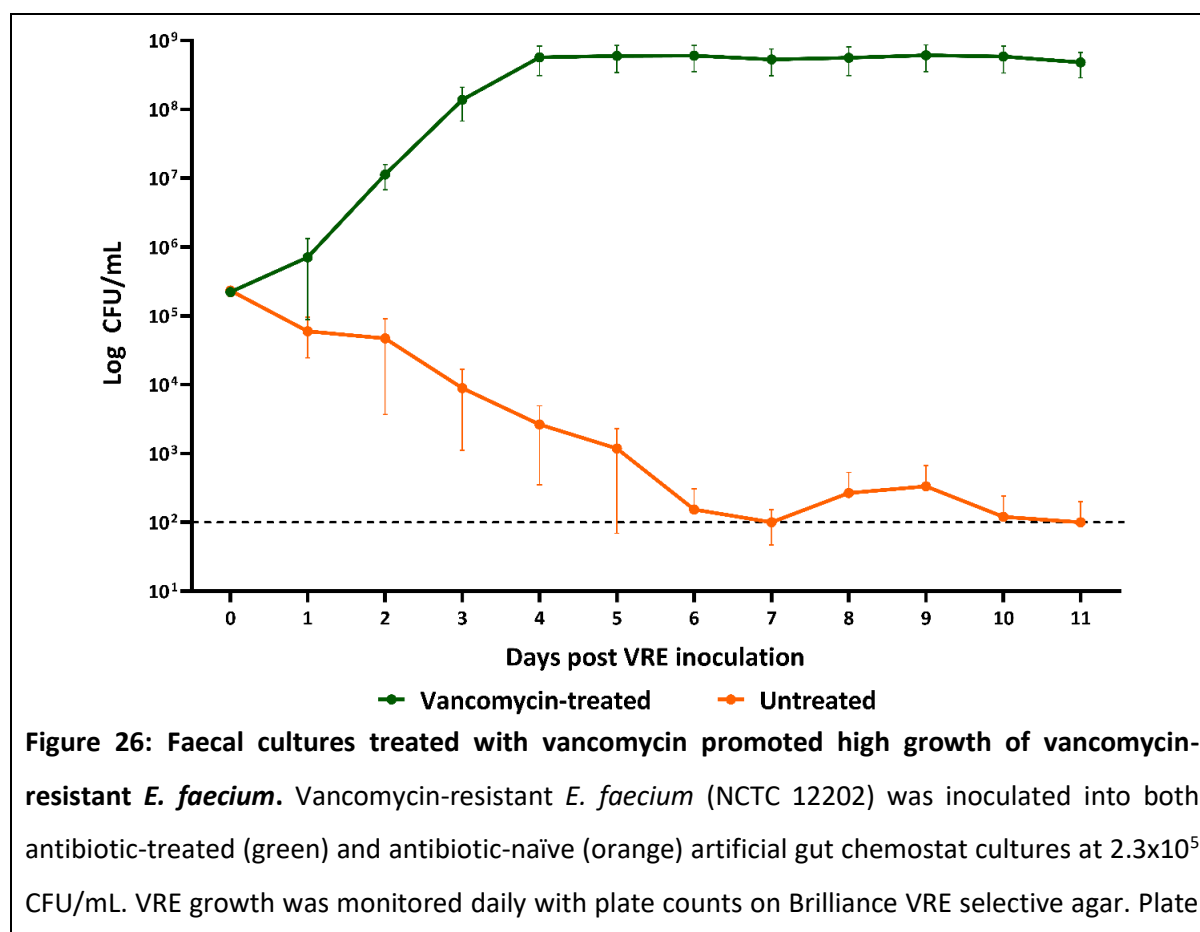
Antibiotic treatment increases nutrient and decreases metabolite concentrations leading to *E. faecium* persistence in artificial gut chemostat models

To investigate the impact of antibiotic treatment on VRE susceptibility in a longitudinal model which more accurately represents the environment of the human gut, artificial gut chemostat model experiments were performed. VAN was selected to test in the artificial gut model because it has been shown to promote VRE intestinal colonisation in both human and murine studies [55, 61, 64, 67, 68, 70, 71, 75, 201-203]. Additionally, the overall effects of VAN treatment on the taxonomic and metabolomic profiles of the gut microbiota was representative of other antibiotics tested (see **Figure 7**, **Figure 11**, and **Figure 13** from Chapter 3). These faecal batch culture experiments showed that VAN treatment caused an increase in nutrient concentration and a decrease in the production of microbial metabolites correlated with decreased diversity and density of the gut microbiota. These experiments were limited in their duration, thus whether these trends were maintained overtime was to be confirmed by the artificial gut chemostat model. To determine what changes following VAN treatment

allowed VRE to proliferate in the artificial gut model, the cultures were analysed with ^1H -NMR spectroscopy and 16S rRNA gene sequencing.

E. faecium was selected for the murine and artificial gut chemostat experiments due to the increasing clinical threat of the species compared to *E. faecalis* [5, 578]. *E. faecium* has also been shown to have greater antibiotic resistance than *E. faecalis*, increasing the difficulty in treating infections in a clinical setting [64, 109-111].

VAN was administered to vessel A and vessel B for 20 days (days 21-40 of the experiment). Vessel C was not treated with VAN (to mimic a healthy gut microbiome). On day 31 (10 days into the vancomycin treatment period) vancomycin-resistant *E. faecium* was inoculated into all three vessels at 10^6 CFU/mL. **Figure 26** shows the daily growth of VRE over three experiments.



counts from vancomycin-treated faecal cultures were averaged from both vessels A and B for all three experiments (n=6 vessels from 3 experiments) and plate counts from antibiotic-naïve faecal cultures were averaged from vessel C only for all three experiments. The limit of detection is shown as a black dashed line. (n=3 vessels from 3 donors).

In the VAN-treated cultures, *E. faecium* steadily grew to 10^9 CFU/mL within four days of inoculation. This trend was seen in all six cultures treated with antibiotics. In the untreated cultures, the VRE decreased to approximately 10^2 CFU/mL. In the first experiment, VRE counts decreased below the limit of detection within two days of inoculation. It took seven days for the VRE to become undetectable in the second experiment and 14 days in the third experiment. Using Short Asynchronous Time-series Analysis (SANTA), the *E. faecium* plate counts from the days where the growth plateaued were compared for the VAN-treated cultures and untreated cultures. The comparison yielded a statistically significant p value of 0.0459.

To account for the difference in VRE growth in the antibiotic-treated vessels compared to the untreated vessels, the metataxonomic and metabonomic profiles of the vessels was assessed using 16S rRNA gene sequencing and ^1H -NMR spectroscopy, respectively. SANTA was used to compare the peak intensity data derived from ^1H -NMR spectroscopy from vessels A and B before and after the initiation of VAN-treatment (**Figure 27**).

The comparison showed that acetate (p=0.014), propionate (p=0.009), butyrate (p=0.01), valerate (p=0.009), isobutyrate (p=0.009), and isovalerate (p=0.01) significantly decreased following antibiotic treatment. 5-aminovalerate (p=0.014) increased with antibiotic treatment. Formate, ethanol, and lactate fluctuated in each vessel overtime and were thus not significantly altered by antibiotic treatment.

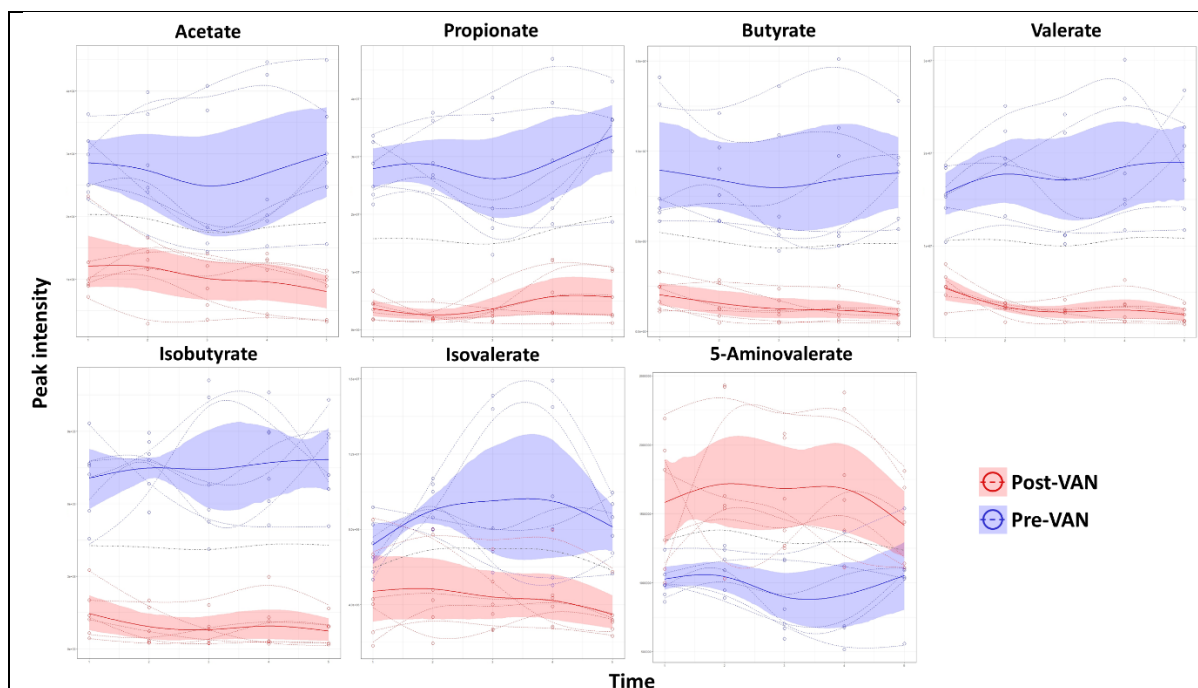


Figure 27: Microbial metabolites decreased in antibiotic-treated faecal cultures grown in an artificial gut model. Metabolite concentrations prior to (day 13-21)(blue) and during (day 23-31)(red) vancomycin (VAN) treatment of the artificial gut cultures were analysed using SANTA analysis with Benjamini-Hochberg (BH) FDR. The generated curves show the mean of individual replicates as a solid line and the range of the replicates as a shaded band. Propionate, valerate and isobutyrate had a p value of 0.009, butyrate and isovalerate had a p value of 0.010, and acetate and 5-aminovalerate had a p value of 0.014. Formate ($p=0.965$), ethanol ($p=0.085$), and lactate ($p=0.164$) were not seen to change. ($n=6$ vessels from 3 independent experiments per group).

Nutrients increased following antibiotic treatment (**Figure 28**). The monosaccharides arabinose ($p=0.009$), fructose ($p=0.009$), fucose ($p=0.009$), galactose ($p=0.009$), glucose ($p=0.029$), mannose ($p=0.009$), and xylose ($p=0.044$) increase during VAN treatment. Likewise, the disaccharides maltose ($p=0.009$), sucrose ($p=0.031$), and trehalose ($p=0.014$) as well as N-acetylglucosamine ($p=0.022$) were elevated in vessel A and B following antibiotic treatment. Amino acids, alanine ($p=0.028$), glutamate ($p=0.021$), glycine ($p=0.013$), isoleucine ($p=0.029$), leucine ($p=0.029$), lysine ($p=0.009$), methionine ($p=0.009$), phenylalanine ($p=0.009$), proline ($p=0.014$), threonine ($p=0.009$), tryptophan ($p=0.009$), tyrosine ($p=0.009$), and valine ($p=0.042$) showed increases following antibiotic treatment. Finally, succinate

($p=0.031$) and uracil ($p=0.009$) were increased in VAN-treated culture. In the antibiotic-naïve vessels, the nutrients remained stable in their concentration.



Hochberg (BH) FDR. The generated curves show the mean of individual replicates as a solid line and the range of the replicates as a shaded band. Arabinose, fructose, fucose, galactose, mannose, maltose, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and uracil ($p=0.009$), glycine ($p=0.013$), trehalose and proline ($p=0.014$), glutamate ($p=0.021$), N-acetylglucosamine ($p=0.022$), alanine ($p=0.028$), glucose, isoleucine, and leucine ($p=0.029$), succinate and sucrose ($p=0.031$), valine ($p=0.042$), and xylose ($p=0.044$) showed a statistically significant change. Nutrients which were not seen to change are not included. ($n=6$ vessels from 3 independent experiments per group).

Vancomycin treatment in artificial gut chemostat cultures decreases the diversity and density of the commensal microbiota

To view the impact of antibiotic treatment on the taxonomic profiles of the artificial gut cultures following VAN treatment, 16S rRNA gene sequencing data run with PacBio and adjusted to absolute abundance with 16S qPCR data was used to generate streamline graphs (**Figure 29**). The data shows a decrease in the total commensal biomass on day 23, shortly after the start of the antibiotic treatment period. This decrease was confirmed by performing a Wilcoxon two-way, matched-pairs, sign ranked t-test on absolute abundance of 16S gene counts from day 21 and day 23 of the VAN-treated cultures ($p=0.0312$). The microbiota recovers in density over the next several days to levels comparable to pre-antibiotic data. By day 25, the absolute abundance of 16S gene counts was no longer statistically different to the pre-VAN counts of day 21 ($p=0.0938$).

The diversity of the cultures were also impacted by the antibiotic treatment. Much like the nutrient and metabolite concentrations, the phyla level changes before and during antibiotics were assessed with SANTA. Prior to antibiotic treatment, the chemostat culture was predominantly made up of members of the Bacteroidota and Bacillota phyla. Lower levels of Pseudomonadota were seen in the vessels prior to treatment. After VAN was administered to vessel A and B, the diversity and abundance of Bacteroidota was significantly decreased

($p=0.0140$) to the point it nearly disappears from the microbiota compared to prior to treatment. At the class level, Bacteroidia was seen to significantly decrease ($p=0.0390$) in all three replicates following VAN administration. While Bacillota as a whole was not significantly decrease, the diversity was negatively impacted by antibiotics with Clostridia being the main class to significantly decrease in abundance ($p=0.0390$). Following the initial decrease in growth after antibiotic treatment, the abundance of Pseudomonadota increased. The Gammaproteobacteria class was shown to have a statistically significant increase in abundance in the three replicates ($p=0.0390$). Desulfobacterota, only seen in notable numbers in the third replicate, were increased in the population following VAN-treatment. Meanwhile, the level of Verrucomicrobiota was suppressed by VAN-treatment in the third replicate. The antibiotic-naïve vessel C culture maintained a stable microbiota without any major or lasting fluctuations. No phyla showed a statistically significant change when reviewed with SANTA. As expected, the *Enterococcus* counts were higher in the antibiotic-treated vessels relative to the antibiotic-naïve vessels.

An rCCA model was generated using the combined 16S rRNA gene sequencing data and the $^1\text{H-NMR}$ spectroscopy data to assess the correlation between bacterial genera, nutrients and metabolites before and after VAN treatment (**Figure 30**). This analysis guided the identification of gut commensals that would make strong candidates for a microbiome therapeutic based on nutrient competition. The days selected before (day 15-21) and after (day 25-31) the initiation of VAN were all prior to the inoculation of VRE to view the antibiotic-induced changes to the microbiome which create environmental conditions that promote VRE intestinal colonisation. Many bacterial genera commonly found in healthy gut microbiota—namely *Bacteroides*, *Faecalibacterium* and *Ruminococcus*—positively correlated with SCFAs

and BCFAs. Conversely, nutrients were negatively correlated with these commensal genera. Some genera, including *Escherichia-Shigella*, *Parasutterella*, and unclassified *Clostridia*, positively correlated with the nutrients and negatively correlated with the metabolites and other commensal genera.

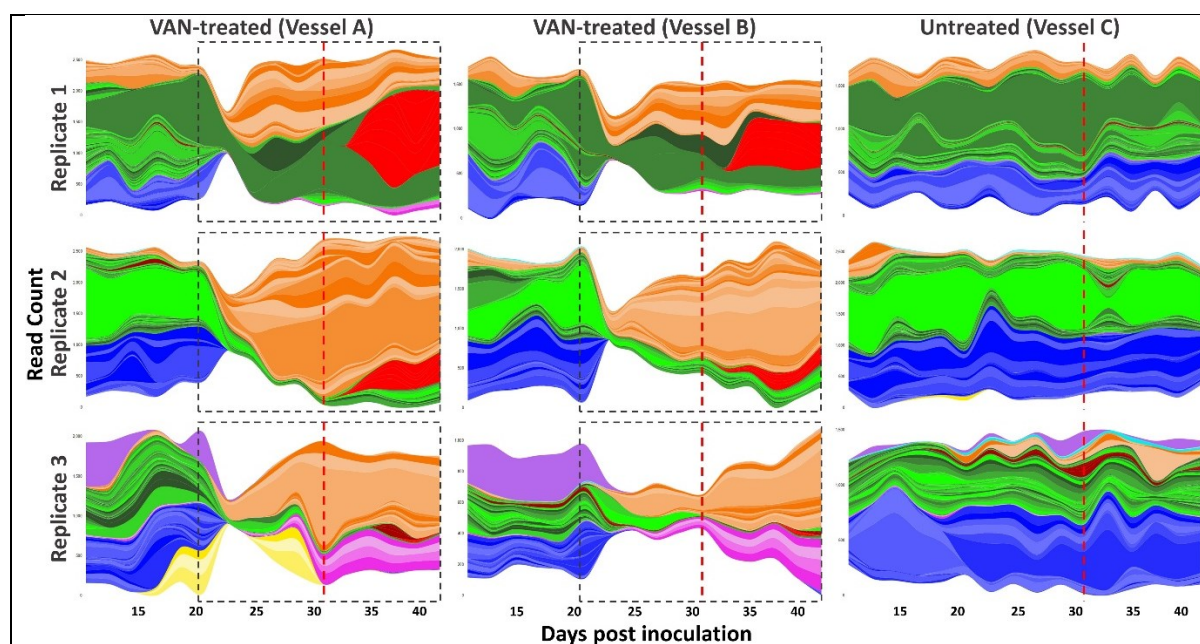
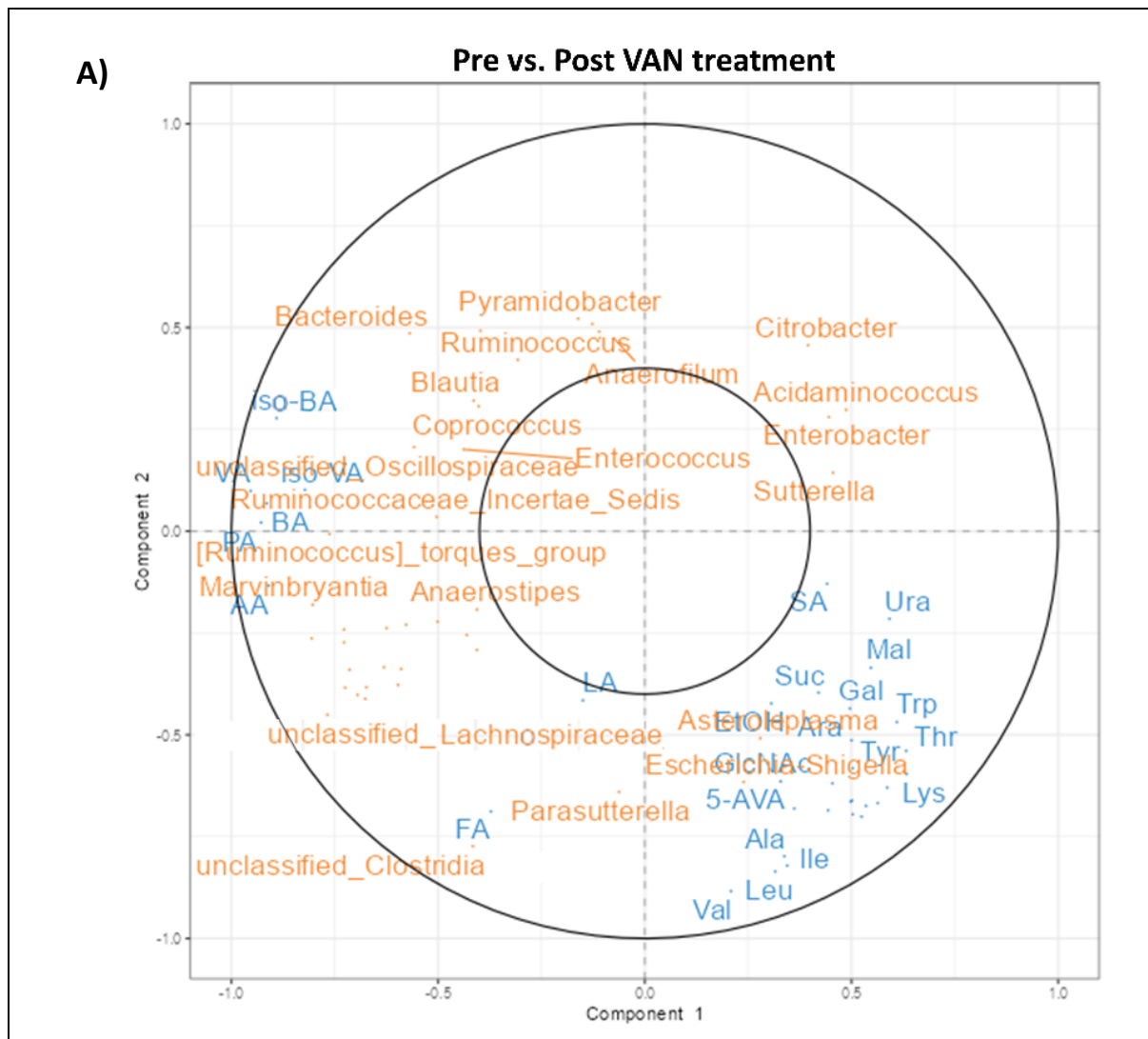
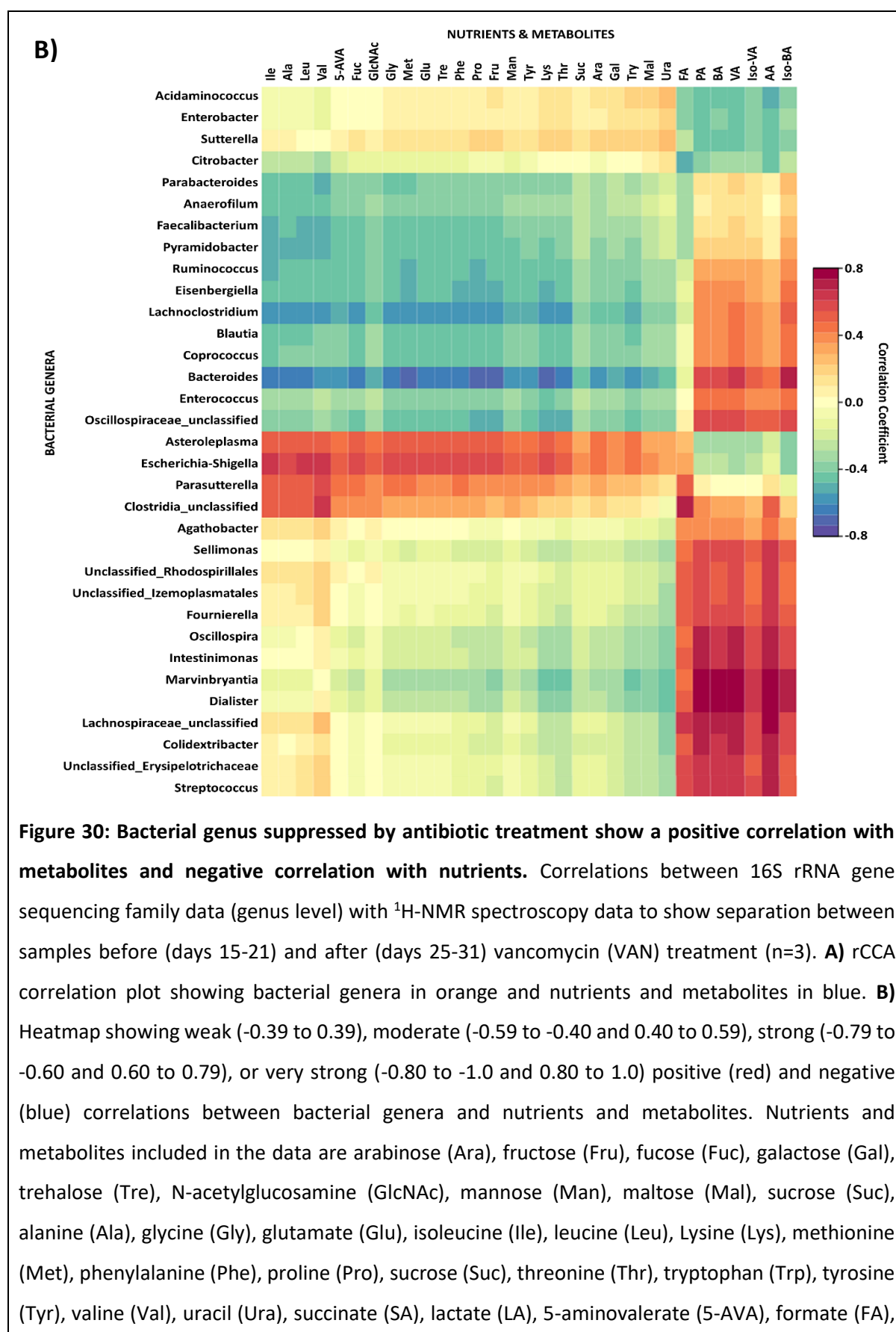


Figure 29: Vancomycin treatment in artificial gut chemostat cultures causes a decrease in the commensal microbiota. The streamgraph shows absolute ASV abundances from 16S rRNA gene sequencing corrected with 16S qPCR data for vancomycin (VAN) treated cultures (vessel A and B) or untreated cultures (vessel C) from day 11 through day 41. The vancomycin treatment days are indicated by the black dashed box. The inoculation of *E. faecium* is indicated by the red dashed line. The streams are grouped by phylum: Actinomycetota (yellow), Bacteroidota (blue), Bacillota (green), and Pseudomonadota (orange), Verrucomicrobiota (purple), Desulfobacterota (pink), Fusobacteriota (brown), Synergistota (cyan), *E. faecium* (bright red), *Enterococcus* species (dark red). The width of each stream represents the ASV abundance on that day.

Next we measured correlations between vancomycin-resistant *E. faecium* plate counts and ¹H-NMR spectroscopy peak integrations to measure correlations between *E. faecium* growth and nutrients and metabolites. Pre-VRE timepoints (days 25-31) were compared to post-VRE timepoints (days 35-41) for the VAN-treated vessels. The Spearman correlation (**Figure 31A**) showed moderate negative correlations between *E. faecium* and uracil ($p=0.0036$), glucose

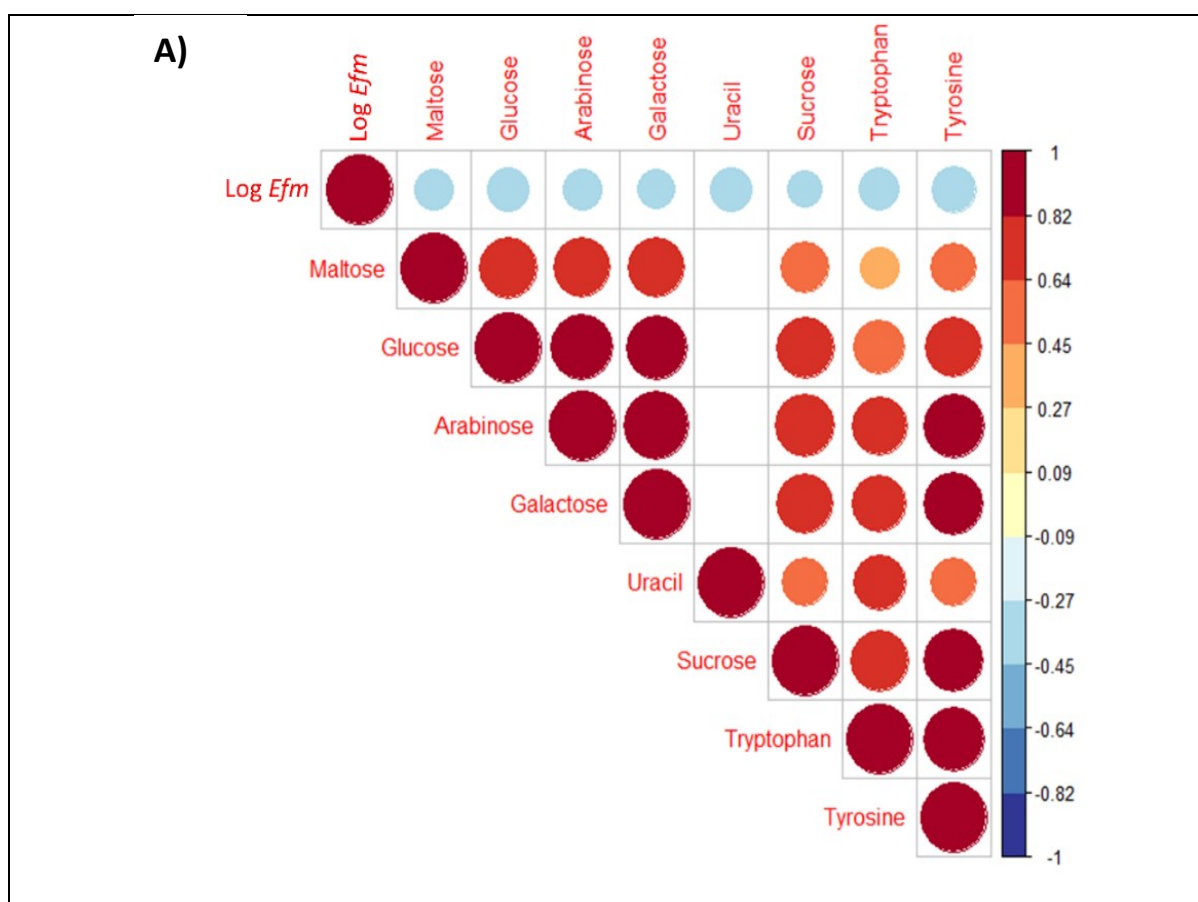
($p=0.0045$), and tyrosine ($p=0.0014$). Weak negative correlations were seen between *E. faecium* and arabinose ($p=0.0149$), galactose ($p=0.0182$), sucrose ($p=0.0432$), tryptophan ($p=0.0088$), and maltose ($p=0.0136$). No nutrients positively correlated with VRE growth. Nutrients that did not have significant correlations are not shown.

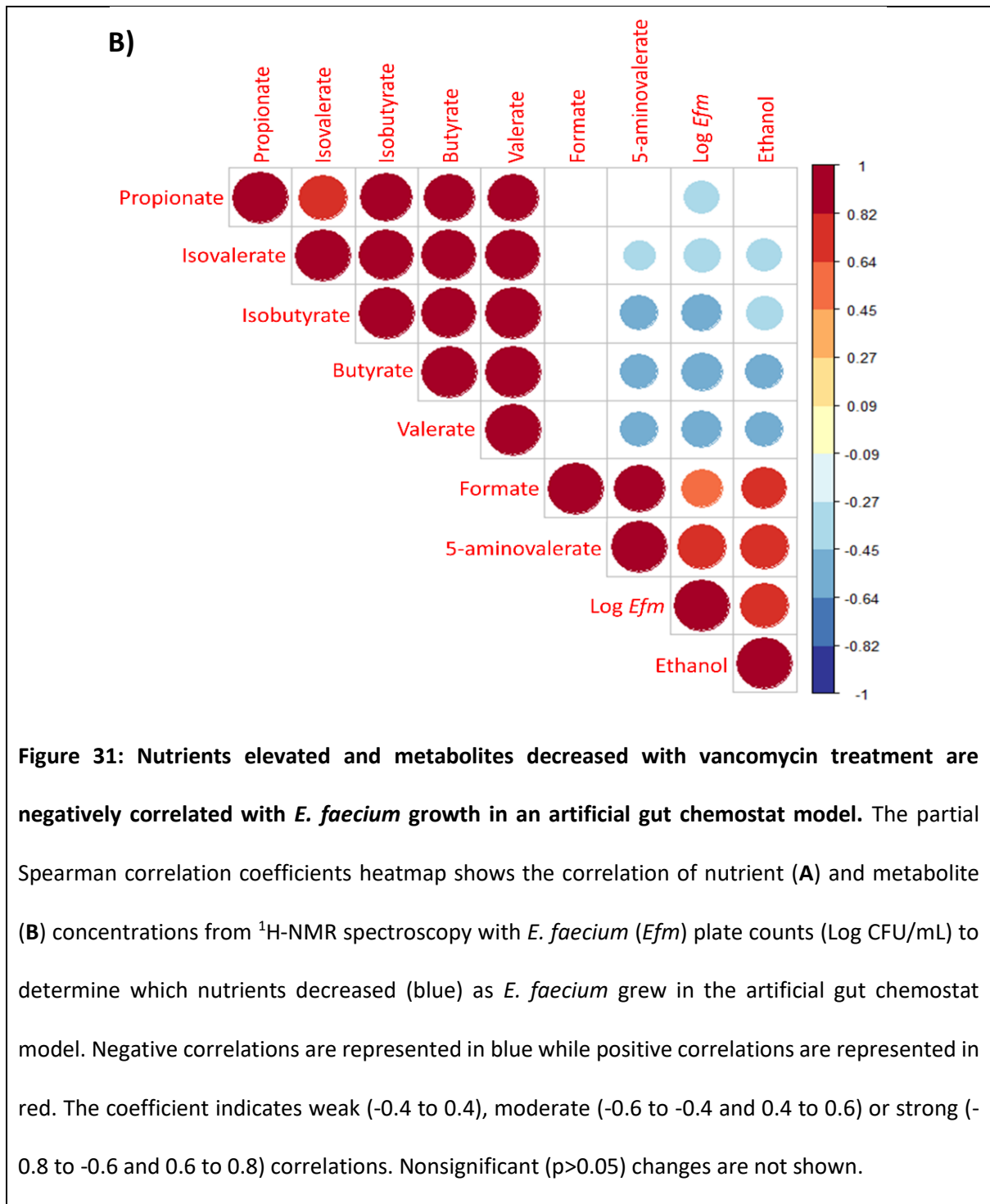




acetate (AA), propionate (PA), butyrate (BA), valerate (VA), isobutyrate (iso-BA), isovalerate (iso-VA), and ethanol (EtOH).

When assessing the interaction between *E. faecium* and metabolite concentrations (**Figure 31B**), moderate negative correlations were seen with propionate ($p=0.0133$), isovalerate ($p=0.0073$), isobutyrate ($p=0.0013$), butyrate ($p=0.0005$), and valerate ($p=0.0009$). A positive moderate correlation was seen between *E. faecium* and 5-aminovalerate ($p<0.0001$), formate ($p=0.0007$), and ethanol ($p<0.0001$). Acetate and lactate did not significantly correlate with *E. faecium*.





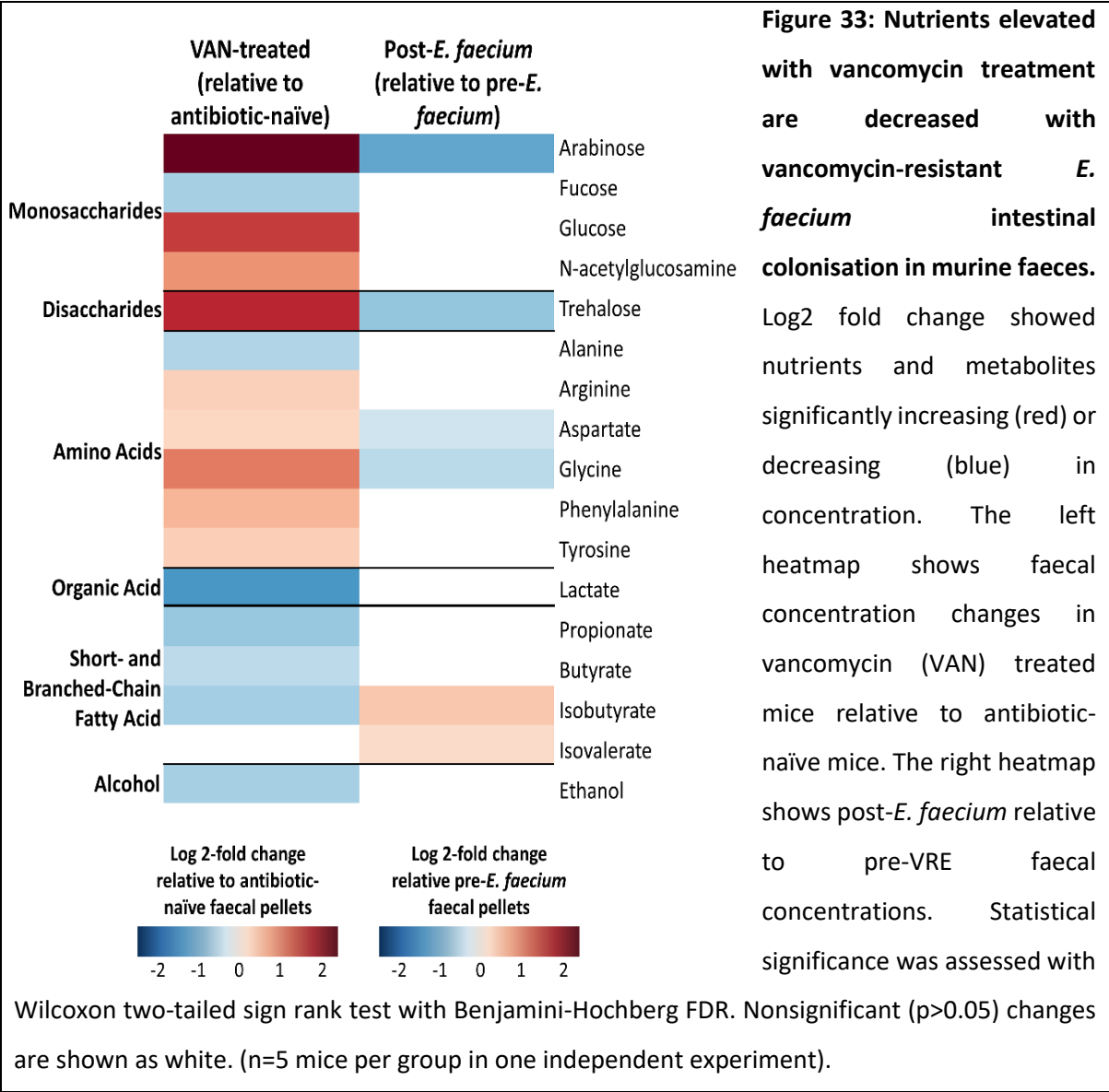
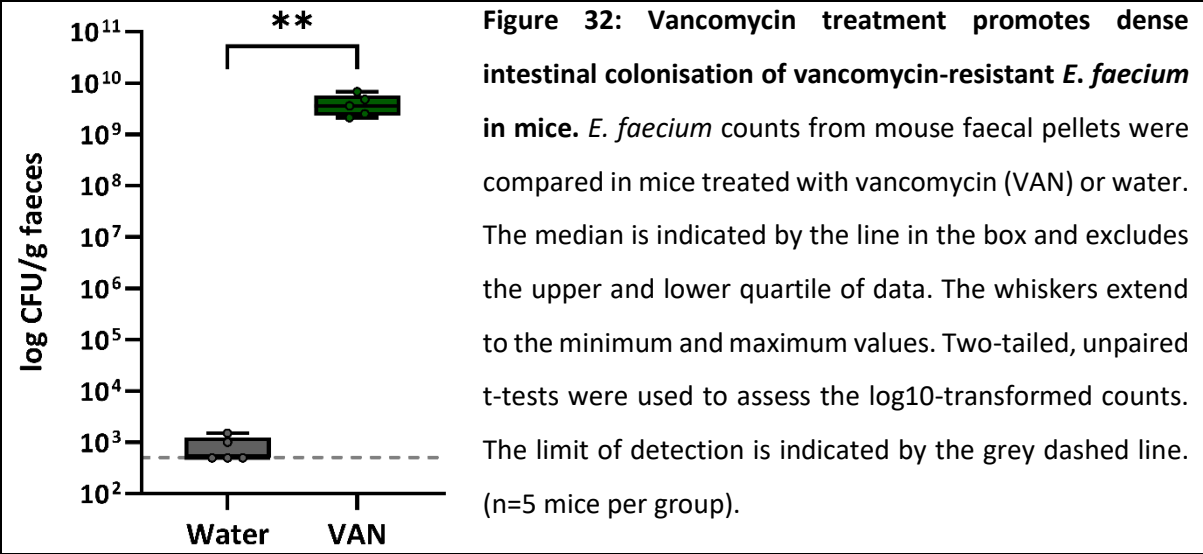
Vancomycin treatment in murine study promotes *E. faecium* gut colonisation

The mouse study was designed to serve as validation of the findings of the artificial gut chemostat in the context of a host. The chemostat data revealed microbe-microbe and microbe-environment interactions. Conducting a murine study allowed confirmation that these general trends would be maintained in a host. Mice were treated with VAN or water to

investigate the effects of antibiotic treatment on nutrient availability and metabolite production. Then, vancomycin-resistant *E. faecium* was administered to demonstrate that it could grow in the antibiotic-treated intestine and to measure nutrient utilisation by *E. faecium*. Changes in nutrient availability and metabolite production were measured in mouse faeces using ¹H-NMR spectroscopy.

Mice which received antibiotic treatment had significantly higher VRE counts in their faeces compared to water-treated mice ($p = 0.0079$; **Figure 32**). The faecal samples collected from the mice treated with water or VAN, as well as those collected pre and post VRE colonisation, were used to determine the impact of antibiotic-treatment and VRE colonisation on nutrient availability and metabolite production using ¹H-NMR spectroscopy. Faeces from antibiotic-treated mice showed significantly higher concentrations of nutrients compared to faeces from antibiotic-naïve mice (**Figure 33**). Sugars (arabinose, fucose, glucose, N-acetylglucosamine, and trehalose), and amino acids (alanine, arginine, aspartate, glycine, phenylalanine and tyrosine) were elevated in antibiotic-treated mice relative to antibiotic-naïve mice. Further the microbial metabolites lactate, propionate, butyrate, isobutyrate, and ethanol were significantly decreased in concentration in the antibiotic-treated mice.

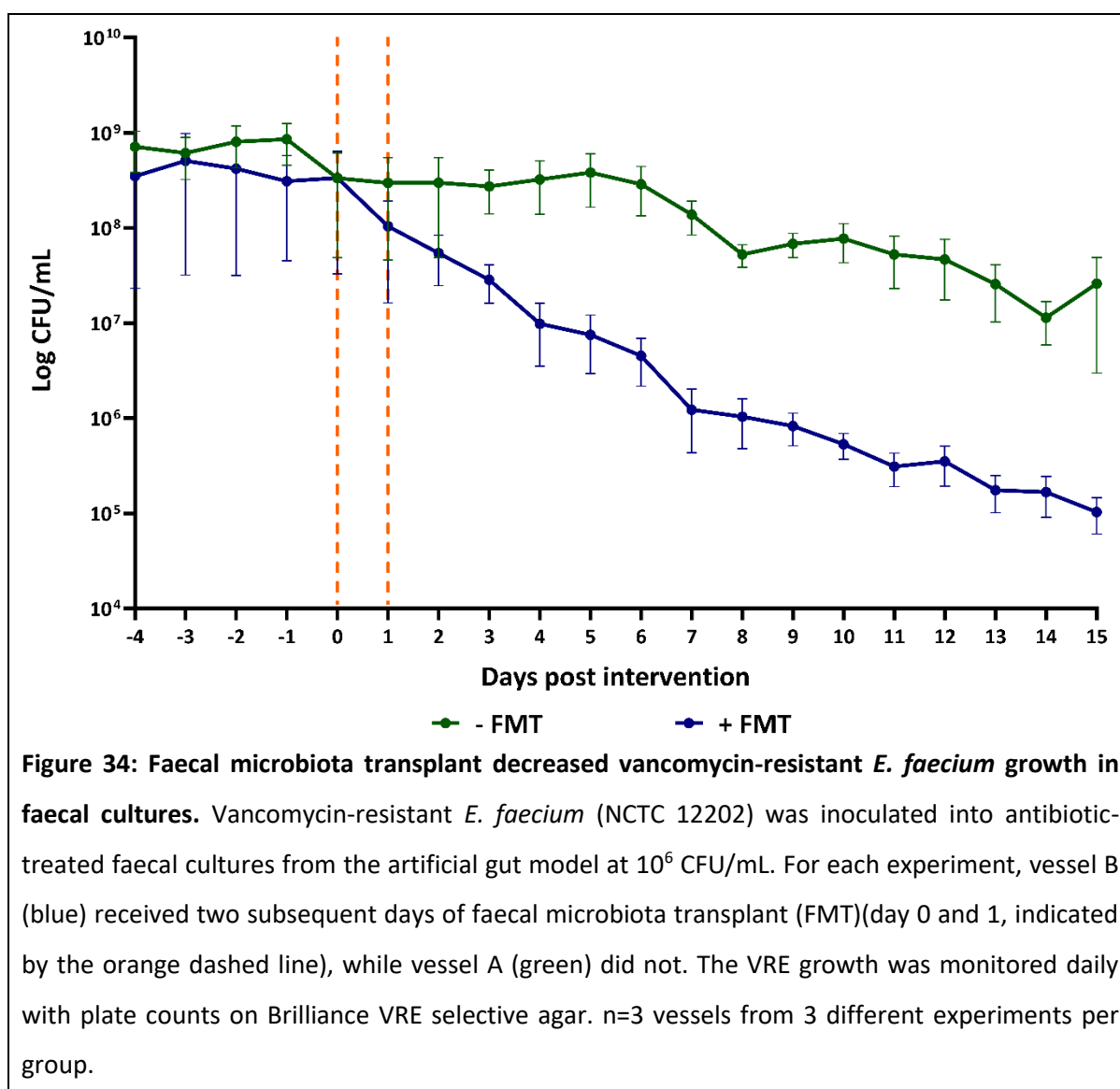
Antibiotic-naïve mice had VRE counts at or just above the limit of detection, while antibiotic-treated mice had high VRE counts ($>10^9$ CFU/g of faeces)(**Figure 32**). Several nutrients that were increased with antibiotic treatment subsequently decreased with *E. faecium* colonisation (**Figure 33**). Arabinose, trehalose, aspartate and glycine were significantly decreased in mouse faeces following *E. faecium* colonisation relative to their concentrations before *E. faecium* colonisation. Moreover, isobutyrate and isovalerate concentrations increased following *E. faecium* colonisation.



Faecal microbiome transplantation depletes nutrients, restores metabolite production, and restricts *E. faecium* growth

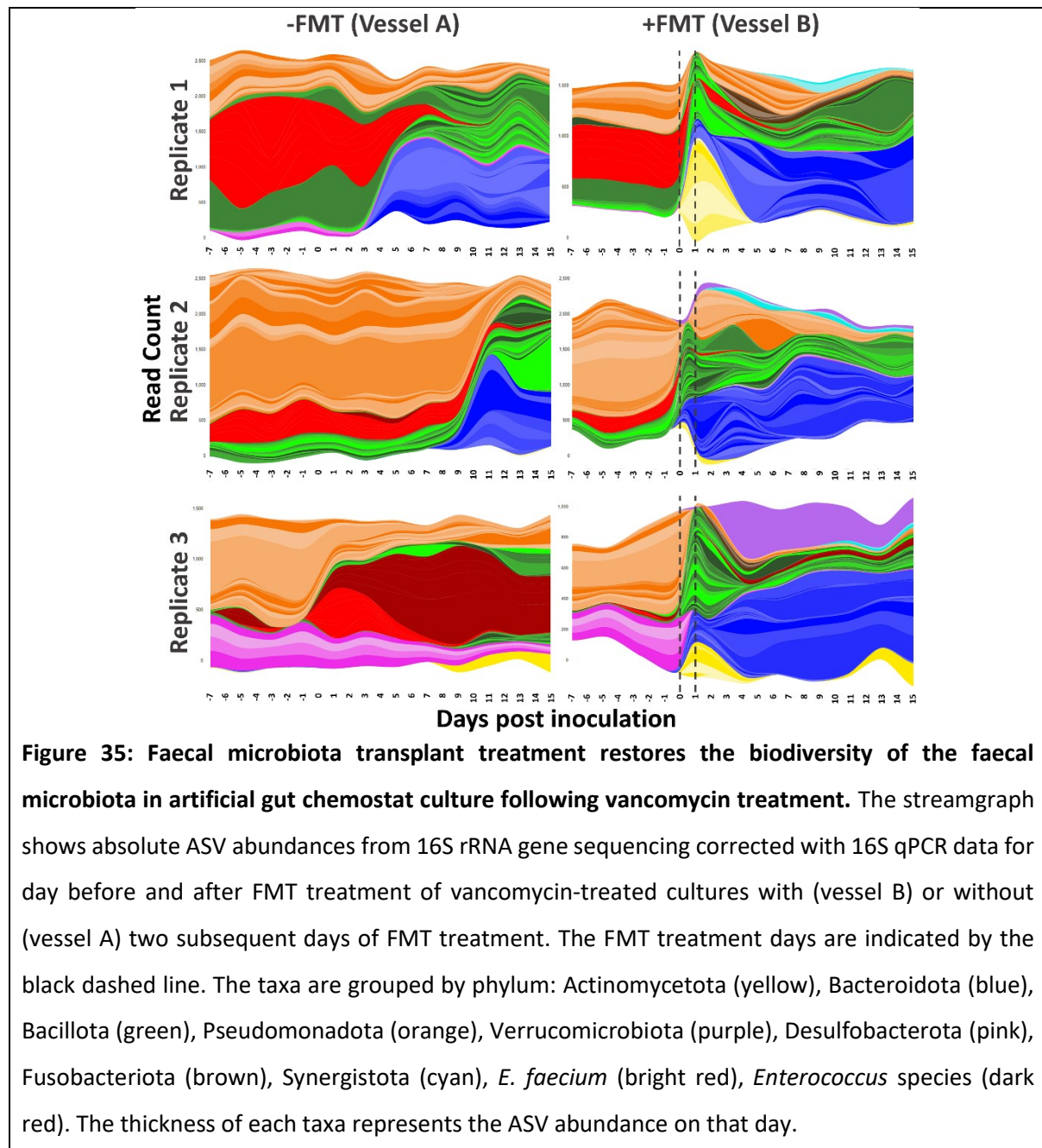
Next, the impact of FMT was measured on faecal cultures following antibiotic treatment and colonisation with vancomycin-resistant *E. faecium*. FMT has been tested as a treatment to restrict VRE in humans [376] and provide insights as to which gut commensals are critical in nutrient metabolism and could act as a microbiome therapeutic. Using faeces from a different healthy donor, FMT was prepared fresh and administered to vessel B on two subsequent days following the cessation of antibiotic treatment. VAN-treated vessel A and antibiotic-naïve vessel C were left without any interventions as a control.

VR-*E. faecium* counts declined steadily following FMT treatment in faecal cultures from vessel B, while counts from vessel A remained relatively stable (**Figure 34**). Prior to FMT treatment, the VRE counts in the two antibiotic-treated vessels were consistent with one another and remained level between 10^8 - 10^9 CFU/mL. After the first FMT treatment (shown as day 0 in **Figure 34**), the VRE counts began to drop. The VRE counts decrease in vessel A as well but at a slower rate. By the end of the experiment, there was a 10^3 CFU/mL difference in the VRE counts between vessel A and B. However, even with the slow decrease in *E. faecium* in vessel A, by the final week of the experiment, there was still a statistically significant difference between the average *E. faecium* counts in vessel A and B (Welch's unpaired t test, $p=0.0030$). By the time FMT treatments were given to vessel B, the VRE counts in vessel C had dropped below the limit of detection and are thus not shown in **Figure 34**.



Next, taxonomic and metabonomic profiles of faecal cultures were measured to determine the effects of FMT on gut commensals, nutrient availability, and metabolite production. The FMT treatments altered the taxonomic composition of the antibiotic-treated faecal microbiota within vessel B, where bacteria that were killed with antibiotics or absent prior to antibiotic treatment colonised within the vessel (**Figure 35**). The data showed that the FMT treatments administered to vessel B caused an immediate increase in the Bacteroidota and Bacillota phyla and decreased the abundance of members of the Psuedomonadota in all three

replicates. In the third replicate, the abundance of Desulfobacterota was decreased while the abundance of Verrucomicrobiota was returned to pre-antibiotic levels.



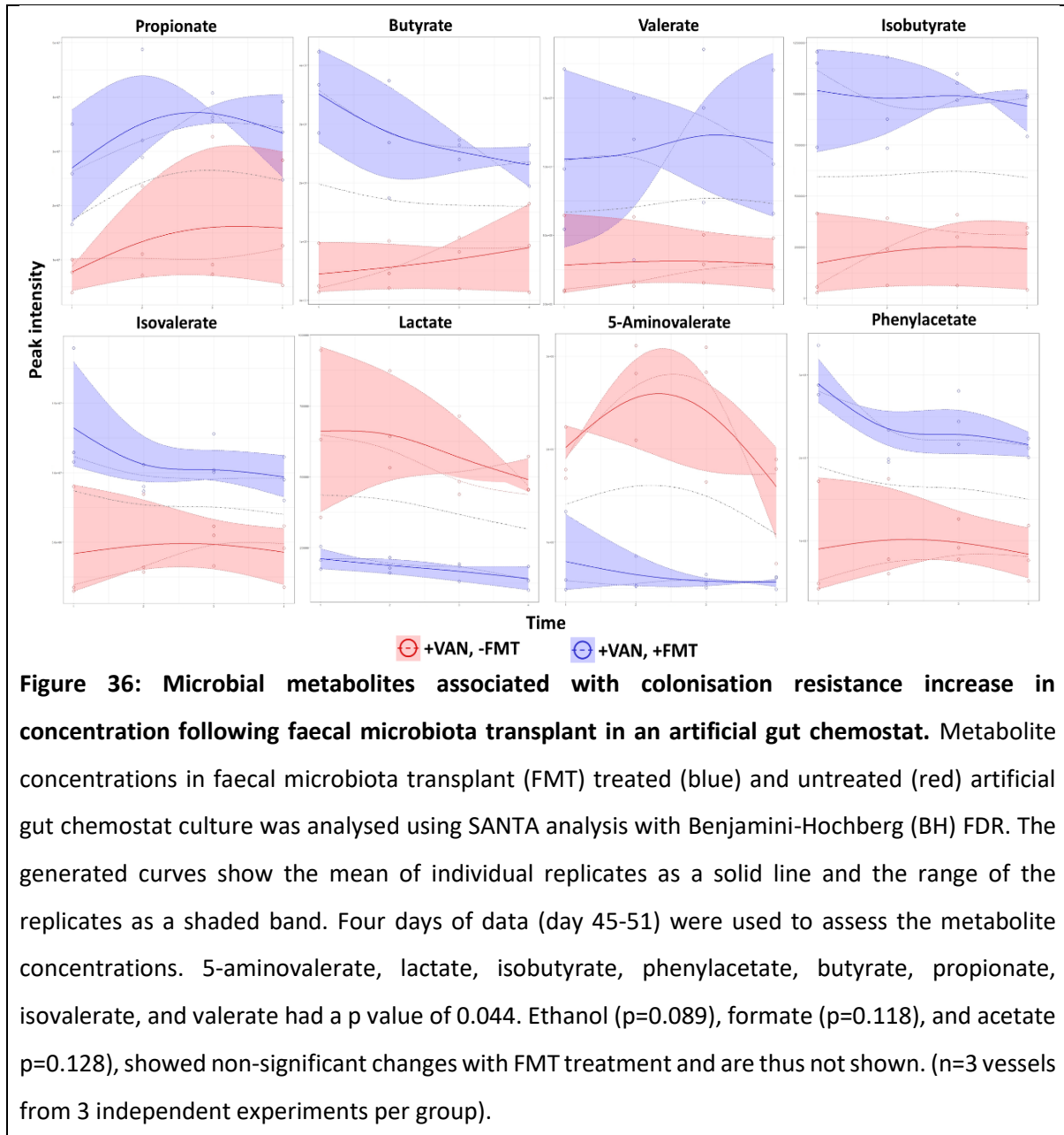
The rate at which the microbiota within the antibiotic-treated vessel A culture, which did not receive FMT treatment, returned to a similarly diverse and dense community as was seen in the pre-antibiotic culture, depended on the initial donor's microbiota. In the first replicate, the abundance of members of the Bacillota and Bacteroidota phyla returned to pre-antibiotic

levels by day 47. In the second replicate, the abundance of Bacteroidota increased by day 53 however, the abundance of Psuedomonadota remained higher than was seen pre-antibiotic. In the third replicate, the bloom of *Enterococcal* species in the culture meant that the dysregulation caused by VAN-treatment and VRE colonisation was maintained till the end of the experiment. Due to differences in both how the initial donor's faecal microbiota reacted to VAN treatment and in the FMT donor's faecal microbiota, statistical analysis of the impact of FMT on the taxonomic profiles between replicates was not possible.

The metabolomic data from the two vessels showed that the FMT treatment restored microbial metabolite concentrations to levels comparable to pre-VAN treatment (**Figure 36**). Using ^1H -NMR spectroscopy data analysed with SANTA, we showed propionate, butyrate, valerate, isobutyrate, isovalerate, and phenylacetate increased compared to the untreated vessel A ($p=0.044$). Lactate and 5-aminovalerate, were shown to decrease significantly with FMT-treatment. The increase in acetate and decrease in formate and ethanol with FMT-treatment were not statistically significant. Nutrients were not significantly changed with FMT due to variation between replicates.

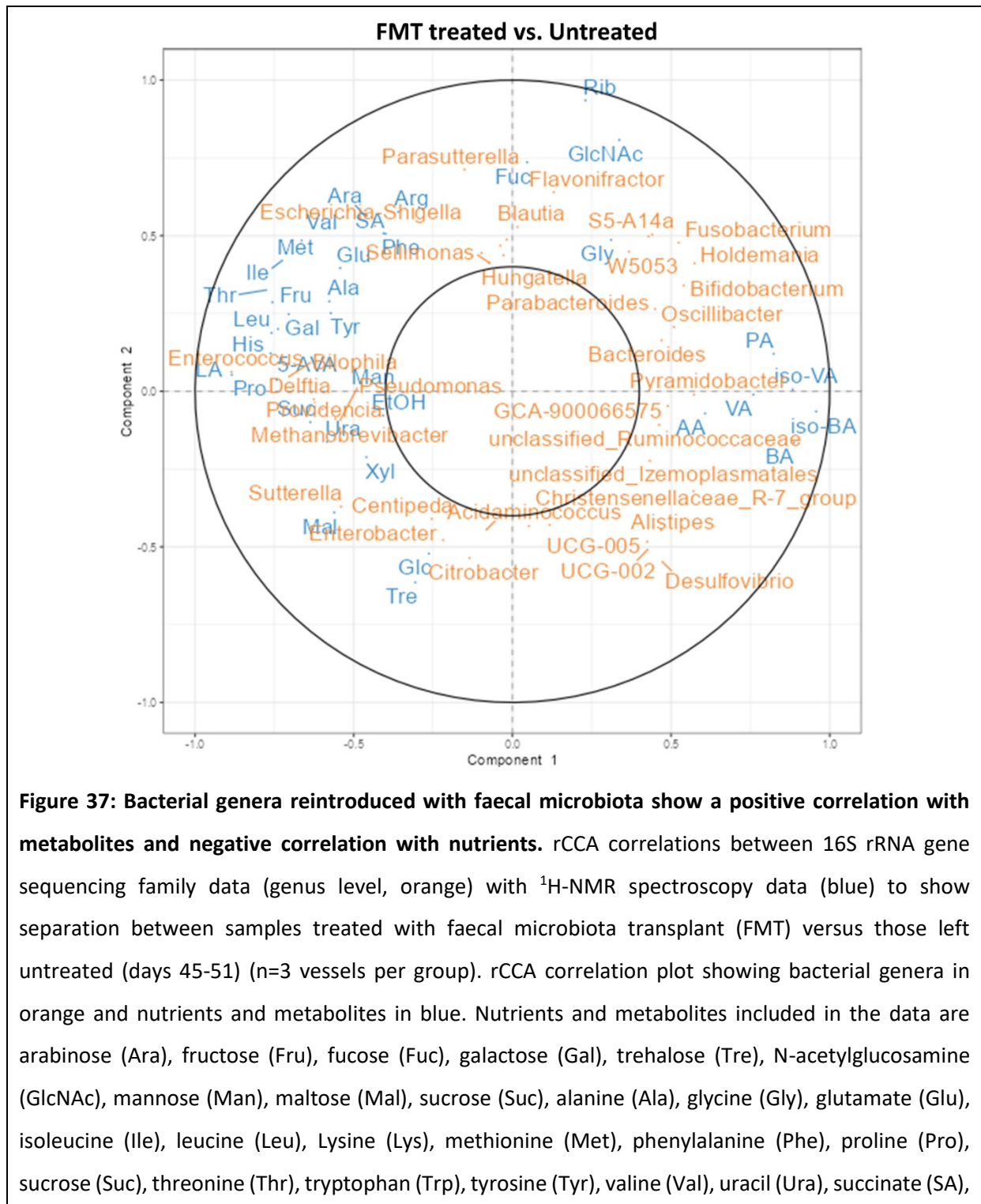
Using an rCCA model combining the taxonomic and metabolomic profiles of the FMT-treated and untreated faecal cultures, it was shown that many bacterial genera positively correlated with metabolite concentrations and negatively correlated with nutrient concentrations (**Figure 37**). Notably, the reintroduction of the faecal microbiota into the VAN-treated faecal culture through FMT resulted in *Bifidobacterium* and *Bacteroides* positively correlating with SCFAs and BCFAs. *Enterococcus* was seen to negatively correlate with these metabolites. As expected, trends seen in the FMT-treated versus non-FMT treated faecal culture correlation

plot (**Figure 37**) were similar to those seen in the plot using data from before and during VAN treatment (**Figure 30A**).



Likewise, when Spearman correlations were used to compare metabolite concentrations to *E. faecium* cell density (**Figure 38**), a strong negative correlation was seen with valerate ($p > 0.0001$) and propionate ($p > 0.0001$) while moderate negative correlations were seen with isovalerate ($p = 0.0012$) and butyrate ($p = 0.003$). Unlike the comparisons seen with VAN treatment (**Figure 31B**), isobutyrate was not significantly correlated with *E. faecium* growth.

A moderate positive correlation was seen between *E. faecium* and acetate ($p=0.0009$) and ethanol ($p=0.00034$). Ethanol was similarly seen to positively correlate with *E. faecium* during VAN treatment.



lactate (LA), 5-aminovalerate (5-AVA), formate (FA), acetate (AA), propionate (PA), butyrate (BA), valerate (VA), isobutyrate (iso-BA), isovalerate (iso-VA), and ethanol (EtOH).

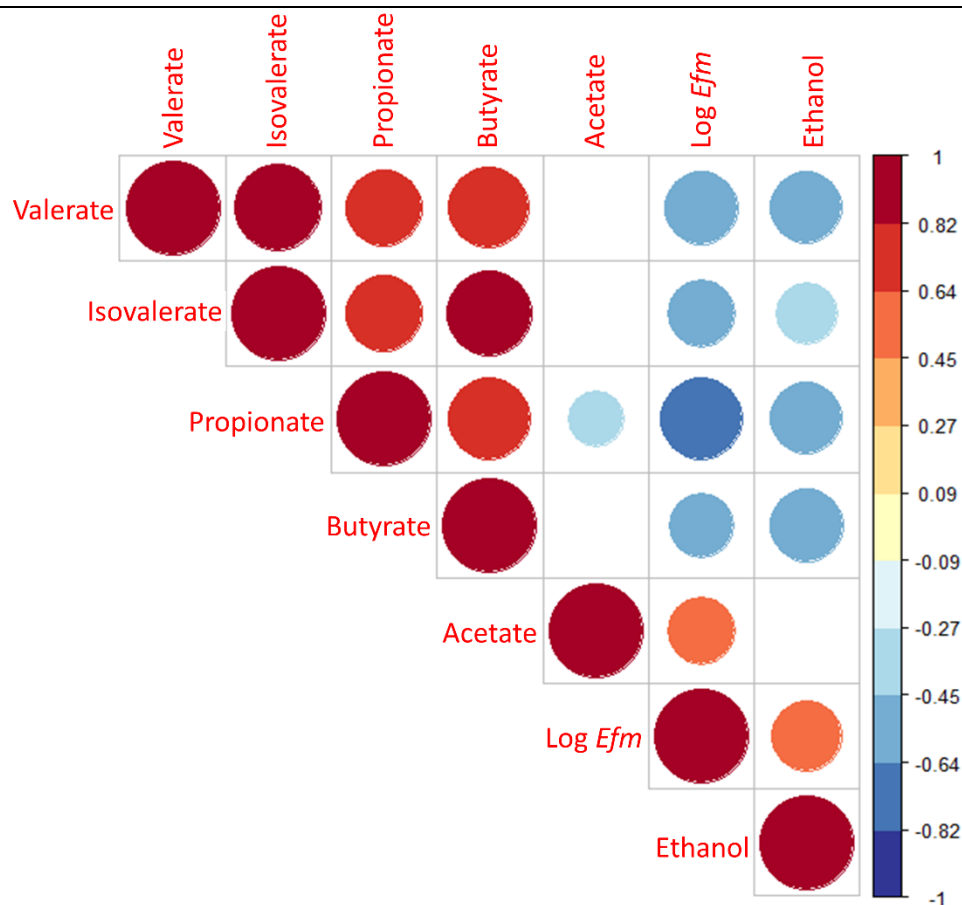


Figure 38: Metabolites elevated with faecal microbiota transplant are negatively correlated with *E. faecium* growth in an artificial gut chemostat model. The partial Spearman correlation coefficients heatmap shows the correlation of metabolite concentrations from ¹H-NMR spectroscopy with *E. faecium* (*Efm*) plate counts (Log CFU/mL) to determine which nutrients decreased (blue) as *E. faecium* grew in the artificial gut chemostat model. Negative correlations are represented in blue while positive correlations are represented in red. The coefficient indicates weak (-0.4 to 0.4), moderate (-0.6 to -0.4 and 0.4 to 0.6) or strong (-0.8 to -0.6 and 0.6 to 0.8) correlations. Nonsignificant ($p > 0.05$) changes are not shown.

Discussion

Intestinal colonisation models, such as artificial gut and mouse models, allow for the longitudinal analysis of the gut microbiota in response to antibiotic treatment and *E. faecium* colonisation. The artificial gut chemostat model allowed for analysis of the taxonomic and

metabonomic profiles comparing (1) VAN-treated or antibiotic-naïve faecal cultures, (2) *E. faecium* colonisation in the presence and absence of antibiotics, and (3) FMT-treated or untreated faecal cultures. For the first time, correlating these findings with the growth of VRE in the artificial gut chemostat communities revealed a better understanding of which gut commensals are killed or suppressed by antibiotics, and how VRE exploits this reduction in competition. This data showed that VRE was able to grow in faecal cultures that had decreased abundances of gut commensals, increased nutrient availability, and decreased metabolite production. A mouse experiment was performed to determine the significance of microbe-microbe interactions compared to host-microbe interactions: if the microbe-microbe interactions were important in the promotion of VRE growth and persistence, the *in vivo* experiments would yield similar findings to the *ex vivo* experiments. Data from the mouse experiment confirmed the findings of the *ex vivo* experiments and confirmed the importance of direct polymicrobial interactions.

The data presented in this chapter was compatible with the findings from Chapter 3 on the impact of vancomycin on the gut microbiota, as well as the results from Chapter 4 showing how vancomycin-resistant *E. faecium* exploited the antibiotic-dysregulated environment. The multilevel analysis of these interactions represented in several models of increasing physiological relevance will prove essential for the informed selection of gut commensals to develop a microbiome therapeutic to restrict VRE growth

Previously published mouse studies have demonstrated that antibiotic treatment increased the duration and density of VRE colonisation compared to those in untreated mice [55, 67, 71, 183]. Rice et al. pretreated mice with VAN to disrupt the microbiome [67]. Next, the mice were inoculated with *E. faecium* and given further treatment with VAN, CLI, and TZP. This

resulted in *E. faecium* growth maintained over 10^9 Log CFU/g faeces [67]. While treating the mice with CRO resulted in a slow decline in *E. faecium* counts, the final cell density was still shown 10^2 Log CFU/g faeces higher than saline treated mice after 19 days. Without the pretreatment with VAN, CRO was shown to promote *E. faecium* growth while TZP was seen to have little effect [67, 71]. These findings supported those presented in this chapter that antibiotic treatment promotes high-density VRE colonisation in mice. In a study by Ubeda et al., it was shown that antibiotic (VAN, MTZ and ampicillin) treatment of mice significantly decreases the intestinal microbial density while simultaneously promoting *E. faecium* growth [55]. As the microbiota recovered, the *E. faecium* counts decreased. This supported the premise of the research in this study that antibiotic promotion of VRE was due to the decrease in gut microbiota diversity and density.

Using simple faecal batch cultures, we showed that the antibiotic treatments tested promoted VRE growth compared to antibiotic-naïve cultures. TZP treatment could not be assessed as the VRE strains used in this study were susceptible to the concentration of TZP tested, and so there was no way to differentiate killing of VRE by the faecal microbiota from killing by the antibiotic. The findings that antibiotic treatment led to higher growth of VRE is also supported by previous human studies which identified antibiotic treatment as a precursor to persistent, high growth of VRE in clinical environments [55, 61, 64, 67, 68, 70, 71, 75, 201-203, 579-583].

In addition to the impact of the antibiotic-treated faecal microbiota on VRE growth, the antibiotics themselves could have varied effect on the survival of the VRE strain. Previous studies have shown that commensal microbes shield pathogens from antimicrobials due to the AMR genes found in the communities, which contribute to antibiotic degradation [584,

585]. This could explain why the VRE strains grew better in VAN-treated faecal culture than in solo culture.

Artificial gut model experiments demonstrated that VAN treatment promoted the growth and stable colonisation of VR-*E. faecium*. In VAN-treated culture, the VRE grew from 10^6 CFU/mL to 10^9 CFU/mL within 4 days of inoculation, slowly decreasing to 10^7 CFU/mL over the course of 26 days without further intervention. This indicated that VAN causes alteration to the microbiota's ability to resist VRE colonisation. In the artificial gut chemostat cultures that were not treated with antibiotics (mimicking a healthy gut microbiota), *E. faecium* plate counts rapidly decreased until they were below the limit of detection. The rate of VRE decolonisation likely differed between experiments due to the unique taxonomic composition of the inoculating donor's faecal microbiota. For all three experiments, *E. faecium* was eliminated within 14 days. A previous study used a continuous-flow competitive exclusion culture model (similar to the artificial gut chemostat model used in this study) to measure *E. faecium* growth in the presence of a healthy human faecal microbiota [376]. This study first inoculated sterile media with VRE and then applied the faecal culture as a treatment to show VRE growth suppression compared to growth in an untreated control. The faecal culture was shown to suppress the growth of *E. faecium* within six to nine days depending on the initial *E. faecium* inoculum. The consistency and level of growth of the VR-*E. faecium* in the chemostat environment was similar to that seen by Donskey et al., despite the experimental design and growth media being different [376].

Similarly, the VAN treatment increased the growth of VRE mice. Four days after inoculation, the VRE counts in three of the five water-treated mice were below the limit of detection while the VAN-treated mice had persistent dense VRE colonisation. In addition to supporting the

findings in this studies batch cultures and artificial gut chemostat model, these results supported previous findings on the impact of antibiotics on VRE colonisation in mouse studies [55, 67, 71, 183].

In the artificial gut model experiments, microbial metabolites (including SCFAs and BCFAs) decreased in concentration following antibiotic treatment (metabolite concentrations were unchanged in the antibiotic-naïve faecal cultures). Likewise, NMR spectra from the faeces of VAN-treated mice showed a decrease several of the same metabolites. Due to differences in the human versus mouse microbiota composition and diet, not all metabolites identified in the chemostat cultures could be identified in the mouse faeces. The decrease in microbial metabolites with antibiotic treatment has been discussed in Chapter 3 of this thesis and in other studies [586-590]. These studies have reported a decrease in the concentration of fatty acids including acetate, butyrate, and propionate, in antibiotic-treated piglets [586, 591], mice [587, 589], and humans [588, 590]. SCFAs and BCFAs produced by gut commensals have been demonstrated to inhibit the growth of pathogens [30, 366, 391, 402, 403, 507, 508]; *C. difficile* was shown to be inhibited by valerate [403]. *E. coli* inhibition by acetate has been reported in multiple studies [391, 402]. Propionate has been shown to inhibit *S. aureus* [366]. Butyrate has been shown to improve barrier function thus protecting against *C. jejuni* [507, 508]. Finally, a mixture of SCFAs was shown to inhibit CRE [30].

Previous studies linked the production of SCFAs and BCFAs to several commensal families including *Bacteroidaceae*, *Lachnospiraceae*, *Eubacteriaceae*, *Acidaminococcaeae*, *Enterobacteriaceae*, *Veillonellaceae*, and *Ruminococcaceae* [520, 523, 526, 533]. Likewise, the antibiotic-treated faecal batch cultures performed in Chapter 3 showed decreased microbial diversity and density of the same key commensal families. Many bacterial genera (including

Bacteroides, *Ruminococcus*, *Anaerostipes*, *Coprococcus* and *Marvinbryantia*) were shown to positively correlate with the concentration of metabolites in the VAN-treated artificial gut chemostat model. This is supported by both previously published correlations [520, 523, 526, 533], as well as the similar analysis performed on the VAN-treated faecal batch cultures from this study which showed *Lachnospiraceae*, *Ruminococcaceae*, and *Bacteroidaceae* positively correlating with the concentrations of SCFAs and BCFAs (see Chapter 3).

Within the first few days of VAN treatment in the artificial gut chemostat cultures, the biomass of the gut microbiota dramatically decreased in the artificial gut chemostat cultures. As the microbiota biomass increases during the antibiotic treatment period, the composition of the community was significantly altered compared to pre-antibiotic conditions. There was a significant decrease in members of the *Bacteroidaceae*, *Lachnospiraceae*, *Enterobacteriaceae*, *Veillonellaceae*, *Ruminococcaceae*, *Rikenellaceae*, *Oscillospiraceae* and *Christensenellaceae* with VAN treatment. These results were supported by those seen in the faecal batch cultures reported in Chapter 3. *Oscillospiraceae* positively correlated with propionate. Members of the *Oscillospiraceae* family are known valerate and propionate producers and are linked to colonisation resistance and decreased inflammation in the gut [523, 524, 531, 592-594]. *Christensenellaceae* also positively correlated with propionate and is a known producer of propionate [595-597]. Although *E. faecium* was not shown to be inhibited by microbial metabolites in pure TSB culture (**Figure 21**, **Figure 22**, Chapter 4), the complex and competitive environment of the chemostat culture could result in stronger susceptibility of the VRE to these metabolites. The VRE growth was negatively correlated with the concentration of microbial metabolites. Further investigation would be required to verify if this relationship is a correlation or causation.

Antibiotic treatment also caused an increase in the concentration of nutrients in both the artificial gut culture and mouse model samples. In the previous chapter, it was shown that *E. faecium* could utilise sugars and amino acids as nutrient sources. Many of these nutrients were shown to increase in concentration following VAN treatment in the chemostat cultures. The combination of sugars and amino acids offer a diverse assortment of nutrient for the VRE to utilise during colonisation. Additionally, though not identifiable in the NMR spectra, complex substrates could serve as viable nutrient sources for *E. faecium* as they were in the DGGM used in the artificial gut chemostat cultures. As previous research in this study has shown VRE to act as both a primary and secondary degrader (**Figure 17**, Chapter 3), the increase in nutrient concentration following antibiotic treatment may be exploited by *E. faecium* to colonise the intestine. Following the inoculation of *E. faecium* into antibiotic-treated artificial gut cultures, the concentration of eight nutrients negatively correlated with *E. faecium* growth (indicating that *E. faecium* may use these nutrients to support its growth). Thus, between the increased concentration of nutrients and the fastidious nature of VRE's wide-ranging nutrient utilisation abilities, *E. faecium* was able to exploit the antibiotic dysregulation of the VAN-treated microbiota.

Previous studies have shown that increased nutrient concentration in the gastrointestinal system—especially mannose and fructose—has contributed to colonisation by VRE in a host's gut [598, 599]. It has been shown that the phosphotransferase system linked with mannose and fructose acquisition in *E. faecium* isolates was enriched in clinical isolates linked with colonisation of antibiotic-treated gastrointestinal systems [598, 599]. Further, deletion of the gene encoding the transporter responsible for the utilisation of fructose and mannose resulted in impaired colonisation in mice [599]. Isaac et al. showed that reestablishing

nutrient competition for fructose led to decreased growth of *E. faecium* in mice [456]. Although *E. faecium* was not shown to be able to utilise mucin and several plant polysaccharides (inulin and pectin) as nutrient sources (**Figure 17**, Chapter 3), one study suggested members of the gut microbiota which act as primary degraders, free up the monosaccharides (arabinose, fucose, galactose, glucose, mannose, N-acetylglucosamine) that *E. faecium* can use to support its growth [490].

The artificial gut model experiments demonstrated that the types and concentrations of the nutrients that were increased following antibiotic treatment varied depending on the composition of the seeding donor's microbiota. Previous studies have demonstrated that nutrient utilisation varies between different gut commensal bacteria [600]. For example, *B. thetaiotaomicron* and *B. fragilis*, though both *Bacteroides* species, have varied nutrient utilisation where *B. fragilis* primarily consumes starch and fructans and *B. thetaiotaomicron* can grow on starch, fructans and pectin nutrient sources [601]. Therefore, the impact of antibiotic treatment on nutrient availability in the intestine will depend on which gut commensals are colonising the donor's intestine, and whether the gut commensals are susceptible or resistant to the antibiotic. Overlap in the nutritional niches of members of the microbiota ("functional redundancy") means that even if certain gut commensals are entirely wiped out by antibiotic treatment, surviving commensals may continue to consume these nutrients [15, 16, 471]. However, as broad-spectrum antibiotics are known to promote VRE intestinal colonisation, it is more likely that a wide range of gut commensals are killed, thus more significantly impacting nutrient utilisation [586, 602, 603].

The 16S rRNA gene sequencing data showed decreases in the number of both primary and secondary degraders of nutrients in the microbiome. Members of the *Bacteroidaceae*,

Ruminococcaceae, *Lachnospiraceae* and *Akkermansiaceae* genus are known to digest complex carbohydrates and polysaccharides, including mucin, pectin and starch [501, 502, 604, 605]. *Bacteroidaceae*, *Ruminococcaceae*, and *Lachnospiraceae* were seen in all three replicates of the artificial gut experiment and decreased in abundance by VAN-treatment. *Akkermansiaceae* was only present in the third replicate but was shown to decrease with antibiotic treatment.

Secondary degraders, such as members of the *Bifidobacterium* and *Collinsella* genera, are known to consume amino acids and monosaccharides in the gut [503-505]. Neither *Bifidobacterium* nor *Collinsella* were seen in the cultures after the first seven days of the experiment. The decrease in these genera may be due to differences in the donor diet and the composition of the growth medium used in the artificial gut model. The DGGM and nutrient competition in the gut chemostat culture may require these genera to utilise less optimal carbon sources, resulting in other gut commensals outcompeting them for the use of these nutrients. Preliminary data from the McDonald lab (not shown) indicates that *Bifidobacteria* species often consume fewer nutrients than other commensals species (such as *Bacteroides* and *Collinsella* species) and grow to lower cell densities. Future research will expand on the competition between commensal strains and how to optimise a commensal consortia where each strain grows well in coculture.

FMT has been tested as a method to decolonise several pathogens within the intestine, including VRE [606-610]. One study reported that, in humans, VRE clearance without further intervention occurred in a median time of 26 weeks [611]. As a clinical treatment in humans, FMT has been shown to have varying degrees of success in suppressing VRE growth [261, 431, 612, 613]. One clinical trial showed clearance of VRE three months after FMT treatment in

one of two patients [606] while another did not detect any residual VRE in the faeces of a patient in the 15 weeks following FMT treatment [612]. In a larger study, 72.7% of patients were VRE negative between one and six months post FMT [614]. To replicate FMT dosing in human clinical studies, in the artificial gut chemostat model, one of the two antibiotic-treated vessels colonised with VR-*E. faecium* was given two subsequent days of FMT treatment which was shown to cause an immediate decrease in VRE counts. The vessel which did not receive FMT treatment maintained high levels of VRE colonisation which only began to decrease after a further seven days.

The FMT treatments were shown to reestablish diversity within the chemostat cultures. Increases in the abundance of members of the Bacteroidota and Bacillota phyla were likely responsible for the decrease in VRE density following FMT treatment. It was seen that the biodiversity of the culture following FMT treatment appeared similar to pre-antibiotic conditions. A previous study which used ampicillin and chemotherapy to disrupt the microbiota of mice showed that FMT treatment increased Bacillota and, to a lesser extent, Bacteroidota to pre-treatment levels, similar to what we demonstrated in the chemostat model [615].

When comparing FMT-treated versus untreated chemostat cultures, the treated vessels were shown to have higher concentrations of metabolites which negatively correlated with *E. faecium* cell density. The increased abundance of bacterial families known to produce metabolites, including *Bacteroidaceae*, *Lachnospiraceae*, *Eubacteriaceae*, *Acidaminococcaceae*, *Enterobacteriaceae*, *Veillonellaceae*, and *Ruminococcaceae*, correlated with higher metabolite concentrations and, potentially, the colonisation resistance granted by these metabolites to the culture [520, 523, 526, 533]. The FMT-driven increase in SCFA

concentrations has also been observed in clinical studies which showed that increased levels of SCFAs were linked with decrease disease symptoms and susceptibility to pathogenic colonisation following FMT [616-619]. Acetate, propionate, and butyrate have been shown to reduce inflammation and apoptosis associated with disease state in the gut [520, 523]. The reintroduction of butyrate-producer, *F. prausnitzii*, was linked with the successful suppression of *C. difficile* and *K. pneumoniae* following FMT in one individual [620]. *K. pneumoniae* inhibition by FMT has also been shown to be associated with *Barnesiella* species which are known producers of acetate [621].

FMT treatment, though shown to effectively suppress the growth of VRE in the gut by restoring the colonisation resistance imbued by biodiversity, has shortcomings. Not all FMT treatments are effective at inhibiting VRE colonisation in humans. In one case, FMT successfully treated a patient's *C. difficile* infection but failed to eradicate the persistence of VRE [622]. Another study reported two patients still had VRE colonisation six months after FMT treatment [261]. While failure to treat VRE infection could be due to a number of reasons (diet, compounding disease, additional infections), repeated courses of antibiotics which prevent the gut commensals from engrafting into the gut has been linked with FMT failure [621]. Further, the risk of unknown variables—including the transmission of a pathogen [436-438]—when implanting a full faecal culture into a compromised gut microbiome can pose a danger to recipients [429, 432-435]. By knowing what gut commensals are killed or suppressed by antibiotic treatment and how the absence of these commensals alters the nutrients and metabolite landscape in the intestine prior to VRE colonisation, a targeted microbiome therapeutic could be designed which mitigates the risk of failure or complications associated with FMT. Based on the results of the artificial gut chemostat model, a probiotic

cocktail containing members of the Bacillota and Bacteroidota phyla could prove an effective combination for the suppression of VRE.

While the artificial gut chemostat model has been shown to successfully culture a wide range of strains from all relevant phyla, some key species showed low to no growth in the faecal cultures. These families negatively correlated with nutrient concentration and positively correlated with microbial metabolites (**Figure 13**, Chapter 3). *Bifidobacterium* and *Collinsella* were seen in the culture of all vessels in all replicates of the artificial gut chemostat model following inoculation. However, they decreased in abundance within the first seven days of the experiment. Despite the absence of these strains, the antibiotic-naïve artificial gut culture (vessel C) still maintained colonisation resistance, suggesting that other genera (such as *Bacteroides*) may play a more important role in colonisation resistance against VRE. Future experiments could look to better optimise the model to retain the growth of more bacterial genera, for example by modifying the growth medium.

Mouse studies are also limited in their physiological relevance to human gut microbiology due to differences in their gut microbiota composition compared to human gut microbiota. Genera including *Oscillopsia*, *Faecalibacterium*, *Lachnospira*, and *Ruminococcus* are found in lower abundance or not at all in the mouse distal gut microbiota [623, 624].

As previously discussed, the complex substrates found in the DGGM media used in the artificial gut chemostat cultures are not currently identifiable by ¹H-NMR spectroscopy and thus, cannot be quantified. An ongoing collaboration between the McDonald lab and the National Phenome Centre aims to develop a mass spectrometry protocol to eliminate this blind spot in future research.

The artificial gut chemostat and murine model showed that VAN treatment increased susceptibility to *E. faecium* by decreasing the abundance of key bacterial genera which both produce microbial metabolites and contribute to nutrient competition. Restoring the diversity of the gut microbiota with FMT treatment in the artificial gut chemostat cultures successfully suppressed *E. faecium* growth. Using the 16S rRNA sequencing data from the artificial gut model, key commensal genera could be selected to create a commensal consortia capable of suppressing VRE strains by restoring the taxonomic profile of the faecal microbiome to pre-antibiotic treatment conditions. Further, competition with this commensal consortia could elucidate which mechanism of colonisation resistance (nutrient competition or metabolite inhibition) has a greater impact on VRE decolonisation.

Chapter 6: Harnessing specific elements of antibiotic naïve colonisation resistance for VRE treatment

Introduction

Colonisation resistance, provided by the commensal microbiota, suppresses pathogenic overgrowth in the gut. In this study, and many previously published [56, 66, 75, 351, 394, 429], it has been shown that antibiotics kill members of the gut microbiota, thus, decreasing the efficiency of colonisation resistance. Although there are many mechanisms involved in the colonisation resistance inferred by the gut microbiome, this study focuses on those mechanisms which are identifiable through 16S rRNA gene sequencing and ¹H-NMR spectroscopy including nutrient competition and metabolite inhibition. Both mechanisms are supported by the diversity and density of the gut microbiota.

The reestablishment of biodiversity in the gut—either naturally overtime, through probiotic therapeutics, or by means of a faecal microbiota transplant has been shown to restore colonisation resistance [69, 75, 403, 430, 625]. However, in nosocomial environments, both waiting for the microbiome to return to a healthy state and forcing diversity through an FMT pose a serious risk to already challenged patients [429, 432-435]. As it is estimated that less than 50% of the gut microbiota members identified with 16S sequencing are culturable [626-628], there remains an element of uncertainty as to what exactly an FMT could transfer to a recipient. The treatment could unintentionally facilitate the transfer of an opportunistic pathogen from the gut of the healthy donor to the already compromised recipient [436-438].

By identifying which species or metabolites present in an FMT are responsible for the suppression of a pathogen, a curated microbiome therapeutic could potentially replace the full faecal culture used in FMT. More targeted administration of a commensal cocktail would

prevent uncontrollable risk without compromising the successful decolonisation of the gastrointestinal pathogen associated with FMT treatment.

The data outlined in Chapter 3 and Chapter 5 showed that antibiotics known to promote VRE intestinal colonisation decrease the abundance of several bacterial phyla—namely families belonging to the Actinomycetota, Bacillota and Bacteroidota phyla. Members of these phyla are associated with nutrient competition and metabolite production and thus have the potential to inhibit pathogenic growth in the gut [9, 14, 30, 367, 458, 475-483]. Decreases in the density of these commensals was associated with the increase in nutrient availability (carbon and nitrogen sources) capable of supporting VRE growth. In faecal cultures not treated with VAN, or those treated with VAN in which the gut commensals were reinoculated by means of FMT, the VRE growth was inhibited. As none of the tested SCFAs, BCFAs, ethanol, lactate or 5-aminovalerate suppressed the growth of *E. faecalis* or *E. faecium* individually or as a group (as shown in Chapter 4) the restoration of metabolite inhibition would be an unlikely primary method of VRE suppression. It is thus postulated that a commensal consortium of species suppressed by antibiotics could reestablish colonisation resistance through nutrient competition. The aim of this chapter was to assess whether a selection of species from these phyla could be assembled which successfully suppressed the growth of VRE with the same efficiency as the complete faecal microbiota.

Results

Commensal species killed by antibiotic treatment consume the same nutrients as VRE strains

The results of the 16S rRNA gene sequencing data from the faecal batch cultures (Chapter 3) and the artificial gut model (Chapter 5), identified 15 commensal strains which decreased with antibiotic treatment and negatively correlated with nutrients utilised by VRE. This

suggested that these species may be involved in nutrient competition with VRE and thus could potentially be responsible for suppressing VRE growth in a healthy gut microbiota. Therefore, the next aim was to test nutrient utilisation by these gut commensal strains and measure their ability to suppress VRE growth through nutrient depletion.

Of the 15 strains, nine were from the *Bacteroides* genus (*B. vulgatus*, *B. thetaiotaomicron*, *B. dorei*, *B. caccae*, *B. xylanisolvens*, *B. uniformis*, *B. stercoris*, *B. ovatus*, and *B. intestinalis*), five were from the *Bifidobacterium* genus (*B. longum*, *B. adolescentis*, *B. bifidum*, *B. catenulatum* and *B. pseudocatenulatum*), and one was from the *Collinsella* genus (*C. aerofaciens*).

In Chapter 4, it was shown that 10 carbon sources support high growth of the VRE strains in solo culture. To determine if the commensal strains were capable of competing for these nutrients with the VRE strains, the carbon utilisation abilities for commensals were individually assessed. The carbon sources were individually supplemented into a gut commensal minimal media and the growth was evaluated with endpoint OD₆₀₀ readings 24 hours after inoculation of the commensal strains.

All of the carbon sources individually supported the growth of multiple strains when tested in a gut commensal minimal media (**Figure 39**). Four *Bacteroides* strains (*B. xylanisolvens*, *B. thetaiotaomicron*, *B. ovatus*, and *B. intestinalis*) and *C. aerofaciens* were shown to grow with all of the tested carbon sources. *B. vulgatus*, *B. stercoris*, and *B. bifidum* used all but one carbon source. Glucose, galactose, and maltose were used by all strains, while ribose and trehalose (which supported the growth of eight and nine of the 15 strains respectively) were used the least of the carbon sources that were tested.

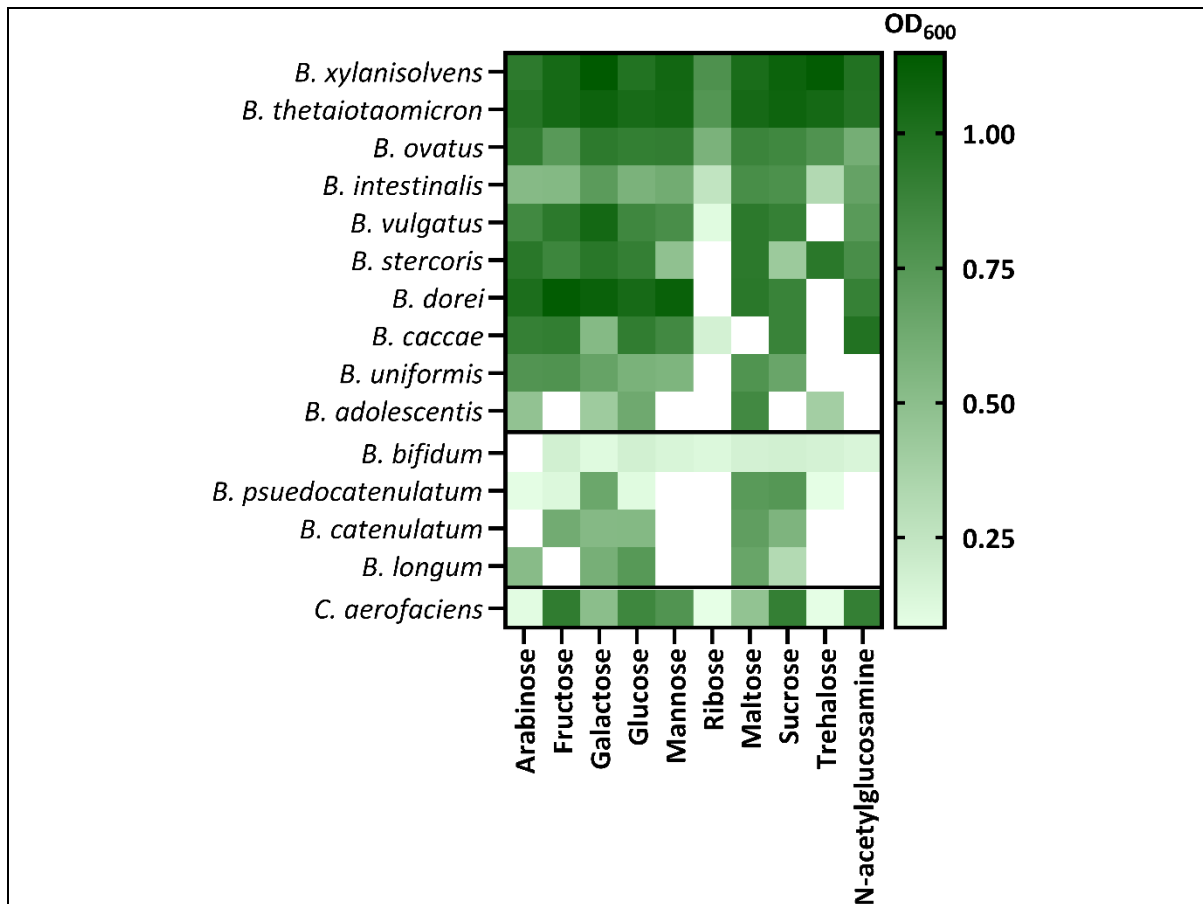
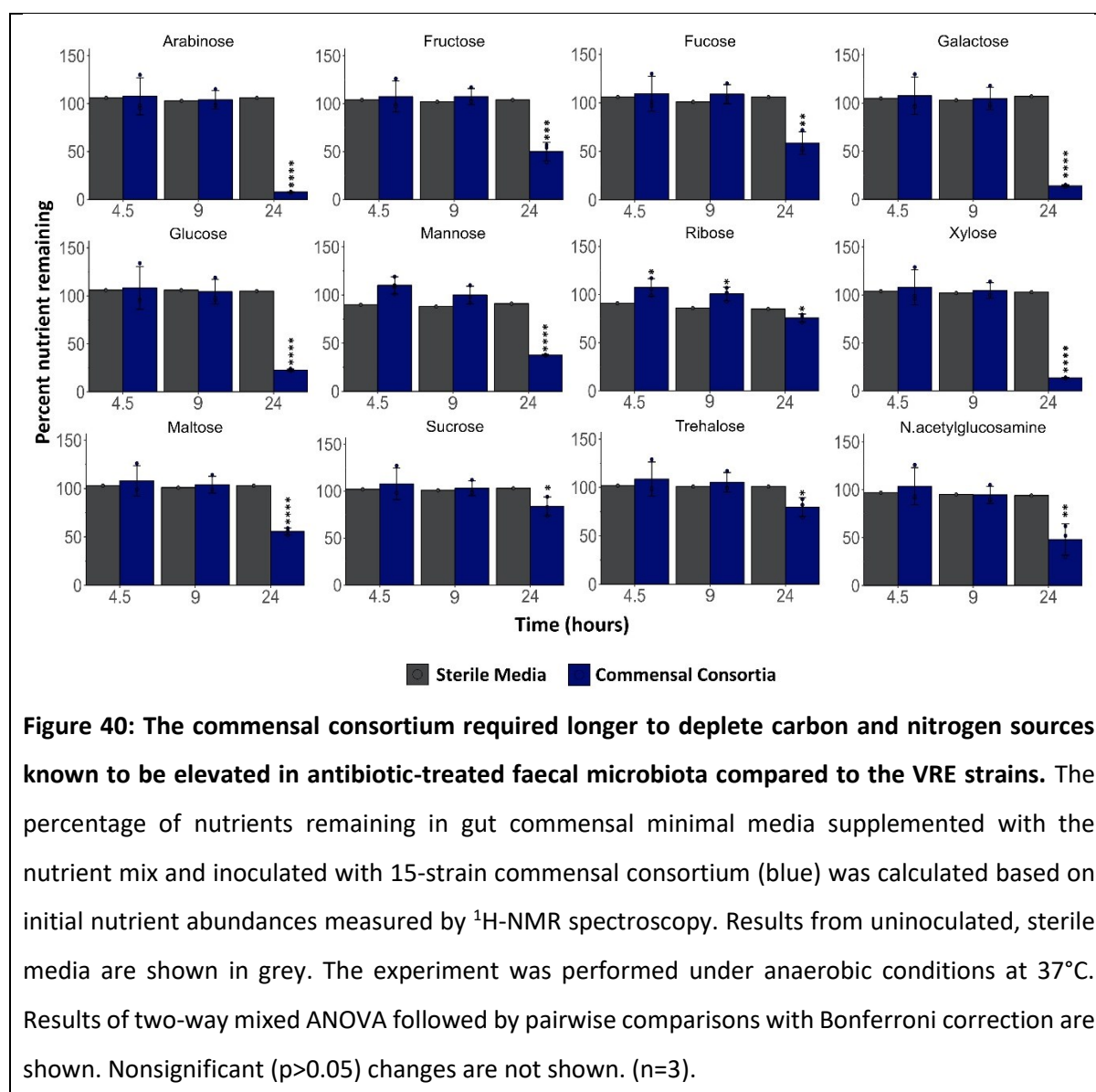


Figure 39: Commensal strains decreased by antibiotic treatment in faecal culture consume the carbon sources necessary for VRE survival. Gut commensal bacteria were assessed for their carbon utilisation abilities, using carbon sources that are also used by VRE isolates. Each carbon source was assessed in gut commensal minimal media. Growth was evaluated with endpoint OD₆₀₀ readings at 24 hours post inoculation which were compared with growth in a media not supplemented with an additional carbon source. The heatmap shows the endpoint OD₆₀₀ readings for sources which resulted in growth of the commensal strains from low growth (light green) to high growth (dark green). Comparison between growth of samples with and without a carbon source were made with one-way ANOVA with Dunnett's multiple comparison test. Nonsignificant ($p > 0.05$) carbon sources are left white. (n=3).

The commensal consortium requires longer than the VRE strains to deplete nutrients elevated by antibiotics

To evaluate if the commensal consortium demonstrated a preference for the nutrients which were seen to increase in antibiotic-treated faecal culture, the commensal consortium was inoculated into gut commensal minimal media supplemented with the antibiotic-

elevated nutrient mix (29 nutrient sources). Data from samples taken 4.5, 9 and 24 hours post inoculation were compared to the data from the samples taken at inoculation (**Figure 40**). The time points were selected to correspond with the mid-exponential, early stationary, and late stationary phase of the VRE strain growth on the same nutrient mix (seen in Chapter 4).



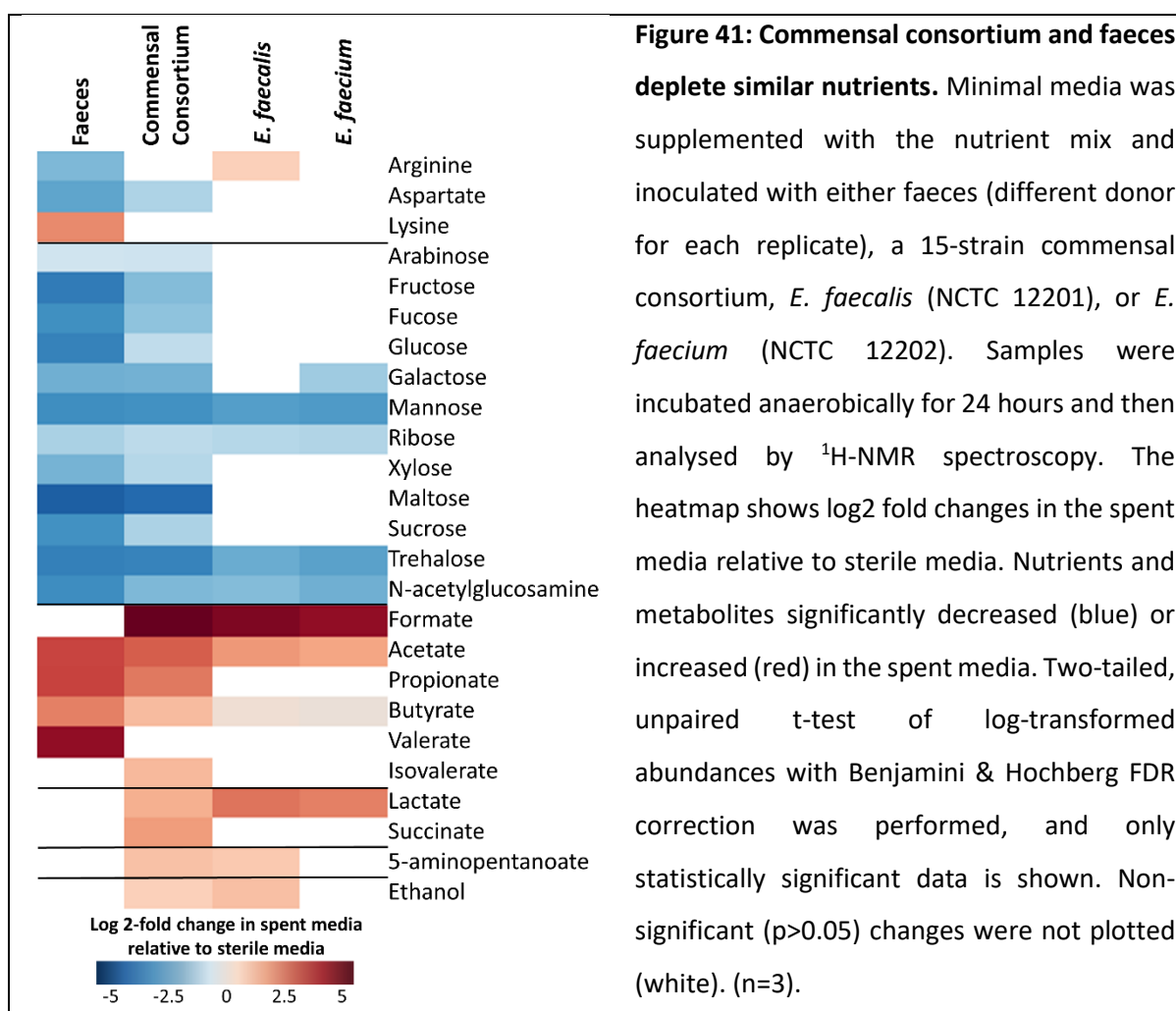
In total, all sugars in the antibiotic-elevated carbon mix were shown to change in concentration over the incubation period. Eight monosaccharides (arabinose, fructose, fucose, galactose, glucose, mannose, ribose, and xylose), three disaccharides (maltose,

sucrose, and trehalose), and N-acetylglucosamine were reduced in concentration by the commensal consortium by the final time point 24 hours post inoculation. Arabinose, galactose, glucose, and xylose were nearly completely depleted from the media. Meanwhile, ribose, sucrose and trehalose were only partially depleted. Nutrients not shown were not decreased to a statistically significant degree over the course of the incubation.

Nutrient depletion by a commensal consortium suppresses the growth of VRE strains

Next, knowing that antibiotic-naïve and FMT-treated antibiotic compromised faecal culture successfully inhibited the growth of VRE in the artificial gut chemostat, the nutrient utilisation of the commensal consortium in the gut commensal minimal media supplemented with the nutrient mix was compared to that of a healthy faecal microbiota, and also to *E. faecalis* or *E. faecium* grown in pure culture. The cultures inoculated with the commensal consortium, faeces, or each VRE strain were incubated for 24 hours before the final nutrient concentrations were compared to sterile supernatant using ¹H-NMR spectroscopy.

The cultures inoculated with faeces and the commensal consortium had a very similar nutrient profile following incubation (**Figure 41**). The concentration of all 12 sugars was significantly decreased to comparable levels. Only two of the amino acids showed significant decreases in the inoculated cultures. Both arginine and aspartate were decreased in concentration in the culture inoculated by faeces whereas only aspartate was decreased by the commensal consortium. Both *E. faecalis* and *E. faecium* were shown to use mannose, ribose, trehalose and N-acetylglucosamine. *E. faecium* also consumed galactose during the incubation. Thus, all nutrients used by the VRE strains during incubation were also used by the commensal consortium and the faecal microbiota.



The $^1\text{H-NMR}$ spectroscopy data showed that both faecal- and commensal-inoculated culture produced acetate, propionate, and butyrate (**Figure 41**). The commensals also produced formate, isovalerate, lactate, succinate, 5-aminovalerate, and ethanol. Valerate was produced in the faecal-inoculated culture but not in the commensal-inoculated culture. The two VRE strains were shown to produce formate, acetate, lactate and low levels of butyrate. *E. faecalis* also produced low levels of 5-aminovalerate.

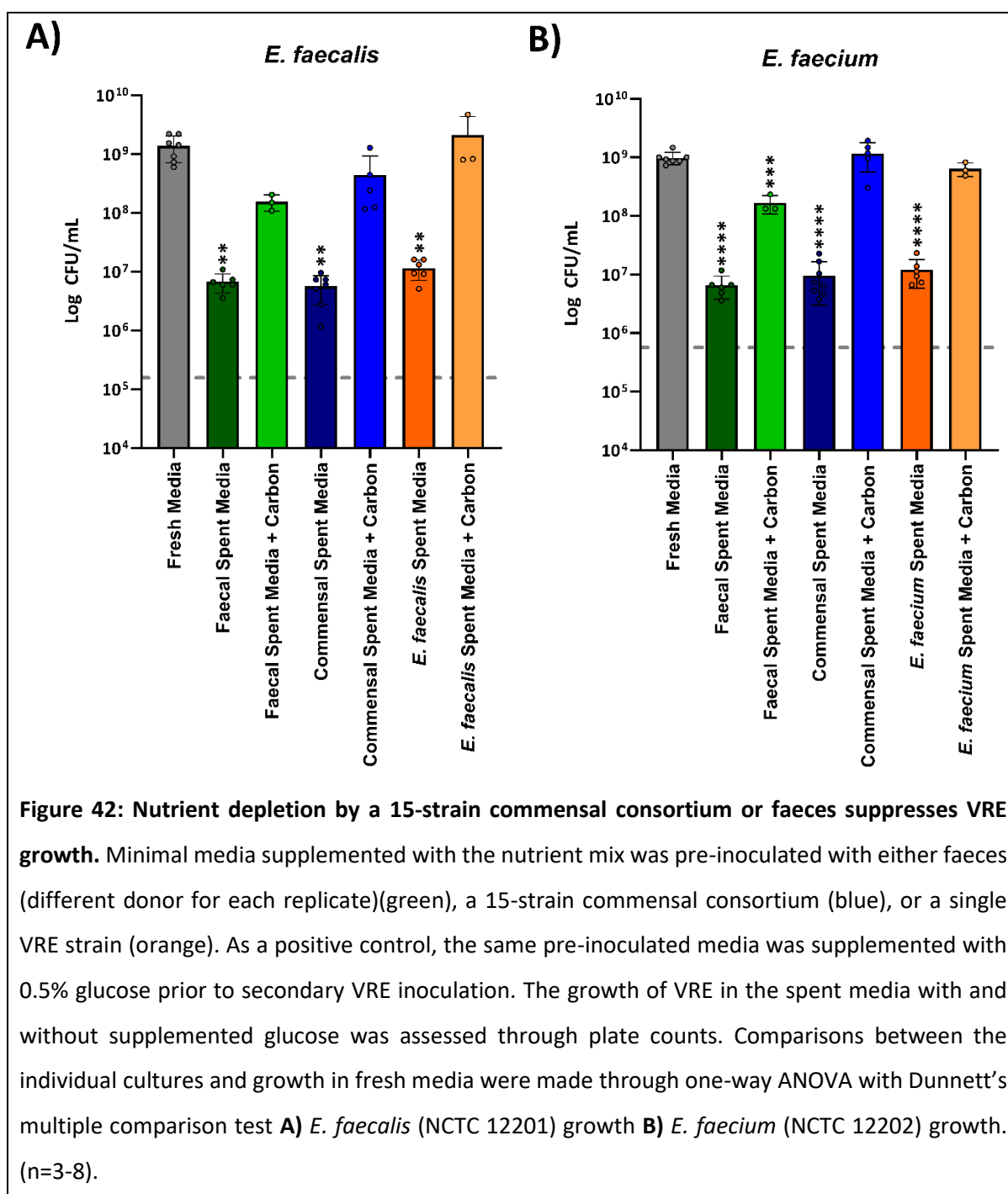
Next, VRE growth was measured in the spent (nutrient depleted), sterilised supernatant from the commensal consortium, faeces, *E. faecium*, and *E. faecalis*. The faeces acted as a control to show the impact of the entire faecal microbiota on the nutrient availability. *E. faecium* and *E. faecalis* acted as a control for the depletion of nutrients known to be used by each isolate

- if the VRE consumed all the nutrients that would support their growth, the same strain would likely not be able to grow in the spent media. After the initial incubation, the pH of the cultures was adjusted to 7 and filter sterilised so only the sterile spent supernatant was used to culture the VRE strains.

The growth of the VRE in the spent supernatant was measured using plate counts (**Figure 42**).

In fresh sterile media that was not incubated with any bacteria, *E. faecalis* and *E. faecium* grew to 10^9 CFU/mL. *E. faecalis* only grew to around 10^7 CFU/mL in spent media initially inoculated with faeces ($p=0.0053$), the commensal consortium ($p=0.0036$), or *E. faecalis* ($p=0.0055$)(**Figure 42A**). Similarly, *E. faecium* only grew to around 10^7 CFU/mL in spent supernatant initially inoculated with faeces ($p<0.0001$), the commensal consortium ($p<0.0001$), or *E. faecium* ($p<0.0001$)(**Figure 42B**). *E. faecalis* and *E. faecium* growth was significantly reduced in all of the spent cultures compared to growth in the fresh media.

The same spent supernatant was supplemented with 0.5% glucose prior to VRE inoculation to determine whether the impact of the spent supernatant on VRE growth was due to nutrient depletion or metabolite inhibition. A two-tailed unpaired, parametric t-test was used to assess the difference in growth of supernatant with and without glucose (not shown in **Figure 42**). For *E. faecalis*, there was a statistically significant increase in growth with the addition of glucose to the spent supernatant from faeces ($p<0.0001$), the commensal consortium ($p=0.0360$), and *E. faecalis* ($p=0.0424$) compared to without the addition (**Figure 42A**). Likewise, the addition of glucose increased the growth of *E. faecium* in supernatant spent by faeces ($p=0.0002$), the commensal consortium ($p=0.0002$), and *E. faecium* ($p<0.0001$)(**Figure 42B**).



In the faecal spent supernatant supplemented with glucose, the growth of the *E. faecalis* ($p=0.0656$, not significant) and *E. faecium* ($p=0.0002$) was still lower than that in fresh media, suggesting the spent supernatant still contained some inhibitory substances. However, in both the commensal consortium spent supernatant supplemented with glucose and the VRE spent supernatant supplemented with glucose, the growth of the VRE strains was not

significantly different to in fresh media, suggesting that nutrient depletion was responsible for restricting VRE growth.

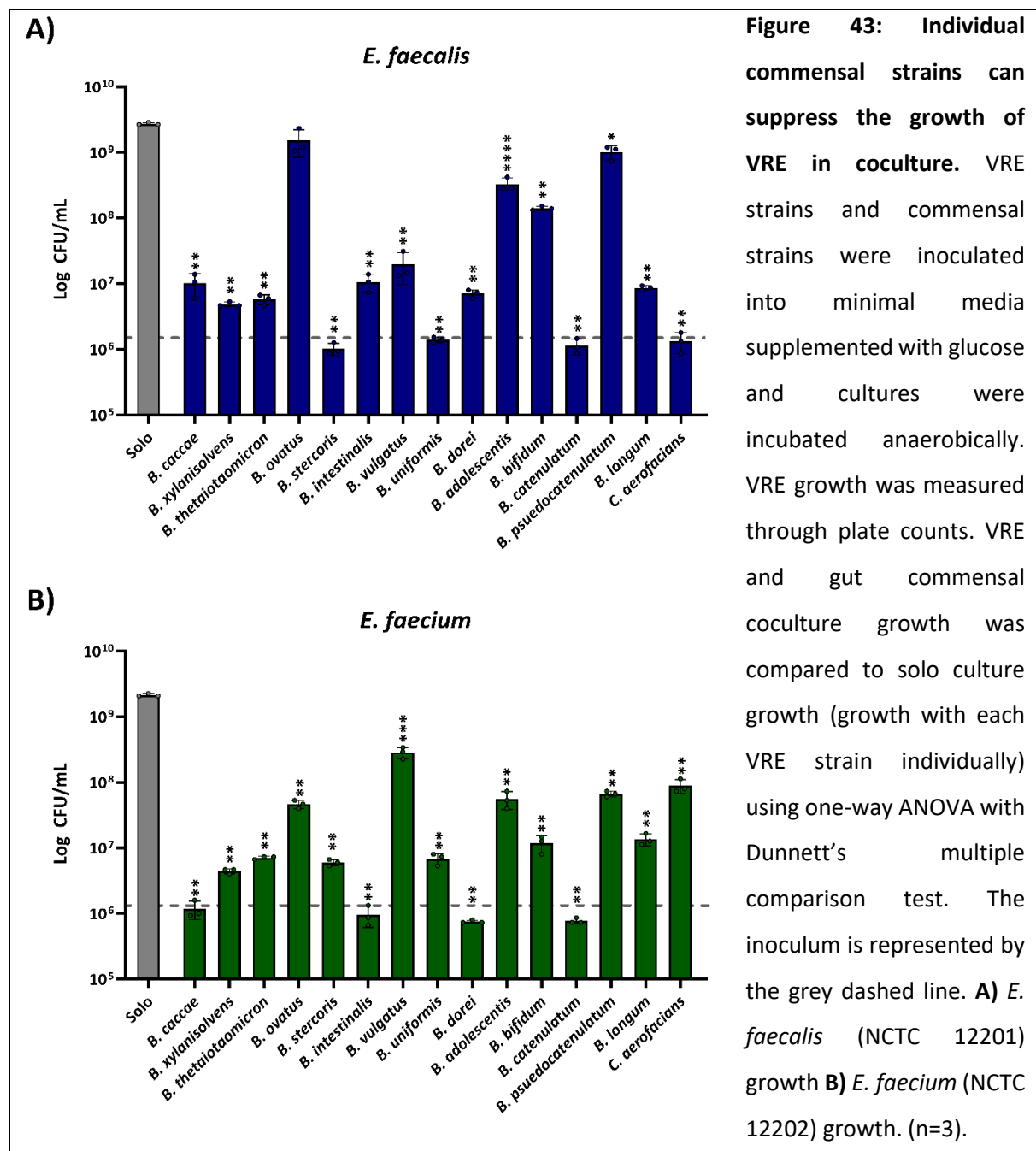
Individual commensal species outcompete VRE for limited nutrients in coculture

Since the mix of 15 commensals indirectly suppressed VRE growth by nutrient depletion in a spent supernatant, direct competition between each commensal strain and the VRE strains was assessed to determine which commensal strains were contributing most to VRE suppression and if any could be eliminated from the consortium in future research. The commensal strains had longer lag times compared to the VRE strains. As such, the commensals were inoculated prior to the VRE so that both strains reach mid-exponential phase simultaneously [629, 630]. The minimal media was supplemented with 0.5% glucose as the carbon source and a mixture of amino acids as the nitrogen sources.

Direct competition between *E. faecalis* and the individual commensal strains showed a statistically significant decrease in VRE growth when incubated with all 15 commensal strains ($p < 0.0001$ for all comparisons)(**Figure 43A**). The lowest growth was seen from incubation with *B. stercoris* (3.43 log decrease, $p = 0.0026$), *B. catenulatum* (3.38 log decrease), *C. aerofaciens* (3.31 log decrease), and *B. uniformis* (3.29 log decrease), in which the VRE strain did not grow to a greater density than was inoculated. *B. xylanisolvens* (2.75 log decrease), *B. thetaiotaomicron* (2.67 log decrease), *B. dorei* (2.58 log decrease), *B. longum* (2.5 log decrease), *B. caccae* (2.43 log decrease), *B. intestinalis* (2.41 log decrease) and *B. vulgatus* (2.14 log decrease) significantly suppressed the growth of *E. faecalis*. Coculture with *B. bifidum* (1.28 log decrease), *B. adolescentis* (0.92 log decrease), and *B. pseudocatenulatum* (0.43 log decrease) produced a significant decrease in VRE growth, but to a lesser degree than

other strains tested. Coculture with *B. ovatus* (0.25 log decrease) was statistically significant but the growth was less inhibited than by any other cocultured strain.

E. faecium was significantly inhibited by all 15 commensals when cocultured ($p < 0.0001$ for all comparisons) (**Figure 43B**). The lowest growth, compared to that achieved in solo culture, was seen from incubation with *B. dorei* (3.45 log decrease), *B. catenulatum* (3.44 log decrease), *B. intestinalis* (3.35 log decrease), and *B. caccae* (3.26 log decrease), in which the strain did not grow in the culture. *B. xylanisolvens* (2.68 log decrease), *B. stercoris* (2.55 log decrease), *B. uniformis* (2.49 log decrease), *B. thetaiotaomicron* (2.48 log decrease), *B. bifidum* (2.26 log decrease), *B. longum* (2.20 log decrease), *B. ovatus* (1.66 log decrease), *B. adolescentis* (1.58 log decrease), *B. pseudocatenulatum* (1.51 log decrease), *C. aerofaciens* (1.38 log decrease), and *B. vulgatus* (0.87 log decrease), suppressed the growth of *E. faecium* more than when the VRE strain was grown in single culture.



Treatment with a commensal consortium inhibited VRE colonisation in VAN-treated mice

A murine model was used to determine if the commensal consortium could protect against VRE colonisation in an environment which more closely replicates the conditions of the human gut microbiome. Two groups of mice were treated with vancomycin for seven days. For the following three days, the mice were either gavaged with saline or the commensal consortium. The next day, the mice were inoculated with vancomycin-resistant *E. faecium*.

The VRE counts were monitored by plating serial dilutions of mouse faecal pellets on Brilliance VRE agar.

One week after the VRE were inoculated, a significant difference in VRE proliferation in the group treated with the commensal consortium compared to the untreated group ($p=0.0159$) was seen (**Figure 44**). The average VRE growth in the commensal inoculated mice was reduced by 91.8% compared to that which was seen in the untreated mice. The data from one day post VRE inoculation also showed lower VRE counts in the mice treated with the commensal consortium compared to the untreated mice, however the difference was not statistically significant until seven days post-*E. faecium* inoculation.

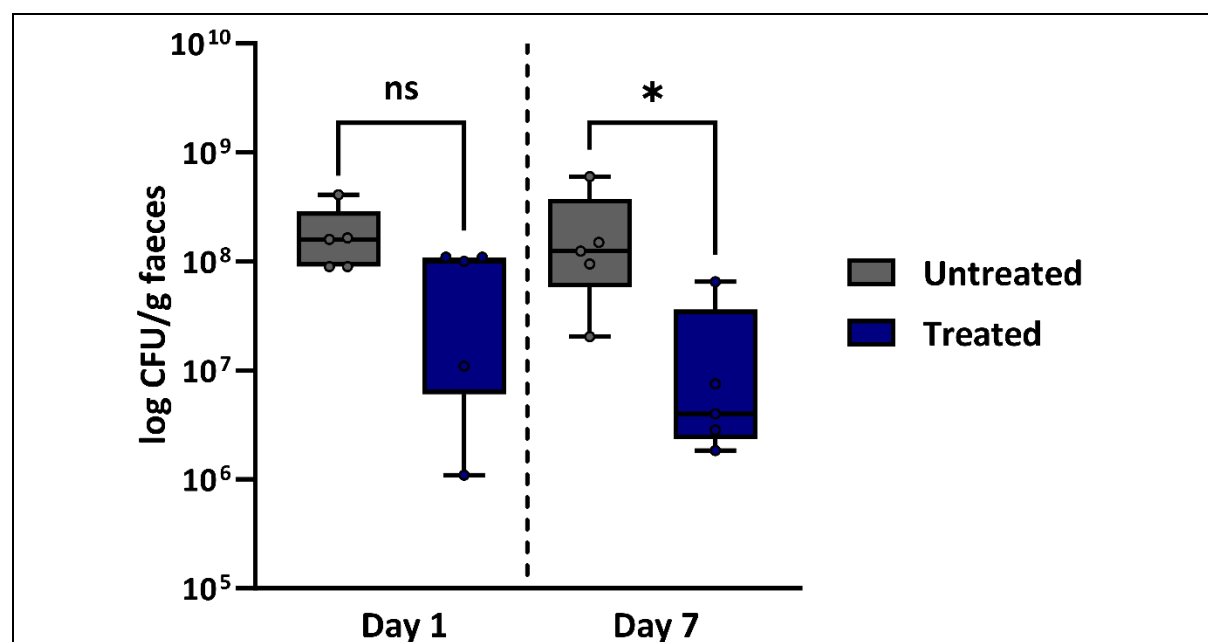
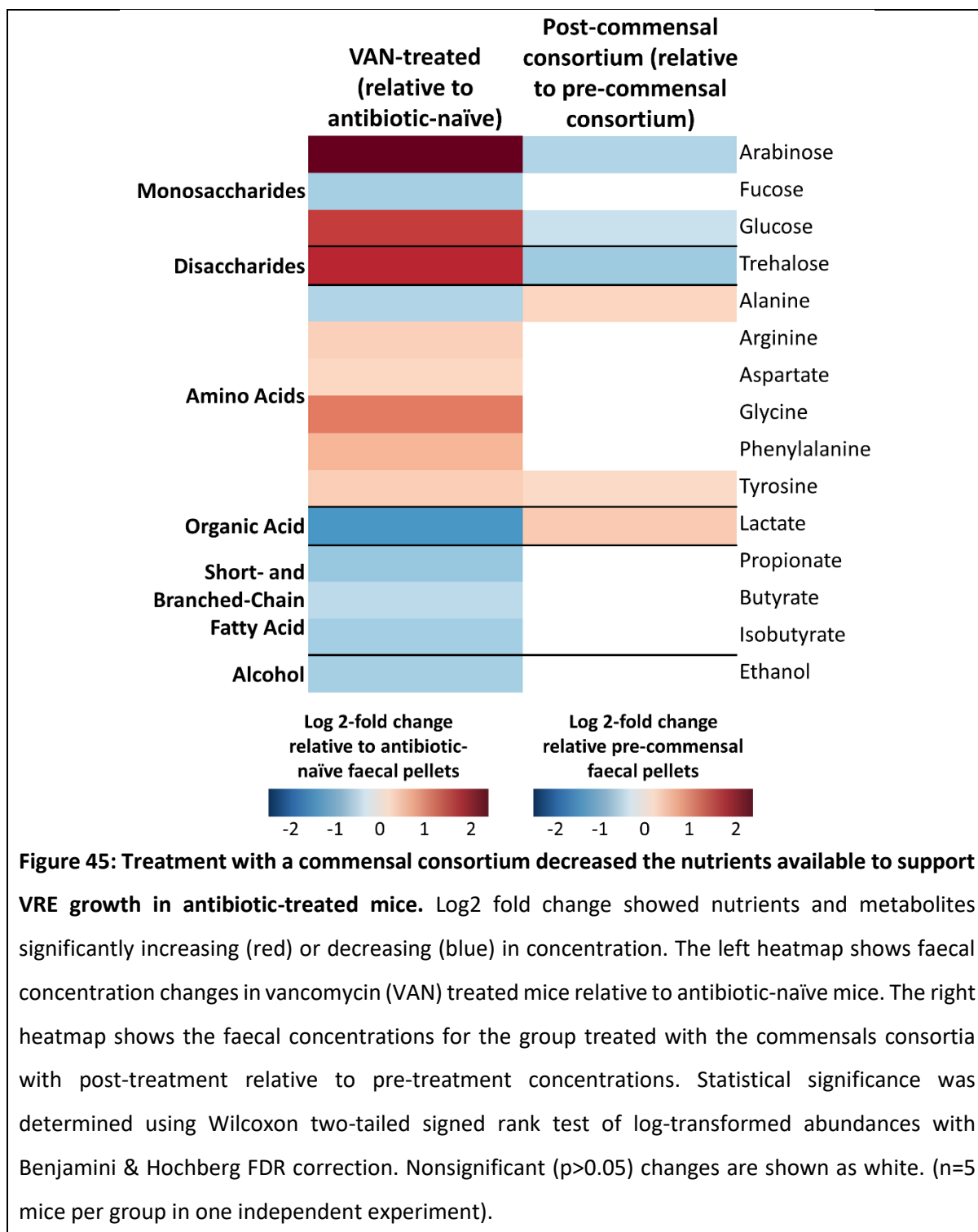


Figure 44: Mice treated with a commensal consortium showed suppression of VRE intestinal colonisation. *E. faecium* (NCTC 12202) counts from mouse faecal pellets with (blue) or without (grey) pretreatment with a probiotic commensal cocktail (containing 15 commensal strains) were compared in vancomycin treated mice on day 1 ($p=0.2143$) and day 7 ($p=0.0159$) post inoculation. The median is indicated by the line in the box and excludes the upper and lower quartile of data. The whiskers extend to the minimum and maximum values. Normality was assessed with a Shapiro-Wilk test before two-tailed, unpaired Mann-Whitney t-test were performed ($n=5$ mice per treatment).



The impact of the commensal consortium on the metabolic profile of the mouse faeces was assessed with ^1H -NMR spectroscopy (**Figure 45**). In antibiotic-treated mice, it was seen that the concentration of arabinose, glucose, arginine, aspartate, glycine, phenylalanine and

tyrosine increased relative to antibiotic-naïve mice. The nutrients, fucose and alanine, as well as the metabolites propionate, butyrate, isobutyrate and ethanol decreased in concentration. Comparing the samples collected before and after the commensal consortium was inoculated, the nutrients arabinose, glucose and trehalose all decrease in concentration. Alanine and tyrosine were seen to increase in concentration following commensal inoculation. Lactate was seen to be increased in commensal inoculated mice. No SCFAs or BCFAs changed following inoculation of the commensal consortium.

Discussion

As with many MDR pathogens, effective antibiotic treatments for VRE are becoming less and less reliable. Since the gastrointestinal system is the primary reservoir for VRE, effectively restricting VRE gut colonisation is essential to prevent the development of systemic—and potentially fatal—VRE infection. VRE, being an opportunistic pathogen, is more common in individuals with a perturbed gut microbiome, either due to disease or prior antibiotic treatment [55, 61, 64, 67, 68, 70, 71, 75, 201-203, 579-583]. While FMT has been shown, in both previous studies and the artificial gut chemostat model data presented in Chapter 5, to restore gut microbiota diversity and suppress VRE growth [261, 431, 606-610, 612, 613], the untargeted nature of FMT exposes the recipient to risk of transmission of an opportunistic pathogen [429, 432-435]. A targeted microbiome therapeutic, which is well characterised and capable of restoring colonisation resistance, could provide a safer, more effective treatment for VRE gut colonisation.

For the first time, data presented in this chapter showed successful inhibition of VRE strains by a 15 commensal consortium which was able to outcompete the opportunistic pathogen for key nutrient sources. The commensal genera used in the consortia were selected based

on data from Chapter 3 and Chapter 5 which indicated that these key commensals were depleted by antibiotic usage and could be critical to colonisation resistance against VRE. Validating the *in vitro* findings from this and previous chapters in an *in vivo* mouse model provided key justification for the importance of exploring nutrient competition based microbiome therapeutics in limiting the risk of VRE colonisation in the gut.

Identifying individual or groups of commensal organisms capable of suppressing the growth of a pathogen through the restoration of nutrient competition has been discussed in previous literature. One group designed a synthetic stool containing 33 commensal species which cured recurrent *C. difficile* infections in two patients [440]. Combinations of commensal *E. coli* strains have been repeatedly shown to outcompete pathogenic *E. coli* O157:H7 for essential proline and sugars [385-387]. Likewise, the suppression of *S. ser.* Typhimurium by commensal *E. coli* strains was achieved due to the depletion of iron and zinc reservoirs by the commensal [388, 389]. Isaac et al. demonstrated that a combination of *Alistipes*, *Barnesiella*, *Olsenella*, *Oscillibacter* and *Flavonifractor*, suppressed VRE growth in a murine model by depleting fructose [456].

Fifteen commensal strains were selected to act as a commensal consortium capable of replicating the inhibitory properties of healthy faeces, including nine *Bacteroides* species, five *Bifidobacterium* species, and *C. aerofaciens*. *Bacteroides* species are known primary degraders in the gut microbiome [501, 502] while *Collinsella* and *Bifidobacterium* are secondary degraders which consume monosaccharides, disaccharides and amino acids [503-505]. *Bifidobacterium* and *Bacteroides* have been shown to participate in crossfeeding in the gut microbiome [506], thus it was theorised that a combination of the two genera in the

consortium would promote a more complete nutrient depletion of both complex and simple nutrients.

Probiotic commensal cocktails aim to restore normal gut microbiome function by seeding in specific commensal species that are already commonly found in the healthy gut microbiota and are capable of suppressing the growth of opportunistic pathogens [625, 631].

Commensal consortia selected for probiotic therapeutics are most commonly Gram-positive species which lack the inflammatory lipopolysaccharide component found in Gram-negative strains [632-635]. Many of the families decreased with antibiotics, including

Bifidobacteriaceae and *Coriobacteriaceae* are Gram-positive and thus made good candidates for a biotherapeutic commensal consortium.

Members of the commensals consortium tested in this chapter have previously been linked with the suppression of other pathogens. Protection against *E. coli* infection in mice has been correlated with high abundance of *Bifidobacterium* [472-474]. *B. longum* has been shown to reduce the pH of the gut through the production of SCFAs [475-477, 557]. One recent study postulated that this species was capable of suppressing VRE growth through production of SCFAs and, to a lesser extent, the competitive consumption of lactulose [557]. Additionally, the decrease in pH of the microenvironment was seen to suppress virulence factor gene abundances. As such, *B. longum* has been administered to infants to prevent infection in clinical research [475-477].

B. longum and *B. thetaiotaomicron* were linked with improving colonisation resistance indirectly by promoting immune mechanisms, including increasing the expression of type I - IFN induced GTPases, downregulating antimicrobial peptide expression and enhancing the expression of REGIII β and REGIII γ in murine studies [295, 636]. Directly, *B. thetaiotaomicron*

has been shown to repress *C. rodentium* by outcompeting the pathogen for monosaccharides [276].

B. adolescentis effectively suppressed the growth of *Candida albicans* in coculture due to the strains ability to produce acetate and lactate [637, 638]. *C. aerofaciens* was shown to negatively correlate with CRE colonisation in the human gut [478]. Members of the *Bacteroidaceae* family have shown to suppress growth of *S. ser. Typhimurium* [367], *C. difficile* [458], and *K. pneumoniae* [479]. *B. dorei*, in particular, has been shown to suppress *C. difficile* by inhibiting biofilm formation [639].

In Chapter 3 and Chapter 5, antibiotic treatments were shown to decrease the microbiota—consequentially increasing the abundance of nutrients which support VRE growth. Thus, the ability for the commensal strains to utilise these nutrients was pivotal to their potential application. The 15 commensal strains assessed all were shown to consume multiple nutrients which VRE rely on for growth. Some of the nutrient utilisation results were supported by previously published studies; Glucose utilisation by *B. adolescentis*, *B. bifidum*, *B. catenulatum*, *B. intestinalis*, *B. pseudocatenulatum*, *B. thetaiotaomicron*, and *B. vulgatus* was shown in a study by Salli et al. [640]. *B. ovatus* was shown to grow in media supplemented with glucose, maltose, sucrose, and trehalose [641]. *Bifidobacterium* species have been shown to utilise many of the monosaccharides which make up human milk oligosaccharides including glucose, galactose, and N-acetylglucosamine [642-645].

It was important that no nutrient was consumed by only a single strain. Functional redundancy has been previously described as an important feature of the gut microbiome [215, 471]. The shared nutrient utilisation profiles of the commensal consortium aimed to ensure full depletion of all available nutrient sources that could support VRE growth.

In two different experiments, it was demonstrated that the commensal consortium reduced the availability of sugars in the gut commensal minimal media supplemented with the nutrient mix. In the first experiment, where samples were taken throughout the incubation period, the commensals reduced the concentration of the mono- and disaccharides in the mix.

Samples from the commensal coculture were taken at 4.5, 9 and 24 hours post inoculation as these were relevant phases of growth for the VRE strains. In Chapter 4, it was shown that the VRE strains reduce the concentration of nutrients as early as 4.5 hours post inoculation. In the commensal cocultures, the decrease in nutrient concentration was only statistically significant at the final timepoint because the commensal consortium had a longer lag phase. This presented issues for simultaneous coculturing of the VRE strains with the commensal consortium as the VRE would grow faster and deplete nutrients quicker than the commensals. In Chapter 4, the growth rate of the VRE strains were seen to vary depending on which nutrient was supplemented. Thus, comparisons of the lag times for the VRE strains and the commensals had to be assessed based on which nutrient(s) were provided in the media. One published study showed that controlling the dilution, and thus the growth rate, of commensal organisms had a significant impact on nutrient utilisation [629]. Thus, to culture the commensals with the VRE strains, a delay in inoculation of the VRE strains was required. Staggering the inoculation so the commensal and the pathogen reached mid-exponential phase simultaneously, could allow the commensals to actively compete for nutrients with the faster growing VRE strains.

Before direct coculture competition was performed, the impact of nutrient depletion on the VRE growth was assessed by growing the VRE strains in spent supernatant. Media

supplemented with the nutrient mix was inoculated with faeces, the commensal consortium, or each VRE strain to better understand how nutrient utilisation differed under each condition. As hypothesised, the nutrient depletion by the commensal consortium was very similar to that of the faecal microbiota. No nutrient depleted by the VRE strains was not also depleted by the commensal consortium. *Bacteroides* and *Bifidobacterium* species have been shown to produce SCFAs which likely accounts for the increased concentration of formate, acetate, propionate, and isovalerate [283, 328, 513, 520-522, 526]. As neither *Bifidobacterium* nor *Bacteroides* species are known producers of butyrate [523, 646], it is possible the butyrate is produced through a crossfeeding interaction. Further experiments would need to be conducted to determine which species play a part in the butyrate production.

In the culture inoculated with the VRE strains, fewer nutrients were seen to be depleted compared to the nutrient preference assay performed in Chapter 4. Most notably, the VRE strains were not shown to deplete fructose, glucose, maltose or sucrose when cultured in the gut commensal minimal media as they were shown to in the VRE minimal media. This indicated that the VRE switch their nutrient utilisation preferences depending on their environment. Similar media-based metabolic preferences have been shown for *E. coli* and *Saccharomyces cerevisiae* which suggested that bacteria can change their metabolism to suit different nutritional contexts [647].

In order to determine if the commensal consortium was capable of restricting VRE growth as effectively as the full faecal microbiota, spent supernatant from the commensal consortium or faeces was used to culture the VRE strains. The VRE strains cultured in the commensal consortium spent supernatant were inhibited to the same extent VRE cultured in the faeces

spent supernatant. Following the initial incubation with the faecal, commensal or VRE inoculum, the spent media was seen to be acidified (pH between 4.2-6). This is due to the production of SCFAs following nutrient consumption [396, 514, 515]. The pH of the spent supernatant was adjusted to 7 prior to inoculation with the VRE strain to ensure that any suppression of growth was due to the nutrient depletion rather than the acidic pH. Since the spent supernatant was filter sterilised, the inhibition was due to metabolic changes to the media rather than contact-dependent mechanisms.

Additional experimental test conditions were necessary to determine whether VRE growth inhibition in spent supernatant was due to nutrient depletion or the production of an inhibitory substance. Microbial metabolites negatively correlated with VRE growth in the artificial gut chemostat and, to a lesser extent, the murine study (Chapter 5). However, data from Chapter 4 showed minimal impact of inhibitory metabolites on VRE growth in solo culture. When glucose was supplemented into the spent supernatant from the commensal consortium, the VRE growth was not statistically different to that in fresh media. This indicated that the suppression seen in the corresponding spent media was due to nutrient depletion. *E. faecium* growth in faeces spent supernatant that was supplemented with glucose was significantly increased compared to the faeces spent supernatant without additional glucose but was still significantly lower than growth in fresh media.

Similar levels of SCFA and BCFA concentrations were seen in spent supernatants from the faecal- and commensal-inoculated cultures. Therefore, it is unlikely that metabolite inhibition with these metabolites was responsible for the difference in growth of these two cultures. Members of the faecal microbiota could be producing bacteriocins or other inhibitory substrates which suppress the growth of the strains even with supplemented

nutrients. Previous research has shown that *Blautia* species are capable of producing a lantibiotic which suppresses VRE growth in murine studies [559]. Additionally, phages present in the faecal microbiota could impact the VRE growth as has been shown in numerous studies [648-650].

After the successful inhibition of the VRE strains in commensal spent supernatant, the VRE strains were cocultured with each individual commensal strain. Glucose was supplemented into the media to assess the competition between the VRE and the commensals for a single nutrient which all the strains were shown to use as a carbon source. In order to overcome the slower growth of the commensal strains, the commensal was added first to line up the two species at mid-exponential phase. This was used as a successful modelling strategy for evaluating direct competition between VRE and *B. longum* in previous research [557]. This experiment was designed with the intention of determining if any commensal strains were unable to outcompete VRE for nutrients thus rendering the strain potentially superfluous in the commensal consortia. All of the commensals reduced the growth of *E. faecium*, and all commensals except *B. ovatus* suppressed the growth of *E. faecalis*. *E. faecalis* was completely suppressed by five strains while *E. faecium* was completely suppressed by four strains. Due to the successful depletion of one or both of the strains by each commensal, no species was eliminated from the consortia at this stage in the research. While some strains were less effective at restricting the VRE strain growth, complex cross-feeding relationships between the commensal consortia will have to be performed if the selected strains were to be optimally refined for clinical application. Future research could expand on this by supplementing different carbon sources into the cocultures.

Growth of the VRE strains was seen to be lower in the individual coculture experiments than in the commensal consortium spent supernatant. This could be due to the pH of the culture at the time of the VRE strain inoculation. The pH of the spent supernatant was adjusted to 7 before the VRE strains were inoculated. In the cocultures, the pH could not be tested and adjusted without risking killing the commensal already in the media. Future research could identify the pH of the culture at the time of VRE inoculation to determine if this negatively impacted growth.

Verifying the potential for the commensal consortium to function as a microbiome therapeutic to restrict VRE growth *in vivo* required testing of this consortium in a mouse model. Following VAN treatment, one group of mice was inoculated with the commensal consortium while the other was inoculated with saline as a control. The commensal-inoculated mice showed lower counts of VRE than the saline-treated mice. This was likely due to the decrease in arabinose, glucose and trehalose concentrations in the mouse gut. Although no nitrogen sources were seen to decrease in the mouse faecal culture following commensal inoculation, the depletion of carbon sources necessary for VRE growth could be sufficient to prevent colonisation.

Fewer sugars were shown to decrease in the mouse faecal culture than in the previous *in vitro* experiments in this chapter. This is likely due to the presence of the mouse microbiota which will influence nutrient depletion differently to the commensal consortium on its own or the human faecal microbiota used in the spent supernatant assay, the artificial gut chemostat model (Chapter 5) or the faecal batch culture (Chapter 3, Chapter 5). This has been shown in previous studies which show differences in the microbiota of humans and mice [623]. One study found that, although both mouse and human microbiota are made up

of predominantly members of the Bacteroidota and Bacillota phyla, 85% of the bacteria found in the mouse microbiota are not found in humans [651]. While germ free mice inoculated with human gut microbiota cultures from faeces could offer a valid avenue for exploration, Imperial College London does not at this time have germ-free facilities thus collaboration with another institution would be necessary.

In Chapter 3 and Chapter 5, VAN treatment was shown to elevate the concentrations of sugars and amino acids in the gut while decreasing the concentration of organic acids and ethanol. Greater nutrient competition in the mouse microbiome may have been responsible for the decrease in VRE growth between the treated and untreated groups. To further explore the potential application of the commensal consortia on VRE suppression, future research should identify whether nutrient competition is, in fact, the primary reason for VRE suppression in the mouse model. This could be accomplished by homogenising faecal pellets from mice which have been treated with VAN and inoculated with the commensal consortia in saline. The faecal culture would need to be filter sterilised and then supplemented with water or glucose. If the VRE strains failed to grow in the faecal culture supplemented with water but grew in the culture supplemented with glucose, then the VRE are likely suppressed by nutrient competition in the mouse model.

In vivo analysis provided far greater obstacles for assessing the efficiency of the commensal consortium compared to *in vitro* experiments. As all members of the commensal consortium were obligate anaerobes, the transfer from the anaerobic chamber followed by the exposure to oxygen during the inoculation into the mice could have resulted in decreased viability of commensals before the commensals even reached the gastrointestinal system. To reach the distal gut, the commensals needed to travel through the upper GI tract which

has significantly lower pH conditions than the commensals encountered *in vitro* assays. Once in the distal gut, the commensal consortium had to compete with the existing microbiota in order to establish growth great enough to impact VRE colonisation. It has been suggested that some probiotics fail to engraft into the host due to bacteriophages and bacteriocins already present in the microbiome of the host [652]. However, one study found that pre-treatment with antibiotic promoted the establishment of *Bifidobacterium* in the host microbiota [653]. Since 16S rRNA gene sequencing was not performed on murine faecal samples due to the amount of faeces that was required for ¹H-NMR spectroscopy and plate counts, we did not measure the degree to which the commensals engrafted themselves into the murine microbiome. Future research should incorporate 16S rRNA gene sequencing into the analysis of the faecal samples of the murine experiments to identify taxonomic changes with the different treatments.

The inoculum of the commensal consortium was based on previous research [455]. Further experimentation to optimise the inoculum could improve the engraftment of the consortium into the murine gut and thus the suppression of VRE colonisation.

Li et al., designed a probiotic mix containing *Lactobacillus murinus* and *Lactobacillus plantarum* which was shown to suppress VRE growth in a murine study [654]. The *Lactobacillus* strains promoted the growth of members of the Bacillota phyla but had little impact on the Bacteroidota phyla. Contrary results have been reported on the efficacy of *Lactobacillus* strains against VRE colonisation in humans with some studies showing decreased density of VRE while others showed no change [655-658].

In order to increase the physiological relevance of the assessment of the commensal consortium, an artificial gut chemostat model could be incorporated into future study.

Comparing parallel vessels which receive either no treatment, FMT treatment, or inoculation with the commensal consortium could allow better understanding of how these commensals incorporate themselves into existing faecal microbiota. Once the commensal consortia was optimised in the artificial chemostat model, follow up mouse model experiments could be designed based on the results of the *in vitro* experiments.

In this chapter, gut commensal minimal media supplemented with a single carbon source or the nutrient mix was used. The previously described VRE minimal media could not be used as it did not support the growth of all 15 commensal strains. The media used in the artificial gut chemostat model, DGGM, could have been used as it supported the growth of the commensal strains and both VRE strains. This media would have served to increase the physiological relevance of the experiment in replicating the nutrients available in the distal gut. However, this media contained complex substrates which could not be evaluated with ^1H -NMR spectroscopy. Without knowing how these complex nutrients were changing in the inoculated cultures, exact understanding of how the nutrient concentrations impacted VRE growth could not be assessed. An ongoing collaboration between the McDonald group and the National Phenome Centre to develop a mass spectrometry protocol for identifying monosaccharides, disaccharides, and polysaccharides will improve the assessment of how sugars and complex nutrients interact with the microbiota and VRE growth.

The development of new microbiome therapeutics is urgently needed to prevent or treat VRE intestinal colonisation before the development of more difficult-to-treat invasive VRE infections. While further research should be performed to verify the efficiency of the commensal consortium against VRE in various models, the initial results show potential for this microbiome therapeutic. For the first time we show that a mixture of *Bacteroides* spp.

Bifidobacterium spp., and a *Collinsella* sp. inhibited the growth of *E. faecium* and *E. faecalis*.

Although this is just the first step to developing a microbiome therapeutic to restrict VRE intestinal colonisation, these results highlight the feasibility of such a therapeutic and lay the groundwork for an optimised consortium that could be further tested in *in vivo* tests or clinical trials

Chapter 7: Conclusion

Summary

Antibiotic usage has increased exponentially in the past century and is still rising at an astonishing rate globally [32, 36]. Between intentional consumption (direct treatment or preventative care) and indirect exposure (agricultural additives and environmental contamination), the elevated contact with antibiotics poses a serious threat to individual and global health [32-34, 42-49]. The prevalence of MDR pathogens is directly linked to increased antibiotic reliance—thus fostering a dangerous system in which the more antibiotics are used, the more they are needed [5, 53]. The clinical obstacles imposed by MDR pathogens lead to increased duration of hospital stays, cost of treatment, rate of transmission, risk of complications, and likelihood of fatality [25, 26, 56, 61, 63, 65-70].

A major consequence of antibiotic exposure is the negative impact to the gut microbiome. The gut microbiome provides colonisation resistance to restrict MDR pathogen growth, which in turn prevents the development of difficult-to-treat invasive infections [56-58]. Data in this study supported previously established conclusions that antibiotic treatment negatively impacts the diversity and density of the gut microbiota, leading to increased susceptibility for colonisation by opportunistic pathogens such as VRE [55, 57, 58].

Treatment with five antibiotics resulted in significant reshaping of faecal microbiota. VAN, MTZ, CRO, CLI and TZP decreased the abundance of key bacterial phyla including Bacillota, Bacteroidota and Actinomycetota. Members of these phyla have been linked to several aspects of colonisation resistance including production of inhibitory metabolites [283, 328, 513, 520-522, 526, 541] and the depletion of nutrients [30, 501-506]. The antibiotic induced changes to the metabonomic and taxonomic profiles of the gut microbiota was evaluated

through the use of both ^1H -NMR spectroscopy and absolute abundance corrected 16S rRNA gene sequencing. The trends were confirmed in established microbiological culturing models including *ex vivo* faecal batch culture models [30, 542-544], *in vitro* artificial gut chemostat models [376, 403, 444, 454], and *in vivo* mouse models [30, 55, 67, 71, 75, 183]. By utilising a variety of culturing techniques, the findings were verified and both microbe-to-microbe and microbe-to-environment interactions were viewed with various degrees of physiological relevance.

The different culturing models were able to highlight different elements of the interaction between antibiotics and the gut microbiota. In faecal batch cultures, a positive correlation was seen between the abundance of commensal bacterial families and the concentration of SCFAs, BCFAs, organic acids and alcohols. Conversely, a negative correlation was seen between these families and the concentration of monosaccharides, disaccharides, and amino acids. In the artificial gut chemostat model, VAN treatment significantly decreased Bacteroidota and Bacillota abundance and diversity while allowing Pseudomonadota to bloom. This consequently decreased the concentration of inhibitory metabolites in the vessel cultures and caused an increase in nutrient concentration which was not seen in antibiotic-naïve artificial gut chemostat culture. Restoration of the diversity of the commensal community through FMT treatment reversed the taxonomic changes in the culture. Similarly, in the murine study, nutrient concentrations increased in the faeces of VAN-treated mice while metabolite concentrations decreased. When a commensal consortium of 15 commensal strains (*Bacteroides*, *Bifidobacterium* and *Collinsella* species) negatively impacted by antibiotic treatment was compared to the full faecal microbiota, the commensal

consortium was shown to reduce the concentration of nutrients and produce SCFAs to a comparable degree.

The consistency of the trends shown in these models supported the hypotheses that 1) antibiotic treatment decreases gut microbiota-mediated colonisation resistance by killing members of the microbiota, which are responsible for producing metabolites and competing for essential nutrients and 2) reestablishing of diversity and density to an antibiotic-compromised gut using key commensal species reversed the impact of the antibiotic treatment to the taxonomic and metabonomic faecal microbiota profiles.

The aim of this study was to identify direct mechanisms of colonisation resistance, and the microbial taxa involved in these mechanisms, that are responsible for restricting VRE growth in a healthy gut microbiome. As a common nosocomial pathogen, VRE is known to cause endocarditis, severe bloodstream, intra-abdominal and urinary tract infections, meningitis and wound infections [63-65]. However, the primary reservoir for VRE is the gut, and intestinal colonisation often precedes the development of invasive infections. Thus, it is important to understand how VRE colonises the gut in order to develop a microbiome therapeutic which can restrict VRE growth.

Initial evaluation focused on how antibiotics disrupt the gut microbiome and promote VRE growth. The nutrient utilisation abilities of *E. faecium* and *E. faecalis* were profiled to determine which of the nutrients (elevated in antibiotic-treated faecal cultures) could support the growth of these strains. In the gut, complex nutrient sources (including starch, inulin, pectin, xylan, arabinogalactan, mucin, peptone and yeast) are broken down into simple nutrient sources by primary degraders [30, 484, 485, 490, 491, 495-497, 501, 502]. The simple nutrients are consumed by secondary degraders [503-506]. Products of the metabolisms of

both primary and secondary degraders can act as nutrient sources (cross feeding) or act as inhibitory substances to other microorganisms in the gut.

Both VRE strains were shown to use a range of mono- and disaccharides as carbon sources. Growth of the VRE strains was not supported on a single nitrogen source tested. Due to amino acid auxotrophies associated with both VRE isolates that were tested in this study, a mix of amino acids was necessary to fulfil the nitrogen requirements and support VRE growth. The VRE strains demonstrated a preference for certain nutrients when provided with a mix of all the tested nutrient sources. The utilisation of these nutrients was influenced by the presence of other nutrients. For example, it was shown that sucrose did not support the growth of *E. faecium* on its own but when the strain was grown in media supplemented with 29 nutrients, sucrose was depleted by *E. faecium*. The same findings were shown for both VRE strains with regards to tyrosine which was utilised only in the presence of other nutrients. Similar findings have been seen with other opportunistic pathogens and indicate that the strains have the flexibility to switch their carbon and nitrogen utilisation based on availability of nutrients in their environment [30, 546, 553]. Further, the VRE strains were shown to be capable of using complex nutrients and metabolic intermediate substrates (i.e. pyruvate and succinate) known to be present in the gut to support growth. Therefore, this data supports the finding that VRE strains were capable of acting as both primary and secondary degraders in the gut microbiome.

SCFAs, BCFAs, organic acids and ethanol are products of metabolic activity in some members of the gut microbiota [11, 66, 391-395]. Since these metabolites have been shown to have inhibitory properties [391, 402, 403, 507, 508] and were shown to decrease with antibiotic treatment, their impact of VRE growth was assessed. The impact of a mix of these metabolites

on the growth of the VRE strains was seen to be dependent of the concentration and pH when the mix was prepared to model various physiologically relevant conditions. Only at the highest concentration and the lowest pH was the mix capable of inhibiting both strains. When assessed individually at low, average and high concentrations at a pH of 6.5, none of the metabolites inhibited the growth of the VRE strains. These findings indicated that nutrient competition is likely more of an obstacle to VRE colonisation in a healthy gut than metabolite inhibition.

Faecal cultures treated with an antibiotic or water were inoculated with VRE to simulate intestinal colonisation. The faecal batch cultures also showed that antibiotic treatment increased the susceptibility of the faecal microbiota to high level colonisation by VRE. Different antibiotics, shown to have varying impacts on the survival of both members of the faecal microbiota and the VRE strains, showed differing levels of VRE growth. In the artificial gut chemostat model, healthy microbial communities restricted vancomycin-resistant *E. faecium* growth. Meanwhile, in VAN-treated faecal cultures, vancomycin-resistant *E. faecium* was maintained at high levels for weeks following inoculation. However, recovery of gut commensals belonging to the Bacteroides and Bacillota phyla resulted in a decrease in *E. faecium* growth. In media pre-conditioned with faeces or the commensal consortium, nutrient depletion by the gut commensals was shown to prevent the subsequent growth of *E. faecalis* and *E. faecium*. The restoration of nutrient concentration completely reversed the inhibition of the VRE strains in the pre-conditioned medium from the commensal consortium. This indicated that the commensal consortium restricted VRE growth through nutrient depletion more so than through inhibitory metabolite production. The data from the mouse experiments also supported this finding. VAN-treated mice showed significantly higher *E.*

faecium counts in their faeces compared to saline-treated mice. Several nutrients which were elevated following VAN treatment were decreased after *E. faecium* inoculation in the mouse faecal pellets, indicating *E. faecium* utilised these nutrients to support its growth. Mice which were pre-colonised with the commensal consortium had lower VRE counts than those which had not received the commensal consortium. Since, like *E. faecium*, the commensal consortium was shown to deplete the availability of arabinose and trehalose in the mouse faecal pellets, it is likely that the decrease in the availability of these nutrients suppressed *E. faecium* growth.

Thus, the initial hypotheses were supported by the data. VRE were shown to exploit the decrease in nutrient competition caused by antibiotic-induced killing of members of the gut microbiota. While it remains unclear if the negative correlation between metabolite concentration and VRE density in complex culturing models is causational or correlational, it is not likely a viable mechanism for the reestablishment of colonisation resistance in the gut microbiota. Increasing nutrient competition through the inoculation of key *Bacteroides*, *Bifidobacterium* and *Collinsella* species was shown to suppress the growth of the VRE strains. This commensal consortium could act as the foundation of a probiotic cocktail capable of successfully reestablishing colonisation resistance in the gut microbiome and suppressing VRE growth without the use of further antibiotic treatments.

Limitations and future work

While diligent effort went into ensuring the research performed in the study was thorough in both its experimental design and analysis, there were limitations which could be addressed in future research. Perhaps the most significant limitation was the use of only two VRE strains. The *E. faecalis* and *E. faecium* strain were selected as representative VRE strains due to their

origin and the availability of preestablished selective and differential media. The two strains were isolated from the faeces of colonised humans [102, 445-448]. This was significant in that the strain has already been proven to grow in the human gut and thus would respond with physiological accuracy to the modelling techniques. The Brilliance VRE selective and differential media allowed the VRE strains to be easily and accurately quantified in coculture. Future research could expand the VRE strain library used in this study to confirm whether these findings are applicable to other clinical VRE isolates. Isolates collected from colonised patients and verified through whole genome sequencing would serve as good candidates to further this research. It would be beneficial to focus on collecting additional *E. faecium* strains rather than other vancomycin-resistant *Enterococcal* strains as *E. faecium* has become the most prevalent and difficult to treat VRE species [5, 578]. *Enterococcal* strains which do not have a VAN resistance gene could also be assessed to determine the link between antibiotic resistance and polymicrobial interactions in the context of the gut microbiota.

In this study, taxonomic analysis was performed with 16S rRNA gene sequencing. As such, only the bacterial members of the gut microbiome were identified, thus overlooking the potential impact of bacteriophages and fungi. However, as fungi have been shown to bloom in the gut following antibiotic treatment, they are unlikely to play a role in VRE suppression [659-662]. Future research could incorporate shotgun sequencing to provide a broader picture of the functional potential of the faecal microbiota and the organisms which interact with VRE during antibiotic treatment and gut colonisation.

As mentioned in previous chapters, the inability to accurately identify complex nutrients in the growth media with ^1H -NMR spectroscopy does limit the physiological relevance of some culturing techniques while providing an incomplete taxonomic assessment of others. The

McDonald lab has an ongoing collaboration with the National Phenome Centre which aims to develop a reliable mass spectroscopy protocol for the identification of polysaccharides. Additionally, initial exploration indicated that the complex nutrient sources used in DGGM media likely contain monosaccharide contamination. Evaluation with mass spectroscopy could confirm complex nutrient utilisation in both the VRE strains and commensal strains.

Using mass spectroscopy to view changes to polysaccharides and other complex nutrients would allow for the artificial gut model to be assessed for both complex nutrient utilisation as well as simple nutrient utilisation. The commensal consortium co-cultures with VRE could be run in DGGM to increase the physiological relevance. If a decrease in suppression was seen, the commensal consortium can be profiled for complex nutrient utilisation and additional strains can be added to fill in the gaps in nutrient utilisation.

Once the commensal consortium is optimised for nutrient competition in DGGM, artificial gut modelling could be performed to compare the efficiency of the commensal consortium to restrict VRE growth, in a similar manner that was performed to test the effects of FMT in this study. The commensal consortium could be compared to FMT treatment and to an untreated control. Tracking VRE growth, the taxonomic profiles, and metabonomic profiles of the cultures would provide evidence for the potential application of the commensal consortium as a novel microbiome therapeutic. Finally, once results from the artificial gut model provide preliminary data on the efficacy of the commensal consortium, a second mouse study could be designed. Confirmation of commensal engraftment through 16S rRNA gene sequencing and qPCR could validate claims that the VRE count changes due to the commensal consortium rather than other uncontrolled factors (such as recovery of the mouse gut microbiome).

Limitations, both present and future, in the treatment of VRE colonisation of the human gut microbiome poses a serious threat. Developing a non-antibiotic therapeutic to decolonise patients requires an understanding of how a healthy gut microbiota suppresses the growth of the pathogen—a knowledge which has yet to be sufficiently obtained. The research presented in this study showed the importance of nutrient competition in VRE intestinal colonisation. Additionally, this study laid the groundwork for the development of a safe, effective and rationally designed microbiome therapeutic which will restrict VRE growth without risking harm to the individual or the further expansion of AMR for VRE.

References

1. Hao, W.L. and Y.K. Lee, *Microflora of the gastrointestinal tract: a review*. Methods Mol Biol, 2004. **268**: p. 491-502.
2. Macfarlane, G.T. and S. Macfarlane, *Bacteria, colonic fermentation, and gastrointestinal health*. J AOAC Int, 2012. **95**(1): p. 50-60.
3. Wu, H.J. and E. Wu, *The role of gut microbiota in immune homeostasis and autoimmunity*. Gut Microbes, 2012. **3**(1): p. 4-14.
4. Prestinaci, F., P. Pezzotti, and A. Pantosti, *Antimicrobial resistance: a global multifaceted phenomenon*. Pathog Glob Health, 2015. **109**(7): p. 309-18.
5. *Transatlantic Taskforce on Antimicrobial Resistance (TATFAR)*. 2020, Centers for Disease Control and Prevention.
6. Johnson, W.M., et al., *Auxotrophic interactions: a stabilizing attribute of aquatic microbial communities?* FEMS Microbiol Ecol, 2020. **96**(11).
7. Martin, E. and R. Hine, *chemoorganotroph*. 2008, Oxford University Press.
8. Ronit, C.-P. and L.K. Dennis, *Anaerobic Infections: General Concepts*, in *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases (Eighth Edition)*, E.B. John, D. Raphael, and J.B. Martin, Editors. 2015, W.B. Saunders: Philadelphia. p. 2736-2743.e1.
9. Lawley, T.D. and A.W. Walker, *Intestinal colonization resistance*. Immunology, 2013. **138**(1): p. 1-11.
10. Bishal, S. and C. Evangeline, *Bacterial metabolites: an unexplored quarry*, in *Volatiles and Metabolites of Microbes*, K. Ajay, S. Joginder, and S. Jastin, Editors. 2021, Academic Press. p. 205-234.
11. Oliphant, K. and E. Allen-Vercoe, *Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health*. Microbiome, 2019. **7**(1): p. 91.
12. King, R.C., W.D. Stansfield, and P.K. Mulligan, *A dictionary of genetics*. 2007, Oxford University Press.
13. Gupta, S., E. Allen-Vercoe, and E.O. Petrof, *Fecal microbiota transplantation: in perspective*. Therap Adv Gastroenterol, 2016. **9**(2): p. 229-39.
14. Tian, L., et al., *Deciphering functional redundancy in the human microbiome*. Nat Commun, 2020. **11**(1): p. 6217.
15. Moya, A. and M. Ferrer, *Functional Redundancy-Induced Stability of Gut Microbiota Subjected to Disturbance*. Trends Microbiol, 2016. **24**(5): p. 402-413.
16. Sommer, F., et al., *The resilience of the intestinal microbiota influences health and disease*. Nat Rev Microbiol, 2017. **15**(10): p. 630-638.
17. Subramanian, M.R., S. Talluri, and L.P. Christopher, *Production of lactic acid using a new homofermentative Enterococcus faecalis isolate*. Microb Biotechnol, 2015. **8**(2): p. 221-9.
18. Ramsden, J.J., *Metabolomics and Metabonomics*, in *Bioinformatics: An Introduction*. 2009, Springer London: London. p. 1-6.
19. Everett, J.R., *A new paradigm for known metabolite identification in metabonomics/metabolomics: metabolite identification efficiency*. Comput Struct Biotechnol J, 2015. **13**: p. 131-44.

20. Shah, N.J., S. Sureshkumar, and D.G. Shewade, *Metabolomics: A Tool Ahead for Understanding Molecular Mechanisms of Drugs and Diseases*. Indian J Clin Biochem, 2015. **30**(3): p. 247-54.
21. Kumar, V., et al., *Cell Injury, Cell Death, and Adaptations*, in *Robbins & Cotran Pathologic Basis of Disease*. 2020, Elsevier. p. 1392.
22. Berg, G., et al., *Microbiome definition re-visited: old concepts and new challenges*. Microbiome, 2020. **8**(1): p. 103.
23. Hou, K., et al., *Microbiota in health and diseases*. Signal Transduct Target Ther, 2022. **7**(1): p. 135.
24. Ursell, L.K., et al., *Defining the human microbiome*. Nutr Rev, 2012. **70 Suppl 1**(Suppl 1): p. S38-44.
25. Harrison, P.F. and J. Lederberg, *IOM*. 1998, National Academy Press, Washington, DC. p. 8-74.
26. Shlaes, D.M., et al. 1997: Infect Control Hosp Epidemiol. p. 275-291.
27. Basak, S., P. Singh, and M. Rajurkar, *Multidrug Resistant and Extensively Drug Resistant Bacteria: A Study*. J Pathog, 2016. **2016**: p. 4065603.
28. Pereira, F.C. and D. Berry, *Microbial nutrient niches in the gut*. Environ Microbiol, 2017. **19**(4): p. 1366-1378.
29. Cronin, P., et al., *Dietary Fibre Modulates the Gut Microbiota*. Nutrients, 2021. **13**(5).
30. Yip, A.Y.G., et al., *Antibiotics promote intestinal growth of carbapenem-resistant Enterobacteriaceae by enriching nutrients and depleting microbial metabolites*. Nat Commun, 2023. **14**(1): p. 5094.
31. Patterson, A.D., F.J. Gonzalez, and J.R. Idle, *Xenobiotic metabolism: a view through the metabolometer*. Chem Res Toxicol, 2010. **23**(5): p. 851-60.
32. Klein, E.Y., et al., *Global increase and geographic convergence in antibiotic consumption between 2000 and 2015*. Proc Natl Acad Sci U S A, 2018. **115**(15): p. E3463-E3470.
33. Research, N.I.f.H., *Antimicrobial Resistance and Modernising Microbiology*. 2021.
34. D'Atri, F., et al., *Targets for the reduction of antibiotic use in humans in the Transatlantic Taskforce on Antimicrobial Resistance (TATFAR) partner countries*. Euro Surveill, 2019. **24**(28).
35. Aminov, R.I., *A brief history of the antibiotic era: lessons learned and challenges for the future*. Front Microbiol, 2010. **1**: p. 134.
36. Browne, A.J., et al., *Global antibiotic consumption and usage in humans, 2000-18: a spatial modelling study*. Lancet Planet Health, 2021. **5**(12): p. e893-e904.
37. Sartelli, M., et al., *Antibiotic Use in Low and Middle-Income Countries and the Challenges of Antimicrobial Resistance in Surgery*. Antibiotics (Basel), 2020. **9**(8).
38. Pokharel, S., S. Raut, and B. Adhikari, *Tackling antimicrobial resistance in low-income and middle-income countries*. BMJ Glob Health, 2019. **4**(6): p. e002104.
39. Raut, S. and B. Adhikari, *Ceftazidime-avibactam in ceftazidime-resistant infections*. Lancet Infect Dis, 2016. **16**(9): p. 997.
40. Kleczka, B., et al., *Using rubber stamps and mobile phones to help understand and change antibiotic prescribing behaviour in private sector primary healthcare clinics in Kenya*. BMJ Glob Health, 2019. **4**(5): p. e001422.
41. Huttner, B., et al., *How to improve antibiotic awareness campaigns: findings of a WHO global survey*. BMJ Glob Health, 2019. **4**(3): p. e001239.

42. Polianciuc, S.I., et al., *Antibiotics in the environment: causes and consequences*. Med Pharm Rep, 2020. **93**(3): p. 231-240.
43. Wohde, M., et al., *Occurrence and transformation of veterinary pharmaceuticals and biocides in manure: a literature review*. Environ Sci Eur, 2016. **28**(1): p. 23.
44. Kinsella, B., et al., *Current trends in sample preparation for growth promoter and veterinary drug residue analysis*. J Chromatogr A, 2009. **1216**(46): p. 7977-8015.
45. Chee-Sanford, J., et al., *Environmental Impacts of Antibiotic Use in the Animal Production Industry*, in *Ecology and Animal Health*. 2012, Ecol Anim Heal Ecosyst Heal Sustain Agric. p. 228-368.
46. Ben, Y., et al., *Human health risk assessment of antibiotic resistance associated with antibiotic residues in the environment: A review*. Environ Res, 2019. **169**: p. 483-493.
47. Van Boeckel, T.P., et al., *Global trends in antimicrobial use in food animals*. Proc Natl Acad Sci U S A, 2015. **112**(18): p. 5649-54.
48. Jechalke, S., et al., *Fate and effects of veterinary antibiotics in soil*. Trends Microbiol, 2014. **22**(9): p. 536-45.
49. Byappanahalli, M.N., et al., *Enterococci in the environment*. Microbiol Mol Biol Rev, 2012. **76**(4): p. 685-706.
50. Cattoir, V., et al., *Unexpected occurrence of plasmid-mediated quinolone resistance determinants in environmental Aeromonas spp.* Emerg Infect Dis, 2008. **14**(2): p. 231-7.
51. Carvalho, I.T. and L. Santos, *Antibiotics in the aquatic environments: A review of the European scenario*. Environ Int, 2016. **94**: p. 736-757.
52. Singer, A.C., et al., *Review of Antimicrobial Resistance in the Environment and Its Relevance to Environmental Regulators*. Front Microbiol, 2016. **7**: p. 1728.
53. *Transatlantic Taskforce on Antimicrobial Resistance (TATFAR)-Actions & Recommendations*. 2020, Centers for Disease Control and Prevention.
54. Ahmed, A., et al., *Current concepts in combination antibiotic therapy for critically ill patients*. Indian J Crit Care Med, 2014. **18**(5): p. 310-4.
55. Ubeda, C., 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): p. 4332-41.
56. Kim, S., A. Covington, and E.G. Pamer, *The intestinal microbiota: Antibiotics, colonization resistance, and enteric pathogens*. Immunol Rev, 2017. **279**(1): p. 90-105.
57. Dethlefsen, L., et al., *The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing*. PLoS Biol, 2008. **6**(11): p. e280.
58. Panda, S., et al., *Short-term effect of antibiotics on human gut microbiota*. PLoS One, 2014. **9**(4): p. e95476.
59. Bohnhoff, M. and C.P. Miller, *Enhanced susceptibility to Salmonella infection in streptomycin-treated mice*. J Infect Dis, 1962. **111**: p. 117-27.
60. van der Waaij, D., J.M. Berghuis-de Vries, and L.-v. Lekkerkerk, *Colonization resistance of the digestive tract in conventional and antibiotic-treated mice*. J Hyg (Lond), 1971. **69**(3): p. 405-11.
61. Miller, W.R., et al., *Vancomycin-Resistant Enterococci: Therapeutic Challenges in the 21st Century*. Infect Dis Clin North Am, 2016. **30**(2): p. 415-439.

62. Weiner, L.M., et al., *Antimicrobial-Resistant Pathogens Associated With Healthcare-Associated Infections: Summary of Data Reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2011-2014*. Infect Control Hosp Epidemiol, 2016. **37**(11): p. 1288-1301.
63. Axelrad, J.E., et al., *Gut colonization with vancomycin-resistant Enterococcus and risk for subsequent enteric infection*. Gut Pathog, 2018. **10**: p. 28.
64. García-Solache, M. and L.B. Rice, *The Enterococcus: a Model of Adaptability to Its Environment*. Clin Microbiol Rev, 2019. **32**(2).
65. Amberpet, R., et al., *Risk factors for intestinal colonization with vancomycin resistant enterococci' A prospective study in a level III pediatric intensive care unit*. J Lab Physicians, 2018. **10**(1): p. 89-94.
66. Ducarmon, Q.R., et al., *Gut Microbiota and Colonization Resistance against Bacterial Enteric Infection*. Microbiol Mol Biol Rev, 2019. **83**(3).
67. Rice, L.B., *The complex dynamics of antimicrobial activity in the human gastrointestinal tract*. Trans Am Clin Climatol Assoc, 2013. **124**: p. 123-32.
68. Al-Nassir, W.N., et al., *Both oral metronidazole and oral vancomycin promote persistent overgrowth of vancomycin-resistant enterococci during treatment of Clostridium difficile-associated disease*. Antimicrob Agents Chemother, 2008. **52**(7): p. 2403-6.
69. Contreras, G.A., J.M. Munita, and C.A. Arias, *Novel Strategies for the Management of Vancomycin-Resistant Enterococcal Infections*. Curr Infect Dis Rep, 2019. **21**(7): p. 22.
70. Banerjee, T., et al., *Vancomycin-resistance Enterococcal Colonization in Hospitalized Patients in Relation to Antibiotic Usage in a Tertiary Care Hospital of North India*. J Lab Physicians, 2015. **7**(2): p. 108-11.
71. Rice, L.B., et al., *Beta-lactam antibiotics and gastrointestinal colonization with vancomycin-resistant enterococci*. J Infect Dis, 2004. **189**(6): p. 1113-8.
72. Palmer, K.L. and M.S. Gilmore, *Multidrug-resistant enterococci lack CRISPR-cas*. mBio, 2010. **1**(4).
73. Murray, B.E., *The life and times of the Enterococcus*. Clin Microbiol Rev, 1990. **3**(1): p. 46-65.
74. Said, M.S., E. Tirthani, and E. Lesho, *Enterococcus Infections*, in StatPearls. 2023: Treasure Island (FL).
75. Gilmore, M.S., et al., *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*. 2014.
76. Fiore, E., D. Van Tyne, and M.S. Gilmore, *Pathogenicity of Enterococci*. Microbiol Spectr, 2019. **7**(4).
77. Zhong, Z., et al., *Comparative genomic analysis of the genus Enterococcus*. Microbiol Res, 2017. **196**: p. 95-105.
78. Lebreton, F., et al., *Tracing the Enterococci from Paleozoic Origins to the Hospital*. Cell, 2017. **169**(5): p. 849-861 e13.
79. Ruoff, K.L., et al., *Species identities of enterococci isolated from clinical specimens*. J Clin Microbiol, 1990. **28**(3): p. 435-7.
80. Gordon, S., et al., *Antimicrobial susceptibility patterns of common and unusual species of enterococci causing infections in the United States*. Enterococcal Study Group. J Clin Microbiol, 1992. **30**(9): p. 2373-8.

81. Facklam, R.R. and M.D. Collins, *Identification of Enterococcus species isolated from human infections by a conventional test scheme*. J Clin Microbiol, 1989. **27**(4): p. 731-4.
82. Mastroianni, A., *Enterococcus raffinosus endocarditis. First case and literature review*. Infez Med, 2009. **17**(1): p. 14-20.
83. Pinkes, M.E., C. White, and C.S. Wong, *Native-valve Enterococcus hirae endocarditis: a case report and review of the literature*. BMC Infect Dis, 2019. **19**(1): p. 891.
84. Monticelli, J., et al., *Clinical management of non-faecium non-faecalis vancomycin-resistant enterococci infection. Focus on Enterococcus gallinarum and Enterococcus casseliflavus/flavescens*. J Infect Chemother, 2018. **24**(4): p. 237-246.
85. Sherman, J.M., *The Streptococci*. Bacteriol Rev, 1937. **1**(1): p. 3-97.
86. Sherman, J.M., J.C. Mauer, and P. Stark, *Streptococcus fecalis*. J Bacteriol, 1937. **33**(3): p. 275-82.
87. Facklam, R.R., *Comparison of several laboratory media for presumptive identification of enterococci and group D streptococci*. Appl Microbiol, 1973. **26**(2): p. 138-45.
88. Fisher, K. and C. Phillips, *The ecology, epidemiology and virulence of Enterococcus*. Microbiology (Reading), 2009. **155**(Pt 6): p. 1749-1757.
89. Klein, G., *Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract*. Int J Food Microbiol, 2003. **88**(2-3): p. 123-31.
90. Gilmore, M.S., F. Lebreton, and W. van Schaik, *Genomic transition of enterococci from gut commensals to leading causes of multidrug-resistant hospital infection in the antibiotic era*. Curr Opin Microbiol, 2013. **16**(1): p. 10-6.
91. Martín, J.D. and J.O. Mundt, *Enterococci in insects*. Applied microbiology, 1972. **24** 4: p. 575-80.
92. Mundt, J.O., *Occurrence of enterococci in animals in a wild environment*. Appl Microbiol, 1963. **11**(2): p. 136-40.
93. Mundt, J.O., *Occurrence of enterococci on plants in a wild environment*. Appl Microbiol, 1963. **11**(2): p. 141-4.
94. Palmer, K.L., et al., *Comparative genomics of enterococci: variation in Enterococcus faecalis, clade structure in E. faecium, and defining characteristics of E. gallinarum and E. casseliflavus*. mBio, 2012. **3**(1): p. e00318-11.
95. Galloway-Peña, J., et al., *Genomic and SNP analyses demonstrate a distant separation of the hospital and community-associated clades of Enterococcus faecium*. PLoS One, 2012. **7**(1): p. e30187.
96. Lebreton, F., et al., *Emergence of epidemic multidrug-resistant Enterococcus faecium from animal and commensal strains*. mBio, 2013. **4**(4).
97. Belloso Daza, M.V., et al., *Genome-based studies indicate that the Enterococcus faecium Clade B strains belong to Enterococcus lactis species and lack of the hospital infection associated markers*. Int J Syst Evol Microbiol, 2021. **71**(8).
98. Thiercelin, M. and L. Jouhaud, *Sur un diplocoque saprophyte de l'intestin susceptible de devenir pathogene*. CR Soc Biol, 1899. **5**(26971.2).
99. Maccallum, W.G. and T.W. Hastings, *A CASE OF ACUTE ENDOCARDITIS CAUSED BY MICROCOCCUS ZYMOGENES (NOV. SPEC.), WITH A DESCRIPTION OF THE MICROORGANISM*. J Exp Med, 1899. **4**(5-6): p. 521-34.
100. Andrewes, F.W. and T.J. Hoder, *A Study of the Streptococci Pathogenic for Man*. The Lancet 1906. **168**(4335): p. 852-855.

101. Orla-Jensen, S., *The lactic acid bacteria*. Memoirs of the Academy of the Royal Society of Denmark, 1919. **85**(Section of Science Series): p. 81-197.
102. Sievert, D.M., et al., *Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009-2010*. Infect Control Hosp Epidemiol, 2013. **34**(1): p. 1-14.
103. Bradley, C.R. and A.P. Fraiese, *Heat and chemical resistance of enterococci*. J Hosp Infect, 1996. **34**(3): p. 191-6.
104. Ramadhan, A.A. and E. Hegedus, *Survivability of vancomycin resistant enterococci and fitness cost of vancomycin resistance acquisition*. J Clin Pathol, 2005. **58**(7): p. 744-6.
105. Hegstad, K., et al., *Mobile genetic elements and their contribution to the emergence of antimicrobial resistant Enterococcus faecalis and Enterococcus faecium*. Clin Microbiol Infect, 2010. **16**(6): p. 541-54.
106. Lam, M.M., et al., *Comparative analysis of the complete genome of an epidemic hospital sequence type 203 clone of vancomycin-resistant Enterococcus faecium*. BMC Genomics, 2013. **14**: p. 595.
107. Horvath, P. and R. Barrangou, *CRISPR/Cas, the immune system of bacteria and archaea*. Science, 2010. **327**(5962): p. 167-70.
108. Alduhaidhawi, A.H.M., et al., *Prevalence of CRISPR-Cas Systems and Their Possible Association with Antibiotic Resistance in Enterococcus faecalis and Enterococcus faecium Collected from Hospital Wastewater*. Infect Drug Resist, 2022. **15**: p. 1143-1154.
109. Dos Santos, B.A., et al., *CRISPR elements and their association with antimicrobial resistance and virulence genes among vancomycin-resistant and vancomycin-susceptible enterococci recovered from human and food sources*. Infect Genet Evol, 2020. **80**: p. 104183.
110. Jabbari Shiadeh, S.M., et al., *Global prevalence of antibiotic resistance in blood-isolated Enterococcus faecalis and Enterococcus faecium: a systematic review and meta-analysis*. Infect Drug Resist, 2019. **12**: p. 2713-2725.
111. Munita, J.M. and C.A. Arias, *Mechanisms of Antibiotic Resistance*. Microbiol Spectr, 2016. **4**(2).
112. Agudelo Higueta, N.I. and M.M. Huycke, *Enterococcal Disease, Epidemiology, and Implications for Treatment*, in *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*, M.S. Gilmore, et al., Editors. 2014: Boston.
113. Johnston, L.M. and L.A. Jaykus, *Antimicrobial resistance of Enterococcus species isolated from produce*. Appl Environ Microbiol, 2004. **70**(5): p. 3133-7.
114. Arias, C.A. and B.E. Murray, *The rise of the Enterococcus: beyond vancomycin resistance*. Nat Rev Microbiol, 2012. **10**(4): p. 266-78.
115. Bush, K. and P.A. Bradford, *beta-Lactams and beta-Lactamase Inhibitors: An Overview*. Cold Spring Harb Perspect Med, 2016. **6**(8).
116. Gutmann, L., *Résistance des entérocoques aux bêta-lactamines et conséquences sur les synergies*. Médecine et maladies infectieuses, 1994. **24**: p. 165-171.
117. Duez, C., et al., *The penicillin resistance of Enterococcus faecalis JH2-2r results from an overproduction of the low-affinity penicillin-binding protein PBP4 and does not involve a psr-like gene*. Microbiology (Reading), 2001. **147**(Pt 9): p. 2561-2569.

118. Infante, V.H., et al., *Evaluation of polymorphisms in pbp4 gene and genetic diversity in penicillin-resistant, ampicillin-susceptible Enterococcus faecalis from hospitals in different states in Brazil*. FEMS Microbiol Lett, 2016. **363**(7).
119. Williamson, R., et al., *One or two low affinity penicillin-binding proteins may be responsible for the range of susceptibility of Enterococcus faecium to benzylpenicillin*. J Gen Microbiol, 1985. **131**(8): p. 1933-40.
120. Tooke, C.L., et al., *beta-Lactamases and beta-Lactamase Inhibitors in the 21st Century*. J Mol Biol, 2019. **431**(18): p. 3472-3500.
121. Ingerman, M., et al., *beta-Lactamase production in experimental endocarditis due to aminoglycoside-resistant Streptococcus faecalis*. J Infect Dis, 1987. **155**(6): p. 1226-32.
122. Armstrong, T., S.J. Fenn, and K.R. Hardie, *JMM Profile: Carbapenems: a broad-spectrum antibiotic*. J Med Microbiol, 2021. **70**(12).
123. Fourmy, D., et al., *Structure of the A site of Escherichia coli 16S ribosomal RNA complexed with an aminoglycoside antibiotic*. Science, 1996. **274**(5291): p. 1367-71.
124. Mingeot-Leclercq, M.P., Y. Glupczynski, and P.M. Tulkens, *Aminoglycosides: activity and resistance*. Antimicrob Agents Chemother, 1999. **43**(4): p. 727-37.
125. Moellering, R.C., Jr. and A.N. Weinberg, *Studies on antibiotic synerism against enterococci. II. Effect of various antibiotics on the uptake of 14 C-labeled streptomycin by enterococci*. J Clin Invest, 1971. **50**(12): p. 2580-4.
126. Groman, R., *Aminoglycosides*. 2015. p. 930-934.
127. Chow, J.W., et al., *Aminoglycoside resistance genes aph(2'')-Ib and aac(6')-Im detected together in strains of both Escherichia coli and Enterococcus faecium*. Antimicrob Agents Chemother, 2001. **45**(10): p. 2691-4.
128. Ferretti, J.J., K.S. Gilmore, and P. Courvalin, *Nucleotide sequence analysis of the gene specifying the bifunctional 6'-aminoglycoside acetyltransferase 2''-aminoglycoside phosphotransferase enzyme in Streptococcus faecalis and identification and cloning of gene regions specifying the two activities*. J Bacteriol, 1986. **167**(2): p. 631-8.
129. Amini, F.M., et al., *Prevalence of Aminoglycoside Resistance Genes in Enterococcus Strains in Kermanshah, Iran*. Iran J Med Sci, 2018. **43**(5): p. 487-493.
130. Kobayashi, N., et al., *Distribution of aminoglycoside resistance genes in recent clinical isolates of Enterococcus faecalis, Enterococcus faecium and Enterococcus avium*. Epidemiol Infect, 2001. **126**(2): p. 197-204.
131. Ounissi, H., et al., *Gene homogeneity for aminoglycoside-modifying enzymes in gram-positive cocci*. Antimicrob Agents Chemother, 1990. **34**(11): p. 2164-8.
132. Singh, K.V., K. Malathum, and B.E. Murray, *Disruption of an Enterococcus faecium species-specific gene, a homologue of acquired macrolide resistance genes of staphylococci, is associated with an increase in macrolide susceptibility*. Antimicrob Agents Chemother, 2001. **45**(1): p. 263-6.
133. Ross, J.I., et al., *Inducible erythromycin resistance in staphylococci is encoded by a member of the ATP-binding transport super-gene family*. Mol Microbiol, 1990. **4**(7): p. 1207-14.
134. Allignet, J., V. Loncle, and N. el Sohl, *Sequence of a staphylococcal plasmid gene, vga, encoding a putative ATP-binding protein involved in resistance to virginiamycin A-like antibiotics*. Gene, 1992. **117**(1): p. 45-51.

135. Singh, K.V., G.M. Weinstock, and B.E. Murray, *An Enterococcus faecalis ABC homologue (Lsa) is required for the resistance of this species to clindamycin and quinupristin-dalfopristin*. Antimicrob Agents Chemother, 2002. **46**(6): p. 1845-50.
136. Dina, J., B. Malbruny, and R. Leclercq, *Nonsense mutations in the lsa-like gene in Enterococcus faecalis isolates susceptible to lincosamides and Streptogramins A*. Antimicrob Agents Chemother, 2003. **47**(7): p. 2307-9.
137. Gleckman, R., N. Blagg, and D.W. Joubert, *Trimethoprim: mechanisms of action, antimicrobial activity, bacterial resistance, pharmacokinetics, adverse reactions, and therapeutic indications*. Pharmacotherapy, 1981. **1**(1): p. 14-20.
138. Khalil, I., et al., *Dihydrofolate reductase and dihydropteroate synthase genotypes associated with in vitro resistance of Plasmodium falciparum to pyrimethamine, trimethoprim, sulfadoxine, and sulfamethoxazole*. Am J Trop Med Hyg, 2003. **68**(5): p. 586-9.
139. Kazanjian, P., et al., *Pneumocystis carinii mutations associated with sulfa and sulfone prophylaxis failures in AIDS patients*. AIDS, 1998. **12**(8): p. 873-8.
140. Zervos, M.J. and D.R. Schaberg, *Reversal of the in vitro susceptibility of enterococci to trimethoprim-sulfamethoxazole by folinic acid*. Antimicrob Agents Chemother, 1985. **28**(3): p. 446-8.
141. Gravet, A., et al., *Predominant Staphylococcus aureus isolated from antibiotic-associated diarrhea is clinically relevant and produces enterotoxin A and the bicomponent toxin LukE-lukD*. J Clin Microbiol, 1999. **37**(12): p. 4012-9.
142. Levine, D.P., *Vancomycin: A History*. Clinical Infectious Diseases, 2006. **42**(Supplement_1): p. S5-S12.
143. Shlaes, D.M., et al., *Inducible, transferable resistance to vancomycin in Enterococcus faecium, D399*. J Antimicrob Chemother, 1989. **23**(4): p. 503-8.
144. Williamson, R., et al., *Inducible resistance to vancomycin in Enterococcus faecium D366*. J Infect Dis, 1989. **159**(6): p. 1095-104.
145. Arthur, M. and P. Courvalin, *Genetics and mechanisms of glycopeptide resistance in enterococci*. Antimicrob Agents Chemother, 1993. **37**(8): p. 1563-71.
146. Arthur, M. and R. Quintiliani, Jr., *Regulation of VanA- and VanB-type glycopeptide resistance in enterococci*. Antimicrob Agents Chemother, 2001. **45**(2): p. 375-81.
147. Courvalin, P., *Genetics of glycopeptide resistance in gram-positive pathogens*. Int J Med Microbiol, 2005. **294**(8): p. 479-86.
148. Courvalin, P., *Vancomycin resistance in gram-positive cocci*. Clin Infect Dis, 2006. **42** Suppl 1: p. S25-34.
149. Jaspán, H.B., et al., *Multidrug-resistant Enterococcus faecium meningitis in a toddler: characterization of the organism and successful treatment with intraventricular daptomycin and intravenous tigecycline*. Pediatr Infect Dis J, 2010. **29**(4): p. 379-81.
150. Depardieu, F., et al., *Modes and modulations of antibiotic resistance gene expression*. Clin Microbiol Rev, 2007. **20**(1): p. 79-114.
151. Vimberg, V., *Teicoplanin-A New Use for an Old Drug in the COVID-19 Era?* Pharmaceuticals (Basel), 2021. **14**(12).
152. Sieradzki, K. and A. Tomasz, *Inhibition of the autolytic system by vancomycin causes mimicry of vancomycin-intermediate Staphylococcus aureus-type resistance, cell concentration dependence of the MIC, and antibiotic tolerance in vancomycin-susceptible S. aureus*. Antimicrob Agents Chemother, 2006. **50**(2): p. 527-33.

153. Wang, F., et al., *Insights into Key Interactions between Vancomycin and Bacterial Cell Wall Structures*. ACS Omega, 2018. **3**(1): p. 37-45.
154. Pryka, R.D., K.A. Rodvold, and J.C. Rotschafer, *Teicoplanin: an investigational glycopeptide antibiotic*. Clin Pharm, 1988. **7**(9): p. 647-58.
155. Zeng, D., et al., *Approved Glycopeptide Antibacterial Drugs: Mechanism of Action and Resistance*. Cold Spring Harb Perspect Med, 2016. **6**(12).
156. Kristich, C.J., L.B. Rice, and C.A. Arias, *Enterococcal Infection-Treatment and Antibiotic Resistance*, in *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*, M.S. Gilmore, et al., Editors. 2014: Boston.
157. Navarro, F. and P. Courvalin, *Analysis of genes encoding D-alanine-D-alanine ligase-related enzymes in Enterococcus casseliflavus and Enterococcus flavescens*. Antimicrob Agents Chemother, 1994. **38**(8): p. 1788-93.
158. Evers, S., D.F. Sahm, and P. Courvalin, *The vanB gene of vancomycin-resistant Enterococcus faecalis V583 is structurally related to genes encoding D-Ala:D-Ala ligases and glycopeptide-resistance proteins VanA and VanC*. Gene, 1993. **124**(1): p. 143-4.
159. Perichon, B., P. Reynolds, and P. Courvalin, *VanD-type glycopeptide-resistant Enterococcus faecium BM4339*. Antimicrob Agents Chemother, 1997. **41**(9): p. 2016-8.
160. Fines, M., et al., *VanE, a new type of acquired glycopeptide resistance in Enterococcus faecalis BM4405*. Antimicrob Agents Chemother, 1999. **43**(9): p. 2161-4.
161. Xu, X., et al., *vanM, a new glycopeptide resistance gene cluster found in Enterococcus faecium*. Antimicrob Agents Chemother, 2010. **54**(11): p. 4643-7.
162. Lebreton, F., et al., *D-Ala-d-Ser VanN-type transferable vancomycin resistance in Enterococcus faecium*. Antimicrob Agents Chemother, 2011. **55**(10): p. 4606-12.
163. Sassi, M., et al., *Genetic characterization of a VanG-type vancomycin-resistant Enterococcus faecium clinical isolate*. J Antimicrob Chemother, 2018. **73**(4): p. 852-855.
164. Konstantinidis, K.T. and J.M. Tiedje, *Genomic insights that advance the species definition for prokaryotes*. Proc Natl Acad Sci U S A, 2005. **102**(7): p. 2567-72.
165. Trieu-Cuot, P., et al., *Study of heterogeneity of chloramphenicol acetyltransferase (CAT) genes in streptococci and enterococci by polymerase chain reaction: characterization of a new CAT determinant*. Antimicrob Agents Chemother, 1993. **37**(12): p. 2593-8.
166. Bannam, T.L. and J.I. Rood, *Relationship between the Clostridium perfringens catQ gene product and chloramphenicol acetyltransferases from other bacteria*. Antimicrob Agents Chemother, 1991. **35**(3): p. 471-6.
167. Huggins, A.S., T.L. Bannam, and J.I. Rood, *Comparative sequence analysis of the catB gene from Clostridium butyricum*. Antimicrob Agents Chemother, 1992. **36**(11): p. 2548-51.
168. Schwarz, S. and M. Cardoso, *Nucleotide sequence and phylogeny of a chloramphenicol acetyltransferase encoded by the plasmid pSCS7 from Staphylococcus aureus*. Antimicrob Agents Chemother, 1991. **35**(8): p. 1551-6.
169. Shaw, W.V., *Chloramphenicol acetyltransferase: enzymology and molecular biology*. CRC Crit Rev Biochem, 1983. **14**(1): p. 1-46.

170. Wang, Y., et al., *A novel gene, optrA, that confers transferable resistance to oxazolidinones and phenicols and its presence in Enterococcus faecalis and Enterococcus faecium of human and animal origin*. J Antimicrob Chemother, 2015. **70**(8): p. 2182-90.
171. Kehrenberg, C., et al., *A new mechanism for chloramphenicol, florfenicol and clindamycin resistance: methylation of 23S ribosomal RNA at A2503*. Mol Microbiol, 2005. **57**(4): p. 1064-73.
172. Vester, B., *The cfr and cfr-like multiple resistance genes*. Res Microbiol, 2018. **169**(2): p. 61-66.
173. Ferrero, L., B. Cameron, and J. Crouzet, *Analysis of gyrA and grlA mutations in stepwise-selected ciprofloxacin-resistant mutants of Staphylococcus aureus*. Antimicrob Agents Chemother, 1995. **39**(7): p. 1554-8.
174. Ferrero, L., et al., *Cloning and primary structure of Staphylococcus aureus DNA topoisomerase IV: a primary target of fluoroquinolones*. Mol Microbiol, 1994. **13**(4): p. 641-53.
175. Ng, E.Y., M. Trucksis, and D.C. Hooper, *Quinolone resistance mutations in topoisomerase IV: relationship to the flqA locus and genetic evidence that topoisomerase IV is the primary target and DNA gyrase is the secondary target of fluoroquinolones in Staphylococcus aureus*. Antimicrob Agents Chemother, 1996. **40**(8): p. 1881-8.
176. Mirzaii, M., et al., *Antibiotic resistance assessment and multi-drug efflux pumps of Enterococcus faecium isolated from clinical specimens*. J Infect Dev Ctries, 2023. **17**(5): p. 649-655.
177. Fernández-Hidalgo, N., et al., *Ampicillin plus ceftriaxone is as effective as ampicillin plus gentamicin for treating enterococcus faecalis infective endocarditis*. Clin Infect Dis, 2013. **56**(9): p. 1261-8.
178. Pericas, J.M., et al., *Changes in the treatment of Enterococcus faecalis infective endocarditis in Spain in the last 15 years: from ampicillin plus gentamicin to ampicillin plus ceftriaxone*. Clin Microbiol Infect, 2014. **20**(12): p. O1075-83.
179. Mainardi, J.L., et al., *Synergistic effect of amoxicillin and cefotaxime against Enterococcus faecalis*. Antimicrob Agents Chemother, 1995. **39**(9): p. 1984-7.
180. Reid, A.B., et al., *Use of pristinamycin for infections by gram-positive bacteria: clinical experience at an Australian hospital*. Antimicrob Agents Chemother, 2010. **54**(9): p. 3949-52.
181. Wade, J., et al., *Pristinamycin for Enterococcus faecium resistant to vancomycin and gentamicin*. Lancet, 1992. **339**(8788): p. 312-3.
182. de Lastours, V., et al., *Ecological impact of ciprofloxacin on commensal enterococci in healthy volunteers*. J Antimicrob Chemother, 2017. **72**(6): p. 1574-1580.
183. Donskey, C.J., et al., *Effect of parenteral fluoroquinolone administration on persistence of vancomycin-resistant Enterococcus faecium in the mouse gastrointestinal tract*. Antimicrob Agents Chemother, 2004. **48**(1): p. 326-8.
184. Zhanel, G.G., et al., *New lipoglycopeptides: a comparative review of dalbavancin, oritavancin and telavancin*. Drugs, 2010. **70**(7): p. 859-86.
185. Florescu, I., et al., *Efficacy and safety of tigecycline compared with vancomycin or linezolid for treatment of serious infections with methicillin-resistant Staphylococcus aureus or vancomycin-resistant enterococci: a Phase 3, multicentre, double-blind, randomized study*. J Antimicrob Chemother, 2008. **62 Suppl 1**: p. i17-28.

186. Roberts, M.C., *Epidemiology of tetracycline-resistance determinants*. Trends Microbiol, 1994. **2**(10): p. 353-7.
187. Mercier, R.C., C. Kennedy, and C. Meadows, *Antimicrobial activity of tigecycline (GAR-936) against Enterococcus faecium and Staphylococcus aureus used alone and in combination*. Pharmacotherapy, 2002. **22**(12): p. 1517-23.
188. Entenza, J.M. and P. Moreillon, *Tigecycline in combination with other antimicrobials: a review of in vitro, animal and case report studies*. Int J Antimicrob Agents, 2009. **34**(1): p. 8 e1-9.
189. Jenkins, I., *Linezolid- and vancomycin-resistant Enterococcus faecium endocarditis: successful treatment with tigecycline and daptomycin*. J Hosp Med, 2007. **2**(5): p. 343-4.
190. Chuang, Y.C., et al., *Daptomycin versus linezolid for treatment of vancomycin-resistant enterococcal bacteremia: systematic review and meta-analysis*. BMC Infect Dis, 2014. **14**: p. 687.
191. Lauridsen, T.K., et al., *Linezolid as rescue treatment for left-sided infective endocarditis: an observational, retrospective, multicenter study*. Eur J Clin Microbiol Infect Dis, 2012. **31**(10): p. 2567-74.
192. Chuang, Y.C., et al., *Daptomycin versus linezolid for the treatment of vancomycin-resistant enterococcal bacteraemia: implications of daptomycin dose*. Clin Microbiol Infect, 2016. **22**(10): p. 890 e1-890 e7.
193. Mishra, N.N., et al., *Daptomycin resistance in enterococci is associated with distinct alterations of cell membrane phospholipid content*. PLoS One, 2012. **7**(8): p. e43958.
194. Montero, C.I., F. Stock, and P.R. Murray, *Mechanisms of resistance to daptomycin in Enterococcus faecium*. Antimicrob Agents Chemother, 2008. **52**(3): p. 1167-70.
195. Munoz-Price, L.S., K. Lolans, and J.P. Quinn, *Emergence of resistance to daptomycin during treatment of vancomycin-resistant Enterococcus faecalis infection*. Clin Infect Dis, 2005. **41**(4): p. 565-6.
196. Sakoulas, G., et al., *Ampicillin enhances daptomycin- and cationic host defense peptide-mediated killing of ampicillin- and vancomycin-resistant Enterococcus faecium*. Antimicrob Agents Chemother, 2012. **56**(2): p. 838-44.
197. Smith, J.R., et al., *β -Lactam combinations with daptomycin provide synergy against vancomycin-resistant Enterococcus faecalis and Enterococcus faecium*. J Antimicrob Chemother, 2015. **70**(6): p. 1738-43.
198. Sakoulas, G., et al., *Ceftaroline restores daptomycin activity against daptomycin-nonsusceptible vancomycin-resistant Enterococcus faecium*. Antimicrob Agents Chemother, 2014. **58**(3): p. 1494-500.
199. Dhand, A., et al., *Use of antistaphylococcal beta-lactams to increase daptomycin activity in eradicating persistent bacteremia due to methicillin-resistant Staphylococcus aureus: role of enhanced daptomycin binding*. Clin Infect Dis, 2011. **53**(2): p. 158-63.
200. Hall Snyder, A., et al., *Evaluation of the novel combination of daptomycin plus ceftriaxone against vancomycin-resistant enterococci in an in vitro pharmacokinetic/pharmacodynamic simulated endocardial vegetation model*. J Antimicrob Chemother, 2014. **69**(8): p. 2148-54.
201. Gonzales, M., et al., *Faecal pharmacokinetics of orally administered vancomycin in patients with suspected Clostridium difficile infection*. BMC Infect Dis, 2010. **10**: p. 363.

202. Thabit, A.K. and D.P. Nicolau, *Impact of vancomycin faecal concentrations on clinical and microbiological outcomes in Clostridium difficile infection*. Int J Antimicrob Agents, 2015. **46**(2): p. 205-8.
203. Sears, P., et al., *Fidaxomicin attains high fecal concentrations with minimal plasma concentrations following oral administration in patients with Clostridium difficile infection*. Clin Infect Dis, 2012. **55 Suppl 2**: p. S116-20.
204. Kuijper, E.J. and J.T. van Dissel, *Spectrum of Clostridium difficile infections outside health care facilities*. CMAJ, 2008. **179**(8): p. 747-8.
205. Wilcox, M.H., A. Brown, and J. Freeman, *Faecal concentrations of piperacillin and tazobactam in elderly patients*. J Antimicrob Chemother, 2001. **48**(1): p. 155-6.
206. Pletz, M.W., et al., *Ertapenem pharmacokinetics and impact on intestinal microflora, in comparison to those of ceftriaxone, after multiple dosing in male and female volunteers*. Antimicrob Agents Chemother, 2004. **48**(10): p. 3765-72.
207. Donskey, C.J., *The role of the intestinal tract as a reservoir and source for transmission of nosocomial pathogens*. Clin Infect Dis, 2004. **39**(2): p. 219-26.
208. Rice, L.B., *Emergence of vancomycin-resistant enterococci*. Emerg Infect Dis, 2001. **7**(2): p. 183-7.
209. Steinbakk, M., et al., *Faecal Concentration of Ten Antibiotics and Influence on Some Microflora-Associated Characteristics (MACs)*. 1992, Microbial Ecology in Health and Disease. p. 269-276.
210. Kager, L., et al., *Concentrations of ampicillin plus sulbactam in serum and intestinal mucosa and effects on the colonic microflora in patients undergoing colorectal surgery*. Eur J Clin Microbiol, 1983. **2**(6): p. 559-63.
211. Amberpet, R., et al., *Screening for Intestinal Colonization with Vancomycin Resistant Enterococci and Associated Risk Factors among Patients Admitted to an Adult Intensive Care Unit of a Large Teaching Hospital*. J Clin Diagn Res, 2016. **10**(9): p. DC06-DC09.
212. Feng, Y., et al., *Antibiotics induced intestinal tight junction barrier dysfunction is associated with microbiota dysbiosis, activated NLRP3 inflammasome and autophagy*. PLoS One, 2019. **14**(6): p. e0218384.
213. Stolfi, C., et al., *Implication of Intestinal Barrier Dysfunction in Gut Dysbiosis and Diseases*. Biomedicines, 2022. **10**(2).
214. Lutgendorff, F., L.M. Akkermans, and J.D. Soderholm, *The role of microbiota and probiotics in stress-induced gastro-intestinal damage*. Curr Mol Med, 2008. **8**(4): p. 282-98.
215. Rowland, I., et al., *Gut microbiota functions: metabolism of nutrients and other food components*. Eur J Nutr, 2018. **57**(1): p. 1-24.
216. Hooper, L.V., T. Midtvedt, and J.I. Gordon, *How host-microbial interactions shape the nutrient environment of the mammalian intestine*. Annu Rev Nutr, 2002. **22**: p. 283-307.
217. Backhed, F., et al., *The gut microbiota as an environmental factor that regulates fat storage*. Proc Natl Acad Sci U S A, 2004. **101**(44): p. 15718-23.
218. Valdes, A.M., et al., *Role of the gut microbiota in nutrition and health*. BMJ, 2018. **361**: p. k2179.
219. Koppel, N., V. Maini Rekdal, and E.P. Balskus, *Chemical transformation of xenobiotics by the human gut microbiota*. Science, 2017. **356**(6344).

220. Abdelsalam, N.A., et al., *Toxicomicrobiomics: The Human Microbiome vs. Pharmaceutical, Dietary, and Environmental Xenobiotics*. Front Pharmacol, 2020. **11**: p. 390.
221. Munoz-Esparza, N.C., et al., *Polyamines in Food*. Front Nutr, 2019. **6**: p. 108.
222. Minois, N., D. Carmona-Gutierrez, and F. Madeo, *Polyamines in aging and disease*. Aging (Albany NY), 2011. **3**(8): p. 716-32.
223. Sagar, N.A., et al., *Polyamines: Functions, Metabolism, and Role in Human Disease Management*. Med Sci (Basel), 2021. **9**(2).
224. O'Hara, A.M. and F. Shanahan, *The gut flora as a forgotten organ*. EMBO Rep, 2006. **7**(7): p. 688-93.
225. Sommer, F. and F. Backhed, *The gut microbiota--masters of host development and physiology*. Nat Rev Microbiol, 2013. **11**(4): p. 227-38.
226. Heiss, C.N. and L.E. Olofsson, *Gut Microbiota-Dependent Modulation of Energy Metabolism*. J Innate Immun, 2018. **10**(3): p. 163-171.
227. Liu, B.N., et al., *Gut microbiota in obesity*. World J Gastroenterol, 2021. **27**(25): p. 3837-3850.
228. Stappenbeck, T.S., L.V. Hooper, and J.I. Gordon, *Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells*. Proc Natl Acad Sci U S A, 2002. **99**(24): p. 15451-5.
229. Rakoff-Nahoum, S., et al., *Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis*. Cell, 2004. **118**(2): p. 229-41.
230. Cebra, J.J., *Influences of microbiota on intestinal immune system development*. Am J Clin Nutr, 1999. **69**(5): p. 1046S-1051S.
231. Vollaard, E.J. and H.A. Clasener, *Colonization resistance*. Antimicrob Agents Chemother, 1994. **38**(3): p. 409-14.
232. Chandra, S., S.S. Sisodia, and R.J. Vassar, *The gut microbiome in Alzheimer's disease: what we know and what remains to be explored*. Mol Neurodegener, 2023. **18**(1): p. 9.
233. Varesi, A., et al., *The Potential Role of Gut Microbiota in Alzheimer's Disease: From Diagnosis to Treatment*. Nutrients, 2022. **14**(3).
234. Chen, C., et al., *Gut microbiota regulate Alzheimer's disease pathologies and cognitive disorders via PUFA-associated neuroinflammation*. Gut, 2022. **71**(11): p. 2233-2252.
235. Adams, J.B., et al., *Microbiota transplant therapy and autism: lessons for the clinic*. Expert Rev Gastroenterol Hepatol, 2019. **13**(11): p. 1033-1037.
236. Krajmalnik-Brown, R., et al., *Gut bacteria in children with autism spectrum disorders: challenges and promise of studying how a complex community influences a complex disease*. Microb Ecol Health Dis, 2015. **26**: p. 26914.
237. Nirmalkar, K., et al., *Shotgun Metagenomics Study Suggests Alteration in Sulfur Metabolism and Oxidative Stress in Children with Autism and Improvement after Microbiota Transfer Therapy*. Int J Mol Sci, 2022. **23**(21).
238. Sharon, G., et al., *Human Gut Microbiota from Autism Spectrum Disorder Promote Behavioral Symptoms in Mice*. Cell, 2019. **177**(6): p. 1600-1618 e17.
239. Radjabzadeh, D., et al., *Gut microbiome-wide association study of depressive symptoms*. Nat Commun, 2022. **13**(1): p. 7128.
240. Limbana, T., F. Khan, and N. Eskander, *Gut Microbiome and Depression: How Microbes Affect the Way We Think*. Cureus, 2020. **12**(8): p. e9966.

241. Clapp, M., et al., *Gut microbiota's effect on mental health: The gut-brain axis*. Clin Pract, 2017. **7**(4): p. 987.
242. Guo, C., et al., *Deficient butyrate-producing capacity in the gut microbiome is associated with bacterial network disturbances and fatigue symptoms in ME/CFS*. Cell Host Microbe, 2023. **31**(2): p. 288-304 e8.
243. Xiong, R., et al., *Multi-'omics of gut microbiome-host interactions in short- and long-term myalgic encephalomyelitis/chronic fatigue syndrome patients*. Cell Host Microbe, 2023. **31**(2): p. 273-287 e5.
244. Troseid, M., et al., *The gut microbiome in coronary artery disease and heart failure: Current knowledge and future directions*. EBioMedicine, 2020. **52**: p. 102649.
245. Rahman, M.M., et al., *The Gut Microbiota (Microbiome) in Cardiovascular Disease and Its Therapeutic Regulation*. Front Cell Infect Microbiol, 2022. **12**: p. 903570.
246. Ochoa-Reparaz, J., T.O. Kirby, and L.H. Kasper, *The Gut Microbiome and Multiple Sclerosis*. Cold Spring Harb Perspect Med, 2018. **8**(6).
247. Thirion, F., et al., *The gut microbiota in multiple sclerosis varies with disease activity*. Genome Med, 2023. **15**(1): p. 1.
248. Jonsson, A.L. and F. Backhed, *Role of gut microbiota in atherosclerosis*. Nat Rev Cardiol, 2017. **14**(2): p. 79-87.
249. Ma, J. and H. Li, *The Role of Gut Microbiota in Atherosclerosis and Hypertension*. Front Pharmacol, 2018. **9**: p. 1082.
250. Lozupone, C.A., et al., *Diversity, stability and resilience of the human gut microbiota*. Nature, 2012. **489**(7415): p. 220-30.
251. Sender, R., S. Fuchs, and R. Milo, *Revised Estimates for the Number of Human and Bacteria Cells in the Body*. PLoS Biol, 2016. **14**(8): p. e1002533.
252. Sender, R., S. Fuchs, and R. Milo, *Are We Really Vastly Outnumbered? Revisiting the Ratio of Bacterial to Host Cells in Humans*. Cell, 2016. **164**(3): p. 337-40.
253. Ley, R.E., et al., *Microbial ecology: human gut microbes associated with obesity*. Nature, 2006. **444**(7122): p. 1022-3.
254. Sadowsky, M.J., et al., *Analysis of gut microbiota - An ever changing landscape*. Gut Microbes, 2017. **8**(3): p. 268-275.
255. Zhang, Q., et al., *Circulating mitochondrial DAMPs cause inflammatory responses to injury*. Nature, 2010. **464**(7285): p. 104-7.
256. Tierney, B.T., et al., *The Landscape of Genetic Content in the Gut and Oral Human Microbiome*. Cell Host Microbe, 2019. **26**(2): p. 283-295 e8.
257. Martinez-Guryn, K., V. Leone, and E.B. Chang, *Regional Diversity of the Gastrointestinal Microbiome*. Cell Host Microbe, 2019. **26**(3): p. 314-324.
258. Badal, V.D., et al., *The Gut Microbiome, Aging, and Longevity: A Systematic Review*. Nutrients, 2020. **12**(12).
259. Sanchez-Alcoholado, L., et al., *Relationships of Gut Microbiota Composition, Short-Chain Fatty Acids and Polyamines with the Pathological Response to Neoadjuvant Radiochemotherapy in Colorectal Cancer Patients*. Int J Mol Sci, 2021. **22**(17).
260. Kim, Y.S., et al., *Sex Differences in Gut Microbiota*. World J Mens Health, 2020. **38**(1): p. 48-60.
261. Dubberke, E.R., et al., *Clearance of vancomycin-resistant enterococcus concomitant with administration of a microbiota-based drug targeted at recurrent Clostridium difficile infection*. Open Forum Infect Dis, 2016. **3**(3): p. ofw133.

262. The Human Microbiome Project Consortium. *Structure, Function and Diversity of the Healthy Human Microbiome*. . Nature, 2012. **486**: p. 207-214.
263. Pickard, J.M., et al., *Gut microbiota: Role in pathogen colonization, immune responses, and inflammatory disease*. Immunol Rev, 2017. **279**(1): p. 70-89.
264. Eckburg, P.B., et al., *Diversity of the human intestinal microbial flora*. Science, 2005. **308**(5728): p. 1635-8.
265. Ley, R.E., et al., *Unexpected diversity and complexity of the Guerrero Negro hypersaline microbial mat*. Appl Environ Microbiol, 2006. **72**(5): p. 3685-95.
266. Donaldson, G.P., S.M. Lee, and S.K. Mazmanian, *Gut biogeography of the bacterial microbiota*. Nat Rev Microbiol, 2016. **14**(1): p. 20-32.
267. de Vos, W.M., et al., *Gut microbiome and health: mechanistic insights*. Gut, 2022. **71**(5): p. 1020-1032.
268. Bowman, J.P., et al., *International Committee on Systematics of Prokaryotes: Subcommittee on the taxonomy of aerobic Bacteroidota. Minutes of the online meeting 25 April 2023*. Int J Syst Evol Microbiol, 2023. **73**(11).
269. Salyers, A.A., A. Gupta, and Y. Wang, *Human intestinal bacteria as reservoirs for antibiotic resistance genes*. Trends Microbiol, 2004. **12**(9): p. 412-6.
270. Thomas, F., et al., *Environmental and gut bacteroidetes: the food connection*. Front Microbiol, 2011. **2**: p. 93.
271. Wexler, H.M., *Bacteroides: the good, the bad, and the nitty-gritty*. Clin Microbiol Rev, 2007. **20**(4): p. 593-621.
272. Zafar, H. and M.H. Saier, Jr., *Gut Bacteroides species in health and disease*. Gut Microbes, 2021. **13**(1): p. 1-20.
273. Bergstrom, K.S. and L. Xia, *Mucin-type O-glycans and their roles in intestinal homeostasis*. Glycobiology, 2013. **23**(9): p. 1026-37.
274. Marcobal, A., et al., *Consumption of human milk oligosaccharides by gut-related microbes*. J Agric Food Chem, 2010. **58**(9): p. 5334-40.
275. Martens, E.C., H.C. Chiang, and J.I. Gordon, *Mucosal glycan foraging enhances fitness and transmission of a saccharolytic human gut bacterial symbiont*. Cell Host Microbe, 2008. **4**(5): p. 447-57.
276. Kamada, N., et al., *Regulated virulence controls the ability of a pathogen to compete with the gut microbiota*. Science, 2012. **336**(6086): p. 1325-9.
277. Wu, S., et al., *A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses*. Nat Med, 2009. **15**(9): p. 1016-22.
278. Sears, C.L., *Enterotoxigenic Bacteroides fragilis: a rogue among symbiotes*. Clin Microbiol Rev, 2009. **22**(2): p. 349-69, Table of Contents.
279. Lopetuso, L.R., et al., *Commensal Clostridia: leading players in the maintenance of gut homeostasis*. Gut Pathog, 2013. **5**(1): p. 23.
280. Bennett, K.W. and A. Eley, *Fusobacteria: new taxonomy and related diseases*. J Med Microbiol, 1993. **39**(4): p. 246-54.
281. Anand, S., H. Kaur, and S.S. Mande, *Comparative In silico Analysis of Butyrate Production Pathways in Gut Commensals and Pathogens*. Front Microbiol, 2016. **7**: p. 1945.
282. Barcenilla, A., et al., *Phylogenetic relationships of butyrate-producing bacteria from the human gut*. Appl Environ Microbiol, 2000. **66**(4): p. 1654-61.
283. Duncan, S.H., et al., *Growth requirements and fermentation products of Fusobacterium prausnitzii, and a proposal to reclassify it as Faecalibacterium*

- prausnitzii* gen. nov., comb. nov. Int J Syst Evol Microbiol, 2002. **52**(Pt 6): p. 2141-2146.
284. Inan, M.S., et al., *The luminal short-chain fatty acid butyrate modulates NF-kappaB activity in a human colonic epithelial cell line*. Gastroenterology, 2000. **118**(4): p. 724-34.
 285. Schwab, M., et al., *Involvement of different nuclear hormone receptors in butyrate-mediated inhibition of inducible NF kappa B signalling*. Mol Immunol, 2007. **44**(15): p. 3625-32.
 286. Klampfer, L., et al., *Inhibition of interferon gamma signaling by the short chain fatty acid butyrate*. Mol Cancer Res, 2003. **1**(11): p. 855-62.
 287. Yang, M., et al., *Pharmaceutical efficacy of novel human-origin Faecalibacterium prausnitzii strains on high-fat-diet-induced obesity and associated metabolic disorders in mice*. Front Endocrinol (Lausanne), 2023. **14**: p. 1220044.
 288. Leylabadlo, H.E., et al., *The critical role of Faecalibacterium prausnitzii in human health: An overview*. Microb Pathog, 2020. **149**: p. 104344.
 289. Archer, S., et al., *Butyrate inhibits colon carcinoma cell growth through two distinct pathways*. Surgery, 1998. **124**(2): p. 248-53.
 290. Christl, S.U., et al., *Antagonistic effects of sulfide and butyrate on proliferation of colonic mucosa: a potential role for these agents in the pathogenesis of ulcerative colitis*. Dig Dis Sci, 1996. **41**(12): p. 2477-81.
 291. Ott, S.J., et al., *Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease*. Gut, 2004. **53**(5): p. 685-93.
 292. Kaser, A., S. Zeissig, and R.S. Blumberg, *Inflammatory bowel disease*. Annu Rev Immunol, 2010. **28**: p. 573-621.
 293. Willing, B.P., et al., *A pyrosequencing study in twins shows that gastrointestinal microbial profiles vary with inflammatory bowel disease phenotypes*. Gastroenterology, 2010. **139**(6): p. 1844-1854 e1.
 294. Sokol, H., 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): p. 16731-6.
 295. Buffie, C.G. and E.G. Pamer, *Microbiota-mediated colonization resistance against intestinal pathogens*. Nat Rev Immunol, 2013. **13**(11): p. 790-801.
 296. Picard, C., et al., *Review article: bifidobacteria as probiotic agents -- physiological effects and clinical benefits*. Aliment Pharmacol Ther, 2005. **22**(6): p. 495-512.
 297. Tanaka, H., et al., *Screening of lactic acid bacteria for bile salt hydrolase activity*. J Dairy Sci, 1999. **82**(12): p. 2530-5.
 298. Tanaka, H., et al., *Bile salt hydrolase of Bifidobacterium longum-biochemical and genetic characterization*. Appl Environ Microbiol, 2000. **66**(6): p. 2502-12.
 299. O'Callaghan, A. and D. van Sinderen, *Bifidobacteria and Their Role as Members of the Human Gut Microbiota*. Front Microbiol, 2016. **7**: p. 925.
 300. Harata, G., et al., *Species- and Age/Generation-Dependent Adherence of Bifidobacterium bifidum to Human Intestinal Mucus In Vitro*. Microorganisms, 2021. **9**(3).
 301. Gryaznova, M., et al., *Dynamics of Changes in the Gut Microbiota of Healthy Mice Fed with Lactic Acid Bacteria and Bifidobacteria*. Microorganisms, 2022. **10**(5).

302. Lindenberg, F., et al., *Expression of immune regulatory genes correlate with the abundance of specific Clostridiales and Verrucomicrobia species in the equine ileum and cecum*. Sci Rep, 2019. **9**(1): p. 12674.
303. Dubourg, G., et al., *High-level colonisation of the human gut by Verrucomicrobia following broad-spectrum antibiotic treatment*. Int J Antimicrob Agents, 2013. **41**(2): p. 149-55.
304. Ulker, I. and H. Yildiran, *The effects of bariatric surgery on gut microbiota in patients with obesity: a review of the literature*. Biosci Microbiota Food Health, 2019. **38**(1): p. 3-9.
305. Fujio-Vejar, S., et al., *The Gut Microbiota of Healthy Chilean Subjects Reveals a High Abundance of the Phylum Verrucomicrobia*. Front Microbiol, 2017. **8**: p. 1221.
306. Lepage, P., et al., *Twin study indicates loss of interaction between microbiota and mucosa of patients with ulcerative colitis*. Gastroenterology, 2011. **141**(1): p. 227-36.
307. Shin, N.R., T.W. Whon, and J.W. Bae, *Proteobacteria: microbial signature of dysbiosis in gut microbiota*. Trends Biotechnol, 2015. **33**(9): p. 496-503.
308. Taur, Y. and E.G. Pamer, *The intestinal microbiota and susceptibility to infection in immunocompromised patients*. Curr Opin Infect Dis, 2013. **26**(4): p. 332-7.
309. Taur, Y., et al., *Intestinal domination and the risk of bacteremia in patients undergoing allogeneic hematopoietic stem cell transplantation*. Clin Infect Dis, 2012. **55**(7): p. 905-14.
310. Kelly, D., L. Yang, and Z. Pei, *Gut Microbiota, Fusobacteria, and Colorectal Cancer*. Diseases, 2018. **6**(4).
311. Afra, K., et al., *Incidence, risk factors, and outcomes of Fusobacterium species bacteremia*. BMC Infect Dis, 2013. **13**: p. 264.
312. Bolstad, A.I., H.B. Jensen, and V. Bakken, *Taxonomy, biology, and periodontal aspects of Fusobacterium nucleatum*. Clin Microbiol Rev, 1996. **9**(1): p. 55-71.
313. Han, Y.W., *Fusobacterium nucleatum: a commensal-turned pathogen*. Curr Opin Microbiol, 2015. **23**: p. 141-7.
314. Su, W., et al., *Fusobacterium nucleatum Promotes the Development of Ulcerative Colitis by Inducing the Autophagic Cell Death of Intestinal Epithelial*. Front Cell Infect Microbiol, 2020. **10**: p. 594806.
315. Strauss, J., et al., *Invasive potential of gut mucosa-derived Fusobacterium nucleatum positively correlates with IBD status of the host*. Inflamm Bowel Dis, 2011. **17**(9): p. 1971-8.
316. Hugenholtz, P., S.D. Hooper, and N.C. Kyrpides, *Focus: Synergistetes*. Environ Microbiol, 2009. **11**(6): p. 1327-9.
317. Jumas-Bilak, E., L. Roudiere, and H. Marchandin, *Description of 'Synergistetes' phyl. nov. and emended description of the phylum 'Deferribacteres' and of the family Syntrophomonadaceae, phylum 'Firmicutes'*. Int J Syst Evol Microbiol, 2009. **59**(Pt 5): p. 1028-35.
318. Murphy, C.L., et al., *Genomic characterization of three novel Desulfobacterota classes expand the metabolic and phylogenetic diversity of the phylum*. Environ Microbiol, 2021. **23**(8): p. 4326-4343.
319. Horz, H.P., et al., *Synergistes group organisms of human origin*. J Clin Microbiol, 2006. **44**(8): p. 2914-20.
320. Vartoukian, S.R., R.M. Palmer, and W.G. Wade, *The division "Synergistes"*. Anaerobe, 2007. **13**(3-4): p. 99-106.

321. Tamargo, A., et al., *PET microplastics affect human gut microbiota communities during simulated gastrointestinal digestion, first evidence of plausible polymer biodegradation during human digestion*. Sci Rep, 2022. **12**(1): p. 528.
322. Figliuolo, V.R., et al., *Sulfate-reducing bacteria stimulate gut immune responses and contribute to inflammation in experimental colitis*. Life Sci, 2017. **189**: p. 29-38.
323. Langwig, M.V., et al., *Large-scale protein level comparison of Deltaproteobacteria reveals cohesive metabolic groups*. ISME J, 2022. **16**(1): p. 307-320.
324. Fallingborg, J., *Intraluminal pH of the human gastrointestinal tract*. Dan Med Bull, 1999. **46**(3): p. 183-96.
325. Payne, A.N., et al., *Advances and perspectives in in vitro human gut fermentation modeling*. Trends Biotechnol, 2012. **30**(1): p. 17-25.
326. Gibson, G.R. and M.B. Roberfroid, *Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics*. J Nutr, 1995. **125**(6): p. 1401-12.
327. Moore, W.E. and L.V. Holdeman, *Human fecal flora: the normal flora of 20 Japanese-Hawaiians*. Appl Microbiol, 1974. **27**(5): p. 961-79.
328. Maier, E., R.C. Anderson, and N.C. Roy, *Understanding how commensal obligate anaerobic bacteria regulate immune functions in the large intestine*. Nutrients, 2014. **7**(1): p. 45-73.
329. Singhal, R. and Y.M. Shah, *Oxygen battle in the gut: Hypoxia and hypoxia-inducible factors in metabolic and inflammatory responses in the intestine*. J Biol Chem, 2020. **295**(30): p. 10493-10505.
330. Rigottier-Gois, L., *Dysbiosis in inflammatory bowel diseases: the oxygen hypothesis*. ISME J, 2013. **7**(7): p. 1256-61.
331. Yoon, M.Y. and S.S. Yoon, *Disruption of the Gut Ecosystem by Antibiotics*. Yonsei Med J, 2018. **59**(1): p. 4-12.
332. Heinzinger, L.R., et al., *Oxygen and Metabolism: Digesting Determinants of Antibiotic Susceptibility in the Gut*. iScience, 2020. **23**(12): p. 101875.
333. Ignacio, A., et al., *Chapter 13 - Role of the Microbiome in Intestinal Barrier Function and Immune Defense*, in *Microbiome and Metabolome in Diagnosis, Therapy, and other Strategic Applications*, J. Faintuch and S. Faintuch, Editors. 2019, Academic Press. p. 127-138.
334. Caballero-Flores, G., J.M. Pickard, and G. Nunez, *Microbiota-mediated colonization resistance: mechanisms and regulation*. Nat Rev Microbiol, 2022.
335. Schroeder, B.O., et al., *Bifidobacteria or Fiber Protects against Diet-Induced Microbiota-Mediated Colonic Mucus Deterioration*. Cell Host Microbe, 2018. **23**(1): p. 27-40 e7.
336. Schroeder, B.O., *Fight them or feed them: how the intestinal mucus layer manages the gut microbiota*. Gastroenterol Rep (Oxf), 2019. **7**(1): p. 3-12.
337. Fernández-Tomé, S., et al., *Gut Microbiota and Dietary Factors as Modulators of the Mucus Layer in Inflammatory Bowel Disease*. Int J Mol Sci, 2021. **22**(19).
338. Johansson, M.E. and G.C. Hansson, *Immunological aspects of intestinal mucus and mucins*. Nat Rev Immunol, 2016. **16**(10): p. 639-49.
339. Kim, Y.S. and S.B. Ho, *Intestinal goblet cells and mucins in health and disease: recent insights and progress*. Curr Gastroenterol Rep, 2010. **12**(5): p. 319-30.
340. Franchi, L., et al., *NLRC4-driven production of IL-1beta discriminates between pathogenic and commensal bacteria and promotes host intestinal defense*. Nat Immunol, 2012. **13**(5): p. 449-56.

341. Strugnell, R.A. and O.L. Wijburg, *The role of secretory antibodies in infection immunity*. Nat Rev Microbiol, 2010. **8**(9): p. 656-67.
342. Frantz, A.L., et al., *Targeted deletion of MyD88 in intestinal epithelial cells results in compromised antibacterial immunity associated with downregulation of polymeric immunoglobulin receptor, mucin-2, and antibacterial peptides*. Mucosal Immunol, 2012. **5**(5): p. 501-12.
343. Fagarasan, S., et al., *Adaptive immune regulation in the gut: T cell-dependent and T cell-independent IgA synthesis*. Annu Rev Immunol, 2010. **28**: p. 243-73.
344. Suzuki, K., et al., *The sensing of environmental stimuli by follicular dendritic cells promotes immunoglobulin A generation in the gut*. Immunity, 2010. **33**(1): p. 71-83.
345. Sassone-Corsi, M. and M. Raffatellu, *No vacancy: how beneficial microbes cooperate with immunity to provide colonization resistance to pathogens*. J Immunol, 2015. **194**(9): p. 4081-7.
346. Khan, I., et al., *Mechanism of the Gut Microbiota Colonization Resistance and Enteric Pathogen Infection*. Front Cell Infect Microbiol, 2021. **11**: p. 716299.
347. Xiao, Y., et al., *Interleukin-33 Promotes REG3gamma Expression in Intestinal Epithelial Cells and Regulates Gut Microbiota*. Cell Mol Gastroenterol Hepatol, 2019. **8**(1): p. 21-36.
348. Satoh-Takayama, N., et al., *Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense*. Immunity, 2008. **29**(6): p. 958-70.
349. Sanos, S.L., et al., *RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells*. Nat Immunol, 2009. **10**(1): p. 83-91.
350. Kiss, E.A., et al., *Natural aryl hydrocarbon receptor ligands control organogenesis of intestinal lymphoid follicles*. Science, 2011. **334**(6062): p. 1561-5.
351. Brandl, K., et al., *Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits*. Nature, 2008. **455**(7214): p. 804-7.
352. Kinnebrew, M.A., et al., *Bacterial flagellin stimulates Toll-like receptor 5-dependent defense against vancomycin-resistant Enterococcus infection*. J Infect Dis, 2010. **201**(4): p. 534-43.
353. Salzman, N.H., M.A. Underwood, and C.L. Bevins, *Paneth cells, defensins, and the commensal microbiota: a hypothesis on intimate interplay at the intestinal mucosa*. Semin Immunol, 2007. **19**(2): p. 70-83.
354. Kamioka, M., et al., *Intestinal commensal microbiota and cytokines regulate Fut2(+) Paneth cells for gut defense*. Proc Natl Acad Sci U S A, 2022. **119**(3).
355. Vital, M., A.C. Howe, and J.M. Tiedje, *Revealing the bacterial butyrate synthesis pathways by analyzing (meta)genomic data*. mBio, 2014. **5**(2): p. e00889.
356. Louis, P. and H.J. Flint, *Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine*. FEMS Microbiol Lett, 2009. **294**(1): p. 1-8.
357. Louis, P., et al., *Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA:acetate CoA-transferase gene*. Environ Microbiol, 2010. **12**(2): p. 304-14.
358. Donohoe, D.R., et al., *Microbial regulation of glucose metabolism and cell-cycle progression in mammalian colonocytes*. PLoS One, 2012. **7**(9): p. e46589.

359. Kelly, C.J., et al., *Crosstalk between Microbiota-Derived Short-Chain Fatty Acids and Intestinal Epithelial HIF Augments Tissue Barrier Function*. *Cell Host Microbe*, 2015. **17**(5): p. 662-71.
360. Espey, M.G., *Role of oxygen gradients in shaping redox relationships between the human intestine and its microbiota*. *Free Radic Biol Med*, 2013. **55**: p. 130-40.
361. Chapman, M.A., et al., *Butyrate oxidation is impaired in the colonic mucosa of sufferers of quiescent ulcerative colitis*. *Gut*, 1994. **35**(1): p. 73-6.
362. Rivera-Chavez, F., et al., *Depletion of Butyrate-Producing Clostridia from the Gut Microbiota Drives an Aerobic Luminal Expansion of Salmonella*. *Cell Host Microbe*, 2016. **19**(4): p. 443-54.
363. Cotter, P.A., et al., *Cytochrome o (cyoABCDE) and d (cydAB) oxidase gene expression in Escherichia coli is regulated by oxygen, pH, and the fnr gene product*. *J Bacteriol*, 1990. **172**(11): p. 6333-8.
364. Cotter, P.A. and R.P. Gunsalus, *Contribution of the fnr and arcA gene products in coordinate regulation of cytochrome o and d oxidase (cyoABCDE and cydAB) genes in Escherichia coli*. *FEMS Microbiol Lett*, 1992. **70**(1): p. 31-6.
365. Alexeeva, S., K.J. Hellingwerf, and M.J. Teixeira de Mattos, *Requirement of ArcA for redox regulation in Escherichia coli under microaerobic but not anaerobic or aerobic conditions*. *J Bacteriol*, 2003. **185**(1): p. 204-9.
366. Alva-Murillo, N., A. Ochoa-Zarzosa, and J.E. Lopez-Meza, *Short chain fatty acids (propionic and hexanoic) decrease Staphylococcus aureus internalization into bovine mammary epithelial cells and modulate antimicrobial peptide expression*. *Vet Microbiol*, 2012. **155**(2-4): p. 324-31.
367. Jacobson, A., et al., *A Gut Commensal-Produced Metabolite Mediates Colonization Resistance to Salmonella Infection*. *Cell Host Microbe*, 2018. **24**(2): p. 296-307 e7.
368. Zheng, T., et al., *The farnesoid X receptor negatively regulates osteoclastogenesis in bone remodeling and pathological bone loss*. *Oncotarget*, 2017. **8**(44): p. 76558-76573.
369. Li, Z., et al., *Dual Targeting of Bile Acid Receptor-1 (TGR5) and Farnesoid X Receptor (FXR) Prevents Estrogen-Dependent Bone Loss in Mice*. *J Bone Miner Res*, 2019. **34**(4): p. 765-776.
370. Gonel, A. and I. Kirhan, *Effects of Broad Spectrum Antibiotics on Measurement of Immunosuppressant Drugs*. *Antiinflamm Antiallergy Agents Med Chem*, 2021. **20**(1): p. 101-105.
371. Shekhar, S. and F.C. Petersen, *The Dark Side of Antibiotics: Adverse Effects on the Infant Immune Defense Against Infection*. *Front Pediatr*, 2020. **8**: p. 544460.
372. Walker, A.W., et al., *Dominant and diet-responsive groups of bacteria within the human colonic microbiota*. *ISME J*, 2011. **5**(2): p. 220-30.
373. David, L.A., et al., *Diet rapidly and reproducibly alters the human gut microbiome*. *Nature*, 2014. **505**(7484): p. 559-63.
374. Salonen, A., et al., *Impact of diet and individual variation on intestinal microbiota composition and fermentation products in obese men*. *ISME J*, 2014. **8**(11): p. 2218-30.
375. Salonen, A., et al., *The adult intestinal core microbiota is determined by analysis depth and health status*. *Clin Microbiol Infect*, 2012. **18 Suppl 4**: p. 16-20.

376. Donskey, C.J., et al., *Inhibition of vancomycin-resistant enterococci by an in vitro continuous-flow competitive exclusion culture containing human stool flora*. J Infect Dis, 2001. **184**(12): p. 1624-7.
377. David, L.A., et al., *Host lifestyle affects human microbiota on daily timescales*. Genome Biol, 2014. **15**(7): p. R89.
378. Leeming, E.R., et al., *Effect of Diet on the Gut Microbiota: Rethinking Intervention Duration*. Nutrients, 2019. **11**(12).
379. Singh, R.K., et al., *Influence of diet on the gut microbiome and implications for human health*. J Transl Med, 2017. **15**(1): p. 73.
380. Reddy, B.S., J.H. Weisburger, and E.L. Wynder, *Effects of high risk and low risk diets for colon carcinogenesis on fecal microflora and steroids in man*. J Nutr, 1975. **105**(7): p. 878-84.
381. Raba, G. and A.S. Luis, *Mucin utilization by gut microbiota: recent advances on characterization of key enzymes*. Essays Biochem, 2023. **67**(3): p. 345-353.
382. Kommineni, S., et al., *Bacteriocin production augments niche competition by enterococci in the mammalian gastrointestinal tract*. Nature, 2015. **526**(7575): p. 719-22.
383. Corr, S.C., et al., *Bacteriocin production as a mechanism for the antiinfective activity of Lactobacillus salivarius UCC118*. Proc Natl Acad Sci U S A, 2007. **104**(18): p. 7617-21.
384. Sassone-Corsi, M., et al., *Microcins mediate competition among Enterobacteriaceae in the inflamed gut*. Nature, 2016. **540**(7632): p. 280-283.
385. Momose, Y., K. Hirayama, and K. Itoh, *Competition for proline between indigenous Escherichia coli and E. coli O157:H7 in gnotobiotic mice associated with infant intestinal microbiota and its contribution to the colonization resistance against E. coli O157:H7*. Antonie Van Leeuwenhoek, 2008. **94**(2): p. 165-71.
386. Fabich, A.J., et al., *Comparison of carbon nutrition for pathogenic and commensal Escherichia coli strains in the mouse intestine*. Infect Immun, 2008. **76**(3): p. 1143-52.
387. Durso, L.M., D. Smith, and R.W. Hutkins, *Measurements of fitness and competition in commensal Escherichia coli and E. coli O157:H7 strains*. Appl Environ Microbiol, 2004. **70**(11): p. 6466-72.
388. Deriu, E., et al., *Probiotic bacteria reduce salmonella typhimurium intestinal colonization by competing for iron*. Cell Host Microbe, 2013. **14**(1): p. 26-37.
389. Behnsen, J., et al., *Siderophore-mediated zinc acquisition enhances enterobacterial colonization of the inflamed gut*. Nat Commun, 2021. **12**(1): p. 7016.
390. den Besten, G., et al., *The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism*. J Lipid Res, 2013. **54**(9): p. 2325-40.
391. Repaske, D.R. and J. Adler, *Change in intracellular pH of Escherichia coli mediates the chemotactic response to certain attractants and repellents*. J Bacteriol, 1981. **145**(3): p. 1196-208.
392. Gorres, K.L., et al., *Activation and repression of Epstein-Barr Virus and Kaposi's sarcoma-associated herpesvirus lytic cycles by short- and medium-chain fatty acids*. J Virol, 2014. **88**(14): p. 8028-44.
393. Layden, B.T., et al., *Short chain fatty acids and their receptors: new metabolic targets*. Transl Res, 2013. **161**(3): p. 131-40.
394. Krautkramer, K.A., J. Fan, and F. Bäckhed, *Gut microbial metabolites as multi-kingdom intermediates*. Nat Rev Microbiol, 2021. **19**(2): p. 77-94.

395. Macfarlane, S. and G.T. Macfarlane, *Regulation of short-chain fatty acid production*. Proc Nutr Soc, 2003. **62**(1): p. 67-72.
396. Cummings, J.H., et al., *Short chain fatty acids in human large intestine, portal, hepatic and venous blood*. Gut, 1987. **28**(10): p. 1221-7.
397. Sellin, J.H., *SCFAs: The Enigma of Weak Electrolyte Transport in the Colon*. News Physiol Sci, 1999. **14**: p. 58-64.
398. Hurst, N.R., et al., *The short chain fatty acids, butyrate and propionate, have differential effects on the motility of the guinea pig colon*. Neurogastroenterol Motil, 2014. **26**(11): p. 1586-96.
399. You, H., et al., *The Therapeutic Effect of SCFA-Mediated Regulation of the Intestinal Environment on Obesity*. Front Nutr, 2022. **9**: p. 886902.
400. Topping, D.L. and P.M. Clifton, *Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides*. Physiol Rev, 2001. **81**(3): p. 1031-64.
401. Szczuko, M., et al., *The Associations of SCFA with Anthropometric Parameters and Carbohydrate Metabolism in Pregnant Women*. Int J Mol Sci, 2020. **21**(23).
402. Roe, A.J., et al., *Inhibition of Escherichia coli growth by acetic acid: a problem with methionine biosynthesis and homocysteine toxicity*. Microbiology (Reading), 2002. **148**(Pt 7): p. 2215-2222.
403. McDonald, J.A.K., et al., *Inhibiting Growth of Clostridioides difficile by Restoring Valerate, Produced by the Intestinal Microbiota*. Gastroenterology, 2018. **155**(5): p. 1495-1507.e15.
404. Gill, P.A., et al., *Review article: short chain fatty acids as potential therapeutic agents in human gastrointestinal and inflammatory disorders*. Aliment Pharmacol Ther, 2018. **48**(1): p. 15-34.
405. Ridlon, J.M., D.J. Kang, and P.B. Hylemon, *Bile salt biotransformations by human intestinal bacteria*. J Lipid Res, 2006. **47**(2): p. 241-59.
406. Keitel, V., R. Kubitz, and D. Haussinger, *Endocrine and paracrine role of bile acids*. World J Gastroenterol, 2008. **14**(37): p. 5620-9.
407. Hylemon, P.B., et al., *Bile acids as regulatory molecules*. J Lipid Res, 2009. **50**(8): p. 1509-20.
408. Monte, M.J., et al., *Bile acids: chemistry, physiology, and pathophysiology*. World J Gastroenterol, 2009. **15**(7): p. 804-16.
409. Sorg, J.A. and A.L. Sonenshein, *Bile salts and glycine as cogerminants for Clostridium difficile spores*. J Bacteriol, 2008. **190**(7): p. 2505-12.
410. Buffie, C.G., et al., *Precision microbiome reconstitution restores bile acid mediated resistance to Clostridium difficile*. Nature, 2015. **517**(7533): p. 205-8.
411. Sannasiddappa, T.H., P.A. Lund, and S.R. Clarke, *In Vitro Antibacterial Activity of Unconjugated and Conjugated Bile Salts on Staphylococcus aureus*. Front Microbiol, 2017. **8**: p. 1581.
412. Watanabe, M., S. Fukiya, and A. Yokota, *Comprehensive evaluation of the bactericidal activities of free bile acids in the large intestine of humans and rodents*. J Lipid Res, 2017. **58**(6): p. 1143-1152.
413. Rea, M.C., et al., *Thuricin CD, a posttranslationally modified bacteriocin with a narrow spectrum of activity against Clostridium difficile*. Proc Natl Acad Sci U S A, 2010. **107**(20): p. 9352-7.

414. Destoumieux-Garzon, D., et al., *Parasitism of iron-siderophore receptors of Escherichia coli by the siderophore-peptide microcin E492m and its unmodified counterpart*. Biometals, 2006. **19**(2): p. 181-91.
415. Mukhopadhyay, J., et al., *Antibacterial peptide microcin J25 inhibits transcription by binding within and obstructing the RNA polymerase secondary channel*. Molecular Cell, 2004. **14**(6): p. 739-751.
416. Cotter, P.D., R.P. Ross, and C. Hill, *Bacteriocins - a viable alternative to antibiotics?* Nature Reviews Microbiology, 2013. **11**(2): p. 95-105.
417. Kumariya, R., et al., *Bacteriocins: Classification, synthesis, mechanism of action and resistance development in food spoilage causing bacteria*. Microb Pathog, 2019. **128**: p. 171-177.
418. Alvarez-Sieiro, P., et al., *Bacteriocins of lactic acid bacteria: extending the family*. Appl Microbiol Biotechnol, 2016. **100**(7): p. 2939-51.
419. Wiedemann, I., et al., *Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity*. J Biol Chem, 2001. **276**(3): p. 1772-9.
420. Stevens, K.A., et al., *Nisin treatment for inactivation of Salmonella species and other gram-negative bacteria*. Appl Environ Microbiol, 1991. **57**(12): p. 3613-5.
421. Gillor, O., L.M. Nigro, and M.A. Riley, *Genetically engineered bacteriocins and their potential as the next generation of antimicrobials*. Curr Pharm Des, 2005. **11**(8): p. 1067-75.
422. Ritchie, J.M., et al., *An Escherichia coli O157-specific engineered pyocin prevents and ameliorates infection by E. coli O157:H7 in an animal model of diarrheal disease*. Antimicrob Agents Chemother, 2011. **55**(12): p. 5469-74.
423. Li, Q., M. Montalban-Lopez, and O.P. Kuipers, *Increasing the Antimicrobial Activity of Nisin-Based Lantibiotics against Gram-Negative Pathogens*. Appl Environ Microbiol, 2018. **84**(12).
424. Bosak, J., et al., *Colicin FY inhibits pathogenic Yersinia enterocolitica in mice*. Sci Rep, 2018. **8**(1): p. 12242.
425. Rea, M.C., et al., *Effect of broad- and narrow-spectrum antimicrobials on Clostridium difficile and microbial diversity in a model of the distal colon*. Proc Natl Acad Sci U S A, 2011. **108 Suppl 1**: p. 4639-44.
426. Rea, M.C., et al., *Bioavailability of the anti-clostridial bacteriocin thuricin CD in gastrointestinal tract*. Microbiology (Reading), 2014. **160**(Pt 2): p. 439-445.
427. Desai, M.S., et al., *A Dietary Fiber-Deprived Gut Microbiota Degrades the Colonic Mucus Barrier and Enhances Pathogen Susceptibility*. Cell, 2016. **167**(5): p. 1339-1353 e21.
428. Geldart, K.G., et al., *Engineered E. coli Nissle 1917 for the reduction of vancomycin-resistant Enterococcus in the intestinal tract*. Bioeng Transl Med, 2018. **3**(3): p. 197-208.
429. Giles, E.M., G.L. D'Adamo, and S.C. Forster, *The future of faecal transplants*. Nat Rev Microbiol, 2019. **17**(12): p. 719.
430. Aaij, R., et al., *Precision measurement of the mass and lifetime of the Xib(-) baryon*. Phys Rev Lett, 2014. **113**(24): p. 242002.
431. Stripling, J., et al., *Loss of Vancomycin-Resistant Enterococcus Fecal Dominance in an Organ Transplant Patient With Clostridium difficile Colitis After Fecal Microbiota Transplant*. Open Forum Infect Dis, 2015. **2**(2): p. ofv078.

432. Park, S.Y. and G.S. Seo, *Fecal Microbiota Transplantation: Is It Safe?* Clin Endosc, 2021. **54**(2): p. 157-160.
433. Baxter, M., et al., *Fatal Aspiration Pneumonia as a Complication of Fecal Microbiota Transplant.* Clin Infect Dis, 2015. **61**(1): p. 136-7.
434. Kelly, C.R., et al., *Fecal microbiota transplant for treatment of Clostridium difficile infection in immunocompromised patients.* Am J Gastroenterol, 2014. **109**(7): p. 1065-71.
435. Solari, P.R., et al., *Tempered enthusiasm for fecal transplant.* Clin Infect Dis, 2014. **59**(2): p. 319.
436. *Fecal Microbiota for Transplantation: Safety Alert - Risk of Serious Adverse Events Likely Due to Transmission of Pathogenic Organisms.* 2020, U.S. Food and Drug Administration.
437. *Important Safety Alert Regarding Use of Fecal Microbiota for Transplantation and Risk of Serious Adverse Reactions Due to Transmission of Multi-Drug Resistant Organisms.* 2019, U.S. Food and Drug Administration.
438. *Safety Alert Regarding Use of Fecal Microbiota for Transplantation and Additional Safety Protections Pertaining to SARSCoV-2 and COVID-19* 2020, U.S. Food and Drug Administration.
439. Gweon, T.G. and S.Y. Na, *Next Generation Fecal Microbiota Transplantation.* Clin Endosc, 2021. **54**(2): p. 152-156.
440. Petrof, E.O., et al., *Stool substitute transplant therapy for the eradication of Clostridium difficile infection: 'RePOOPulating' the gut.* Microbiome, 2013. **1**(1): p. 3.
441. Ott, S.J., et al., *Efficacy of Sterile Fecal Filtrate Transfer for Treating Patients With Clostridium difficile Infection.* Gastroenterology, 2017. **152**(4): p. 799-811 e7.
442. Macfarlane, G.T., S. Macfarlane, and G.R. Gibson, *Validation of a Three-Stage Compound Continuous Culture System for Investigating the Effect of Retention Time on the Ecology and Metabolism of Bacteria in the Human Colon.* Microb Ecol, 1998. **35**(2): p. 180-7.
443. Lesmes, U., et al., *Effects of resistant starch type III polymorphs on human colon microbiota and short chain fatty acids in human gut models.* J Agric Food Chem, 2008. **56**(13): p. 5415-21.
444. McDonald, J.A., et al., *Evaluation of microbial community reproducibility, stability and composition in a human distal gut chemostat model.* J Microbiol Methods, 2013. **95**(2): p. 167-74.
445. Uttley, A.H., et al., *Vancomycin-resistant enterococci.* Lancet, 1988. **1**(8575-6): p. 57-8.
446. Uttley, A.H., et al., *High-level vancomycin-resistant enterococci causing hospital infections.* Epidemiol Infect, 1989. **103**(1): p. 173-81.
447. Leclercq, R., et al., *Plasmid-mediated resistance to vancomycin and teicoplanin in Enterococcus faecium.* N Engl J Med, 1988. **319**(3): p. 157-61.
448. Dicks, J., et al., *NCTC3000: a century of bacterial strain collecting leads to a rich genomic data resource.* Microb Genom, 2023. **9**(5).
449. Woodford, N., *Glycopeptide-resistant enterococci: a decade of experience.* J Med Microbiol, 1998. **47**(10): p. 849-62.
450. Kuijper, E.J. and M.H. Wilcox, *Decreased effectiveness of metronidazole for the treatment of Clostridium difficile infection?* Clin Infect Dis, 2008. **47**(1): p. 63-5.

451. European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters, version 13.0. 2023; Available from: http://www.eucast.org/clinical_breakpoints/.
452. Wiegand, I., K. Hilpert, and R.E. Hancock, *Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances*. Nat Protoc, 2008. **3**(2): p. 163-75.
453. Miles, A.A., S.S. Misra, and J.O. Irwin, *The estimation of the bactericidal power of the blood*. J Hyg (Lond), 1938. **38**(6): p. 732-49.
454. Freter, R., et al., *Mechanisms that control bacterial populations in continuous-flow culture models of mouse large intestinal flora*. Infect Immun, 1983. **39**(2): p. 676-85.
455. Caballero, S., et al., *Cooperating Commensals Restore Colonization Resistance to Vancomycin-Resistant Enterococcus faecium*. Cell Host Microbe, 2017. **21**(5): p. 592-602 e4.
456. Isaac, S., et al., *Microbiome-mediated fructose depletion restricts murine gut colonization by vancomycin-resistant Enterococcus*. Nat Commun, 2022. **13**(1): p. 7718.
457. Erikstrup, L.T., et al., *Treatment of Clostridium difficile infection in mice with vancomycin alone is as effective as treatment with vancomycin and metronidazole in combination*. BMJ Open Gastroenterol, 2015. **2**(1): p. e000038.
458. Mullish, B.H., et al., *Functional microbiomics: Evaluation of gut microbiota-bile acid metabolism interactions in health and disease*. Methods, 2018. **149**: p. 49-58.
459. Rhoads, A. and K.F. Au, *PacBio Sequencing and Its Applications*. Genomics Proteomics Bioinformatics, 2015. **13**(5): p. 278-89.
460. *Template Preparation and Sequencing Guide*. 2010, Pacific Biosciences of California, Inc.
461. Slizovskiy, I.B., et al., *Target-enriched long-read sequencing (TELSeq) contextualizes antimicrobial resistance genes in metagenomes*. Microbiome, 2022. **10**(1): p. 185.
462. Callahan, B.J., et al., *Bioconductor Workflow for Microbiome Data Analysis: from raw reads to community analyses*. F1000Res, 2016. **5**: p. 1492.
463. McLaren, M.R., et al., *Implications of taxonomic bias for microbial differential-abundance analysis*. bioRxiv, 2022: p. 2022.08.19.504330.
464. mixOmics, *mixOmics:Omics Data Integration Project*. 2017, K. Le Cao, F. Rohart, I. Gonzalez and S. Dejean. p. R package.
465. Leekha, S., C.L. Terrell, and R.S. Edson, *General principles of antimicrobial therapy*. Mayo Clin Proc, 2011. **86**(2): p. 156-67.
466. Worley, B. and R. Powers, *PCA as a practical indicator of OPLS-DA model reliability*. Curr Metabolomics, 2016. **4**(2): p. 97-103.
467. Le Cao, K., et al., *mixOmicx:omics data integration project*. 2017.
468. Aaboud, M., et al., *Search for minimal supersymmetric standard model Higgs Bosons H/A and for a [Formula: see text] boson in the [Formula: see text] final state produced in pp collisions at [Formula: see text] TeV with the ATLAS detector*. Eur Phys J C Part Fields, 2016. **76**(11): p. 585.
469. Gonzalez, I., et al., *Visualising associations between paired 'omics' data sets*. BioData Min, 2012. **5**(1): p. 19.
470. Gloor, G.B., et al., *Microbiome Datasets Are Compositional: And This Is Not Optional*. Front Microbiol, 2017. **8**: p. 2224.

471. Horrocks, V., et al., *Role of the gut microbiota in nutrient competition and protection against intestinal pathogen colonization*. Microbiology (Reading), 2023. **169**(8).
472. Fukuda, S., et al., *Bifidobacteria can protect from enteropathogenic infection through production of acetate*. Nature, 2011. **469**(7331): p. 543-7.
473. Aw, W. and S. Fukuda, *Protective effects of bifidobacteria against enteropathogens*. Microb Biotechnol, 2019. **12**(6): p. 1097-1100.
474. Asahara, T., et al., *Probiotic bifidobacteria protect mice from lethal infection with Shiga toxin-producing Escherichia coli O157:H7*. Infect Immun, 2004. **72**(4): p. 2240-7.
475. Duar, R.M., D. Kyle, and G. Casaburi, *Colonization Resistance in the Infant Gut: The Role of B. infantis in Reducing pH and Preventing Pathogen Growth*. High Throughput, 2020. **9**(2).
476. Firrman, J., et al., *The impact of environmental pH on the gut microbiota community structure and short chain fatty acid production*. FEMS Microbiol Ecol, 2022. **98**(5).
477. Frese, S.A., et al., *Persistence of Supplemented Bifidobacterium longum subsp. infantis EVC001 in Breastfed Infants*. mSphere, 2017. **2**(6).
478. Kang, J.T.L., et al., *Long-term ecological and evolutionary dynamics in the gut microbiomes of carbapenemase-producing Enterobacteriaceae colonized subjects*. Nat Microbiol, 2022. **7**(10): p. 1516-1524.
479. Sequeira, R.P., et al., *Commensal Bacteroidetes protect against Klebsiella pneumoniae colonization and transmission through IL-36 signalling*. Nat Microbiol, 2020. **5**(2): p. 304-313.
480. Le Guern, R., et al., *Gut colonisation with multidrug-resistant Klebsiella pneumoniae worsens Pseudomonas aeruginosa lung infection*. Nat Commun, 2023. **14**(1): p. 78.
481. Antharam, V.C., et al., *Intestinal dysbiosis and depletion of butyrogenic bacteria in Clostridium difficile infection and nosocomial diarrhea*. J Clin Microbiol, 2013. **51**(9): p. 2884-92.
482. Perez-Cobas, A.E., et al., *Structural and functional changes in the gut microbiota associated to Clostridium difficile infection*. Front Microbiol, 2014. **5**: p. 335.
483. Perez-Cobas, A.E., et al., *Colonization Resistance of the Gut Microbiota against Clostridium difficile*. Antibiotics (Basel), 2015. **4**(3): p. 337-57.
484. Chen, O., et al., *Effect of arabinogalactan on the gut microbiome: A randomized, double-blind, placebo-controlled, crossover trial in healthy adults*. Nutrition, 2021. **90**: p. 111273.
485. Sun, Y., et al., *Prebiotic characteristics of arabinogalactans during in vitro fermentation through multi-omics analysis*. Food Chem Toxicol, 2021. **156**: p. 112522.
486. Aslam, H., et al., *The effects of dairy and dairy derivatives on the gut microbiota: a systematic literature review*. Gut Microbes, 2020. **12**(1): p. 1799533.
487. Smid, E.J., B. Poolman, and W.N. Konings, *Casein utilization by lactococci*. Appl Environ Microbiol, 1991. **57**(9): p. 2447-52.
488. Niness, K.R., *Inulin and oligofructose: what are they?* J Nutr, 1999. **129**(7 Suppl): p. 1402S-6S.
489. Correa, R.O., et al., *Inulin diet uncovers complex diet-microbiota-immune cell interactions remodeling the gut epithelium*. Microbiome, 2023. **11**(1): p. 90.
490. Pultz, N.J., L.C. Hoskins, and C.J. Donskey, *Vancomycin-resistant Enterococci may obtain nutritional support by scavenging carbohydrate fragments generated during*

- mucin degradation by the anaerobic microbiota of the colon*. Microb Drug Resist, 2006. **12**(1): p. 63-7.
491. Pan, M., N. Barua, and M. Ip, *Mucin-degrading gut commensals isolated from healthy faecal donor suppress intestinal epithelial inflammation and regulate tight junction barrier function*. Front Immunol, 2022. **13**: p. 1021094.
 492. Kaya, M., et al., *Characterization of citrus pectin samples extracted under different conditions: influence of acid type and pH of extraction*. Ann Bot, 2014. **114**(6): p. 1319-26.
 493. Elshahed, M.S., et al., *Pectin in diet: Interactions with the human microbiome, role in gut homeostasis, and nutrient-drug interactions*. Carbohydr Polym, 2021. **255**: p. 117388.
 494. Assaf, A., et al., *Evaluation of the impact of buffered peptone water composition on the discrimination between Salmonella enterica and Escherichia coli by Raman spectroscopy*. Anal Bioanal Chem, 2020. **412**(15): p. 3595-3604.
 495. Vital, M., et al., *Metagenomic Insights into the Degradation of Resistant Starch by Human Gut Microbiota*. Appl Environ Microbiol, 2018. **84**(23).
 496. Cerqueira, F.M., et al., *Starch Digestion by Gut Bacteria: Crowdsourcing for Carbs*. Trends Microbiol, 2020. **28**(2): p. 95-108.
 497. DeMartino, P. and D.W. Cockburn, *Resistant starch: impact on the gut microbiome and health*. Curr Opin Biotechnol, 2020. **61**: p. 66-71.
 498. Flint, H.J., et al., *Microbial degradation of complex carbohydrates in the gut*. Gut Microbes, 2012. **3**(4): p. 289-306.
 499. Bastawde, K.B., *Xylan structure, microbial xylanases, and their mode of action*. World J Microbiol Biotechnol, 1992. **8**(4): p. 353-68.
 500. Cockburn, D.W. and N.M. Koropatkin, *Polysaccharide Degradation by the Intestinal Microbiota and Its Influence on Human Health and Disease*. J Mol Biol, 2016. **428**(16): p. 3230-3252.
 501. Raimondi, S., et al., *Identification of mucin degraders of the human gut microbiota*. Sci Rep, 2021. **11**(1): p. 11094.
 502. Amaretti, A., et al., *Profiling of Protein Degradors in Cultures of Human Gut Microbiota*. Front Microbiol, 2019. **10**: p. 2614.
 503. Glowacki, R.W.P. and E.C. Martens, *If You Eat It or Secrete It, They Will Grow: the Expanding List of Nutrients Utilized by Human Gut Bacteria*. Journal of Bacteriology, 2021. **203**(9): p. 10.1128/jb.00481-20.
 504. Clavel, T., et al., *Intestinal microbiota in metabolic diseases: from bacterial community structure and functions to species of pathophysiological relevance*. Gut Microbes, 2014. **5**(4): p. 544-51.
 505. Kageyama, A., Y. Benno, and T. Nakase, *Phylogenetic and phenotypic evidence for the transfer of Eubacterium aerofaciens to the genus Collinsella as Collinsella aerofaciens gen. nov., comb. nov.* Int J Syst Bacteriol, 1999. **49 Pt 2**: p. 557-65.
 506. Fernandez-Julia, P., et al., *Cross-feeding interactions between human gut commensals belonging to the Bacteroides and Bifidobacterium genera when grown on dietary glycans*. Microbiome Res Rep, 2022. **1**(2): p. 1-12.
 507. Van Deun, K., et al., *Butyrate protects Caco-2 cells from Campylobacter jejuni invasion and translocation*. Br J Nutr, 2008. **100**(3): p. 480-4.

508. Peng, L., et al., *Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers*. J Nutr, 2009. **139**(9): p. 1619-25.
509. Hughes, E.R., et al., *Microbial Respiration and Formate Oxidation as Metabolic Signatures of Inflammation-Associated Dysbiosis*. Cell Host Microbe, 2017. **21**(2): p. 208-219.
510. Patel, M.S., et al., *The pyruvate dehydrogenase complexes: structure-based function and regulation*. J Biol Chem, 2014. **289**(24): p. 16615-23.
511. Ragsdale, S.W., *Pyruvate ferredoxin oxidoreductase and its radical intermediate*. Chem Rev, 2003. **103**(6): p. 2333-46.
512. Sawers, R.G. and D.P. Clark, *Fermentative Pyruvate and Acetyl-Coenzyme A Metabolism*. EcoSal Plus, 2004. **1**(1).
513. Catlett, J.L., et al., *Metabolic Feedback Inhibition Influences Metabolite Secretion by the Human Gut Symbiont Bacteroides thetaiotaomicron*. mSystems, 2020. **5**(5).
514. Nogal, A., et al., *Circulating Levels of the Short-Chain Fatty Acid Acetate Mediate the Effect of the Gut Microbiome on Visceral Fat*. Front Microbiol, 2021. **12**: p. 711359.
515. Hernandez, M.A.G., et al., *The Short-Chain Fatty Acid Acetate in Body Weight Control and Insulin Sensitivity*. Nutrients, 2019. **11**(8).
516. Schwiertz, A., et al., *Microbiota and SCFA in lean and overweight healthy subjects*. Obesity (Silver Spring), 2010. **18**(1): p. 190-5.
517. Cummings, J.H., *Short chain fatty acids in the human colon*. Gut, 1981. **22**(9): p. 763-79.
518. Wolin, M.J., et al., *Formate-dependent growth and homoacetogenic fermentation by a bacterium from human feces: description of Bryantella formatexigens gen. nov., sp. nov.* Appl Environ Microbiol, 2003. **69**(10): p. 6321-6.
519. Rey, F.E., et al., *Dissecting the in vivo metabolic potential of two human gut acetogens*. J Biol Chem, 2010. **285**(29): p. 22082-90.
520. Ohira, H., W. Tsutsui, and Y. Fujioka, *Are Short Chain Fatty Acids in Gut Microbiota Defensive Players for Inflammation and Atherosclerosis?* J Atheroscler Thromb, 2017. **24**(7): p. 660-672.
521. Sanders, M.E., et al., *Shared mechanisms among probiotic taxa: implications for general probiotic claims*. Curr Opin Biotechnol, 2018. **49**: p. 207-216.
522. Gupta, R.S., A. Nanda, and B. Khadka, *Novel molecular, structural and evolutionary characteristics of the phosphoketolases from bifidobacteria and Coriobacteriales*. PLoS One, 2017. **12**(2): p. e0172176.
523. Louis, P., G.L. Hold, and H.J. Flint, *The gut microbiota, bacterial metabolites and colorectal cancer*. Nat Rev Microbiol, 2014. **12**(10): p. 661-72.
524. Scott, K.P., et al., *Whole-genome transcription profiling reveals genes up-regulated by growth on fucose in the human gut bacterium "Roseburia inulinivorans"*. J Bacteriol, 2006. **188**(12): p. 4340-9.
525. Reichardt, N., et al., *Phylogenetic distribution of three pathways for propionate production within the human gut microbiota*. ISME J, 2014. **8**(6): p. 1323-35.
526. Koh, A., et al., *From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites*. Cell, 2016. **165**(6): p. 1332-1345.
527. Liu, H., et al., *Butyrate: A Double-Edged Sword for Health?* Adv Nutr, 2018. **9**(1): p. 21-29.

528. Vecchia, M.G., et al., *Acetate and propionate potentiate the antiproliferative effect of butyrate on RBL-2H3 growth*. Gen Pharmacol, 1997. **29**(5): p. 725-8.
529. Milovic, V., et al., *Effect of structural analogues of propionate and butyrate on colon cancer cell growth*. Int J Colorectal Dis, 2000. **15**(5-6): p. 264-70.
530. Roediger, W.E., *Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man*. Gut, 1980. **21**(9): p. 793-8.
531. Duncan, S.H., et al., *Acetate utilization and butyryl coenzyme A (CoA):acetate-CoA transferase in butyrate-producing bacteria from the human large intestine*. Appl Environ Microbiol, 2002. **68**(10): p. 5186-90.
532. Gao, G., et al., *Effects of valerate on intestinal barrier function in cultured Caco-2 epithelial cell monolayers*. Mol Biol Rep, 2022. **49**(3): p. 1817-1825.
533. Hinnebusch, B.F., et al., *The effects of short-chain fatty acids on human colon cancer cell phenotype are associated with histone hyperacetylation*. J Nutr, 2002. **132**(5): p. 1012-7.
534. de Smit, S.M., et al., *Continuous n-valerate formation from propionate and methanol in an anaerobic chain elongation open-culture bioreactor*. Biotechnol Biofuels, 2019. **12**: p. 132.
535. Seto, B. and T.C. Stadtman, *Purification and properties of proline reductase from Clostridium sticklandii*. J Biol Chem, 1976. **251**(8): p. 2435-9.
536. Nisman, B., *The Stickland reaction*. Bacteriol Rev, 1954. **18**(1): p. 16-42.
537. Theriot, C.M., et al., *Antibiotic-induced shifts in the mouse gut microbiome and metabolome increase susceptibility to Clostridium difficile infection*. Nat Commun, 2014. **5**: p. 3114.
538. Rios-Covian, D., et al., *An Overview on Fecal Branched Short-Chain Fatty Acids Along Human Life and as Related With Body Mass Index: Associated Dietary and Anthropometric Factors*. Front Microbiol, 2020. **11**: p. 973.
539. Morita, N., et al., *GPR31-dependent dendrite protrusion of intestinal CX3CR1(+) cells by bacterial metabolites*. Nature, 2019. **566**(7742): p. 110-114.
540. Luethy, P.M., et al., *Microbiota-Derived Short-Chain Fatty Acids Modulate Expression of Campylobacter jejuni Determinants Required for Commensalism and Virulence*. mBio, 2017. **8**(3).
541. Louis, P., et al., *Microbial lactate utilisation and the stability of the gut microbiome*. Gut Microbiome, 2022. **3**(E3).
542. Ladirat, S.E., et al., *High-throughput analysis of the impact of antibiotics on the human intestinal microbiota composition*. J Microbiol Methods, 2013. **92**(3): p. 387-97.
543. Lim, H. and H. Shin, *Introduction to Fed-Batch Cultures*, in *Fed-Batch Cultures: Principles and Applications of Semi-Batch Bioreactors*. 2013, Cambridge University Press: Cambridge. p. 1-18.
544. Pompei, A., et al., *In vitro comparison of the prebiotic effects of two inulin-type fructans*. Anaerobe, 2008. **14**(5): p. 280-6.
545. *Point prevalence survey of healthcare-associated infections and antimicrobial use in European acute care hospitals*, ECDC, Editor. 2013.
546. Autieri, S.M., et al., *L-fucose stimulates utilization of D-ribose by Escherichia coli MG1655 DeltafucAO and E. coli Nissle 1917 DeltafucAO mutants in the mouse intestine and in M9 minimal medium*. Infect Immun, 2007. **75**(11): p. 5465-75.

547. Shelton, C.D., et al., *Salmonella enterica* serovar Typhimurium uses anaerobic respiration to overcome propionate-mediated colonization resistance. *Cell Rep*, 2022. **38**(1): p. 110180.
548. Sorbara, M.T., et al., *Inhibiting antibiotic-resistant Enterobacteriaceae by microbiota-mediated intracellular acidification*. *J Exp Med*, 2019. **216**(1): p. 84-98.
549. Liu, W., L. Zhang, and H. Yi, *Development of a Chemically Defined Medium for Better Yield and Purification of Enterocin Y31 from Enterococcus faecium Y31*. *Journal of Food Quality*, 2017. **2017**.
550. McCoy, T.A. and S.H. Wender, *Some factors affecting the nutritional requirements of streptococcus faecalis*. *J Bacteriol*, 1952. **65**(6): p. 5.
551. Zhang, G., D.A. Mills, and D.E. Block, *Development of chemically defined media supporting high-cell-density growth of lactococci, enterococci, and streptococci*. *Appl Environ Microbiol*, 2009. **75**(4): p. 1080-7.
552. Rivera-Chavez, F., C.A. Lopez, and A.J. Baumler, *Oxygen as a driver of gut dysbiosis*. *Free Radic Biol Med*, 2017. **105**: p. 93-101.
553. Chang, D.E., et al., *Carbon nutrition of Escherichia coli in the mouse intestine*. *Proc Natl Acad Sci U S A*, 2004. **101**(19): p. 7427-32.
554. Turner, C., et al., *Rectal pH in well and unwell infants*. *J Trop Pediatr*, 2012. **58**(4): p. 311-3.
555. Yamamura, R., et al., *Intestinal and fecal pH in human health*. *Frontiers in Microbiomes*, 2023. **2**.
556. Ilhan, Z.E., et al., *pH-Mediated Microbial and Metabolic Interactions in Fecal Enrichment Cultures*. *mSphere*, 2017. **2**(3).
557. Odenwald, M.A., et al., *Bifidobacteria metabolize lactulose to optimize gut metabolites and prevent systemic infection in patients with liver disease*. *Nature Microbiology*, 2023. **8**(11): p. 2033-2049.
558. Pultz, N.J., et al., *Mechanisms by which anaerobic microbiota inhibit the establishment in mice of intestinal colonization by vancomycin-resistant Enterococcus*. *J Infect Dis*, 2005. **191**(6): p. 949-56.
559. Kim, S.G., et al., *Microbiota-derived lantibiotic restores resistance against vancomycin-resistant Enterococcus*. *Nature*, 2019. **572**(7771): p. 665-669.
560. Ramsey, M., A. Hartke, and M. Huycke, *The Physiology and Metabolism of Enterococci*, in *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*, M.S. Gilmore, et al., Editors. 2014: Boston.
561. Palmer, K.L., et al., *Enterococcal Genomics*, in *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection*, M.S. Gilmore, et al., Editors. 2014: Boston.
562. McKenney, P.T., et al., *Intestinal Bile Acids Induce a Morphotype Switch in Vancomycin-Resistant Enterococcus that Facilitates Intestinal Colonization*. *Cell Host Microbe*, 2019. **25**(5): p. 695-705 e5.
563. Molly, K., M. Vande Woestyne, and W. Verstraete, *Development of a 5-step multi-chamber reactor as a simulation of the human intestinal microbial ecosystem*. *Appl Microbiol Biotechnol*, 1993. **39**(2): p. 254-8.
564. Barrila, J., et al., *Three-dimensional organotypic co-culture model of intestinal epithelial cells and macrophages to study Salmonella enterica colonization patterns*. *NPJ Microgravity*, 2017. **3**: p. 10.

565. Barrila, J., et al., *Organotypic 3D cell culture models: using the rotating wall vessel to study host-pathogen interactions*. Nat Rev Microbiol, 2010. **8**(11): p. 791-801.
566. Drummond, C.G., C.A. Nickerson, and C.B. Coyne, *A Three-Dimensional Cell Culture Model To Study Enterovirus Infection of Polarized Intestinal Epithelial Cells*. mSphere, 2016. **1**(1).
567. Ingber, D.E., *Human organs-on-chips for disease modelling, drug development and personalized medicine*. Nat Rev Genet, 2022. **23**(8): p. 467-491.
568. Xiang, Y., et al., *Gut-on-chip: Recreating human intestine in vitro*. J Tissue Eng, 2020. **11**: p. 2041731420965318.
569. Rycroft, C.E., et al., *A comparative in vitro evaluation of the fermentation properties of prebiotic oligosaccharides*. J Appl Microbiol, 2001. **91**(5): p. 878-87.
570. Vulevic, J., R.A. Rastall, and G.R. Gibson, *Developing a quantitative approach for determining the in vitro prebiotic potential of dietary oligosaccharides*. FEMS Microbiol Lett, 2004. **236**(1): p. 153-9.
571. Allison, C., C. McFarlan, and G.T. MacFarlane, *Studies on mixed populations of human intestinal bacteria grown in single-stage and multistage continuous culture systems*. Appl Environ Microbiol, 1989. **55**(3): p. 672-8.
572. Minekus, M., et al., *A computer-controlled system to simulate conditions of the large intestine with peristaltic mixing, water absorption and absorption of fermentation products*. Appl Microbiol Biotechnol, 1999. **53**(1): p. 108-14.
573. Vujkovic-Cvijin, I., et al., *Host variables confound gut microbiota studies of human disease*. Nature, 2020. **587**(7834): p. 448-454.
574. Miyoshi, J., et al., *Minimizing confounders and increasing data quality in murine models for studies of the gut microbiome*. PeerJ, 2018. **6**: p. e5166.
575. Gianetto-Hill, C.M., et al., *The Robogut: A Bioreactor Model of the Human Colon for Evaluation of Gut Microbial Community Ecology and Function*. Curr Protoc, 2023. **3**(4): p. e737.
576. Moura, I.B., et al., *Method comparison for the direct enumeration of bacterial species using a chemostat model of the human colon*. BMC Microbiol, 2020. **20**(1): p. 2.
577. Crowther, G.S., et al., *Development and validation of a chemostat gut model to study both planktonic and biofilm modes of growth of Clostridium difficile and human microbiota*. PLoS One, 2014. **9**(2): p. e88396.
578. *WHO publishes list of bacteria for which new antibiotics are urgently needed*. 2017, World Health Organization
579. Morris, J.G., Jr., et al., *Enterococci resistant to multiple antimicrobial agents, including vancomycin. Establishment of endemicity in a university medical center*. Ann Intern Med, 1995. **123**(4): p. 250-9.
580. Moreno, F., et al., *Clinical and molecular epidemiology of vancomycin-resistant Enterococcus faecium during its emergence in a city in southern Texas*. Clin Infect Dis, 1995. **21**(5): p. 1234-7.
581. Dahms, R.A., et al., *Third-generation cephalosporins and vancomycin as risk factors for postoperative vancomycin-resistant enterococcus infection*. Arch Surg, 1998. **133**(12): p. 1343-6.
582. Tornieporth, N.G., et al., *Risk factors associated with vancomycin-resistant Enterococcus faecium infection or colonization in 145 matched case patients and control patients*. Clin Infect Dis, 1996. **23**(4): p. 767-72.

583. Cetinkaya, Y., P. Falk, and C.G. Mayhall, *Vancomycin-resistant enterococci*. Clin Microbiol Rev, 2000. **13**(4): p. 686-707.
584. Gjonbalaj, M., et al., *Antibiotic Degradation by Commensal Microbes Shields Pathogens*. Infect Immun, 2020. **88**(4).
585. Jernberg, C., et al., *Long-term ecological impacts of antibiotic administration on the human intestinal microbiota*. ISME J, 2007. **1**(1): p. 56-66.
586. Patangia, D.V., et al., *Impact of antibiotics on the human microbiome and consequences for host health*. Microbiologyopen, 2022. **11**(1): p. e1260.
587. Guinan, J., et al., *Antibiotic-induced decreases in the levels of microbial-derived short-chain fatty acids correlate with increased gastrointestinal colonization of Candida albicans*. Sci Rep, 2019. **9**(1): p. 8872.
588. Ramirez, J., et al., *Antibiotics as Major Disruptors of Gut Microbiota*. Front Cell Infect Microbiol, 2020. **10**: p. 572912.
589. Shen, B., et al., *Antibiotics exacerbated colitis by affecting the microbiota, Treg cells and SCFAs in IL10-deficient mice*. Biomed Pharmacother, 2019. **114**: p. 108849.
590. Khan, T., et al., *Association of gut dysbiosis with intestinal metabolites in response to antibiotic treatment*. Human Microbiome Journal, 2019. **11**.
591. Mu, C., et al., *Alteration of metabolomic markers of amino-acid metabolism in piglets with in-feed antibiotics*. Amino Acids, 2017. **49**(4): p. 771-781.
592. Yang, J., et al., *Oscillospira - a candidate for the next-generation probiotics*. Gut Microbes, 2021. **13**(1): p. 1987783.
593. Piekarska, J., et al., *Trichinella spiralis: the influence of short chain fatty acids on the proliferation of lymphocytes, the goblet cell count and apoptosis in the mouse intestine*. Exp Parasitol, 2011. **128**(4): p. 419-26.
594. Chen, R., et al., *Puerariae Lobatae Radix with chuanxiong Rhizoma for treatment of cerebral ischemic stroke by remodeling gut microbiota to regulate the brain-gut barriers*. J Nutr Biochem, 2019. **65**: p. 101-114.
595. Upadhyaya, B., et al., *Impact of dietary resistant starch type 4 on human gut microbiota and immunometabolic functions*. Sci Rep, 2016. **6**: p. 28797.
596. Oki, K., et al., *Comprehensive analysis of the fecal microbiota of healthy Japanese adults reveals a new bacterial lineage associated with a phenotype characterized by a high frequency of bowel movements and a lean body type*. BMC Microbiol, 2016. **16**(1): p. 284.
597. Waters, J.L. and R.E. Ley, *The human gut bacteria Christensenellaceae are widespread, heritable, and associated with health*. BMC Biol, 2019. **17**(1): p. 83.
598. Banla, L.I., N.H. Salzman, and C.J. Kristich, *Colonization of the mammalian intestinal tract by enterococci*. Curr Opin Microbiol, 2019. **47**: p. 26-31.
599. Zhang, X., et al., *Identification of a genetic determinant in clinical Enterococcus faecium strains that contributes to intestinal colonization during antibiotic treatment*. J Infect Dis, 2013. **207**(11): p. 1780-6.
600. Tuncil, Y.E., et al., *Reciprocal Prioritization to Dietary Glycans by Gut Bacteria in a Competitive Environment Promotes Stable Coexistence*. mBio, 2017. **8**(5).
601. Pudlo, N.A., et al., *Phenotypic and Genomic Diversification in Complex Carbohydrate-Degrading Human Gut Bacteria*. mSystems, 2022. **7**(1): p. e0094721.
602. Blaser, M., *Antibiotic overuse: Stop the killing of beneficial bacteria*. Nature, 2011. **476**(7361): p. 393-4.

603. Dubourg, G., et al., *Culturomics and pyrosequencing evidence of the reduction in gut microbiota diversity in patients with broad-spectrum antibiotics*. Int J Antimicrob Agents, 2014. **44**(2): p. 117-24.
604. Engevik, M.A., et al., *Mucin-Degrading Microbes Release Monosaccharides That Chemoattract Clostridioides difficile and Facilitate Colonization of the Human Intestinal Mucus Layer*. ACS Infect Dis, 2021. **7**(5): p. 1126-1142.
605. Wang, H., et al., *Daily fluctuation of colonic microbiome in response to nutrient substrates in a pig model*. NPJ Biofilms Microbiomes, 2023. **9**(1): p. 85.
606. Davido, B., et al., *Fecal microbiota transplantation to eradicate vancomycin-resistant enterococci colonization in case of an outbreak*. Med Mal Infect, 2019. **49**(3): p. 214-218.
607. Shin, J., et al., *Efficacy and Safety of Fecal Microbiota Transplantation for Clearance of Multidrug-Resistant Organisms under Multiple Comorbidities: A Prospective Comparative Trial*. Biomedicines, 2022. **10**(10).
608. Hyun, J., et al., *Faecal microbiota transplantation reduces amounts of antibiotic resistance genes in patients with multidrug-resistant organisms*. Antimicrob Resist Infect Control, 2022. **11**(1): p. 20.
609. Woodworth, M.H., et al., *The Role of Fecal Microbiota Transplantation in Reducing Intestinal Colonization With Antibiotic-Resistant Organisms: The Current Landscape and Future Directions*. Open Forum Infect Dis, 2019. **6**(7).
610. Davido, B., et al., *Impact of faecal microbiota transplantation to eradicate vancomycin-resistant enterococci (VRE) colonization in humans*. J Infect, 2017. **75**(4): p. 376-377.
611. Shenoy, E.S., et al., *Natural history of colonization with methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant Enterococcus (VRE): a systematic review*. BMC Infect Dis, 2014. **14**: p. 177.
612. Crum-Cianflone, N.F., E. Sullivan, and G. Ballon-Landa, *Fecal microbiota transplantation and successful resolution of multidrug-resistant-organism colonization*. J Clin Microbiol, 2015. **53**(6): p. 1986-9.
613. Davido, B., et al., *Is faecal microbiota transplantation an option to eradicate highly drug-resistant enteric bacteria carriage?* J Hosp Infect, 2017. **95**(4): p. 433-437.
614. Dubberke, E.R., et al., *Clearance of Vancomycin-Resistant Enterococcus Concomitant With Administration of a Microbiota-Based Drug Targeted at Recurrent Clostridium difficile Infection*. Open Forum Infect Dis, 2016. **3**(3): p. ofw133.
615. Le Bastard, Q., et al., *Fecal microbiota transplantation reverses antibiotic and chemotherapy-induced gut dysbiosis in mice*. Sci Rep, 2018. **8**(1): p. 6219.
616. Zhang, J., Y. Guo, and L. Duan, *Features of Gut Microbiome Associated With Responses to Fecal Microbiota Transplantation for Inflammatory Bowel Disease: A Systematic Review*. Front Med (Lausanne), 2022. **9**: p. 773105.
617. Lou, X., et al., *Fecal microbiota transplantation and short-chain fatty acids reduce sepsis mortality by remodeling antibiotic-induced gut microbiota disturbances*. Front Immunol, 2022. **13**: p. 1063543.
618. El-Salhy, M., et al., *Changes in fecal short-chain fatty acids following fecal microbiota transplantation in patients with irritable bowel syndrome*. Neurogastroenterol Motil, 2021. **33**(2): p. e13983.
619. Su, S.H., et al., *Fecal microbiota transplantation and replenishment of short-chain fatty acids protect against chronic cerebral hypoperfusion-induced colonic*

- dysfunction by regulating gut microbiota, differentiation of Th17 cells, and mitochondrial energy metabolism.* J Neuroinflammation, 2022. **19**(1): p. 313.
620. Bahl, M.I., et al., *Faecal microbiota transplantation for eradication of co-infection with Clostridioides difficile and extensively drug-resistant KPC-producing Klebsiella pneumoniae.* Scand J Gastroenterol, 2020. **55**(5): p. 626-630.
 621. Bilinski, J., 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): p. 364-370.
 622. Jang, M.O., et al., *Refractory Clostridium difficile Infection Cured With Fecal Microbiota Transplantation in Vancomycin-Resistant Enterococcus Colonized Patient.* Intest Res, 2015. **13**(1): p. 80-4.
 623. Nguyen, T.L., et al., *How informative is the mouse for human gut microbiota research?* Dis Model Mech, 2015. **8**(1): p. 1-16.
 624. Park, J.C. and S.H. Im, *Of men in mice: the development and application of a humanized gnotobiotic mouse model for microbiome therapeutics.* Exp Mol Med, 2020. **52**(9): p. 1383-1396.
 625. Weiss, G., *Intestinal irony: how probiotic bacteria outcompete bad bugs.* Cell Host Microbe, 2013. **14**(1): p. 3-4.
 626. Sommer, M.O., *Advancing gut microbiome research using cultivation.* Curr Opin Microbiol, 2015. **27**: p. 127-32.
 627. Goodman, A.L., et al., *Extensive personal human gut microbiota culture collections characterized and manipulated in gnotobiotic mice.* Proc Natl Acad Sci U S A, 2011. **108**(15): p. 6252-7.
 628. Rettedal, E.A., H. Gumpert, and M.O. Sommer, *Cultivation-based multiplex phenotyping of human gut microbiota allows targeted recovery of previously uncultured bacteria.* Nat Commun, 2014. **5**: p. 4714.
 629. Adamberg, K. and S. Adamberg, *Selection of fast and slow growing bacteria from fecal microbiota using continuous culture with changing dilution rate.* Microb Ecol Health Dis, 2018. **29**(1): p. 1549922.
 630. Hudson, A.W., et al., *Klebsiella pneumoniae l-Fucose Metabolism Promotes Gastrointestinal Colonization and Modulates Its Virulence Determinants.* Infect Immun, 2022. **90**(10): p. e0020622.
 631. Nagpal, R., et al., *Human-origin probiotic cocktail increases short-chain fatty acid production via modulation of mice and human gut microbiome.* Sci Rep, 2018. **8**(1): p. 12649.
 632. Behnsen, J., et al., *Probiotics: properties, examples, and specific applications.* Cold Spring Harb Perspect Med, 2013. **3**(3): p. a010074.
 633. Marco, M.L., S. Pavan, and M. Kleerebezem, *Towards understanding molecular modes of probiotic action.* Curr Opin Biotechnol, 2006. **17**(2): p. 204-10.
 634. Wang, X. and P.J. Quinn, *Endotoxins: lipopolysaccharides of gram-negative bacteria.* Subcell Biochem, 2010. **53**: p. 3-25.
 635. Cross, M.L., et al., *Patterns of cytokine induction by gram-positive and gram-negative probiotic bacteria.* FEMS Immunol Med Microbiol, 2004. **42**(2): p. 173-80.
 636. Sonnenburg, J.L., C.T. Chen, and J.I. Gordon, *Genomic and metabolic studies of the impact of probiotics on a model gut symbiont and host.* PLoS Biol, 2006. **4**(12): p. e413.

637. Ricci, L., et al., *Human gut bifidobacteria inhibit the growth of the opportunistic fungal pathogen Candida albicans*. FEMS Microbiol Ecol, 2022. **98**(10).
638. Ricci, L., et al., *Bifidobacterium adolescentis shows potential to strengthen host defence against gastrointestinal infection via inhibition of the opportunistic pathogen Candida albicans and stimulation of human-isolated macrophages killing capacity in vitro*. Access Microbiology, 2020. **2**(7A).
639. Hassall, J., J.K.J. Cheng, and M. Unnikrishnan, *Dissecting Individual Interactions between Pathogenic and Commensal Bacteria within a Multispecies Gut Microbial Community*. mSphere, 2021. **6**(2).
640. Salli, K., et al., *Selective Utilization of the Human Milk Oligosaccharides 2'-Fucosyllactose, 3-Fucosyllactose, and Difucosyllactose by Various Probiotic and Pathogenic Bacteria*. J Agric Food Chem, 2021. **69**(1): p. 170-182.
641. Fultz, R., et al., *Unraveling the Metabolic Requirements of the Gut Commensal Bacteroides ovatus*. Front Microbiol, 2021. **12**: p. 745469.
642. Lawson, M.A.E., et al., *Breast milk-derived human milk oligosaccharides promote Bifidobacterium interactions within a single ecosystem*. ISME J, 2020. **14**(2): p. 635-648.
643. Li, S., et al., *Bifidobacterium infantis utilizes N-acetylglucosamine-containing human milk oligosaccharides as a nitrogen source*. Gut Microbes, 2023. **15**(2): p. 2244721.
644. Zivkovic, A.M., et al., *Human milk glycometabolism and its impact on the infant gastrointestinal microbiota*. Proc Natl Acad Sci U S A, 2011. **108** Suppl 1(Suppl 1): p. 4653-8.
645. Avershina, E., et al., *Bifidobacterial succession and correlation networks in a large unselected cohort of mothers and their children*. Appl Environ Microbiol, 2013. **79**(2): p. 497-507.
646. Laureys, D. and L. De Vuyst, *Microbial species diversity, community dynamics, and metabolite kinetics of water kefir fermentation*. Appl Environ Microbiol, 2014. **80**(8): p. 2564-72.
647. Kim, J. and K.H. Kim, *Effects of minimal media vs. complex media on the metabolite profiles of Escherichia coli and Saccharomyces cerevisiae*. Process Biochemistry, 2017. **57**: p. 64-71.
648. Wandro, S., et al., *Phage Cocktails Constrain the Growth of Enterococcus*. mSystems, 2022. **7**(4): p. e0001922.
649. Shlezinger, M., et al., *Eradication of Vancomycin-Resistant Enterococci by Combining Phage and Vancomycin*. Viruses, 2019. **11**(10).
650. Pradal, I., et al., *Enterococcus faecium Bacteriophage vB_EfaH_163, a New Member of the Herelleviridae Family, Reduces the Mortality Associated with an E. faecium vanR Clinical Isolate in a Galleria mellonella Animal Model*. Viruses, 2023. **15**(1).
651. Ley, R.E., et al., *Obesity alters gut microbial ecology*. Proc Natl Acad Sci U S A, 2005. **102**(31): p. 11070-5.
652. Walter, J., M.X. Maldonado-Gomez, and I. Martinez, *To engraft or not to engraft: an ecological framework for gut microbiome modulation with live microbes*. Curr Opin Biotechnol, 2018. **49**: p. 129-139.
653. Freitag, T.L., et al., *Minor Effect of Antibiotic Pre-treatment on the Engraftment of Donor Microbiota in Fecal Transplantation in Mice*. Front Microbiol, 2019. **10**: p. 2685.

654. Li, X., et al., *Two Strains of Lactobacilli Effectively Decrease the Colonization of VRE in a Mouse Model*. Front Cell Infect Microbiol, 2019. **9**: p. 6.
655. Manley, K.J., et al., *Probiotic treatment of vancomycin-resistant enterococci: a randomised controlled trial*. Med J Aust, 2007. **186**(9): p. 454-7.
656. Szachta, P., I. Ignys, and W. Cichy, *An evaluation of the ability of the probiotic strain Lactobacillus rhamnosus GG to eliminate the gastrointestinal carrier state of vancomycin-resistant enterococci in colonized children*. J Clin Gastroenterol, 2011. **45**(10): p. 872-7.
657. Vidal, M., et al., *Probiotics and intestinal colonization by vancomycin-resistant enterococci in mice and humans*. J Clin Microbiol, 2010. **48**(7): p. 2595-8.
658. de Regt, M.J., et al., *Effects of probiotics on acquisition and spread of multiresistant enterococci*. Antimicrob Agents Chemother, 2010. **54**(7): p. 2801-5.
659. Samonis, G., et al., *Prospective evaluation of effects of broad-spectrum antibiotics on gastrointestinal yeast colonization of humans*. Antimicrob Agents Chemother, 1993. **37**(1): p. 51-3.
660. Mulligan, M.E., et al., *Impact of cefoperazone therapy on fecal flora*. Antimicrob Agents Chemother, 1982. **22**(2): p. 226-30.
661. Dollive, S., et al., *Fungi of the murine gut: episodic variation and proliferation during antibiotic treatment*. PLoS One, 2013. **8**(8): p. e71806.
662. Spatz, M., et al., *Antibiotic treatment using amoxicillin-clavulanic acid impairs gut mycobiota development through modification of the bacterial ecosystem*. Microbiome, 2023. **11**(1): p. 73.