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Impact of Antibiotics on the Gut and Milk Microbiome

Thesis presented by

Dhrati Patangia, M.tech

for the degree of

Doctor of Philosophy

UCC student number: 118221884

University College Cork

School of Microbiology, University College Cork, Cork, Ireland

Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

APC Microbiome Ireland, University College Cork, Cork, Ireland

Supervisor(s): Prof. Catherine Stanton, Prof. R. Paul Ross

Head of School/Department: Dr David Clarke

March 2023





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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.

Contributions

Chapter 3: Microbiota and resistome analysis of colostrum and milk from dairy cows: Involved in conceptualisation of the study along with supervisors. Performed DNA extractions, shotgun sequencing library preparation of colostrum and milk samples collected as part of the study. Also processed and analysed the shotgun sequenced metagenomics samples for microbial and resistome analysis in collaboration with Dr Ghjuvan Grimaud.

Chapter 4: Early life antibiotic exposure has an immediate and persistent effect on infant gut microbiome and Resistome: Involved in looking for previous DNA and stool samples, carried out DNA extractions. Performed shotgun sequencing library preparation for two of the three runs of shotgun sequencing data included in this study. Was involved in bioinformatics analysis of shotgun sequencing data to carry out microbial and resistome profiling in collaboration with Dr Ghjuvan Grimaud. Involved in conceptualisation along with supervisors and writing.

Chapter 5: Global antibiotic resistance gene prevalence in infants – A meta-analysis: Involved in conceptualisation of the study with supervisors. Performed searching the PubMed database for finding studies, reviewing papers thoroughly to match the inclusion criteria and short listing studies for further analysis. Further, was involved in matching the metadata to samples from each study and processing the samples in collaboration with Dr Shaopu Wang. Performed further downstream analysis in collaboration with Dr Ghjuvan Grimaud.

Chapter 6: Gut Microbiome and Resistome Profile of Cystic Fibrosis Individuals - A Pilot Study: Used publicly available shotgun sequencing data from a previous study by Fiona Fouhy et al., (2017). Performed bioinformatics and statistical analysis along with the help of Dr Raul Cabrera Rubio.

Appendix paper: A compendium of 32,277 metagenome-assembled genomes and over 80 million genes from the early-life human gut microbiome: Involved in conceptualisation of the study along with supervisors. Completed a comprehensive PubMed search to short-list studies for the meta-analysis. Performed shotgun-sequence

data and metadata downloading and matching the metadata to samples from each study and preparing for further downstream processing.

Signature: ____  _____

Date: _____03/03/2023_____

Student number: 118221884

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Abstract

While antibiotics are lifesaving, they come at the cost of antimicrobial resistance (AMR) and collateral damage to microbiome. Using single-sample and longitudinal study designs, this thesis aimed to study antibiotic resistance genes (ARGs) spread using shotgun metagenomics sequencing. ARGs developed in animals can enter humans through the food chain. We examined the effect of single antibiotic use on microbiome and resistome in milk from dairy cows longitudinally. The microbial diversity increased from colostrum to later time-points in the antibiotic groups which was not prominent in the no-ab group. Further, microbial composition in all groups was different, leading to distinct clustering of no-antibiotic from antibiotic groups. All groups had a wide resistome profile, with antibiotic groups showing higher ARG abundance. Also, high abundance of mastitic pathogens was absent in the no-ab group. The results show that prophylactic antibiotics during DCT is not essential. We next longitudinally studied microbiome and resistome in infants divided into three groups [CSab (C-section/antibiotic), CSnoab (C-section/antibiotic naive) and VDnoab (Vaginal delivered/antibiotic naive)] based on delivery mode and antibiotic use during early life. CSab group showed low initial microbial diversity, which increased gradually and slowly. CSab group demonstrated significant associations to antibiotic classes corresponding to the antibiotic administered to infants in this group. Taxa belonging to Gammaproteobacteria were dominant carriers of ARGs, most being non persisters. The results show that early antibiotic exposure can have immediate and long-term effects on the infant microbiome. Next, publicly available shotgun-sequencing data was downloaded, analysed, resulting in a catalogue of early-life gut genomes of infants below three years of age which was used to study the global resistome. Gram-negative bacteria such as *Escherichia, Enterobacter, Citrobacter, Klebsiella* had highest ARG abundance with Glycopeptides, fluoroquinolone, macrolides, tetracyclines being most abundant classes. High abundance of ARGs was positively related to the socioeconomic status and healthcare access index of a country. These results confirm infant gut microbiota as ARG reservoir. Lastly, the gut microbial and resistome profile of adult cystic fibrosis (CF) patients was studied due to their chronic antibiotic use. The CF group had a microbial composition significantly distinct from controls with higher abundance of ARGs and virulence factors. CF group showed strong association to antibiotic classes administered to individuals in this group. Our results demonstrate the need to investigate the resistome and functional profile in this patient group; as antibiotic overuse can lead to MDR, aggravating the health status. This thesis sheds light on the microbial and resistome profile in previously unexplored manners and provides a baseline for researchers and policy makers to design pre-emptive and proactive measures to maximise health restoration and minimise ARG development.

Publications

Patangia, D.V.; Ryan, C.A.; Dempsey, E.; Ross, R.P.; Stanton, C. Impact of antibiotics on the human microbiome and consequences for host health. MicrobiologyOpen 2022, 11, e1260. doi: 10.1002/mbo3.1260.

Patangia, D.V.; Ryan, C.A.; Dempsey, E.; Ross, R.P.; Stanton, C. Vertical transfer of antibiotics and antibiotic resistant strains across the mother/baby axis. Trend in Microbiology 2022, 30, P47-56. DOI:https://doi.org/10.1016/j.tim.2021.05.006.

Co-authored publications

Zeng, Shuqin; **Patangia, Dhrati**; Almeida, Alexandre; Zhou, Zhemin; Mu, Dezhi; Ross, R. Paul; Stanton, Catherine; Wang, Shaopu. Early-life human gut metagenome-assembled genomes and proteins catalogs. Nat comm., 2022

K.M. Lynch, C.R. Strain, C. Johnson, **D. Patangia**, C. Stanton, F. Koc, ..., E.K. Arendt. Extraction and characterisation of arabinoxylan from brewers spent grain and investigation of microbiome modulation potential. European Journal of Nutrition, 60 (8) (2021), pp. 4393-4411.

Sami A., Elimairi I., **Patangia D.,** Watkins C., Ryan C.A., Ross R.P., Stanton C. The ultra-structural, metabolomic and metagenomic characterisation of the Sudanese smokeless tobacco 'Toombak'. Toxicol. Rep. 2021;8:1498–1512.

Mary I Butler, Thomaz F S Bastiaanssen, Caitriona Long-Smith, Sabrina Morkl, Kirsten Berding, Nathaniel L. Ritz, Conall Strain, **Dhrati Patangia**, Shriram Patel, Catherine Stanton, Siobhain M O'Mahony, John F Cryan, Gerard Clarke, Timothy G Dinan. The Gut Microbiome in Social Anxiety Disorder; Evidence of altered composition and function. Accepted in Translational Psychiatry.

Conferences

1. Attended the 1st Annual Scientific Meeting of the One Health European Joint Programme OHEJP on foodborne zoonoses, antimicrobial resistance and emerging threats. (Dublin, Ireland: 22nd-24th May 2019).

2. Attended and presented a three minute elevator pitch at the annual APC Symposium 2020, University College Cork.

3. Attended and presented at the FEMS conference on Microbiology (Belgrade, Serbia: 30th June – 2nd July 2022). "*Impact of early life antibiotic exposure and delivery mode on infant microbiome*"
Received the FEMS conference attendance grant.

4. Attended and presented at the Dublin University Microbiological Society (DUMS) Focused meeting on 'Microbiomes and Human Health'. (Dublin, Ireland: 13th June 2022). "*Microbiota and resistome analysis of colostrum and milk longitudinally in dairy cows*"
Won the best poster prize in the DUMS focused meeting.

5. Attended and presented a three minute lightning talk at the annual APC Symposium 2022, University College Cork. "*Impact of early life antibiotic exposure on infant gut microbiome*"
Won the first prize for lightning talk at the APC symposium 2022.

6. Attended and presented a poster at the International Human Microbiome Conference (IHMC) (Kobe, Japan: 8th – 10th November 2022). "*Impact of early life antibiotic exposure and delivery mode on infant microbiome*".

Courses attended

1. EMBL Course: Introduction to Bioinformatics with R and Bioconductor. (Heidelberg, Germany: 1st – 4th April 2019).
Received EMBL travel grant to attend the course.

2. Workshop on Metagenomics, metatranscriptomics, and multi'omics for microbial community studies by Physalia courses with Huttenhower lab. (Online, 24th – 28th May 2021).

Abbreviations

AAD: Antibiotic-associated diarrhoea

α: Alpha

Ab: Antibiotic

AMR: Antimicrobial resistance

ANI: Average nucleotide identity

ANOVA: Analysis of Variance

ARG: Antibiotic resistance gene

ATM: Amplicon tagment mix

β: Beta

BH: Benjamini-Hochberg

BLAST: Basic Local Alignment Search Tool

BM: Breast milk

bp: Base Pair

bwa: Burrows-Wheeler Aligner

CARD: Comprehensive Antibiotic Resistance Database

CAZymes: Carbohydrate-Active enZYmes

CF: Cystic Fibrosis

CFTR: Cystic fibrosis transmembrane conductance regulator

Con: Control

cpm: copies per million

CS: Caesarean section

°C: Degree Celsius

DCT: Dry Cow Therapy

db: Database

ds: double stranded

DNA: Deoxyribonucleic acid

e.g.: Example

EAMMS: End Anchored Max Scoring Segments

EFSA: European Food Safety Authority

ELGG: Early-life gut genomes

ESKAPE: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp.

ESVAC: European Surveillance of Veterinary Antimicrobial Consumption

FDR: False discovery rate

FOS: Fructo-Oligosaccharides

g: Gravitational force or gram

GBS: Group B Streptococcus

GH: Glycoside Hydrolase

GOS: Galacto-Oligosaccharides

GTDB: Genome Taxonomy Database

h: Hour

HAQ: Healthcare access and quality

HGT: Horizontal gene transfer

HMO: Human Milk Oligosaccharides

IAP: Intrapartum antimicrobial prophylaxis

Ig: Immunoglobulin

IL: Interleukin

I.M.: Intramuscularly

IM: InfantMet

Kbp: Kilobase pair

KEGG: Kyoto Encyclopedia of Genes and Genomes

KO: KEGG Orthology

KU: kiloUnit

L: litre

LCA: Lithocholic acid

LDA: Linear discriminant analysis

LEfSe: Linear discriminant analysis Effect Size

LGT: Lateral gene transfer

LPS: Lipopolysaccharides

MaAsLin: Microbiome Multivariable Association with Linear Models

mAU: milli-absorbance unit

MAG: Metagenome-Assembled Genome

MDR: Multidrug resistant

M: Month

MIC: Minimum inhibitory concentration

min: Minutes

mg: milligram

MGE: Mobile genetic element

MNG: MyNewGut

NCBI: National Centre for Biotechnology Information

NEC: Necrotising enterocolitis

NICU: Neonatal intensive care unit

NMDS: Non-metric multidimensional scaling

NRPS: Non-ribosomal peptides synthetases

NT: Neutralise Tagment Buffer

ORF: Open reading frame

PBS: Phosphate buffered saline

PCoA: Principal Coordinate Analysis

PCR: polymerase chain reaction

PERMANOVA: Permutational multivariate ANOVA

PRR: Pattern recognition receptors

Refs: Reference

RGI: Resistance Gene Identifier

RPM: Revolutions Per Minute

SCFA: Short-chain fatty acids

spp.: species

SRA: Sequence Read Archive

t/T: timepoint

TD: Tagment DNA

TLR: Toll like receptor

µg: microgram

µl: microliter

UK: United Kingdom

USA: United States of America

UKDILAS: UK Drugs in Lactation Advisory Service

UTI: Urinary tract infections

v: Version

vs.: versus

VD: Vaginal delivery

VF: Virulence factor

VFDB: Virulence Factor Database

w: Weeks

WAAFLE: Workflow to Annotate Assemblies and Find LGT Events

WHO: World Health Organisation

Chapter 1

Mini review

Vertical transfer of antibiotics and antibiotic resistant strains across the mother/baby axis

Dhrati V. Patangia, C. Anthony Ryan, Eugene Dempsey, Catherine Stanton, R. Paul Ross

Dhrati Patangia: Conceptualization (lead); writing – original draft (lead), writing – review and editing (equal), **Paul Ross**: Supervision (equal); conceptualization (supporting); writing – review and editing (equal), **Catherine Stanton**: Supervision (equal); conceptualization (supporting); writing – review and editing (equal), **Tony Ryan**: Supervision (equal); conceptualization (supporting); writing – review and editing (equal), **Eugene Dempsey**: Supervision (equal); conceptualization (supporting); writing – review and editing (equal).

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1.1 Abstract

Antibiotic resistance is a health and socioeconomic crisis recognised as a serious threat affecting humans worldwide. Overuse of antibiotics enhances the spread of multidrug-resistant bacteria, causing drug-resistant infections which can be difficult to treat. This resistance, mostly of the acquired type, is thus a major clinical issue. Acquired resistance can occur by horizontal transfer of genes between bacteria (community settings), by vertical transmission that can occur between mother and her offspring at birth and during lactation or spontaneously due to antibiotic exposure. While there have been multiple studies about the horizontal transfer of antibiotic resistance genes, not many studies have been conducted to study their vertical transmission. Vertical transmission is of importance as the early bacterial colonisation of infants has an impact on their health and immune programing throughout life. This review discusses some possible mechanisms of mother-to-infant transmission of antibiotics and antibiotic-resistant strains and addresses the knowledge gaps for further studies.

Keywords: Vertical transmission, Antibiotics, Antibiotic resistance, mother-infants

Highlights:

- Antibiotic resistance is a global issue and transfer of antibiotic resistance genes can occur through horizontal and vertical transmission.
- Mother is a major source of microbiome for infants, with occurrence of vertical transmission from mother to infants now established.
- Transfer of antibiotic resistance genes through vertical transmission is not well studied, despite the usage of antibiotics during pregnancy and infancy.
- Human microbiota and antibiotic resistance is of importance due to its role in health, metabolism and immune programming.
- The negative impacts of antibiotic-resistant strains on the infant gut microbiota could result in drug-resistant infections which are difficult to treat.

Outstanding Questions:

- Can the use of prophylactic antibiotics be reduced during pregnancy and infancy?

- Do antibiotics reach mothers milk at a concentration high enough to affect the milk microbiome?
- Does antibiotic treatment during pregnancy and lactation affect the milk microbiome composition and resistome?
- Does antibiotic treatment of the mother during lactation affect the infant gut microbial diversity over time?
- Can antibiotics administered during lactation transfer to infants in milk in sufficient amounts to affect the resistome and microbiome profile in infants?
- Can alternatives to antibiotics be used instead of antibiotics during these crucial stages of life with the same effectiveness?

1.2 Introduction

Antibiotics have revolutionised medicine, saving millions of lives and increasing life expectancy by an estimated eight years. However, the misuse and overuse of antibiotics, and their improper disposal, have led to a major health and environmental issue, namely the rise of antimicrobial resistance (AMR) (Ventola et al., 2015). AMR is estimated to be responsible for over 750 000 deaths annually[i]. Furthermore, increasing appreciation of the role of the gut microbiome for human health means that antimicrobials should ideally be target-specific, inflicting minimal collateral damage to the surrounding microbiome (Willing et al., 2011). Antibiotic resistance can spread through horizontal gene transfer in bacterial communities and can be disseminated through various niches, including food, animals, plants, the environment, and specifically hospital settings. By contrast, vertical mother/ baby transmission either at birth or via lactation has been far less studied (Figure 1.1A,B) (Pärnänen et al., 2018).

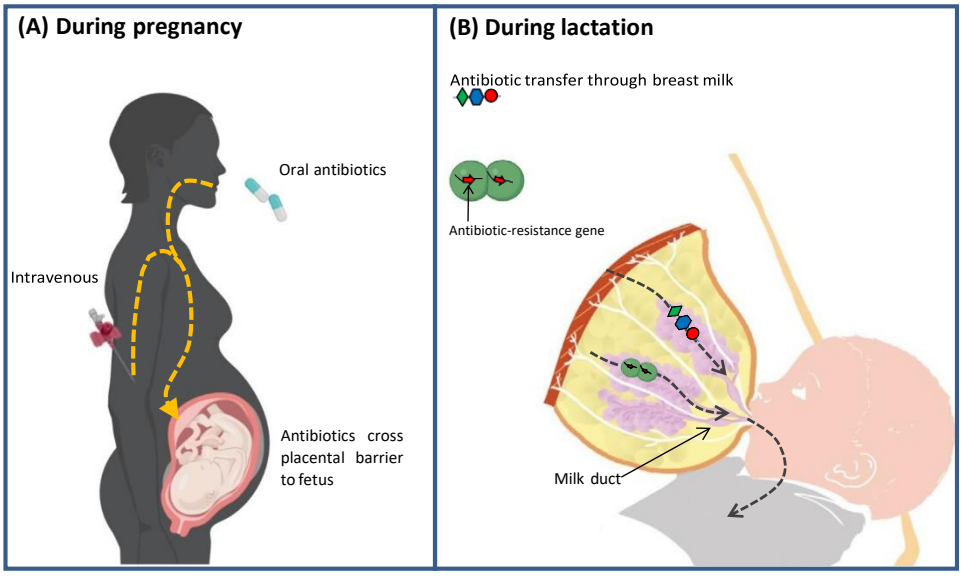


Figure 1.1. Vertical transmission of antibiotics and antibiotic-resistant bacteria from mother to infant. (A) Antibiotic residues administered during pregnancy can cross the placental barrier and circulate in the fetus (created with BioRender). (B) Antibiotic residues and antibiotic-resistant bacteria can transfer from mother to infant during lactation.

The advancements of sequencing technologies have led to increased research interest in studying microbiome vertical transmission from mother to infant, demonstrating that the

mother is the major influencer of the infant's gut microbiome, particularly following vaginal delivery (Table 1.1). As shown in Table 1.1, some studies focus on genera and species level transfer which is not as strong an indicator of vertical transmission as strain-level transfer; thus, more studies examining strain-level transfer in future will further strengthen the notion of vertical transfer. With the increased use of antibiotics during pregnancy and lactation, there is a risk for the emergence of antibiotic-resistant bacteria within the maternal gut and breast milk which can be passed to the infant (Tapiainen et al., 2019; Pärnänen et al., 2018). Indeed, a mouse model study has demonstrated that maternal antibiotic intake can affect the overall maternal and infant gut microbiota (Gonzalez-Perez et al., 2016). Studies have suggested that antibiotics administered to mothers during pregnancy can cross the placental barrier and pass to the fetus, and during lactation they can enter the breast milk and pass to the new-born, due to the low-molecular-weight and high lipid solubility of these compounds (Kelsey, 2016). Most studies to date focus on the harmful effects of these antibiotics on the mother or the fetus; fewer studies have focused on the antimicrobial resistance aspects (Nahum et al., 2006; Passmore et al., 1988) (Lactmed)[ii]. The presence of antibiotic-resistance genes in bacteria present in the infant gut, in the absence of antibiotic exposure, might point toward the passage of these strains from mother to infant (Nogacka et al., 2017; Zhang et al., 2011). Thus, there is a need to characterise the species and strains carrying these resistance genes as the microbiota established in early life plays a vital role in infant health, immune development, metabolism, and other biological activities (Houghteling and Walker, 2015) and could compromise the efficacy of antibiotic treatment on the individual later in life. In particular, the following questions need to be answered. Can passage of the antibiotic via breast milk in mothers affect the milk microbiome and resistome? Is the absorbed drug present in sufficient quantities to be bactericidal in the infant gut? As a consequence, can resistance develop in the infant gut microbiome? Can antibiotic uptake affect the maternal gut microbiome and resistome – and thus the infant gut microbiome? If the answer to any of these questions is in the affirmative, then special care will have to be taken before prescribing antibiotics during pregnancy and lactation as the repercussions could be long-lasting in terms of infant health and infection.

Table 1.1. Vertical transmission of microbes

Putative vertical transmission of bacteria	Sample analysed	Methodology used and resolution obtained	Refs
i) Nonantibiotic group *Bacteroides* sp., *Bacteroides vulgatus, Bacteroides fragilis, Bacteroides ovatus, Bacteroides salanitronis, Bifidobacterium longum*, and *Parabacteroides distasonis* ii) Antibiotic-treated group *Faecalibacterium prausnitzii Eubacterium rectale, Anaerostipes hadrus, Bacteroides ovatus, B. vulgatus, Bacteroides* spp., *Flavonifractor plautii, Roseburia hominis, Roseburia intestinalis*, and *Ruminococcus* spp.	Fecal sample	Shotgun sequencing (bacterial species and exclusively shared genes)	Li et al., 2020
Bacteroides spp., *Parabacteroides* spp., *E. coli*, and *Bifidobacterium* spp.	Fecal sample	Shotgun sequencing (species level)	Shao et al., 2019
63% infant microbiome and 15% virome	Fecal sample	16S rRNA sequencing (shared family, genus and	Maqsood et al., 2019

		Amplicon Sequence Variants)	
Bifidobacterium, Bacteroides, Escherichia, and *Streptococcus*	Milk and fecal sample	Shotgun sequencing (shared genus, species and strains)	Pärnänen et al., 2018
Bacteroides uniformis, Bacteroides vulgatus, and *Bacteroides dorei*, *Bifidobacterium adolescentis* and *Bifidobacterium longum, and Escherichia coli*	Fecal sample	Shotgun sequencing (shared species and strains)	Yassour et al., 2018
Actinobacteria and Bacteroidia	Fecal sample	Shotgun sequencing (shared strains using single-nucleotide variants)	Korpela et al., 2018
Phyla Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria	Vaginal swabs and fecal sample	Shotgun sequencing (shared genus, species and strains)	Wampach et al., 2018
Transmission of 62 strains, depending on site and time point	Skin, oral and vaginal swab, milk and fecal samples	Shotgun sequencing (shared species and strains)	Ferretti et al., 2018
Bifidobacterium bifidum, *Coprococcus comes*, and *Ruminococcus bromii*	Milk and fecal sample	Shotgun sequencing (shared species and strains)	Asnicar et al., 2017

Bifidobacterium longum, *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Bifidobacterium adolescentis*, and *Bifidobacterium pseudocatenulatum*	Milk and fecal sample	Internal Transcribed Spacer gene amplification and 16S rRNA sequencing (shared species and strains)	Duranti et al., 2017
Streptococcus, *Veillonella*, and *Rothia*	Milk and fecal sample	16S rRNA sequencing (shared genus)	Pannaraj et al., 2017
Bifidobacterium adolescentis, *Bifidobacterium angulatum*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium dentium*, *Bifidobacterium longum*, *Bifidobacterium pseudolongum*, and *Bifidobacterium thermacidophilum*	Milk and fecal sample	ITS gene amplification and 16S rRNA sequencing (shared species and strains)	Milani et al., 2015
Escherichia/Shigella, *Bifidobacterium longum*, *Enterococcus faecalis*, *Bacteroides fragilis*, *Bacteroides thetaiotaomicron*, and *Bilophila wadsworthia*	Fecal sample	Shotgun sequencing (shared genus and species)	Bäckhed et al., 2015

The resistome can be either acquired (by point mutations/horizontal gene transfer) or intrinsic (various cellular mechanisms which make bacteria resistant); this contributes to the resistance profile of the bacteria and plays a role in bacterial metabolism and physiology. This highlights the importance of understanding the evolution of intrinsic AMR as it can also become mobile over time (Singh et al., 2019).

In this short review, the various avenues for AMR inheritance across the mother/baby axis are discussed with a view to a greater understanding of the frequency and significance of such events. Firstly, transfer of antibiotics from the mother to the infant either *in utero* or through breastfeeding is discussed, while, secondly, vertical transfer of the AMR strains themselves is considered.

1.3 Transfer of antibiotics *in utero*

Antibiotics are given during selected vaginal deliveries as prophylaxis against group B Streptococcus (GBS), while evidence-based medicine recommends that all women undergoing Caesarean section (CS) receive prophylactic antibiotics to reduce wound infection (Smaill and Gyte, 2014). Some studies report that a single dose of prophylactic antibiotic is beneficial post-vaginal-delivery and encourage its use to prevent maternal infections (Knight et al., 2019). Almost all drugs cross the placenta after therapeutic treatment during pregnancy and may be in the maternal and newborn circulation at the time of birth, with implications for microbiota transfer. Nahum et al. studied 11 broad-spectrum antibiotics (amoxicillin, chloramphenicol, ciprofloxacin, clindamycin, doxycycline, gentamicin, levofloxacin, penicillin G, penicillin VK, rifampin, vancomycin) that are used in pregnancy and lactation and reported that all the antibiotics were capable of crossing the placenta and reaching the unborn infant (Nahum et al., 2006). While not many studies have calculated the concentration of antibiotics in infant serum after maternal antibiotic treatment, high concentrations of antibiotics used during pregnancy have been associated with their presence in umbilical cord serum, suggesting the possibility of transfer (Viel-Theriault et al., 2019). It has been reported that oral or intravenous doses of 250 mg to 1 g of ampicillin resulted in accumulation in amniotic fluid (Kraybill et al., 1980; Blecher et al., 1966). The concentration in neonatal serum exceeded the minimal inhibitory concentration against *Escherichia coli*, GBS, and *Listeria monocytogenes*, and the peaks remained for 2–4 h in cord blood after which it

declined after maternal treatment (Kraybill et al., 1980; Blecher et al., 1966). Muller *et al*. demonstrated that amoxicillin was capable of being transferred and detected in umbilical cord sera (18% of maternal concentration) and neonatal serum (Muller et al., 2009). It has been reported that gentamicin was present at 34% (Yoshioka et al., 1972) and 42% (Weinstein et al., 1976) in umbilical cord blood of the associated maternal blood concentrations. In other studies, the cord blood concentration of 4.7 μg/ml, and the cord-blood-to-maternal-blood ratio of 0.71 at delivery, were reported when pregnant women with chorioamnionitis at term were treated with ampicillin (Maberry et al., 1992; Gilstrap et al., 1988). This strongly indicates that maternal antibiotic treatment during pregnancy reaches the infant in sufficient quantities to potentially influence their resistome profiles.

1.4 Transfer of antibiotics in breast milk

Breast milk is the optimum food for newborn infants but it can allow the passage of unwanted components, such as antibiotics, to the infant (Bar-Oz et al., 2003). Factors such as the concentration of the drug in breast milk, the pharmacokinetics and pharmacodynamic properties of the drug, as well as the concentration received by the infant need to be taken into consideration to predict the potential adverse effects in the infant (Hotham, 2015). Generally, an antibiotic that can be safely administered directly to an infant is considered safe for the mother during breastfeeding. Many antibiotics are regarded as safe for use during lactation by the American Academy of Paediatrics. However, there is some controversy in this area as cautionary use is advised for many of the same drugs or it is advised that they be used only if clinically appropriate during lactation by the UK Drugs in Lactation Advisory Service (UKDILAS); this controversy is due to insufficient data about the harmful effects many antibiotics can cause during lactation[iii]. However, the impact of most of these drugs on the gut microbiota is as yet unknown (Lactmed)[ii]. In fact, no drug, no matter how low its concentration in breast milk, is absolutely safe because of potential idiosyncratic reactions in the infant, allergic sensitisation, or due to its ability to result in resistant strains at subtherapeutic levels (Mathew, 2004).

Nearly all drugs transfer to breast milk to some extent (Mathew, 2004; Kafetzis et al., 1981), and many studies have reported the presence of antibiotics in breast milk (Table 1.2). The small doses received by the infant could cause direct gastrointestinal and oral

candida infections, diarrhoea, and malabsorption of nutrients due to gut microbiota alterations and modifications to gut defence mechanisms (Goldstein et al., 2009; Ito et al., 1993). It has been demonstrated that even low levels of maternal antibiotic use can result in the formation of antibiotic-resistant strains (Wistrand-Yuen et al., 2018; Kohanski et al., 2010).

Recent studies have investigated the effect of maternal oral antibiotic use during pregnancy and lactation in modulating the infant oral or gut microbial diversity. Maternal intrapartum antimicrobial prophylaxis (IAP) has been associated with decreased numbers and arrested growth of *Bifidobacterium* and *Bacteroides* species (Nogacka et al., 2017; Rachel et al., 2017; Azad et al., 2016; Aloisio et al., 2014; Fouhy et al., 2012) and increased levels of members of the family Enterobacteriaceae and *Enterococcus faecalis* in the infant gut (Gonzalez-Perez et al., 2016; Arboleya et al., 2015). Indeed, studies suggest that oral maternal antibiotic intake has effects on infant gut microbial diversity similar to those of infant antibiotic consumption (Tanaka et al., 2009).

The presence of antibiotics in breast milk may blunt their positive effects due to the negative impact on the infant gut and possibly milk microbiota. Prolonged IAP can also result in infants suffering from AMR strain infections which can be even more difficult to treat (Edwards et al., 2002) and increase the risk of late-onset neonatal infections (Didier et al., 2012).

Table 1.2. Excretion of antibiotics in breast milk

Drugs	Concentration (average) in breast milk after the first dose	Refs
Amoxicillin (1 g oral dose)	0.69 mg/l at 4 h and 0.81 mg/l at 5 h	Kafetzis et al., 1981
Cephalosporins i)Cephalexin 1 g oral dose	0.51 mg/l after 4-5h	Kafetzis et al., 1981, 1980

ii)Cefotaxime 1g dose intravenously	 0.32 mg/l after 2h	
Clindamycin 150 mg orally 150 mg three times daily 600 mg intravenously	1.3 mg/l after 4 h 1.2 mg/l after 6 h 1.03 mg/ after 2 h	Zhang et al., 1997; Steen and Rane, 1982; Matsuda et al., 1969
Gentamicin 80 mg I.M.[a] 3 times a day	0.48 and 0.49 after 3 and 5h	Celiloglu et al., 1994
Metronidazole oral dose of 200 mg 400 mg orally 3 times a day	 3.4 mg/l at 4 h, 2.8 mg/l after 8 h 12.9 mg/l after 4 h	Gray et al., 1961 Passmore et al., 1988
Erythromycin 500 mg orally 500 mg dose intravenously	1.2 after 4 h 2.5 mg/l after 2 h	Zhang et al., 1997; Matsuda et al., 1984

[a]Abbreviation: I.M., intramuscularly.

1.5 Transfer of AMR strains *in utero* and during birth

1.5.1 Placenta

Placenta acts as a barrier between the mother and unborn baby, allowing passage of specific nutrients. Although controversial, some studies have demonstrated that the placenta is not sterile and harbors a unique set (and low number) of microbes that may be transferred to the infant (Aagaard et al., 2014; Stout et al., 2013). However, a more recent study demonstrated that placenta does not have a microbiome but can harbor potential pathogens; it reported the presence of *Streptococcus agalactiae* (de Goffau et al., 2019).

These potential pathogens, such as GBS, could be antibiotic-resistant microorganisms that can transfer resistance genes to infant microbes and complicate further treatments in infants. If the placenta contains live bacteria, theoretically, this could be a route for vertical transfer *in utero* – however, given the small numbers of bacteria which would be involved, we would view the risk as being extremely low.

1.5.2 Vagina

Natural childbirth results in the infant being exposed to the mother's gut and vaginal microbiota. *Lactobacillus* spp. dominate the vaginal landscape and can inhibit the growth of pathogenic bacteria. However, under certain conditions, the vagina can harbor potential pathogens which are, in many cases, responsible for adverse pregnancy outcomes due to their transfer. GBS can reach the amniotic cavity ascending from the vagina and can also cross the placental barrier (Patras and Nizet, 2018). This is of clinical significance as *S. agalactiae* is a GBS that is associated with neonatal infections resulting in approximately 57,000 stillbirths and 90 000 infant deaths worldwide as of 2015 (Seale et al., 2017). Also, there has been a trend for increasing antibiotic resistance in GBS with multidrug resistance causing fatal sepsis (Gao et al., 2018, Ji et al., 2017). The use of intrapartum antibiotics during pregnancy and labor has decreased adverse outcomes due to pathogen exposure; however, it might result in the emergence of drug-resistant pathogens. Vaginal colonisation of extended-spectrum-β-lactamase- (ESBL)-producing bacteria during pregnancy can result in adverse outcomes and premature birth. This can also lead to colonisation of infants with ESBL-producing bacteria, thus increasing the prevalence of resistant strains (Foessleitner et al., 2020). Studies have reported the presence in the vagina, and transfer from mothers to their infants, of ampicillin-, cefotaxime- and multidrug-resistant *E. coli* (Sáez-López et al., 2016; Barman et al., 2014; Dubois et al., 2010), penicillin-resistant *Streptococcus* (McDonald et al., 2003), and tetracycline-resistant bacteria, including *Lactobacillus, Prevotella, Ureaplasma, Gardnerella* carrying the *tet*(M) gene, *Lactobacillus* and *Corynebacterium* carrying the *tet*(O) gene, and *tet*(W) carried by *Staphylococcus* and *Corynebacterium* (Alicea-Serrano et al., 2013).

Further studies are required to investigate transfer of resistant bacteria *in utero* at strain level, as it can affect initial infant gut colonisation, and to shed light on risk factors for

transmission, which will aid in the development of preventive strategies for healthier outcomes.

1.6 Transfer of AMR strains through breast milk

Breast milk is considered the gold standard when it comes to nutrition for infants, providing optimal nutrition for neonates and influencing development of immunity and the infant gut microbiota. Studies have reported that human breast milk is a rich source of sugars, antibodies, human milk oligosaccharides (HMOs), and a diverse population of bacteria for infants (Murphy et al., 2017; Walker, 2010).

The more serious outcome of prolonged antibiotic use in lactating mothers can be selection of antibiotic-resistant strains in mothers that can be transferred to the infant (Kawada et al., 2003), leading to neonatal sepsis and infant morbidity (Terrone et al., 1999). The bacteria in mother's milk, such as lactobacilli and bifidobacteria, can be beneficial (Murphy et al., 2017; Milani et al., 2015; Jost et al., 2014) or can be a serious source of infectious microorganisms such as GBS (Gagneur et al., 2009; Dinger et al., 2002). It has been shown that antibiotics can alter the breast milk microbiota, with higher microbial richness observed in mothers receiving intrapartum antibiotics, for reasons yet unknown. The authors suggest that intrapartum antibiotics may have impacted the breast milk microbiota in the earlier stages of lactation. This could be due to low bacterial loads reported in breast milk (Hermansson et al., 2019). Some studies also report that *Bifidobacterium* were present in low abundance, or absent, in mothers who received intrapartum antibiotics (Hermansson et al., 2019). This is of significance as bifidobacteria are reported to promote gut health and colonisation resistance. Further studies evaluating breast milk microbial changes at multiple time points, and before and after antibiotic uptake, are needed to understand the effects of antibiotics on the milk microbiome and its consequent resistome.

Zhang *et al.* observed that the infant gut microbiota can be a reservoir of antibiotic-resistance genes even in the absence of previous antibiotic exposure. Further, they observed antibiotic- resistant bacteria in breast milk but none in infant formula and foods, suggesting that breast milk is a possible source of AMR strains in infants (Zhang et al., 2011). Didier et al. reported that antenatal antibiotics can increase the risk of antibiotic-

resistant infections (Didier et al., 2012). Kozak et al. (2015) demonstrated that highly similar isolates (based on genetic barcode matches) of antibiotic-resistant *Bifidobacterium longum*, *Lactobacillus fermentum*, *Lactobacillus gasseri*, and *Enterococcus faecalis* were isolated from five infant–mother pairs. This suggests the passage of AMR strains from mothers to infants in early life. Studies have reported the presence of *Staphylococcus* and *Streptococcus* strains resistant to multiple antibiotics in human milk of healthy donors and their passage to infants by breastfeeding (Li et al., 2018; Chen et al., 2016; Behari et al., 2004). This is of importance as *Staphylococcus aureus* is known to be a leading cause of hospital and community infections (Mediavilla et al., 2012) and because *Staphylococcus* and *Streptococcus* are considered to be constituents of the core breast milk microbiome (Murphy et al., 2017), suggesting their possible transfer to infants. Huang et al. (2019) demonstrated that the majority of bacterial strains isolated from human milk samples of 30 mothers in Taiwan were multiresistant strains. Resistance was observed to some commonly used antibiotics, such as tetracycline, chloramphenicol, streptomycin, gentamicin, and quinupristin. Acquisition of resistant strains of bacteria from other persons is uncommon in infancy and so mothers are a source of resistant bacteria for infants (de Vries et al., 2011; Prelog et al., 2009). Pärnänen et al. (2018) reported that infants share gut resistomes and mobile genetic elements with their own mother's gut and breast milk microbiota, suggesting that the mother's history of antibiotic use or acquired antibiotic-resistant strains influence the infant's gut resistome. Inheritance of resistance genes – such as those against tetracycline, macrolide–lincosamide–streptogramin B, trimethoprim, and colistin – was observed. Within the infant gut resistome, *E. coli* and Gammaproteobacteria correlated positively with resistance load while *Bifidobacterium* correlated negatively. Thus, given that breastfeeding reduces the abundance of Gammaproteobacteria and increases bifidobacterial load, the authors suggest that breastfeeding could also be promoted on the grounds of reducing antibiotic resistance load in the infant gut. Indeed, termination of breastfeeding before six months of age was associated with increased enrichment of many antibiotic-resistance genes and mobile genetic elements in infants.

1.7 Preterm births and antibiotic exposure

It is thought that many preterm births may be associated with maternal infections or inflammation (Pararas et al., 2006). These cases are accompanied by antibiotic

administration to the mothers as well as the infants, prophylactically in many cases. The World Health Organisation (WHO) recommends administration of antibiotics to infants at risk of developing early-onset infections as a prophylactic measure during the first few days of life (Fuchs et al., 2018) and preterm infants are at a high risk of developing infections (Henderickx et al., 2019). This can alter the preterm gut microbiota and create a reservoir of resistant strains. Preterm infants acquire a distinct microbial structure, compared to full-term infants in the first weeks of life (Hill et al., 2017) due to a variety of factors such as CS birth mode, extended hospital stays, and exposure to multiple antibiotics. Pathogens such as *Klebsiella*, *Enterobacter*, and *Enterococcus* are predominantly present in preterm infants compared to term infants, while levels of commensals such as *Bifidobacterium* and *Lactobacillus* are generally decreased in the preterm infants' gut (Henderickx et al., 2019; Gibson et al., 2016) – but this could be due to low bacterial numbers in preterm infants. This, along with the immature immune system of preterm infants, increases the risk of developing necrotising enterocolitis, late-onset sepsis, allergies, and later, asthma. Thus, more detailed studies that look into the spread of antimicrobial resistance in this patient group are needed. Furthermore, it is reported that the gut microbiota is involved in modulating and producing many neurotransmitters, allowing communication at the gut–brain axis (Dinan and Cryan, 2017). Disruption of gut microbiota by antibiotics could impair microbial function and impact the gut–brain axis.

1.8 Concluding remarks and future scope

Vertical transmission of antibiotics and antibiotic-resistant microorganisms from mother to infant negatively impacts the development and succession of the infant gut microbiota and may result in the enrichment of disease-associated microorganisms and aggravate treatment(s). The growing concern related to the consequences of antibiotic administration requires better diagnostic and risk assessment strategies to promote better antibiotic stewardship in clinical practice. Currently, antibiotic stewardship strategies, including surveillance and assessment of all antibiotic use, identifying scenarios where antibiotic use can be reduced, and implementing interventions while monitoring safety, are the only effective ways to reduce antibiotic use in clinical settings. Furthermore, there should be a focus on narrow-spectrum antimicrobials once the target organism has been identified. These strategies may reduce the rate of gene transfer and antibiotic resistance

development but will not prevent its occurrence (Makri et al., 2018). Therefore, studies in this area are urgently required and should be a major influencing factor toward the prescription of antibiotics during pregnancy and lactation. This will help us to understand the origin and spread of AMR genes and aid in developing effective control strategies to limit vertical transfer across the mother/ baby interface (see Outstanding questions). Other alternative therapies for fighting bacteria should be investigated with a view to developing the most promising ones for clinical use. These could include bacteriophage therapy, the benefits of which address the shortcomings of antibiotics (Furfaro et al., 2018); and bacteriocin therapy (Cotter et al., 2013). Another potential therapy is the use of probiotics; though not a replacement for antibiotics, their use may reduce maternal and neonatal adverse outcomes (Martín et al., 2019; Huurre et al., 2008). These substitute therapies can be the protagonists of the postantibiotic era which may help to reduce health-related complications that occur due to the use of antibiotics during pregnancy, lactation, and infancy.

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1.10 Declaration of interests

There are no interests to declare.

1.11 Resources

[i]www.daghammarskjold.se/publication/when-the-drugs-dont-work-antibiotic-resistance-as-a-global-development-problem/

[ii]www.ncbi.nlm.nih.gov/books/NBK501922/

[iii]www.hse.ie/eng/services/publications/clinical-strategy-and-programmes/antimicrobial-safety-in-pregnancy-and-lactation.pdf

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Chapter 2

2 LITERATURE REVIEW

IMPACT OF ANTIBIOTICS ON THE HUMAN MICROBIOME AND CONSEQUENCES FOR HOST HEALTH

Dhrati V. Patangia, C. Anthony Ryan, Eugene Dempsey, R. Paul Ross, Catherine Stanton

Dhrati Patangia: Conceptualization (lead); writing – original draft (lead), writing – review and editing (equal), **Paul Ross**: Supervision (equal); conceptualization (supporting); writing – review and editing (equal), **Catherine Stanton**: Supervision (equal); conceptualization (supporting); writing – review and editing (equal), **Tony Ryan**: Supervision (equal); conceptualization (supporting); writing – review and editing (equal), **Eugene Dempsey**: Supervision (equal); conceptualization (supporting); writing – review and editing (equal).

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2.1 Abstract

It is well established that the gut microbiota plays an important role in host health and is perturbed by several factors including antibiotics. Antibiotic-induced changes in microbial composition can have a negative impact on host health including reduced microbial diversity, changes in functional attributes of the microbiota, formation and selection of antibiotic-resistant strains making hosts more susceptible to infection with pathogens such as *Clostidioides difficile*. Antibiotic resistance is a global crisis and the increased use of antibiotics over time warrants investigation into its effects on microbiota and health. In this review, we discuss the adverse effects of antibiotics on the gut microbiota and thus host health, and suggest alternative approaches to antibiotic use.

2.2 Introduction

Since their discovery, antibiotics have revolutionised the treatment of infectious diseases on a global scale. They are recognised as one of the contributing factors to increased life expectancy in the 20th century owing to the decline in infectious disease mortality (Adedeji, 2016). However, their overuse and misuse in human and veterinary medicine and animal husbandry have resulted in the current global antibiotic resistance crisis (Vidovic and Vidovic, 2020; Lorr and Bjerrum, 2014) which is exacerbated by the slow rate of new drug development (Simpkin et al., 2017). Despite this, antibiotics are still widely prescribed in disease treatment and studies have reported increased consumption of antibiotics in certain countries in the past number of years (Klein et al., 2018; Adriaenssens et al., 2011).

More recently, scientists have uncovered the detrimental impact of broad-spectrum antibiotics on the gut microbiota. Home to bacteria, archaea, micro-eukaryotes and viruses, the gut microbiota plays a fundamental role in human health. It prevents pathogen colonisation, regulates gut immunity, provides essential nutrients and bioactive metabolites, and is involved in energy homeostasis (Mills et al., 2019). In infants, the gut microbiota is acquired during birth and thereafter plays an essential role in the development of infant gut immunity. Evidence to date strongly suggests that balanced microbiota composition and rich species diversity are essential to its optimal functioning (Heiman and Greenway, 2016), which can be compromised in disease states (Mosca et

al., 2016). Likewise, reduced diversity and imbalanced microbiota composition in the infant gut are associated with intestinal illnesses and a predisposition to certain diseases later in life (Volkova et al., 2021; Milani et al., 2017).

Broad-spectrum antibiotics reduce gut microbiota diversity (Dubourg et al., 2014), and as well as killing the pathogen of concern can eradicate beneficial microbes (Blaser, 2011), with deleterious consequences for the host. Despite this, in Western countries, up to 35% of women are exposed to an antibiotic during pregnancy and delivery, and antibiotics comprise 80% of the drugs a woman is exposed to during pregnancy (Kuperman & Koren, 2016; Stokholm et al., 2013). Mothers are frequently prescribed intrapartum antibiotics prophylactically to prevent and treat infections (Verani et al., 2010).

Clostridioides difficile (formerly known as *Clostridium difficile*) infection is an example of a disease brought about directly through antibiotic disruption of the gut microbiota (Theriot et al., 2014). Illness ranges from mild diarrhoea to death (Guh & Kutty, 2018). Antibiotic eradication of beneficial bacteria in the gut enables *C. difficile* to flourish (Rea et al., 2011). A recent study also concluded that oral antibiotic use is associated with increased risk of colon cancer (Zhang et al., 2019). These are just some of the examples of how antibiotic therapy can compromise health.

This review thus focuses on the negative impacts of antibiotics on human health from pregnancy through to adulthood, most of which are microbiota-dependant, although we also provide evidence of non-microbiota associated negative impacts. We discuss the changes to microbiota composition and functionality and the consequences for host health (Figure 2.1). We look at the impact of antibiotics at the single bacterial cell level, and how antibiotic use and misuse result in antibiotic resistance development. Further, we consider alternative approaches to antibiotic therapy and discuss therapeutics that can be used to maintain and improve host health and minimise the effects of antibiotics when used.

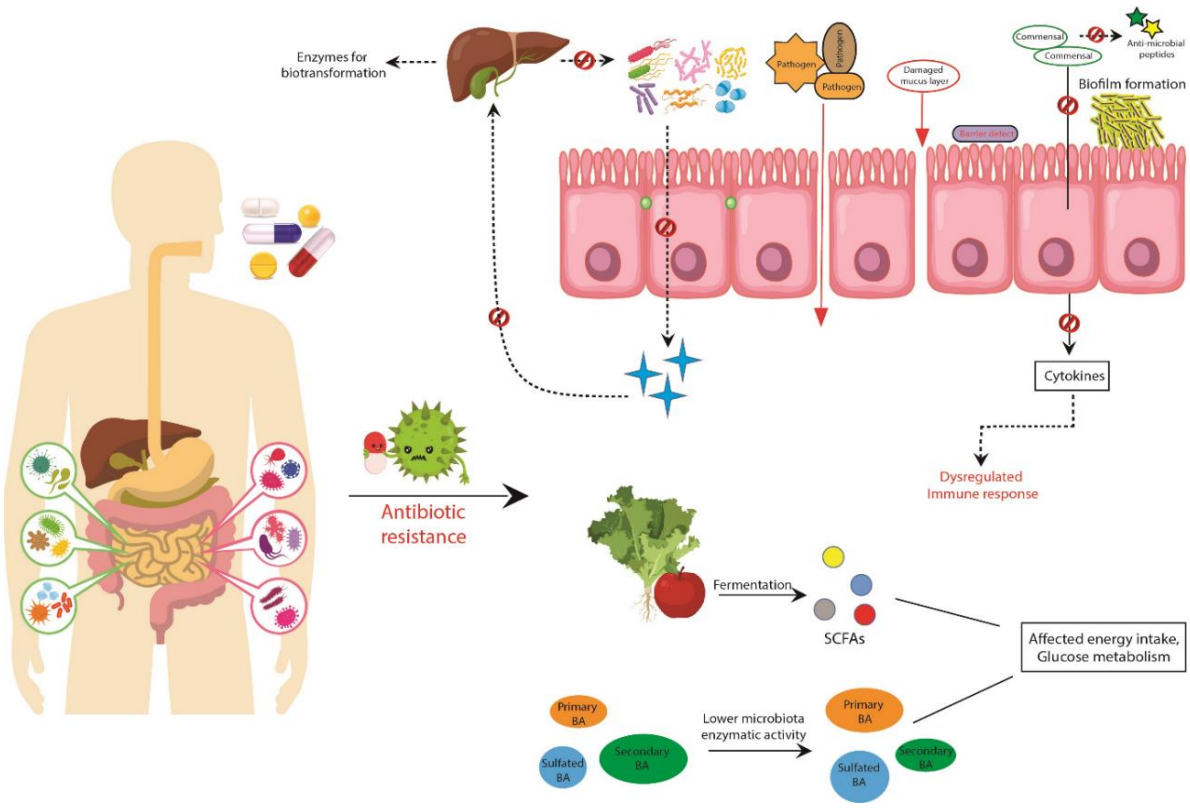


Figure 2.1: Figure shows the negative impacts that can occur on host health due to overuse and misuse of antibiotics. Figure was created using freepik.

2.3 Introduction to Microbiota composition from Infants to Adulthood

It was previously believed that infants are protected in the mother's womb which is a sterile environment, but studies have now demonstrated that amniotic fluid samples, placenta from mothers, and meconium samples from infants contain bacterial DNA suggesting the early exposure of infants to bacteria (Stinson et al., 2019; de Goffau et al., 2019; Aagaard et al., 2014; Moles et al., 2013). However, this is much debated (Perez-Muñoz et al., 2017) due to issues with contamination and varying interpretations, thus we emphasize on microbiota development from infancy to adults in this review.

The gut microbiota in the early stages of life becomes more diverse until it reaches a stable adult-like composition by two to four years of age. Following birth, the gut is colonised by facultative anaerobes due to the partially aerobic or microaerophilic environment. These then generate the appropriate atmosphere for the development of anaerobes by consuming the available oxygen. Thereafter, followed by early exposure to

food (breast milk or infant formula), this composition changes and facultative anaerobes such as *Bifidobacterium, Bacteroides,* and *Clostridium* dominate (Voreades et al., 2014). An initial decrease in Proteobacteria and Enterobacteriaceae accompanied by increases in Bacteroidetes and bifidobacteria have been reported in many studies (Bokulich et al., 2016; Bäckhed et al., 2015). The establishment of the adult-like microbiota occurs at two-four years of age, which is represented by high relative abundance of Bacteroidetes and Firmicutes (Fouhy et al., 2019).

It is largely accepted that mother is the most important source of the gut microbiota for infants (Ferretti et al., 2018; Asnicar et al., 2017). The establishment of the healthy infant gut microbiota and its subsequent development is a continuous process that is influenced by several factors. Mode of delivery is one of the first factors that influences the infant gut microbiota, with vaginally-delivered infants having microbiota that is more diverse and similar to their mothers vaginal microbiota while caesarean section-born infants are deprived of this exposure and thus have gut microbiota similar to their mothers' skin and the hospital environment. These differences are significant and studies have demonstrated an increased association of *Propionibacterium, Corynebacterium, Staphylococcus, C. difficile* and *Streptococcus* and lower abundances of bifidobacteria and *Bacteroides* with caesarean-born neonates, whereas *Lactobacillus*, *Prevotella*, and *Sneathia* spp. are associated with vaginally-delivered neonates (Azad et al., 2013; Dominguez-Bello et al., 2010, Penders et al., 2006). Feeding habit is another crucial factor affecting the infant gut microbiota composition. Because of the presence of oligosaccharides in human milk (human milk oligosaccharides, HMOs) that are largely used by bifidobacteria, breastfed infants show higher levels of bifidobacteria compared to formula-fed infants, and the proportions remain high even post-weaning (Bezirtzoglou et al., 2011; Fallani et al., 2011). *Bacteroides, Streptococcus,* and *Lactobacillus* have also been reported in breastfed infants (Harmsen et al., 2000). Formula-fed infants present higher abundances of *Escherichia coli, C difficile,* the *Bacteroides fragilis* group, and lactobacilli than their breastfed counterparts (Penders et al., 2006). Gestational age is another factor that affects the gut microbiota composition with preterm infants showing lower diversity, higher abundance of Proteobacteria and reduced levels of obligate anaerobes such as *Bifidobacterium, Bacteroides,* and *Atopobium* compared to full-term infants (Hill et al.,

2017, Moles, 2013; Arboleya et al., 2012). Another factor is antibiotic administration, as shown in Figure 2.2 and is discussed below.

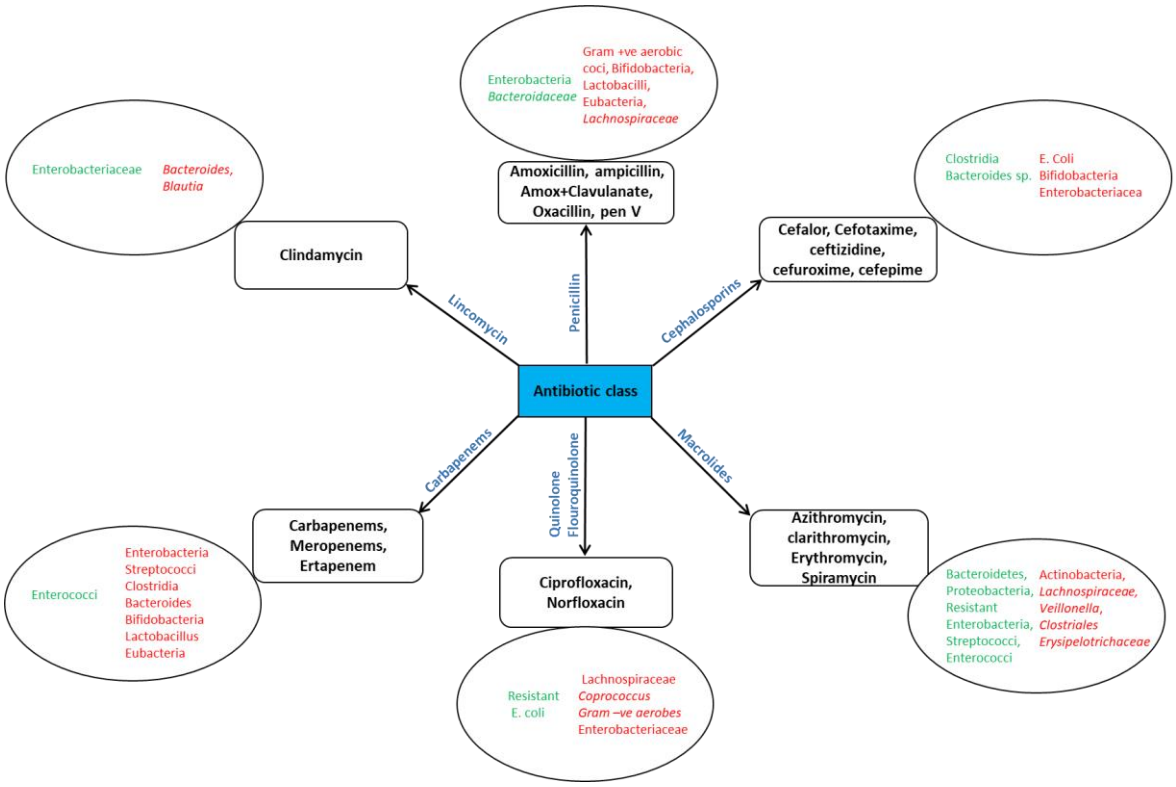


Figure 2.2: Diagrammatic representation of effect of various antibiotics on human gut microbiota (green denotes increasing levels, while red indicates decrease).

The gut microbial composition changes throughout pregnancy and a healthy pregnancy is characterised by an increase in the bacterial load and profound alterations in the composition of gut microbiota (Nuriel-Ohayon et al., 2016). During the first to the third trimester of pregnancy, major changes such as an overall increase in Proteobacteria and Actinobacteria and reduced richness have been reported owing to the major physiological changes (Koren et al., 2012). Also, the vaginal microbiota during pregnancy has been reported to change – represented by high bacterial load with high *Lactobacillus* abundance, low richness and diversity compared to the vaginal microbiota of non-pregnant females (Freitas et al., 2017; Aagaard et al., 2012).

The gut microbiota of healthy adults is predominantly composed of the phyla Firmicutes and Bacteroidetes, representing the majority, followed by Actinobacteria, Proteobacteria and Verrucomicrobia (Arumugam et al., 2011). Indeed, the gut microbiome can be perturbed by short-term use or even low-doses of antibiotics that can have long-term effects on health (Jernberg et al., 2010), this cautions against the misuse and overuse of antibiotics, particularly in pregnant women and young children.

2.4 Antibiotic Types Commonly Administered

The use of antibiotic therapy during pregnancy and lactation varies depending on the underlying condition, country and medical guidelines, but some of the most commonly prescribed antibiotics during pregnancy are beta-lactam antibacterials (Petersen et al., 2010). Some of the other antibiotic classes prescribed include sulphonamides/trimethoprim and macrolides/lincosamides/streptogramins (Jonge et al., 2014). Common infections for which antibiotics are prescribed during pregnancy include urinary tract infections (UTI), respiratory tract infections, skin or ear infections, bacterial vaginosis and fever of unknown origin (Petersen et al., 2010; Heikkila, 1993). Antibiotics are frequently administered to mothers during labour to prevent Group B *Streptococcus* transmission, to reduce and prevent infections in the endometrium and to prevent wound infections (Braye et al., 2019); though the WHO advises against the prophylactic use of antibiotics post uncomplicated delivery. This is of concern as infant antibiotic exposure through intrapartum antibiotic prophylaxis (IAP) has been shown to alter infant gut microbial diversity (Tapiainen et al., 2019). Further, antibiotics are commonly prescribed to newborns, owing to their high susceptibility to infections and lowered immunity, particularly in premature infants (Clark et al., 2006; Vergnano et al., 2005). The most commonly used antibiotics for infants include amoxicillin, co-amoxiclav, benzylpenicillin, cephalosporins, gentamycin, vancomycin, clindamycin and azithromycin. These antibiotics are indicated in respiratory and ear infections, bronchitis, pharyngitis and high temperature (Centre for Disease Control, 2017). Table 2.1 summarises the commonly used antibiotics and their indication for use.

Table 2.1: Summary of the various antibiotics used commonly, their mode of action and indications when used.

Antibiotic	**Class of antibiotic**	**Broad/narrow spectrum**	**Mode of action**	**Target pathogen**	**Reference**
Amoxicillin, Ampicillin	Beta lactam	Broad spectrum	Bactericidal	Rhinosinusitis, respiratory and genitourinary tract infections, septicemia	Akhavan et al., 2020; Peechakara et al., 2020
Cephalosporin	Beta lactam	Broad spectrum	Bactericidal	Urinary and respiratory tract infection, gram-negative bacteria	Bui and Preuss 2020
Azithromycin/Erythromycin	Macrolides	Broad spectrum	Bactericidal	Sinusitis, pneumonia, respiratory tract and skin infection, urogenital and chlamydial infection	Pitsouni et al., 2007; Patel and Hashmi 2021
Metronidazole	Nitroimidazole	Broad spectrum	Bactericidal	Protozoal infections, *C. difficile*	Rineh et al., 2014; Weir

				infections, gram-negative bacterial infections	and Le 2020.
Gentamycin	Aminoglycoside	Broad spectrum	Bactericidal	Urinary tract infection, gram-negative bacterial infections	Habak and Griggs, 2020; Chaves and Tadi, 2021.

2.5 Impact of Antibiotics on Gut Microbial Composition

2.5.1 Impact of antibiotics during pregnancy and lactation

Perinatal and peripartum antibiotic use can impact gut microbial colonisation and the resistome profile in infants (Wong et al., 2020; Zhou et al., 2020). To understand the potential impact of antibiotic administration on offspring during pregnancy, scientists examined the temporal impact of cefoperazone, on both maternal and offspring microbiota when administered during the peripartum period in an IL-10-deficient murine model of colitis (Miyoshi et al., 2017). Offspring from cefoperazone-exposed dams developed altered gut microbial communities into adulthood and had increased susceptibility to spontaneous and chemically-induced colitis (Miyoshi et al., 2017). Similar results were demonstrated by Schulfer et al. (2018), who inoculated germ-free pregnant mice with an antibiotic-altered microbial community. They observed that the altered microbial community was transmitted to the IL-10-deficient offspring which resulted in the development of markedly increased colitis (Schulfer et al., 2018). Another study demonstrated that uptake of antibiotics during pregnancy can lead to alterations in the vaginal microbial composition before birth (Stokholm et al., 2014); this can impact the microbial composition infants receive at birth (Dobbler et al., 2019). Maternal

antibiotic uptake during pregnancy has been reported to be associated with altered microbial composition depending on the antibiotic type (Azad et al., 2016, Coker et al., 2019). It is also associated with increased risk of asthma and allergy in the infant (Stokholm et al., 2014), although there is some controversy around this (Kim et al., 2019), as well as with functional impairment in development and cognition (Kenyon et al., 2008), obesity (Mueller et al.,2015), immunological alterations and development of diabetes (Tormo-Badia et al., 2014) in offspring.

Many studies have demonstrated that IAP-exposed infants in the first weeks of life have lower proportions of Actinobacteria and Bacteroidetes (Nogacka et al., 2017, Aloisio et al., 2016), high oral Proteobacteria levels (Gomez-Arango et al., 2017) and lower levels of bifidobacteria (Mazzola et al., 2016). At 3 months, they show under-representation of *Bacteroides*, *Parabacteroides*, and higher *Enterococcus* and *Clostridium* (Azad et al., 2016), as well as higher abundance of Enterobacteriaceae (Mazzola et al., 2016), as compared to non-antibiotic exposed infants. Similar results were reported in a recent study including 22 newborns which demonstrated that maternal intrapartum antibiotics can affect the infant oral microbiota with phyla Actinobacteria, Bacteroidetes and Proteobacteria being more abundant in infants of antibiotic-treated mothers (Li et al., 2019). Another study in 28 preterm infants (with fecal samples collected on day 7 and 14 from birth) demonstrated similar results with decreases in Bacteroidetes and *Bifidobacterium* in prenatal antibiotic-exposed infants and suggested that the altered microbiota resembled the resistant bacteria in the neonatal intensive care unit (NICU) during the same period (Zou et al., 2018). Many of these effects are similar to those observed following postnatal antibiotic administration (Tapiainen et al., 2019). Ampicillin use as an intrapartum prophylactic drug in mothers against group B *Streptococcus* is also demonstrated to reduce the levels of bifidobacteria in infants (Aloisio et al., 2014). Similar effects of perinatal antibiotic exposure have been observed in preterm infants (n=40); marked by increased levels of Enterobacteriaceae species and reduced levels of *Bacteroidaceae* (Arboleya et al., 2016).

Maternal antibiotic administration during lactation also influences the milk microbiota (Hermansson et al., 2019; Soto et al., 2014), which can in turn influence the infant gut microbial composition.

2.5.2 Impact of antibiotic administration directly to infants on the infant gut microbiota

Premature infants are very often treated with antibiotics owing to their health conditions, and antibiotics are one of the most commonly prescribed drugs in the NICU (Clark, 2006). Many of the prophylactic antibiotics are broad-spectrum and thus affect a huge proportion of the gut bacterial community, leading to many alterations in the early establishment pattern. Studies have reported that very preterm infants who received prolonged antibiotic treatment had less diverse bacterial populations and reduced species richness in their gut and more antibiotic resistance genes (Gasparrini et al., 2019; Gibson et al., 2016). Both short-term and long-term exposure of preterm infants to antibiotics can alter their gastrointestinal microbiota. These changes include decreases in bifidobacteria and *Bacteroidetes* relative abundance, and an increase in *Enterococcus* abundance (Gasparrini et al., 2019; Zwittink et al., 2018; Zou et al., 2018). Extended antibiotic treatment in premature infants can result in increased risk of developing late-onset sepsis (primarily caused by group B *Streptococcus*), necrotising enterocolitis and overall mortality (Esaiassen et al., 2017; Kuppala et al., 2011).

Amoxicillin treatment in infants (n=31) for seven days was shown to completely eradicate *Bifidobacterium adolescentis* species which was accompanied by decreased diversity of the bifidobacteria population (Mangin et al., 2010). In a cohort of 18 infants, we have previously found that the use of ampicillin and gentamicin in early life resulted in higher levels of Proteobacteria and lower proportions of *Bifidobacterium* and *Lactobacillus* four and eight weeks after the treatment (Fouhy et al., 2012). A study reporting on the effects of administering the broad-spectrum antibiotic, cefalexin, in 26 infants, in the first four days of life revealed that the gut microbiota of antibiotic-treated infants showed less diversity than the control antibiotic-free infants (Tanaka et al., 2009). The antibiotic also arrested the growth of some bacterial groups such as bifidobacteria and resulted in unusual colonisation of *Enterococcus* in the first week. Further to this, a high Enterobacteriaceae population was observed one month following antibiotic treatment as compared to the control antibiotic-free infants (Tanaka et al., 2009). Antibiotics change the dominant members of the bacterial community which could have profound effects on

immune development, metabolism and growth of the infant (Cox et al., 2014, Nobel et al., 2015).

Similar results of reduced gut microbiota diversity have been observed in children (n=39, monthly sampling in the first three years of life) who have undergone antibiotic therapy. The authors also reported the presence of Antibiotic Resistance Genes (ARG) on mobile genetic elements long after cessation of treatment (Yassour et al., 2016). Antibiotic use during infancy and childhood has been associated with altered microbial composition and metabolic functions (Korpela et al., 2016), higher risk of asthma and allergy development (Ni et al., 2019; Yamamoto-Hanada et al., 2017) and obesity (Bailey et al., 2014) in later life.

2.5.3 Impact of antibiotics on the gut and oral microbiota in adults

To investigate the long term effects of antibiotics on the healthy state microbial composition, the antibiotics amoxicillin (n=6) (Cochetière et al., 2005), ciprofloxacin (n=3) (Les Dethlefsen 2008) and cefprozil (n=24) (Raymond et al., 2016) were administered to healthy individuals. These studies reported changes in microbial composition persisting for up to 12 weeks after treatment had ended with incomplete restoration of microbial composition and emergence of antibiotic-resistant strains. In one study, the authors examined the distal gut microbiota of three individuals over 10 months post-administration of the antibiotic ciprofloxacin. They reported that the effect of ciprofloxacin on the gut microbiota was profound and rapid with a decrease in richness and diversity of microbiota accompanied by shifts in levels of Bacteroidetes, *Lachnospiraceae* and the *Ruminococcaceae*. By one week after the end of each course, communities began to return to their initial state, but the return was incomplete and variable from the initial stage (Dethlefsen & Relman 2011). Many studies have investigated the long-term impact on gut microbiota following a course of antibiotics. A short-term course of clindamycin (seven days) resulted in significant disturbances in the bacterial community such as a sharp decline in *Bacteroides* (Jernberg et al., 2007, Löfmark et al., 2006) and enterococcal colonies (Lindgren et al., 2009) that remained for up to two years post-treatment and was accompanied by increased levels of antibiotic resistance genes and strains (Lindgren et al., 2009; Jernberg et al., 2007; Löfmark et al., 2006). Another study (n=10) that analysed adult fecal samples from 11 days to 12 months

post-antibiotic treatment demonstrated that the use of ciprofloxacin for 10 days resulted in lower abundances of *Bifidobacterium* but did not affect *Lactobacillus* and *Bacteroides* levels, but clindamycin treatment for the same number of days caused *Lactobacillus* and *Bifidobacterium* to decrease and *Bifidobacterium* did not normalise until one-year post-treatment, showing that different bacterial groups require different times for normalisation post-treatment (Rashid et al., 2015).

Perez Cobas et al. (2013) also reported that the type of antibiotic, whether static or cidal, had varying effects on the gut microbiota which was reflected at the functional level. For instance in a study of four subjects, a bacteriostatic drug resulted in the flourishing of gram-negative bacteria which was related to an increase in the number of genes involved in lipopolysaccharide (LPS) synthesis, while the bactericidal drug was associated with an increase in gram-positive bacteria which was accompanied by an over-representation of genes involved in endospore formation (Pérez-Cobas et al., 2013). Antibiotic administration for the eradication of *Helicobacter pylori* demonstrated that the antibiotics can affect the indigenous microbiota and lead to the development of resistant strains which can persist for years after treatment (Jakobsson et al., 2010; Sjölund et al., 2003).

Furthermore, many antibiotics are used for dentistry procedures routinely. These antibiotics can increase the number of resistant strains present orally, can increase the minimum inhibitory concentrations (MICs) and can also eliminate the non-pathogenic strains (Ready et al., 2004; Harrison et al., 1985), which can lead to systemic infections and inflammation.

2.6 Consequences of Antibiotic-Induced Microbiota Changes for Health and Disease

2.6.1 In Adults

Due to the role of the microbiota in host metabolism and physiology, many studies postulate that microbial imbalances can be related to obesity (Scott et al., 2016; Riley et al., 2013), diabetes (Mikkelsen et al., 2015; Boursi et al., 2015), and asthma (Arrieta et al., 2015; Kozyrskyj et al., 2007). Blaser and Falkow (2009) have suggested a link between the “missing microbes” and modern conditions such as obesity and juvenile diabetes. These multifactorial conditions can be controlled by identifying the controllable

factors such as the microbiota component and dietary habits, thus preventing them from occurring if possible.

Studies have reported a link between antibiotic usage and obesity (Fiol et al., 2018). Some studies suggest that an increased ratio of Firmicutes to Bacteriodetes rather than specific levels is associated with obesity (Kasai et al., 2015), though results are conflicting (Schwiertz et al., 2010; Duncan et al., 2008). While the microbial component of obesity is debated, studies have reported a common change at functional microbial levels. Indeed, obese individuals have higher short-chain fatty acid (SCFA) content compared to lean individuals (Schwiertz et al., 2010; Turnbaugh et al., 2006). Furthermore, obesity is associated with metabolic alterations related to glucose homeostasis and insulin resistance and linked to development of diabetes (Cani et al., 2012). In a study in 96 humans (48 each antibiotic group and controls), researchers reported significant and persistent weight gain after an episode of infectious endocarditis in patients who had been treated with vancomycin and gentamycin (Thuny et al., 2010).

An association between antibiotic-induced changes in microbial colonisation and type 1 diabetes in male mice was reported (Candon et al., 2015). A combination of broad-spectrum antibiotics or vancomycin alone was given to neonatal non-obese diabetic mice that spontaneously developed autoimmune type-1 diabetes. The microbiota was significantly altered with an increase in *Escherichia* and *Lactobacillus* species and a decrease of the Clostridiales order compared to controls. A major reduction of IL-17-producing cells was also observed in the lamina propria of the ileum and the colon of vancomycin-treated mice (Candon et al., 2015), which can affect host defence mechanisms. Some studies in human populations also suggest a link between repeated use of broad-spectrum antibiotics and diabetes (Boursi et al., 2015, Mikkelsen et al., 2016), while some suggest a protective and preventative role of antibiotics and diet in diabetes development in diabetes-prone animals partly due to lowering of specific antigenic load or development of tolerogenic APCs (Hu et al., 2015; Brugman, 2006).

Associations between altered microbial composition and type-2 diabetes are more established, with decreased levels of butyrate-producing bacteria reported in type-2 diabetic patients (Gurung et al., 2020). Zhang et al. (2013) studied 121 subjects with

normal glucose tolerance, prediabetes and newly diagnosed diabetes and reported that there is modulation of the gut microbial composition at the prediabetes stage which can act as marker for development of diabetes state (Zhang et al., 2013).

Antibiotics can lead to antibiotic-associated diarrhoea (AAD) and studies have demonstrated that clindamycin can result in alteration of the microbial community which can promote the colonisation of potential pathogens such as *C. difficile* which can lead to diarrhoea and colitis (Buffie et al., 2012, McDonald, 2017). Another study in a mouse model reported that antibiotic treatment resulted in decreased alpha and beta diversity, which potentially caused a decrease in levels of serotonin, tryptophan hydrolase and secondary bile acids which can further affect gut motility and metabolism (Ge et al., 2017).

2.6.2 During pregnancy and infancy

Extrinsic factors such as antibiotics can alter the diversity of the maternal microbiota that can affect the infant's gut microbiota diversity, immunity and disease development in later life, both directly and indirectly (Tapiainen et al., 2019; Nyangahu et al., 2018; Azad et al., 2016; Tormo-Badia et al., 2014). According to the hygiene hypothesis, if the host is not exposed to a diverse range of microbiota early in childhood or in the developing stages, immune-related disorders may develop such as asthma and allergic sensitisation. Antibiotics can have a similar effect when administered during infancy. Preterm infants are often exposed to antibiotics which results in an altered microbial composition (Zou et al., 2018; Gibson et al., 2016; Clark, 2006), predisposing them to probable infections such as necrotizing enterocolitis (NEC), and invasive fungal infections (Esaiassen et al., 2017). Animal studies have reported that administration of low dose or sub-therapeutic concentrations of antibiotics in early life can disturb the microbial composition, affecting the expression of genes involved in immunity and carbohydrate metabolism, and can alter metabolic homeostasis predisposing the host to adiposity later in life (Schulfer et al., 2019; Cox et al., 2014; Cho et al., 2012). Obesity has been widely linked with altered microbial colonisation during early life owing to the role of the gut microbiota in dietary metabolism. Studies have demonstrated that early-life antibiotic exposure has potential links to increases in body mass index, overweight and central adiposity, and this can be gender-specific affecting males more than females (Azad et al., 2014; Murphy et al., 2014). Both murine models (Cox et al., 2014; Cho et al., 2012) and human studies have

reported that antibiotic exposure in the first few months of life is associated with increases in body mass index and risk of overweight (Scott et al., 2016; Bailey et al., 2014; Trasande et al., 2013) and asthma (Risnes et al., 2011; Kozyrskyj et al., 2007) in childhood. Similarly, studies have reported that antibiotic administration in early life can be associated with heightened risk of asthma, allergy and atopic dermatitis and IBD (Ni et al., 2019; Yamamoto-Hanada et al., 2017; Kronman et al., 2012; Johnson et al., 2005). NEC and AAD have also been associated with prolonged or prophylactic antibiotic uptake in early life (Kuppala et al., 2011; Alexander et al., 2011; Cotten et al., 2009).

2.6.3 Changes in Immune response

The immune system is trained to fight pathogens during infancy, and this is the time when microbial colonisation takes place. Any disturbance to microbial colonisation has been shown to affect immune maturation due to this co-developmental process. Studies in germ-free mice have confirmed that the absence of microbes in the gut results in both physiological and immunological changes to the gut environment. These changes include alterations in mucus thickness and composition (Szentkuti et al., 1990), reduced gastric motility (Abrams and Bishop, 1967), improper development and functioning of intestinal cells and immune cells (Cahenzli et al., 2013; Vaishnava et al., 2008) and improper development of the immune system (Macpherson & Uhr, 2004; Bauer et al., 1963). Antibiotic treatment has been shown to reduce the thickness of the colonic mucus layer thus increasing the risk of pathogen invasion and intestinal inflammation in eight to ten week old mice (Wlodarska et al., 2011). Another study in mice reported that antibiotic-induced alterations in the microbiota shift the TH1/TH2 balance toward TH2-dominant immunity – which leads to atopy development, accompanied by a reduced number of lymphocytes (Oyama et al., 2001). Altered microbial composition, and altered gene maturation profile such as down-regulation of genes coding for MHC class 1b and class II proteins and products of paneth cells such as defensins were seen post-clamoxyl treatment in neonatal rats, which could affect mucosal barrier development (Schumann et al., 2005).

A study demonstrated that certain molecules produced by bacteria in the gut are involved in immune system maturation. Indeed, germ-free mice colonised with *B. fragilis*

producing a bacteria polysaccharide resulted in correcting T-cell-deficiencies and improving T(H)1/T(H)2 imbalances along with promoting lymphoid organogenesis; this was not observed in the case of the non-polysaccharide-producing mutant *B. fragilis* (Mazmanian et al., 2005). Studies have reported that the secretion of antimicrobial peptides by intestinal epithelial cells is regulated by the microbiota in the microenvironment. Germ-free mice colonised with conventional or human microbiota or specific probiotic species or lipopolysaccharides demonstrated increased production of antimicrobial peptides like REGIII-γ, boosting the innate immune response (Natividad et al., 2013; Brandl et al., 2008; Cash et al., 2006).

A study in mice demonstrated that prenatal antibiotics not only altered the pattern of microbiota colonisation in infant mice but also negatively affected the activity of CD8+ T lymphocytes towards viral infections affecting their immune responses (Gonzalez-Perez et al., 2016). Further, they also observed that infant mice were more susceptible to infection when born in stricter hygienic facilities (Gonzalez-Perez et al., 2016). Similar results were reported post-antibiotic treatment in another murine model with low bacterial diversity alongside reduced cytokine production by CD4+ T lymphocytes and reduced production of interferon-γ (Hill et al., 2010). In a study involving influenza patients, the authors reported that subjects with low titres of infection and treated with antibiotics had low immune responses accompanied with microbiota loss, low immunoglobulin (Ig) G1, IgA, and secondary bile acid levels against the infection (Hagan et al., 2019).
These studies demonstrate the complex relationship between the microbiota and the host immune response, and the impact of antibiotics on this interaction which needs to be further studied. It can also impact the effectiveness of vaccines used post-antibiotic treatment. This is more relevant now than ever when the global population is being vaccinated to protect against COVID-19 infection.

2.7 Influence of Antibiotic-Induced Changes on Microbiota Functionality and Bacterial Behaviour at Single-Cell Level

2.7.1 Changes in metabolites

The gut microbiota is responsible for the production of many essential metabolites including SCFAs and amino acids (Mills et al., 2019). Studies have reported that

commensal-produced butyrate and propionate have anti-inflammatory roles, promoting the generation and differentiation of regulatory T cells (Arpaia et al., 2013; Furusawa et al., 2013), with roles in energy metabolism (De Vadder et al., 2014; den Besten et al., 2013). By impacting the composition of the microbial community, antibiotics also alter microbiota functionality and thus the metabolites produced (Ferrer et al., 2017).

For example, metabolomics profiles were analysed in antibiotic-treated piglets fed a corn-soy basal diet with or without in-feed antibiotics from postnatal day 7 to day 42. The antibiotic-treated group had higher concentrations of metabolites associated with amino acid metabolism, decreasing the concentration of amino acids. A reduction in SCFA production was also reported as levels of butyrate and propionate were decreased (Mu et al., 2017). Another study in piglets (day 7 to 21) demonstrated that fecal microbiota transplant and antibiotic (amoxicillin) treatment both resulted in lowering of fatty acid oxidative catabolism and amino acid biosynthesis, though antibiotics had a more significant effect (Wan et al., 2019).

Multiple studies performed in mouse models have elucidated the effects of antibiotic treatment on host metabolic functions. A study in antibiotic (streptomycin)-treated mice reported that antibiotic administration can affect pathways of hormone synthesis such as steroids and eicosanoids, along with altering the pathways involved in sugar, bile acids, and amino acid metabolism, thus suggesting the role of the microbiota in these pathways (Antunes et al., 2011). Lin Sun et al. reported that mice treated with antibiotics (enrofloxacin, vancomycin, and polymixin B sulfate) showed up-regulated gene expression of various cytokines in the colon, with significant metabolic shifts in valine, leucine, and isoleucine biosynthesis pathways. These alterations correlated to changes in microbial composition (Sun et al., 2019). One study reported that clindamycin treatment in mice resulted in significant changes in metabolite composition (30% of the compounds analysed); the restoration of which was associated with recovery of the altered microbiota (Jump et al., 2014). Zhao et al. reported that antibiotic treatment altered the products of bacterial metabolism. This included decreased levels of SCFAs, amino acids (correlated with abundance of *Prevotella*, *Alistipes* and *Barnesiella*) and increased precursors like bile acids and oligosaccharides (associated with high levels of facultative anaerobic bacteria, *Enterococcus faecalis*, *Enterococcus faecium* and *Mycoplasma*) (Zhao et al.,

2013). Microbial depletion in mice due to antibiotic uptake decreased baseline serum glucose levels, improved insulin sensitivity (Zarrinpar et al., 2018), altered systemic glucose metabolism along with changes in expressions of the genes in the liver and ileum involved in glucose and bile acid metabolism (Rodrigues et al., 2017). This was reported to be accompanied by reduced levels of SCFAs and secondary bile acid pools (Zarrinpar et al., 2018). Similar results were reported following vancomycin treatment (Vrieze et al., 2014). This can lead to impairment of barrier function (Kelly et al., 2015); act as a causative factor in the development of ulcerative colitis (Machiels et al., 2014), and *Salmonella* infection (Gillis et al., 2017).

Studies have also reported that antibiotic uptake can result in changes in protein expression, energy metabolism in the microbiota, with a slight increase following antibiotic therapy, which may be as a coping mechanism to antibiotic stress but decreased at later stages and post-antibiotic usage (Pérez-Cobas et al., 2013). Another study demonstrated that antibiotics have a sex-dependant effect on host metabolism. They reported that vancomycin and ciprofloxacin-metronidazole treatment resulted in significant reductions of Firmicutes and SCFAs in female mice, which was only observed after vancomycin treatment in males. They also reported that both antibiotic exposures significantly decreased the levels of alanine, branched-chain amino acids (BCAAs; leucine, isoleucine, and valine) and aromatic amino acids in colonic contents of female mice but not in male mice (Gao et al., 2019).

2.7.2 Accumulation of metabolites/xenobiotics

Xenobiotics (including antibiotics, heavy metals, and environmental chemicals) have an impact on gut microbial composition. The effect here is cyclical in that the microbiota is necessary for xenobiotic biotransformation (Figure 2.3). The metabolism of xenobiotics before it reaches its target organ site is largely dependent on the microbiota. The gut microbiota can affect the xenobiotics half-life in the host, the extent to which they reach the target receptor and may also influence the host's capacity to metabolize xenobiotics (Koppel et al., 2017). Both *in vivo* and *in vitro* studies have shown that the gut microbiota is involved in the biotransformation of xenobiotics (Lu et al., 2013). Studies using germ-free and conventional rodents reported that the absence of gut microbiota-affected gene expression of many liver enzymes including active androstane receptor and host

detoxifying enzymes such as glutathione peroxidases, sulfotransferase, epoxide hydrolases, and N-acteyltransferases (Björkholm et al., 2009; Meinl et al., 2009). However, the absence of microbes necessary for metabolising particular compounds can result in their accumulation in the host which may lead to toxicity. Ciprofloxacin administration to SPF and germ-free mice revealed decreases in hepatic Cyp3a11 expression, this was associated to the gut microbiota. The lower lithocholic acid (LCA) levels due to decreases in LCA-producing bacteria post-antibiotic administration could be responsible for decreases in expression of Cyp3a11. Similar effects in humans can result in less clearance of multiple CYP3A4 (human analogue of Cyp3a11)-dependant medications (Lynch & Price, 2007).

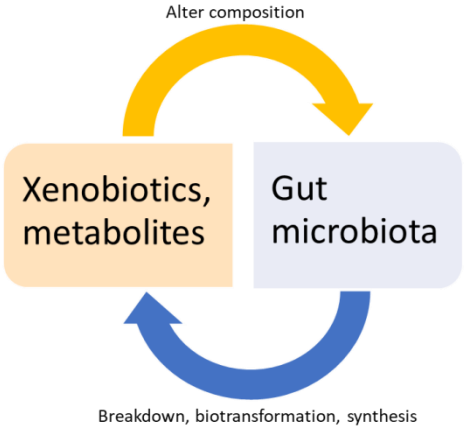


Figure 2.3: Figure demonstrating cyclical relation between gut microbiota, xenobiotics and metabolites

Another study speculated that increased levels of hepatic lipid accumulation and TG levels in mice were due to antibiotic treatment and the altered gut microbial composition (Jin et al., 2016). Studies have shown that antibiotic treatment in mice increases the levels of sialic acid and succinate which increase the susceptibility of the host to *Salmonella* and *C. difficile* infections (Ferreyra et al., 2014; Ng et al., 2013). In the same manner, an antibiotic-altered microbial composition can result in deficiencies of certain metabolites or vitamins that are solely produced by bacteria. For example, one study reported that antibiotic-induced changes in the gut microbiota of mice resulted in shifts in copper (Cu) metabolism. This can have consequences for immunity and the intestinal barrier due to the role of Cu in these functions (Miller et al., 2019).

Furthermore, cross-feeding is a significant feature of the gut microbiota. For example, *Bifidobacterium adolescentis* produces lactate and acetate by utilising fructo-oligosaccharides and starch. Butyrate-producing anaerobes cannot utilise fructo-oligosaccharides and starch but rely on lactate and acetate as growth substrates. Therefore, *B. adolescentis* indirectly facilitates the proliferation and expansion of butyrate-producing species through cross-feeding. This type of dependency is also seen in many other bacterial groups (Rowland et al., 2018; Heinken & Thiele, 2015). This co-dependency can be disturbed by antibiotic use leading to increased accumulation or deficiency of some metabolites/compounds. For example, a study reported a decrease in abundance of gram-negative bacteria in the gut by vancomycin which is a gram-positive targeting antibiotic (Ubeda et al., 2010). This can be due to the interdependency of bacteria in their community.

2.7.3 Changes in Bacterial signalling pattern

Antibiotics can alter the transcription of several major functional genes such as those encoding transport proteins, genes involved in metabolism of carbohydrates, and protein synthesis (Lin et al., 2005; Goh et al., 2002). A study demonstrated induced expression of virulence-associated genes in *P. aeruginosa* leading to higher secretion of rhamnolipids and phenazines on exposure to sub-inhibitory concentrations of antibiotics (Shen et al., 2008). Many studies have reported that aminoglycosides (Hoffman et al., 2005), Beta lactams (Ng et al., 2013; Kaplan et al., 2012), vancomycin and oxacillin (Mirani & Jamil, 2010) can induce biofilm formation even at sub-lethal concentrations. These biofilms then act as reservoirs of antibiotic resistance. This confers additional resistance to bacteria against several antibiotics and host defence, which can make treatment difficult in humans and can cause several issues such as blockage of pipes/equipment in healthcare settings and food industries (Muhammad et al., 2020).

Bacteria interact with their host using pattern recognition receptors (PRR) via signalling through the production of bile acids, SCFAs, fatty acids, amino acids, lipopolysaccharides, lipoteichoic acid, flagellin, CpG DNA and peptidoglycan. These signalling molecules either serve as a source of energy for other cells, regulate or

modulate the function of immune cells such as monocytes, macrophages, T cells via G-protein coupled receptor and nuclear receptor family, free fatty acid receptors (Brestoff & Artis, 2013). Antibiotic use results in a reduction of these bacteria and hence the PRRs such as TLR signalling and downstream regulation of innate defences (Willing et al., 2011). A study reported that an antibiotic-mediated decrease in butyrate-producing bacteria resulted in reduced epithelial signalling through the intracellular butyrate sensor peroxisome proliferator-activated receptor γ (PPAR-γ) (Byndloss et al., 2017). Similarly, antibiotic disruption of the commensal microbiota in newborn mice increases their susceptibility to pneumonia, due to interrupted migration of interleukin 22 producing lymphoid cells (IL-22+ILC3) (Gray et al., 2017). This effect could be reversed by transfer of commensal microbiota to mice at birth (Gray et al., 2017). Another study reported the importance of commensals in protecting against colon injury and maintaining intestinal homeostasis (SethRakoff-Nahoum 2004). In this case, commensals induced release of protective factors via TLRs; these factors were not released in mice treated with antibiotics lacking the commensal bacteria (SethRakoff-Nahoum 2004). Antibiotics can thus impact complex host-microbial interactions due to changes in microbial community composition.

2.7.4 Antibiotic resistance gene reservoir

Antibiotic resistance genes are now reported to be found in the environment including oceans and freshwater bodies (Hatosy & Martiny, 2015), soil (Cycoń et al., 2019), glaciers (Segawa et al., 2013), the food chain, and also within humans. Apart from bacteria, viruses have also been reported to be carriers of ARGs (Debroas & Siguret, 2019). Some of this spread of antibiotic resistance genes is historical such as in some untouched/uncontaminated environments (Van Goethem, 2018) but much of it is because of the wide use of antibiotics by humans.

For instance, apart from their use in treating infections in humans, antibiotics have been widely used as growth promoters for weight gain in animals (Butaye et al., 2003) and to treat and control infections (Ding, 2014; McManus et al., 2002). They have also been used in aquaculture for similar reasons (Lulijwa et al., 2019; Cabello, 2006). Some of these antibiotics are the same as, or are structurally similar to the ones used to treat human

infections such as erythromycin, gentamycin, enrofloxacin, neomycin, streptomycin (Marshall & Levy 2011). The development of antibiotic-resistant bacteria in agriculture and aquaculture is a serious concern as such bacteria can enter humans through the food chain promoting cross-resistance and also reduce the susceptibility of infectious bacteria to antibiotic treatment.

Humans are reservoirs of antibiotic resistance genes (Clemente et al., 2015; Bailey et al., 2010; Sommer et al., 2010) and the gut has been pinpointed as the epicentre of antibiotic resistance (Carlet et al., 2012). Intriguingly, some studies have reported the presence of ARGs in humans from remote communities who have had very limited exposure to antibiotic therapy. They reported high levels of acquired resistance to antibiotics such as tetracycline, ampicillin, trimethoprim/sulfamethoxazole, streptomycin and chloramphenicol (Bartoloni et al., 2009). Similarly, ARGs in healthy humans have been reported despite the absence of antibiotic use (Palme et al., 2015; de Vries et al., 2011; Sommer et al., 2009). Studies in healthy infants and children who have never been exposed to antibiotics report the presence of genes that confer resistance to beta-lactams, fluoroquinolones, tetracycline, macrolide, sulphonamide or multiple drug classes. The major ARG carriers were found to be *Enterococcus* spp., *Staphylococcus* spp., *Klebsiella* spp., *Streptococcus* spp., and *Escherichia*/*Shigella* spp (Casaburi et al., 2019; Moore et al., 2013; Zhang et al., 2011; Karami et al., 2006).

In the gut, bacteria can horizontally and vertically transmit genes to other related or unrelated bacteria due to their proximity via mobile genetic elements (Table 2.2). ARGs in the infant gut microbiota can be derived from that of their mothers, as AR bacteria can be transmitted from mother to infant through breastfeeding (Parnanen et al., 2018). The presence of ARGs in humans is a global crisis because it makes the treatment of infections more difficult, costly and inefficient.

Table 2.2: Various mobile genetic elements that can be used for transfer of ARGs

Mobile elements and transfer of resistance genes

Vehicle element	Description	Some Antibiotic resistance genes transported
Plasmids (Rozwandowicz et al., 2018)	Extra chromosomal material	Colistin resistance, extended spectrum β-lactams, β-lactams, aminoglycoside, quinolone, sulphonamides, tetracycline, chloramphenicol, trimethoprim.
Integrons (Partridge et al., 2009)	Genetic elements with site-specific recombination system	Sulphonamide resistance (sulI), aminoglycosides, β-lactams, quinolones, chloramphenicol and trimethoprim
Transposons (Lupski 1987)	Mobile elements that need integration into chromosome or plasmid	β-lactams, Macrolides, aminoglycoside, chloramphenicol, Tetracycline

2.8 Non-Microbiota Associated Effects of Antibiotics

2.8.1 In Pregnancy

Associations between antibiotic usage during pregnancy with neonatal and congenital abnormalities have been reported. For example, studies have reported an increased risk of developing cerebral palsy, epilepsy, cardiac and genital malformations in infants born to mothers treated with macrolides during pregnancy and which are more harmful when consumed during the first trimester (Meeraus et al., 2015; Källén & Danielsson 2014; Kenyon et al., 2008). Similarly, amoxicillin use during the first trimester was linked to cleft lip and cleft palate development, and though reported in only a small number of cases, it exemplifies the adverse effects of antibiotic use during pregnancy (Lin et al., 2012). Birth defects such as micro-ophthalmos, hypoplastic left heart syndrome, atrial septal defects, and cleft lip with cleft palate have also been associated with the use of sulfonamides and nitrofurantoin during the first trimester of pregnancy (Crider et al., 2009). Similarly, trimethoprim-sulphonamide use during pregnancy is associated with higher risk of cardiovascular malformations (Czeizel et al., 2001). The evidence in all

cases is mixed (Muanda et al., 2017), and is usually linked with consumption during the early months of pregnancy. This could be due to the anti-bacterial impacting the neonate during the organogenesis and early development stages *in utero*. Though the examples cited represent potential associations and not causal relationships, a reappraisal of antibiotic usage during pregnancy is warranted.

2.8.2 General Effects

Studies have reported that antibiotics can exert direct toxic effects on host tissues such as mitochondrial damage, suppressed ribosomal gene expression (Morgun et al., 2015) and oxidative tissue damage in mammalian cells (Kalghatgi et al., 2013). Though controversial, some studies suggest that antibiotic use can be related to increased risk of breast cancer (Tamim et al., 2008; Velicer et al., 2004) and increased risk of miscarriage (Fan et al., 2019). Sometimes, antibiotics can worsen the condition they are meant to treat. The bactericidal action of many beta-lactam antibiotics has been reported to increase toxin production such as Shiga toxin which is released from entero-haemorrhagic *Escherichia coli*, predisposing the host to a higher risk of haemolytic uremic syndrome *(*Wong et al., 2000; Kimmitt et al., 1999). Antibiotics can also affect host metabolism directly without microbes as a mediator, while making the targeted pathogen less susceptible to the antibiotic. These changes in host metabolites are mostly local to the site of infection and include high levels of AMP which decrease the efficacy of antibiotics and also increase phagocytic activity. This was accompanied by impaired immune function because of the inhibitory effect of antibiotics on the respiratory activity of immune cells (Yang et al., 2017).

2.9 Antibiotic alternatives and use of probiotics for restoring the microbial community and betterment of health.

It is now well established that antibiotic use results in changes in microbial composition, the consequences of which can be detrimental for the host. Certain approaches can be used along with or post-antibiotic therapy to restore the microbial composition faster (Figure 2.4). Probiotics are widely used for this purpose and have been shown to increase the abundance of beneficial microbes, stabilise the microbial community and thus alleviate the effects of antibiotics (Korpela et al., 2018, 2016; Cha et al., 2012). Probiotics exert their effects by promoting antimicrobial peptide production, producing bacteriocins,

suppressing the growth of non-commensals via competing for nutrients and receptors on intestinal mucosa, enhancing barrier function in the gut, and modulating immunity (Cazorla et al., 2018; Xue et al., 2017; O'Shea et al., 2012; Bron et al., 2011; Collado et al., 2007), but the use of probiotics may not lead to complete restoration of the gut microbiota.

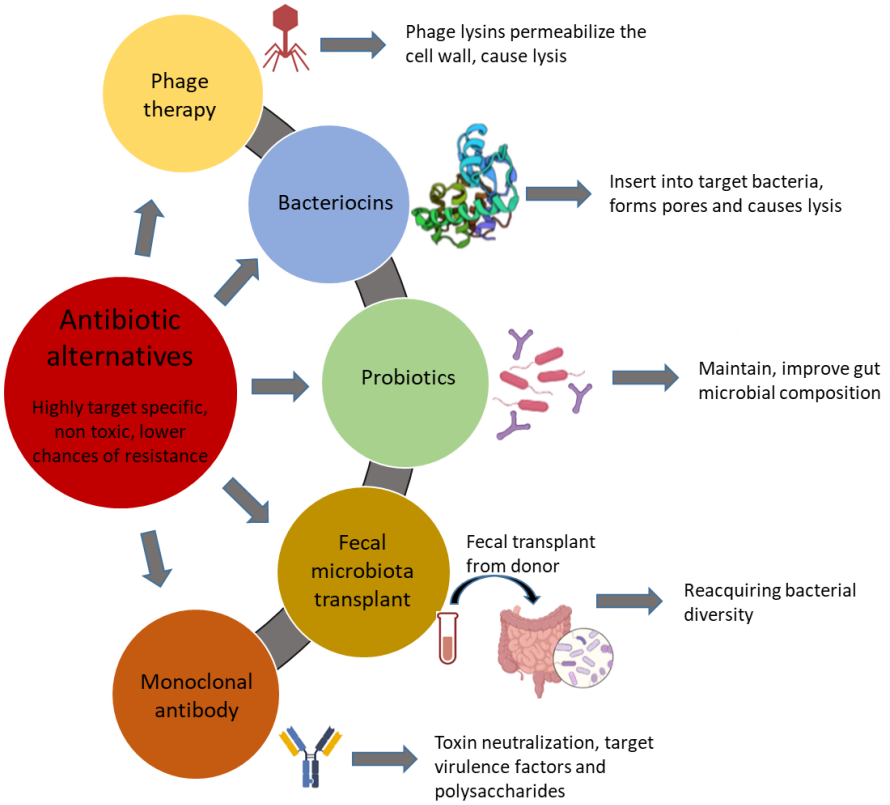


Figure 2.4: Figure demonstrating various alternatives to antibiotics that can be used by itself or in some cases in conjunction with antibiotic treatment.

Fecal microbiota transplant (FMT) can be more beneficial for regaining microbial balance in the gut (Suez et al., 2018). FMT has been widely used therapeutically for rebalancing the microbiota of *C. difficile*-infected patients; restoring their microbial and metabolic activity (Weingarden et al., 2014). FMT can also utilise donor fecal material from the patient itself before antibiotic therapy, known as autologous FMT. Many factors such as efficacy, cost and suitability make FMT an attractive option but detailed studies are

needed to optimise the process and understand other probable therapeutic applications beyond gut disorders (Ramai et al., 2019; Allegretti et al., 2019).

One of the major issues of antibiotic use is development of bacterial resistance. Alternatives such as bacteriophage (phage) therapy and bacteriocins are now being investigated as substitutes for antibiotics or complementary therapies with antibiotics to overcome the issue of resistance. Phage therapy was first described and used in the early 1900s, but due to the introduction of antibiotics, phage therapy was dismissed in western medicine (Lin et al., 2017), although it is still practised in certain regions of the world including Georgia, Russia and Poland (Hill et al., 2018). With the growing antibiotic resistance crisis, phage therapy is now being revisited. The use of phage therapy to reduce biofilm and treat lung infections has been studied in mouse models and it has been demonstrated that phage therapy successfully treats respiratory *P. aeruginosa* infection (Fong et al., 2017; Waters et al., 2016; Cao et al., 2015). A report based on using personalised phage therapy for treating a patient with MDR *A. baumannii* reported clearance of infection and success of the treatment (Schooley et al., 2017). Furthermore, case reports of patients with bacterial prostatitis and septicaemia and acute kidney injury reported pathogen eradication and decrease in clinical symptoms post phage therapy (Letkiewicz et al., 2009, Jennes et al., 2017). Another study reported that 67 of 96 patients' wounds and ulcers healed post phage therapy; healing was associated with reduction in pathogens (Markoishvili et al., 2002). Use of engineered bacteriophages to treat drug-resistant *Mycobacterium abscessus* was reported to show clinical improvement in a patient with cystic fibrosis (Dedrick et al., 2019). Another promising approach is the use of phage lytic proteins as antimicrobial compounds (Mondal et al., 2020), making phages a strong antibacterial contender of antibiotics.

Bacteriocins represent another category of potential antibiotic alternative. Bacteriocins are ribosomally-produced antibacterial peptides produced by bacteria which themselves are immune to the killing peptide due to specific immunity mechanisms. To date, bacteriocins have been mainly used in the food industry as food safety and preservative agents (Silva et al., 2018). Bacteriocins have shown promising results as antimicrobials in animal studies. For example, mouse model studies have reported the successful use of pyocin to treat *P. aeruginosa* lung infections with high efficacy and without any adverse

effects (McCaughey et al., 2016; Merrikin & Terry, 1972). Another study in mice reported that administration of nisin and pediocin-producing *L. lactis* and *P. acidilactici* strains helped reduce intestinal vancomycin-resistant enterococci colonisation (Millette et al., 2008). Bacteriocins have been successfully used to treat and prevent bovine mastitis with a comparable efficacy to antibiotics (Kitching et al., 2019; Cao et al., 2007; Crispie et al., 2004). Furthermore, nisin has proven to be effective in treating mastitis caused by *Staphylococcus* in eight lactating females (Fernández et al., 2008).

Another effective way of addressing the problem of antibiotic resistance is with the use of monoclonal antibodies as alternatives or in conjunction with antibiotics. Monoclonal antibodies bypass the complications of toxicity, resistance development and early clearance by the immune system which is seen in the case of antibiotics. The use of monoclonal antibodies for treating bacterial infections is emerging in the past few years, before which monoclonal antibodies were mostly used for treating cancer, autoimmune diseases or viral infections (Zurawski and McLendon, 2020). A recent study in rabbits demonstrated the success of use of monoclonal antibody obiltoxaximab against anthrax protective antigen. The authors reported that use of obiltoxaximab improved survival of rabbits that received a lethal dose of *B. anthracis* spores (Henning et al., 2018). In a recent clinical trial of 2655 participants, authors reported that use of bezlotoxumab (monoclonal antibody against *C. difficile* toxin) for treating *C. difficile* infection resulted in lower recurrence of infection (Wilcox et al., 2017). In another recent study, Watson et al. (2021) generated a monoclonal antibody from B cells of a patient to be used against *Mycobacterium tuberculosis* infection in mice. Monoclonal antibodies are costlier than producing antibiotics but have many benefits and more studies in this direction will help can help transform medicine.

One of the major advantages of bacteriocins, phages and their endolysins, and monoclonal antibodies is that they can be highly target-specific thus rendering minimal if any, collateral damage to the microbiota.

2.10 Conclusion

This review has summarised the importance of the gut microbiota in host metabolism and immune functions such as immunity development, colonisation resistance, cell signalling,

and with the help of advanced omics technologies, the complex interactions between host and microbiota are now becoming clear. Antibiotics disrupt the microbial balance and hence the networking within the bacterial community, and that with the host. The resulting AMR bacteria makes clinical treatment difficult. Due to this complex link between the host and microbiota, the current usage of antibiotics requires careful stewardship, with an emphasis on the application of antibiotic alternatives, while limiting collateral damage. To this end, we need to design, develop and translate new antibiotic alternatives from bench to bedside, in addition to methodologies that are efficient in conserving and restoring the microbial community after antibiotic-associated perturbations.

2.11 Author contributions

Dhrati Patangia: Conceptualisation (lead); writing – original draft (lead), Writing – review and editing (equal), **Paul Ross**: Supervision (equal); Conceptualisation (supporting); Writing – review and editing (equal), **Catherine Stanton:** Supervision (equal); Conceptualisation (supporting); Writing – review and editing (equal), **Tony Ryan**: Supervision (equal); Conceptualisation (supporting); Writing – review and editing (equal), **Eugene Dempsey**: Supervision (equal); Conceptualisation (supporting); Writing – review and editing (equal),

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2.13 Ethics statement

None required

2.14 Conflict of interest

None declared

2.15 Data Availability statement

Not applicable

2.16 Bibliography

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Chapter 3

3 Microbiota and resistome analysis of colostrum and milk from dairy cows

3.1 Abstract

Most studies in dairy cows are focused on inspecting the microbial composition of milk with respect to mastitis or dairy product shelf-life. This study investigated the longitudinal impact of dry cow therapy (DCT) in healthy cows with and without antibiotics on the microbial composition and resistome profile in colostrum and milk for up to six months after DCT treatment. Using whole genome shotgun sequencing, we investigated the microbiome in first milk samples at lactation (colostrum) (M0 – month 0) and milk samples at months 2 (M2), 4 (M4) and 6 (M6) months from dairy cows (n=24). Lactating dairy cows were prophylactically administered antibiotics six to eight weeks before starting lactation – called DCT. Cows negative for mastitis from five Irish farms were selected and divided into three groups based on antibiotic treatment during DCT: No antibiotic (noab), Cephaguard DC (cef - Cefquinome) and Ubro Red Dry Cow Intramammary Suspension (ubro - Framycetin Sulfate, Penethamate Hydriodide and

Procaine Penicillin) treatment. The microbial diversity of milk from antibiotic-treated groups was different and higher than noab group. This difference was more evident in milk samples compared to colostrum, with increasing diversity seen over time only in antibiotic-treated groups (Alpha diversity). The microbiome of antibiotic-treated groups clustered separately from the non-antibiotic group at M2-, M4- and M6- milk samples, showing the effect of antibiotic treatment on between-group (beta) diversity. The phyla Actinobacteria, Proteobacteria and Firmicutes and genera *Actinoallotecihus*, *Rhodococcus*, and *Corynebacterium* were observed in high relative abundance in milk from all three groups. Noab group did not show high relative abundance of mastitis-causing pathogens like *Escherichia* and *Staphylococcus* in milk during early lactation, while *Corynebacterium* whose role depends on the species present was abundant in this group. Similarly noab group was more associated to genera such as *Psychrobacter, Serratia, Gordonibacter, Brevibacterium* and *Sphingobacterium*. High relative abundance of ARGs was observed in the milk of antibiotic-treated groups with the cephaguard group showing significantly high abundance of genes conferring resistance to cephalosporin, aminoglycoside and penam classes. The milk microbiome of non-antibiotic treated cows during DCT had different microbial composition from antibiotic-treated groups at all time points after colostrum over the first six months of lactation, pointing towards a potential long-lasting effect of antibiotics used during the drying off period. These data did not show high abundance of mastitis-causing pathogens such as *Escherichia, Staphylococcus* in the no-antibiotic group, with different microbial composition and more abundant ARG profile of the antibiotic-treated groups compared to no-antibiotic group. The data support the use of non-antibiotic alternatives for DCT and encourage the new European legislation discouraging the use of blanket DCT.

Keywords: cow milk microbiota, resistome, shotgun metagenomics, longitudinal

3.2 Introduction

The first milk produced after the non-lactating period, colostrum, is highly nutrient-rich, provides the calf with both nutritional and immune benefits, and shapes the calf gut microbiota (Song et al., 2019). It helps in the education, maturation and development of the immune, organ system and intestinal function (Lopez and Heinrichs et al., 2022; Hammon et al., 2020). This is crucial for the calf as it is born agammaglobulinemic and depends on the colostrum for passive immunity and gut microbiota development (Song

et al., 2019). Colostrum and stable gut microbiota colonisation are important in early life for ruminant development from the monogastric stage at birth, and impact the overall health of the animal (Amin and Seifert et al., 2021). The mother to neonate microbial transfer is now widely studied both in humans and animals (Zhu et al., 2021; Wang et al., 2020). Similarly, due to the importance of animal health and its relation to dairy products, research is now focused on bovine colostrum and milk microbiota composition and its association with disease (Oikonomou et al., 2020; Porcellato et al., 2020; Ganda et al., 2016; Quigley et al., 2013). Most of the milk microbiota studies in bovines have focused on the variation in milk microbial composition along with its treatment for the production of dairy products (Fasse et al., 2021). Reports are now emerging that focus on the health status of the cow and milk microbial composition (Pang et al., 2018; Oikonomou et al., 2012, 2014).

In cows, before the milking begins there is a period called the dry period (six to eight weeks long) which allows the teats to dry up. This can be done either by allowing the drying to occur naturally, using teat sealants or by using medications and antibiotics to ensure the teats are free of any infection, called dry cow therapy (DCT). The objectives of DCT are to eliminate infections presenting from previous lactation and to avoid new udder infections during the dry period – when mammary glands are highly susceptible to new infections (Berry & Hillerton 2002). The use of DCT can be either selective or for the entire herd. In the second approach, called the 'blanket' approach, the entire herd is treated with antibiotics for preventive measures. It helps to achieve the goal of attaining high quantities of good quality milk (Crispie et al., 2004; Berry & Hillerton 2002), however, prophylactic treatment of animals with antibiotics is increasingly being frowned upon due to problems associated with antibiotic resistance development.

Many dairy advisors recommend that all quarters of cow udder be treated with antibiotics approved for use in the drying period. The use of antimicrobials for DCT is common globally (More et al., 2017; Sneeringer et al., 2015; Lam et al., 2013; Dufour et al., 2012; USDA 2008) although a declining trend is now observed in many European countries (ESVAC, 2020). Some countries use selective therapy as opposed to the blanket approach with changing guidelines (Huey et al., 2021; Scherpenzeel et al., 2016). Many antibiotics used in livestock are medically relevant and based on the same anti-microbial compound used in human medicine. Commonly used antibiotics include penicillin alone or in

combination with aminoglycosides and cephalosporins, tetracyclines, fluoroquinolones, macrolides, and sulfonamides (ESVAC, 2020; EFSA Panel on Biological Hazards; Ricci et al., 2017). Indiscriminate use of antibiotics can cause loss of effectiveness in treating further infections in dairy cows as well as humans (Phillips et al., 2003).

Milk from cows treated with prophylactic antibiotics usually contains significant antibiotic residues (Sachi et al., 2019; Kurjogi et al., 2019; Layada et al., 2016). Sub-MIC (minimum inhibitory concentration) levels of antibiotics can form resistant bacteria, which can be more problematic than those selected at higher doses (Andersson and Hughes, 2012; Davies and Davies, 2010). Colostrum is suggested as a potential source of ARGs for calf gut microbiota in the absence of antibiotic use in calves (Liu et al., 2019; Thames et al., 2012). Further, reports suggest the presence of antibiotic resistance genes (ARGs) and significantly altered microbial and functional profile in calf gut when fed with milk containing antibiotic residues (Liu et al., 2019; Pereira et al., 2018; Maynou et al., 2016, 2017; Van Vleck Pereira et al., 2016; Thames et al., 2012). Antibiotic resistance in dairy milk is also an area of interest because of human consumption of raw milk or other dairy products made from contaminated raw milk and issues of environmental pollution with ARGs making it a One-Health issue (Di Rocco et al., 2021; Zaheer et al., 2019; Astorga et al., 2019; Mungai et al., 2015; Oikonomou et al., 2014; Tanaka et al., 2009; Thames et al., 2012; Kyselková et al., 2015).

Moreover, the presence of ARGs is not limited to pathogenic bacteria but may also occur in commensals. Mostly, the antibiotic resistance of pathogens in bovine milk is studied due to its role in mastitis (Boireau et al., 2018; Saini et al., 2012; Kalmus et al., 2011; Erskine et al., 2002), but not many studies have been conducted to study the resistance pattern of the overall milk microbiota. Also, most studies focus on the resistance patterns of pathogens at one time point, thus, temporal effects of antibiotics on the resistance pattern in colostrum and milk are yet to be investigated. We hypothesised that antibiotic use in the Cephaguard (cef) and Ubro red (ubro) group during DCT will result in varying abundance and composition of microbiota and resistance genes as compared to the non-antibiotic group (noab). Thus, in this study, shotgun metagenomics sequencing was used to investigate the longitudinal microbial composition and resistome profiles of colostrum and milk samples obtained from healthy cows throughout lactation, that were previously treated with and without antibiotics (cephaguard and Ubro red) during DCT.

3.3 Methods

3.3.1 Enrolment criteria and treatment for cows

Colostrum and subsequent milk samples taken throughout lactation at M2, M4 and M6 were collected from healthy cows (negative for mastitis with no visible infections) from five different Irish farms between February and September 2020 as detailed below. All cows were housed in different herds in farms located in County Cork, Ireland (Detailed metadata: Table S3.1). Cows were divided into three different groups based on the DCT treatment provided on the five farms. Cows on all farms were treated with a teat sealant-Boviseal- in addition to antibiotics as described during the drying-off period. Noab did not use any antibiotics during the drying-off period (natural drying off using teat sealant) and was assigned as the control group. Ubro-red group was treated with Ubro Red Dry Cow Intramammary Suspension at drying off; of which the active ingredients are Framycetin Sulfate, Penethamate Hydriodide and Procaine Penicillin. Cef group was treated with Cephaguard DC 150 mg intramammary ointment at drying off; of which the active ingredient is Cefquinome (a fourth-generation cephalosporin). Twenty-four cows divided into three groups were included in the study (noab:n=9, cef:n=8, ubro:n=7), with four samples collected longitudinally from each cow throughout the first six months of lactation. Cows showing signs of mastitis or any other infections were excluded from the study and milk samples were collected only from healthy cows. Cows were not subjected to antibiotic treatment during the time interval of sample collection.

3.3.2 Sample and data collection

Serial milk samples from twenty-four cows in the study were collected by personnel on the farms through the first six months of lactation. Instructions to farmers were provided for sterile sample collection. Briefly, teats were disinfected by dipping in iodine tincture followed by tapping and wiping them dry. The teat ends were then cleaned with alcohol wipes and approximately 15 ml of colostrum and milk were collected into sterile 15 ml falcon tubes without preservatives. The samples were immediately frozen at -20°C on the farms before being transported to the laboratory, where they were stored at -80°C until further use. Metadata regarding the parity of cows, any previous infections, the treatment used for DCT and any other medications were collected from the farms (Table S3.1).

3.3.3 DNA extraction

Milk samples were thawed by placing them at 4°C the night prior to DNA extractions. The samples were homogenised by inverting the tubes and centrifuged at 4000 g for 30 min at 4°C. The fatty/cream layer that accumulated at the top was discarded using sterile cotton swabs and the supernatant was discarded. DNA was extracted from the pellet according to the manufacturer's instructions using the PowerFood microbial DNA Isolation Kit (MoBIO Laboratories Inc., Carlsbad, CA) with modifications as follows: Briefly, the cell pellets were washed with PBS (phosphate-buffered saline) (Sigma Aldrich) and centrifuged at 13,000 x g for one min at 20°C to discard the supernatants. This process was repeated until the supernatant was no longer cloudy. The pellet was then re-suspended in PBS and treated with 90µl of 50 mg/ml lysozyme (Sigma Aldrich, Lysozyme activity: ≥=40,000 units/mg protein) and 50µl of 5 KU/ml mutanolysin (Sigma Aldrich) followed by 15 min incubation at 55°C with bump vortexing at intervals of 5 min. Further, samples were treated with Proteinase k (Qiagen, UK, 28 µl of 20 mg/ml, >600 mAU/ml), incubated at 55°C for 15 min and then treated according to the manufacturer's instructions using the PowerFood microbial DNA Isolation Kit protocol. The extracted DNA was stored at -30°C until further use.

3.3.4 Amplification of DNA, library preparation and shotgun metagenomics sequencing

The quantification of DNA was performed using the Qubit double-stranded DNA (dsDNA) high sensitivity assay kit (Invitrogen). Some samples had less than 0.2 ng/µl of DNA, thus a modified low-input Nextera XP protocol was used for shotgun sequencing library preparation as described previously (Rinke et al., 2016). Briefly, 5µl of DNA was used for the tagmentation run along with 10ul of Tagment DNA (TD) buffer and 5µl of 1:10 diluted Amplicon Tagment Mix (ATM), making the total reaction volume 20µl. The samples were incubated at 55°C for 5 min on the thermal cycler for tagmentation reaction, with the immediate addition of 5µl of NT (Neutralise Tagment) buffer to stop the tagmentation. Subsequent amplification was performed using 20 amplification cycles to ensure high quantities of amplified product for downstream reactions. 1.6x ratio of Ampure XP beads was used to purify the libraries and the purified product was eluted in 20µl of re-suspension buffer. The quality of purified DNA was determined using the High Sensitivity DNA kit on the Agilent Bioanalyzer. The amplified DNA was quantified using

Qubit and the samples were then pooled and outsourced for sequencing to the Teagasc Next Generation DNA Sequencing Facility (Teagasc, Ireland).

3.3.5 Bioinformatics and statistical analysis

Raw metagenomics shotgun reads received post-sequencing were quality-checked using FastQC (0.11.8) and MultiQC (v1.9). The reads were then filtered and trimmed using *Trim galore* (v0.6.1) and Bowtie2 (v 2.4.4) using the *Bos taurus* reference genome database from NCBI (Genome assembly ARS-UCD1.3) to remove any host contaminant reads. Reads obtained post-host removal step were considered microbial and were used for further analysis. The filtered and trimmed reads were then used for taxonomic assignment with Kraken2 (v2.1.1) and kraken-biom (https://github.com/smdabdoub/kraken-biom). Further, antibiotic resistance analysis was performed using RGI (Resistance Gene Identifier) (RGI-bwt) (v3.1.4) and CARD (Comprehensive Antibiotic Resistance Database) (McArthur et al., 2013). The phyloseq object (McMurdie and Holmes, 2013) formed from Kraken2 output was normalised and filtered to keep reads with abundance greater than 5e-4 reads per taxa, and those belonging to viruses, Euryarchaeota and Archaea and not classified at phylum level were removed. Initially, 5194 taxa were obtained which were filtered down to 235 taxa. Stringent filtering parameters were applied to make sure that no taxa arising as contaminants/singletons remain in downstream analysis. Further, decontam package (v1.14.0) in R was used to look for contaminants in samples and remove them. Using the default parameters, no PCR blank reads were observed in any of the study samples. The negative control sample (with less than 500 reads) was thus excluded from further analysis and the phyloseq object with all study samples was used for further analysis. Downstream analysis was performed in R (v4.0.2) using packages phyloseq (v1.38.0), microbiome (v1.16.0) and ggplot2 (v3.3.2). Statistical analysis was also performed in R using Wilcoxon test and Dunn test with the function stat_compare_means and stat_pwc from package ggpubr (v0.4.0) and Permanova using pairwiseAdonis (v0.4) package.

3.4 Results

3.4.1 Microbiota diversity is influenced by antibiotic treatment

Shotgun sequencing of the raw bovine colostrum and milk samples yielded 494,107,934 reads. Post trimming and host removal, the number of microbial reads remaining were

108,774,030 with an average of 1,121,382 reads (median = 310,854, min = 40,371, max = 6,941,451). Shannon and Chao1 diversity indices were used to calculate the alpha diversity of the microbiota within the groups. Shannon diversity index provides details about richness and evenness while the Chao1 index estimates total richness only. Wilcoxon test was performed to test if the difference between any two groups or time points was significant. It was observed that both Shannon and Chao1 diversity indices did not vary between time points within the no-antibiotic group (Figure 3.1A, Figure S3.1A). However, significant differences were observed for both Shannon and Chao1 diversity indices between M0 (colostrum) and M2 (2 month milk) (For Ubro: Shannon p-value=0.026, Chao1 p-value=0.011, For Cef: Shannon p-value=0.00016, Chao1 p-value=0.003; Wilcoxon test), M0 and M4 (4 month milk) (For Ubro: Shannon p-value=0.00058, Chao1 p-value=0.0021, For Cef: Shannon p-value=0.00031, Chao1 p-value=0.0061; Wilcoxon test), M0 and M6 (6 month milk) (For Ubro: Shannon p-value=0.00058, Chao1 p-value=0.002, For Cef: Shannon p-value=0.00016, Chao1 p-value=0.0044; Wilcoxon test) in both cephaguard (cef) and ubro red (ubro) treated groups (Figure 3.1A, Figure S3.1A). Additionally, a significant difference between milk obtained at M2 and M6 was observed for the Chao1 index in the Ubro group (Figure S3.1A). Concerning individual time points, no significant differences were observed between the Shannon and Chao1 indices of the three groups at M0, while cef and noab groups showed differences at M2 (2 month milk); ubro and noab at M4 (4 month milk) and both antibiotics groups to noab at M6 (6 month milk) (Figure 3.1B, Figure S3.1B). Beta diversity of milk microbiota was calculated using Bray-Curtis distance dissimilarity matrix and observed using PCoA (Figure 3.1C, 3.1D). Beta diversity of milk microbiota between the noab and antibiotic groups (ubro and cef) demonstrated distinct clustering overall and at all time-points after M0 (colostrum) (Figure 3.1C, 3.1D). Apart from M0, at each time point, noab to cef and noab to ubro groups demonstrated discrete grouping with significant p-values obtained with pairwiseAdonis test (p.adj. values at M2, noab vs cef 0.0030, noab vs ubro 0.0045; at M4, noab vs cef 0.0015, noab vs ubro 0.0015; at M6, noab vs cef 0.0015, noab vs ubro 0.0015, Wilcoxon test). Furthermore, discrete clustering of samples was observed based on time points, with samples at M0 clustering separately from all later time points (Figure 3.1D).

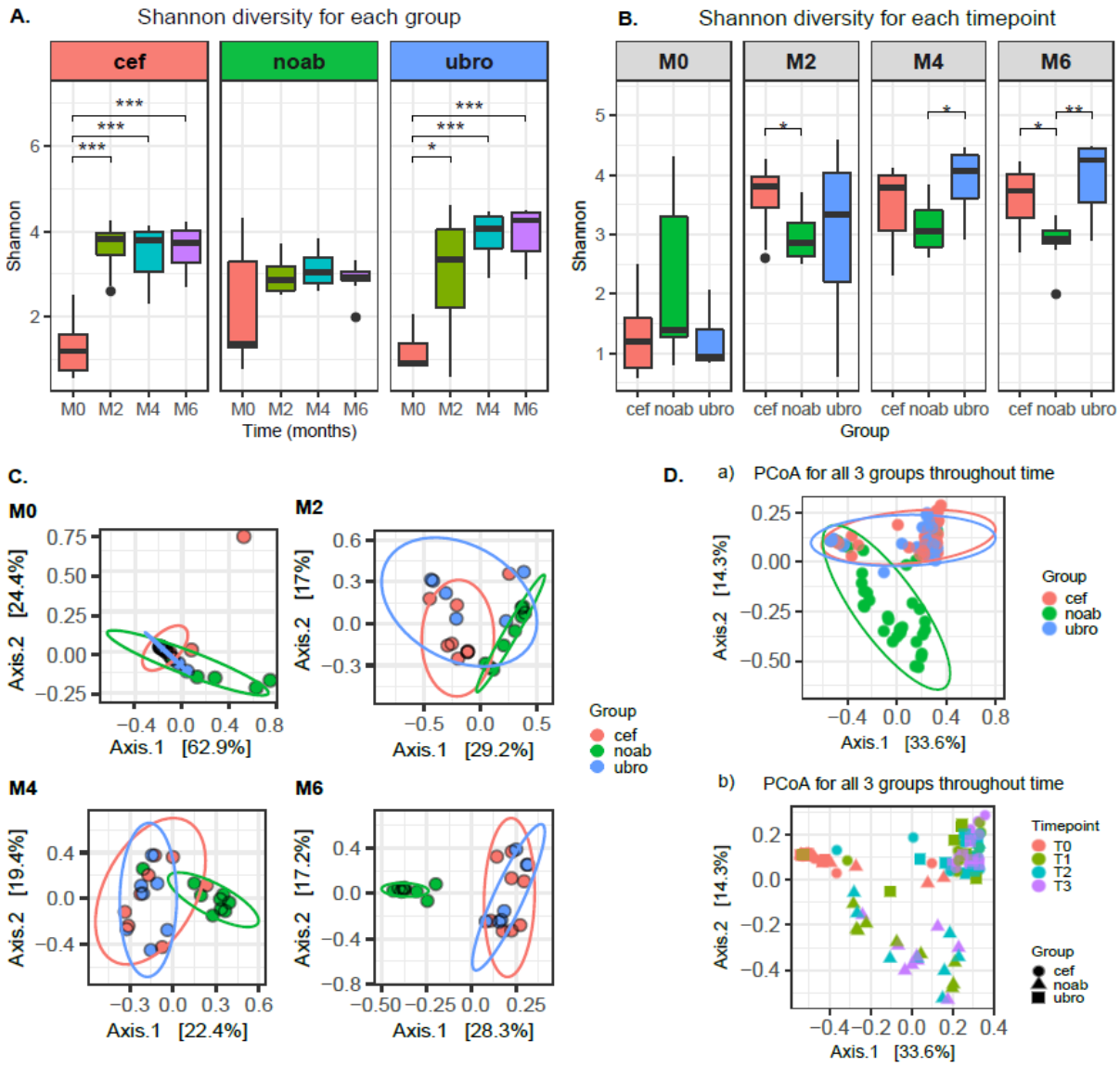


Figure 3.1: A. Alpha diversity of milk microbiota using Shannon index between all time-points for each group. The plot shows significant differences in diversity between milk obtained at M0 and M2, M4, M6 for both the antibiotic-treated groups (ubro and cef). B. Alpha diversity using Shannon index between the three groups (noab, ubro and cef) at all time-points. Plot shows no significant difference between groups at M0, while significance between antibiotic and no-antibiotic group was seen at later time points. C. PCoA plot using Bray-Curtis distance matrix between the three groups at all time-points; shows distinct clustering between groups. D. PCoA plot using Bray-Curtis distances a) showing distinct clustering of all three groups; with antibiotic groups clustering discretely

compared to the no-antibiotic group and; plot b) showing separate grouping of points between groups and time-points.

3.4.2 Taxonomic composition is associated to dry cow therapy treatment

Differences in the relative abundance of the microbial composition of milk between the three groups at all time points were observed using plots at phyla and genera levels (Figure 3.2). Overall, the phylum Actinobacteria was observed to have high relative abundance in both colostrum and milk samples in all groups with highest relative abundance in the noab group which was seen to decrease over time. The next most abundant phylum was Proteobacteria, followed by Firmicutes and Bacteroidetes. The relative abundance of Bacteroidetes was very low in the antibiotic-treated groups as compared to the noab group (Figure 3.2A).

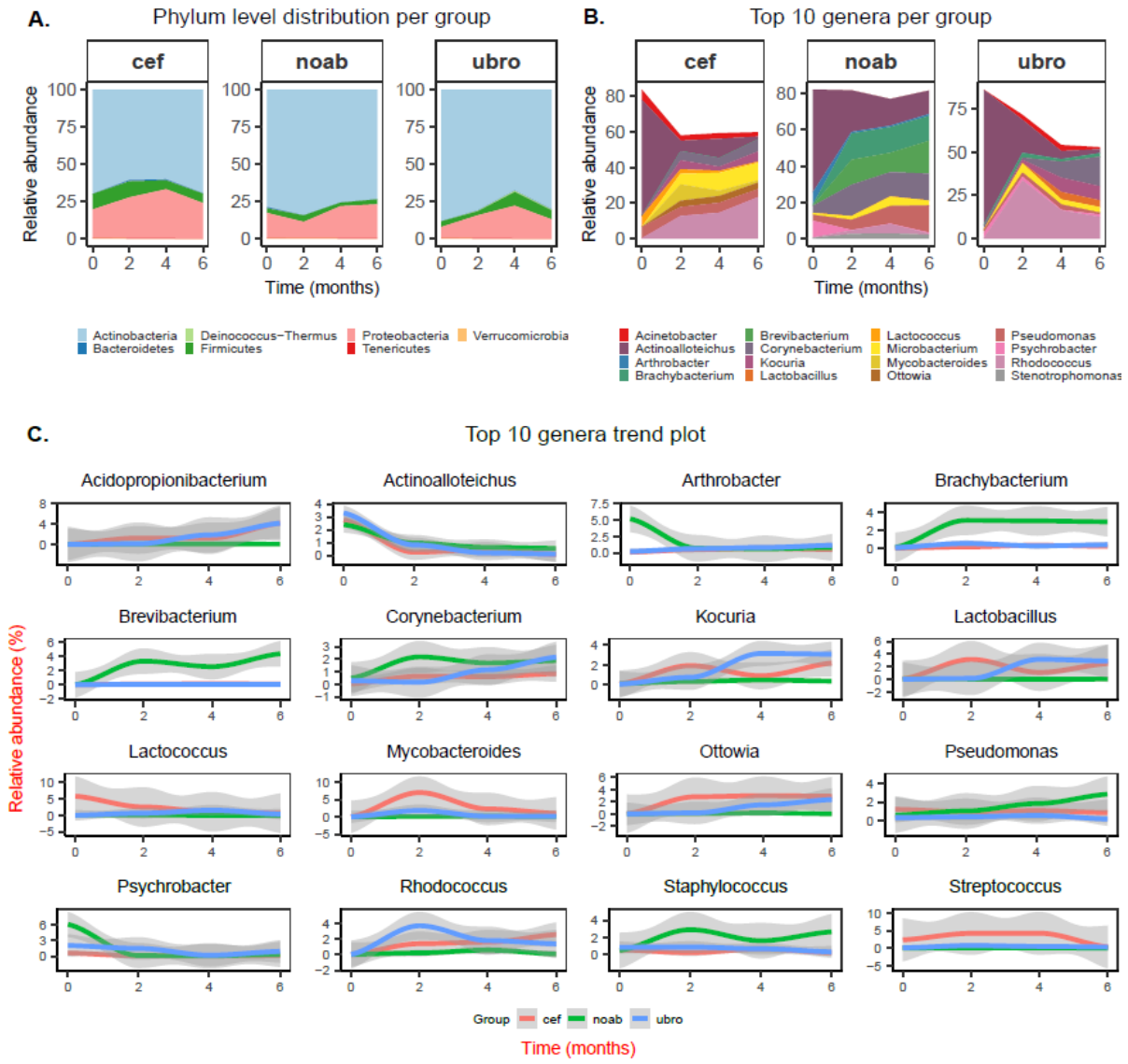


Figure 3.2: A) Phylum level distribution of taxa in all three groups across all time points. B) Overall top 10 genera in all three groups (noab, ubro and cef) at all time-points. C) Trend plots at genera level, showing relative abundance of top 10 genera from all three groups (noab, ubro and cef) over time with confidence interval of 95% (with different Y-axis scale for each taxa).

The top 10 genera found in milk from each group over all time points were examined and several genera identified in cow milk in previous studies were also observed in this study such as *Acinetobacter, Brevibacterium, Corynebacterium, Lactobacillus, Lactococcus, Microbacterium, Pseudomonas, Propionibacterium, Kocuria* and *Staphylococcus* (Figure 3.2B) (Porcellato et al., 2021; Rubiola et al., 2020; McHugh et al., 2020; Bonsaglia et al., 2017; Zhang et al., 2015; Oikonomou et al., 2014; Gulbe & Valdovska 2014; Masoud et al., 2012). In the noab group, the genera *Actinoallotecihus, Corynebacterium, Brachybacterium, and Microbacterium* were amongst the top 10 over all time points. *Brachybacterium* and *Brevibacterium* showed an increase in the no-antibiotic group, compared with the antibiotic-treated groups. Further, *Corynebacterium* was found in both antibiotic-administered and noab groups with higher initial abundance in the noab group. *Actinoalloteichus* was also present in all three groups in high relative abundance at early time points and showed a declining trend with time of lactation (Figure 3.2C). Higher abundance of *Acinetobacter* was observed in the antibiotic-administered groups compared to the no-antibiotic group. Additionally, *Rhodococcus, Ottowia* and *Lactobacillus* were observed in higher relative abundance in both the antibiotic-treated groups and demonstrated an increasing trend over time of lactation. *Pseudomonas, Microbacterium, Corynebacterium,* and *Kocuria* were also present at all time points in all three groups, with *Kocuria* and *Microbacterium* increasing over lactation time in the antibiotic-treated groups. A closer look at the relative abundance of major mastitis-causing pathogens in dairy cows revealed lack of high relative abundance of potential pathogens such as *Escherichia, Staphylococcus* in the non-antibiotic group as M0 and M2 (Figure 3.3A). Relative abundance of *Streptococcus* was close to zero in all three groups (plot not shown). The relative abundance of *Corynebacterium* was higher in the noab group than the Cef group at M0, and higher than both antibiotic-treated groups at M2 (Figure 3.3A).

3.4.3 Differential microbial composition in antibiotic vs non-antibiotic groups

Relative associations of genera to each group were estimated with Songbird (Morton et al., 2019) using the formula: C(Group, Treatment('noab')). The differentials obtained (Table S3.2) were ordered by rank and the top 10 most positive and negative genera were plotted and visualised using R (Figure 3.3B). Negative coefficient values denote stronger associations of the feature (genera here) with the reference. Upon comparing the antibiotic-treated group with the no-antibiotic group, *Brevibacterium, Brachybacterium, Serratia, Gordonibacter* were more associated to the noab group, while *Ottowia, Kocuria, Rhododcoccus, Microbacterium, Pseudomonas* were more associated to the antibiotic-treated groups. A comparison of the differentially abundant genera between the antibiotic-treated groups (ubro and cef) did not show any pattern highlighting a stronger association of any genera to a particular antibiotic group (Figure not shown).

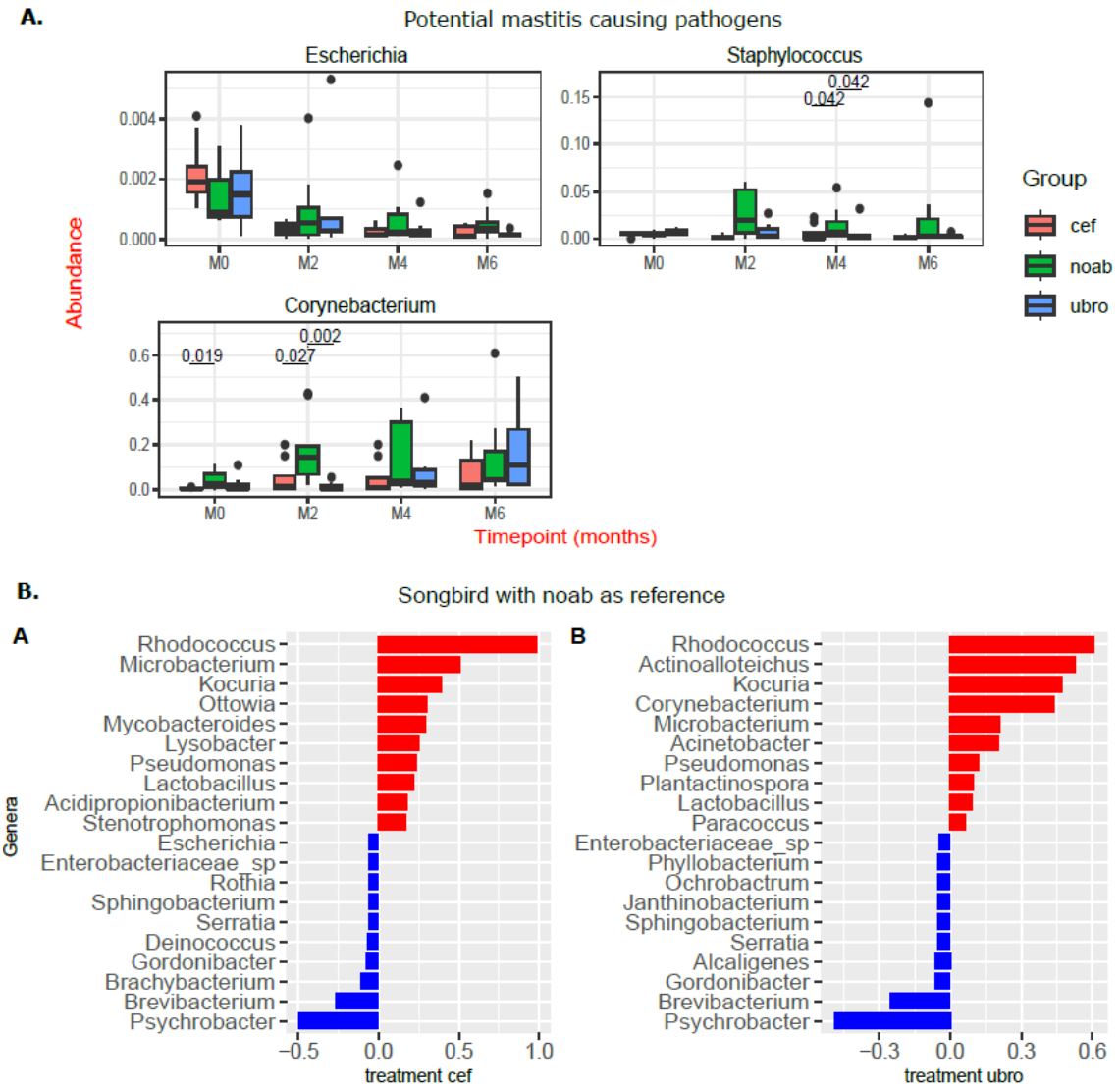


Figure 3.3: A. Boxplots showing abundance of potential mastitis-causing pathogens in milk of all three groups (noab, ubro and cef) over time of lactation. Significance was determined using Wilcoxon test in R, and p.adj values <0.05 are considered significant. B. Songbird differentials obtained using the formula C(Group, Treatment('noab')) were sorted by ranks and the top 10 positive and negative features are plotted and depicted using bar charts with noab groups as reference (Negative here is more associated to reference group – noab here).

3.4.4 Impact of dry cow therapy on antibiotic resistance gene reservoir

RGI-bwt was used to predict antibiotic resistance genes in filtered and quality-controlled shotgun sequencing reads. The results were normalised to copies per million based on read counts using all mapped reads from the gene mapping output file of RGI-bwt. The results were further log-transformed and the abundance of ARGs at each time point was visualised using heatmap, boxplots and bar plot in R. ARGs belonging to 189 AMR gene families conferring resistance to 43 different classes of antibiotics were found. Of these, several genes conferred resistance to more than one class and genes conferring resistance to three or more classes were termed multi-drug resistance (MDR) genes (Figure 3.4A). Further, resistance was also observed for classes not administered in the present cycle of DCT with a very high abundance of MDR genes (Figure 3.4B). Both previous and current antibiotic use may result in the selection of bacteria which carry resistance genes, thus increasing the overall resistome profile diversity of the microbiota.

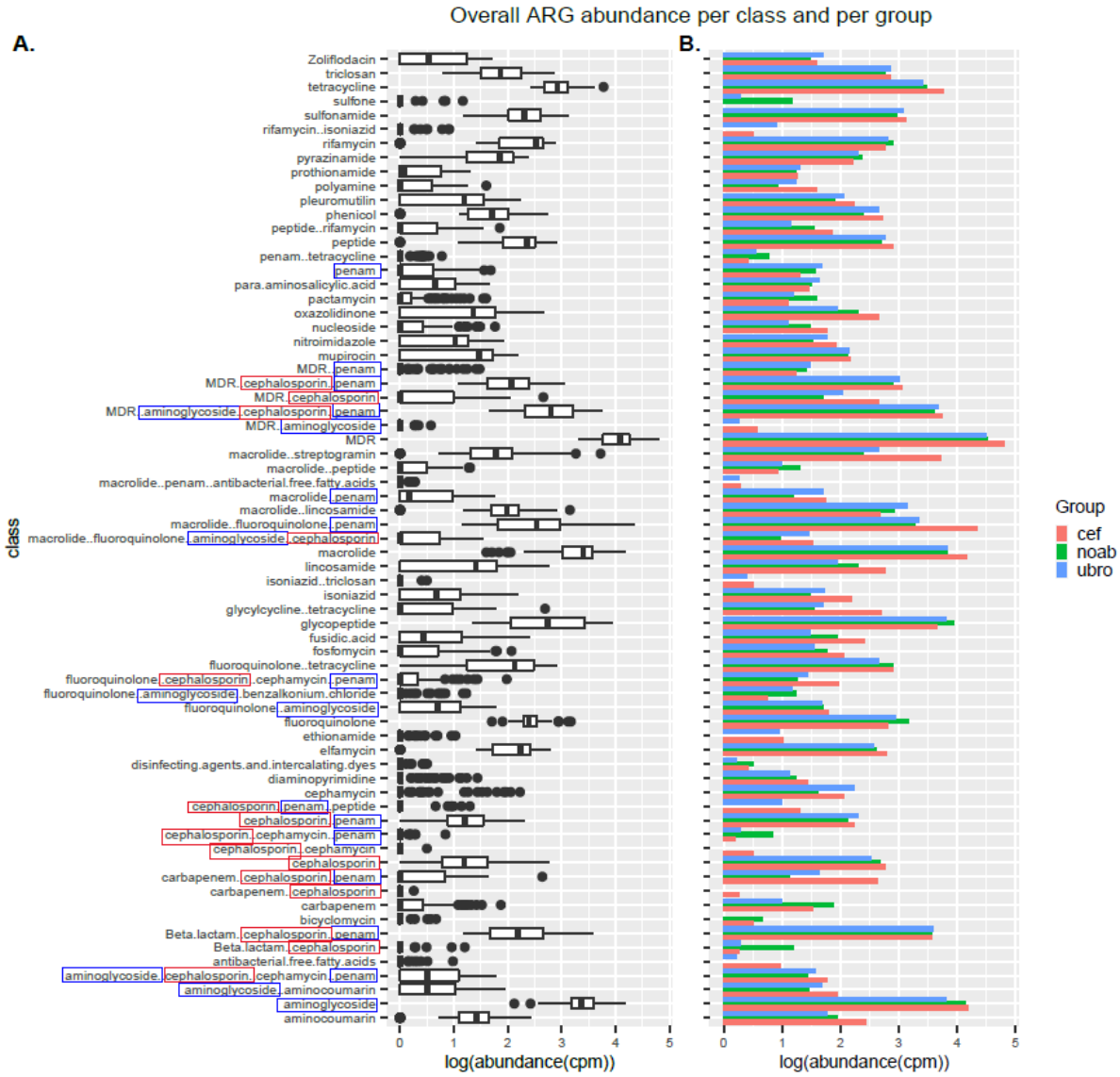


Figure 3.4: A) Boxplot showing log-transformed abundance of the different classes of antibiotics to which resistance was observed in this study. B) Bar plot showing representation of ARGs in all three groups (noab, ubro and cef) over all time-points. The classes highlighted in blue boxes correspond to the class of antibiotics administered to the ubro group, while those in red boxes correspond to the cef group.

It was observed that alpha diversity of ARGs over time was not different in milk samples from all groups (Shannon index), while the cef and noab groups showed significant differences (Chao1 index) pointing toward higher richness in the cef group. No significant differences were observed between groups at all time points or between time points in individual groups (Figure 3.5A). Beta diversity analysis showed discrete clustering of

milk microbiota between the cef and noab groups (p.adj=0.03, pairwise Adonis) (Figure 3.5B). No significant clustering was observed at any individual time points except M6 (p.adj values: noab vs cef: 0.039, noab vs ubro: 0.015, pairwiseAdonis) (Figure 3.5C).

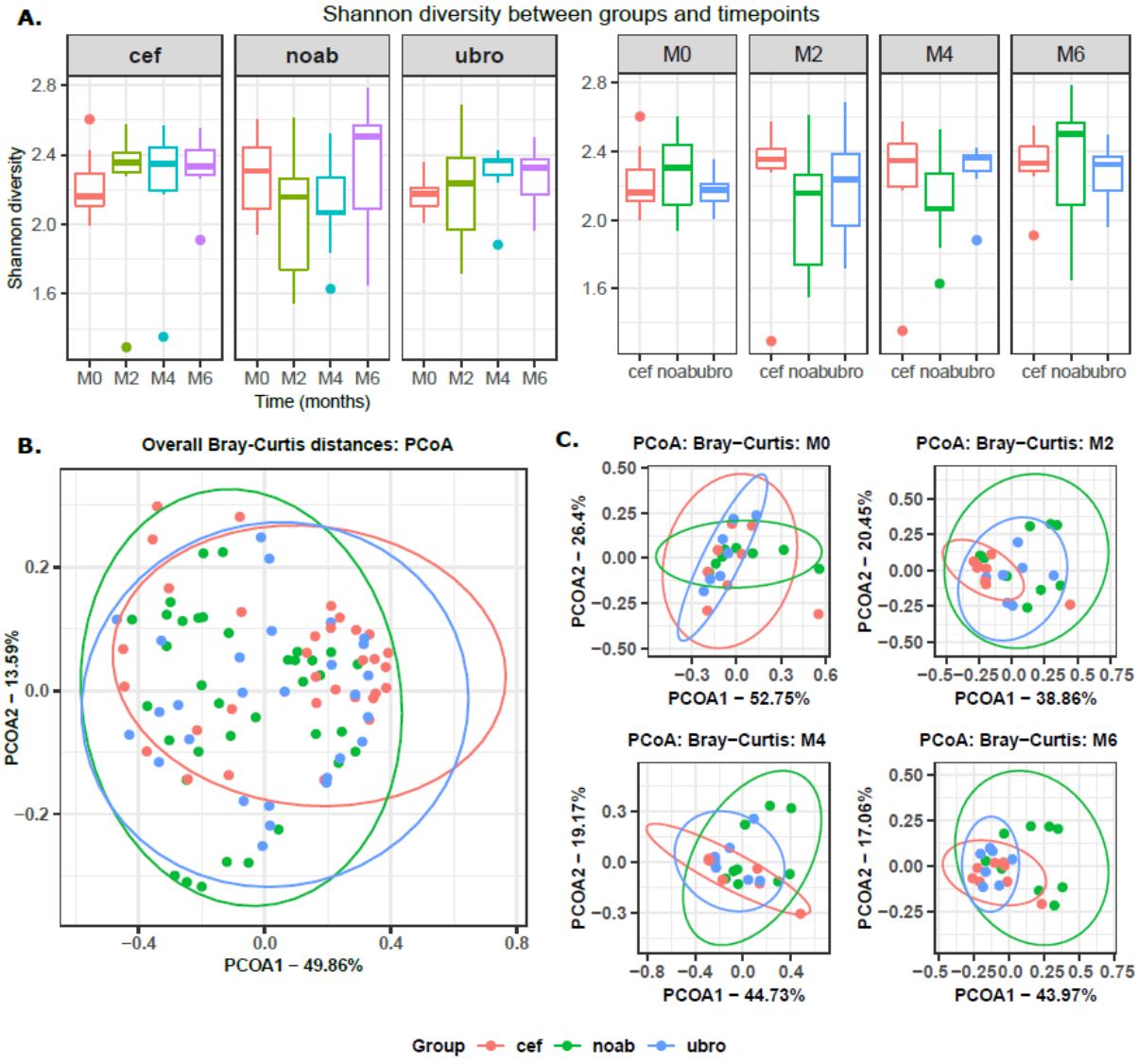


Figure 3.5: A) Alpha diversity with Shannon index shows no difference between groups (noab, ubro and cef) at any time points for ARG abundance. B) Beta diversity of ARGs using PCoA and Bray-Curtis distance. C) Beta diversity of ARGs at each time point using PCoA plot with Bray-Curtis distance.

Cef group showed highest ARG abundance, with both antibiotic-treated groups showing higher ARG abundance compared to noab group (Figure S3.2). The abundance of genes conferring resistance to the drug class cephalosporin at M0 (colostrum) was higher in

abundance in the cef group. Multiple comparisons between groups (noab group as control) were performed for each class and high resistance was found in the antibiotic groups at all time points (Figure 3.6).

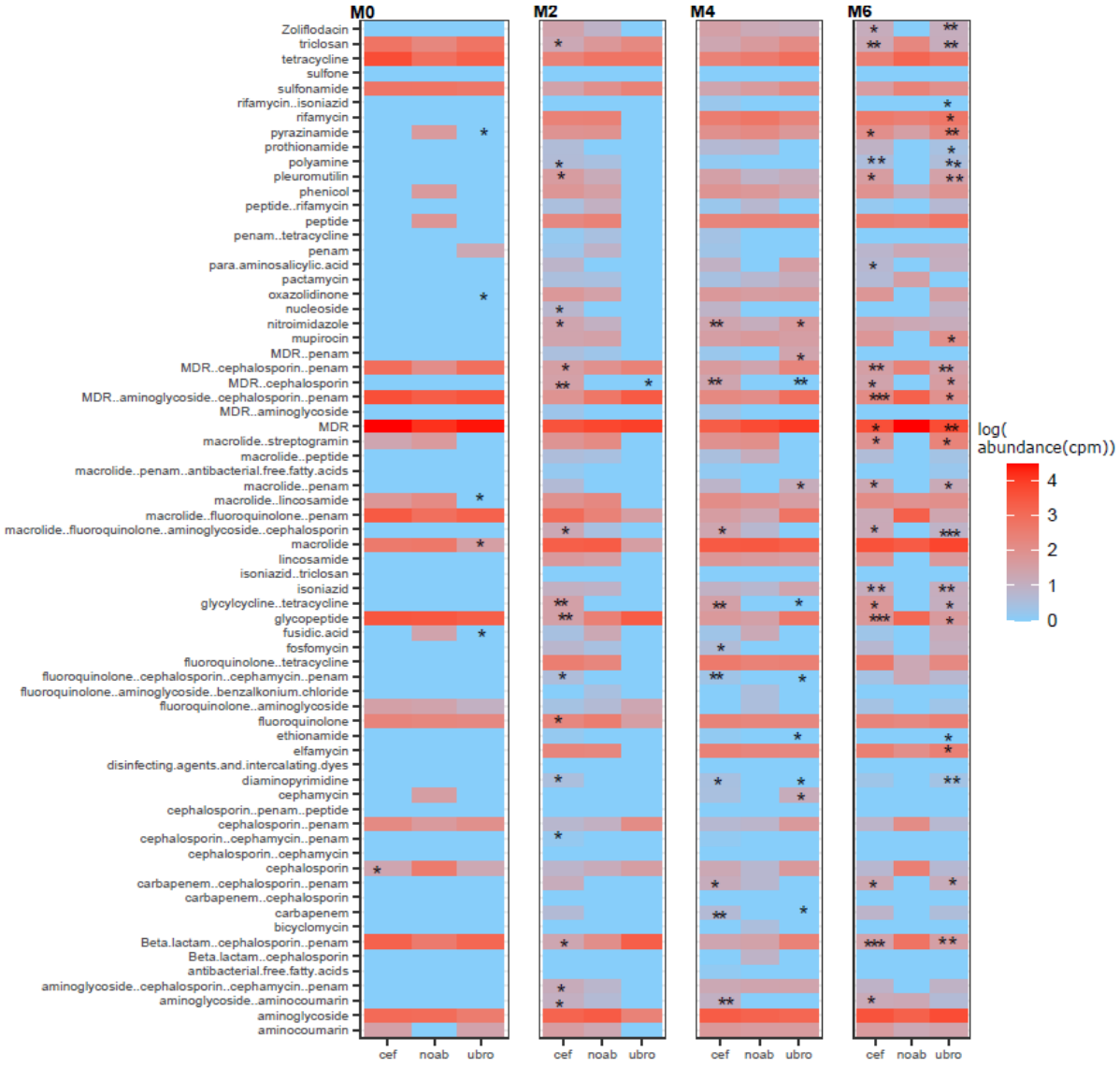


Figure 3.6: Heatmap showing log-transformed abundance (cpm) of various ARG classes per group at each timepoint. Significance was calculated with noab group as reference group against each antibiotic-treated groups using Dunn test for multiple comparisons. P.adj values below 0.05 were considered significant.

3.5 Discussion

In this study, we compared the microbial and resistome profiles of colostrum and milk from healthy cows throughout lactation, separated into three groups treated with differing dry cow therapies – two antibiotic-treated groups and one non-antibiotic treated group using shotgun metagenomics. Studying the bovine milk microbiome is considered challenging as it is a low biomass sample, difficult to collect without contamination, along with the possibility of kit contaminants, with inter-individual variation of microbiota (Porcellato et al., 2020), different farming practices (Metzger et al., 2018) and lack of studies to develop a standardised protocol. Shotgun metagenomics is rarely used to study the resistance pattern of the microbiota in dairy colostrum and milk, however, this approach provides high resolution and allows functional profiling (Jovel et al., 2017). The antibiotics used were cephaguard and Ubro red and are broad-spectrum antibiotics. Their target species include major mastitis-causing organisms such as *Streptococcus* spp. (*uberis, dysgalactiae, agalactiae*), *Staphylococcus aureus*, coagulase-negative staphylococci for cephaguard, while ubro red targets *Staphylococcus* spp., *Streptococcus* spp., *Corynebacterium* spp., *Escherichia* spp., *Klebsiella* and *Pseudomonas* spp.

This study demonstrated significant differences in the diversity of microbial genera within the antibiotic-treated groups over the time points (Shannon alpha diversity) similar to Derakhshani et al. (2018). However, the transition from colostrum to milk in the non-antibiotic group did not demonstrate significant changes in microbial diversity over time. This suggests that low initial microbial diversity in the antibiotic-groups could be due to antibiotic use. Further, diversity between groups was significantly different at M2, M4 and M6 (beta diversity - PCoA), with noab group clustering distinctly compared to antibiotic-treated groups. Additionally, we do not report high prevalence of mastitis-causing pathogens such as *Escherichia* spp, *Streptococcus* spp, *Staphylococcus* spp. in the groups, especially in the noab group. Similar to other studies, (Biscarini et al., 2020; Derakhshani et al., 2018; Bonsaglia et al., 2017; Parker et al., 2008) our data support the use of only teat sealants for drying off reducing antibiotic use.

Acinetobacter was high in antibiotic-treated groups while *Corynebacterium* was higher in the no-antibiotic group. The presence of *Corynebacterium* in the top 10 throughout lactation in the noab group is not unusual as it is thought to be controlled by various anti-sepsis measures (Jeffrey L. Watts 1990, S.C. Nickerson 2002 -

https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/corynebacterium-bovis). Its high prevalence could also be as it is observed to be a dominant bacterium on teat skin (Frétin et al., 2018). Some studies suggest an association between *Corynebacterium* and intramammary infections suggesting its role as a mastitis-causing pathogen though mainly this association is with *Corynebacterium bovis* while most other species are reported to be present in environmental niches (Woudstra et al., 2023). Others suggest that the high abundance of *Corynebacterium* may provide protective effects against infections caused by major mammary gland pathogens such as *Staphylococcus* and *Streptococcus* (Lücken et al., 2021; Watts et al., 2000). To confirm the role of high relative abundance of *Corynebacterium* in the noab group, further studies including a mix of culture and sequencing-based methods are needed. *Actinoalloteichus* was observed to have high relative abundance in all groups in the study. *Actinoalloteichus* was found in milk aliquots in a recent study (Rubiola et al., 2020), but not many other studies have reported its presence. *Actinoalloteichus* is reported to be isolated from marine sponges (Zhang et al., 2006), seashores (Xie et al., 2020) and soil (Boudjelal et al., 2015) and is considered to be a source of secondary metabolites due to the presence of several secondary metabolite biosynthetic gene clusters (Schaffert et al., 2016). The high abundance of this genus in all three groups is surprising, and further studies are needed on Irish farms to study its origins.

Microbial diversity in milk from cows is affected by several factors. For instance, Porcellato et al. (2021) demonstrated changes in microbial diversity longitudinally. The authors observed an increased abundance of *Streptococcus* and *Kocuria* over the summer months. The antibiotic-treated groups in our study show an increasing trend of *Kocuria* over lactation time, and these time points overlap with summer-time in the given geographical area. Similarly, parity of the cow (Lima et al., 2017) and seasonal housing (indoor or outdoor sampling and housing) (Doyle et al., 2017) can impact the milk microbial composition. Stage of lactation, milking hygiene, bedding material, and feeding habits affect cow milk microbial composition and diversity (Derakhshani et al., 2018; Frétin et al., 2018; Metzger et al., 2018; Zhang et al., 2015). For instance, Doyle et al. (2017) observed that *Acinetobacter* and *Pseudomonas* were in lower proportions in milk from indoor-housed animals as compared to outdoor milk samples. An increase in these genera over M2 and M4 was observed in this study, which could be attributed to the fact

that once the cows calve and are put on grass they are outdoors full time (later time points – M4, M6 in our study correspond to outdoor samples). However, *Acinetobacter* and *Pseudomonas* are also psychrotrophic and observed in stored bulk milk samples (McHugh et al., 2020) and are common dairy environment contaminants, thus further studies are needed to investigate their origin in raw quarter milk samples. Another possible explanation as described by Oikonomou et al. (2020), is that the milk microbiota was not alive and DNA from dead bacteria was thus not affected by treatments.

Changes in ARG diversity over time were not observed in any group in this study. Some ARGs present in the noab group, while other ARGs not belonging to classes of antibiotics used could be due to previous antibiotic exposures, or environmental sources such as contaminated manure and water – and horizontal gene transfer within the herd. Antibiotic use must be reduced overall on farms as this can lead to an increase in the numbers and spread of ARGs. Further, a high abundance of ARGs in the antibiotic-treated groups was observed. Certain genes conferring resistance to classes like beta-lactams, penams and cephalosporin and MDR genes were observed. The presence of ARGs (those that confer resistance to the antibiotic in question) in the antibiotic-treated groups can be justified due to the use of antibiotics, while many other resistance genes conferring resistance to drug classes like tetracycline, intercalating dye and disinfecting agents, triclosan, glycopeptide antibiotic could be due to contaminating bacteria from environmental sources such as irrigation (Blaustein et al., 2016) and groundwater (Li et al., 2014; Pan & Chu 2017), slurry waste (Baker et al., 2022), manure, and interaction with other animals in the herd (Pitta et al., 2020; Sawant et al., 2007). These ARGs detected could also be a result of selection of MDR bacteria post-antibiotic use. Certain resistance could be due to previous antibiotic use and might be horizontally transferred between bacteria over time. The antibiotics used by farms in this study were Ubro red (active ingredient Framycetin sulfate, Penethamate hydroiodide and Procaine Penicillin) and Cephaguard (cefquinome – 4th generation cephalosporin). The European Medicine Agency (EMA) classifies cefquinome as a category B (restricted use) antibiotic, while Framycetin is to be used with caution (https://www.gov.ie/en/collection/45f45-antimicrobial-resistance-amr/?referrer=http://www.agriculture.gov.ie/amr/). Thus, this study lends further support to legislation that recommends reducing the use of antimicrobials in dairy cows as well

as their cautionary use in other farm practices, to reduce the development and spread of antibiotic-resistant bacteria.

The method used for DCT can impact the microbial composition and resistome profile and studies have reported a relationship between milk production in the previous lactation and the development of infection in the next lactation period. Pinedo et al. (2011, 2012) observed that compared to high milk-producing cows, lower milk production in the previous lactation poses a higher risk of mastitis after calving (Pinedo et al., 2011, 2012). Dias and Allaire (1982) reported that age, calving intervals and prior milk yield data can be used to maximise milk yield in the next lactation, with younger cows and high milk-producing cows needing a longer dry period. Further, studies have reported that the length of DCT needs to be critically assessed as it can affect the occurrence of new infections (Berry and Hillerton 2007; Natzke et al., 1975). Such observations can be used for selective DCT enabling the development of strategies for prudent use of antibiotics (Cameron et al., 2015; Scherpenzeel et al., 2014). Use of boviseal – an inert teat sealant is an alternative to antibiotic use (Crispie et al., 2004; Bonsaglia et al., 2017) containing bismuth subnitrate, colloidal anhydrous silica as the main ingredients. A recent study revealed that for reasons yet unknown bismuth subnitrate has an inhibitory effect on bacterial growth and further studies are needed to completely understand the inhibitory effect of bismuth subnitrate, as bismuth is a heavy metal compound and certain effects might be due to the antimicrobial nature of some heavy metals (Notcovich et al., 2020). Another alternative to antibiotics is the process of teat dipping using a disinfectant; thus protecting from new infections during the dry period. Some of these approaches can be used in combination with good hygienic practices, clean bedding areas and field conditions to protect cows from new infections. Nutritional management and development of new vaccines and probiotics will also enable farmers to keep herds healthy and boost their immunity (Pellegrino et al., 2017). These techniques along with maximising the awareness of farmers will help in reducing the use of antibiotics and will support the one health initiative.

The healthcare costs of treating patients with AMR cost the Health Service Executive an additional €12 million in 2019 (https://www.hiqa.ie/sites/default/files/2021-07/Report-of-Economic-Burden-of-AMR.pdf) with more funds being allocated by the government to tackle AMR issues in the health service. Prophylactic use of antibiotics on dairy farms

is an important contributor to AMR spread in the environment. Thus, along with EU legislation to cease blanket DCT, other national laws to reduce antibiotic use in other livestock and dairy activities might help lower the development and spread of ARGs. This is of importance as AMR development on dairy farms is a One-health problem, with possibilities of ARG transfer to humans through the food chain.

The limitations of this study include the relatively small number of animals included per group, although we would emphasise that these animals were all followed longitudinally for six months of lactation. We suggest that this study demonstrates the value of doing temporal microbial and resistome studies on bovine colostrum and milk microbiota to examine the effect of DCT and antibiotic use. More studies with larger cohort sizes and shorter between-sample intervals are needed to understand the longitudinal effect of antibiotics on bovine colostrum and milk samples. This will help reduce antibiotic use in livestock and decrease the generation and transfer of ARGs.

3.6 Conclusion

Antibiotic treatment during DCT was associated with high abundances of ARGs in milk compared to non-antibiotic control group, and altered microbial composition throughout lactation. Using teat sealant without antibiotics did not lead to the presence of high abundance of mastitis-causing pathogens. Furthermore, the microbial diversity (Alpha diversity) in antibiotic-treated groups increased over time, which was not observed in the non-antibiotic group and suggests the possible microbiota-suppressing effect of antibiotic use during DCT on bovine colostrum and milk. The presence of ARGs at high abundance in cow colostrum and milk is alarming because of the threat of passage of ARGs to the calf and to humans through the food chain. It is challenging to trace the source of bacteria and thus the resistance genes in question, thus preventive and protective laws to reduce the use of antibiotics on farms and associated resources is crucial.

3.7 Acknowledgements

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3.8 Ethics statement

None required/ Not applicable

3.9 Conflict of interest

None declared

3.10 References

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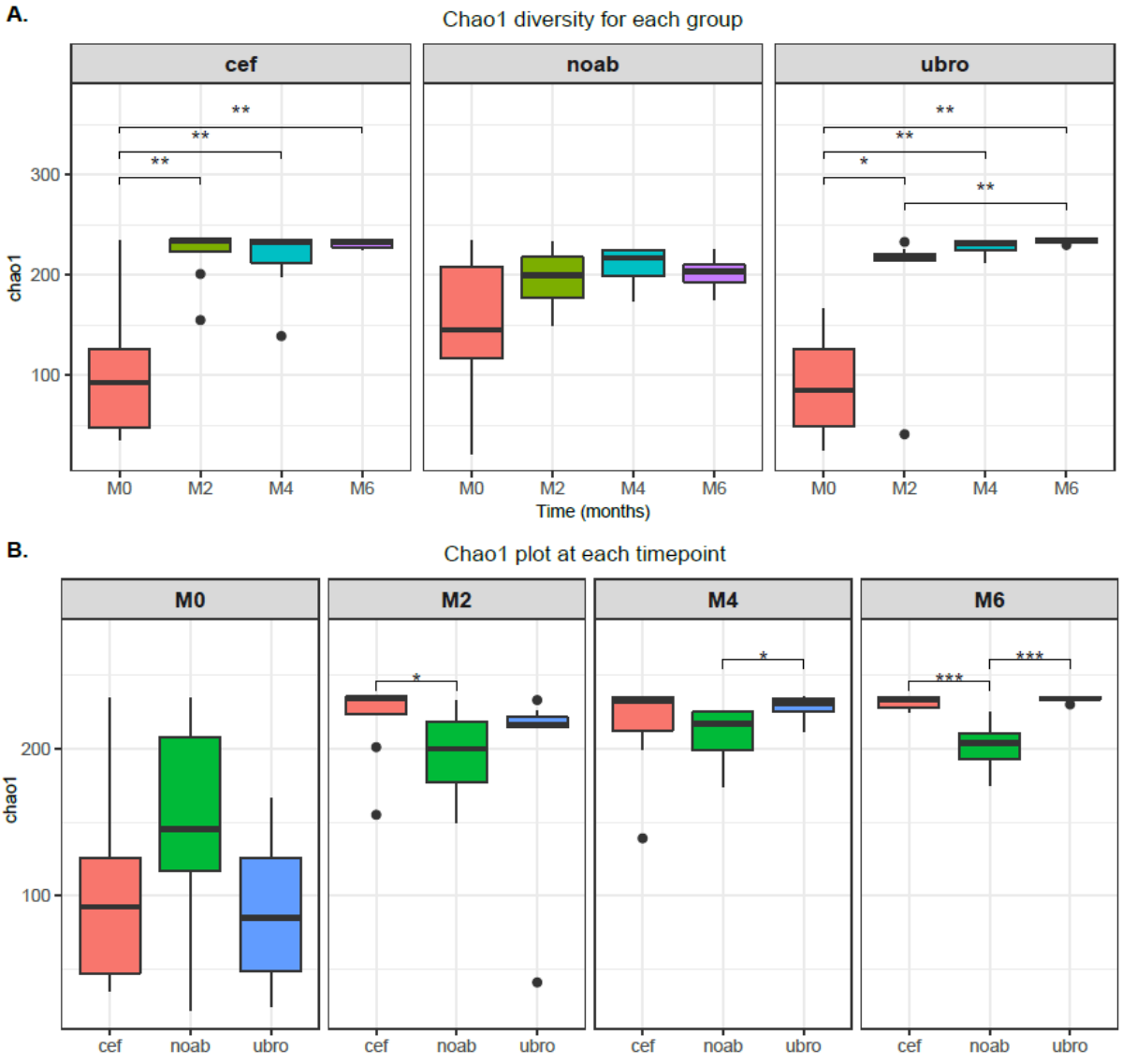
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3.11 Supplementary material

Supplementary figures:

Figure S3.1:



Supplementary figure S3.1: Alpha diversity as calculated by Chao1 index A) Between groups and B) Between time points.

Figure S3.2:

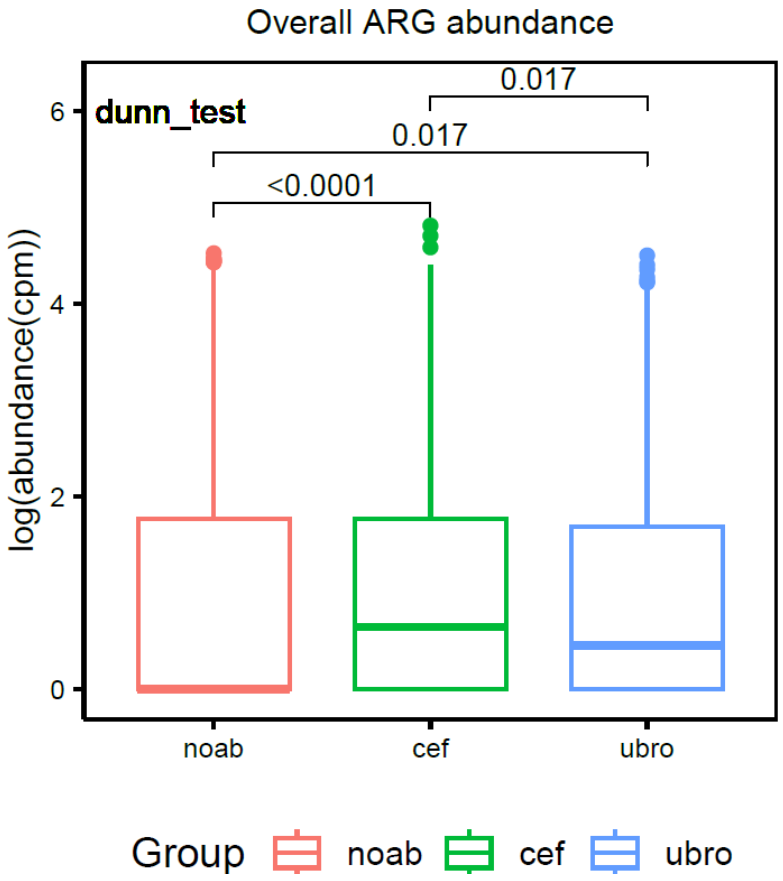


Figure S3.2: Boxplot showing overall ARG abundance in all three groups. P.adj values were calculated using Dunn test in R, and p.adj values below 0.05 were considered significant.

Supplementary tables:

Table S3.1: Metadata file showing details of all cows included in this study.

SampleID	Group	Age	Timepoint	Parity	Parous
D16T1	cef	4	T1	2	Multiparous
L10C	ubro	5	T0	3	Multiparous
T8T3	ubro	5	T3	3	Multiparous
P10T2	cef	3	T2	1	Primiparous
T17T3	ubro	3	T3	1	Primiparous
T17C	ubro	3	T0	1	Primiparous
C16T1	noab	2	T1	0	Nulliparous
C16C	noab	2	T0	0	Nulliparous
D13C	cef	3	T0	1	Primiparous

C12T1	noab	3	T1	1	Primiparous
D16T3	cef	4	T3	2	Multiparous
C19C	noab	2	T0	0	Nulliparous
L6T1	ubro	3	T1	1	Primiparous
P10C	cef	3	T0	1	Primiparous
L11C	ubro	5	T0	3	Multiparous
L5C	ubro	3	T0	1	Primiparous
C12T3	noab	3	T3	1	Primiparous
C16T3	noab	2	T3	0	Nulliparous
C1T2	noab	3	T2	1	Primiparous
L6T2	ubro	3	T2	1	Primiparous
C16T2	noab	2	T2	0	Nulliparous
C3T1	noab	3	T1	1	Primiparous
P9T1	cef	3	T1	1	Primiparous
L10T1	ubro	5	T1	3	Multiparous
L6T3	ubro	3	T3	1	Primiparous
C5T1	noab	2	T1	0	Nulliparous
C5T3	noab	2	T3	0	Nulliparous
P9C	cef	3	T0	1	Primiparous
D17T3	cef	4	T3	2	Multiparous
P10T3	cef	3	T3	1	Primiparous
C4T3	noab	2	T3	0	Nulliparous
L13T2	ubro	3	T2	1	Primiparous
P20T1	cef	3	T1	1	Primiparous
L5T1	ubro	3	T1	1	Primiparous
D17C	cef	4	T0	2	Multiparous
L11T1	ubro	5	T1	3	Multiparous
C2T1	noab	2	T1	0	Nulliparous
C12C	noab	3	T0	1	Primiparous
C7T3	noab	3	T3	1	Primiparous
L11T3	ubro	5	T3	3	Multiparous
T8T1	ubro	5	T1	3	Multiparous

D16T2	cef	4	T2	2	Multiparous
L11T2	ubro	5	T2	3	Multiparous
D17T1	cef	4	T1	2	Multiparous
D9C	cef	3	T0	1	Primiparous
P20C	cef	3	T0	1	Primiparous
P10T1	cef	3	T1	1	Primiparous
C19T1	noab	2	T1	0	Nulliparous
C3C	noab	3	T0	1	Primiparous
C1T3	noab	3	T3	1	Primiparous
L13T1	ubro	3	T1	1	Primiparous
C7T1	noab	3	T1	1	Primiparous
C5T2	noab	2	T2	0	Nulliparous
C2C	noab	2	T0	0	Nulliparous
C1T1	noab	3	T1	1	Primiparous
T17T2	ubro	3	T2	1	Primiparous
L13C	ubro	3	T0	1	Primiparous
D17T2	cef	4	T2	2	Multiparous
L6C	ubro	3	T0	1	Primiparous
L10T3	ubro	5	T3	3	Multiparous
D13T3	cef	3	T3	1	Primiparous
D13T1	cef	3	T1	1	Primiparous
C3T2	noab	3	T2	1	Primiparous
C19T2	noab	2	T2	0	Nulliparous
P19C	cef	3	T0	1	Primiparous
P19T3	cef	3	T3	1	Primiparous
C1C	noab	3	T0	1	Primiparous
L13T3	ubro	3	T3	1	Primiparous
P9T3	cef	3	T3	1	Primiparous
L10T2	ubro	5	T2	3	Multiparous
P9T2	cef	3	T2	1	Primiparous
C7T2	noab	3	T2	1	Primiparous
D16C	cef	4	T0	2	Multiparous

C4T1	noab	2	T1	0	Nulliparous
T8C	ubro	5	T0	3	Multiparous
P20T2	cef	3	T2	1	Primiparous
C4C	noab	2	T0	0	Nulliparous
P19T1	cef	3	T1	1	Primiparous
C7C	noab	3	T0	1	Primiparous
D9T2	cef	3	T2	1	Primiparous
T8T2	ubro	5	T2	3	Multiparous
C5C	noab	2	T0	0	Nulliparous
L5T3	ubro	3	T3	1	Primiparous
L5T2	ubro	3	T2	1	Primiparous
C2T3	noab	2	T3	0	Nulliparous
T17T1	ubro	3	T1	1	Primiparous
P20T3	cef	3	T3	1	Primiparous
C19T3	noab	2	T3	0	Nulliparous
C12T2	noab	3	T2	1	Primiparous
D9T1	cef	3	T1	1	Primiparous
C4T2	noab	2	T2	0	Nulliparous
D13T2	cef	3	T2	1	Primiparous
D9T3	cef	3	T3	1	Primiparous
C3T3	noab	3	T3	1	Primiparous
C2T2	noab	2	T2	0	Nulliparous
P19T2	cef	3	T2	1	Primiparous

Table S3.2: Table with coefficients obtained from differential abundance analysis using Songbird tool with formula C(Group, Treatment('noab')).

featureid	Intercept	C(Group2, Treatment('noab'))[T.cef]	C(Group2, Treatment('noab'))[T.ubro]

k__Bacteria|p__Proteobacteria|c__Gammaproteobacteria|o__Pseudomonadales|f__Moraxellaceae|g__*Psychrobacter*	-0.48714	-0.49549	-0.49071
k__Bacteria|p__Proteobacteria|c__Gammaproteobacteria|o__Pseudomonadales|f__Moraxellaceae|g__*Acinetobacter*	0.104539	0.021822	0.20377
k__Bacteria|p__Proteobacteria|c__Gammaproteobacteria|o__Pseudomonadales|f__Moraxellaceae|g__*Moraxella*	-0.18873	-0.06052	-0.03973
k__Bacteria|p__Proteobacteria|c__Gammaproteobacteria|o__Pseudomonadales|f__Pseudomonadaceae|g__*Pseudomonas*	0.822938	0.23601	0.118122
k__Bacteria|p__Proteobacteria|c__Gammaproteobacteria|o__Enterobacterales|f__Enterobacteriaceae|g__	-0.22715	-0.06166	-0.05006
k__Bacteria|p__Proteobacteria|c__Gammaproteobacteria|o__Enterobacterales|f__Enterobacteriaceae|g__*Escherichia*	-0.21432	-0.06126	-0.04628
k__Bacteria|p__Proteobacteria|c__Gammaproteobacteria|o__Enterobacterales|f__Yersiniaceae|g__*Serratia*	-0.2413	-0.06436	-0.05548
k__Bacteria|p__Proteobacteria|c__Gammaproteobacteria|o__Enterobacterales|f__Erwiniaceae|g__*Buchnera*	-0.21317	-0.06081	-0.04642
k__Bacteria|p__Proteobacteria|c__Gammaproteobacteria|o__Enterobacterales|f__Pectobacteriaceae|g__*Sodalis*	-0.21525	-0.05879	-0.04835
k__Bacteria|p__Proteobacteria|c__Gammaproteobacteria|o__Xanthomonadales|f__Xanthomonadaceae|g__	-0.20194	-0.05346	-0.04568

k__Bacteria|p__Proteobacteria|c__Gammaproteobacteria|o__Xanthomonadales|f__Xanthomonadaceae|g__*Stenotrophomonas*	0.340684	0.166177	0.065002
k__Bacteria|p__Proteobacteria|c__Gammaproteobacteria|o__Xanthomonadales|f__Xanthomonadaceae|g__*Lysobacter*	0.259635	0.25292	-0.01833
k__Bacteria|p__Proteobacteria|c__Gammaproteobacteria|o__Methylococcales|f__Methylococcaceae|g__*Methylovulum*	-0.21223	-0.0552	-0.03209
k__Bacteria|p__Proteobacteria|c__Alphaproteobacteria|o__Rhodobacterales|f__Rhodobacteraceae|g__	-0.19415	-0.06025	-0.02359
k__Bacteria|p__Proteobacteria|c__Alphaproteobacteria|o__Rhodobacterales|f__Rhodobacteraceae|g__*Paracoccus*	-0.11266	-0.05799	0.06665
k__Bacteria|p__Proteobacteria|c__Alphaproteobacteria|o__Rhodobacterales|f__Rhodobacteraceae|g__*Rhodobacter*	-0.21346	-0.06027	-0.03847
k__Bacteria|p__Proteobacteria|c__Alphaproteobacteria|o__Rhizobiales|f__|g__	-0.15449	-0.02776	-0.02639
k__Bacteria|p__Proteobacteria|c__Alphaproteobacteria|o__Rhizobiales|f__Phyllobacteriaceae|g__*Mesorhizobium*	-0.19888	-0.04567	-0.04165
k__Bacteria|p__Proteobacteria|c__Alphaproteobacteria|o__Rhizobiales|f__Phyllobacteriaceae|g__*Aminobacter*	-0.20662	-0.04857	-0.04445
k__Bacteria|p__Proteobacteria|c__Alphaproteobacteria|o__Rhizobiales|f__Phyllobacteriaceae|g__*Phyllobacterium*	-0.21616	-0.04196	-0.05048
k__Bacteria|p__Proteobacteria|c__Alphaproteobacteria|o__Rhizobiales|f__Bradyrhizobiaceae|g__*Rhodopseudomonas*	-0.2002	-0.04949	-0.04099

k__Bacteria|p__Proteobacteria|c__Alphaproteobacteria|o__Rhizobiales|f__Brucellaceae|g__*Ochrobactrum*	-0.18801	-0.0127	-0.05095
k__Bacteria|p__Proteobacteria|c__Alphaproteobacteria|o__Caulobacterales|f__Caulobacteraceae|g__*Brevundimonas*	-0.21691	-0.05234	-0.04544
k__Bacteria|p__Proteobacteria|c__Betaproteobacteria|o__Burkholderiales|f__|g__	-0.17826	-0.03499	-0.0341
k__Bacteria|p__Proteobacteria|c__Betaproteobacteria|o__Burkholderiales|f__Comamonadaceae|g__	-0.16555	-0.01551	-0.03083
k__Bacteria|p__Proteobacteria|c__Betaproteobacteria|o__Burkholderiales|f__Comamonadaceae|g__*Comamonas*	-0.20775	-0.04027	-0.04723
k__Bacteria|p__Proteobacteria|c__Betaproteobacteria|o__Burkholderiales|f__Comamonadaceae|g__*Variovorax*	-0.1943	-0.05054	-0.03201
k__Bacteria|p__Proteobacteria|c__Betaproteobacteria|o__Burkholderiales|f__Comamonadaceae|g__*Delftia*	-0.01807	0.130668	-0.03337
k__Bacteria|p__Proteobacteria|c__Betaproteobacteria|o__Burkholderiales|f__Comamonadaceae|g__*Ottowia*	0.187391	0.303989	0.04468
k__Bacteria|p__Proteobacteria|c__Betaproteobacteria|o__Burkholderiales|f__Alcaligenaceae|g__*Alcaligenes*	-0.09802	0.096201	-0.06153
k__Bacteria|p__Proteobacteria|c__Betaproteobacteria|o__Burkholderiales|f__Burkholderiaceae|g__*Polynucleobacter*	0.202905	0.07999	0.046725
k__Bacteria|p__Proteobacteria|c__Betaproteobacteria|o__Burkholderiales|f__Oxalobacteraceae|g__*Janthinobacterium*	-0.20971	-0.03783	-0.05259

k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__|g__	-0.02247	-0.01838	0.018418
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Micrococcaceae|g__	-0.13686	-0.04669	-0.02187
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Micrococcaceae|g__*Arthrobacter*	0.206154	-0.02504	0.030991
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Micrococcaceae|g__*Glutamicibacter*	-0.14841	-0.05916	-0.02827
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Micrococcaceae|g__*Kocuria*	0.727719	0.393234	0.473597
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Micrococcaceae|g__*Rothia*	-0.21063	-0.06299	-0.04428
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Micrococcaceae|g__*Micrococcus*	-0.13219	-0.05269	0.026983
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Micrococcaceae|g__*Sinomonas*	-0.2013	-0.05532	-0.03988
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Microbacteriaceae|g__	-0.16104	-0.04075	-0.02757
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Microbacteriaceae|g__*Microbacterium*	0.878039	0.503077	0.209511
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Microbacteriaceae|g__*Agromyces*	-0.20839	-0.05587	-0.04294
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Microbacteriaceae|g__*Clavibacter*	-0.20502	-0.05873	-0.04458

k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Microbacteriaceae|g__*Leifsonia*	-0.19225	-0.05052	-0.03915
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Microbacteriaceae|g__*Microterricola*	-0.21058	-0.05393	-0.04648
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Microbacteriaceae|g__*Frondihabitans*	-0.21036	-0.05969	-0.04549
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Dermabacteraceae|g__*Brachybacterium*	1.100255	-0.10812	-0.01632
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Intrasporangiaceae|g__*Janibacter*	-0.1361	-0.05692	0.026611
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Intrasporangiaceae|g__*Intrasporangium*	-0.18586	-0.05871	-0.03145
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Intrasporangiaceae|g__*Serinicoccus*	-0.18811	-0.05803	-0.02364
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Intrasporangiaceae|g__*Arsenicicoccus*	-0.18527	-0.05417	-0.02229
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Brevibacteriaceae|g__*Brevibacterium*	1.510723	-0.26926	-0.25562
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Dermacoccaceae|g__*Luteipulveratus*	-0.2025	-0.05754	-0.03512

k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Dermacoccaceae|g__*Kytococcus*	-0.20443	-0.05523	-0.03523
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Dermacoccaceae|g__*Dermacoccus*	-0.19912	-0.05235	-0.03468
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Beutenbergiaceae|g__*Beutenbergia*	-0.21159	-0.0552	-0.04207
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Beutenbergiaceae|g__*Miniimonas*	-0.19863	-0.05425	-0.0283
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micrococcales|f__Sanguibacteraceae|g__*Sanguibacter*	-0.20739	-0.0561	-0.0427
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Corynebacteriales|f__|g__	-0.10821	-0.01763	-0.00379
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Corynebacteriales|f__Corynebacteriaceae|g__*Corynebacterium*	1.661905	-0.00768	0.438415
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Corynebacteriales|f__Nocardiaceae|g__*Rhodococcus*	1.499408	0.988738	0.605951
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Corynebacteriales|f__Dietziaceae|g__*Dietzia*	-0.11913	-0.04926	0.010988
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Corynebacteriales|f__Mycobacteriaceae|g__	-0.1052	0.019298	-0.01502
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Corynebacteriales|f__Mycobacteriaceae|g__*Mycobacterium*	-0.07432	0.0247	-0.00822

k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Corynebacteriales|f__Mycobacteriaceae|g__*Mycobacteroides*	0.210905	0.297149	0.065613
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Corynebacteriales|f__Tsukamurellaceae|g__*Tsukamurella*	-0.17258	-0.01866	-0.04098
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Streptomycetales|f__Streptomycetaceae|g__*Streptomyces*	0.04393	0.025371	0.039756
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Pseudonocardiales|f__Pseudonocardiaceae|g__*Actinoalloteichus*	3.228573	0.130644	0.527985
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Pseudonocardiales|f__Pseudonocardiaceae|g__*Pseudonocardia*	-0.20553	-0.05877	-0.03884
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Propionibacteriales|f__Nocardioidaceae|g__*Nocardioides*	-0.08152	-0.03465	0.019309
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Propionibacteriales|f__Nocardioidaceae|g__*Aeromicrobium*	-0.20082	-0.05963	-0.04363
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Propionibacteriales|f__Nocardioidaceae|g__*Pimelobacter*	-0.18516	-0.05011	-0.03011
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Propionibacteriales|f__Nocardioidaceae|g__*Kribbella*	-0.21425	-0.05734	-0.04205
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Propionibacteriales|f__Nocardioidaceae|g__*Micropruina*	-0.1825	-0.04948	-0.02635

k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Propionibacteriales|f__Propionibacteriaceae|g__	-0.17764	-0.02195	-0.03498
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Propionibacteriales|f__Propionibacteriaceae|g__*Tessaracoccus*	-0.05011	-0.00429	0.034666
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Propionibacteriales|f__Propionibacteriaceae|g__*Acidipropionibacterium*	0.096559	0.175055	0.057006
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Propionibacteriales|f__Propionibacteriaceae|g__*Cutibacterium*	-0.15491	-0.03241	-0.03512
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Propionibacteriales|f__Propionibacteriaceae|g__*Propionibacterium*	-0.0024	0.079033	0.044894
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Propionibacteriales|f__Propionibacteriaceae|g__*Microlunatus*	-0.18241	-0.05756	-0.01455
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Micromonosporales|f__Micromonosporaceae|g__*Plantactinospora*	0.278867	0.040809	0.097554
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Bifidobacteriales|f__Bifidobacteriaceae|g__*Bifidobacterium*	0.103772	0.083442	0.033794
k__Bacteria|p__Actinobacteria|c__Actinobacteria|o__Nakamurellales|f__Nakamurellaceae|g__*Nakamurella*	-0.19973	-0.04952	-0.03668
k__Bacteria|p__Actinobacteria|c__Coriobacteriia|o__Eggerthellales|f__Eggerthellaceae|g__*Gordonibacter*	-0.0631	-0.07634	-0.06444

k__Bacteria|p__Actinobacteria|c__Thermoleophilia|o__Solirubrobacterales|f__Conexibacteraceae|g__*Conexibacter*	-0.1998	-0.03787	-0.04831
k__Bacteria|p__Firmicutes|c__Bacilli|o__Lactobacillales|f__|g__	-0.20243	-0.04009	-0.04365
k__Bacteria|p__Firmicutes|c__Bacilli|o__Lactobacillales|f__Streptococcaceae|g__*Streptococcus*	-0.17518	-0.00236	-0.04769
k__Bacteria|p__Firmicutes|c__Bacilli|o__Lactobacillales|f__Streptococcaceae|g__*Lactococcus*	-0.06575	0.08604	-0.02159
k__Bacteria|p__Firmicutes|c__Bacilli|o__Lactobacillales|f__Lactobacillaceae|g__	-0.22052	-0.04908	-0.04804
k__Bacteria|p__Firmicutes|c__Bacilli|o__Lactobacillales|f__Lactobacillaceae|g__*Lactobacillus*	0.155053	0.21733	0.090622
k__Bacteria|p__Firmicutes|c__Bacilli|o__Lactobacillales|f__Enterococcaceae|g__*Enterococcus*	-0.20221	-0.04571	-0.03201
k__Bacteria|p__Firmicutes|c__Bacilli|o__Lactobacillales|f__Leuconostocaceae|g__*Leuconostoc*	-0.17699	-0.02222	-0.03086
k__Bacteria|p__Firmicutes|c__Bacilli|o__Bacillales|f__Staphylococcaceae|g__*Staphylococcus*	0.241502	-0.05603	-0.02029
k__Bacteria|p__Firmicutes|c__Clostridia|o__Clostridiales|f__Clostridiaceae|g__*Clostridium*	-0.14928	-0.0416	-0.03513
k__Bacteria|p__Firmicutes|c__Negativicutes|o__Veillonellales|f__Veillonellaceae|g__*Negativicoccus*	0.166914	0.013254	0.064588
k__Bacteria|p__Deinococcus-Thermus|c__Deinococci|o__Deinococcales|f__Deinococcaceae|g__*Deinococcus*	-0.21875	-0.06637	-0.02945
k__Bacteria|p__Tenericutes|c__Mollicutes|o__Mycoplasmatales|f__Mycoplasmataceae|g__*Mycoplasma*	-0.20723	-0.05714	-0.04786

k__Bacteria|p__Bacteroidetes|c__Sphingobacteriia|o__Sphingobacteriales|f__Sphingobacteriaceae|g__*Sphingobacterium*	-0.22744	-0.06315	-0.05452
k__Bacteria|p__Bacteroidetes|c__Flavobacteriia|o__Flavobacteriales|f__Flavobacteriaceae|g__*Chryseobacterium*	-0.16978	0.000731	-0.0474
k__Bacteria|p__Verrucomicrobia|c__|o__|f__|g__	-0.20056	-0.05548	-0.04549

Chapter 4

4 Early life antibiotic exposure has an immediate and persistent effect on infant gut microbiome and resistome

4.1 Abstract

Human gut microbiota colonisation begins in infancy and coevolves with the host, with modulation by early life factors such as mode of delivery, feeding habit and antibiotic exposure influencing its development. Infant gut microbiota is highly malleable, but the long-term longitudinal impact of antibiotic uptake in early life, together with mode of delivery on infant gut microbiota and resistome, is not extensively studied. Two hundred and eight samples from 45 infants collected from birth until two years of age over five time points (week 1, 4, 8, 24, year 2) were analysed. Based on shotgun metagenomics, the gut microbial composition and resistome profile was compared in early life of infants divided into three groups: vaginal delivery/no-antibiotic in first four days of life (VDnoab); C-Section/no-antibiotic in first four days of life (CSnoab); C-Section/antibiotic exposed in first four days of life (CSab). Gentamycin and benzylpenicillin were the most commonly administered antibiotics during the first week of life in this cohort.

Newborn gut microbial composition differed in all three groups, with higher diversity and stable composition seen at two years of age, compared to week 1. An increase in microbial diversity from week 1 to week 4 only in the CSab group reflects the effect of antibiotic use, with a gradual increase thereafter. Overall relative abundance of Actinobacteria and *Bacteroides* was significantly higher in VDnoab while Proteobacteria was significantly higher in CSab. Strains from species belonging to *Bifidobacterium* and Bacteroidetes were generally persisters, with *Bifidobacterium breve* and *B. bifidum* species being the major persisters in all three groups. *Bacteroides* persistence was dominant in the VDnoab group, with species *B. ovatus* and *Phocaeicola vulgatus* found to be persisters in the no-antibiotic groups. Most strains carrying antibiotic-resistance genes belonged to phyla Proteobacteria and Firmicutes, with the CSab group presenting higher frequency of ARGs. Given the high abundance of ARGs in the three groups, subsequent longitudinal studies determining the effect of antibiotic alternatives in early life are needed to examine their influence on the long-term presence of ARGs and the functionality of the microbiota.

Keywords: Infant gut, gut microbiota, shotgun metagenomics, resistome profile, strain persistence

4.2 Introduction

Early life is a crucial window for development of the neonate as the foundations for later life are laid. The timing and source of colonisation of the microbiota play an indispensable role in developing optimal health. These initial colonisers perform important functions such as the breakdown of complex compounds, e.g. the role of bifidobacteria in the breakdown of human milk oligosaccharides (HMOs) to harvest energy and synthesise vitamins and metabolites such as vitamins B, and K and short-chain fatty acids (SCFAs) (Arrieta et al., 2014). They protect against pathogen colonisation - a phenomenon referred to as colonisation resistance (Pickard et al., 2017) and they are involved in neural development and immune programming as the neurocognitive and immunity maturation occur during this period (Dinan and Cryan, 2017, Walker, 2013, Zheng et al., 2020).

Infants have a simple gut microbial structure, comprised of fewer bacterial species than adults, which stabilises by three-four years of age towards adult life. This early-life gut microbiota is influenced by several factors that include but are not limited to gestational age (Cong et al., 2016), mode of delivery (vaginal vs C-section) (Dominguez-Bello et al., 2010), feeding habits (breastfeeding vs formula feeding) (Parnanen et al., 2022) and antibiotic uptake, both by the infant and the mother (Arboleya et al., 2022; Patangia et al., 2022a; Tapiainen et al., 2019; Parnanen et al., 2018).

The infant gut is considered an antibiotic-resistance gene (ARG) reservoir (Li et al., 2021; Klassert et al., 2020). This high ARG abundance is associated with Gammaproteobacteria, owing to their intrinsic resistance and high colonisation levels in early life (Oliveira & Reygaert, 2022, Gibson et al., 2016, Gosalbes et al., 2016, Backhed et al., 2015, Moore et al., 2013). C-section (CS) born infants are colonised by a high abundance of opportunistic pathogens belonging to taxa such as *Escherichia coli*, *Klebsiella*, and *Enterobacter*, often resulting in higher antibiotic use and ARG carriage in these infants (Reyman et al., 2019; Shao et al., 2019). Antibiotic resistance is a global health concern (WHO, AMR report, 2014). Septic infections caused by antibiotic-resistant bacteria are responsible for 214,000 neonatal deaths each year, of which multidrug-resistant (MDR) bacteria cause 30% (Laxminarayan et al., 2016). The immediate effect after antibiotic treatment is an overall decrease in gut microbiota diversity, including commensals, benefiting the colonisation of antimicrobial-resistant (AMR) strains due to selective pressure. Increasing evidence has demonstrated the

association between antibiotic use and altered microbiota in early life, the rise in ARGs (Tapiainen et al., 2019, Fjalstad et al., 2017), and its linkage to the development of disease conditions in later life (Aversa et al., 2021).

CS and antibiotic use are common medical practices (Betrán et al., 2016; Liu et al., 2016), but it disturbs the natural microbial colonisation process, impacting long-term health of the host (Patangia et al., 2022b). However, there are still important gaps in the understanding of how disruption of microbiota by differing modes of delivery and antibiotic use can enhance or reduce the gut resistome as the infant ages. In this study, the combined effects of CS and antibiotic uptake on the meta-taxonomic diversity, and frequency of ARGs over the first few weeks of life were investigated by comparing it to CS-born and vaginally-delivered control groups without antibiotics. Furthermore, the effect of antibiotic exposure in early life on the resistome and functional profile of infant gut microbiota longitudinally up to two years of age was investigated. CS delivery and antibiotic exposure in early life were seen to pose a twofold disadvantage, resulting in significantly lower microbial diversity and higher ARG frequency with antibiotic use being the dominant influencing factor.

4.3 Methods

4.3.1 Study group participants

In this study, DNA extracted from faecal samples from full term infants from two study cohorts- INFANTMET and MYNEWGUT- were used to investigate the evolution of the infant gut resistome using shotgun metagenomics throughout the first 24 weeks of life up to two years of age. Briefly, MyNewGut (MNG) recruited full term CS born infants administered antibiotics in the first four days of life. InfantMet (IM) included infants born at full term both via CS and vaginal delivery and were not administered any antibiotic in the first week of life (Hill et al., 2017). A subset of subjects from these two studies (n=45) was selected and divided in three groups. Group 1 (MNG+ab/CSab) included infants born by CS and given antibiotics during the first week of life. Group 2 (IM-Ab/CSnoab) contains infants born by CS, not administered antibiotics, and group 3 (IM-Ab/VDnoab) has infants born via vaginal delivery not administered antibiotics in the first few days of life. The cohorts and control groups were matched by gestational age (> 35 weeks gestation), birth mode (CS and VD), antibiotic treatment (i.e. benzylpenicillin and

gentamicin), diet (mostly breastfed or combination of breast and formula feeding), and time points of sample collection. The demographics for these three infant groups are as follows (Table 4.1).

Table 4.1: Metadata for infants included in this study.

Summary statistics (n=45)				
Characteristics	**MNG-CS (CSab)**	**IM-CS (CSnoab)**	**IM-VD (VDnoab)**	
Number of infants, n (%)	10 (22.22)	17 (37.77)	18 (40)	
Number of samples, n (%)	50 (24)	78 (37.5)	80 (38.4)	
Time points, n				
Week 1	10 (%)	17	17	
Week 4	10	15	17	
Week 8	10	17	15	
Week 24	10	17	17	
Year 2	10	12	14	
Gender, n (%)				
Male	4 (40)	9 (52.9)	9 (50)	
Female	6 (60)	8 (47)	9 (50)	
Mode of Delivery, n (%)				
Vaginal	0	0	18 (100)	
C-section	10 (100)	17 (100)	0	
Gestational age (days)	*280(273,287)	*273(272,28)	278(277,287)	1 missing
Birth weight (g)	4002+- 646.61	3543.5+- 463.88	3499.4+- 402.9	

Antibiotic uptake in the first 4 days of life	Yes	No	No	
Feeding, n (%)				
Breast	1 (1)	3 (17.6)	4 (25)	
Combine	8 (8)	10 (58.8)	14 (77.7)	
NA	1 (1)	4 (23.5)	0 (0)	
Maternal age at infant birth (years) - average	NA	34	33	
Gravidity	1.5(1,2)	2(2,4)	2(1.25,2.75)	
Parity	1(1,1)	2(2,3)	2(1,2.75)	
APGAR score				
Initial	9(8.25,9)	9(9,9)	9(8.75,9)	
Second	10(9,10)	10(9,10)	10(9,10)	

4.3.2 DNA extraction

Metagenomic DNA was extracted from fecal samples using the QIAmp Fast DNA Stool Mini kit (Qiagen, UK) with a modified protocol combined with repeated bead beating method (Zhongtang Yu & Mark Morrison 2004). Briefly, 1 ml of lysis buffer was added to the stool sample in the bead beating tube. The samples were homogenised with the lysis buffer using a mini bead-beater, incubated at 70°C for 15 minutes with mixing at regular intervals, followed by another 15-minute incubation at 37°C and then centrifuged at 4°C for five min at 16,000× g. The supernatant was then treated with ammonium acetate to remove impurities, and incubated on ice for 5 min. This was followed by another centrifugation step at 4°C for 10 min at 16,000× g and then the DNA was precipitated by combining with equal volume of iso-propanol and stored overnight at -20°C. On the next day, the DNA was pelleted by centrifugation at 4°C for 15 min at 16,000× g, washed with ethanol and Tris-EDTA, treated with Proteinase-k and RNAse and purified according the manufacturer's instructions (QIAmp Fast DNA Stool Mini kit; Qiagen, UK). The DNA was quantified using qubit and stored at -30°C until further use.

4.3.3 Shotgun sequencing Library Preparation

Concentration of DNA was determined using qubit and diluted to 0.2ng/µl as per the manufacturer's instructions. Library prep for shotgun sequencing was performed using Nextera XT kit following the Nextera XT DNA Library Preparation Guide from Illumina. Briefly, genomic DNA (0.2ng/µl) was tagmented in 20µl PCR reaction (containing 5µl of gDNA, 10µl of tagment DNA buffer and 5µl of Amplicon Tagment Mix) volume at 55°C. The tagmented DNA was then amplified using Illumina index primers, following which the libraries were cleaned using AMPure beads. Quality of the library was then evaluated by running it on the Bioanalyzer using the Agilent High Sensitivity DNA chip. The DNA was quantified using Qubit, normalised, pooled and outsourced to the Teagasc sequencing facility for sequencing. Samples were sequenced as part of three sequencing runs along with one single sample (I159) ran on a new run making it part of a fourth sequencing run.

4.3.4 Filtering, Taxonomic and Functional analysis of metagenomics reads

The quality of raw reads from metagenomic sequencing was assessed using FastQC (v0.11.8) and MultiQC (v1.9). Further, trimming, filtering and host genome decontamination of the metagenomics reads was done using Trimmomatic (v0.39) and bowtie2 with *Homo sapiens* reference genome using the default parameters via the KneadData (v0.7.2) program from Huttenhower lab (https://github.com/biobakery/kneaddata). Further, HUMAnN3 (v3.0) was used for functional assignment of the reads providing microbial gene and pathway abundance information (Beghini et al., 2021). While MetaPhlAn3 was used to provide taxonomic assignment to the filtered and trimmed reads using default parameters. MetaPhlAn3 output was processed using the microbiomics (v0.0.0.9000) and phyloseq (v1.38.0) package in R and relative abundance and mean relative abundance data was used for plotting figures in R.

4.3.5 Metagenomics assembly and contig binning

Post KneadData filtering, the quality-filtered sequencing reads were first fixed and sorted using bbmap (v38.22). The metagenomes were then assembled using metaSPAdes (v3.14) (Nurk et al., 2017) with default parameters. The scaffolds generated were filtered to obtain resulting scaffolds with minimum length of 1000 bp (1kbp). Metagenomic bins

were generated from the filtered scaffolds using three metagenomic binning tools (MetaBAT v2.12.1, MaxBin v2.2.622, and CONCOCT v1.0.023) using metaWRAP (v1.3.1) (Uritskiy et al., 2018) with default parameters. The bins were then refined with Bin_refinement module of metaWRAP with options'-c 50 -x 10', corresponding to the criterion of medium-quality draft MAGs. The quality (estimated completeness and contamination) of bins was evaluated with CheckM (v1.0.18) (Parks et al., 2015), implemented in metaWRAP. This resulted in formation of 1449 medium quality Metagenome-Assembled Genomes (MAGs) which were used for further analysis.

4.3.6 Taxonomic and functional annotation of MAGs

The resulting MAGs were then provided taxonomic annotation with GTDB-Tk (v1.5.0) using 'classify_wf' workflow with default settings. The phylogenetic tree for the 1449 MAGs was generated using PhyloPhlAn (v3.0.2) with options'-diversity low' and '-fast' (Asnicar et al., 2020). The protein coding genes from MAGs were predicted using Prokka (v1.14) using default parameters. dbCAN (v3.0.1) (Huang et al., 2018) (run_dbcan.py) was used to assign carbohydrate active enzymes (CAZymes) to all the MAGs using default parameters. Only certain CAZymes as reported to be involved in HMO, FOS metabolism were filtered and examined in downstream analysis. inStrain (Olm et al., 2021) was used for identification of identical strains from MAGs, briefly MAGs were dereplicated using drep (v3.2.0) resulting in 598 unique MAGs which were then mapped on filtered and trimmed shotgun reads using the inStrain profile command using Bowtie2 (v2.3.4). The inStrain profiles are then processed with inStrain compare, where the profiles are compared and those with 99.99% population level - average nucleotide identity (popANI) are termed to be the same strain, belonging to the same cluster and thus considered belonging to the same sample. The resulting strains were then classified as early or late colonisers, if they appeared during the first eight weeks of life or later. Early colonisers were further classified as persisters and non-persisters. The definition for persisters was adapted from Lou et al. (2022) and persisters were defined as early colonisers that persisted for 24 weeks (six months) or more, otherwise the strain was termed as non-persister. Possible contaminants were examined and detected by looking for strains present in different clusters, marking and removing them from further analysis. Some strains were present in samples from two different plates and thus they could be classified as non-contaminants. However for a cluster, where two samples I08 and I18

were considered, some of the samples were on different plates, some showed persistence and thus we infer that these could either be contaminants or samples from siblings. However, additional metadata to study this in further details was not available. Two other MAGs were found to be part of three same samples (M15, M20, M25) which were all sequenced as part of the same plate and thus could be possible contaminants. Either way all the MAGs flagged as possible contaminants were removed from further analysis.

4.3.7 Resistome and virulence analysis

MAGs generated were then processed through ABRicate (https://github.com/tseemann/abricate) using default settings with CARD (Comprehensive Antibiotic Resistance Database) (Jia et al., 2017), VFDB (Virulence Factor Database) (Chen et al., 2016), Plasmid finder (Carattoli et al., 2014) databases. Results from CARD and VFDB were used further unless otherwise specified. RGI-bwt along with the CARD (v3.1.4) (Alcock et al., 2020) was used to predict ARGs in the clean and filtered metagenomics reads using default parameters. The RGI-bwt read mapping results at gene level were normalised by the read counts per sample and converted to copies per million (cpm) of ARGs per sample and used for downstream analysis. ARG names from ABRicate and RGI output were curated to antibiotic class names based of phenotype from CARD. Any gene that conferred resistance to three or more antibiotic classes was termed as multi-drug resistant (MDR) in downstream analysis. However, the antibiotic classes of interest in the study (classes of antibiotics administered to infants in the first week of like in CSab group - Aminoglycoside and or Penams) where appeared are spelled out along with MDR for better visualisation purposes.

4.3.8 Differential abundance analysis

Analysis of which taxa or ARG classes drive the difference between the three groups in the present study was done using MaAsLin2 (Mallick et al., 2021) and Songbird (Morton et al., 2019). Songbird for differential abundance analysis of longitudinal data was performed using default parameters to study the bacterial species most associated with each group and driving the differences in the groups. Only the top 10 associations (both positive and negative top10) were used for further analysis.

4.3.9 Statistical analysis and data representation

Downstream analysis of the data was done in R (v4.1.0) using packages including Vegan (v2.5.7) (Oksanen et al., 2020), Phyloseq (v1.38.0) (Mcmurdie & Holmes, 2013), ggplot2 (v3.3.5) (Wickham, 2016), microbiome utilities (v1.0.16) and ggpubR (v0.4.0). Statistical analysis was performed in R using Permanova to check the proportion of explained variance and significance of each variable using pairwiseAdonis (v0.4). Wilcoxon sum tests, paired t-tests, Dunn test and ANOVA as mentioned throughout the text were used to verify statistical significance. Spearman correlation coefficient was used to determine the correlation between relative abundance over time for species for each group and top 10 positive and negative results were plotted in R. Statistical significance was determined by 999 permutations and p-values were corrected using FDR; p-values below 0.05 were considered significant.

4.4 Results

To characterise the microbial and resistome development in early life, metagenomic data of two hundred and eight stool samples from 45 infants were divided into three groups based on their mode of delivery and antibiotic uptake in the first week of life, i.e. vaginal delivery/no-antibiotic (VDnoab); C-Section/no-antibiotic (CSnoab); C-Section/antibiotic (CSab). This study included samples at five time points from week 1, week 4, week 8, week 24 and year 2 of age. Altogether 10 infants were born by CS and administered antibiotics in the first four days of life, with gentamycin and Benzyl-Penicillin being the most commonly administered. Gestational age and birth weight were similar across all groups (Table 4.1).

4.4.1 Antibiotics limit microbial composition development and decrease diversity

Metagenomic analysis revealed increased richness and diversity over time in all groups (Shannon index, Figure 4.1A, 4.1B), with significant differences between all early time points (week 1, week 4, week 8 and week 24) and the final time-point, year 2. The significant difference (p-value=0.015) in alpha diversity (Shannon index) observed between week 1 and week 4 in the CSab group, which was not the case in the other groups, shows a gradual increase and high variation in microbial diversity in early life due to the effect of antibiotic administration (Figure 4.1A). The CSab group had low initial microbial diversity (Shannon index) when compared with the other groups (Figure

S4.1A), with a significant difference observed at week 1 between CSab and CSnoab (p-value=0.01) groups and at week 24 between CSab and CSnoab (p-value=0.013) and CSab and VDnoab (p-value=0.04) groups, however, no significant differences were observed otherwise (Figure S4.1A).

Principal coordinate analysis (PCoA) was used along with Bray-Curtis dissimilarity metrics and UniFrac distance to plot microbial composition profile differences between the three groups, giving similar results for each variable tested (Figure 4.1C). Using Bray-Curtis distances, CSab group showed a distinct microbial composition compared to CSnoab (p-value=0.0105, PERMANOVA) and VDnoab (p-value=0.0060, PERMANOVA) groups. Beta diversity also varied between time points (p-value=0.001, R2=0.11; Adonis2), by mode of delivery (p-value=0.003, R2=0.013; Adonis2) and antibiotic use (p-value=0.002, R2=0.016; Adonis2) in early life; with antibiotic use explaining slightly higher variation between the groups after the time variable. Microbial communities were mainly structured by time-point, and significant differences were observed between the CSab and VDnoab groups at week 1, week 4 and week 8 (p-values=0.024, 0.048, 0.018 respectively, Adonis2) and between VDnoab and CSnoab groups at week 1 (p-value=0.075) (Figure S4.2A). To determine the effect of delivery mode and antibiotic use on the microbial composition, PERMANOVA was performed using pairwiseAdonis in R on microbial composition data at each time-point. At week 1, delivery mode and antibiotic usage caused significant variations in the microbial composition of infants, with delivery mode having a higher effect size. The effect of mode of delivery decreased over time, but antibiotic use in the first week of life was a significant variable affecting microbial composition in infants up to week 24 (Figure S4.2C).

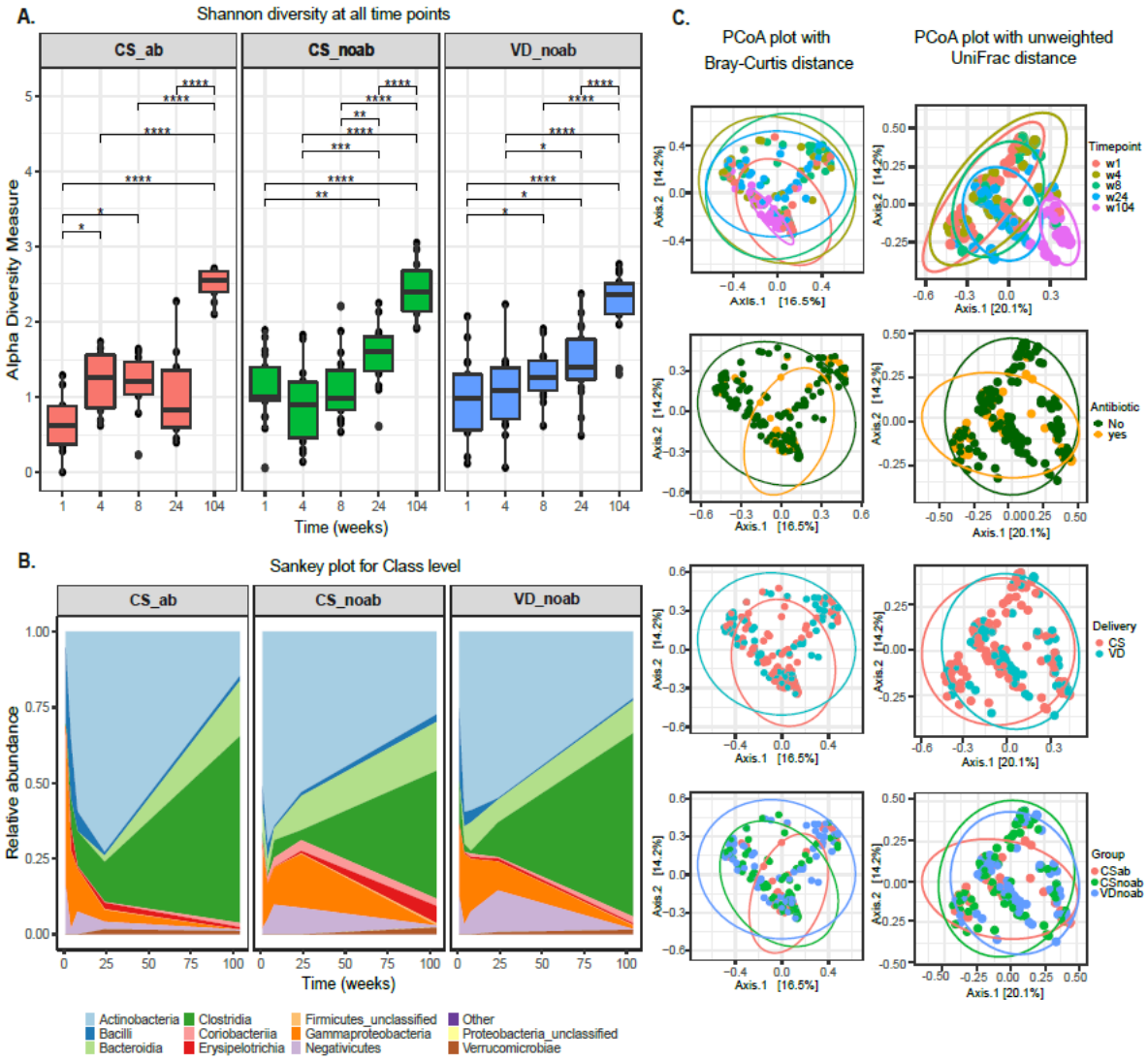


Figure 4.1: A. Alpha diversity as measured by Shannon's diversity index between all time points in the three study groups. P-values were calculated using the Wilcoxon test with stat_compare_means, with $p < 0.05$ used as the significance threshold. B. Taxonomic distribution of infant gut microbiota at class level using relative abundance for each group over time from week 1 to year 2. C. PCoA plot using Bray-Curtis and unweighted UniFrac distance metrics; with ellipses drawn with each grouping variable namely Time-point, Antibiotic, Delivery and Study groups. P-value and R2 values were generated using PERMANOVA, with $p < 0.05$ considered as significant.

4.4.2 Microbiota composition is strongly influenced by antibiotics following CS delivery in early life

Given the significant effect of delivery mode and early antibiotic exposure on microbial diversity, the colonisation pattern in the three groups by read and assembly-based

approaches from shotgun sequencing data was examined. The phyla Firmicutes and Proteobacteria were most prevalent in CS-born infants, with highest abundance in the CSab group (Figure S4.1B). Actinobacteria and Bacteroides abundance was high in VD infants, with high relative abundance in the no-antibiotic-treated groups and lowest in the CSab group from week 1. Detailed analysis at each time point demonstrated the absence of phylum Bacteroidetes in the CS-born infants at week 1. At week 4, Bacteroidetes were present in the no-antibiotic groups (CSnoab) but not in CSab until week 24 (Figure S4.1B). Phylogenetic analysis using 1448 MAGs demonstrated a grouping where the majority of the no-antibiotic-treated group-based MAGs were present in the tree's upper half, dominated by Actinobacteria, Bacteroidetes and Verrucomicrobiota. This upper half of the tree also was associated with VD infants, with scarce abundance of MAGs belonging to the CSab group. A distinct clustering of MAGs at year 2 was seen and this was mostly associated with Firmicutes_A, pointing towards increasing Firmicutes abundance with increasing age (Figure 4.2).

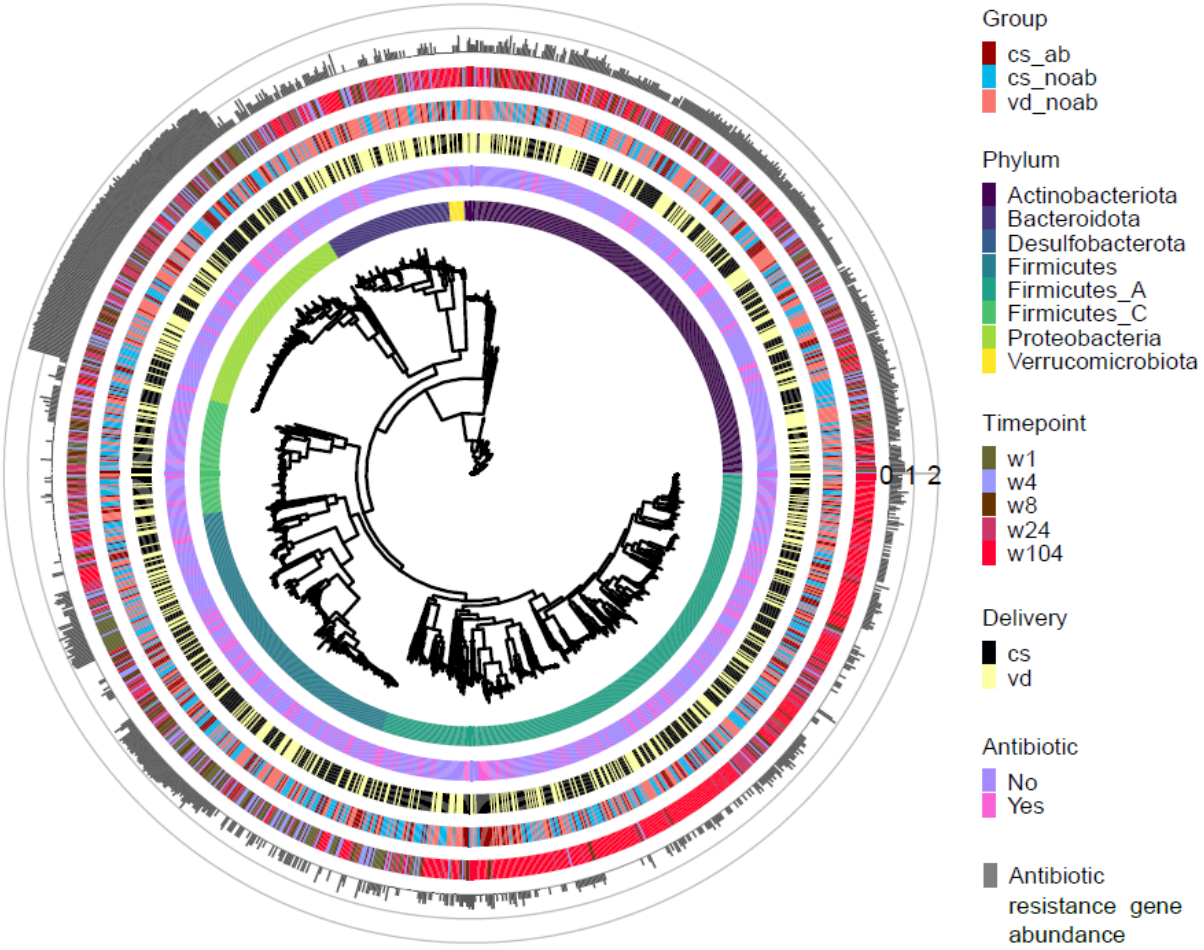


Figure 4.2: Phylogenetic tree created using Phylophlan from 1448 medium-quality MAGs generated in this study. Each branch represents a MAG with (from innermost to outermost ring): phylum level distribution of the MAGs, antibiotic use in early life and delivery mode, groups variable in study, succeeded by time-point and counts of ARG abundance per MAG as examined using ABRicate with CARD.

We further examined the mean relative abundances of the classes (Figure 4.1B) and top 10 most abundant species for all groups longitudinally (Figure 4.3A). Commensals such as *Bifidobacterium* species including *B. bifidum*, *B. breve*, *B. longum*, *B. kashiwanohense*, *Bacteroides* (*Bacteroides vulgatus*), *Collinsella aerofaciens* and *Escherichia coli* abundances were highest in the VDnoab group at week 1 and week 4, and generally, an increasing trend was seen for all groups till week 24. Interestingly, *Bifidobacterium dentium* abundance was highest in the CSab group, granting this species an undisclosed

benefit in adverse circumstances like the administration of antibiotics. Low prevalence of commensals was observed in CS-born infants, with higher initial abundance of opportunistic bacteria in the CSab group belonging to Proteobacteria and Firmicutes such as *Klebsiella pneumoniae*, *Ruminococcus gnavus*, *Veillonella parvula*, *Raoultella ornithinolytica*, *Enterobacter cloacae* complex, which decreased over time (Figure 4.3 A,C).

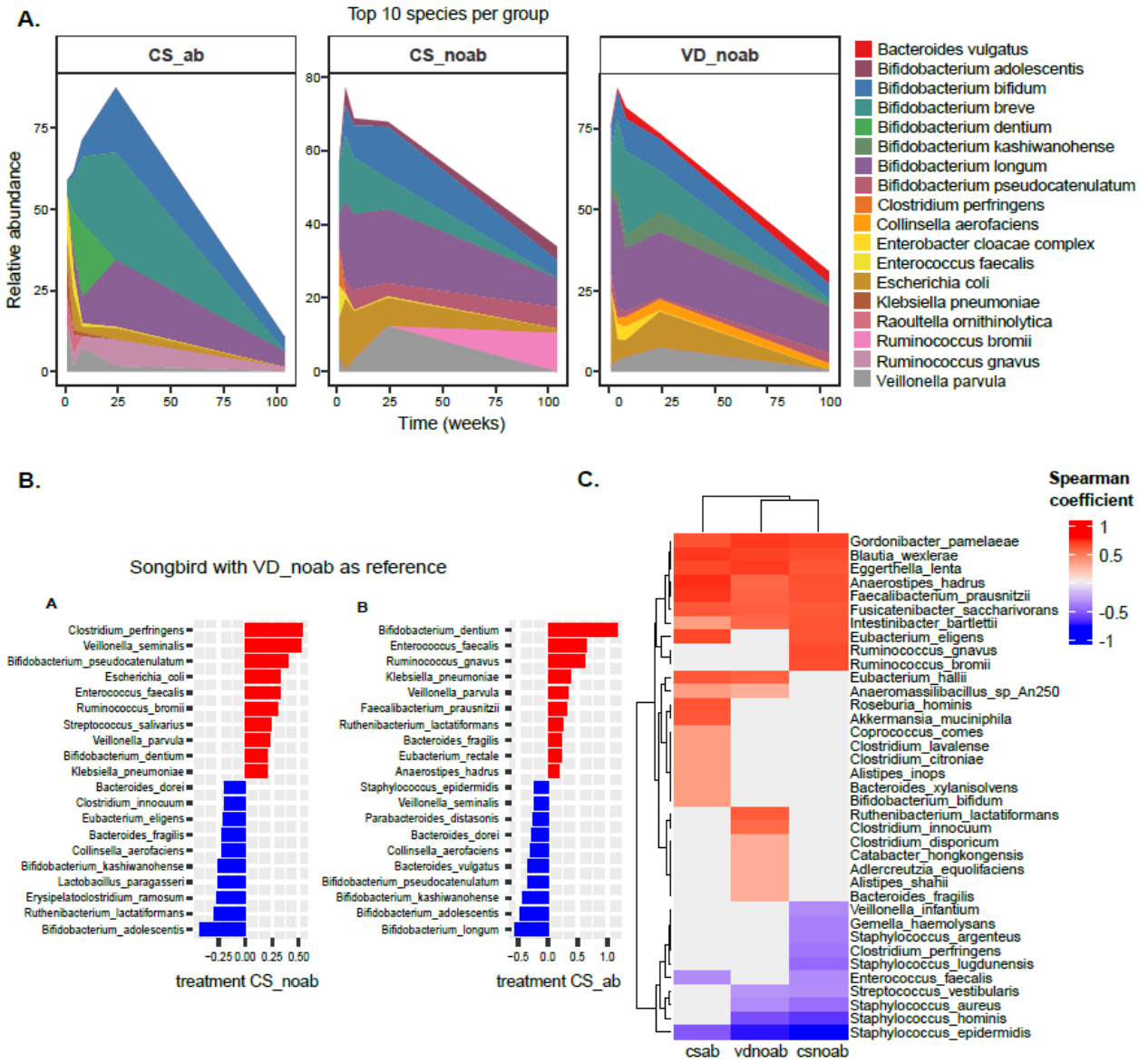

A.
Top 10 species per group
CS_ab
CS_noab
VD_noab
Relative abundance
Time (weeks)
Bacteroides vulgatus
Bifidobacterium adolescentis
Bifidobacterium bifidum
Bifidobacterium breve
Bifidobacterium dentium
Bifidobacterium kashiwanohense
Bifidobacterium longum
Bifidobacterium pseudocatenulatum
Clostridium perfringens
Collinsella aerofaciens
Enterobacter cloacae complex
Enterococcus faecalis
Escherichia coli
Klebsiella pneumoniae
Raoultella ornithinolytica
Ruminococcus bromii
Ruminococcus gnavus
Veillonella parvula
B.
Songbird with VD_noab as reference
A
Clostridium_perfringens
Veillonella_seminalis
Bifidobacterium_pseudocatenulatum
Escherichia_coli
Enterococcus_faecalis
Ruminococcus_bromii
Streptococcus_salivarius
Veillonella_parvula
Bifidobacterium_dentium
Klebsiella_pneumoniae
Bacteroides_dorei
Clostridium_innocuum
Eubacterium_eligens
Bacteroides_fragilis
Collinsella_aerofaciens
Bifidobacterium_kashiwanohense
Lactobacillus_paragasseri
Erysipelatoclostridium_ramosum
Ruthenibacterium_lactatiformans
Bifidobacterium_adolescentis
treatment CS_noab
B
Bifidobacterium_dentium
Enterococcus_faecalis
Ruminococcus_gnavus
Klebsiella_pneumoniae
Veillonella_parvula
Faecalibacterium_prausnitzii
Ruthenibacterium_lactatiformans
Bacteroides_fragilis
Eubacterium_rectale
Anaerostipes_hadrus
Staphylococcus_epidermidis
Veillonella_seminalis
Parabacteroides_distasonis
Bacteroides_dorei
Collinsella_aerofaciens
Bacteroides_vulgatus
Bifidobacterium_pseudocatenulatum
Bifidobacterium_kashiwanohense
Bifidobacterium_adolescentis
Bifidobacterium_longum
treatment CS_ab
C.
Spearman coefficient
Gordonibacter_pamelaeae
Blautia_wexlerae
Eggerthella_lenta
Anaerostipes_hadrus
Faecalibacterium_prausnitzii
Fusicatenibacter_saccharivorans
Intestinibacter_bartlettii
Eubacterium_eligens
Ruminococcus_gnavus
Ruminococcus_bromii
Eubacterium_hallii
Anaeromassilibacillus_sp_An250
Roseburia_hominis
Akkermansia_muciniphila
Coprococcus_comes
Clostridium_lavalense
Clostridium_citroniae
Alistipes_inops
Bacteroides_xylanisolvens
Bifidobacterium_bifidum
Ruthenibacterium_lactatiformans
Clostridium_innocuum
Clostridium_disporicum
Catabacter_hongkongensis
Adlercreutzia_equolifaciens
Alistipes_shahii
Bacteroides_fragilis
Veillonella_infantium
Gemella_haemolysans
Staphylococcus_argenteus
Clostridium_perfringens
Staphylococcus_lugdunensis
Enterococcus_faecalis
Streptococcus_vestibularis
Staphylococcus_aureus
Staphylococcus_hominis
Staphylococcus_epidermidis
csab
vdnoab
csnoab

Figure 4.3: A. Relative abundance showing top 10 species per group over time from week 1 up to year 2, where time is in weeks. B. Plots show differential abundance analysis using Songbird with VDnoab group as reference run using the formula C(Group, Treatment('VDnoab')). Plot on the left side (B.A) corresponds to CSnoab group as treatment while that on right (B.B) depicts CSab group as treatment against the reference group. In both cases negative value (Blue bars) represent the association to the reference group (here VDnoab) while positive values (red bars) represent the association to the treatment group; here CSnoab (on left plot) and CSab (right side plot). C. Heatmap representing the association of bacterial abundance with time. Results from Spearman correlation analysis in R, run using relative abundance of species for each group to time-point. P-adj values < 0.05 were considered significant and corresponding species and their coefficients plotted. In the plot, blue bands denote decreasing with time (negative coefficient) while red bands denote positive coefficient which means increasing over time. While grey means NA denoting not detected significantly as top 10 in that group.

4.4.3 Stronger association and colonisation of CS-born babies with pathobionts

To further determine the association of each group and time with bacterial taxa, microbial abundance (species) data were associated with longitudinal time-point using Spearman correlation for each group (Figure 4.3C). The top 10 positive and negative coefficients derived after filtering for significant p.adjusted values were then plotted. In general, facultative anaerobes (such as *Staphylococcus*, *Streptococcus*, *Enterococcus*) known to colonise the gut in the first week of life showed a decreasing trend over time, while obligate anaerobes (*Blautia*, *Bifidobacterium*, *Anaerostipes*, *Bacteroides*, *Eggerthella*, *Ruminococcus*, *Eubacterium*) showed a growing trend with time. The taxa *Bifidobacterium bifidum*, *Faecalibacterium prausnitzii*, *Blautia* (*Blautia wexlerae*), *Bacteroides* (*Bacteroides fragilis*), *Eubacterium* (*Eubacterium rectale*, *Eubacterium hallii*), *Anaerostipes* (*Anaerostipes hadrus*) and *Ruminococcus* (*Ruminococcus bromii*) were found to have positive associations and increase in all three groups over time (Figure 4.3C).

Genera that were significantly different between any two groups overall were identified using Songbird differential abundance analysis tool. Associations of species with specific groups from Songbird demonstrated stronger association of *Bifidobacterium* species to the VDnoab group, but when CSnoab to CSab were compared, *Bifidobacterium* species

were more associated to the CSnoab group (Table S4.1). When VDnoab group was used as reference (Figure 4.3B), several commensals were associated to the VDnoab group, some of which are involved in folate, lactate and butyrate production such as *Bifidobacterium adolescentis, Ruthenibacterium lactatiformans, Erysipelatoclostridium ramosum, Bifidobacterium longum, Lactobacillus paragasseri, Bifidobacterium kashiwanohense, Collinsella aerofaciens, Bacteroides fragilis, Parabacteroides distasonis, Bacteroides dorei*. While a mix of opportunistic bacteria and commensals including *Clostridium perfringens, Veillonella* (sp *seminalis* and *parvula*), *Bifidobacterium pseudocatenulatum, Streptococcus salivarius, Escherichia coli, Enterococcus faecalis, Ruminococcus bromii* was associated with the CSnoab group. Similarly, the CSab group was more associated to *Bifidobacterium dentium, Ruminococcus gnavus, Enterococcus faecalis, Veillonella parvula, Faecalibacterium prausnitzii, Klebsiella pneumoniae*. While comparing associations between CSnoab and CSab groups (Figure S4.2B) *Bifidobacterium adolescentis, Bifidobacterium pseudocatenulatum, Bifidobacterium kashiwanohense, Bacteroides vulgatus, Veillonella seminalis, Bifidobacterium longum, Parabacteroides distasonis, Bacteroides dorei* were associated to the CSnoab group. At the same time, *Bifidobacterium dentium, Veillonella parvula, Klebsiella pneumoniae, Faecalibacterium prausnitzii, Bifidobacterium breve, Enterococcus faecalis, Ruminococcus gnavus* – a direct mix of commensals and opportunists was associated to the CSab group (Figure S4.2B).

4.4.4 Antibiotic administration in early life is strongly associated to the resistome profile

Using CARD with ABRicate, the presence of 213 ARGs in 1024 MAGs was detected. Resistance was observed against 26 different classes of antibiotics in the present infant cohort; which included antimicrobials belonging to 'critically important', 'highly important' and 'important' categories (as categorised by WHO: https://apps.who.int/iris/bitstream/handle/10665/312266/9789241515528-eng.pdf).

Highest ARG distribution was in the phylum Proteobacteria followed by Actinobacteria and Firmicutes (Figure 4.2), with Proteobacteria possessing the highest abundance of unique ARGs (182). The majority of genera contributing to this ARG abundance included several taxa belonging to Gammaproteobacteria namely *Escherichia, Klebsiella,*

Enterobacter, *Raoultella*, *Citrobacter* and others including *Enterococcus*, *Staphylococcus*, *Streptococcus* and bifidobacteria. Interestingly, species belonging to *Escherichia* contributed to the largest number of ARG abundance accounting for 56% of the total number of ARGs detected (146 different ARGs present out of 213 detected) (Figure S4.4A). Results from the read-based approach using RGI with CARD further confirmed the resistome profile distribution in infants with a high abundance of resistance gene mapping observed to aminoglycoside, MDR, macrolide, Beta-lactam, tetracycline and fluoroquinolone antibiotics (Figure 4.4A). Functional characterisation of ARGs showed that antibiotic target alteration was the most common type, followed by antibiotic inactivation and antibiotic efflux as mechanisms for antibiotic resistance. The distribution and abundance pattern of ARGs in the study's three groups was investigated, and a decrease in alpha diversity from week 1 to year 2 (p-values: For CSab=0.001, For CSnoab=0.003 and for VDnoab=0.0001) and week 24 to year 2 (p-values: For CSab=0.02, For CSnoab=2.6e-05 and for VDnoab=0.0001) was observed; however, no other differences were seen (Figure 4.4B). PERMANOVA demonstrated discrete clustering between the CSab and VDnoab groups (p-value=0.03, Adonis2) using NMDS plot with Bray-Curtis distance matrix (Figure S4.4 B). Antibiotic use in early life and time, both had significant impact on the ARG diversity, leading to significant differences (antibiotic exposure: p-value=0.007, time: p-value=0.001, PERMANOVA); with no significant effect of mode of delivery. Significant p-values were also observed between CSnoab and CSab (p-value=0.05) at week 1 and between CSnoab and CSab and VDnoab and CSab at week 4 (p-value=0.02 and 0.03 respectively, PERMANOVA).

Additionally, 20 classes of differentially abundant antibiotic resistance classes were detected using MaAsLin2. Overall, and at week 1 and week 4, the CSab group was strongly (positively) associated with genes conferring resistance to aminoglycoside, Beta lactams, penams, MDR, elfamycin, fluoroquinolone, tetracycline, macrolide among others when compared with the other groups. Some of these antibiotic classes such as penams, aminoglycosides are the same class as those administered to infants in this group during the first four days of life (Figure 4.4C). Interestingly, infants in the other two groups, CSnoab and VDnoab also showed positive associations to certain antibiotic classes even at week 1 and week 4 when no antibiotics were administered during the first week of life. Further, the antibiotic resistance for all the classes observed in the study

groups over time was examined by normalising the data to cpm and plotted the log-transformed results, with VDnoab group as reference (Figure S4.3). The no-antibiotic groups clustered together at each time point, with CSab group showing resistance to several antibiotic classes overall and at week 1 and week 4 including aminoglycoside, beta lactams and penams, MDR including aminoglycosides, which were drugs, administered to the infants during the first week of life. Interestingly, at week 4, CSab showed significantly high resistance to several antibiotic classes with reference to VDnoab group while CSnoab group showed none. These differences could be due to the transient nature of the many taxa that colonised during early life in the CSnoab group, which might have reduced in abundance over time. However, in the CSab group, antibiotic exposure in the first four days of life resulted in selective pressure that might result in the presence of these ARG-containing bacteria, which were detected at week 4, and then possibly decreasing over time (Figure S4.3).

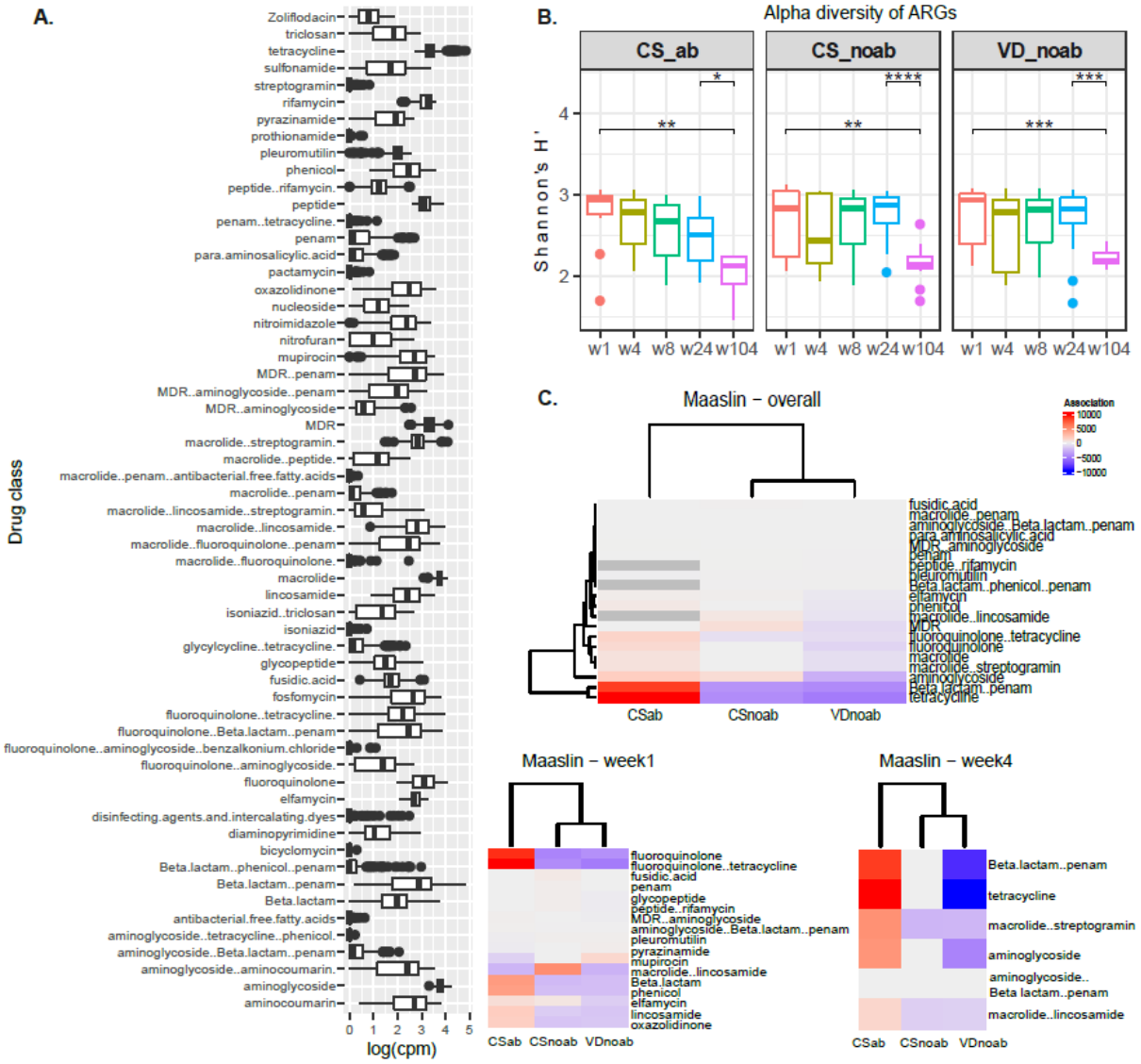


Figure 4.4: A. Box plot representing the total resistome profile of the infants included in this study, with antibiotic class names of penams and aminoglycosides retained in MDR classes to facilitate understanding, as these are the classes of antibiotics administered to infants in the CSab group. Antibiotic classes were detected using RGI with CARD. The abundance of antibiotic classes was normalised to cpm and log-transformed. B. Alpha diversity plot using Shannon diversity index for ARG diversity for each group between all the time points. P-value of < 0.05 was considered significant (Wilcoxon test). C. MaAsLin2 differential abundance analysis coefficients obtained by running MaAsLin2 overall and at week 1 and week 4 for each group using the parameters min_prevalence = 0.1 and min_abundance = 0.

4.4.5 Strain persistence is increased in vaginal delivery and antibiotic naïve groups

To understand the prevalence patterns of the resistome in the infant gut, the antibiotic resistance observed due to early life antibiotic exposure was investigated to check if it persists in bacterial strains to two years of age. inStrains was used with MAGs to identify identical strains and examine their persistence in infants in early life. Briefly, bins were dereplicated at 98% whole-genome average nucleotide identity (gANI) to obtain unique stains, which were mapped onto shotgun reads. If the genomic region demonstrated more than 99.99% population-level ANI (popANI) identity in two samples, the same strain was considered identical in the two samples. Based on the classification of persisters, non-persisters and early colonisers (adapted from Lou et al., 2021), a higher number of early colonisers were present in the no-antibiotic groups, with highest detected in the VDnoab group (Figure 4.5B). Further, 98% of the detected strains were early colonisers, of which 45% were non-persisters. Of the early colonisers, 38% in CSnoab group, 50% in the VDnoab group and 48% in the CSab group were non-persisters (Figure 4.5B). Not surprisingly, the highest number of persisters that remained until two years of age was in the VDnoab group, which could be due to the higher number of strains inherited maternally by vertical transmission such as those belonging to the Bacteroidetes phylum in this group. Overall, due to their abundant appearance in early life in healthy infants and critical role in carbohydrate metabolism in early life, bifidobacteria constituted the genera that contributed to the maximum percentage of persisters. *Bacteroides* were generally persisters, with very few non-persisters. While majorly, Proteobacteria along with some Actinobacteria and Firmicutes were among the top non-persisters (Figure S4.5). Based on the stringent criteria to define strains, we consider that ARG abundance detected within persisters are the same genes that appeared in early life and persisted until year 2. Investigating the top 10 strains carrying antibiotic resistance genes revealed that all strains in the no-antibiotic groups belonged to the genera *Escherichia*, while those in the CSab group belonged to a mixture of taxa from Gammaproteobacteria; including *Escherichia, Klebsiella and Raoultella*; with none persisting up to two years of age. A closer look at the top 10 strains per group and antibiotic class showed a wide variety of early colonisers belonging to genera *Escherichia, Enterococcus, Bifidobacterium, Streptococcus, Collinsella, Phocaeicola* and *Bacteroides* among others; the majority of these strains were non-persisters, with most present till week 8 and some till week 24 (Figure 4.5A).

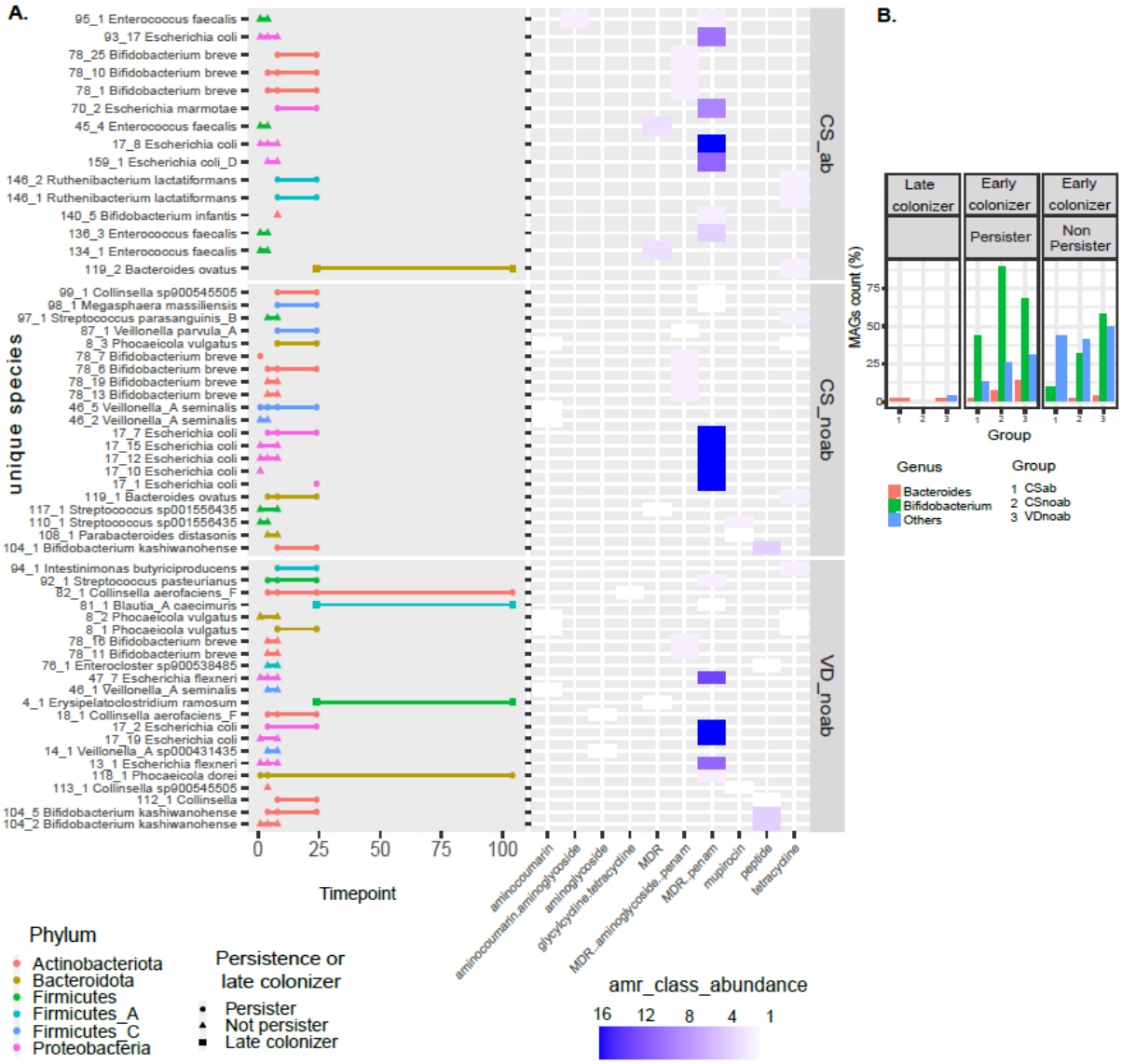


Figure 4.5: A. Persistence of strains carrying the top 10 highest abundance of AMR per group and class in infants up to two years of. Right side of the plot depicts abundance as detected by ABRicate in the form of a heatmap. The strains are colour coded based on the phylum which they belong to, as detected by GTDB. B. Bar plot showing percent distribution of the genera bifidobacteria, *Bacteroides* and others as early colonisers (further divided into persisters and non-persisters) and late colonisers for each of the three groups in the study.

4.4.6 Functional profiles of the microbiota in infants is influenced by antibiotic exposure and age

Differences in microbial composition, abundance and resistome might infer variation in functional capabilities of the microbiota. Thus, next, differences in the functional ability of gut microbiota were examined in the three study groups VDnoab, CSnoab and CSab. Carbohydrate metabolism including HMO and GOS degradation is an important ability for infant gut microbiota in early life especially when fed breast milk. Overall CAZyme (GH) abundance was significantly higher in the VDnoab group as compared to CSnoab and CSab groups (p-value=1.9e-15 and 0.0028 respectively; Wilcoxon test) and CSnoab group had higher GH abundance compared to the CSab group (p-value=0.0033) at week 1. At week 4, significant difference was observed between VDnoab and CSnoab and VDnoab and CSab (p-value=3.3e-06, 9.7e-06 respectively). CAZyme analysis was restricted to GH related to HMO, GOS and FOS degradation enzyme families in early life due to their importance in early life. The phylum Actinobacteria and Bacteroidota had the highest percent contribution to GH abundance and it was highest in the VDnoab group. At the same time, Firmicutes and Proteobacteria contributed to a higher percentage in the CS-born infants (Figure 4.6A). Further, GH abundance related to HMOs and GOS decreased over time in all three groups and the VDnoab group had significantly higher abundance than CSnoab group (p-value=0.0035, Wilcoxon test). Pathway abundance data from HUMAnN3 was analysed by clustering the pathway to higher order MetaCyc pathways. Based on MetaCyc pathways from HUMAnN3 results (Figure 4.6B), the antibiotic-treated infants showed stronger association to pyrimidine nucleotide biosynthesis, ubiquinol (electron carrier biosynthesis) and aromatic compound degradation (toluene, catechol, protocatechuate) pathways, with essential pathways such as glycolysis, phospholipid biosynthesis, nucleic acid processing, carbohydrate degradation, amino acid biosynthesis increased over time (Table 4.2). Similarly, NMDS analysis with Bray-Curtis showed significant difference between week 1 and all later time points, and year 2 and all early time points (significant p-values, Adonis2).

Table 4.2: Table presenting significant p-values obtained from MaAsLin2 differential abundance analysis for unstratified data from HUMAnN3.

feature	Antibiotic	coef	p-value

PWY.7211..superpathway.of.pyrimidine.deoxyribonucleotides.de.novo.biosynthesis	yes	961.594	0.00019
PPGPPMET.PWY..ppGpp.biosynthesis	yes	759.515	0.00064
PWY.6545..pyrimidine.deoxyribonucleotides.de.novo.biosynthesis.III	yes	750.848	0.00117
CATECHOL.ORTHO.CLEAVAGE.PWY..catechol.degradation.to..beta..ketoadipate	yes	194.333	0.00614
PROTOCATECHUATE.ORTHO.CLEAVAGE.PWY..protocatechuate.degradation.II..ortho.cleavage.pathway.	yes	278.919	0.00382
PWY.5181..toluene.degradation.III..aerobic...via.p.cresol.	yes	218.475	0.00547
PWY.5417..catechol.degradation.III..ortho.cleavage.pathway.	yes	246.549	0.00621
PWY.5431..aromatic.compounds.degradation.via..beta..ketoadipate	yes	246.549	0.00621
PWY.5855..ubiquinol.7.biosynthesis..prokaryotic.	yes	359.876	0.00680
PWY.5856..ubiquinol.9.biosynthesis..prokaryotic.	yes	359.876	0.00680
PWY.5857..ubiquinol.10.biosynthesis..prokaryotic.	yes	359.876	0.00680
PWY.6318..L.phenylalanine.degradation.IV..mammalian..via.side.chain.	yes	320.314	0.00409
PWY.6562..norspermidine.biosynthesis	yes	68.1263	0.00334
PWY.6708..ubiquinol.8.biosynthesis..prokaryotic.	yes	359.876	0.00680
PWY.7210..pyrimidine.deoxyribonucleotides.biosynthesis.from.CTP	yes	638.424	0.00441
PWY0.1297..superpathway.of.purine.deoxyribonucleosides.degradation	yes	937.509	0.00576

PWY.6182..superpathway.of.salicylate.degradation	yes	190.901	0.00862
ECASYN.PWY..enterobacterial.common.antigen.biosynthesis	yes	380.688	0.01017
P185.PWY..formaldehyde.assimilation.III..dihydroxyacetone.cycle.	yes	453.982	0.01145
PWY.7199..pyrimidine.deoxyribonucleosides.salvage	yes	918.564	0.01226

A closer look at the stratified counterpart of pathway abundance data from HUMAnN3 showed study variables including groups, delivery mode, antibiotic uptake in early life, time-point having an effect on pathway abundance with significant clustering observed between VDnoab and CSab and between CSnoab and CSab groups (Figure 4.6C) (pairwise Adonis, PERMANOVA: p-values=0.003 and 0.045 respectively for groups, p-value=0.01 by mode of delivery, p-value=0.001 by early antibiotic uptake and p-value=0.001 by time point, PERMANOVA). Based on R2 values from Adonis, time variable and early antibiotic exposure (R2: 0.15, 0.015 respectively) had the highest influence on separating samples based on pathway abundance; this along with distinct clustering of the CSab group from the no-antibiotic groups points towards the impact of antibiotics in early life on the functionality of the microbiota. Many pathways including those related to sugar metabolism, purine and pyrimidine biosynthesis, and amino acid biosynthesis were associated to the antibiotic-treated group. These differentially abundant pathways were mostly driven by *Bifidobacterium dentium* species, *Ruminococcus* and *Faecalibacterium* species (Table 4.3).

Table 4.3: Table presenting significant p-values obtained from MaAsLin2 differential abundance analysis for stratified data from HUMAnN3.

feature	Antibiotic	coef	p-value
ARGSYNBSUB.PWY..L.arginine.biosynthesis.II..acetyl.cycle..g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	398.3997	0.0006

BRANCHED.CHAIN.AA.SYN.PWY..superpathway.of.branched.amino.acid.biosynthesis.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	455.515	0.0010
COA.PWY.1..coenzyme.A.biosynthesis.II..mammalian..g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	427.7017	0.0007
COA.PWY..coenzyme.A.biosynthesis.I.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	386.625	0.0007
DTDPRHAMSYN.PWY..dTDP.L.rhamnose.biosynthesis.I.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	488.6735	0.0009
HISTSYN.PWY..L.histidine.biosynthesis.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	380.7538	0.0009
ILEUSYN.PWY..L.isoleucine.biosynthesis.I..from.threonine..g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	473.6178	0.0011
NONMEVIPP.PWY..methylerythritol.phosphate.pathway.I.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	397.1707	0.0008
OANTIGEN.PWY..O.antigen.building.blocks.biosynthesis..*E..coli*..g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	396.0303	0.0010
P124.PWY..Bifidobacterium.shunt.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	401.8314	0.0009
PWY.2942..L.lysine.biosynthesis.III.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	488.1304	0.0007
PWY.3001..superpathway.of.L.isoleucine.biosynthesis.I.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	440.3852	0.0008
PWY.3841..folate.transformations.II.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	375.2278	0.0006

PWY.4242..pantothenate.and.coenzyme.A.biosynthesis.III.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	389.7976	0.0007
PWY.5097..L.lysine.biosynthesis.VI.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	429.1196	0.0008
PWY.5103..L.isoleucine.biosynthesis.III.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	442.9995	0.0009
PWY.5384..sucrose.degradation.IV..sucrose.phosphorylase..g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	517.8944	0.0007
PWY.5659..GDP.mannose.biosynthesis.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	390.9609	0.0007
PWY.5686..UMP.biosynthesis.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	536.4589	0.0007
PWY.6121..5.aminoimidazole.ribonucleotide.biosynthesis.I.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	340.0982	0.0008
PWY.6122..5.aminoimidazole.ribonucleotide.biosynthesis.II.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	369.1303	0.0008
PWY.6123..inosine.5..phosphate.biosynthesis.I.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	365.3022	0.0007
PWY.6124..inosine.5..phosphate.biosynthesis.II.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	344.4034	0.0007
PWY.6151..S.adenosyl.L.methionine.cycle.I.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	430.8586	0.0008
PWY.6277..superpathway.of.5.aminoimidazole.ribonucleotide.biosynthesis.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	369.1303	0.0008
PWY.6385..peptidoglycan.biosynthesis.III..mycobacteria..g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	363.3126	0.0007

PWY.6387..UDP.N.acetylmuramoyl.pentapeptide.biosynthesis.I..meso.diaminopimelate.containing..g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	342.8993	0.0007
PWY.6737..starch.degradation.V.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	538.29	0.0008
PWY.7111..pyruvate.fermentation.to.isobutanol..engineered..g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	474.6266	0.0010
PWY.7219..adenosine.ribonucleotides.de.novo.biosynthesis.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	438.3994	0.0007
PWY.7221..guanosine.ribonucleotides.de.novo.biosynthesis.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	611.7984	0.0007
PWY.7234..inosine.5..phosphate.biosynthesis.III.g__*Bifidobacterium*.s__Bifidobacterium_dentium	yes	365.5587	0.0007
PWY.724..superpathway.of.L.lysine..L.threonine.and.L.methionine.biosynthesis.II.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	413.5308	0.0007
THRESYN.PWY..superpathway.of.L.threonine.biosynthesis.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	424.5996	0.0007
UDPNAGSYN.PWY..UDP.N.acetyl.D.glucosamine.biosynthesis.I.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	369.8979	0.0010
VALSYN.PWY..L.valine.biosynthesis.g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	473.4299	0.0011
X1CMET2.PWY..N10.formyl.tetrahydrofolate.biosynthesis.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	126.3377	0.0030
ARGSYN.PWY..L.arginine.biosynthesis.I..via.L.ornithine..g__*Blautia*.s__*Ruminococcus_gnavus*	yes	155.6105	0.0030

ARGSYNBSUB.PWY..L.arginine.biosynthesis.II..acetyl.cycle..g__*Blautia*.s__*Ruminococcus_gnavus*	yes	184.2446	0.0023
BRANCHED.CHAIN.AA.SYN.PWY..superpathway.of.branched.amino.acid.biosynthesis.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	154.5044	0.0031
COA.PWY.1..coenzyme.A.biosynthesis.II..mammalian..g__*Blautia*.s__*Ruminococcus_gnavus*	yes	152.4818	0.0024
COA.PWY..coenzyme.A.biosynthesis.I.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	150.0988	0.0026
DTDPRHAMSYN.PWY..dTDP.L.rhamnose.biosynthesis.I.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	146.8284	0.0015
GLUTORN.PWY..L.ornithine.biosynthesis.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	129.8241	0.0031
PEPTIDOGLYCANSYN.PWY..peptidoglycan.biosynthesis.I..meso.diaminopimelate.containing..g__*Blautia*.s__*Ruminococcus_gnavus*	yes	156.6033	0.0026
PWY.1042..glycolysis.IV..plant.cytosol..g__*Blautia*.s__*Ruminococcus_gnavus*	yes	170.2212	0.0024
PWY.2942..L.lysine.biosynthesis.III.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	168.046	0.0026
PWY.3841..folate.transformations.II.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	135.7699	0.0025
PWY.4242..pantothenate.and.coenzyme.A.biosynthesis.III.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	148.9855	0.0021
PWY.5097..L.lysine.biosynthesis.VI.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	158.8982	0.0028
PWY.5103..L.isoleucine.biosynthesis.III.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	153.8428	0.0029
PWY.5667..CDP.diacylglycerol.biosynthesis.I.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	137.6698	0.0031

PWY.5686..UMP.biosynthesis.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	153.4085	0.0027
PWY.6121..5.aminoimidazole.ribonucleotide.biosynthesis.I.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	150.544	0.0028
PWY.6122..5.aminoimidazole.ribonucleotide.biosynthesis.II.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	146.362	0.0029
PWY.6126..superpathway.of.adenosine.nucleotides.de.novo.biosynthesis.II.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	191.424	0.0024
PWY.6151..S.adenosyl.L.methionine.cycle.I.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	148.139	0.0031
PWY.6168..flavin.biosynthesis.III..fungi..g__*Faecalibacterium*.s__*Faecalibacterium_prausnitzii*	yes	165.249	0.0026
PWY.621..sucrose.degradation.III..sucrose.invertase..g__*Bifidobacterium*.s__*Bifidobacterium_dentium*	yes	577.1506	0.0016
PWY.6277..superpathway.of.5.aminoimidazole.ribonucleotide.biosynthesis.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	146.3629	0.0029
PWY.6385..peptidoglycan.biosynthesis.III..mycobacteria..g__*Blautia*.s__*Ruminococcus_gnavus*	yes	146.0853	0.0030
PWY.6386..UDP.N.acetylmuramoyl.pentapeptide.biosynthesis.II..lysine.containing..g__*Blautia*.s__*Ruminococcus_gnavus*	yes	159.5831	0.0028
PWY.6387..UDP.N.acetylmuramoyl.pentapeptide.biosynthesis.I..meso.diaminopimelate.containing..g__*Blautia*.s__*Ruminococcus_gnavus*	yes	159.868	0.0025
PWY.6527..stachyose.degradation.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	173.832	0.0029
PWY.6609..adenine.and.adenosine.salvage.III.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	171.398	0.0016

PWY.6700..queuosine.biosynthesis.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	110.115	0.0026
PWY.6737..starch.degradation.V.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	167.253	0.0018
PWY.6936..seleno.amino.acid.biosynthesis.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	120.057	0.0025
PWY.7199..pyrimidine.deoxyribonucleosides.salvage.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	133.736	0.0026
PWY.7219..adenosine.ribonucleotides.de.novo.biosynthesis.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	182.6095	0.0026
PWY.7220..adenosine.deoxyribonucleotides.de.novo.biosynthesis.II.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	277.0192	0.0027
PWY.7221..guanosine.ribonucleotides.de.novo.biosynthesis.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	141.313	0.0024
PWY.7222..guanosine.deoxyribonucleotides.de.novo.biosynthesis.II.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	277.019	0.0027
PWY.7229..superpathway.of.adenosine.nucleotides.de.novo.biosynthesis.I.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	204.081	0.0026
PWY.7357..thiamin.formation.from.pyrithiamine.and.oxythiamine..yeast..g__*Blautia*.s__*Ruminococcus_gnavus*	yes	151.121	0.0026
PWY.7400..L.arginine.biosynthesis.IV..archaebacteria..g__*Blautia*.s__*Ruminococcus_gnavus*	yes	154.296	0.0030
PWY0.1296..purine.ribonucleosides.degradation.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	161.093	0.0014
PWY0.1319..CDP.diacylglycerol.biosynthesis.II.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	137.669	0.0031
RHAMCAT.PWY..L.rhamnose.degradation.I.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	149.167	0.0026

RIBOSYN2.PWY..flavin.biosynthesis.I..bacteria.and.plants..g__*Faecalibacterium*.s__*Faecalibacterium_prausnitzii*	yes	167.321	0.0025
THISYNARA.PWY..superpathway.of.thiamin.diphosphate.biosynthesis.III..eukaryotes..g__*Blautia*.s__*Ruminococcus_gnavus*	yes	144.268	0.0027
TRPSYN.PWY..L.tryptophan.biosynthesis.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	173.449	0.0018
ILEUSYN.PWY..L.isoleucine.biosynthesis.I..from.threonine..g__*Blautia*.s__*Ruminococcus_gnavus*	yes	163.440	0.0034
PWY.7111..pyruvate.fermentation.to.isobutanol..engineered..g__*Blautia*.s__Ruminococcus_gnavus	yes	199.953	0.0034
PYRIDNUCSYN.PWY..NAD.biosynthesis.I..from.aspartate..g__*Blautia*.s__*Ruminococcus_gnavus*	yes	129.144	0.00352
PWY4FS.7..phosphatidylglycerol.biosynthesis.I..plastidic..g__*Blautia*.s__*Ruminococcus_gnavus*	yes	119.961	0.00364
PWY4FS.8..phosphatidylglycerol.biosynthesis.II..non.plastidic..g__*Blautia*.s__*Ruminococcus_gnavus*	yes	119.961	0.00364
PHOSLIPSYN.PWY..superpathway.of.phospholipid.biosynthesis.I..bacteria..g__*Blautia*.s__*Ruminococcus_gnavus*	yes	128.775	0.00371
VALSYN.PWY..L.valine.biosynthesis.g__*Blautia*.s__*Ruminococcus_gnavus*	yes	160.932	0.00398
PWY.7220..adenosine.deoxyribonucleotides.de.novo.biosynthesis.II.g__*Faecalibacterium*.s__*Faecalibacterium_prausnitzii*	yes	120.3413	0.00441
PWY.7222..guanosine.deoxyribonucleotides.de.novo.biosynthesis.II.g__*Faecalibacterium*.s__*Faecalibacterium_prausnitzii*	yes	120.3413	0.00441

PWY.7242..D.fructuronate.degradation.g__*Faecalibacterium*.s__*Faecalibacterium_prausnitzii*	yes	120.7312	0.00481
GALACTUROCAT.PWY..D.galacturonate.degradation.I.g__*Faecalibacterium*.s__*Faecalibacterium_prausnitzii*	yes	129.3251	0.00544
PWY.6507..4.deoxy.L.threo.hex.4.enopyranuronate.degradation.g__*Faecalibacterium*.s__*Faecalibacterium_prausnitzii*	yes	114.3616	0.00554
PWY.6897..thiamin.salvage.II.g__*Faecalibacterium*.s__*Faecalibacterium_prausnitzii*	yes	100.8492	0.00608
PWY.5177..glutaryl.CoA.degradation.g__*Faecalibacterium*.s__*Faecalibacterium_prausnitzii*	yes	143.8907	0.00643
PWY.5695..urate.biosynthesis.inosine.5..phosphate.degradation.g__*Faecalibacterium*.s__*Faecalibacterium_prausnitzii*	yes	102.5299	0.00711
PWY.6737..starch.degradation.V.g__*Faecalibacterium*.s__*Faecalibacterium_prausnitzii*	yes	183.4145	0.00748

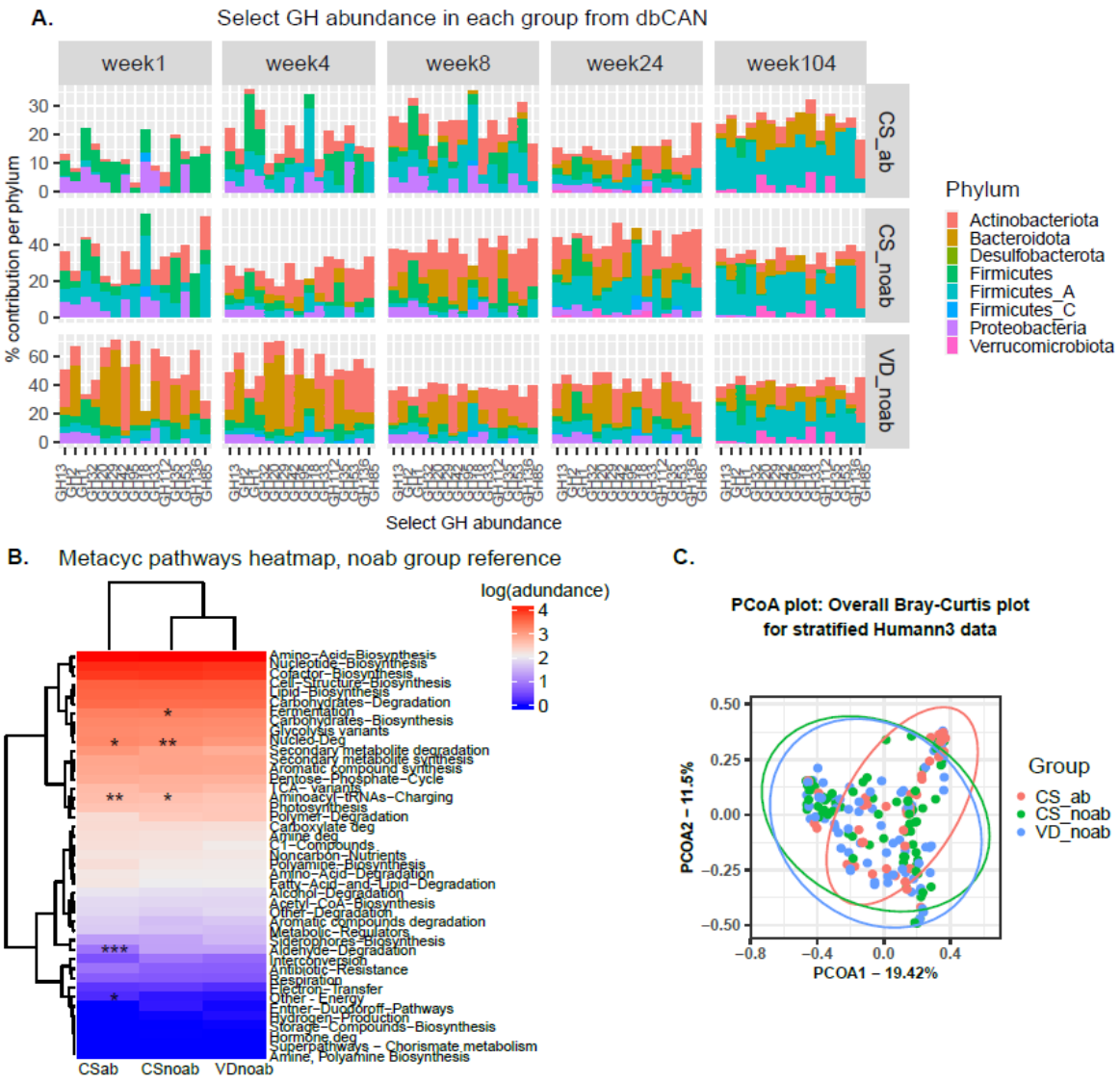


Figure 4.6: A. Bar plot depicting percent contribution of each of the detected phyla to abundance of CAZymes important for HMO, GOS and FOS degradation in infants. The plot presents the percent distribution for each phyla for log-transformed abundance at each time point for all the three groups VDnoab, CSnoab and CSab. B. Pathway abundance data for unstratified pathways as detected from HUMAnN3 was categorised into higher MetaCyc pathways, represented using ComplexHeatmap in R. cpm abundance was log-transformed, and significant difference in abundance between the groups was calculated using Wilcoxon test with VDnoab group as reference. P-values < 0.05 are considered significant. C. PCoA plot using Bray-Curtis distance for stratified pathway abundance data from HUMAnN3; showing distinct clustering of CSab from the other two groups.

4.5 Discussion

Previous studies have reported that changes in early-life gut microbiota composition are influenced by several factors including delivery mode, feeding habits and antibiotic exposure. In this study, antibiotic exposure in early life and mode of delivery were seen to influence microbial diversity and richness and influence microbial diversity between samples longitudinally, with antibiotic exposure having the greatest effect.

Similar to previous reports, a stronger association and higher prevalence of *Bifidobacterium* and *Bacteroidetes* were detected in vaginally-delivered infants, while CS-born infants had higher abundance of potential pathogens including *Enterococcus* spp. and species of Enterobacteriaceae (Reyman et al., 2019; Shao et al., 2019; Backhed et al., 2015; Dominguez-Bello et al., 2010; Alderberth et al., 2006). CS-born infants are more prone to negative health outcomes such as allergy, asthma, obesity, and diabetes development in later life (Słabuszewska-Jóźwiak et al., 2020; Cardwell et al., 2008). Additionally, colonisation patterns of CS infants with bacteria belonging to the category of ESKAPE pathogens is of concern as most bacteria in this group are MDR and are known to act as potential pathogens or are pathobionts (Ma et al., 2020; Santajit and Indrawattana, 2016); thus making any futuristic treatments more difficult to curtail in later life. The source of these species and their pathogenic potential in CS-born infants should be examined to help restore the gut microbial community and develop new and better-suited therapeutic approaches. Further, many *Bifidobacterium* and *Bacteroidetes* spp. showed lower or delayed colonisation in CS-born infants, with a pronounced effect in the CSab group, which is analogous to previous reports (Backhed et al., 2015; Dogra et al., 2015; Jakobsson et al., 2014), except for *Bifidobacterium dentium* which was seen to have high relative abundance in the CSab group. Recent studies have shown *B. dentium* in high abundance in infants belonging to the intrapartum antibiotic prophylaxis (IAP) group (Saturio et al., 2021) or those fed with donor breast milk (Arboleya et al., 2020); our result showing high *B. dentium* in the CSab group is in agreement with these reports and further studies are needed to understand the advantageous growth of this spp. under sub-optimal circumstances. The beneficial and protective roles of *Bifidobacterium* and Bacteroidetes in the infant gut, such as fermenting HMOs, maintaining barrier function, enhancing immune responses, colonisation resistance and competing for nutrients against pathogens are well established (Zafar & Saier, 2021; Saturio et al., 2021; Tamana et al., 2021;

Stewart et al., 2017; O'Callaghan & van Sinderen, 2016, Jakobsson et al., 2014). Decreased colonisation of these beneficial taxa is associated with adverse outcomes such as necrotising enterocolitis and late-onset sepsis (Dobbler et al., 2017; Patel and Denning, 2015); thus demanding further attention for studies in infants born by CS and exposed to antibiotics.

Further, an increase in diversity from week 1 to week 4 only in CSab but not in CSnoab and VDnoab groups was observed, emphasising the negative effect of antibiotics on microbial diversity in early life. A diverse range of ARGs and MDR bacteria was detected in all three groups, with decreasing ARG diversity seen over time. The most common antibiotic classes observed were MDR, which includes resistance to penams, fluoroquinolones, peptides, aminocoumarin, aminoglycosides and tetracyclines. This is in line with many reports suggesting the infant gut microbiota is a reservoir for ARGs (Tapiainen et al., 2019; Parnanen et al., 2018; Moore et al., 2015). The top abundance of ARGs was detected in *Escherichia, Klebsiella, Enterobacter*, *Bifidobacterium* and *Raoultella* and other Proteobacteria and Firmicutes species, with the highest association of antibiotic classes to the CSab group. Our findings are consistent with previous studies that report high abundance of ARGs in several taxa belonging to Gammaproteobacteria (Lebeaux et al., 2021, Gasparrini et al., 2019; Casaburi et al., 2019; Parnanen et al., 2018; Bailey et al., 2010). The CSab group also showed the presence of ARGs and stronger associations with classes of antibiotics administered to the infants in this group, clustering separately from the no-antibiotic groups, further pointing towards the fact that antibiotic exposure contributed to ARG abundance more than delivery mode.

This study found a higher frequency of ARGs in the CSab group, followed by CSnoab and VDnoab group, primarily due to this group's antibiotic exposure in early life. This can be attributed to the fact that several commensals and pathobionts harbour a set of ARGs, which could be inherited or transferred by horizontal gene transfer in the highly populated environment in the gut. Additionally, the use of antibiotics in early life alters the microbial composition and results in the selection of bacteria whose ARG reservoir includes genes capable of conferring resistance to the class of antibiotic in question. These pathobionts can switch to their opportunistic pathogenic side and can lead to infections that are difficult to treat due to the plethora of ARGs they contain. Disturbed microbial colonisation due to delivery mode and early antibiotic exposure can lead to colonisation

by more hospital-acquired strains, which would mean lower colonisation of strains from the mother. These nosocomial strains are enriched with a diverse resistome profile (Wu et al., 2022, Sukhum et al., 2022; Raplee et al., 2021; Lerminiaux et al., 2018). Furthermore, the high frequency of ARGs found in this study was associated with their high abundance in Proteobacteria. Increased abundance of specific taxa in this group (such as some *Klebsiella*, *Enterobacter*, *Raoultella* species) is reported to be associated with several functional variations and difficult-to-treat infections (Bradley and Pollard., 2017; Litvak et al., 2017; Pammi et al., 2017; Shin et al., 2015). Establishment of such strains from infancy in the gut can lead to complications in treatment in later life; thus, highly regulated antibiotic stewardship programmes are necessary along with probable therapeutic administration of probiotics where possible. Further, due to the stringent parameters used to define a strain we were able to examine persistence of resistance in the strains, and observed that the top 10 strains per class and group showed very little to no persistence up to two years of age. Moreover, *Bifidobacterium* strains and *Bacteroidetes* strains were observed to be major persisters, which sustain up to two years of age.

Though overall GH abundance was highest in the VDnoab group and lowest in the CSab group at week 1 and week 4, this difference was not prominent when selective GH related to HMO, FOS and GOS enzyme families were considered. This could be because functions like HMO degradation are essential and redundant in early life and colonisation is not solely dependent on feeding habits, mode of delivery or antibiotic exposure. While *Bifidobacterium* and *Bacteroides* are recognised as proficient HMO degraders (Marcobal et al., 2011; Kunz et al., 2000), their scarcity or non-existence in infants does not negate the critical role of HMO degradation, which can still be performed by other bacterial species present in these infants. Furthermore, a strong association of perinatal factors such as antibiotic treatment with aromatic compound degradation pathways, amino acid biosynthesis, cofactor and coenzyme biosynthesis, nucleotide biosynthesis (both purine and pyrimidine), and carbohydrate degradation pathways was observed. Similar associations such as an increase in nucleotide biosynthesis pathways, and carbohydrate degradation, were reported earlier in antibiotic-treated groups (Vanni et al., 2021; Chu et al., 2017). Such changes in metabolic potential can affect children's health in later life, and detailed studies will help understand these changes and their influence.

Some strengths of this study include examining several factors such as delivery mode, antibiotic use in early life and age on the dynamic nature of microbial colonisation pattern with read- and assembly-based approaches using shotgun sequencing. We also inspected and observed the taxa in infants that act as persisters and colonise the gut up to two years of age from early life. This study was limited due to a small cohort of 45 infants, and limited sample numbers between time points. Further, clinical metadata of antibiotic exposure relating to all time points of sample collection or similar data from the mother was missing; which narrows down the hypothesis that could be validated in the study. Thus, future studies with detailed clinical metadata and more time points with smaller intervals will help unfold the complex relationship between study variables, microbial and resistome profile and functionality of the microbiota.

In conclusion, this study highlights the effects of antibiotic exposure in early life and mode of delivery on infant gut microbiota in a longitudinal setting. Antibiotic exposure in early life has a stronger influence over the type and order of microbial colonisation in infants in early life than delivery mode. Such disturbed colonisation, along with a diverse resistance reservoir impacts the functional capability of the infant microbiota; including but not limited to the impairment that can be caused to other crucial functional capacities such as colonisation resistance, metabolism, maintaining gut permeability and a healthy immune response. Thus, more studies with larger cohorts are needed to better understand ARGs' persistence and impact on microbial functional capability with more certainty. Moreover, new alternatives/strategies need to be developed and used where needed to restore the microbial ecosystem, maintain a healthy microenvironment, restore their functional powers and reduce the use of prophylactic antibiotics during the crucial infancy stages.

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4.7 Ethics statement

The infants in this study were recruited as part of INFANTMET and MYNEWGUT studies. The ethics were approved by the Cork University Hospital Research Ethics Committee.

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4.9 Competing interests

The authors declare no competing interests.

4.10 Supplementary material

Supplementary figures:

Figure S4.1:

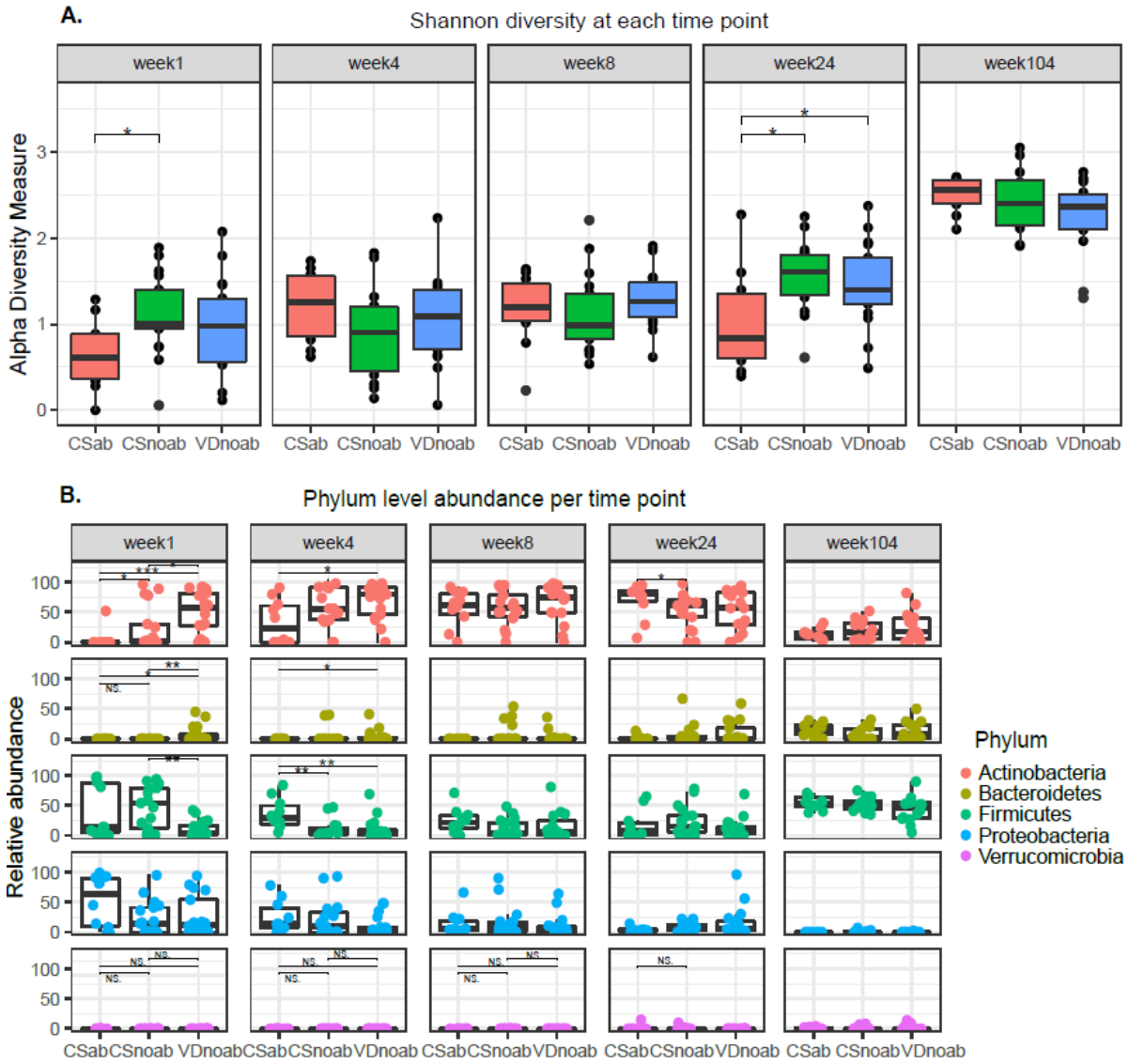


Figure S4.1: A. Microbial diversity of the infant gut as measured using Shannon diversity index between all three groups at each time-point in our study. B. Relative abundance plot showing phylum level distribution in all the study groups at each time-point.

Figure S4.2:

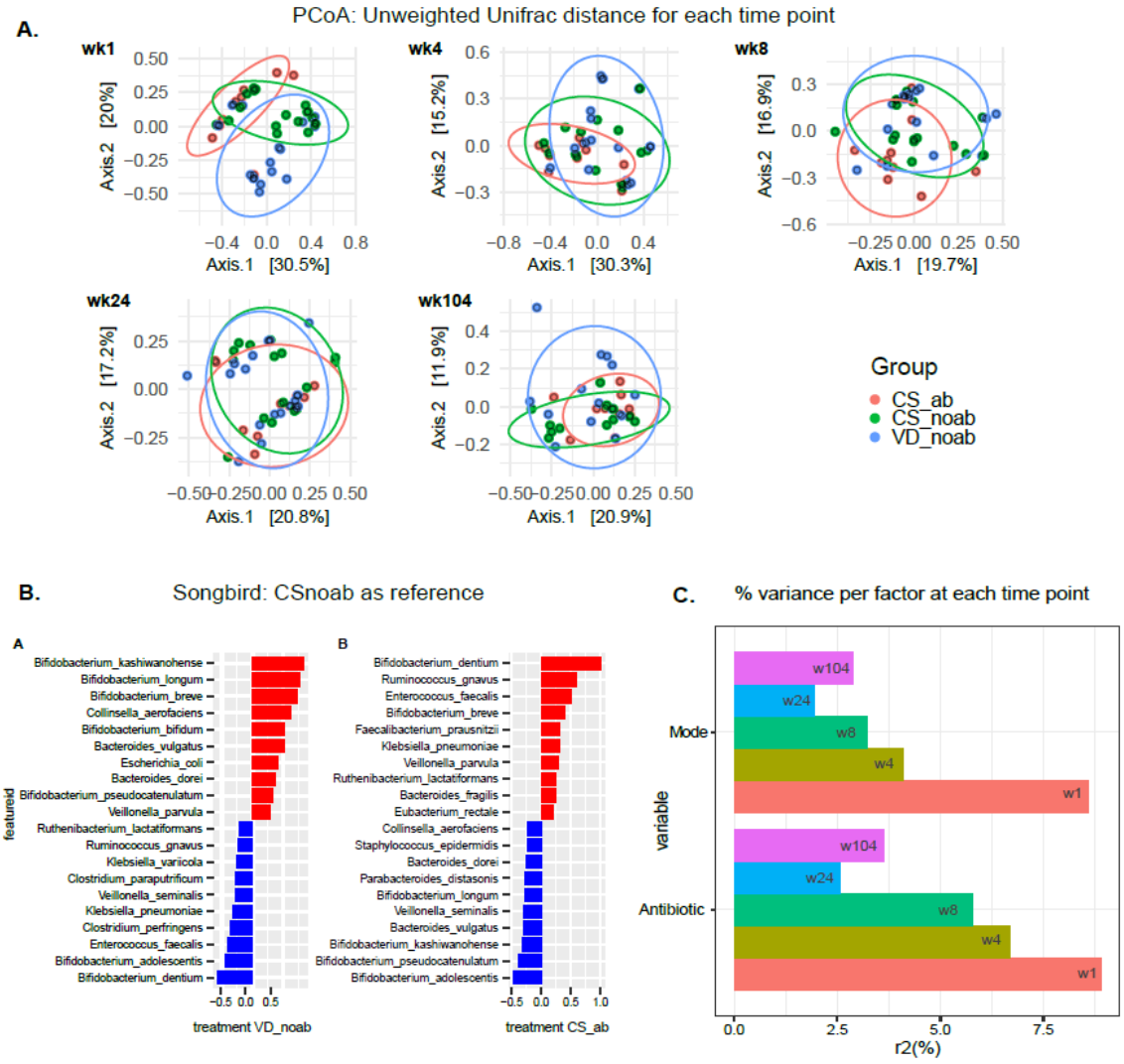


Figure S4.2: A. Beta diversity using unweighted Unifrac distance as depicted using PCoA at each time point. B. Plots shows differential abundance analysis using Songbird with CSnoab group as reference run using the formula C(Group, Treatment('CSnoab')). Plot on the left side corresponds to VDnoab group as treatment while that on right depicts CSab group as treatment against the reference group. In both cases negative value (Blue bars) represent the association to the reference group (here CSnoab) while positive values (red bars) represent the association to the treatment group; here VDnoab (on left plot) and CSab (right side plot). C. Percent variance for each time point plotted using R2 values as generated from PERMANOVA for Antibiotic and Mode of delivery variables in the study using unweighted Unifrac distance.

Figure S4.3:

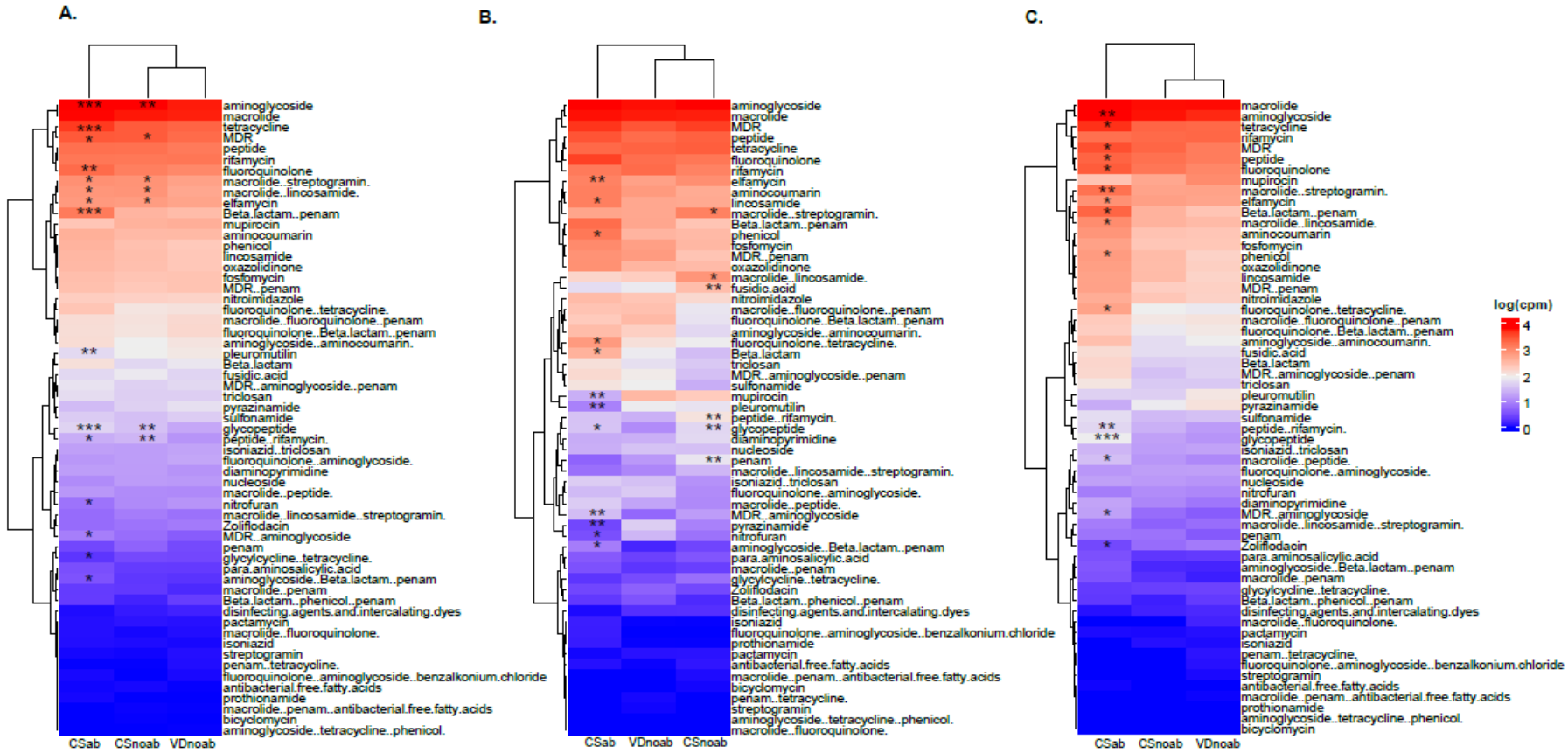

A.
aminoglycoside
macrolide
tetracycline
MDR
peptide
rifamycin
fluoroquinolone
macrolide..streptogramin.
macrolide..lincosamide.
elfamycin
Beta.lactam..penam
mupirocin
aminocoumarin
phenicol
lincosamide
oxazolidinone
fosfomycin
MDR..penam
nitroimidazole
fluoroquinolone..tetracycline.
macrolide..fluoroquinolone..penam
fluoroquinolone..Beta.lactam..penam
aminoglycoside..aminocoumarin.
pleuromutilin
Beta.lactam
fusidic.acid
MDR..aminoglycoside..penam
triclosan
pyrazinamide
sulfonamide
glycopeptide
peptide..rifamycin.
isoniazid..triclosan
fluoroquinolone..aminoglycoside.
diaminopyrimidine
nucleoside
macrolide..peptide.
nitrofuran
macrolide..lincosamide..streptogramin.
Zoliflodacin
MDR..aminoglycoside
penam
glycylcycline..tetracycline.
para.aminosalicylic.acid
aminoglycoside..Beta.lactam..penam
macrolide..penam
Beta.lactam..phenicol..penam
disinfecting.agents.and.intercalating.dyes
pactamycin
macrolide..fluoroquinolone.
isoniazid
streptogramin
penam..tetracycline.
fluoroquinolone..aminoglycoside..benzalkonium.chloride
antibacterial.free.fatty.acids
prothionamide
macrolide..penam..antibacterial.free.fatty.acids
bicyclomycin
aminoglycoside..tetracycline..phenicol.
CSab CSnoab VDnoab
B.
aminoglycoside
macrolide
MDR
peptide
tetracycline
fluoroquinolone
rifamycin
elfamycin
aminocoumarin
lincosamide
macrolide..streptogramin.
Beta.lactam..penam
phenicol
fosfomycin
MDR..penam
oxazolidinone
macrolide..lincosamide.
fusidic.acid
nitroimidazole
macrolide..fluoroquinolone..penam
fluoroquinolone..Beta.lactam..penam
aminoglycoside..aminocoumarin.
fluoroquinolone..tetracycline.
Beta.lactam
triclosan
MDR..aminoglycoside..penam
sulfonamide
mupirocin
pleuromutilin
peptide..rifamycin.
glycopeptide
diaminopyrimidine
nucleoside
penam
macrolide..lincosamide..streptogramin.
isoniazid..triclosan
fluoroquinolone..aminoglycoside.
macrolide..peptide.
MDR..aminoglycoside
pyrazinamide
nitrofuran
aminoglycoside..Beta.lactam..penam
para.aminosalicylic.acid
macrolide..penam
glycylcycline..tetracycline.
Zoliflodacin
Beta.lactam..phenicol..penam
disinfecting.agents.and.intercalating.dyes
isoniazid
fluoroquinolone..aminoglycoside..benzalkonium.chloride
prothionamide
pactamycin
antibacterial.free.fatty.acids
macrolide..penam..antibacterial.free.fatty.acids
bicyclomycin
penam..tetracycline.
streptogramin
aminoglycoside..tetracycline..phenicol.
macrolide..fluoroquinolone.
CSab VDnoab CSnoab
C.
macrolide
aminoglycoside
tetracycline
rifamycin
MDR
peptide
fluoroquinolone
mupirocin
macrolide..streptogramin.
elfamycin
Beta.lactam..penam
macrolide..lincosamide.
aminocoumarin
fosfomycin
phenicol
oxazolidinone
lincosamide
MDR..penam
nitroimidazole
fluoroquinolone..tetracycline.
macrolide..fluoroquinolone..penam
fluoroquinolone..Beta.lactam..penam
aminoglycoside..aminocoumarin.
fusidic.acid
Beta.lactam
MDR..aminoglycoside..penam
triclosan
pleuromutilin
pyrazinamide
sulfonamide
peptide..rifamycin.
glycopeptide
isoniazid..triclosan
macrolide..peptide.
fluoroquinolone..aminoglycoside.
nucleoside
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diaminopyrimidine
MDR..aminoglycoside
macrolide..lincosamide..streptogramin.
penam
Zoliflodacin
para.aminosalicylic.acid
aminoglycoside..Beta.lactam..penam
macrolide..penam
glycylcycline..tetracycline.
Beta.lactam..phenicol..penam
disinfecting.agents.and.intercalating.dyes
macrolide..fluoroquinolone.
pactamycin
isoniazid
penam..tetracycline.
fluoroquinolone..aminoglycoside..benzalkonium.chloride
streptogramin
antibacterial.free.fatty.acids
macrolide..penam..antibacterial.free.fatty.acids
prothionamide
aminoglycoside..tetracycline..phenicol.
bicyclomycin
CSab CSnoab VDnoab
log(cpm)
4
3
2
1
0

Figure S4.3: All antibiotic classes as detected using RGI were used to plot heatmap in R depicting correlation of each group to AMR class abundance at A. Overall, B. week 1 and C. at week 4. P-values < 0.05 were considered significant and were generated using Wilcoxon test with VDnoab group as reference.

Figure S4.4:

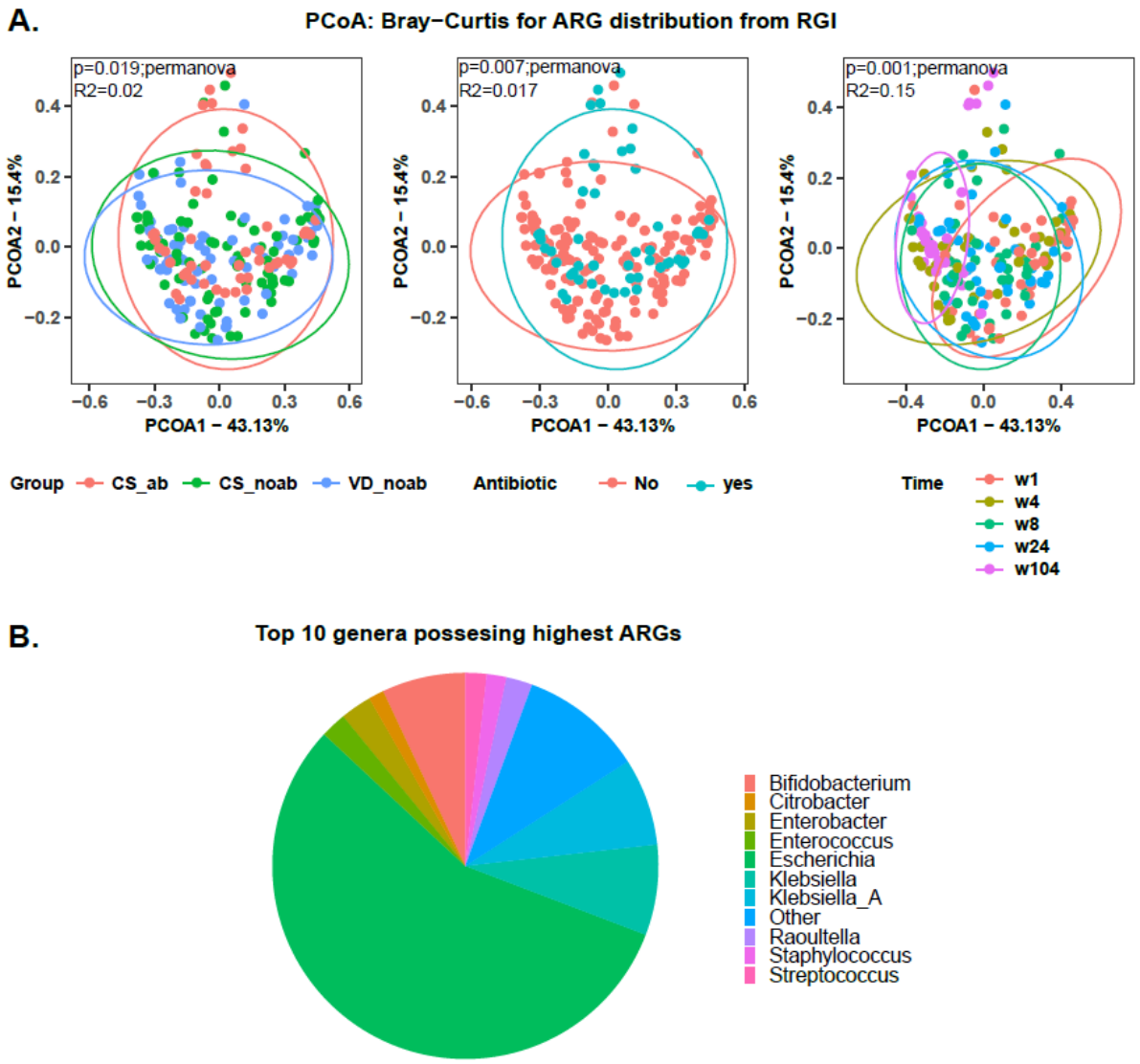


Figure S4.4: A. Beta diversity computed for ARG distribution from RGI using Bray-Curtis distance metrics and plotted using PCoA shows distinct clustering between 1. Groups, 2. Samples based on antibiotic exposure in first four days of life and 3. study time-points. B. Plot showing top 10 genera possessing highest abundance of ARGs as detected using ABRicate.

Figure S4.5:

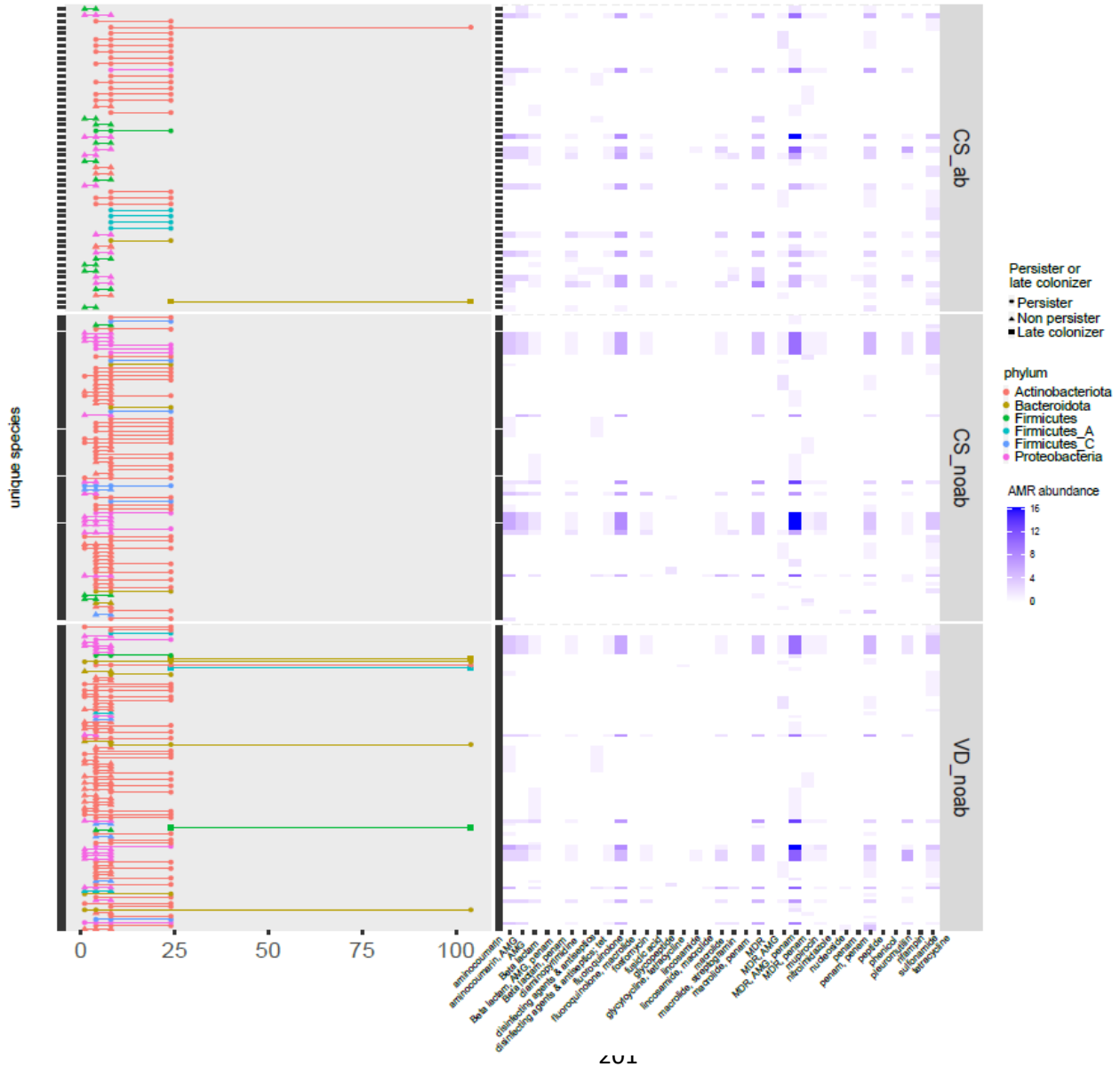

unique species
0
25
50
75
100
CS_ab
CS_noab
VD_noab
aminocoumarin
aminocoumarin, AMG
Beta lactam
Beta lactam, AMG
Beta lactam, peram
disinfecting agents & antiseptics
disinfecting agents & antiseptics, tet
fluoroquinolone
fluoroquinolone, macrolide
fosfomycin
fusidic acid
glycopeptide
glycolipopeptide, tetracycline
lincosamide, macrolide
macrolide
macrolide, streptogramin
macrolide, peram
MDR
MDR, AMG
MDR, AMG, peram
MDR, peram
nitroimidazole
nucleoside
nucleoside, peram
peram
peram, peram
peptide
phenicol
pleuromutilin
rifampin
sulfonamide
tetracycline
Persister or late colonizer
Persister
Non persister
Late colonizer
phylum
Actinobacteriota
Bacteroidota
Firmicutes
Firmicutes_A
Firmicutes_C
Proteobacteria
AMR abundance
16
12
8
4
0

Figure S4.5: Persistence of all unique strains as observed from inStrains. Right side of the plot depicts abundance of ARGs as detected by ABRicate in the form of heatmap. Left side of the plot depicts persistence of strains up to two years of age. The strains are colour coded based on the phylum which they belong to, as detected by GTDB.

Supplementary tables:

Table S4.1: Table shows coefficients obtained by running differential abundance analysis using Songbird with CSnoab group as reference run using the formula C(Group, Treatment('CSnoab')).

featureid	C(Group, Treatment('CS_noab'))[T.CS_ab]	C(Group, Treatment('CS_noab'))[T.VD_noab]
s__*Bifidobacterium_adolescentis*	-0.461624786	-0.44888641
s__*Bifidobacterium_pseudocatenulatum*	-0.376452371	0.334038301
s__*Bifidobacterium_kashiwanohense*	-0.319553137	0.823212488
s__*Bacteroides_vulgatus*	-0.304268077	0.513280137
s__*Veillonella_seminalis*	-0.29253386	-0.280086697
s__*Bifidobacterium_longum*	-0.285144448	0.768663629
s__*Parabacteroides_distasonis*	-0.264707908	0.119381948
s__*Bacteroides_dorei*	-0.243102014	0.363738581
s__*Staphylococcus_epidermidis*	-0.239515215	-0.04300128
s__*Collinsella_aerofaciens*	-0.227144569	0.618961438
s__*Escherichia_coli*	-0.218357757	0.41331832
s__*Staphylococcus_hominis*	-0.188115552	-0.132084594
s__*Veillonella_atypica*	-0.185183957	0.0767114
s__*Streptococcus_parasanguinis*	-0.185049698	-0.192258553

s__*Clostridium_perfringens*	-0.173089847	-0.374569745
s__*Ruminococcus_lactaris*	-0.146253064	-0.169122056
s__*Clostridium_paraputrificum*	-0.141725525	-0.278720648
s__*Veillonella_dispar*	-0.140300497	-0.192313359
s__*Roseburia_faecis*	-0.139978066	-0.110566572
s__*Eubacterium_hallii*	-0.139586702	-0.012941704
s__*Dorea_longicatena*	-0.118079945	-0.164423929
s__*Roseburia_inulinivorans*	-0.116796508	-0.209173308
s__*Fusicatenibacter_saccharivorans*	-0.091681316	-0.032283531
s__*Ruminococcus_bromii*	-0.073511198	-0.13761212
s__*Bacteroides_ovatus*	-0.060816064	-0.101639614
s__*Flavonifractor_plautii*	-0.056162164	-0.210859493
s__*Ruminococcus_torques*	-0.046746746	-0.085351364
s__*Bacteroides_uniformis*	-0.03068395	0.068745299
s__*Akkermansia_muciniphila*	-0.027762488	-0.154325889
s__*Eubacterium_eligens*	0.046054617	-0.109816806
s__*Erysipelatoclostridium_ramosum*	0.06502445	0.209913655
s__*Klebsiella_variicola*	0.073789433	-0.26101406
s__*Lactobacillus_paragasseri*	0.111511782	-0.180475161
s__*Streptococcus_salivarius*	0.125989512	-0.155585305
s__*Clostridium_innocuum*	0.126462594	-0.117592559
s__*Blautia_wexlerae*	0.12969543	0.108885272
s__*Streptococcus_vestibularis*	0.13077648	-0.109347896
s__*Bifidobacterium_bifidum*	0.156286195	0.518355413
s__*Anaerostipes_hadrus*	0.175960198	0.089932545
s__*Eubacterium_rectale*	0.212511137	-0.119487659
s__*Bacteroides_fragilis*	0.24164857	0.090509995
s__*Ruthenibacterium_lactatiformans*	0.251872912	-0.220478044

s__*Veillonella_parvula*	0.300057068	0.290145083
s__*Klebsiella_pneumoniae*	0.315716937	-0.316212238
s__*Faecalibacterium_prausnitzii*	0.315744594	-0.010124699
s__*Bifidobacterium_breve*	0.408126071	0.731155856
s__*Enterococcus_faecalis*	0.50914903	-0.409636241
s__*Ruminococcus_gnavus*	0.588117793	-0.232703791
s__*Bifidobacterium_dentium*	1.009432629	-0.566254036

5 Chapter 5

5 Global antibiotic resistance gene prevalence in infants – A meta-analysis

5.1 Abstract

Antibiotic resistance is a growing global concern with scientific evidence showing high abundance of antibiotic resistance genes (ARGs) in all environments including the human gut. With increasing indications of ARGs in infants, we aimed to achieve a global representation of antibiotic resistance during early life. An extensive literature search on NCBI was performed, and 26 studies were selected, including infant fecal samples sequenced up to three years of age. The 32,277 MAGs (Metagenome-Assembled Genome) generated from paired-end shotgun sequencing reads were used for resistome analysis using RGI with CARD (Comprehensive Antibiotic Resistance Database). By screening the MAGs generated from 26 publicly available study samples, the distribution of ARGs across the globe was found to be similar compositionally with varying diversity. The composition and relative abundance of ARGs varies by socioeconomic status and the healthcare access and quality (HAQ) index across the globe, with countries having lower socioeconomic index and lower HAQ index showing lower ARG abundance. Gram-negative genera including *Escherichia, Enterobacter, Citrobacter,* and *Klebsiella* were carriers of the most abundant ARGs which included classes that belong to reserve (last-resort) categories such as glycopeptides, fluoroquinolone, sulphonamides, macrolides, tetracyclines. A direct correlation with decrease in ARG abundance with time as infants age was also detected. Many of the high ARG abundant species observed are well-known pathobionts in early life. Highly abundant and diverse ARGs in their genomes points towards the infant gut acting as an ARG reservoir. This is of concern and further studies are needed to examine its consequences on long-term health.

Keywords: meta-analysis, ARG reservoir, infant gut, resistome

5.2 Introduction

Early life gut microbial colonisation has been studied extensively, thanks to the advancements in metagenomics sequencing technologies. Increasing advances and decreasing costs have resulted in several studies identifying and establishing the role and importance of early-life gut microbiota colonisation in health, innate and adaptive immunity development, and disease development in later life (Zhuang et al., 2019; Yang et al., 2016; Fouhy et al., 2019; Derrien et al., 2019). Infant gut microbiota is highly

dynamic and stabilises by the age of three years. In the past decade, several studies have delineated the effect of factors such as mode of delivery, feeding habits, gestational age, environmental factors, and antibiotic consumption on early-life microbial colonisation (Tapiainen et al., 2019; Fouhy et al., 2019; Hill et al., 2017; Martin et al., 2016). Antibiotics are among the most frequently prescribed drugs to infants, with studies reporting frequent and prolonged antibiotic use and misuse in neonates and children prophylactically and for precise reasons (Prusakov et al., 2021; Fink et al., 2020). Several adverse effects, including altered initial gut microbial colonisation, which increases the likelihood of allergic and metabolic disorders in later life (Patangia et al., 2022a), accompany the life-saving benefits of antibiotics. The worst adverse outcome of antibiotic use is antibiotic resistance development among the gut microbiota, which is considered a global threat and is responsible for the deaths of hundreds of thousands of people each year (Antimicrobial resistance collaborators., 2022; WHO., 2014 report; Aminov., 2010); reported to reach an estimate of 10 million lives at risk by 2050 unless solutions are found (O' Neill., 2016).

The number of studies reporting the presence and source of antibiotic resistance genes (ARGs) in infants has increased drastically due to the dire situation created by ARGs' increasing spread, abundance and ubiquitous nature. Recent studies have shed light on ARG abundance and the extent to which several factors affect this abundance (Parnanen 2022, 2018; Gasparrini et al., 2019; Casaburi et al., 2019). Studies have demonstrated a high abundance of ARGs in infants irrespective of antibiotic exposure; making the infant gut a reservoir of ARGs (Klassert et al., 2020; Tapiainen et al., 2019; Nogacka et al., 2017; Yassour et al., 2016). This is a serious concern as ARGs can be transmitted within bacterial communities by horizontal gene transfer, the possibility of which is high in a densely populated environment such as the gut. Furthermore, ARGs can be transferred between bacteria from different species by mobile genetic elements and, in some cases, from antibiotic-producing bacteria using conjugative methods (Jiang et al., 2017; Hu et al., 2016; Gosalbes et al., 2016; Broaders et al., 2013). The use of antibiotics in infancy disturbs the microbial composition, results in high antibiotic resistance, and can positively select for taxa with a high abundance of ARGs. Studies have further reported that bacteria belonging to Gammaproteobacteria have the highest abundance of ARGs (Lebeaux et al., 2021; Parnanen et al., 2018; Hu et al., 2013); notably, several taxa within this group are

pathobionts or potential pathogens. Infants can also acquire antibiotic-resistant strains from mothers through vertical transmission (Patangia et al., 2022b). These resistant bacteria increase the chances of disease, resulting in harder-to-treat infections in later life, increase the cost of medical care and can result in mortality (O' Neill., 2016; Aminov., 2010).

Reports of the presence and persistence of ARGs in the antibiotic naïve infant gut suggest the complex nature of this issue. And it is acknowledged that factors such as antibiotic exposure, age of life, feeding habits and mode of delivery all impact microbial composition and therefore impact the resistome profile (Parnanen 2022, 2018; Gasparrini et al., 2019; Casaburi et al., 2019). Geographic location is another factor affecting microbiota development, but not many reports have studied this factor and its association to the infant gut ARG profile. We hypothesise that this ARG reservoir could possibly update as the infant grows due to the varying environmental factors, which can add to the repertoire. We thus sought to perform a meta-analysis by selecting studies that include shotgun-sequencing data for infants under three years of age. We examined the effect of geography, mode of delivery, age, socioeconomic status, healthcare access and quality index (HAQ) on the infants' resistome profile, and provide a comprehensive picture of the global ARG burden in the infant gut. Such monitoring will provide a representation of the global ARG pool in infants in early life and help strategise antibiotic use and its alternatives.

5.3 Methods

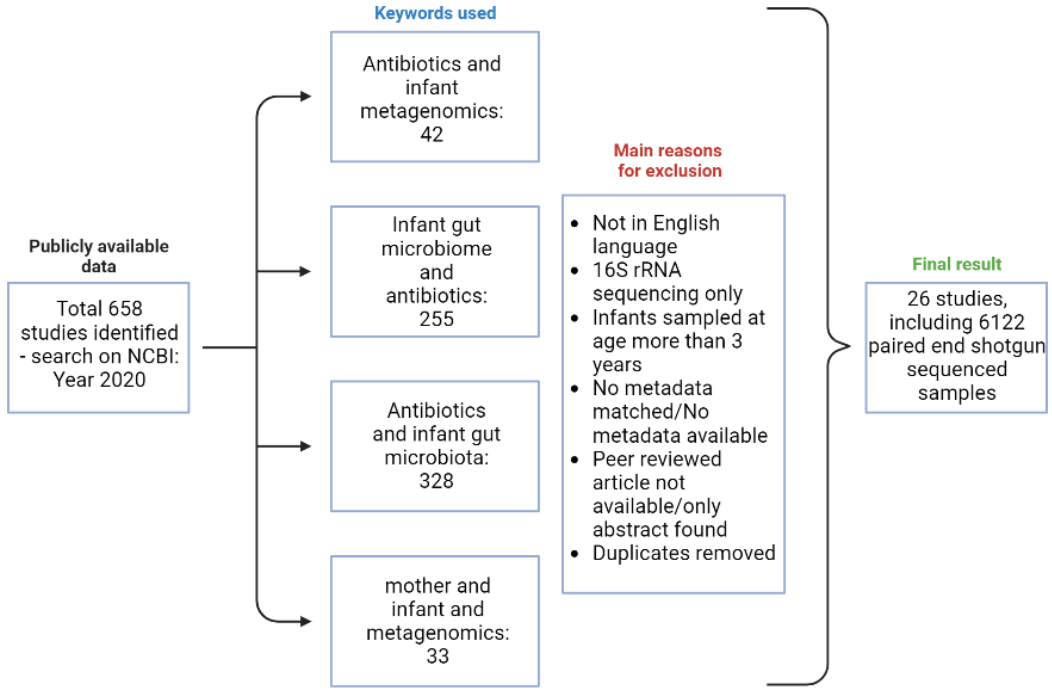


Figure 5.1: Diagrammatic representation of the study design delineating the inclusion and exclusion criteria for studies included in the meta-analysis. The figure was created using Biorender.

5.3.1 Publicly available data selection and retrieval

PubMed database using NCBI was searched to identify studies including shotgun sequencing data generation/analysis in the year 2020. This search was restricted up to June 2020 and was carried out using the following combinations of keywords: "antibiotics and infant metagenomics", "infant gut microbiome and antibiotics", "Antibiotics and infant gut microbiota" and "mother and infant metagenomics". The inclusion of papers was limited to the English language and peer-reviewed papers (Figure 5.1). 26 papers matching the inclusion criteria with available metadata were thus selected, resulting in 6122 samples, which were matched to the metadata from each paper. The metadata extracted from each paper included delivery mode, infant and maternal antibiotic information, day of life of sample collection, gender, accession ID and country of study. The shotgun sequencing data for the 6122 samples was downloaded using NCBI SRA by matching the accession IDs published for each study using Prefetch. Fastq-dump with "–

split-3" from SRA tools (v2.9.2_1) was then used to convert the downloaded SRA files to paired-end FASTQ files.

5.3.2 Data processing and antibiotic resistance analysis

FASTQ files were first trimmed and filtered to remove any human host contamination (Homo sapiens database) using KneadData (v0.7.2) with default parameters. The quality-controlled reads were then concatenated and used for taxonomic and functional characterisation using HUMAnN3 (v3.0) and MetaPhlAn3 as part of the HUMAnN3 run (Beghini et al., 2021).

Using the publicly available data from 26 studies, another study "A compendium of 32,277 metagenome-assembled genomes and over 80 million genes from the early-life human gut microbiome" was published (Zeng et al., 2022, attached as appendix to the thesis). The early-life gut genome MAGs generated as part of this study are also available online and are used in this study for resistome analysis. To examine the ARG profile globally in the infant gut, RGI main (v6.0.0) with CARD (Alcock et al., 2020) was used using default parameters on the MAGs and considered only "perfect" and "strict" hits for further analysis.

5.3.3 Bioinformatics and statistical analysis

Results from RGI were either used directly as count/richness of ARGs/antibiotic classes per MAG or after normalising to copies per million (cpm). The information about the socioeconomic statuses of countries was sourced from the World Bank (https://www.worldbank.org/en/home), while HAQ index data was sourced from GBD 2016 Healthcare Access and Quality Collaborators et al. (2018) and the data for each country included in this study is summarised in Table 5.1. Downstream processing of the results along with visualisation was performed in R using several packages including ggtree, maps, ggplot2, ComplexHeatmap, vegan and scatterpie. Statistical analysis was performed in R using packages including pairwiseAdonis, ggpubR and ggsignify.

Table 5.1: Table showing summary of socioeconomic status and healthcare access and quality index for the year 2016 for each country included in the analysis.

Country	Low-middle or High income (World bank classification 2016)	Healthcare access and quality (HAQ) index – for 2016, absolute change from 2000-2016
Bangladesh	Lower middle	47.6(44.3-50.9), 20.1 (16.3-23.8)
Estonia	High	85·9 (83·6 to 88·3), 14·3 (11·8 to 17·0)
Finland	High	95·9 (94·6 to 96·9), 8·1 (6·7 to 9·5)
Italy	High	94·9 (93·4 to 96·0), 6·1 (4·7 to 7·4)
Luxembourg	High	96·0 (94·4 to 97·3), 5·7 (3·9 to 7·4)
New Zealand	High	92·4 (90·3 to 94·3), 5·4 (3·1 to 7·4)
Russia	Upper middle income	75·1 (67·7 to 81·7), 12·6 (5·0 to 19·4)*
Singapore	High	90·6 (87·2 to 93·3), 10·9 (7·1 to 14·8)*
Sweden	High	95·5 (93·4 to 97·2), 3·1 (1·0 to 5·0)
UK	High	90·5 (89·6 to 91·3), 6·5 (5·9 to 7·2)
USA	High	88·7 (88·0 to 89·4), 1·9 (1·4 to 2·5)

5.4 Results

This meta-analysis dataset included 26 studies altogether, comprising a very comprehensive dataset. The meta-analysis included studies conducted across the globe including North America (2420 infants), the United Kingdom (1519), New Zealand (646), Europe (1162), Asia (70) and Russia (305), spanning four continents. Of the 6122 infants included in the meta-analysis, 2685 (43.85%) were born by Caesarean-section birth mode and 3427 (55.97%) were vaginally delivered with no information about 10 (0.16%). Other variables considered include the socioeconomic status of the country and the Healthcare Access and Quality (HAQ) index. The HAQ index obtained on a scale of 1 to 100 (Table

5.1) was then transformed into the following categories: very low/below (<=50), low/below (<=80) and above (>80). Resistance to several antibiotic classes belonging to each of the AWaRe classes as classified by the World Health Organisation (WHO) including glycopeptide, fluoroquinolone, tetracycline, aminoglycosides, rifamycin to name a few along with multi-drug resistance (MDR) was observed.

5.4.1 Global antibiotic resistance distribution in infants

Of the 6122 infants included, samples corresponding to 5886 infants demonstrated resistance to at least one antibiotic class and were included for downstream analysis. The presence of 102 different classes of antibiotics including several MDR classes using RGI with CARD were detected. The MAGs ARG abundance data was normalised to copies per million (cpm) and filtered to include ARG classes having mean abundance of more than five and represented as a Heatmap (Figure 5.2). Resistance to several antibiotics including glycopeptides, peptides, fluoroquinolone, tetracyclines, macrolides and rifamycin was seen in all countries. To gain a more detailed understanding, the top five most abundant antibiotic classes per country were examined (Figure 5.3) and glycopeptide was found to be the most abundant ARG class and was present in all countries except Singapore. The other ARGs that are seen in all countries provided resistance to fluoroquinolone antibiotic, and a MDR class (which confers resistance to fluoroquinolone, cephalosporin, glycycline, penam, tetracycline, rifamycin, phenicol antibiotic, disinfecting agents and antiseptics). Other classes observed in the top five are aminocoumarin, macrolide & fluoroquinolone, rifamycin, tetracycline, phosphonic acid antibiotic, fluoroquinolone & tetracycline, and peptide antibiotic. Further, the radius of each pie corresponds to the total ARG abundance and countries like Luxembourg, the USA, and the UK show very high ARG abundance.


Continent
glycopeptide
peptide
FQ;cephalosporin;glycylcycline;penam;TET;rifamycin;phenicol;disinfecting agents & antiseptics
FQ
macrolide;FQ;penam
TET
AMG;AMC
AMG
nucleoside;disinfecting agents & antiseptics
AMC
phosphonicacid
macrolide;AMG;cephalosporin;TET;peptide;rifamycin;disinfecting agents & antiseptics
FQ;monobactam;carbapenem;cephalosporin;glycylcycline;cephamycin;penam;TET;rifamycin;phenicol;penem;disinfecting agents & antiseptics
rifamycin
FQ;TET
macrolide;FQ;AMG;carbapenem;cephalosporin;penam;peptide;penem
FQ;glycylcycline;TET;DAP;nitrofuran
penam
FQ;cephalosporin;penam;TET;peptide;disinfecting agents & antiseptics
macrolide;streptogramin;streptograminB
FQ;lincosamide;nucleoside;phenicol;disinfecting agents & antiseptics
macrolide;FQ;rifamycin
lincosamide;streptogramin;streptograminA;streptograminB;pleuromutilin
SN
cephamycin
carbapenem;peptide;AMC;rifamycin
mupirocin−like
macrolide;lincosamide;streptogramin;streptograminA;streptograminB
carbapenem;cephalosporin;cephamycin;penam
monobactam;cephalosporin;penam;penem
nucleoside
glycylcycline;TET
fusidane
elfamycin
carbapenem;cephalosporin;penam
monobactam;carbapenem;cephalosporin;cephamycin;penam;penem
monobactam;cephalosporin;penam
cephalosporin;penam;penem
FQ;AMG;penam;TET;disinfecting agents & antiseptics
phenicol
macrolide;FQ;monobactam;carbapenem;cephalosporin;cephamycin;penam;TET;peptide;AMC;DAP;SN;phenicol;penem
macrolide;FQ;AMG;cephalosporin;penam;TET;AMC;DAP;phenicol
FQ;TET;disinfecting agents & antiseptics
macrolide;monobactam;TET;AMC
cephalosporin;penam
macrolide;carbapenem;TET;DAP;phenicol;disinfecting agents & antiseptics
macrolide;TET;disinfecting agents & antiseptics
macrolide;FQ;TET;phenicol;disinfecting agents & antiseptics
macrolide;FQ;AMG;carbapenem;cephalosporin;cephamycin;penam;TET;phenicol;disinfecting agents & antiseptics
macrolide;FQ;monobactam;AMG;carbapenem;cephalosporin;cephamycin;penam;TET;phenicol;penem;disinfecting agents & antiseptics
lincosamide
macrolide;AMG;oxazolidinone;DAP;phenicol
carbapenem
cephalosporin;cephamycin
FQ;AMG
macrolide;FQ;lincosamide;carbapenem;cephalosporin;TET;rifamycin;DAP;phenicol;penem
macrolide;FQ
disinfecting agents & antiseptics;isoniazid−like
macrolide;lincosamide
FQ;cephalosporin;glycylcycline;penam;TET;oxazolidinone;glycopeptide;rifamycin;phenicol;disinfecting agents & antiseptics
macrolide;FQ;monobactam;AMG;carbapenem;cephalosporin;cephamycin;penam;TET;peptide;AMC;DAP;SN;phenicol;penem
bicyclomycin−like
macrolide;FQ;monobactam;AMG;carbapenem;cephalosporin;cephamycin;penam;TET;peptide;AMC;DAP;SN;phenicol;penem;disinfecting agents & antiseptics
FQ;AMG;disinfecting agents & antiseptics
FQ;cephalosporin;cephamycin;penam
macrolide;FQ;penam;TET
cephalosporin;cephamycin;penam
nitroimidazole
FQ;DAP;phenicol
TET;disinfecting agents & antiseptics
macrolide
DAP
cephalosporin
macrolide;FQ;cephalosporin;cephamycin;penam;TET
macrolide;FQ;AMG;carbapenem;cephalosporin;glycylcycline;cephamycin;penam;TET;peptide;AMC;rifamycin;phenicol;penem;disinfecting agents & antiseptics
FQ;cephalosporin;glycylcycline;cephamycin;penam;TET;rifamycin;phenicol;disinfecting agents & antiseptics
disinfecting agents & antiseptics
FQ;disinfecting agents & antiseptics
Luxembourg
US
Italy
UK
Singapore
Finland
Sweden
Estonia
New_Zealand
Bangladesh
Russia
Mean(ARG abundance (cpm)), filter > 5
2000
1500
1000
500
0
Continent
Asia
Europe
North America
Oceania

Figure 5.2: Heatmap demonstrating all the classes of antibiotics per country which have mean abundance as more than five cpm. Columns in the heatmap represent country while rows represent antibiotic classes with top row annotation showing continents. Some of the antibiotic classes are abbreviated in the heatmap as follows: AMG = "aminoglycoside", TET = "tetracycline", RIF = "rifampin", FQ = "fluoroquinolone", DAP = "diaminopyrimidine", SN = "sulfonamide" and AMC = "aminocoumarin".

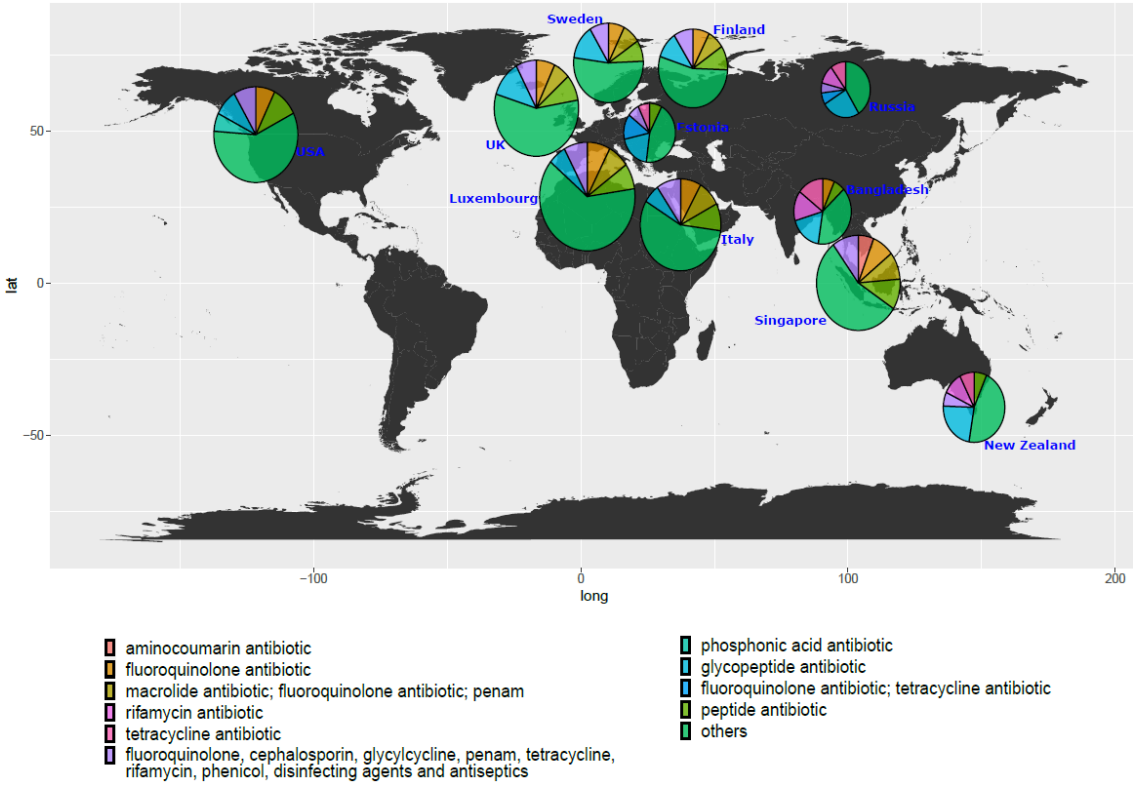


Figure 5.3: World map showing the top five antibiotic resistance classes per country while the remaining classes are represented as "others" in the pie charts for each country. The abundance depicted is normalised to cpm. The radius of each pie corresponds to the square root of square root of the total ARG abundance per country.

5.4.2 Most abundant ARG classes are carried by gram-negative bacteria

Next, we examined the top 20 species globally that carry the highest ARG abundance, and found that the majority of these species are gram-negative bacteria (Figure 5.4). Both

by normalising the data to cpm and by using raw count data, similar results were seen in that species belonging to the genera *Bacillus, Citrobacter, Enterobacter, Erwinia, Escherichia, Haemophilus, Klebsiella, Pseudomonas* and *Salmonella* carry high abundance of ARG. Further, inspecting the top five species per country carrying the highest ARG abundance (Figure S5.1) showed that species belonging to genera *Escherichia, Enterobacter, Klebsiella* were in this category for most countries while species belonging to genera *Pseudoescherichia* and *Bifidobacterium* were present in some countries like New Zealand, Bangladesh and the UK.

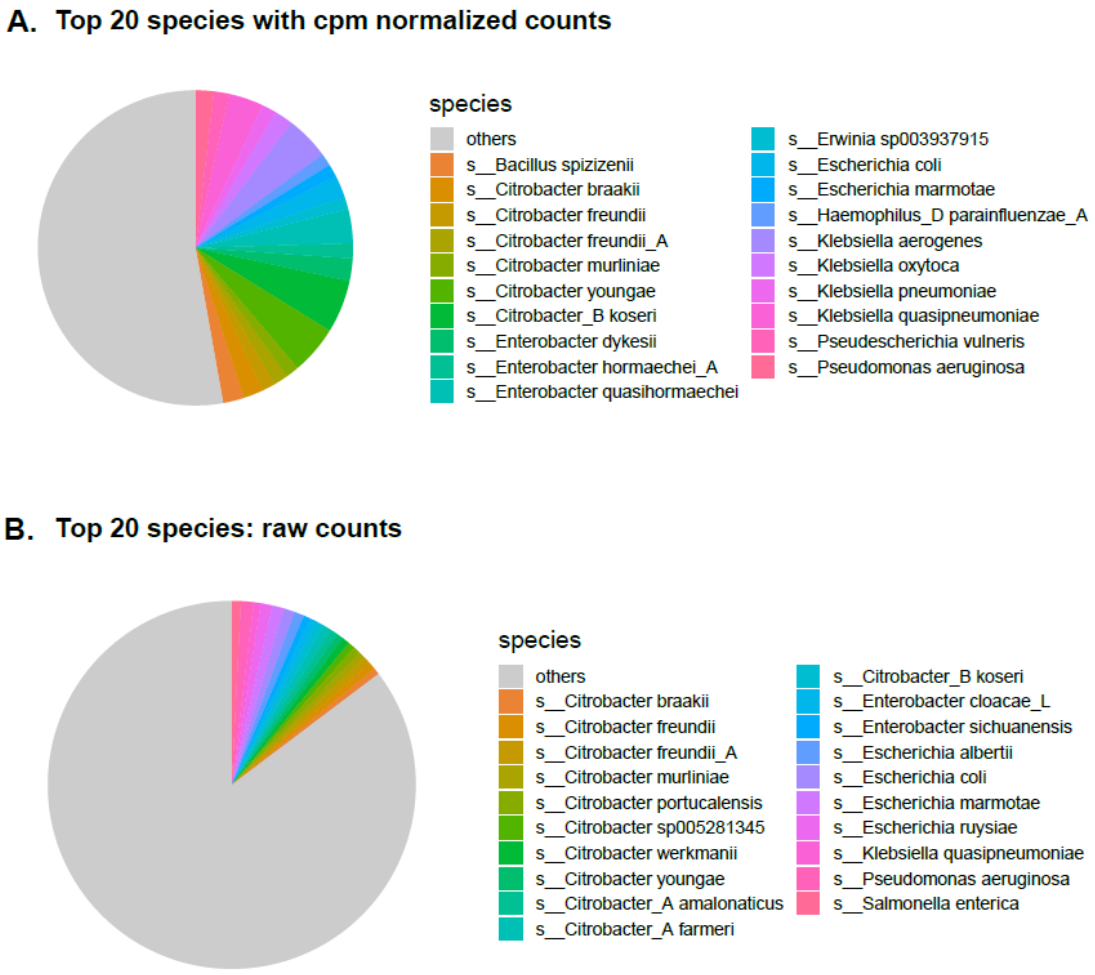


Figure 5.4: Pie chart represents the top 20 species that carry highest ARG abundance globally in infants under three years of age using A. abundance normalised to cpm and B. raw counts. With the remained of the classes represented as “others”.

Furthermore, we sought to examine the top five most abundant AR classes per country and the species carrying those classes (Figure 5.5). The most abundant ARG classes and genera results were complimentary to the above results, with the top five species belonging to the genera *Bifidobacterium*, *Citrobacter*, *Erysipelatoclostridium*, *Escherichia*, *Pseudomonas* and *Staphylococcus* and AR classes being glycopeptide, fluoroquinolone, tetracycline, rifamycin among others along with MDR.

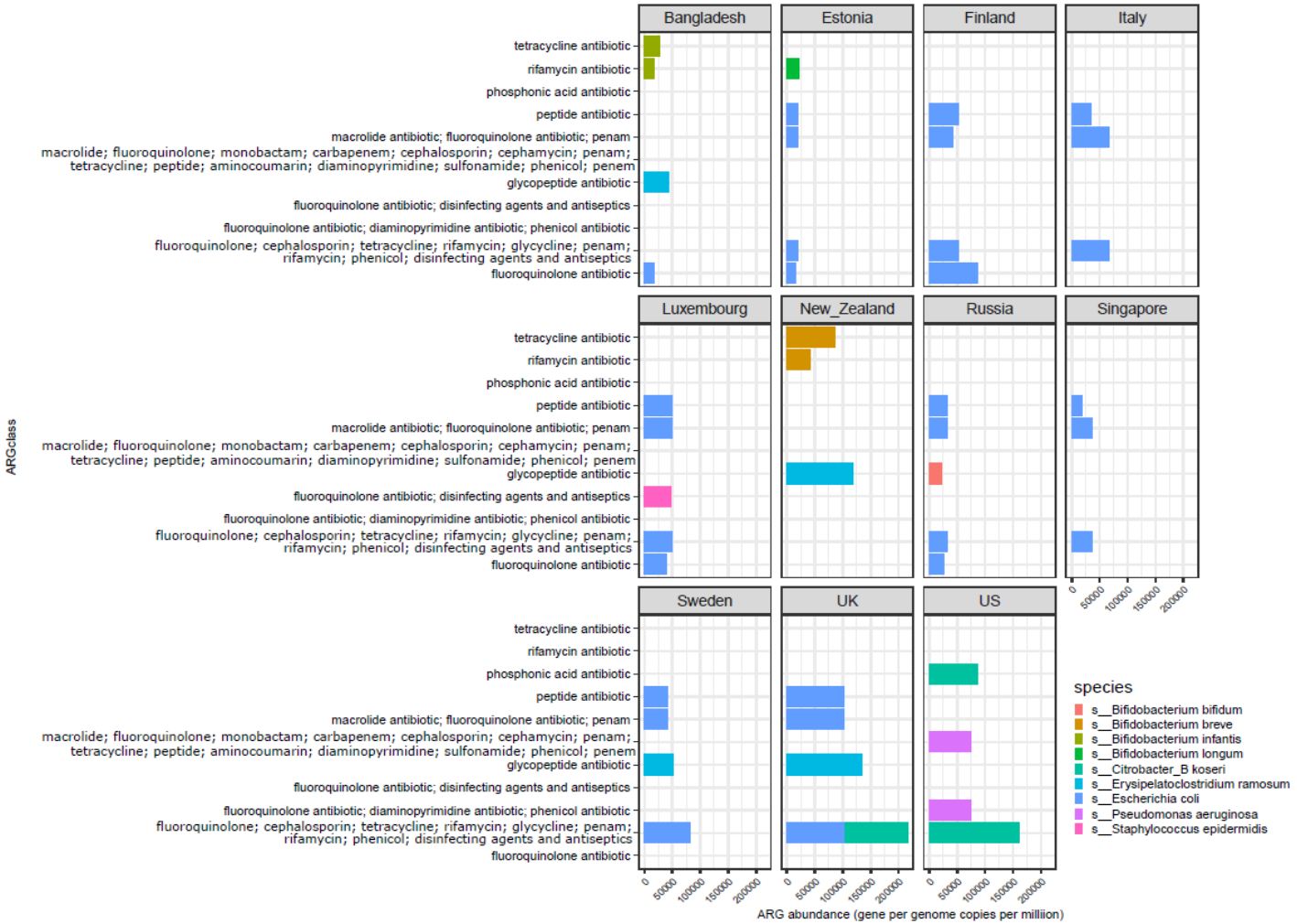


Figure 5.5: Bar plot shows the top five antibiotic resistance classes per country and the species carrying them. The ARG abundance is normalised to cpm.

5.4.3 Antibiotic class abundance and diversity vary by country, continent, socioeconomic status and HAQ index

The relation between ARG class abundance and age of infants in months, socioeconomic status and the healthcare access and quality (HAQ) index was assessed (Figure 5.6). Significant differences in the abundance of ARG classes were found, based on these variables as measured by the Wilcoxon test. As a general trend, higher abundance of ARGs was seen in very early life which decreased with time (Figure 5.6A), and a significant p-value showing direct correlation was seen (p-value=2.2e-16, spearman correlation). While the lowest ARG abundance was observed in countries corresponding to very low HAQ index (Figure 5.6B), no significant correlation between ARG abundance and HAQ index using spearman correlation was observed (p=0.09). Furthermore, the ARG abundance also varied by the country's socioeconomic status to which the HAQ index is associated; with lower- and middle-income countries having low HAQ and thus lower ARG abundance.

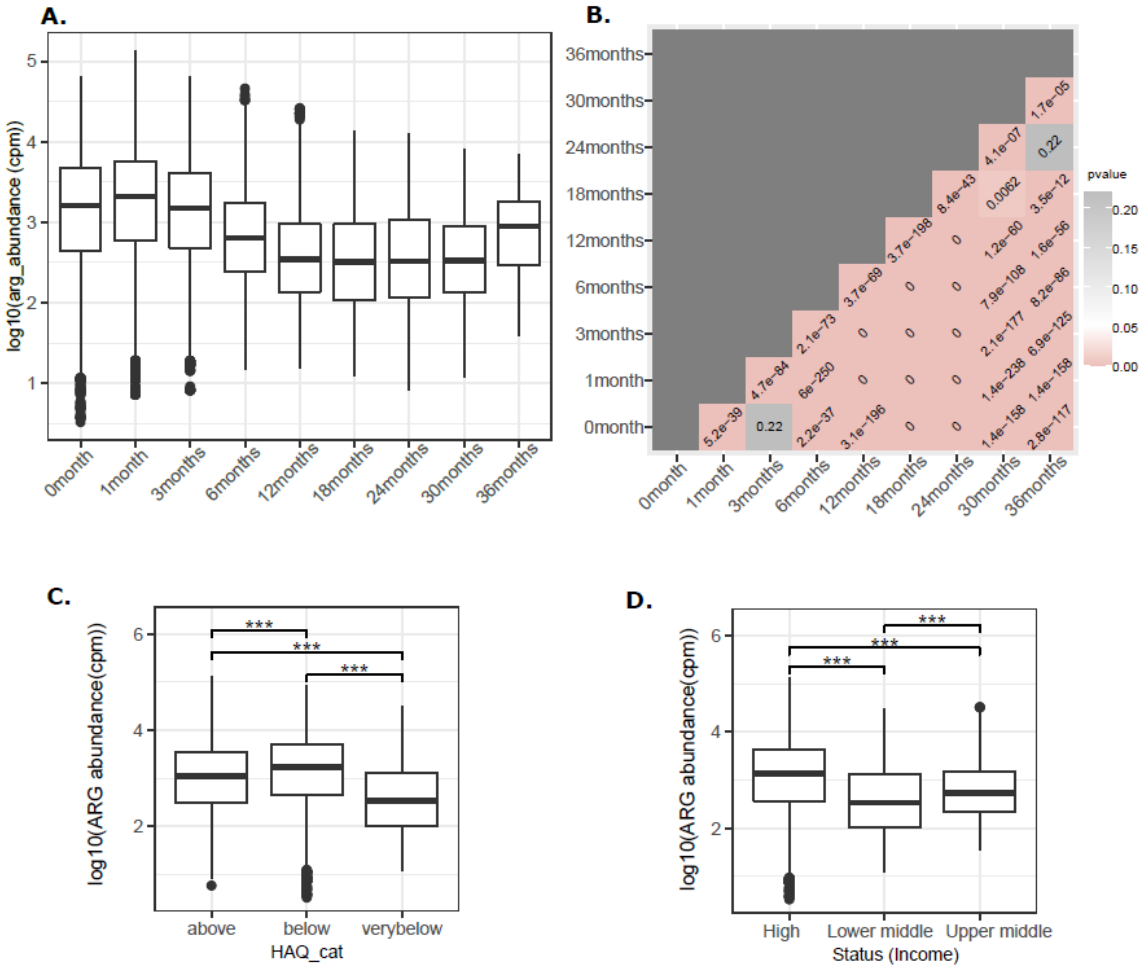


Figure 5.6: Box plot depicting the association between log of ARG abundance as normalised in cpm to A. Age in months, B. Heatmap showing p.adj values obtained from Wilcoxon test comparing two time points, with the formula arg_abundance ~ month with ggpubr package in R. Box plot showing the association between log-transformed ARG abundance normalised to cpm and C. Healthcare access and quality (HAQ) index and D. the socioeconomic status of countries. Significance was determined using the ggpubr package in R using Wilcoxon test.

To understand the relationship between antibiotic resistance class diversity and study variables, alpha and beta diversity analysis was performed. Alpha diversity analysis using Shannon's index for evenness and richness presented that countries including Luxembourg and Singapore have high ARG abundance with low variance (Figure 5.7), while countries such as the UK, USA and Italy show high relative abundance and high variance in the Shannon diversity index. Between country alpha diversity using Shannon index is significantly different between Luxembourg, Singapore and all other countries and for the UK and the USA to the majority of other countries (p-values using the Wilcoxon test: Table S5.1).

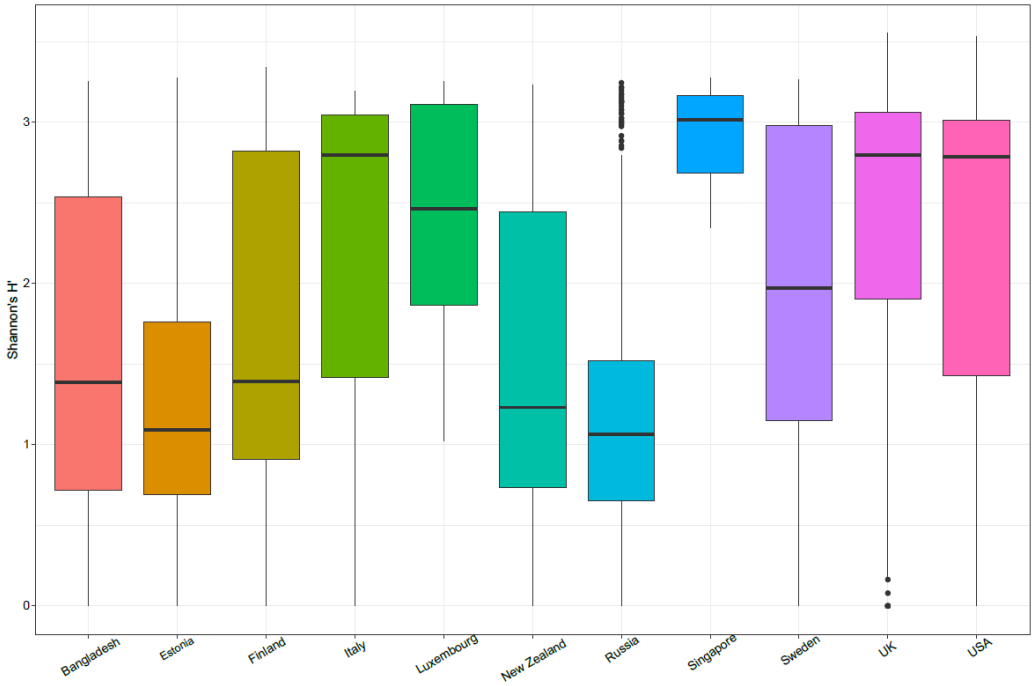


Figure 5.7: Box plots show alpha diversity analysis on cpm normalised AR abundance data using Shannon's index.

Beta diversity analysis (Figure 5.8) using Jaccard distances along with Adonis and pairwiseAdonis test in R confirmed that variables such as socioeconomic status of the country (p-value=0.001), continent (p-value=0.001), healthcare access and quality index (p-value=0.001) have a significant impact on the AR class diversity in infants in early life. PairwiseAdonis test in R confirmed significant clustering based on HAQ index, socioeconomic status and continent pairs except for Asia and Europe (Table 5.2).

Table 5.2: Results from PairwiseAdonis test in R showing p-value and p.adjusted values for comparison for beta diversity analysis.

HAQ category							
pairs	Df	SumsOfSqs	F.Model	R2	p-value	p.adj	sig
verybelow vs below	1	1.177258	5.218574	0.00184	0.002	0.006	*
verybelow vs above	1	0.809732	3.972389	0.001284	0.014	0.042	.
below vs above	1	9.47106	44.24523	0.007512	0.001	0.003	*
Status							
Lower middle income vs high income	1	1.042999	4.95991	0.000884	0.002	0.006	*
Lower middle income vs upper middle income	1	2.581248	11.99847	0.03647	0.001	0.003	*
High income vs upper middle income	1	31.34937	149.0585	0.024864	0.001	0.003	*
Continent							
Asia vs Europe	1	0.949689	4.509252	0.001535	0.01	0.06	
Asia vs Oceania	1	2.958477	13.98324	0.020091	0.001	0.006	*
Asia vs North America	1	0.988634	4.850663	0.002026	0.002	0.012	.

Europe vs Oceanic	1	19.86468	93.87106	0.026171	0.001	0.006	*
Europe vs North America	1	14.57863	70.07686	0.013297	0.001	0.006	*
Oceania vs North America	1	36.42061	176.5437	0.056484	0.001	0.006	*

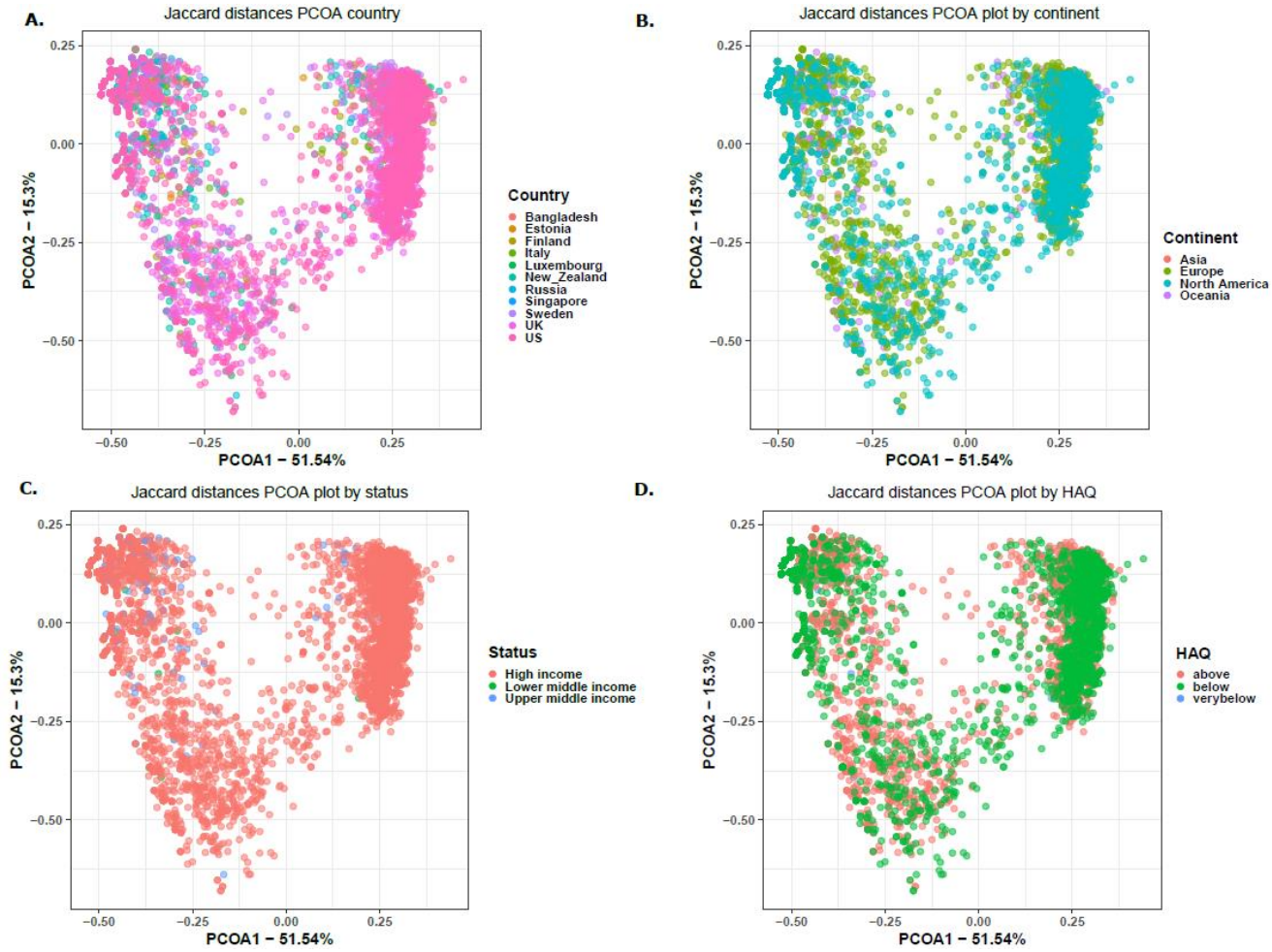


Figure 5.8: Beta diversity as represented using PCoA computed using Jaccard distances showing clustering by A. Country, B. Continent, C. Socioeconomic status and D. HAQ index category.

5.4.4 Antibiotic efflux is the most common resistance mechanism in infants

The antibiotic resistance mechanisms that were most common in infants under three years of age globally were investigated. Antibiotic efflux was the most common mechanism of resistance which was followed by antibiotic target alteration and inactivation overall globally and per country (Figure 5.9, Figure S5.2).

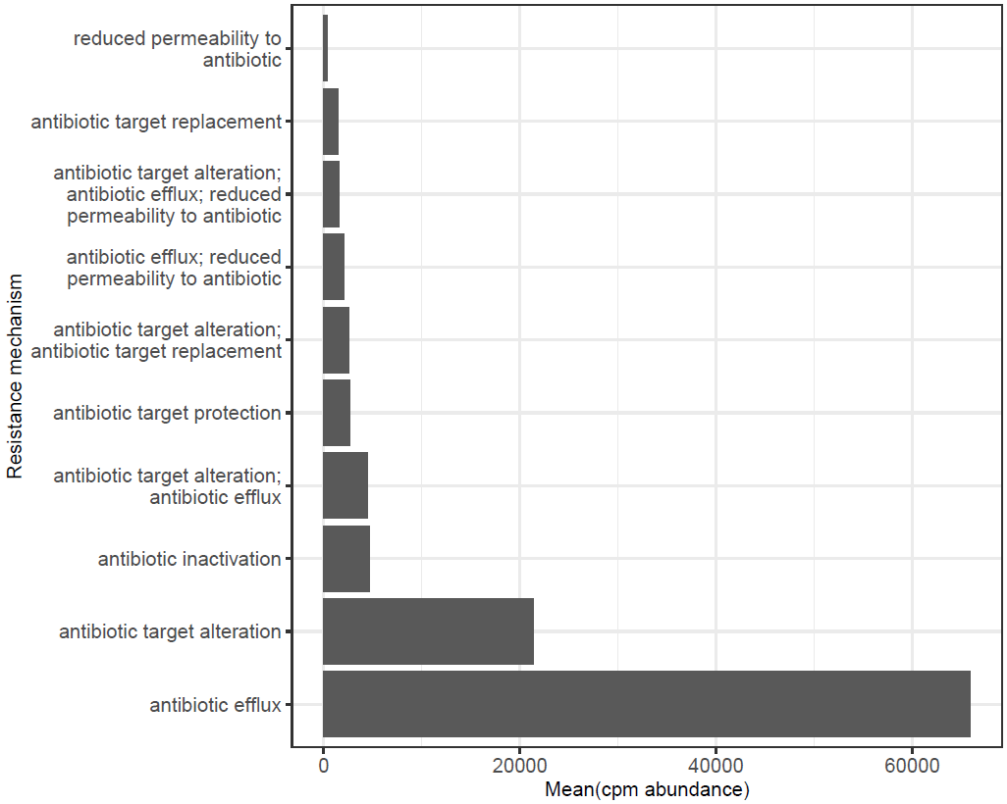


Figure 5.9: Barplot depicting the various antibiotic resistance mechanisms observed in infants under three years of age as detected using RGI with CARD. The abundance is shown as an average of normalised values.

5.5 Discussion

Several studies to date have reported the global trends of antibiotic use over the years, associating it with age group, disease type and income status of the country (Prusakov et al., 2021; Browne et al., 2021; Brown et al., 2020; Klein et al., 2018). These studies provide a comprehensive view of the worldwide antibiotic consumption and report increasing use of last-resort drugs such as carbapenems, glycyclines, and 3rd generation cephalosporins. Thus, enabling policy makers to make informed decisions to tackle the AMR issue. However, very few studies have investigated the global resistome profile of

the infant gut microbiome. The rise and spread of AMR, which is many fold higher than the antibiotic consumption, is due to over-use and misuse of antibiotics, and increasing globalisation. In this study, we analysed shotgun-sequencing data from infants under three years of age to study the worldwide resistome profile in early life. Our objective was to summarise AMR data in multiple countries, which along with already available antibiotic use data will help in forming better antibiotic stewardship programs. The resistance to several drug classes categorised as "reserved" by the WHO was observed to be high in all the countries included in this study. This included antibiotic classes such as glycopeptides, tetracyclines, and aminoglycosides to name a few. These antibiotic classes are reserved and suggested to be used as last resort antibiotics, the presence of resistance for reserve category antibiotics in early life is alarming.

Antibiotic consumption profile have been reported to differ between low vs high-income countries, with low and middle-income countries showing a high and increasing trend for antibiotic consumption between 2005-2017. Indeed, in some low-middle-income countries, the rate of antibiotic use was reported to increase from 2010-2015 but it varied by antibiotic type, and the overall global rates were high (Brown et al., 2020; Jackson et al., 2019). A high rate of antibiotic consumption in high-income and upper-middle-income countries has also been reported (Browne et al., 2021). The socio-demographic status of a country is shown to be associated with healthcare access and quality (GBD 2016 Healthcare Access and Quality Collaborators, 2018) and a small hike in the misuse of antibiotics is observed in middle and high-income countries (Mallah et al., 2022). This could be because people in high-income countries have easier access to antibiotics.

From the countries included in our study, as per the 2016 World Bank classification, only Bangladesh was classified as lower-middle income country and Russia as an upper-middle-income country while all other countries were high-income (https://datatopics.worldbank.org/world-development-indicators/the-world-by-income-and-region.html). These socioeconomic statuses correspond to the HAQ status with Bangladesh having the lowest HAQ (47.6), and Russia being second with 75.1 in 2016 amongst the countries included in our study (GBD 2016 Healthcare Access and Quality Collaborators, 2018). Our results are in line with the published reports as we also observed a difference in abundance and diversity of ARG classes based on these variables with low HAQ index showing lower relative ARG abundance, without any significant

correlation. A similar relationship was observed regarding socioeconomic status where high-income countries showed higher relative ARG class abundance, followed by upper- and lower-middle countries in our study.

Furthermore, a decrease in ARG class abundance with age in months was identified, this correlation can be linked to the developing dynamic infant gut microbiota in early life. The species carrying the highest ARG abundance in our study were predominantly gram-negative and belonged to the genera *Citrobacter, Escherichia, Enterobacter, Pseudomonas,* and *Klebsiella* which are all known pathobionts in infants which can cause sepsis in infants (Camacho-Gonzalez et al., 2013). Some *Bifidobacterium* species also showed high ARG abundance. Further study is required to understand the relationship between this high ARG abundance and *Bifidobacterium* functional profile.

Certain advantages of the current meta-analysis is that it used the most updated global early life gut genome (ELGG) reservoir generated from studies in multiple countries. Our analysis adds on to our previous study (Zeng et al., 2022) but is independent of antibiotic use or the health status of the individuals as this metadata was absent in many cases. Certain limitations are that as MAGs are generated only from shotgun-sequencing data we were restricted to the number of studies/countries passing the inclusion criteria and included in the analysis. Further, the depth of sequencing, timepoints of longitudinal sample collection, varying time duration of the studies involved are all factors which restrict the analysis.

In conclusion, antibiotic resistance was high in infants in all the countries included in our study and was associated to the geography, age and demographics of the country. Further, studies that combine antibiotic use data with resistance data in infants are needed to help limit the use of antibiotics in early life. Indeed, it has been reported that the presence of specific antibiotic stewardship programs reduces the use of antibiotics in Neonatal intensive care units (NICU) (Prusakov et al., 2021). This study emphasises the urgent need for stricter guidelines and regularly updated antibiotic stewardship programs for infant groups globally.

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5.8 Supplementary material

Supplementary figures:

Figure S5.1:



Figure S5.1: Barplot depicts top 5 species per country carrying highest ARG abundance. The ARG abundance is depicted as mean of cpm normalised abundance values.

Figure S5.2:

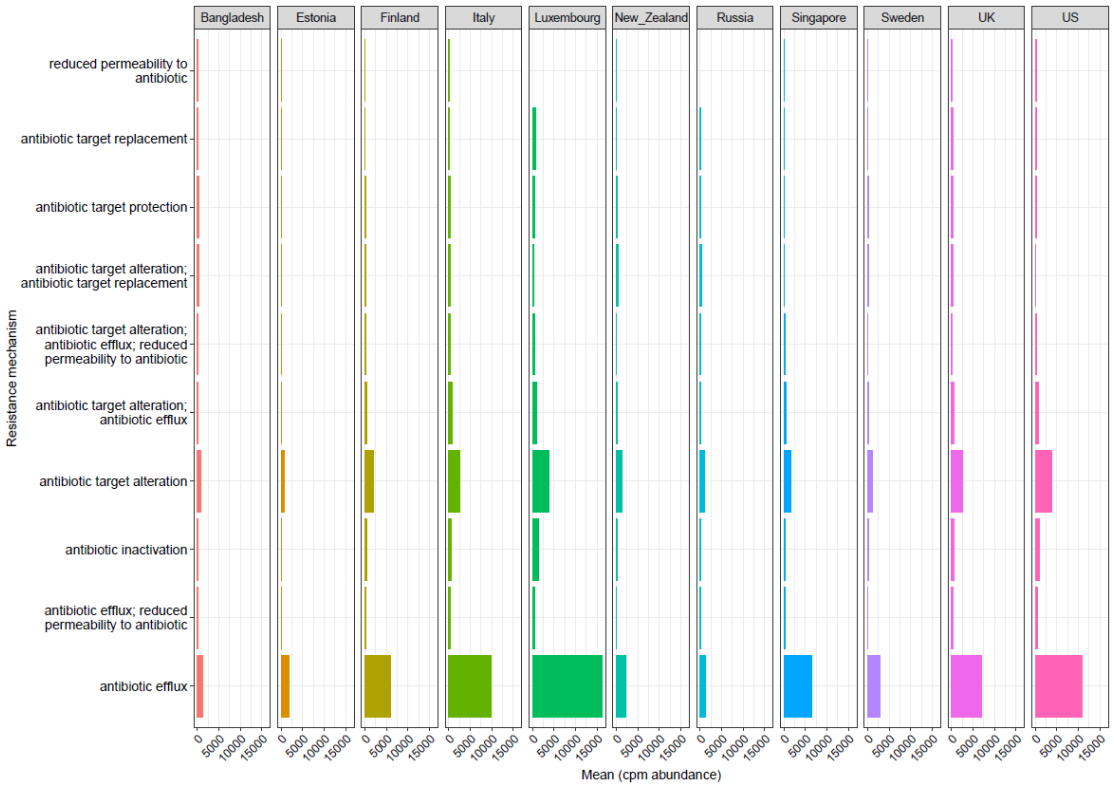


Figure S5.2: Bar plot showing common antibiotic resistance mechanisms observed in all the countries included in our meta-analysis. The abundance is shown as mean of normalised cpm values.

Supplementary table:

Table S5.1: Presents p-values and p.adjusted values based on Wilcoxon test for each country pair comparison accompanying Alpha diversity analysis.

group1	group2	p-value	p.adj
Bangladesh	Estonia	0.226949	1
Bangladesh	Finland	0.529961	1
Bangladesh	Italy	0.000673	0.013
Bangladesh	Luxembourg	0.000275	0.0055
Bangladesh	New_Zealand	0.702701	1
Bangladesh	Russia	0.048663	0.58

Bangladesh	Singapore	1.55E-06	4.30E-05
Bangladesh	Sweden	0.012612	0.18
Bangladesh	UK	5.39E-07	1.60E-05
Bangladesh	US	0.000157	0.0034
Estonia	Finland	0.00014	0.0032
Estonia	Italy	5.20E-12	2.10E-10
Estonia	Luxembourg	1.78E-09	6.40E-08
Estonia	New_Zealand	0.06707	0.67
Estonia	Russia	0.249198	1
Estonia	Singapore	1.58E-10	5.90E-09
Estonia	Sweden	1.32E-13	5.70E-12
Estonia	UK	6.08E-42	3.00E-40
Estonia	US	8.29E-27	4.10E-25
Finland	Italy	3.54E-07	1.10E-05
Finland	Luxembourg	3.98E-06	0.00011
Finland	New_Zealand	0.003437	0.052
Finland	Russia	4.04E-09	1.40E-07
Finland	Singapore	1.22E-08	4.20E-07
Finland	Sweden	7.79E-06	2.00E-04
Finland	UK	3.67E-45	1.90E-43
Finland	US	7.50E-25	3.60E-23
Italy	Luxembourg	0.157284	1
Italy	New_Zealand	2.83E-12	1.20E-10
Italy	Russia	1.15E-16	5.30E-15
Italy	Singapore	0.00108	0.019
Italy	Sweden	0.013352	0.18
Italy	UK	0.115483	1
Italy	US	0.612954	1
Luxembourg	New_Zealand	1.88E-08	6.20E-07
Luxembourg	Russia	2.54E-11	9.90E-10
Luxembourg	Singapore	0.050678	0.58
Luxembourg	Sweden	0.001603	0.027

Luxembourg	UK	0.680141	1
Luxembourg	US	0.119232	1
New_Zealand	Russia	9.43E-05	0.0023
New_Zealand	Singapore	7.97E-11	3.00E-09
New_Zealand	Sweden	1.96E-14	8.80E-13
New_Zealand	UK	8.27E-86	4.50E-84
New_Zealand	US	1.05E-55	5.60E-54
Russia	Singapore	3.20E-12	1.30E-10
Russia	Sweden	3.28E-22	1.50E-20
Russia	UK	1.98E-71	1.10E-69
Russia	US	7.31E-49	3.80E-47
Singapore	Sweden	1.05E-06	3.00E-05
Singapore	UK	0.001773	0.028
Singapore	US	0.000219	0.0046
Sweden	UK	2.61E-14	1.10E-12
Sweden	US	8.41E-05	0.0021
UK	US	1.91E-08	6.20E-07

Chapter 6

6 Gut Microbiome and Resistome Profile of Cystic Fibrosis Individuals - A Pilot Study

6.1 Abstract

Cystic fibrosis (CF) individuals are prone to bacterial infections due to the build-up of large amounts of mucus in their lungs making them chronic antibiotic users. The airway microbiota in CF individuals is altered during the course of disease and treatment. Disruption of Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene function along with antibiotic use including last-line antibiotics, causes variations in the gut microbiota and resistome profile. However, the gut resistome of CF individuals has not been extensively studied. The aim of this study was to examine the effect of chronic antibiotic exposure on the CF gut microbial and resistome profiles. Shotgun sequencing data from six CF and six non-CF healthy controls from a previous study were used. Downstream processing was performed using Kraken2 for taxonomic classification and ABRicate with Comprehensive Antibiotic Resistance Database (CARD) and Virulence Factor Database (VFDB) for resistance and virulence analysis. CF and control groups had significant differences in beta diversity showing distinct clustering (p-value=0.006 at genera level, and p-value=0.009 at species level); with increased abundance of Firmicutes and decreased Bacteroidetes in the CF group. Differences between the two groups showed several taxa including *Clostridium* spp., *Enterococcus*, *Blautia*, and *Eggerthela* among others to be more associated to the CF group while *Faecalibacterium*, *Coprococcus*, *Subdoligranulum* were related to controls. The resistome profile of CF and control groups differed significantly, with the CF group presenting a more diverse (p-value=0.002, Shannon index) and abundant resistance gene reservoir (Wilcoxon test: p-value=8.5e-11) compared to controls. Several antibiotic classes, including those administered to the CF subjects in this study, such as macrolide, tetracycline, fluoroquinolone, oxazolidinone, and sulfonamide, were more associated with the CF group. The virulence factor (Wilcoxon test: p-value=6.5e-06) profile in CF was also higher, with changes in functional profiles reported previously. The varying microbial composition along with the presence of high numbers of virulence factors and resistance genes in CF individuals is of concern as altered functionality of the gut microbial ecosystem and antibiotic-resistant pathogens can make treatment more complicated by lowering effectiveness of future antibiotic therapies.

Keywords: Antibiotic resistance gene, Cystic fibrosis, gut microbiota, resistome.

6.2 Introduction

Cystic fibrosis (CF) individuals are subjected to chronic antibiotic exposure due to their underlying condition and polymicrobial infections owing to increasing multidrug-resistant bacteria (Taccetti et al., 2021). CF is a genetic disorder caused due to mutation in the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) gene thus leading to a reduction in functional CFTR proteins. This affects the transport of chloride and bicarbonate ions, which causes hypersecretion of viscous mucus in the airways and digestive tract. Impaired mucociliary clearance leads to infections, inflammation and respiratory distress. The presence of the CFTR protein throughout the body on the apical layer of epithelial cells leads to multiple complications including pancreatic insufficiency, impact on growth and gastrointestinal dysfunction (Modolell et al., 2002; Van Elburg et al., 1996). This is accompanied by alterations in the microbiota of the lungs and respiratory tract as their habitat environment changes; with changes in gut microbiota in individuals with CF altering the intestinal microenvironment (Vernocchi et al., 2018; Schippa et al., 2013). The bi-directional nature of the gut-lung axis partly implies that an alteration in gut microbial composition can presage changes in lung microbiota (Price & O' Toole 2021; Madan et al., 2012) and alter lung and gut immune responses (Anand & Mande, 2018; Shukla et al., 2017; Keely et al., 2012).

The gut bacteria of CF patients have been profiled, and bacterial richness and diversity show a decrease in CF patients relative to healthy controls (Burke et al., 2017; Nielsen et al., 2016; Duytschaever et al., 2011). In CF patients, a reduction in levels of *Bacteroides*, *Faecalibacterium*, *Roseburia*, *Bifidobacterium,* and *Ruminococcaceae* along with increased levels of Firmicutes, *Clostridium difficile*, *Escherichia*, *Shigella*, *Pseudomonas aeruginosa*, and *Klebsiella*, which can be linked to intestinal inflammation, is observed (Vernocchi et al., 2018; de Freitas et al., 2018; Burke et al., 2017; Francesco et al., 2017; Fouhy et al., 2017; Nielsen et al., 2016; Hoffman et al., 2014; Duytschaever et al., 2011, 2013; Schippa et al., 2013). It has been reported that altered microbial composition such as changes (reduction) in Bacteroidetes levels affects lung inflammation in CF (Antosca et al., 2019) and plays a role in progression of CF. Studies have demonstrated altered functional capacity of the microbiota in CF patients (Fouhy et al., 2017, Manor et al., 2016). Bacterial groups altered include short-chain fatty acid (SCFA) producers and

bacteria involved in lipid and xenobiotic metabolism (Fouhy et al., 2017, Manor et al., 2017; Debyser et al., 2016).

Many other factors such as increased mucus secretion, altered intestinal permeability and barrier function affect gut microbial composition (Flass et al., 2015; Modolell et al., 2002; Van Elburg et al., 1996). Gut microbiota alteration is also due to antibiotic use and prolonged medication in CF patients due to recurrent pulmonary infections (Vernocchi et al., 2018). Antibiotics are known to alter gut microbial composition in disease and healthy states (Patangia et al., 2022). Patients with CF often require long-term antibiotic treatment, the dosage and regularity are further altered depending on the patient's ability to clear the drug and severity of infection (Abbott et al., 2019, Sorgel 1996). Antibiotics in CF patients are used to treat exacerbations and also to suppress bacterial growth such that pulmonary functions are improved (Høiby et al., 2011).

Despite the benefits of using antibiotics to curb infections, early and prolonged antibiotic usage has been linked to the development of allergies, decreased colonisation resistance and promotion of the formation of antibiotic-resistant bacteria (Kidd et al., 2018; Sriramulu et al., 2013; Parmer & Nasser 2005). These antibiotic-resistant bacteria can make treatment more complex and may be associated with other respiratory infections. Many gram-negative bacteria involved in pathogenesis of CF are intrinsically resistant with some capable of forming biofilms, while antibiotic treatment adds to the number of resistant bacteria (Kidd et al., 2018; Carla López-Causapé et al., 2015). Antibiotics cause the loss of metabolically beneficial bacteria while allowing the growth of pathogens and resistant bacteria (Francino, 2016). Selective pressure due to continuous and prolonged antibiotic use in CF patients can promote the transfer of antibiotic-resistant genes (ARGs) among bacteria. It allows the bacteria to undergo genetic mutations and evade the host immune response to survive, and result in chronic infections (Smith et al., 2006). Delaying pathogen colonisation can help reduce the severity of the CF condition; hence, it is important to understand the resistome profile of the microbiota present in this patient group.

Studies have reported the resistome profile of lung and sputum showing increased transmissible macrolide, lincosamide, and streptogramin gene abundance, but only a few studies have focussed on the gut resistome in CF patients (Taylor et al., 2018; Feigelman et al., 2017). Due to the significance of the gut microbiome in the development and

progression of CF, it is clinically important to gain insight into the gut resistome profile of patient groups suffering from chronic diseases and long-term antibiotic exposure such as CF. The aim of this study was to examine the microbiota and resistome patterns of the intestinal microbiota of CF patients compared to healthy adults, using a shotgun sequencing approach (Dataset used by Fouhy et al., 2017).

6.3 Methods

A total of 12 samples available from the CFMATTERS study and previously analysed by Fouhy et al. (2017) were used for the analysis of the antibiotic resistance profile of the microbiota in this study. DNA extracted from six stool samples each from CF subjects (four out of the six CF subjects were pancreatic insufficient) and non-CF controls were subjected to Illumina Miseq shotgun sequencing. Controls were non-CF healthy individuals with no antibiotic exposure in the 28 days before sample collection. CF individuals received multiple antibiotic courses in the previous 12 months. For more details about the CFMATTERS cohort and selection criteria used, please refer to https://www.nature.com/articles/s41598-017-06880-y#Sec2.

6.3.1 Quality check and trimming

The quality of shotgun metagenomic data was first evaluated using FastQC (v0.11.8). The reads were then pre-processed by filtering out low-quality reads using Prinseq (Schmieder & Edwards, 2011). Reads with a mean quality score lower than 25 for a sliding window of 5 base pairs at the 3' end were trimmed. Bowtie2 (v2.3.4) and Samtools (Langmead & Salzberg, 2012; Li et al., 2009) was then used to eliminate any host reads (human contamination) present in the data set by screening the filtered sequences against the reference human genome (Homo sapiens) database from NCBI. Read duplicates from the sequences were then removed using PICARD tools (v2.18.23-0) ((https://broadinstitute.github.io/picard/, 2016), the output being files with unique reads only. This was followed by final quality checking and trimming of the dataset using trimBWAstyle.usingBam.pl. from the Bioinformatics Core at UC Davis Genome Center (https://github.com/genome/genome/blob/master/lib/perl/Genome/Site/TGI/Hmp/HmpSraProcess/trimBWAstyle.usingBam.pl). The script was used to trim off bases with a quality value of three or lower. This threshold was chosen to delete all the bases with an uncertain quality, as defined by Illumina's EAMMS (End Anchored Max Scoring

Segments) filter. Additionally, reads trimmed to less than 200 bp were also removed. Quality checks of the filtered and trimmed reads using FastQC (v0.11.8) and MultiQC (v1.9) demonstrated that the mean quality value across each base position in the read was more than 20.

6.3.2 Taxonomic and functional profiling, Assembly

The microbial composition was analysed using the MetaPhlAn2 (v2.7.5) species classifier (Segata et al., 2012). Taxa with a relative abundance of <0.01% were categorised as "Other" for each classifier. Subsequently, the functions were assigned with HUMAnN2 (v2.8.1) tool (Franzosa et al., 2018), based on ChocoPhlan and UniRef databases (Suzek et al., 2007). Metagenome assembly was performed using metaSPAdes (kmers 25-110), keeping those contigs with length above 500 bp. Taxonomic assignment to contigs was performed using Kraken2 (v2.0.8).

6.3.3 Antimicrobial resistance, virulence factors and taxonomic assignment of ARGs, secondary metabolites

To determine the resistome profile of the samples, ABRicate with CARD (Comprehensive Antibiotic Resistance Database) and VFDB (Virulence Factor DataBase) were used (Seemann T, Abricate, Github https://github.com/tseemann/abricate). Contigs were used as input to determine antibiotic resistance and output from CARD (Jia et al., 2017) and VFDB (Chen et al., 2016) was used throughout for further analysis (doi:10.1093/nar/gkw1004) (doi:10.1093/nar/gkv1239). Taxonomic assignment of the contigs was used to assign and correlate bacterial phyla to the ARGs. ABRicate results were normalised to copies per million (cpm) using the read counts from filtered and trimmed shotgun reads. The ARGs list was further curated into different antibiotic classes based on CARD. Similarly, the gene names encoding for virulence factors were modified and curated to virulence factor classes based on VFDB. antiSMASH (v 4.1.0) was used on contigs to detect and understand the profile of biosynthetic gene clusters responsible for secondary metabolites in both groups. Further, the contigs were analysed to look for genes possibly resulting from a lateral gene transfer event by using the WAAFLE tool from Huttenhower lab (Tiffany et al., manuscript in process). Matching the genes showing lateral gene transfer to those genes that were assigned as ARGs using CARD from ABRicate helped us to understand the nature of ARG transfer in our dataset. Similarly, MEfinder on contigs was

used to detect the presence of mobile genetic elements and identify if ARGs as detected from ABRicate are involved in transfer using mobile genetic elements.

6.3.4 Visualisation and Statistical analysis

To determine the association between antibiotic resistance and antibiotic use associated with disease condition, our dataset had two major groups; CF and healthy controls. All analyses were performed using R v4.0.2. Alpha diversity was estimated using Shannon index, Simpson index, evenness and richness indices using the vegan package (v2.5.7) in R. Significance between alpha diversity estimates was calculated using t-test. Beta diversity was visualised using the metaMDS function with the vegan and Phyloseq (v1.38) Package in R using NMDS with Bray-Curtis dissimilarity index and PCoA with Jaccard and Bray-Curtis distances. The significance between groups was tested using Adonis and anosim tests. These analyses were also conducted at genera and species levels for microbiome characterisation. Beta diversity calculations were done on relative abundance data. Further visualisations of the differentially abundant features in microbiome, resistome and pathway abundance data from trimmed and filtered reads were also made using LEfSe through the galaxy server online using default parameters (Threshold on the logarithmic LDA score for discriminative features: 2.0, p-value cutoff: 0.05) (Segata et al., 2011) and MaAsLin2 (minimum prevalence = 0.1, minimum abundance = 0.001, max significance for q value: 0.250) (v1.7.3) (Mallick et al., 2021). A list of AMR genes and virulence factors was extracted and grouped into antibiotic drug classes to which resistance was conferred, or to virulence category and the assignments were viewed using boxplots, heatmaps using ComplexHeatmap and circlize package in R (Gu et al., 2016, 2014). Other plots in R were produced using the ggplot2 (v3.3.5), microbiomics (v0.0.0.9) package. A p-value higher than 0.05 was considered non-significant.

6.4 Results

6.4.1 Diversity analysis for antibiotic resistance genes and gut microbiota

In total, 12 samples from 12 different participants were included in this study. The participants were divided into two groups; six samples from individuals with CF who have chronic antibiotic exposure and six from healthy controls. ARG abundance was determined using CARD in ABRicate. Alpha diversity of the resistome was calculated

for the two groups using Shannon, Simpson, pileus evenness and richness indices. Significantly higher ARG diversity and richness were observed in the CF group than in the controls (Figure 6.1A). Species richness index considers only the abundance and was found to be significant between the two groups (p-value=0.005), while Chao1 index which also considers rare features was also significant (p-value=0.0043). Both Shannon and Simpson alpha diversity indices account for the feature's diversity, abundance and evenness and showed that ARGs were significantly lower in healthy controls compared to the CF group (p-value=0.002 for Shannon, p-value=0.0022 for Simpson) (Figure 6.1A). This showed a strong association of the resistome with antibiotic treatment in the CF group. Further, alpha diversity characterisation at genera and species levels for the gut microbiota showed no significant differences in the diversity and richness of the two groups (Figure 6.2 A, B).

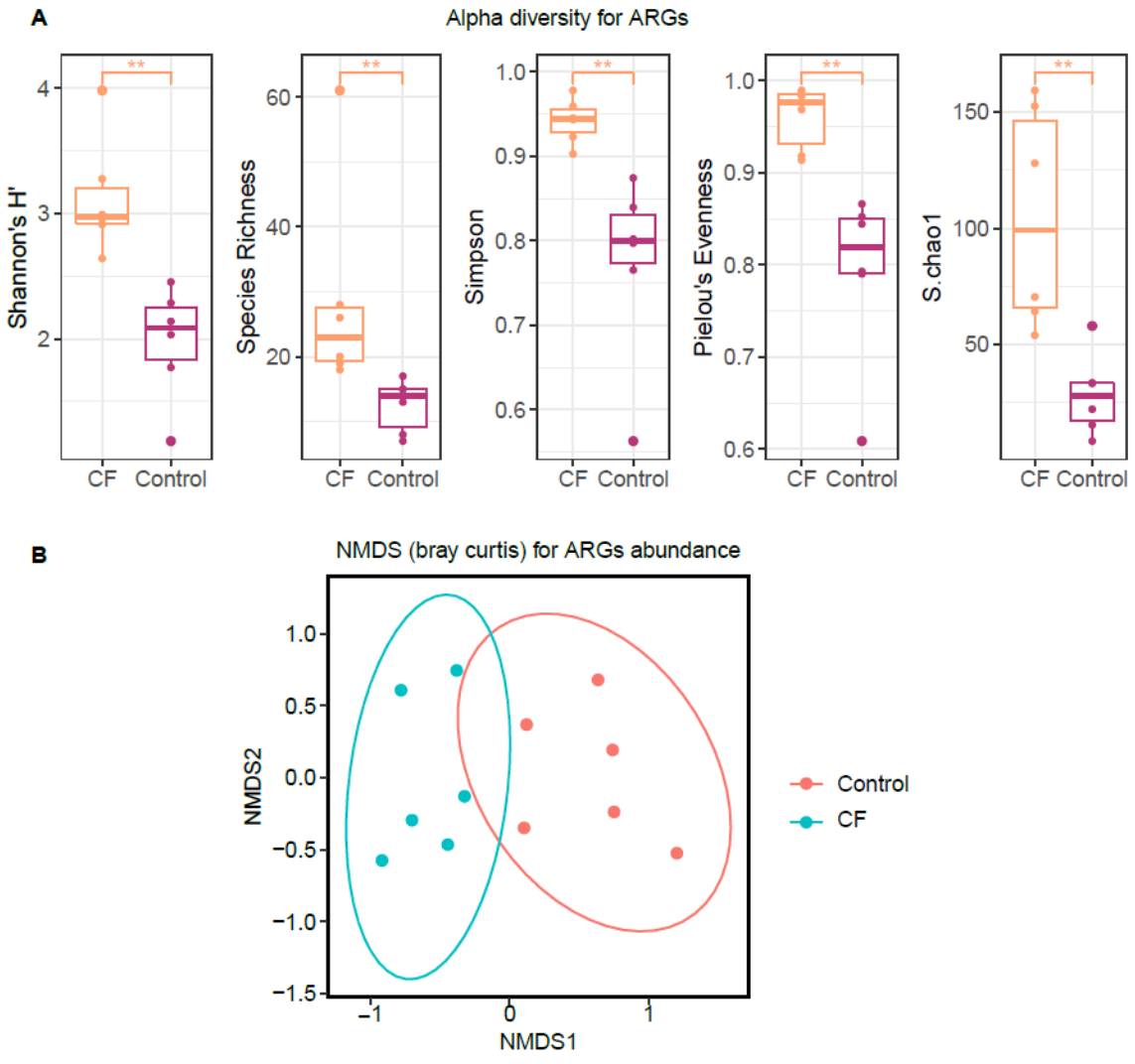


Figure 6.1: Based on resistome profile: A. Alpha diversity of CF and Controls as measured by Shannon, Species Richness, Simpson, Pieleou's evenness and S.Chao1. indices and B. Beta diversity between CF and Controls as measured by Bray-Curtis distance. P-values < 0.5 were considered significant.

Beta diversity showed clustering of CF samples separately from controls (Figure 6.1B). The effect of the type of sample and antibiotic use on sample dissimilarities was determined by permutational multivariate analysis of variance (PERMANOVA) using distance matrices with Adonis function. Beta diversity for ARGs gave a significant p-value (0.005) with Adonis test (R^2: 0.31) (Figure 6.1B). Similarly, Adonis for taxonomy (genera level from MetaPhlAn2) resulted in a significant p-value (p-value=0.01, R^2 = 0.23) (Figure 6.3A). Adonis presented significant results for taxonomy at species level derived from MetaPhlAn2 (p-value= 0.007, R^2=0.19) (Figure 6.3B); with a high R2 value explaining the effect of chronic antibiotic exposure on the microbiota and ARG diversity between the two groups (Figure 6.3C).

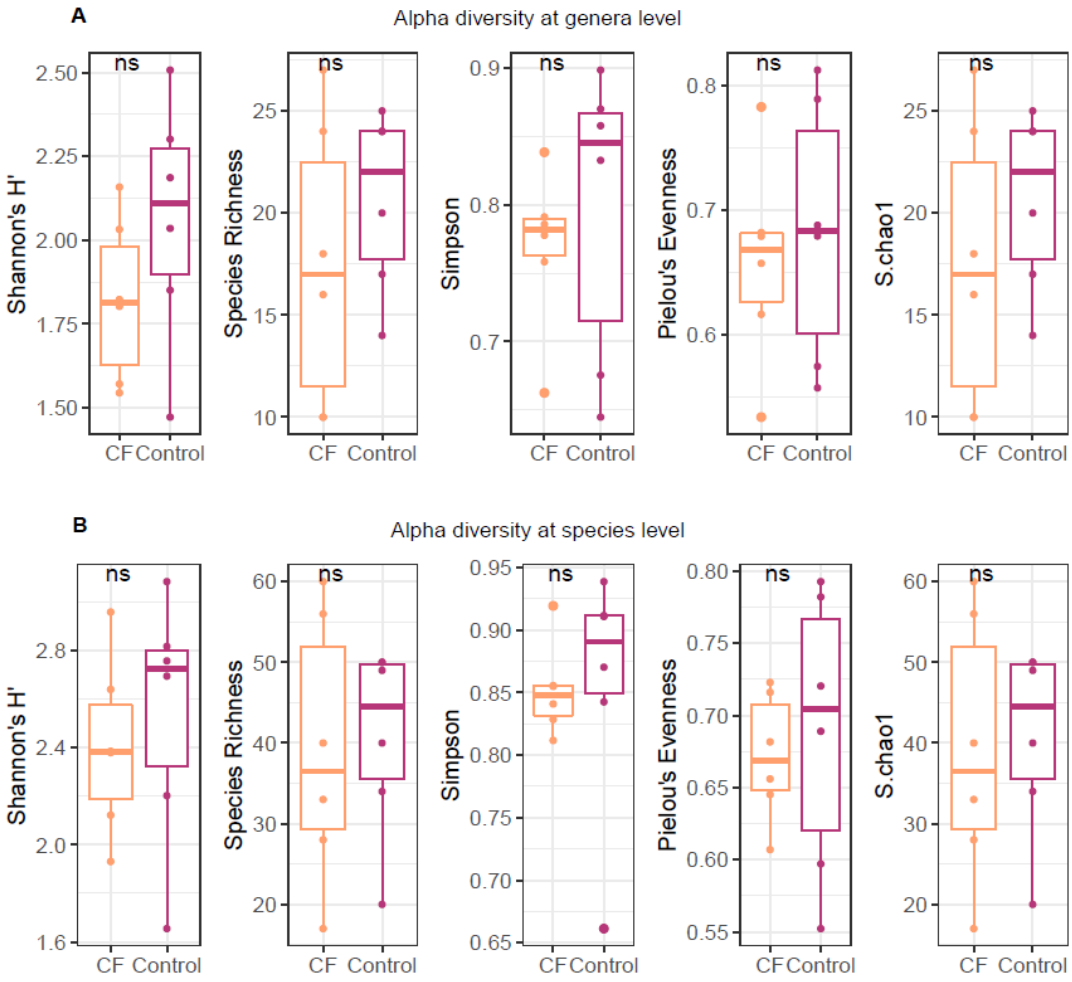


Figure 6.2: Diversity estimates based on microbiome profile using Alpha diversity with Shannon, Species Richness, Simpson, Pieleou's evenness and S.Chao1 indices at A. genera and B. species level. P-values < 0.05 were considered significant.

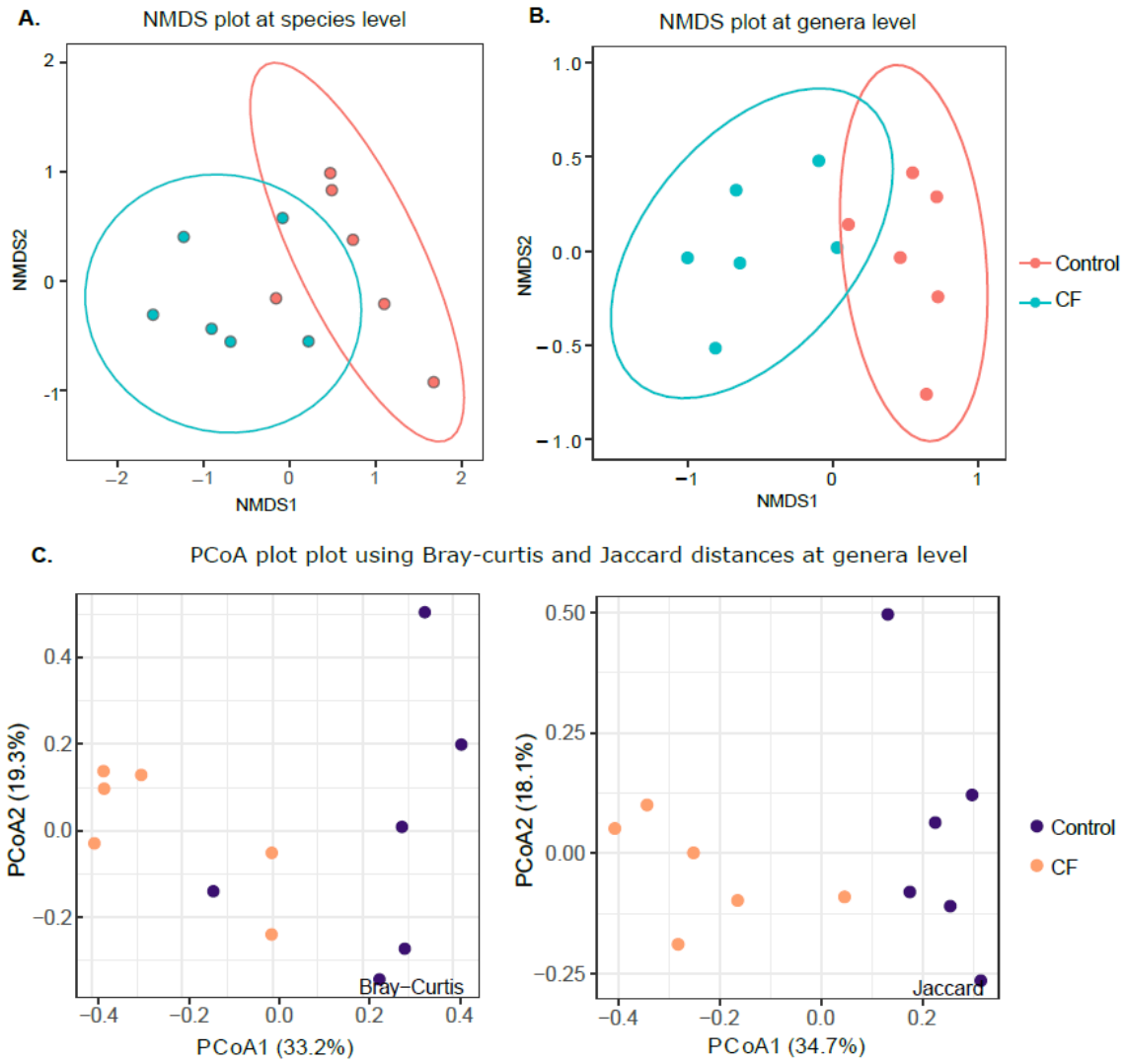


Figure 6.3: Beta diversity with NMDS plot based on Bray-Curtis distances at A. Species level (ellipse at 90% confidence), and B. Genera level (ellipse at 90% confidence), and C. PCoA plot based on Jaccard and Bray-Curtis distances at genera level.

6.4.2 Microbiota changes are influenced by chronic antibiotic use

MetaPhlAn2 was used to assign taxonomy to the filtered and trimmed reads. Mean relative abundance scores were used to plot phyla-level abundances of the two groups. Firmicutes were increased in the CF group, while Bacteroidetes, Actinobacteria and Proteobacteria decreased compared to controls (Figure 6.4A).

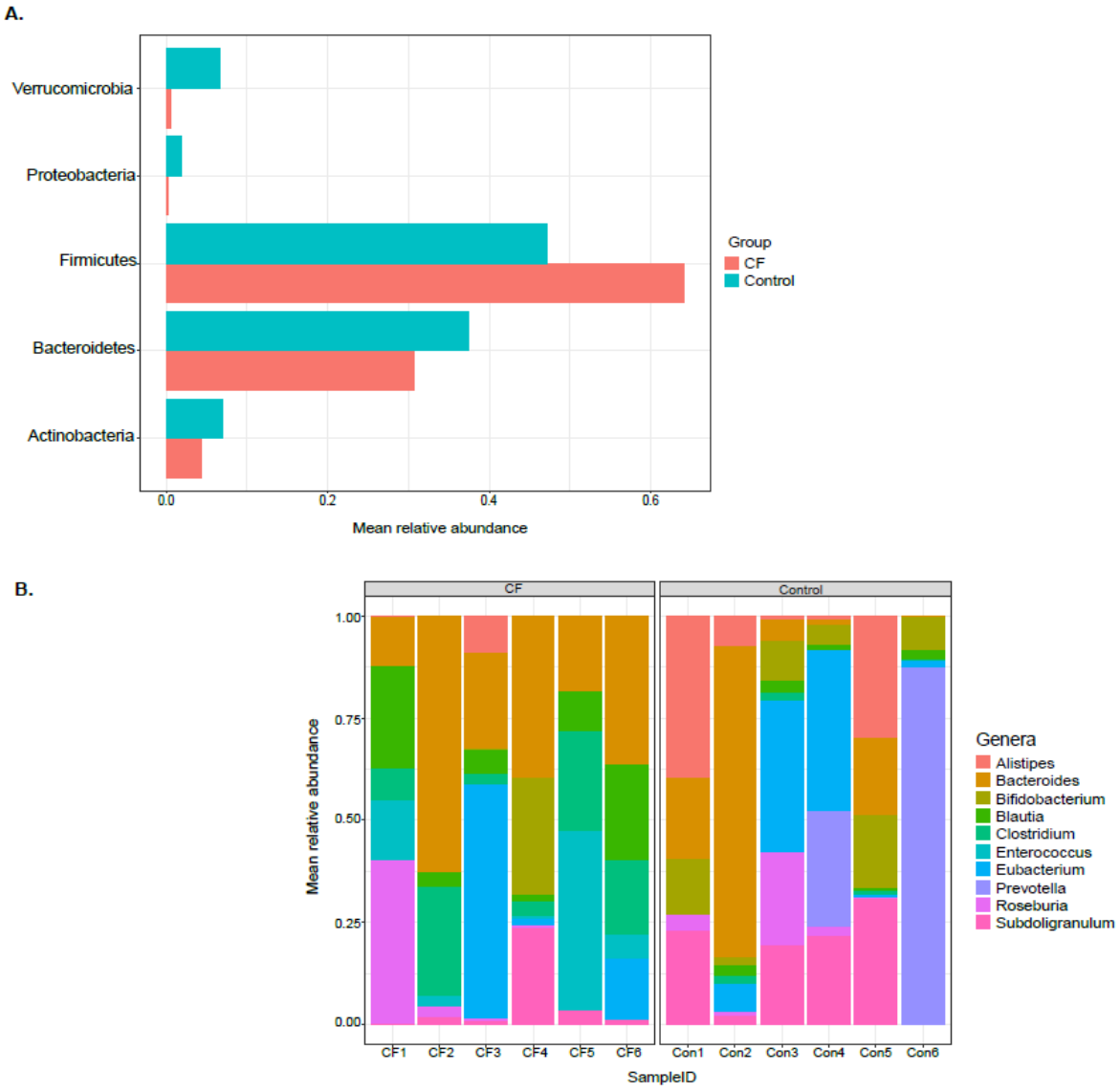


Figure 6.4: Mean relative abundance at A. Phylum level and B. Barplot depicting mean relative abundance of top 10 genera in CF and control (Con) groups.

The mean relative abundance of the top 10 bacterial genera in CF showed higher levels of *Blautia*, *Enterococcus*, *Bacteroides*, and *Clostridium* compared with controls while higher levels of *Subdoligranulum*, *Alistipes* and *Bifidobacterium* were found in controls (Figure 6.4B). For a complete representation of the differences in relative abundance of bacterial genera in both groups, a heatmap was generated by performing log transformation on relative abundance data for visualisation (Figure 6.5). Similarly, the species level top 10 relative abundance plot showed higher abundance of *Ruminococcus*

gnavus, *Eubacterium rectale*, *Enterococcus faecalis*, *Bacteroides vulgatus* and *Bacteroides uniformis* in CF individuals (Figure 6.6A).

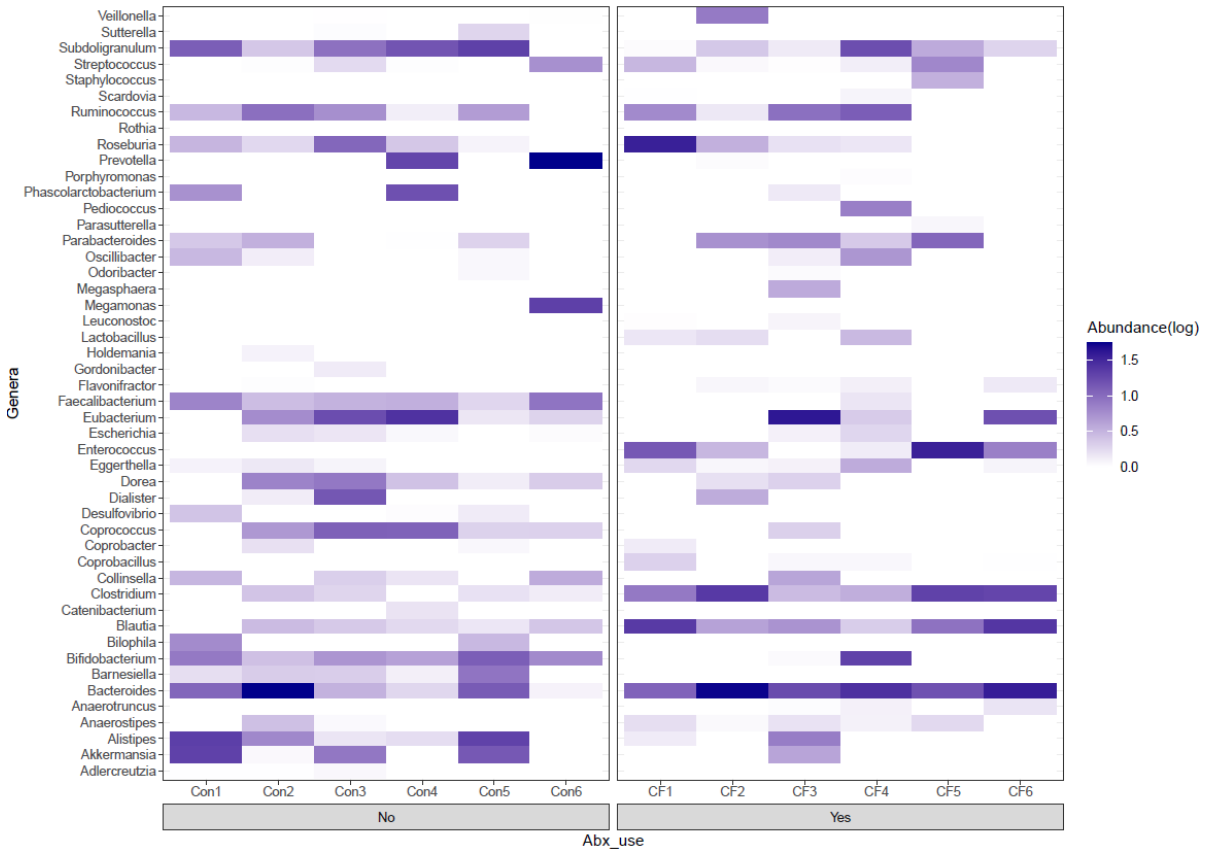


Figure 6.5: Heatmap demonstrating log-transformed abundance of total genera profile in CF and control (Con) group.

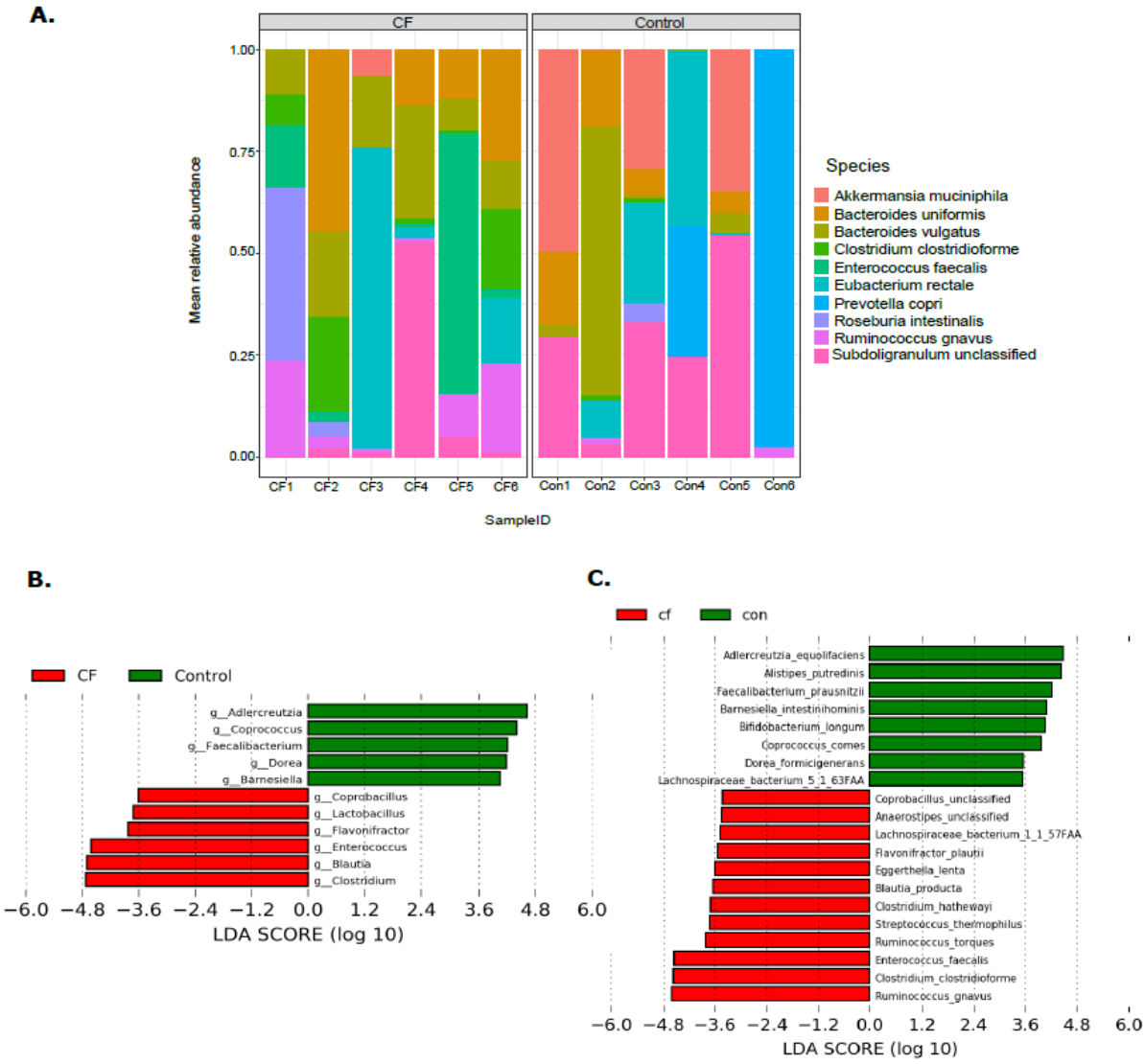


Figure 6.6: A. Mean relative abundance plot from top 10 species from MetaPhlAn2 output. B. Histogram of LDA scores computed for differentially abundant genera and C) species in the CF and healthy controls (Con).

Using LEfSe and MaAsLin2, the most differentially abundant taxa in the two groups were determined. Results from LEfSe showed that the bacterial taxa with significantly differential abundance in controls included high *Alistipes*, *Aldercruetzia*, *Coprococcus*, *Faecalibacterium*, *Dorea* and *Barnesiella*; while CF subjects were more associated to the genera *Coprobacillus*, *Lactobacillus*, *Flavonifractor*, *Enterococcus*, *Blautia* and *Clostridium* (Figure 6.6B and C, refer to Table S6.1 and S6.2 for p-values and LDA score).

Further, multivariate linear models were used to compare the taxonomic composition at the genera and species levels in the two groups using MaAsLin2. Based on FDR values from MaAsLin2 at the genera level, the CF group showed similar results to previous tests and showed a higher abundance of *Clostridium*, *Blautia*, *Enterococcus*, *Coprobacillus*, *Flavonifractor*, *Lactobacillus* and *Bacteroides*. Similarly, LEfSe and MaAsLin2 analysis was performed on species-level data. Species-level differential testing demonstrated that *Faecalibacterium prausnitzii*, *Barnesiella intestinihominis*, *Alistipes putredinis*, *Coprococcus comes*, *Bifidobacterium longum* and *Lacnospiraecea* are more associated to controls, showing negative association to the CF patient group. *Rumunicoccus gnavus*, *Clostridium clostridioforme*, *Clostridium hathewayi*, and *Enterococcus faecalis* were more closely associated to the CF group (Tables 6.1 and 6.2).

Table 6.1: MaAsLin2 significant results from genera level run using default parameters and minimum abundance of 0.001.

feature	metadata	value	coef	q-value
Bifidobacterium	Diagnosis	Control	5.225782	0.061835
Faecalibacterium	Diagnosis	Control	3.298034	0.002403
Barnesiella	Diagnosis	Control	2.552116	0.072445
Dorea	Diagnosis	Control	2.206713	0.177698
Coprococcus	Diagnosis	Control	2.144477	0.102917
Adlercreutzia	Diagnosis	Control	1.696687	0.177698
Flavonifractor	Diagnosis	Control	-2.14709	0.102917
Bacteroides	Diagnosis	Control	-2.60153	0.185961
Blautia	Diagnosis	Control	-2.99821	0.061302
Enterococcus	Diagnosis	Control	-3.96316	0.070155

Coprobacillus	Diagnosis	Control	-4.07215	0.075185
Lactobacillus	Diagnosis	Control	-4.09814	0.14336
Clostridium	Diagnosis	Control	-4.21516	0.010268

Table 6.2: MaAsLin2 significant results from species level run using default parameters and minimum abundance of 0.001.

feature	metadata	value	coef	q-value
Clostridium_clostridioforme	Antibiotic	Yes	4.783623	0.196041
Clostridium_hathewayi	Antibiotic	Yes	4.257744	0.196041
Coprobacillus_unclassified	Antibiotic	Yes	4.072152	0.196041
Enterococcus_faecalis	Antibiotic	Yes	3.663978	0.196041
Ruminococcus_gnavus	Antibiotic	Yes	3.448063	0.203126
Bacteroides_vulgatus	Antibiotic	Yes	3.437633	0.203126
Ruminococcus_torques	Antibiotic	Yes	3.109445	0.163316
Eggerthella_lenta	Antibiotic	Yes	3.083378	0.196041
Flavonifractor_plautii	Antibiotic	Yes	2.147088	0.203126
Lachnospiraceae_bacterium_1_1_57FAA	Antibiotic	Yes	2.011794	0.196041
Anaerostipes_unclassified	Antibiotic	Yes	1.901939	0.218427
Coprococcus_comes	Antibiotic	Yes	-1.67113	0.218427
Lachnospiraceae_bacterium_5_1_63FAA	Antibiotic	Yes	-1.86003	0.248814
Barnesiella_intestinihominis	Antibiotic	Yes	-2.55212	0.196041
Alistipes_putredinis	Antibiotic	Yes	-2.93847	0.218427

Faecalibacterium_prausnitzii	Antibiotic	Yes	-3.29803	0.006324
Bifidobacterium_longum	Antibiotic	Yes	-3.71335	0.233812

6.4.3 Functional characterisation of the antibiotic vs non-antibiotic group

The functionality of the microbiome varied in the CF group as compared to controls. HUMAnN2 output data was renormalised to cpm by excluding special features and the results visualised using Heatmap in R. Sample type had a profound effect on the functional profile of the microbiota (based on unstratified pathway abundance data from HUMAnN2) and samples clustered significantly based on sample type (CF and controls), over the pancreatic insufficiency status of CF subjects (Figure 6.7 A, B). More detailed analysis of differences in functional profile of the two groups is demonstrated in detail in the original study (Fouhy et al., 2017). Further, antiSMASH (v4.1.0) was used to determine the secondary metabolite composition between the two groups. The biosynthetic gene cluster (BGC) belonging to NRPS (non-ribosomal peptide synthetases), was significantly enriched in CF individuals (Figure 6.8A). NRPs are secondary metabolites that help microbes survive in stressful conditions. This explains their high abundance in the CF group as many of the secondary metabolites in this class act as signalling molecules, immune-suppressants, and virulence factors and also help to grow in low iron conditions acting as siderophores, and are involved in motility and biofilm formation (Gulick 2017; Tyrrell & Callaghan 2016; Nikolouli & Mossialos 2012; Fischbach & Walsh, 2006; Cane &Walsh, 1999).

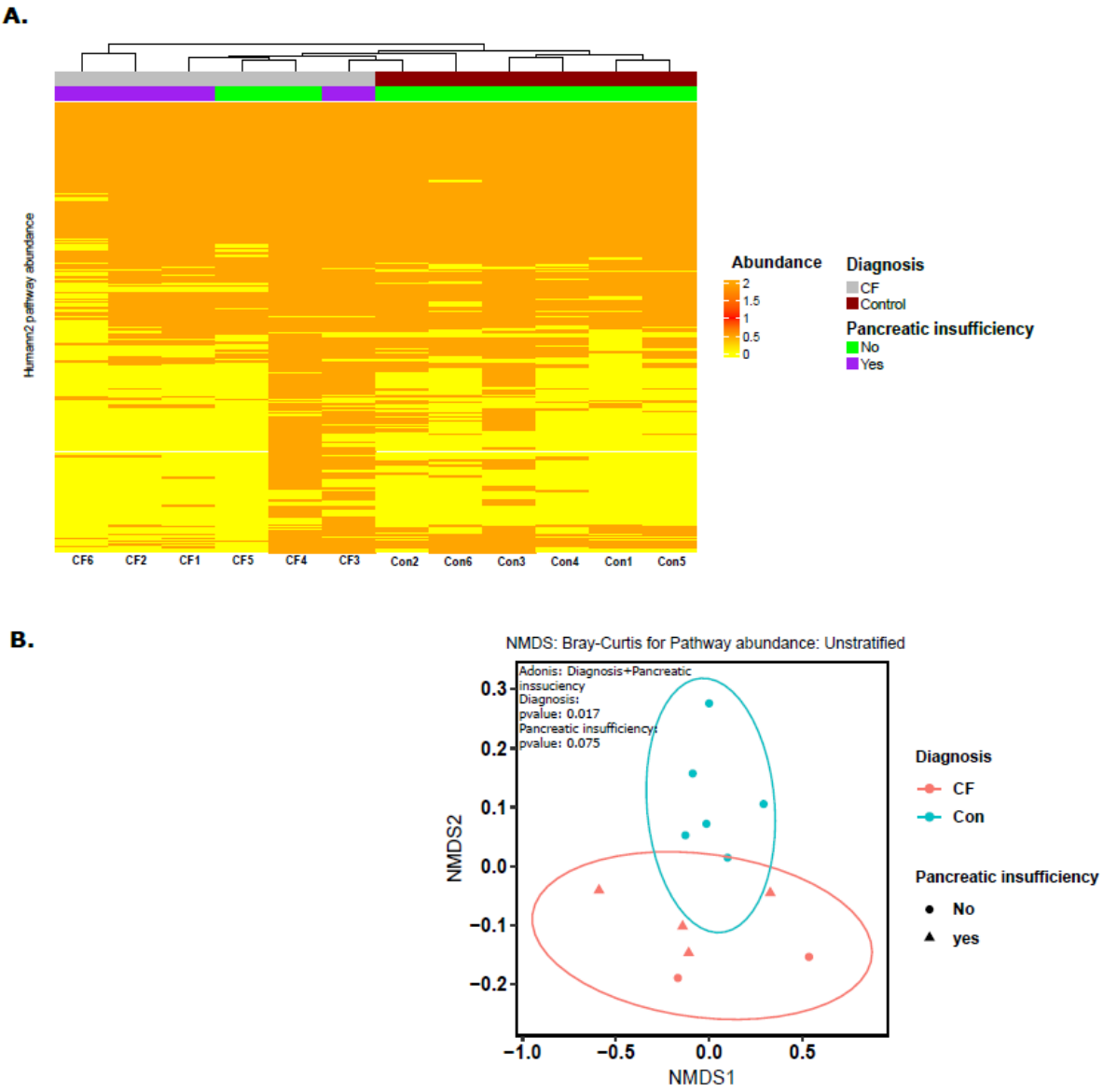


Figure 6.7: A. Heatmap showing distinct clustering to samples based on unstratified pathway abundance data from HUMAnN2. B. Beta diversity (NMDS plot using Bray-Curtis distance matrix) plot on unstratified pathway abundance data, shows distinct clustering of sample based on sample type over pancreatic insufficiency (Ellipse at 90% confidence interval).

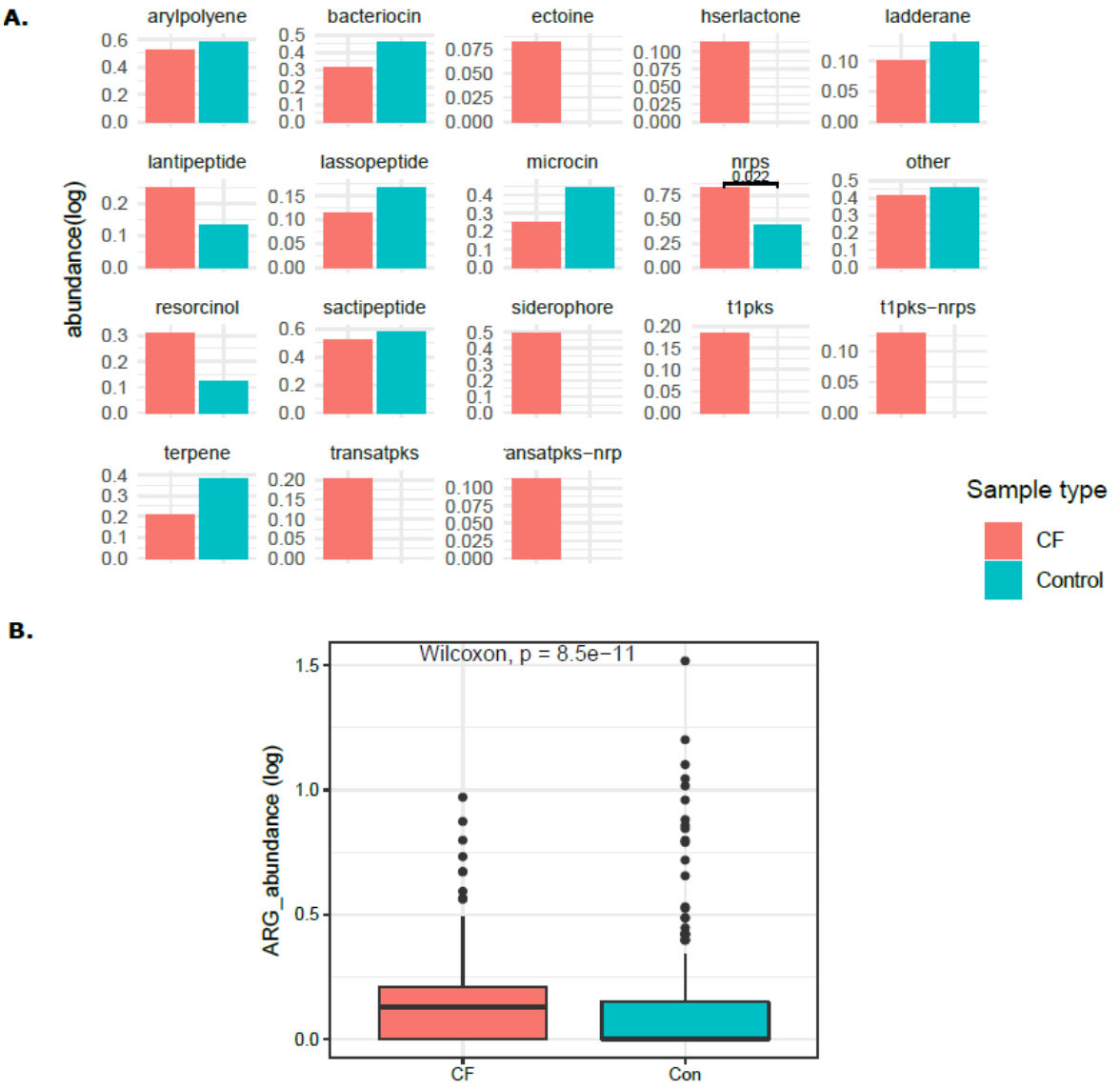


Figure 6.8: A. Comparative bar plots showing BGCs detected by antiSMASH in the 2 groups. B. Boxplot showing ARG abundance in both groups, with higher overall abundance observed in the CF group.

6.4.4 ARG abundance and profiling

The ARGs detected using ABRicate were manually assigned antibiotic classes based on the phenotype from CARD. Further, any ARG that confers resistance to three or more antibiotics was termed as multi-drug resistant (MDR), except for drug classes belonging to the antibiotics administered to CF individuals in the study. Shotgun data analysis of the CF and control groups resulted in the identification of 172 ARGs. These genes

included rifamycin, tetracycline, multi-drug resistance, MLSP, aminoglycoside, beta-lactam, glycopeptide, diaminopyrimidine and mupirocin resistance genes among others. The total number of ARGs detected in both groups was visualised by filtering for ARGs present in at least 30% of the samples, and it was observed that the ARG abundance differed significantly with higher amounts present in the CF group (Figure 6.8B, p=8.56e-11, Wilcoxon test).

A heatmap using log-transformed abundance was generated for antibiotic resistance classes, which reveals higher ARGs in CF subjects as compared to controls (Figure 6.9B). Further, the microbial composition was associated with raw ARG counts from ABRicate based on taxonomic classification of contigs from Kraken2. Most ARGs in the CF group were present in phyla Firmicutes, Proteobacteria and Bacteroidetes while those in control were in the phyla Firmicutes, Proteobacteria and Actinobacteria but at lower abundances (Figure 6.9A). Differential abundance testing using MaAsLin2 on ARG abundance data showed a positive correlation of the CF group to many antibiotic classes such as aminoglycosides, oxazolidinone, tetracycline, and fluoroquinolone, which were administered to the CF individuals throughout their treatment (Table 6.3, MaAsLin coef. Correlation table: positive coefficient means positive relation to the CF group: Underlined classes denote antibiotics administered to CF individuals).

Table 6.3: Table showing MaAsLin2 significant results, where MaAsLin2 was run with default parameters and with minimum abundance of 0.001. (Positive coefficient means positive relation to the CF group: underlined antibiotic classes denote antibiotics used by CF individuals)

feature	metadata	value	coef	q-value
aminoglycoside	Antibiotic	Yes	3.405736	0.0051
MDR, macrolide, oxazolidinone, tetracycline	Antibiotic	Yes	2.670728	0.005425
Lincosamide, macrolide, streptogramin	Antibiotic	Yes	2.003744	0.099103

Fluoroquinolone, macrolide, rifamycin	Antibiotic	Yes	1.864142	0.023119
diaminopyrimidine	Antibiotic	Yes	1.806078	0.099103
Acridine dye	Antibiotic	Yes	1.741314	0.029626
nucleoside	Antibiotic	Yes	1.656453	0.042762
lincosamide	Antibiotic	Yes	1.395792	0.075989
Sulfonamide, sulfone	Antibiotic	Yes	1.304193	0.099103
tetracycline	Antibiotic	Yes	0.973884	0.068514
rifamycin	Antibiotic	Yes	-1.63983	0.046152
mupirocin	Antibiotic	Yes	-3.38061	0.0051

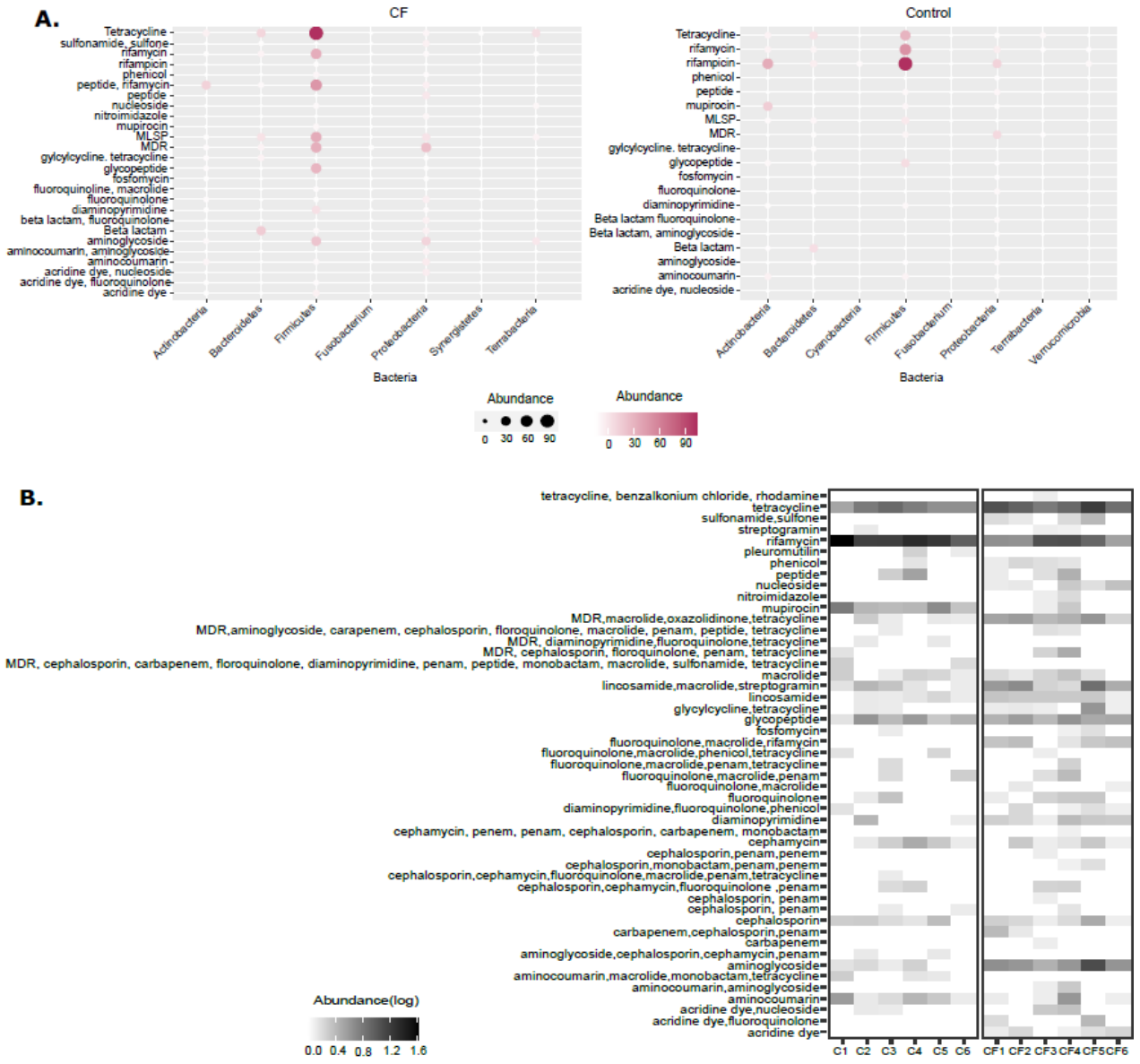

A.
CF
Control
Tetracycline
sulfonamide, sulfone
rifamycin
rifampicin
phenicol
peptide, rifamycin
peptide
nucleoside
nitroimidazole
mupirocin
MLSP
MDR
glycylcycline. tetracycline
glycopeptide
fosfomycin
fluoroquinoline, macrolide
fluoroquinolone
diaminopyrimidine
beta lactam, fluoroquinolone
Beta lactam
aminoglycoside
aminocoumarin, aminoglycoside
aminocoumarin
acridine dye, nucleoside
acridine dye, fluoroquinolone
acridine dye
Beta lactam fluoroquinolone
Beta lactam, aminoglycoside
aminoglycoside
Actinobacteria
Bacteroidetes
Firmicutes
Fusobacterium
Proteobacteria
Synergistetes
Terrabacteria
Cyanobacteria
Verrucomicrobia
Bacteria
Abundance
0 30 60 90
B.
tetracycline, benzalkonium chloride, rhodamine
sulfonamide,sulfone
streptogramin
pleuromutilin
MDR,macrolide,oxazolidinone,tetracycline
MDR,aminoglycoside, carapenem, cephalosporin, floroquinolone, macrolide, penam, peptide, tetracycline
MDR, diaminopyrimidine,fluoroquinolone,tetracycline
MDR, cephalosporin, floroquinolone, penam, tetracycline
MDR, cephalosporin, carbapenem, floroquinolone, diaminopyrimidine, penam, peptide, monobactam, macrolide, sulfonamide, tetracycline
lincosamide,macrolide,streptogramin
lincosamide
glycylcycline,tetracycline
fosfomycin
fluoroquinolone,macrolide,rifamycin
fluoroquinolone,macrolide,phenicol,tetracycline
fluoroquinolone,macrolide,penam,tetracycline
fluoroquinolone,macrolide,penam
fluoroquinolone,macrolide
fluoroquinolone
diaminopyrimidine,fluoroquinolone,phenicol
diaminopyrimidine
cephamycin, penem, penam, cephalosporin, carbapenem, monobactam
cephamycin
cephalosporin,penam,penem
cephalosporin,monobactam,penam,penem
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aminoglycoside
aminocoumarin,macrolide,monobactam,tetracycline
aminocoumarin,aminoglycoside
aminocoumarin
acridine dye,nucleoside
acridine dye,fluoroquinolone
acridine dye
C1 C2 C3 C4 C5 C6 CF1 CF2 CF3 CF4 CF5 CF6
Abundance(log)
0.0 0.4 0.8 1.2 1.6

Figure 6.9: A. Circle heatmap showing correlation between ARG and bacterial phyla, approx. 13% of the contigs conferring resistance to ARGs were removed from the below circle heatmap as they were categorised as unclassified by Kraken2; B. Heatmap based on log-transformed abundance values for antibiotic resistance classes between the two groups CF (CF) and Controls (C).

6.4.5 Virulence Factors, lateral gene transfer and mobile genetic elements

VFDB in ABRicate was used to determine virulence factors in the dataset. The results from ABRicate were normalised based on read counts of filtered and trimmed shotgun reads. Shotgun data analysis of the dataset revealed the presence of 289 genes encoding for virulence factors, which were then grouped into higher virulence factor classes based on VFDB. The total number of virulence factors was higher in the CF group compared to the control group (Figure 6.10A, p-value=6.5e-06, Wilcoxon test). The virulence factors curated into classes were log-transformed and used to generate a heatmap for comprehensive visualisation (Figure 6.10B). Novel lateral gene transfers were examined using WAAFLE (v1.0) on metagenomic contigs. The contigs identified as participating in lateral gene transfer events by WAAFLE were then matched with contigs coding for ARGs as identified by ABRicate. No ARGs were found to show novel lateral gene transfer in controls while a few genes namely, *ErmG*, *tet(W/N//W)*, *Bifidobacterium adolescentis rpoB* conferring resistance to rifampicin, *ErmB*, *InuA*, *Bifidobacteria intrinsic ileS* conferring resistance to mupirocin, and *Campylobacter coli* chloramphenicol acetyltransferase, *sul2*, contributed to lateral gene transfer in the CF group as detected by WAAFLE. Mefinder (Mobile element finder: https://pypi.org/project/MobileElementFinder/) was used to predict the presence of mobile genetic elements in contigs generated from shotgun sequence data. The output was cpm normalised and no significant difference in the total abundance of mobile genetic elements in the two groups was observed, however, a significant difference was observed in the abundance of insertion sequence type mobile genetic elements between the two groups (Figure 6.10C) where the CF group showed greater abundance (p-value=0.011). Some of the mobile genetic elements in the CF group belonged to the same contigs as ARGs as predicted by CARD in ABRicate, but none in the control group.

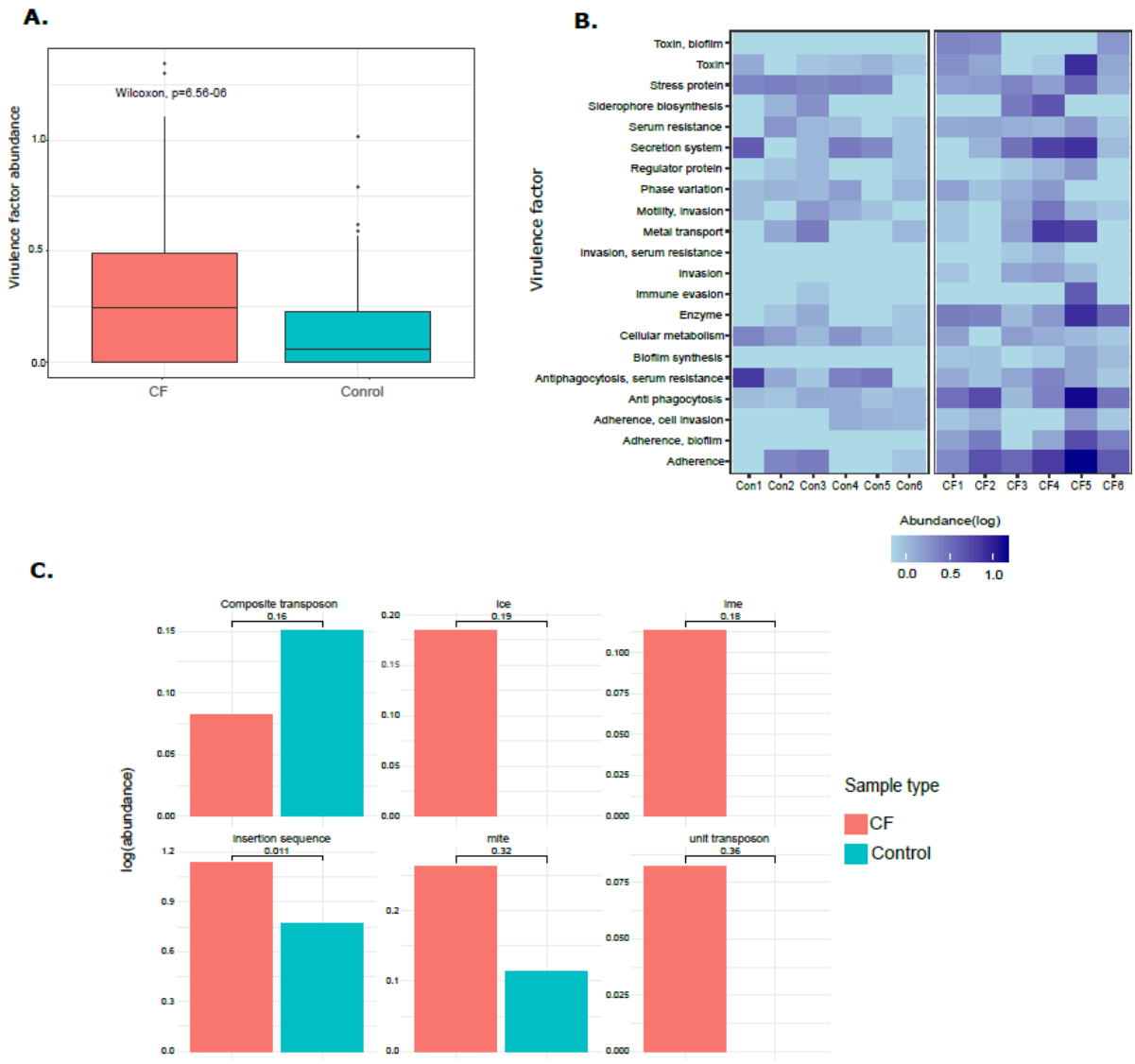


Figure 6.10: A. Boxplot for virulence factors between CF and Control groups and B. Heatmap of log-transformed abundance values of virulence factors between the two groups and C. Bar plot showing the presence of different mobile genetic elements in both groups.

6.5 Discussion

The gut resistome of CF individuals has not been studied extensively. In this study, the shotgun sequencing data from six CF and six control adult individuals who were enrolled as a part of the CFMATTERS study (Fouhy et al., 2017) was analysed. CF individuals are exposed to continuous antibiotics for treatment and prophylactic purposes. This

improves the quality of life and has several benefits for CF individuals, however, the risk associated with the use of antibiotics, such as development of antibiotic-resistant bacteria, cannot be neglected. Since the condition of CF primarily affects the respiratory tract, most studies have focussed on the microbiome and resistome profiles of the airways. However recent developments have shown the importance of studying the gut microbiome and resistome profile in CF individuals due to the multi-organ nature of the disease (Thavamani et al., 2021; Burke et al., 2017).

The gut microbial community composition (Beta diversity) of CF individuals was altered as compared to controls and this was evident from the distinct clustering of the two groups in PCoA and NMDS plot based on Jaccard and Bray-Curtis distances. As observed by alpha diversity measures, the microbial diversity did not differ within the two groups. Our results are contradictory to other studies examining gut microbial diversity in CF individuals compared to controls and reporting lower gut microbial diversity in CF individuals (Coffey et al., 2019; Burke et al., 2017, Nielsen et al., 2016, Scanlan et al., 2012). These differences could be due to the different phenotypic conditions and age groups of individuals used in each study. For instance, Coffey et al. (2019) and Nielsen et al. (2016) reported lower gut microbial diversity in children, as opposed to adults in the current study. While Burke et al. (2017) identified differences in gut microbial diversity within CF groups varying by lung dysfunction. However, differences in microbial abundance profiles at phylum, genera and species levels were seen. For instance, the genera *Clostridium*, *Blautia*, *Enterococcus*, *Coprobacillus*, *Flavonifractor*, and *Bacteroides* were found to be higher in CF individuals while bifidobacteria, *Dorea*, and *Faecalibacterium* were lower in CF individuals as compared to healthy controls. These results support findings from previous studies which report decreased abundance of *Faecalibacterium* and *Bifidobacterium* in CF individuals (Burke et al., 2017; Duytschaever et al., 2013; Scanlan et al., 2012; Schippa et al., 2013). *Faecalibacterium prausnitzii* is one of the main butyrate-producing bacteria in the human gut, and thus a decrease in abundance of *Faecaibacterium prausnitzii* may be associated with the intestinal inflammation observed in CF individuals given its anti-inflammatory properties (Sokol et al., 2008).

The data revealed that in CF individual's bacteria belonging to the phylum Firmicutes have high abundance of ARGs. Manor et al. (2016) reported that in children with cystic

fibrosis, the Firmicutes members *Enterococcus faecalis* and *Enterococcus faecium* possess ARGs. Mostly bacteria belonging to the *Ruminococcaceae* family, *Blautia*, *Faecalibacterium*, and *Bifidobacterium* spp. (Parada Venegas et al., 2019), carry out SCFA production by carbohydrate fermentation; all of which were reduced in CF individuals as reported in previous studies (Manor et al., 2016). In contrast, high abundance of *Roseburia* and *Eubacterium* in CF individuals in our data set was found. Burke et al. (2017) reported that CF individuals who had the highest percentage of predicted Forced expiratory volume in 1 s (a measure of lung function) had increased *Roseburia* levels, suggesting disease intensity can impact gut microbiota composition.

Secondary metabolites produced by the gut microbial community also change as microbial composition changes. The significantly high levels of NRPs observed in CF individuals in this study could be a result of the stressful environment that the microbiota is exposed to in this patient group such as continuous antibiotic exposure. Several ESKAPE pathogens show the presence of NRPS biosynthetic gene clusters and their secondary metabolites that are involved in virulence. Indeed, a recent study also reported a positive correlation between NRPs and the virulence profile of *Burkholderia* species (Gulick, 2017; Pearson et al., 2020). Furthermore, an *in-vitro* study using synthetic CF sputum medium revealed the positive role of the CF disease environment and antibiotic use in upregulating the formation of secondary metabolites such as siderophores, antimicrobials, and quorum-sensing signals in three *Burkholderia cenocepacia* strains (Jaiyesimi et al., 2021). More studies are needed to examine the secondary metabolite profiles in patients with chronic antibiotic exposure, to understand the mechanism of resistance development and virulence in pathogens involved, helping towards better and targeted therapy, with effective pathogen clearance.

The CF individuals in this study were exposed to various antibiotics including inhaled, intravenous and oral antibiotics for treatment and prophylaxis during the 12 months before sample collection. Consequently, this patient group was anticipated to contain high abundance of ARGs in the resistome profile. The classes of ARGs were highly diverse in the CF group compared to controls, and the total abundance of ARGs varied significantly between the two groups. The presence of ARG-containing bacteria is of concern as studies suggest that a third of CF patients infected with *Pseudomonas* carry MDR bacteria (Oliver et al., 2000). *Pseudomonas aeruginosa* can evade antibiotic treatment by several

methods such as biofilm formation and carries several virulence factors (Malhotra et al., 2019).

WAAFLE-based analysis showed the presence of a few novel lateral ARG transfer events in contigs from the CF group, while none were found in the control group. The genes that were laterally transferred in the CF group confer resistance to streptogramin antibiotic, macrolide antibiotic, lincosamide antibiotic, tetracycline, rifamycin, mupirocin, phenicol and sulfonamide antibiotic. Many of these genes confer resistance to antibiotics administered to CF individuals, which is of concern as transfer of resistance genes in the bacterial community can contribute to the development of multidrug resistance in pathogens. Examining the presence of mobile genetic elements in the two groups revealed that insertions sequences were significantly higher in CF individuals. Insertion sequences are associated with transfer of ARGs between bacterial communities (Razavi et al., 2020). Some mobile genetic elements in our analysis were anticipated to be part of the same contig as those of ARGs as predicted by ABRicate in the CF group, though only very few. This number might be small as many of the resistance genes are present inherently in the bacteria, making the patient group reservoirs of ARGs. In addition, ABRicate only detects acquired mutations, and there could be many unrecognized ARGs – this leads a scope for future research to study the context of insertion sequences and ARGs to better understand their association in patients with chronic antibiotic exposure and an enriched resistome profile is observed.

The current dataset derived from a previous study (Fouhy et al., 2017) is a pilot-scale investigation but has some limitations, predominantly the small cohort size which makes it difficult to make firm conclusions. Further studies with larger cohort sizes, individuals at different stages of infection (newly diagnosed vs being treated long term), who differ in disease severity and condition will help in better understanding the gut microbiome and resistome profile of this patient group. Further, due to the chronic antibiotic exposure in CF care, it would be helpful to study the longitudinal microbiome and resistome profile in CF individuals of all age groups, which will help to study resistance persistence and changes over time as studies report decreasing gut microbiota diversity with age (Nielsen et al., 2016). Our analyses focused only on resistome profile differences of the two groups and did not look at single nucleotide polymorphisms or point mutations, which could also be a contributing factor worth studying due to its association with antibiotic resistance.

However, this study highlights the importance of studying gut resistome and microbiome profiles in patients with chronic antibiotic exposure, so that careful antibiotic stewardship measures can be developed by considering the risk-benefit ratio for the patient group and the community. The examination of CF individuals' gut microbiota and resistome profile presents a prospect for the development of probiotics, which can be used to limit the changes in microbial composition, thus limiting the alterations in metabolite production affecting patient health. Studies have reported that probiotic administration to CF individuals reduces intestinal inflammation, hospital admissions and pulmonary exacerbations (Bruzzese et al., 2007, 2014; Weiss et al., 2010). Further multi-omic studies will help develop personalised precision medicines and treatments for CF individuals while simultaneously fighting the resistome issue.

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6.7 Ethics statement

The individuals in this study were recruited as part of CFMATTERS project. Ethics approval was provided by Clinical Research Ethics Committee of the Cork Teaching Hospitals, Cork, Ireland.

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6.9 Supplementary tables

Table S6.1: LEfSe LDA effect size score and p-value for significant features at the genera level.

feature	log of highest class average	feature_enriched_cohort	LDA effect size	p-value
g__*Coprococcus*	4.683911245	Control	4.401867	0.03263
g__*Flavonifractor*	3.178697195	CF	3.829288	0.04951
g__*Coprobacillus*	3.335287449	CF	3.610929	0.007397
g__*Lactobacillus*	3.707529962	CF	3.716033	0.02223
g__*Barnesiella*	4.27730536	Control	4.048424	0.007397
g__*Blautia*	5.017799064	CF	4.698476	0.016309
g__*Enterococcus*	4.978101063	CF	4.614802	0.007397
g__*Faecalibacterium*	4.549470041	Control	4.206192	0.002802
g__*Adlercreutzia*	2.471921648	Control	4.623487	0.02223
g__*Clostridium*	5.076356818	CF	4.729345	0.003885
g__*Dorea*	4.442866638	Control	4.175714	0.04632

Table S6.2: LEfSe LDA effect size score and p-value for significant features at the species level.

feature	log of highest class average	Feature enriched cohort	LDA effect size	p-value
s__*Bifidobacterium_longum*	4.53231	Control	3.961777	0.04632

s__*Adlercreutzia_equolifaciens*	2.450487	Control	4.49664	0.02223
s__*Barnesiella_intestinihominis*	4.254815	Control	4.049568	0.007397
s__*Faecalibacterium_prausnitzii*	4.538161	Control	4.224192	0.002802
s__*Enterococcus_faecalis*	4.936658	CF	4.551878	0.007397
s__*Ruminococcus_gnavus*	4.898391	CF	4.604546	0.022487
s__*Flavonifractor_plautii*	3.171377	CF	3.669287	0.04951
s__*Clostridium_hathewayi*	3.486421	CF	3.57922	0.020096
s__*Lachnospiraceae_bacterium_1_1_57FAA*	3.520937	CF	3.473938	0.007397
s__*Blautia_producta*	3.881229	CF	3.676417	0.02223
s__*Lachnospiraceae_bacterium_5_1_63FAA*	3.643299	Control	3.62877	0.04951
s__*Clostridium_clostridioforme*	4.821948	CF	4.570319	0.022487
s__*Dorea_formicigenerans*	3.880082	Control	3.668189	0.049336
s__*Anaerostipes_unclassified*	3.464199	CF	3.476088	0.02223
s__*Coprobacillus_unclassified*	3.32171	CF	3.36148	0.007397
s__*Streptococcus_thermophilus*	4.1133	CF	3.706251	0.049336
s__*Alistipes_putredinis*	4.62819	Control	4.348902	0.04951
s__*Coprococcus_comes*	4.374467	Control	4.054879	0.021025
s__*Ruminococcus_torques*	4.093516	CF	3.822113	0.020096
s__*Eggerthella_lenta*	3.148434	CF	3.502648	0.021025

7 Chapter 7

General Discussion

7.1 Discussion

Growing resistance and slowed manufacturing of new antibiotics mean we must use the available antibiotics cautiously. However, antibiotics are often misused, such as their use in viral infections (Houten et al., 2019) and overused, such as prophylactic use in farm animals (Oikonoumou et al., 2020). Antibiotic resistance development data can help design antibiotic stewardship programs and aid in the development of new antimicrobials.

It is now known that antibiotic resistance genes (ARGs) produced in one environment can be easily transferred to another – moving between humans, animals, and the environment, making it a One-Health issue (Kim & Cha, 2021). Antibiotics and antibiotic-resistant strains can lead to the formation of multi-drug resistant (MDR) strains, which can complicate patient care, increase the cost and decrease the effectiveness of treatments, and affect the host microbiome composition, which can adversely influence host immune and metabolic functions. Detailed studies are thus required to examine the prevalence and source of ARGs in antibiotic-exposed and naïve settings, allowing medical workers and policymakers to make informed decisions promoting the judicial use of antibiotics.

Studies included in this thesis focused on the presence and persistence of ARGs in various settings such as bovine colostrum and milk following dry cow therapy (DCT) with and without antibiotics, the infant gut microbiome following different modes of delivery and antibiotic treatment or not, and the adult cystic fibrosis (CF) gut microbiome which is invariably exposed to chronic antibiotic treatment.

Chapter 1 reviewed and described in detail the potential modes of vertical transfer of antibiotics and antimicrobial-resistant (AMR) strains to infants, as mother is considered to be the most influential source of the microbiome for infants. Studies have reported passage of antibiotics and/or AMR strains through breast milk and in-utero, which is alarming as the infant microbiota is highly fragile, and alterations in early life can have long-term impressions on health.

Chapter 2 further discussed and provided a detailed overview of the negative impact of antibiotics on host health both with and without mediation by the microbiota.

One of the major risk factors for accelerated AMR is the routine veterinary use of medically important classes of antibiotics in treatments such as DCT. ARGs in animal-

associated microbiomes can be transferred from one bacterium to another by horizontal gene transfer and can enter humans through the food chain (Founou et al., 2016). We thus examined the effect of prophylactic antibiotic use in cows before lactation, a process termed dry cow therapy, on milk microbial and resistome profiles (Chapter 3). Bovine milk was chosen as it can be involved in the spread of ARGs through the food chain via human consumption. Based on the analysis, ARGs were present in colostrum and milk microorganisms from all farms under investigation with higher abundance in antibiotic-treated groups, thus acknowledging the need for further studies to examine the source of these ARGs. We further found that the microbial composition varied from colostrum to milk with increasing diversity over time in antibiotic-treated groups. Such a difference in alpha diversity in the antibiotic-treated group suggests the negative impact of antibiotics on the milk microbiome. The microbial composition of the non-antibiotic-treated group was significantly different from the antibiotic-treated group (Beta diversity), though several common milk genera were found in all the groups, similar to that reported by Basbas et al. (2022). *Brachybacterium, Brevibacterium, Microbacterium, Kocuria, Corynebacterium* were observed in all three groups (control group – no antibiotics, Ubro Red, Cephaguard DC) with some genera including *Lactobacillus, Corynebacterium, Ottowia, Kocuria and Microbacterium* showing increases over time in antibiotic-treated groups. Several factors including farm hygiene, season, and indoor vs. outdoor housing can affect the microbial composition and thus resistome profile and are factors that must be considered in future studies (Derakhshani et al., 2018). Furthermore, recent studies have reported a slight change in milk microbial composition during the transition to mastitis, suggesting a mastitis-related microbiota (Taponen et al., 2019; Pang et al., 2018; Lima et al., 2017). In the present study, mastitis preventative treatments (use of antibiotics during DCT) did not show any significant variations in microbial diversity compared to the control group, but high abundance of ARGs was observed. Mastitis-causing pathogens were also not abundantly detected in the non-antibiotic-treated group. Thus, these data support using only teat sealants for DCT and reducing antibiotic treatments for probable infections without any diagnosis. The presence of ARGs in cow milk is of concern due to the risk of its transfer to humans through the food chain. Inclusion of swabs from the environment including bedding and water pipes in future studies will also help to examine the source of ARGs in colostrum and milk of cows unexposed to antibiotics. Further studies with both cow milk samples and corresponding calf fecal

samples will enhance our understanding of the vertical transmission of ARGs in this setting and help understand the impact of ARGs in early life in calves.

The neonatal period and infancy is the most crucial stage from the viewpoint of microbiota and immune functioning development – with both processes working hand in hand. Thus, the early life microbiota forms the foundation of health in adult life and the microbiome co-evolves with the host through childhood, with several factors such as gestational age, delivery mode, feeding habits and antibiotic uptake affecting/shaping it (Arrieta et al., 2014). C-section infants often have an altered gut microbial composition and are associated with a higher risk of hospitalisation and infections (Christensen et al., 2018), this could mean higher antibiotic administration to avoid sepsis and other probable infections in early life. Chapter 4 thus examined infants' microbiota and resistome development in early life from week 1 to year 2. This study included three groups based on antibiotic exposure during the first four days of life and mode of delivery (vaginally-delivered/no-antibiotics [VDnoab]; caesarean delivery with antibiotics [Csab]; caesarean delivery/no-antibiotics [CSnoab]). It was observed that antibiotic use in early life had a strong and long-term effect, which was more prominent than delivery mode on the microbiota, in line with the results of Reyman et al. (2022). Furthermore, CSab group had a double disadvantage - as the infants in this group are born by CS and administered antibiotics – consequently showing higher abundance of pathobionts with delayed colonisation of Bacteroidetes. VD infants had higher abundance and prevalence of taxa belonging to phyla Actinobacteria and Bacteroidota. These results are in accordance with previous studies (Reyman et al., 2019; Parnenen et al., 2018; Dominguez-Bello et al., 2010). A high abundance of ARGs in infants in early life linked to Gammaproteobacteria taxa similar to Parnanen et al., (2018) was also observed. The CSab group was positively associated to various antibiotic classes including aminoglycosides, fluoroquinolones, penams, tetracyclines, MDR – some of which are the same class of drugs administered to infants in this group. Similar to recent studies (Lebeaux et al., 2022; Li et al., 2021; Parnanen et al., 2018), *Escherichia coli* was found to be highly correlated to ARGs. Based on the 1449 MAGs generated as part of the assembly-based analysis, the persistence of bacteria and ARGs was detected using stringent parameters. Persistence analysis of the microbiota and ARGs showed that taxa belonging to the genus *Bifidobacterium* and *Bacteroides* were generally persisters in all infants with Proteobacteria as persisters

observed in the CSab group. Most of the ARGs did not persist up to two years of age and were lost with the transition of bacteria from early life to age two. CS-birth and antibiotic use is associated with colonisation by several pathobionts; possibly the use of antibiotics in CS infants resulted in the positive selection of these taxa and, consequently, the high ARG association, which were lost over time as the microbiota developed.

Chapter 5 investigated the resistome profile of infants under three years of age, by conducting a meta-analysis to study the global burden of ARGs on the infant gut. After a comprehensive search, shotgun-sequencing data was downloaded from 26 studies including infants under three years of age and used for microbial and resistome data analysis. These data were also used as part of another study (Zeng et al., 2022 – attached in appendix) to generate a compendium of 32,277 early-life gut genomes, which were used in the present study for antibiotic resistance analysis. This analysis showed a high overall ARG abundance in the infant gut globally, with antibiotic classes namely glycopeptides, fluoroquinolones, tetracycline, and macrolides being the most abundant. The highly abundant antibiotic classes were carried mostly by gram-negative bacteria, many of which are known pathobionts in early life, such as *Escherichia, Enterobacter, Citrobactera, and Klebsiella*. Further, building on the reported relationship between the socioeconomic status of a country and its healthcare access and quality (HAQ) index (Mallah et al., 2022; GBD 2016 Healthcare Access and Quality Collaborators), we also observed a direct relationship between status and HAQ index to ARG abundance – with countries that have a higher socioeconomic status and receive better healthcare access presenting higher ARG abundance in infants in early life. An association between delivery mode and ARGs, with CS infants showing higher ARG abundance (as observed in Chapter 4), was also observed. Similar to our previous results (Chapter 4), a decrease in ARG abundance with increasing age was found. This could be attributed to the highly dynamic and evolving microbial profile in infants in early life.

Results from Chapters 4 and 5 provide a picture of the global ARG abundances in infants below three years of age, showing high levels of ARGs even in antibiotic-naïve infants. MAGs used in Chapters 4 and 5 allow thorough analysis of the microbial and resistome profile. The presence of high abundance of ARGs, even in infants not exposed to antibiotics is alarming as this could affect the functionality of the microbiota. Strategies like stricter antibiotic stewardship programs, guidelines for antibiotic use in pregnancy,

and probiotic use in infants can help reduce the overall ARG burden on the infant gut microbiome (Abo et al., 2022; Guitor et al., 2022; Casaburi et al., 2019). Further longitudinal studies with deeper sequencing depth in early life are needed to better understand the impact of prenatal and postnatal prophylactic antibiotic use in later life. This will allow for generation of larger and higher quality MAGs that can be used for detailed strain and ARG tracking. Further, this will add to the Early-life gut genome reservoir, which can help in identifying potential probiotic strains.

While the work presented in earlier chapters focused on the gut and milk resistome and microbiota profile in infants or in a situation where antibiotic use is acute and preventable, Chapter 6 was designed to examine the resistome and microbial profile when chronic antibiotic use is inevitable. Chapter 6 was designed to examine the effect of chronic antibiotic exposure to treat respiratory infections in adult CF patients on the gut resistome. This study was planned as an extension of a previous study (Fouhy et al., 2017) with the aim to study the abundance of ARGs and virulence factors – ARGs and virulence factors such as biofilm formation can lead to MDR bacteria, which can complicate treatments in this group. Interestingly, Fouhy et al. (2017) observed a difference in the gut microbiota functionality of CF subjects recruited as part of the CFMATTERS cohort vs. healthy controls. The present analysis demonstrated a higher abundance of total ARGs and virulence factors in subjects with CF similar to previously reported results by Taylor et al. (2021). Differential abundance testing on the antibiotic resistance classes using MaAsLin2 showed a positive association of the CF group to antibiotic classes such as aminoglycosides, MDR, macrolide, oxazolidinone, tetracycline, fluoroquinolone, streptogramin, diaminopyrimidine, sulfonamide, sulfone, tetracycline, lincosamide, mupirocin – most of which were drug classes administered to CF individuals in this study. Microbial analysis revealed the prevalence and stronger association of commensals such as *Dorea, Coprococcus, Faecalibacterium, Barnesiella* and *Bifidobacterium* with healthy controls, while a mix of commensals and pathobionts such as *Eggerthella, Clostridium, Streptococcus, Flavonifractor, Enteroccous* was associated with CF individuals. This difference in microbial composition was associated with the overall health status of the individuals. It influenced the abundance of non-ribosomal peptide synthetases (NRPS) secondary metabolites that are generally high in stressful conditions. However, no significant difference in microbial diversity was observed between the two groups, in

contrast to that reported in a previous study of children with CF (Coffey et al., 2019). This could be because the adult gut microbiota is more stable and resilient than that of children, and may have adapted to the long-term antibiotic usage in this study. Given the high abundance of *P. aeruginosa* and other gram-negative bacteria in CF individuals, detailed longitudinal studies with larger cohorts are needed to examine the transmission potential of ARGs. Indeed, such bacteria are reported to have high abundances of inherited and acquired ARGs in their genomes, which can be transferred to the wider community (Woodford et al., 2011). While recent studies have reported the gut microbiota composition in individuals with CF (van Dorst et al., 2022), the present study sheds light on CF individuals' previously unexplored gut resistome profile. Future studies involving baseline samples based on genetic determination of CF along with samples post CF detection will help in understanding the transition of bacteria and resistome profile before and after chronic antibiotic exposure in this patient group.

The results from this thesis provide a comprehensive picture of the effects of single-dose and chronic antibiotic usage on microbiota and resistome profiles by generating and/or analysing metagenomics shotgun sequencing data. Irrespective of the dosage of antibiotic and the environment, we observed high ARG abundance in the bacterial composition analysed. Though there are several advantages of using shotgun metagenomics sequencing (Ranjan et al., 2016), a major drawback is that only the abundance of genes responsible for the underlying function can be calculated and the answer to whether the gene is functional or not remains unknown. In addition, when using genomic data to predict results, it is unknown if the organism is alive or dead; thus, results can lead to false positives. Using culture-based techniques, along with meta-transcriptomics and metabolomics analyses can help overcome this issue. Further, short-read shotgun sequencing data can lead to shorter contigs during genome assembly and, thus, smaller genome sizes, which can mean lower chances of matching with a reference genome. More recent long-read sequencing platforms, such as PacBio, and Oxford Nanopore platform, combined with short-read sequencing can allow detailed distinction of reads between taxa without compromising sequencing accuracy. Studies using mixed sequencing along with other omics techniques can help to attain in-depth knowledge of densely populated environments such as the gut and sparsely populated samples such as milk.

Further studies that focus on the virome profile of infant gut and bovine milk along with the bacterial profile will help better understand the transfer of ARGs within the microbiota. Future studies will also benefit from using baseline samples before antibiotic use and following up with subjects for samples post-antibiotic use, this will allow the tracking of ARG development and spread. Furthermore, studies that focus on microbiota's functional profile and resistance pattern will help delineate the relationship between microbial resistome and functional profile. Overall, this thesis's results will help formulate several research questions for future studies such as; what is the origin of ARGs in bovine milk in the absence of antibiotic exposure on Irish farms? How does the high initial ARG diversity impact the long-term functional profiling of the microbiota? Can ARG abundance be reversed by probiotics or other microbial reinforcements in early life?

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APPENDIX - A compendium of 32,277 metagenome-assembled genomes and over 80 million genes from the early-life human gut microbiome (Nature communications, 2022)

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A compendium of 32,277 metagenome-assembled genomes and over 80 million genes from the early-life human gut microbiome

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Shuqin Zeng[1], Dhrati Patangia[2,3,4], Alexandre Almeida[5,6], Zhemin Zhou[7], Dezhi Mu[1], R. Paul Ross[2,4], Catherine Stanton[2,3] & Shaopu Wang[1]

Age-specific reference genomes of the human gut microbiome can provide higher resolution for metagenomic analyses including taxonomic classification, strain-level genomic investigation and functional characterization. We present the Early-Life Gut Genomes (ELGG) catalog with 32,277 genomes representing 2172 species from 6122 fecal metagenomes collected from children under 3 years old spanning delivery mode, gestational age, feeding pattern, and geography. The ELGG substantially expanded the phylogenetic diversity by 38% over the isolate microbial genomes, and the genomic landscape of the early-life microbiome by increasing recruitment of metagenomic reads to 82.8%. More than 60% of the ELGG species lack an isolate representative. The conspecific genomes of the most abundant species from children differed in gene diversity and functions compared to adults. The ELGG genomes encode over 80 million protein sequences, forming the Early-Life Gut Proteins (ELGP) catalog with over four million protein clusters, 29.5% of which lacked functional annotations. The ELGG and ELGP references provided new insights into the early-life human gut microbiome and will facilitate studies to understand the development and mechanisms of disturbances of the human gut microbiome in early life.

The human gut microbiome—the vast microbial ecosystem present in the gastrointestinal tract—has been suggested to play diverse and crucial roles in host health and various diseases throughout the course of life[1,2]. The acquisition and development of the gut microbiome in early life have long-lasting effects on the structure and function of this microbial community later in life[3]. Despite the increasing number of studies providing substantial insights into the early-life gut microbiome[4–9], extensive genome-resolved metagenomic analyses of the early-life gut microbiome remain scarce. Having high-quality and extensive reference genomes of the early-life human gut microbiome can improve the resolution and accuracy of taxonomic and functional analyses, which is essential for driving future early-life microbiome studies.

Tremendous efforts have been undertaken to increase the number of isolate reference genomes from the human gut, such as the Human Microbiome Project (HMP)[10], the Human Gastrointestinal

[1]Key Laboratory of Birth Defects and Related Diseases of Women and Children (Sichuan University), Ministry of Education, Department of Pediatrics, West China Second University Hospital, Sichuan University, Chengdu, China. [2]APC Microbiome Ireland, University College Cork, Cork, Ireland. [3]Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland. [4]School of Microbiology, University College Cork, Cork, Ireland. [5]Department of Veterinary Medicine, University of Cambridge, Cambridge, UK. [6]European Bioinformatics Institute (EMBL–EBI), Wellcome Genome Campus, Hinxton, UK. [7]Pasteurien College, Medical College of Soochow University, Soochow University, Suzhou, China. e-mail: shaopu.wang@scu.edu.cn

Bacteria Genome Collection (HGG)[11], and Culturable Genome Reference (CGR)[12], however, the currently available reference genomes representing the human gut microbiome are still underrepresented[13,14]. Therefore, in parallel to culturing, de novo assembly of shotgun metagenomic reads and binning into metagenome-assembled genomes (MAGs)—a culturing-independent and reference-free approach—is thought to be a useful strategy to efficiently discover the potential microbial diversity that is recalcitrant to the current culturing approaches in the laboratory. Using MAGs has provided massive expansion of the tree of life from different environmental niches[15–17]. Studies have described some of the dynamic microbiome changes including taxonomic composition and strain-specific functional adaptation that occur during early life compared to adulthood[18]. For instance, most strains of *Bifidobacterium* that dominated the gut microbiome during breastfeeding and dissipated later in life typically carry high numbers of gene cluster responsible for human milk oligosaccharides (HMOs) utilization; whereas these gene cluster are no longer present in most bifidobacterial strains after weaning[19,20]. Understanding strain-specific differences in gene content and function also requires representative genomes of the early-life gut microbiome. Previous MAGs studies either analyzed samples exclusively from non-human gut source[21], or from the human gut but with a relatively low proportion of early-life fecal samples[13,15]. Additionally, unifying the human gut genomes including MAGs and isolate genomes has indeed substantially provided novel insights into the richness, diversity and cultivability of gut microbiome at various taxonomic and functional levels[22]. However, there is currently no large-scale catalog of MAGs available specifically designed for the gut microbiome in early life.

Therefore, in order to fill this gap, we specifically analyzed 6122 fecal metagenomes from children under the first 3 years of life, and generated a set of 32,277 MAGs clustered into 2172 species-level clusters together with 86,678,654 genes representing 4,036,936 gene clusters, forming the Early-Life Gut Genomes (ELGG) and Proteins (ELGP) catalogs, respectively. With these comprehensive sequence collections, we characterized the taxonomic and functional profile of the early-life gut microbiome at the genome level and interrogated the genomic variations present in the gut microbiome of children associated with various clinical factors.

Results

Recovering 32,277 microbial genomes from over 6000 early-life gut metagenomes

To elucidate differences in the early-life gut microbiome at the genome level and also to expand the genomes for novel human gut lineages during early life, we employed a combination of metagenomic assembly and binning on 6122 multi-country distributed metagenomes across four continents from children from birth to three years old (Fig. 1a; Supplementary Data 1). Compared to the metagenomes that were used to build the Unified Human Gastrointestinal Genome (UHGG)[22], 1904 metagenomes overlapped. The MAGs were produced by three different binning tools (i.e., MetaBAT[23], MaxBin[24], and CONCOCT[25]), and then integrated and refined to remove duplicates and improve the quality of assembled genomes with metaWRAP[26] (Fig. 1b). Following this pipeline, a total of 42,054 MAGs were met or exceeded the medium-quality (≥50% completeness and <10% contamination) based on the "Minimum information about a metagenome-assembled genome" (MIMAG) standard[27]. In order to provide stricter genome quality control, we selected those genomes having completeness >50% and contamination <5% together with genome quality score (defined as completeness−5×contamination, QS) > 50 and free of chimerism (passed by GUNC[28]), resulting in 32,277 MAGs for subsequent analyses, which we referred to as the ELGG catalog (Fig. 1c, d; Supplementary Data 2). The median size of the 32,277 MAGs was 2.59 megabases (Mb) (interquartile range, IQR = 2.08–3.75 MB) with N50 values between 1.7 kilobases and 2.8 Mb. Among the ELGG catalog, 25,303 MAGs (accounting for 78.4% of the total dataset) were >90% complete (IQR = 97.3–99.7%) and <5% contaminated (IQR = 0.00–1.04%), hereafter referred to as 'near-complete' genomes. A subset of 4614 MAGs (18.2% of near-complete genomes) had 5 S, 16 S and 23 S rRNA genes as well as at least 18 of the standard tRNAs, which can be classified as the 'high quality' draft genomes based on the MIMAG standard[27]. The relatively low proportion of high quality recovered MAGs was comparable with previous large-scale studies of human gut MAGs[13,22] due to the typical challenge in the MAGs assembled from metagenomes with short reads. The rest of the ELGG catalog consists of 6974 medium-quality MAGs (>50% completeness and <5% contamination) (Fig. 1d). The other genome statistics (including contig number and N50, genome depth, and relative abundance) supported the consistent high quality of near-complete MAGs compared to medium-quality MAGs even when the latter were stratified based on the QS at the threshold of 75 (Fig. 1c).

In line with previous studies[15,22], the ELGG catalog was further investigated at the level of strain heterogeneity per genome by using CMSeq[15], which has been suggested to represent a useful measure to assess genome quality. We found that the median strain heterogeneity (proportion of polymorphic positions) of genomes from the ELGG catalog was 0.005% (IQR = 0.001–0.031%; Fig. 1c), which is much lower than the UHGG catalog (0.06%) that included the human gut samples covering all ages[22]. The near-complete genomes displayed a lower level of strain heterogeneity compared to the medium-quality genomes from the ELGG catalog (Fig. 1c).

A reference protein catalog for the gut microbiome early in life

To expand our understanding of the functions of early-life gut microbiome, the protein-coding sequences (CDS) for each of the 32,277 MAGs were predicted, resulting in a total of 86,678,654 genes. This accounted for 54.9% of all genes when taking the unbinned contigs from the 6122 metagenomic samples into account. After clustering the protein sequences at 95% amino acid identity, we obtained 4,036,936 protein clusters, forming the ELGP catalog. Rarefaction analysis indicated a saturation point was still not reached as the number of ELGP clusters steadily increased as a function of the number of MAGs included (Fig. 2a), and this pattern was also observed with the inclusion of all contigs from 6122 samples (Supplementary Fig. 1a), which was in line with pervious observations[22,29]. However, when removing protein clusters with one protein sequence, the number of protein clusters approached saturation (Fig. 2a; Supplementary Fig. 1a). This may suggest that although the microbial genes from children gut microbiome are still underestimated, the majority of undiscovered genes are likely to be rare. We further compared our early-life gene catalog to the large protein database—Unified Human Gastrointestinal Protein (UHGP)—that mainly includes microbial genes from the gut of adults and clustered at 95% protein identity (n = 20,239,340)[22]. This revealed that 2.9 million gene clusters from the ELGP overlapped with the UHGP catalog, but there was a large proportion (27.3%, n = 1,076,116) from the ELGP not represented in UHGP, and the total number of proteins from 1,076,116 clusters accounted for 5.4% when taking all 86,678,654 genes into consideration, underlying the uniqueness of the gut microbiome of children. Among those protein cluster representatives exclusively from ELGP or UHGP, 27.6% (n = 296,624) and 30.1% (n = 3,972,835) of representatives were respectively annotated with a known function, and the rest of the clusters were either putative or hypothetical proteins (Fig. 2b). Therefore, our results provide a comprehensive collection of the gut microbiome protein space early in life that may serve as a reference for early-life gut microbiome research.

To better elucidate the functional diversity of the early-life gut microbiome, we annotated gene functions of the ELGP catalog with currently available databases, including Clusters of Orthologous Genes (COGs), KEGG modules, level-4 Enzyme Commission categories (ECs),

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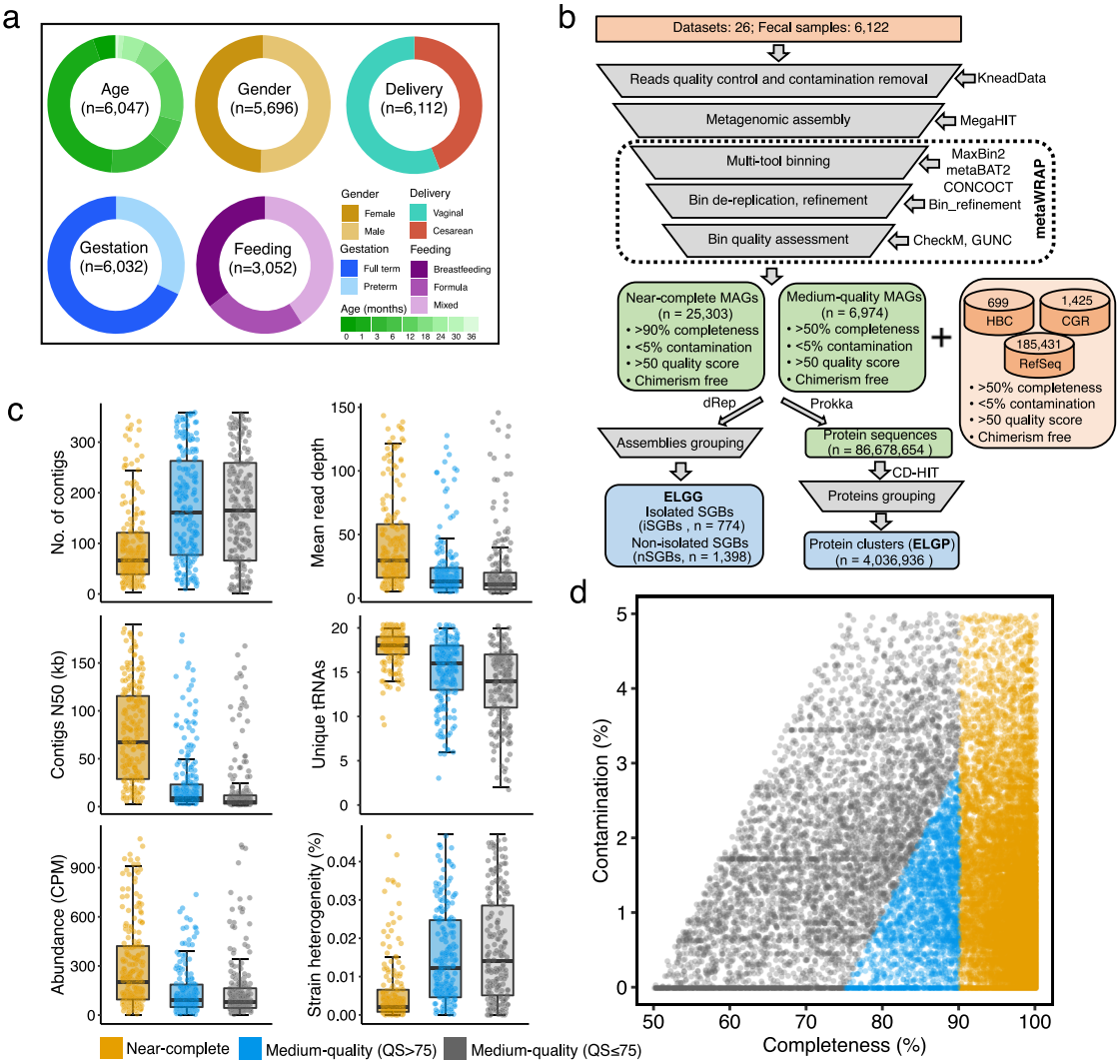


Fig. 1 | The reconstruction of sequence catalog from the early-life human gut microbiome. a The number and proportion of fecal metagenomes stratified by clinical features including age, gender, delivery mode, gestational age, and feeding patterns. **b** Overview of the computational pipeline to generate ELGG and ELGP catalogs. **c** Quality metrics across near-complete ($n = 25,303$), medium with quality score (QS) > 75 ($n = 2063$) and medium with QS ≤ 75 ($n = 4911$) MAGs. CPM copies per million reads. Boxes show the interquartile range (IQR), with the horizonal line as the median, the whiskers indicating the range of the data (up to 1.5× IQR), and points beyond the whiskers as outliers. **d** Completeness and contamination scores for each of 32,277 genomes. QS = completeness−5 × contamination.

Gene Ontologies (GOs), and carbohydrate-active enzymes (CAZy). We found that a total of 70.5% of genes from the ELGP had a match to at least one of the databases of COGs ($n = 2,844,021$ genes across 24 functional categories), ECs ($n = 722,946$ genes matching 2658 enzymes), KEGG ($n = 533,759$ genes from 674 modules), GOs ($n = 256,861$ genes from 10,461 orthologous groups), and CAZy ($n = 46,392$ genes matching 104 families) (Fig. 2c). These results showed that a median of 88.7% (IQR = 85.9–91.0%) of genes per genome in the ELGG were annotated, and this rate was lower in genomes from children at 36 months of age (a median of 89.1% at birth vs. 86.5% at 36 months; linear model, $p < 0.0001$) (Fig. 2d). Based on the distribution of COGs functions that matched the largest number of ELGP genes, the most abundant genes with a known function present in the ELGP were involved in transcription, replication/recombination/repair, cell wall/membrane/envelope biogenesis, and carbohydrate transport and metabolism (Fig. 2e). The most highly represented families of ECs, KEGG, and GOs were DNA helicase (EC: 3.6.4.12), M00178 (ribosome, bacteria) and biological process (GO: 0008150). The predominant glycoside hydrolase family in the ELGP catalog was GH13, targeting the hydrolysis of a wide range of simple and complex glycans including di-, oligo-, and polysaccharides as well as related substrates, such as starch, amylose, and pullulan[30] (Supplementary Fig. 1b). We again observed that the majority of the investigated COGs categories (11/19) were well-characterized at the first few months, and then gradually decreased as children aged (i.e., Wilcoxon test, FDR < 0.05, when compared to the annotated gene per genomes at birth to that from ≥36 months) (Fig. 2f).

Early-life MAGs belonging to 2172 species-level clusters

To explore the number of culturable species that were included in the ELGG catalog, we clustered 32,277 MAGs together with 187,555 isolate reference genomes from NCBI RefSeq and two human gut culturing studies[11,12]. The species-level clusters (SGBs for species-level genome bins) were computed by using a multistep distance-based approach with at least 95% average nucleotide identity (ANI) and at least 30% overlap of alignment fraction (AF) (Methods). A total of 23,307 SGBs were generated, and the MAGs from the ELGG catalog were distributed into 2172 SGBs (Fig. 3; Supplementary Data 3). Among the 2172 SGBs, only 774 SGBs contained isolate reference genomes (denoted as cSGBs for cultured SGBs) containing 86,283 isolate reference genomes and 29,367 MAGs. A large proportion of 99.8% ($n = 86,132$) of 86,238 isolate reference genomes were near-complete (Supplementary Fig. 2). The

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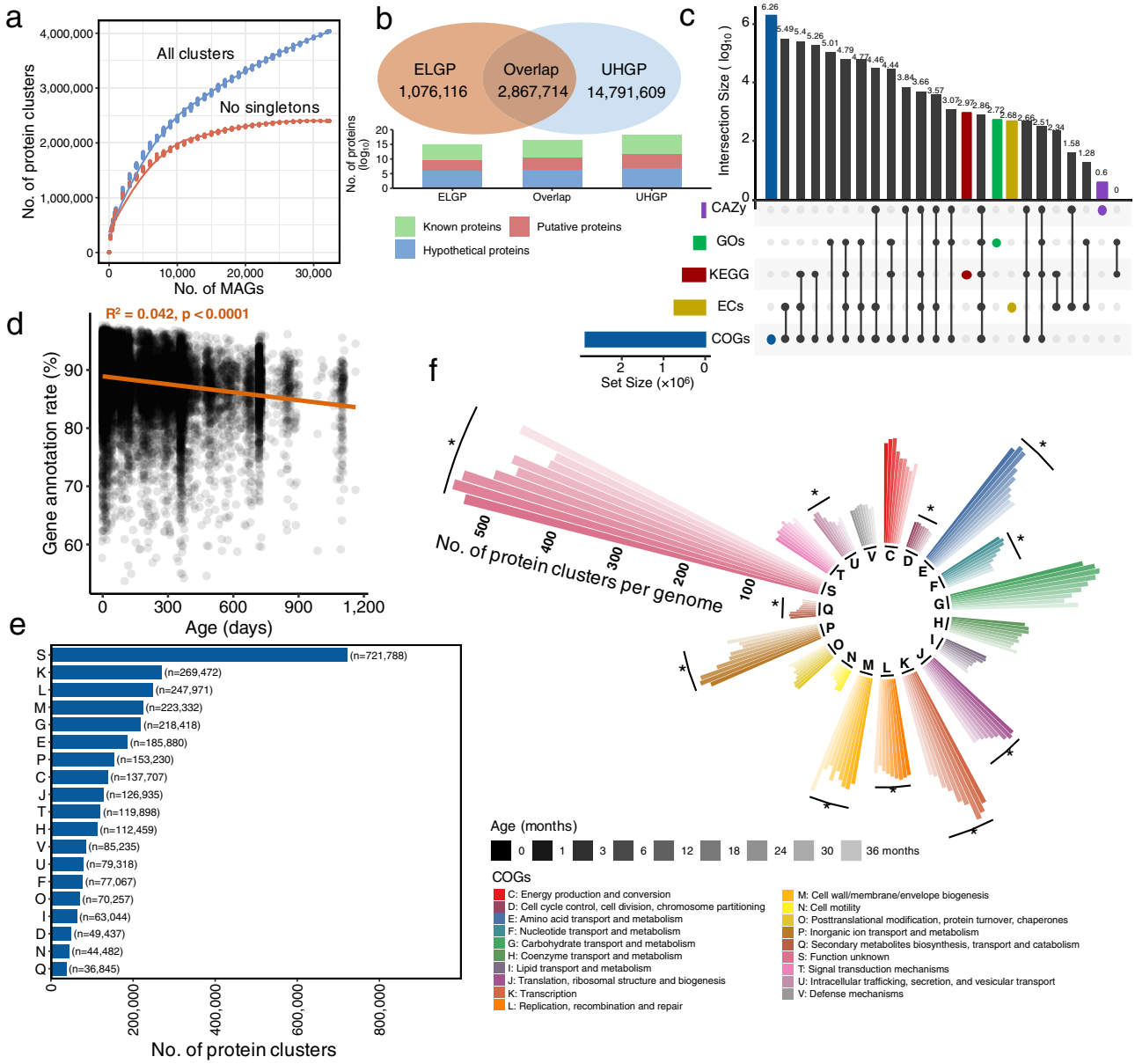


Fig. 2 | The ELGP catalog and functional characterization. a Rarefaction analysis of the number of protein clusters of early-life gut microbiome at 95% amino acid identity as a function of the number of genomes included. Curves are depicted for all the protein clusters and after excluding singleton protein clusters (containing only one protein sequence). **b** Overlap between the ELGP (orange) and UHGP (blue), both clustered at 95% amino acid identity. The bars at bottom indicate the number of proteins that the cluster representatives from three categories (ELGP exclusive, Overlap, and UHGP exclusive) encode, stratified as known, putative, and hypothetical proteins. **c** Number of proteins with functional annotation across the five functional categories and their degree of overlap. Vertical bars represent the number of proteins unique (color) to each functional category or shared (black) between the specific functional categories. Horizontal bars in the lower panel indicate the total number of proteins with functional annotation in each functional category. **d** Dynamics of the rate of protein characterization of ELGP along with the age of children. **e** COG functional annotation of the ELGP catalog clustered at 95% amino acid identity. Only functions with >5000 genes are plotted. **f** Dynamics of the rate of COG functional annotation of ELGP catalog clustered at 95% amino acid identity in response to the age of children. Vertical bars from left to right represent the age of children at 0, 1, 3, 6, 12, 18, 24, 30, and 36 months. Asterisk (*) indicates the significant difference (two-tailed Wilcoxon test, FDR < 0.05) between the rate of COG functional annotation of ELGP catalog at birth and 36 months old children.

other 1398 SGBs contained exclusively 2910 MAGs in total (denoted uSGBs for uncultured SGBs), indicating that 64.4% of the ELGG SGBs (9% of total MAGs) lack isolate genomes (Fig. 3a). When compared to the 4644 representatives of the UHGG using a distance cutoff of 0.05 (95% ANI), 13.4% of ELGG SGBs lacked a match to the UHGG. By counting the number of MAGs within each SGBs, it was observed that cSGBs represented the largest clusters, while uSGBs tended to be the rarest, with 1003 of uSGBs represented by a single genome, which was in line with the previous studies reconstructing MAGs from the environmental and host-associated microbiota[15,16,22]. Interestingly, cSGBs with >50% MAGs outnumbered uSGBs with 0–50% MAGs for clusters containing three or more genomes, underscoring the discovery power of large metagenomic cohorts (Fig. 3b). The early-life human microbial phylogenetic diversity of the 2171 bacterial SGBs was increased by 38% with the uSGBs, indicating the utility of these genomes to improve the classification of sequences from the early-life microbiome (Fig. 3c). The median pairwise distances of genomes within SGBs was 0.020 (IQR = 0.014–0.029) when including references and MAGs and 0.020 (IQR = 0.013–0.029) when only considering MAGs.

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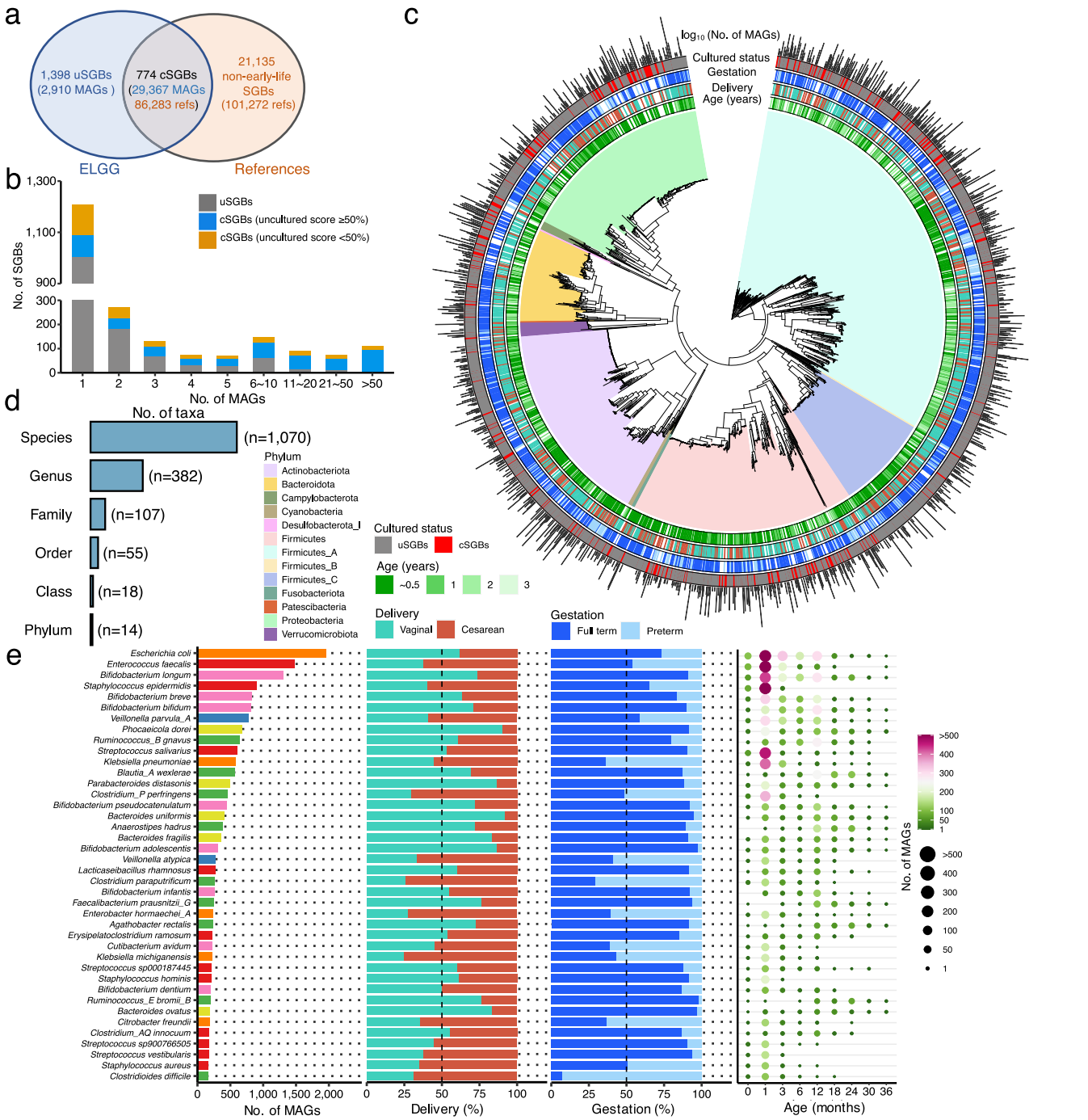


Fig. 3 | A total of 2172 species-level clusters (SGBs) obtained from 32,277 early-life MAGs. a Overlap of SGBs containing both MAGs and isolate reference genomes. SGBs containing MAGs and reference genomes are denoted as cultured SGBs (cSGBs), SGBs without reference genomes are denoted as uncultured SGBs (uSGBs), and those exclusively containing reference genomes are denoted as non-early-life SGBs. **b** The number of cSGBs and uSGBs as a function of the genome number within each SGBs. The uncultured score is calculated as the proportion of MAGs in the total genomes belonging to that SGB. **c** The phylogenetic tree of early-life gut microbiome built with 2171 bacterial representative genomes of the ELGG catalog. **d** The number of cultured taxa at different resolutions from 2172 representative genomes. **e** The number of MAGs in each SGBs, and only the top 40 most represented SGBs were displayed. The clinical factor (i.e., delivery mode, gestational age, and age) related to the MAGs per species are plotted.

Taxonomic landscape of the gut microbiome early in life

We further taxonomically annotated each species representative using the Genome Taxonomy Database Toolkit (GTDB-Tk) based on the GTDB database consisting of >311,000 bacterial and >6000 archaeal genomes that are comprised of isolate genomes, MAGs, and single-amplified genomes. We found that the ELGG catalog covered 14 known phyla (13 for bacteria and 1 for archaea), 18 known classes (17 for bacteria and 1 for archaea), 55 known orders (54 for bacteria and 1 for archaea), and 382 known genera (381 for bacteria and 1 for archaea) (Fig. 3d; Supplementary Data 3). Additionally, there were still 214 uSGBs including 339 MAGs that were not classified at the species level, indicating the lack of microbial representation in the current GTDB database. The top five uSGB classified genera were *Collinsella* (71 uSGBs with 143 MAGs), *Streptococcus* (33 uSGBs with 43 MAGs), *Haemophilus* D (13 uSGBs with 17 MAGs), *Veillonella* (13 uSGBs with 14 MAGs), and *Bifidobacterium* (9 uSGBs with 16 MAGs). Compared to the

UHGG collection that is mainly comprised of microbial genomes from adults[22], the phylum Firmicutes_A (705 SGBs with 7,765 MAGs in ELGG catalog) took up the largest proportion of SGBs in both children and adult gut microbiomes, followed by Firmicutes (390 SGBs, 7102 MAGs), Actinobacteriota (359 SGBs, 6188 MAGs), Proteobacteria (336 SGBs, 5409 MAGs), and Firmicutes_C (165 SGBs, 2,007 MAGs) (Supplementary Fig. 3a). All these top five phyla in children gut microbiome were represented by over 60% of uSGBs (Supplementary Fig. 3b). When compared at higher taxonomic resolution, a distinct difference was observed between children and adults gut microbiota. The MAGs assembled from children gut microbiome mainly consisted of the genus *Streptococcus* (164 SGBs, 2112 MAGs), *Collinsella* (129 SGBs, 534 MAGs), *Veillonella* (89 SGBs, 1501 MAGs), *Haemophilus* D (78 SGBs, 418 MAGs), and *Bifidobacterium* (58 SGBs, 4,604 MAGs) (Supplementary Fig. 3a); while the top genera from the UHGG catalog were *Collinsella*, *Prevotella*, *Streptococcus*, *Bacteroides*, and *Alistipes*.

At species level, the most represented SGBs in the ELGG catalog were *Escherichia coli*, *Enterococcus faecalis*, *Bifidobacterium longum*, *Staphylococcus epidermidis*, and *Bifidobacterium breve*, which completely differed from the genomes of the UHGG catalog (Fig. 3e). We further stratified the MAGs within each species according to delivery mode [vaginal and cesarean section (C-section)], gestational age (full-term and preterm) and the age of children at sampling. The MAGs belonging to species *E. faecalis*, *S. epidermidis*, *Clostridium* spp., *Veillonella* spp., *Klebsiella* spp., and *Streptococcus vestibularis* were mainly reconstructed from children born by C-section and/or preterm children. These species are potentially pathogenic and commonly associated with the hospital environment[4,31]. The majority of these MAGs were derived from fecal samples collected within the first year of life, highlighting the specificity of the ELGG catalog for the early-life gut microbiome. Notably, some MAGs were not reconstructed from the first few months after birth, but obtained at a later time, such as *Anaerostipes hadrus* and *Ruminococcus_E bromli_B*.

Rarefaction analysis of the total number of SGBs as a function of the number of MAGs indicated that the species from the ELGG catalog has not approached saturation, highlighting that more species remain to be discovered in the gut microbiome of children (Supplementary Fig. 3c). However, in line with the rarefaction analysis based on genomes from the UHGG catalog[22], this unsaturated status was mainly attributed to rare members of the gut microbiota, as there were 1206 SGBs with only one MAG from the ELGG catalog (Supplementary Fig. 3d). When only considering SGBs containing at least two conspecific MAGs, the number of species was much closer to saturation (Supplementary Fig. 3c). When looking into the geographic prevalence of SGBs in each continent (i.e., Asia, Europe, North America, and Oceania), the most prevalent species worldwide included *E. coli*, *B. longum*, and *E. faecalis* (Supplementary Fig. 4). Meanwhile, there were a number of SGBs with various rates of prevalence in each continent. For example, species of *Clostridium* spp., *Klebsiella michiganensis*, *Citrobacter freundii*, and *Clostridioides difficile* were more prevalent in the samples of North America, which may be attributable to the high proportion (77%) of fecal samples collected from preterm children.

Comparison of SGBs across studies with the same metagenomic datasets

To investigate the reproducibility of SGBs from the ELGG catalog, we clustered the subset of MAGs with >50% genome completeness and <5% contamination and free of chimerism from a common set of 941 metagenomes from Bäckhed et al.[32] and Vatanen et al.[33] that were available in another two previous human gut MAG studies (i.e., Nayfach et al.[13] and Pasolli et al.[15]) (Supplementary Data 4). Different assembly and binning approaches were applied in the three studies, i.e., Pasolli *et al.* assembled and binned with metaSPAdes and MetaBAT2; Nayfach et al. used MegaHIT and a combination of MaxBin2, MetaBAT2, CONCOCT and DAS Tool for assembling, binning and refinement. We observed that the pattern of MAG number produced from each sample was consistent across the three studies, but a slight increase (Wilcoxon test, $p < 0.01$) in the total number of MAGs was observed with our pipeline ($n = 5203$) compared to Nayfach et al. ($n = 4284$) and Pasolli et al. ($n = 4728$), respectively (Supplementary Fig. 5a). By calculating the proportion of shared SGBs on a per-sample basis with one other study (referred to as SGBs similarity, Methods), the median of SGBs similarity of the current study compared to the other two previous studies reached 100% for both Nayfach et al., and Pasolli et al. (Supplementary Fig. 5b). In addition, conspecific MAGs reconstructed from the same samples by different studies had a median ANI and AF of 99.9% and 93.9%, respectively (95.0% AF with near-complete MAGs and 85.3% AF with medium-quality MAGs; Supplementary Fig. 5c). These results suggest a high reproducibility of popular assembly and binning tools used in large-scale genome reconstructions, in line with previous comparisons[22].

Enlargement of the pan-genomes of key *Bifidobacterium* spp. early in life

Bifidobacterium represents the dominant genus in the gut microbiota of children and is known as the pioneering microbial member that influences microbiota succession and the capability of the host to utilize prebiotic HMOs early in life. A depletion of *Bifidobacterium* or their genes for the utilization of HMOs has recently been indicated to be involved in host systemic inflammation and immune imbalance[34]. Based on the GTDB annotation, we greatly expanded the diversity of *Bifidobacterium* intraspecies diversity by a range of 4 (*B. longum*) to 12 (*Bifidobacterium kashiwanohense*) times compared to the reference genomes belonging to the top eight *Bifidobacterium* SGBs that contained more than 100 MAGs. The largest SGB is *B. longum* with 296 reference genomes and 1306 added MAGs, followed by *B. breve* (107 reference genomes; 830 MAGs), *Bifidobacterium bifidum* (91 reference genomes; 823 MAGs), and *Bifidobacterium pseudocatenulatum* (77 reference genomes; 446 MAGs) (Fig. 4a). The pan-genome of each SGB is defined as the sum of the genes including core and accessory genes of all the genomes within that SGB[35]. The ELGG increased the size of the pan-genome per species up to a range of 5385 (*Bifidobacterium dentium* with 2337 exclusively from MAGs) to 10,759 (*B. longum* with 3522 exclusively from MAGs) that were higher than the reference genomes (Fig. 4b). This may indicate the large proportion of bifidobacterial metabolic functions that have not been uncovered based on current culturing approaches. By quantifying the abundance of these genomes in the metagenomic samples, we found that the relative abundance of bifidobacterial species decreased as children aged from birth to 3 years old (Fig. 4d). In addition, we found a lower level of strain heterogeneity in samples from early life (first 6 months), which may reflect the relatively simple dietary components (e.g., breastfeeding) in this period.

Next, we functionally annotated the pan-genomes of each *Bifidobacterium* species by mapping them against the broad range of databases including COGs, KEGG, GOs, ECs, and CAZy, and found that a proportion of genes between 30.9% (for *B. dentium*) and 39.2% (for *Bifidobacterium adolescentis*) lacked a match to any database. When we stratified the genes as core and accessory, the majority of unmatched genes were accessory (only a proportion of 56.0–64.6% genes matched), and over 92% of core genes were annotated (Fig. 4c). According to COG categories, the replication/recombination/repair, carbohydrate transport and metabolism, transcription, and amino acid transport and metabolism were the most prevalent known functions (Supplementary Fig. 6a). In addition, a total of 271 KEGG modules were encoded by the eight bifidobacterial species present in the ELGG (Supplementary Fig. 6b; Supplementary Data 5), with the main functions relating to multiple sugar transport system (M00207), ribosomal structure (M00178), putative ABC transport system (M00258), and raffinose/stachyose/melibiose transport system (M00196), reflecting their high capabilities of carbohydrate metabolism.

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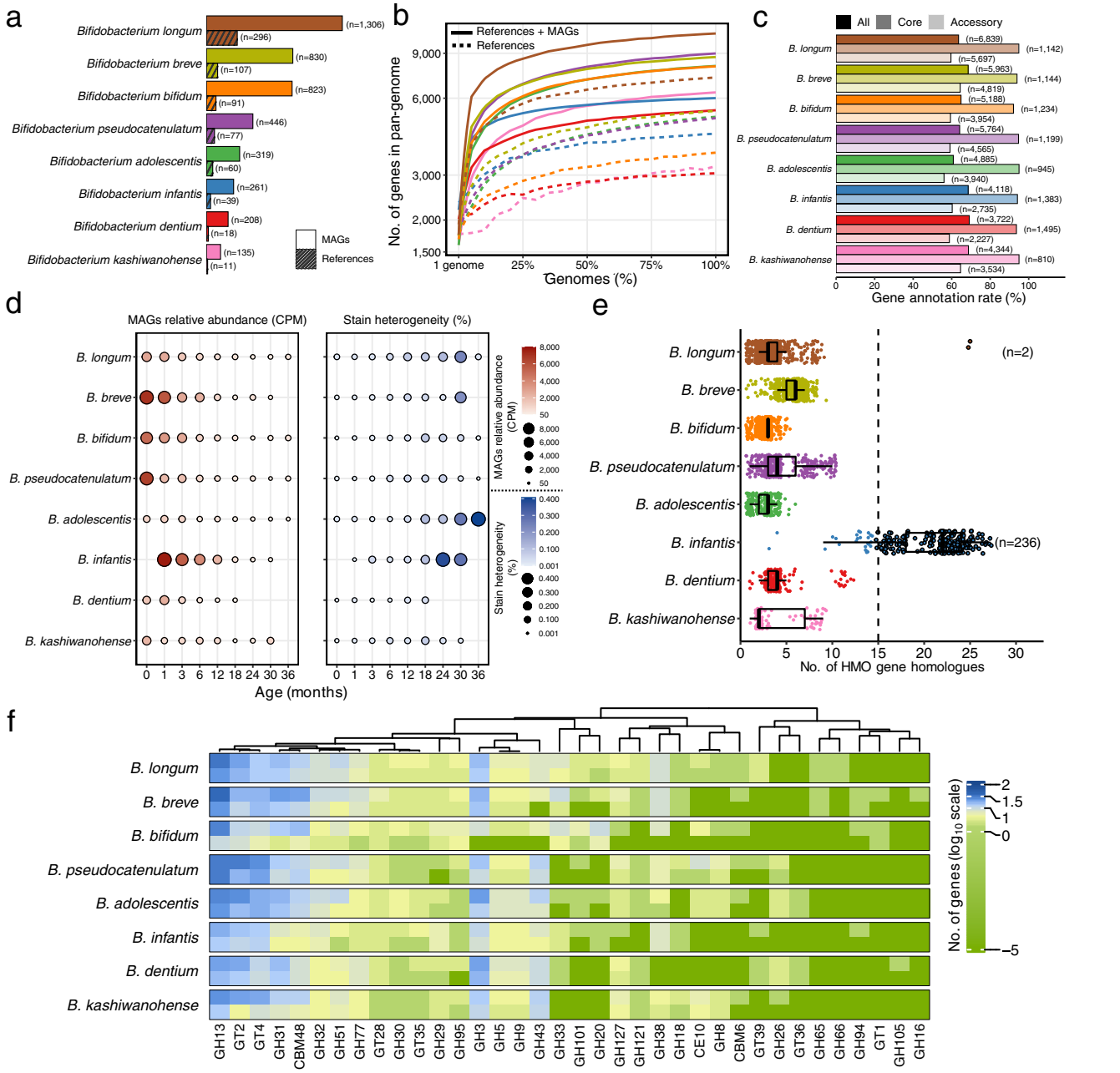


Fig. 4 | Characterization of key early-life *Bifidobacterium* spp. from ELGG catalog. a The number of genomes stratified by MAGs and reference genomes. **b** The pan-genome plot represented by the accumulated number of genes as a function of the number of genomes stratified by MAGs and reference genomes. **c** The rate of functional annotation across databases of COGs, KEGG, GOs, ECs, and CAZy for each species stratified by core and accessory genes. The number in parentheses indicates the number of genes with functional annotation. **d** Dynamics of the relative abundance and strain heterogeneity of MAGs in response to the age of children. **e** The number of gene homologs matched to a well-characterized gene cluster responsible for HMOs utilization from each species. Boxes show the interquartile range (IQR), with the vertical line as the median, the whiskers indicating the range of the data (up to 1.5× IQR), and points beyond the whiskers as outliers. **f** The glycobiome (columns) colored by the number of genes per genome (rows) of each species annotated with the CAZy database. The $\log_{10}$ scaled value (after adding a pseudocount of 1×10^{-5} to avoid non-finite values resulting from zero gene) is plotted.

As the main microbial degraders of carbohydrates in the gastrointestinal tract early in life, we further profiled the glycobiome of bifidobacterial species based on the CAZy profiles (Fig. 4f). A total of 26 glycoside hydrolases (GH), 7 glycosyl transferases (GT), two carbohydrate-binding modules (CBM), and one carbohydrate esterase (CE) were observed across eight bifidobacterial species including reference genomes and MAGs. Notably, GH13 (followed by GT2, GT4, GH3, and GH31) were the most prevalent CAZy families within the bifidobacterial glycobiome, which has been proven to have the capacity to break down a wide range of carbohydrates dominant in the diet[30]. Compared to reference genomes, MAGs in the ELGG were annotated with higher and/or distinct gene families involved in carbohydrate metabolism. For instance, the MAGs from *B. bifidum* contain 27 CAZy families, 10 of which were not found in reference isolate genomes. The CAZy families present in MAGs but absent from reference isolate genomes included GH3, GH5, GH9, GH43, GH127, GH38, CE10, GH8, CBM6, and GH94. Considering breastfeeding during infancy, we further explored the functional potential of the MAGs in terms of HMO utilization by investigating the presence of gene cluster described as involved in HMO transport and degradation in *B. infantis*

(ATCC 15967). MAGs from *B. longum* subspecies clade, *B. infantis*, carried a high number of HMO homologs, (236 out of 261 MAGs had at least 15 homologs, accounting for 50% of the HMO gene cluster) (Fig. 4e), while only two MAGs from *B. longum* carried gene cluster related to HMO metabolism, indicating the distinct capacity in HMO utilization of bifidobacterial species. When comparing the relative abundance of *B. infantis* with other B. *longum* genomes, a higher (Wilcoxon test, $p < 0.0001$) abundance of *B. infantis* was observed in all continents except for Oceania (Supplementary Fig. 6c), indicating the competitive advantage of *B. infantis* strains in early life that may be conferred by the presence of HMO gene cluster.

Over 80% of early-life gut metagenomic sequences represented in the ELGG

To assess how representative the ELGG is as a genomic reference for metagenomes from the human gut in early life, we compared the mapping rate of 353 child fecal samples aged within the first 3 years against the ELGG catalog and another two large-scale reference collections, i.e., CIBIO ($n = 4930$)[15] and UHGG ($n = 4644$)[22]. Using Bowtie2, we obtained a median mapping rate of 82.8% (IQR = 72.7–88.8%) with the ELGG catalog. This level of classification was higher than that obtained with the CIBIO and UHGG catalogs [69.5% (IQR = 61.1–76.4%) and 71.2% (IQR = 62.1–77.8%) respectively; Wilcoxon test, $p < 0.0001$] (Supplementary Fig. 7a; Supplementary Data 6). Additional evidence to support the specificity of the ELGG for classification of the early-life gut microbiome was the slightly lower mapping rate [a median of 66.7% (IQR = 60.2–73.2%, ELGG) compared to 72.1% (IQR = 69.2–75.0%, CIBIO) and 73.2% (IQR = 69.5–75.5%, UHGG); Wilcoxon test, $p < 0.0001$] when aligning metagenomic sequencing reads from the adult fecal samples ($n = 510$) against each catalog (Supplementary Fig. 7b; Supplementary Data 6).

Conspecific genomic diversity associated with delivery mode

Children born by C-section display a significantly distinct gut microbial acquisition and development in the first few years compared to children born vaginally[4,6], and several studies have attempted to restore the gut microbiota by probiotic supplements[36], vaginal swabbing[37], or fecal microbiota transplantation[38] due to this disordered microbiome being positively linked with various diseases later in life[39]. We, therefore, leveraged the ELGG catalog together with the available metadata to address the taxonomic and functional differences associated with C-section at a genome level. A total of 18,836 and 13,412 MAGs were obtained from vaginally ($n = 3299$ samples) and C-section ($n = 2612$ samples) born children, respectively, with 1 to 38 MAGs per sample (mean ± SD: 5.71 ± 4.18) for the former and 1 to 27 MAGs per sample for the latter (5.13 ± 3.37) (Wilcoxon test, $p < 0.0001$). When adjusting by the sequencing depth, the number of MAGs per million paired reads differed (0.32 ± 0.35 for vaginal and 0.37 ± 0.24 for C-section; Wilcoxon test, $p < 0.001$) (Supplementary Fig. 8a). The majority of MAGs for either delivery mode were annotated as phyla of Firmicutes/_A/_C, Actinobacteriota, Proteobacteria, Bacteroidota, and Verrucomicrobiota (Supplementary Fig. 8b). When stratified by children's age, the prevalence of the genera *Bacteroides*/*Phocaeicola* and *Parabacteroides* belonging to the Bacteroidota phylum present in C-section born children were at lower levels (Wilcoxon test blocked by children age, $p < 0.05$), while the genera *Veillonella* and *Klebsiella* were higher (Wilcoxon test blocked by children age, $p = 0.035$ and $p = 0.056$, respectively) than those born vaginally, in particular in the first few months of life (Fig. 5a). This observation confirms and expands the previous results obtained with the read-based analysis[4,40].

Beyond the observed differential taxa, the reconstructed genomes enabled us to explore the intraspecies genetic and genomic diversity of the gut microbiome associated with delivery mode in early life. Only SGBs with at least 10 conspecific near-complete genomes (>90% completeness and <5% contamination) from both vaginal and C-section born children were considered in this part of the analysis. A total of 116 species were retained, covering the phyla Firmicutes/_A/_C ($n = 30/34/7$), Proteobacteria ($n = 20$), Actinobacteriota ($n = 16$), Bacteroidota ($n = 7$), and Verrucomicrobiota ($n = 2$), totaling 20,816 genomes (82% of all near-complete genomes of ELGG) (Supplementary Data 7). When looking into the intraspecies genomic diversity, the average pairwise genetic distances of core genes for each SGB was below 5% (typically used as a threshold to define bacterial species) (Supplementary Data 7). When setting the threshold of ANI at a higher level based on whole genomes, a number of subspecies from 1 to 88 and a range of 2 to 596 were obtained at a cutoff of 97% and 99%, respectively, suggesting the existence of diverse subspecies populations (Supplementary Data 7). We further sought to determine to what extent delivery mode contributed to these variances. The intraspecies variation within the core genomes of 46 species, and the genomic distances (based on gene presence/absence) of 64 species were significantly (PERMANOVA, FDR < 0.05) influenced by delivery mode with effect size up to 18.4% and 17.3%, respectively (Fig. 5b; Supplementary Fig. 8c). Notably, *Streptococcus agalactiae* also known as group B streptococci was highly sensitive to genetically associate with delivery mode.

The pan-genome size of the 116 species here analyzed ranged from 1788 (*Negativicoccus succinicivorans*, $n = 58$ genomes) to 25,698 (*Phocaeicola dorei*, $n = 677$ genomes) (Supplementary Data 7). A total of 31,976 unique genes across 116 species were observed with varying levels of prevalence among genomes from children born vaginally or via C-section. Functions encoded by the genes prevalent (>70%) in C-section born children but not children born vaginally (<30%) were mainly involved in carbohydrate transport/metabolism, cell motility, transcription, and cell wall/membrane/envelope biogenesis (Fig. 5c). The majority of differentially prevalent genes were not related to HMO degradation and utilization as only 4738 unique genes (out of 31,976) were matched with a HMO gene cluster from strain *B. infantis* ATCC 15697.

Mothers who give birth by C-section usually undergo antibiotic treatment, which may result in different antibiotic resistance profiles reflected in the gut microbiome of children. We thus functionally annotated the genomes with antibiotic resistance genes (ARGs) based on the Comprehensive Antibiotic Resistance Database (CARD). The average ARG richness per genome from C-section born children was higher (Wilcoxon test, $p < 0.0001$) than that of vaginally born children (11.6 vs. 10.0 type of ARGs), however, both distributions of ARG richness of genomes from either delivery mode were clearly trimodal (Fig. 5d), with a larger peak at only one ARG, and the other two smaller peaks at 31 and 50 genes, respectively. The origins of ARGs within each peak differed among children born by different delivery modes. In the second peak, genera *Klebsiella*, *Enterobacter*, and *Citrobacter* were the main contributors in children born by C-section; while the third peak was mainly contributed by *E. coli* that was more prevalent in vaginally born children. Apart from *E. coli*, 73 MAGs from *Pseudomonas aeruginosa* were found to carry higher (Wilcoxon test, $p < 0.0001$) richness of ARGs (58.8 ± 1.38) than *E. coli* (50.2 ± 2.27). Among these 73 MAGs, 68 were reconstructed from preterm children within the first 6 months (62 genomes within the first month). As children aged, the richness of ARGs in the gut microbiome generally decreased, from an average of 42.6 at one month to 6.8 ARGs at over 36 months old (Fig. 5e). Notably, the richness of ARGs present in the gut microbiomes of children born by C-section was overall higher than that of vaginally born children (an average of 36.9 vs. 32.5 AGRs; Wilcoxon test, $p < 0.0001$). When comparing the genomes within the same species from children born differently in terms of ARG richness, 15 species showed differential ARG richness, and 12 species contained higher numbers (Wilcoxon test, $p < 0.05$) of ARGs in C-section born children than those born vaginally, while three species (*Pauljensenia radingae_A*, *Clostridium paraputrificum*, and *Clostridium_P perfringens*) exhibited opposite patterns

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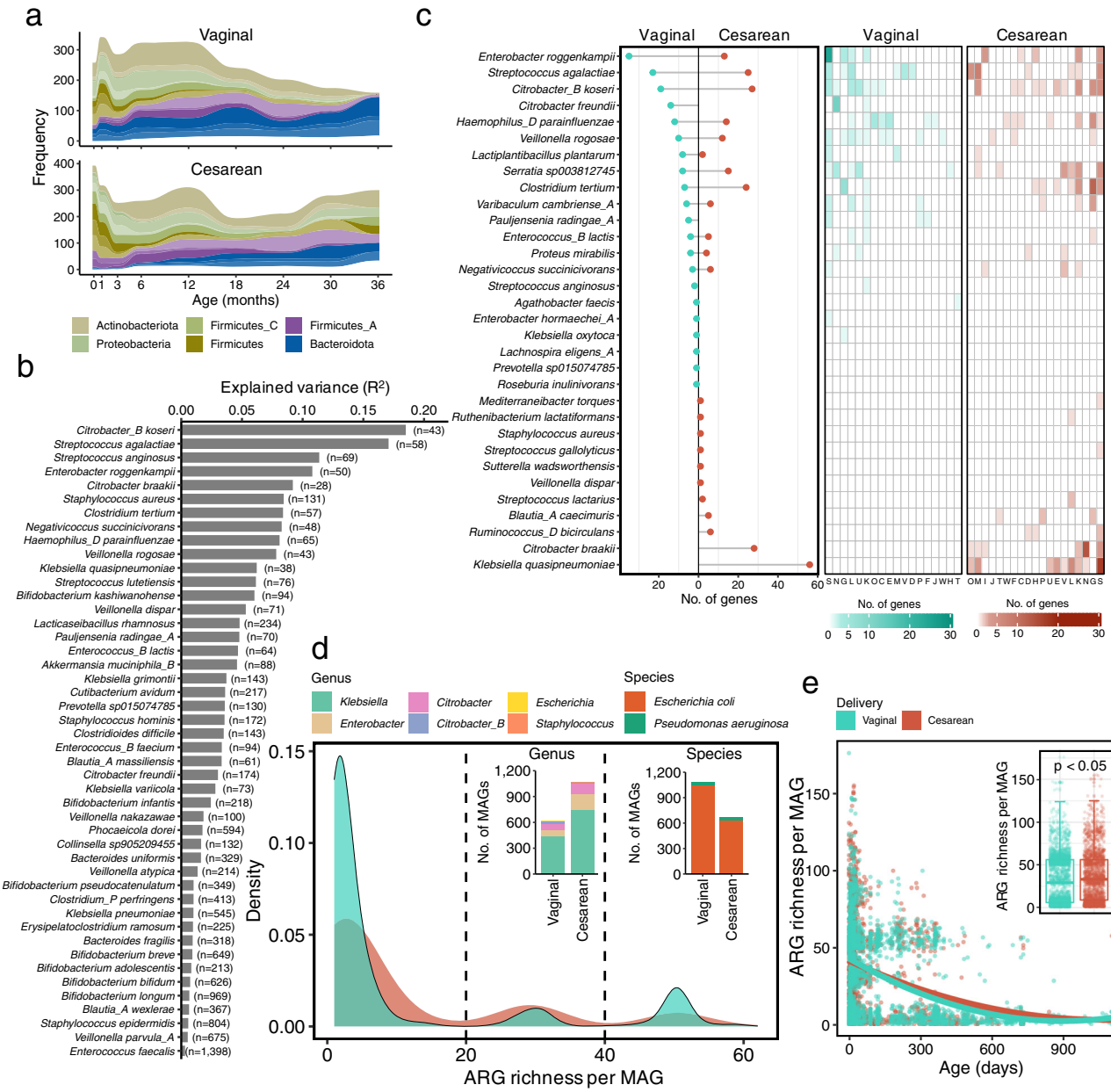


Fig. 5 | Influences of delivery mode on early-life gut microbiome at a genome-resolved level. a Prevalence of 16 bacterial genera in children stratified by delivery mode over time, where each genus was colored by its phylum. Only genera with >10% prevalence in children born by any of delivery modes are plotted. **b** The explained variance (R^2) contributed by delivery mode of 46 species that were significantly (PERMANOVA, FDR < 0.05) associated with delivery mode based on the hamming distance of core genes per species. The number in parentheses indicate the number of MAGs of this species. **c** The number of genes that were prevalent in C-section born children or vaginally born children (>70% in C-section born children and <30% in vaginally born children, and vice versa) for each species and their associated functions annotated by COGs database. **d** The density of antibiotic resistance genes (ARGs) richness in each genome of ELGG, and the taxonomic assignment of the genomes at genus (left inset) and species (right inset) level. **e** The dynamics of ARGs richness from the early-life human gut microbiome in response to the age of children. The gut microbiome from children born by C-section carried higher (two-tailed Wilcoxon test, $p < 0.05$, inset) number of ARGs than that of children born vaginally. The inset boxes show the interquartile range (IQR), with the horizonal line as the median, the whiskers indicating the range of the data (up to 1.5× IQR), and points beyond the whiskers as outliers.

(Supplementary Fig. 8d). The most common mechanisms of antibiotic resistance discovered in the 20,816 genomes included antibiotic efflux, antibiotic target alteration, and antibiotic inactivation (Supplementary Fig. 8e).

Comparisons of gut microbiome between children and adults

The comprehensive catalog of the early-life microbiome enabled us to explore the taxonomic and functional differences between the children and adult gut microbiomes at a genome level. We thus compared the five most represented genera in children (≤3 years) and adults (≥18 years) (i.e., *Alistipes*, *Bacteroides*, *Bifidobacterium*, *Prevotella*, *Streptococcus*, *Veillonella*) based on ELGG and UHGG catalogs[22], totaling 12 species with >60 near-complete genomes (>90% completeness and <5% contamination). The pan-genome size was positively associated with the number of included genomes, but none of the species reached a plateau, even *Bacteroides uniformis* with the highest number of genomes ($n = 1087$) containing 32,215 genes in adults. Species of *Streptococcus thermophilus* had the lowest pan-genome size with 2572 for adults and 2639 for children from 143 and 136 genomes, respectively (Fig. 6a). This suggests additional genomes from each species

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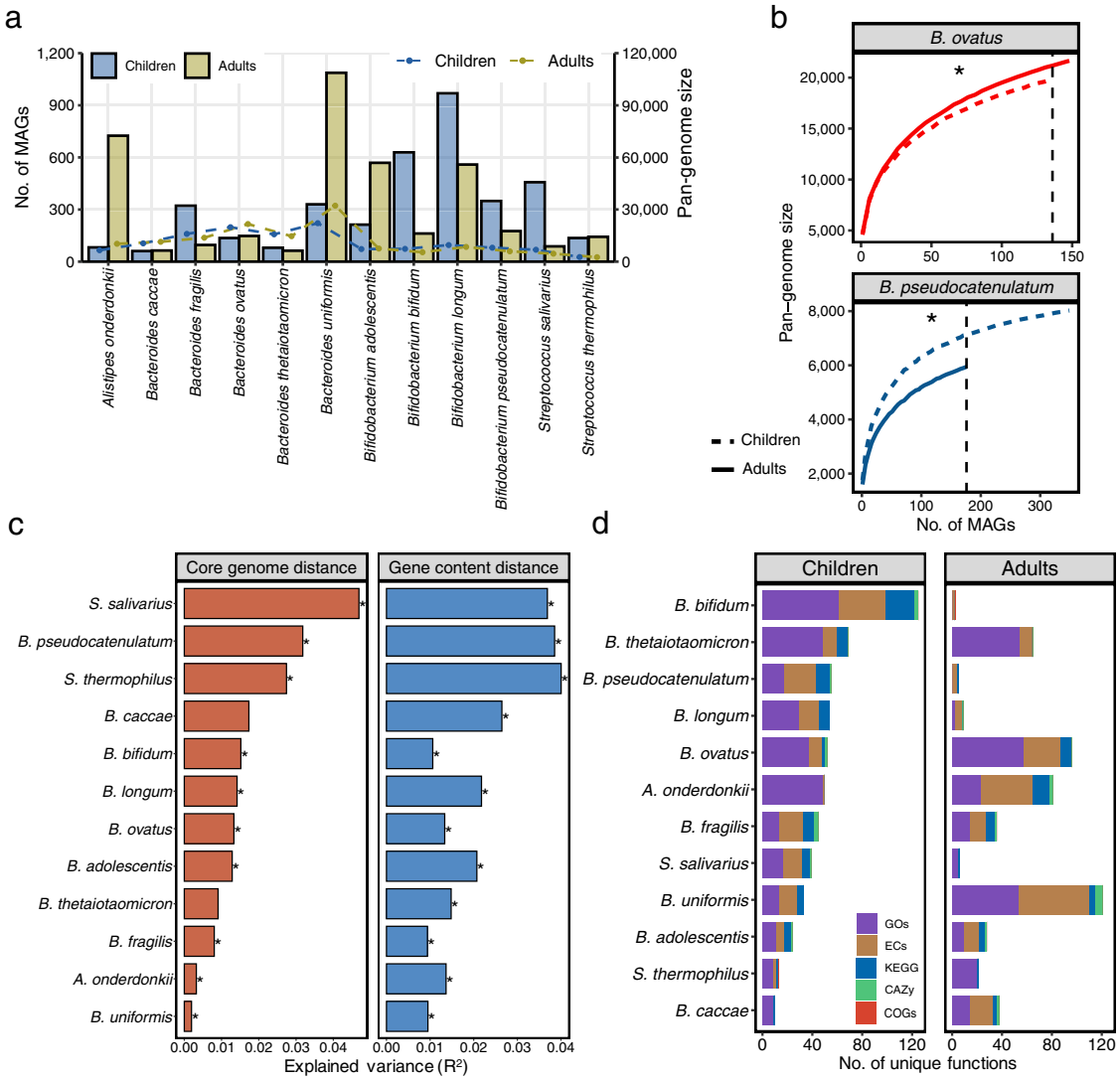


Fig. 6 | Comparisons of gut microbiome between children and adults. a Number of genomes (bar plot) and pan-genome size of each species from children and adults. **b** Pan-genome plot represented by the accumulated number of genes against the number of genomes of *B. ovatus* and *B. pesudocatenulatum* stratified by children and adults (two-tailed Wilcoxon test, *FDR < 0.05). **c** The explained variance (R^2) contributed by age (children and adults) based on the hamming distance of core genes per species and Jaccard distance of presence/absence genes (two-tailed Wilcoxon test, *FDR < 0.05). **d** The unique functional annotations belonging to either children or adults categorized by databases of COGs, KEGG, GOs, ECs, and CAZy.

remain to be discovered across populations. In the genus *Bacteroides*, genomes from adults contained a higher number of unique genes than those from children when considering the same number of genomes (Wilcoxon test, FDR < 0.05). In contrast, gene numbers of *Alistipes onderdonkii*, *B. adolescentis*, *B. longum*, *B. pseudocatenulatum*, and *Streptococcus salivarius* were higher (Wilcoxon test, FDR < 0.05) in children (Fig. 6b; Supplementary Fig. 9).

Notably, when looking into gene diversity (estimated by the Jaccard distance based on the presence/absence of genes per genome), genomes from adults showed higher (Wilcoxon test, FDR < 0.05) gene diversity on average than that of children for 5 out of 12 species, including *Bacteroides fragilis*, *Bacteroides ovatus*, *B. bifidum*, *S. salivarius*, and *S. thermophilus* (Supplementary Fig. 10a). These results indicate that genomes within these species in early life are more conserved, and more specific genes are acquired by the microorganisms later in life. On the contrary, the enriched species *B. longum* showed higher (FDR < 0.05) gene diversity than that of adults. We also explored the effect size and significance of age (≤3 years for children and ≥18 years for adults) on the gene diversity of each species. The results showed the distinct contribution of age (PERMANOVA, FDR < 0.05) to the genetic variation of species between children and adults. *S. salivarius* ($R^2 = 0.047$ for hamming distance and $R^2 = 0.037$ for Jaccard distance), *B. pseudocatenulatum* ($R^2 = 0.032$; $R^2 = 0.039$), and *S. thermophilus* ($R^2 = 0.027$; $R^2 = 0.040$) were the species most significantly associated with age (Fig. 6c).

Based on the multiple functional annotation schemes as ELGP, the pan-genome of species showed comparable rates of gene annotations between children and adults, but differed across species, namely, *B. adolescentis* with the lowest rates of 62.4% and 64.6% for children and adults respectively, and *S. thermophilus* with the highest respective rates of 84.6% and 85.3% for children and adults (Supplementary Fig. 10b). Based on the CAZy annotation of the pan-genomes, we found that gut microorganisms from children harbored a higher (Wilcoxon test, FDR < 0.05) number of specific CAZy families, most notably GH13, GT4, GT2, GH43, and GH3 (Supplementary Fig. 10c). Additionally, we sought to determine the functions that were unique to either children or adults. We found a large number of EC families among species ($n = 1–38$ in children and 0–57 in adults), KEGG modules ($n = 0–23$ in children and 0–13 in adults), CAZy families ($n = 0–4$ in children and 0–7 in adults), COGs ($n = 0–1$ for both children and adults) and GOs

categories (n = 8–61 in children and 0–57 adults) to be specific to either children or adults (Fig. 6d; Supplementary Data 8). Within EC families of *B. bifidum*, the enzymes of GlfT2 (EC 2.4.1.288; n = 27 genomes), asparagine synthase (EC 6.3.5.4; 17 genomes), and D-xylulose reductase (EC 1.1.1.9; 12 genomes) were the most prevalent in children; and the top families of CAZy from children included GH127 (5 genomes), GH94 (4 genomes), and CBM6 (1 genomes). Within EC families of *B. uniformis*, the enzymes of dTDP-6-deoxy-L-talose 4-dehydrogenase (EC 1.1.1.34; 13 genomes), thiamine kinase (EC 2.7.1.89; 13 genomes), and histidine decarboxylase (EC 4.1.1.22; 13 genomes) were the most prevalent in adults; and the top families of CAZy from adults included PL4 (7 genomes), PL11 (3 genomes), AA10 (2 genomes), and CBM73 (2 genomes).

Discussion

We provide a large-scale sequence resource of the human gut microbiome in early life, representing 32,277 newly reconstructed genomes and over 80 million protein sequences, and spanning multiple clinical factors including age, delivery mode, gestational age, and feeding patterns with significant influences on the early-life gut microbiome[4,7,19,32]. The ELGG catalog considerably expands the phylogenetic diversity of the early-life gut microbiome by increasing the classification rate of metagenomic sequencing reads (over 82%) and uncovering genomes without a cultured representative. Although the gut microbiome community is relatively simple in early life compared to adults, over 64% of the 2172 species-level clusters from ELGG lack a cultured representative, suggesting the importance and need to experimentally isolate and characterize the gut microbiome from children. Having this ELGG catalog can serve as a reference for future studies specifically for early-life gut microbiome research and help prioritize targets for experimental isolation and cultivation. Characterizing newly isolate strains from children may aid in the development of dietary supplements to help restore the gut microbiome composition perturbed by extrinsic or intrinsic factors, such as C-section delivery and antibiotic intervention.

With the establishment of the ELGG catalog, the strain dynamics, pan-genome and genomic diversity of the main *Bifidobacterium* species from the human gut in early life have been investigated at a genome level. The pan-genome of these bifidobacterial species has been greatly expanded by up to 12 times in comparison to the reference genomes only, substantially contributing to the genomic landscape of bifidobacterial species. However, nearly 40% of the pan-genome across bifidobacterial species remains uncharacterized functionally. Given significant influences of delivery mode on the development of the early-life gut microbiome, we further compared the gut microbiome from children born by C-section or vaginally at multiple levels of resolution and identified a set of species and genomic variations linked to delivery mode. Notably, the richness of ARGs gradually decreased as children aged, and microbial genomes from C-section-born children carried higher ARGs than that from children born vaginally. We speculate this pattern could be related either with the antibiotic treatment typically administered to mothers undergoing C-sections or with antibiotic interventions in children after birth. Moving from early life to adulthood, it has been known that along with changes in diet, physiological functions, and the immune system, the gut microbiome gradually approaches maturity as children grow[3,19]. Consistent with the initial hypothesis of the existence of age-specific microbial communities with distinct genomic and functional patterns[18,32], we found that some microbial genomes from adults possessed higher gene diversity, suggesting that gut microorganisms in adults gain unique genes later in life. The genomes from either children or adults showed comparable rates of functional annotations across species, however, the most prevalent functions differed between children and adults, highlighting the importance of developing age-specific reference genomes targeted at particular populations.

Currently, linkages between the early-life human gut microbiome and health have been well recognized, and enhanced resolution and accuracy of taxonomic classification and microbial genomic adaptation will require more culture-based and bioinformatic work targeting specific periods of life or clinical contexts. Our newly reconstructed genomes represent a key step for the early-life human gut microbiome and provide new insights into the taxonomic, functional and genomic diversity of this period of life. The establishment of ELGG and ELGP catalogs will substantially improve our ability to understand the development and mechanisms of disturbances of the early-life gut microbiome. Our knowledge of the human gut microbiome composition and function in early life will have a profound impact on promoting or maintaining human health throughout the course of life.

Methods

Publicly available early-life gut metagenomic datasets and quality control

A total of 6122 paired-end sequencing runs collected by 26 studies for early-life gut metagenomes were downloaded from the NCBI SRA according to the accession numbers published in each included study. FASTQ files were retrieved by using fastq-dump v2.9.1 with the option "-split-3" from SRA Toolkit v2.9.1. These samples were distributed among 11 countries across four continents, with the United States, United Kingdom, and New Zealand being the top three represented. All metagenomic sequencing data were quality controlled and human contamination (hg19 human reference genome) was filtered by using KneadData v0.7.2 with default parameters, resulting in 1.3×10^{11} paired reads (87% of the raw sequencing reads).

Metagenomic assembly and contig binning

The quality-filtered sequencing reads from each of 6122 metagenomes were assembled using MegaHIT v1.1.3[41] with option "-min-contig-len 1000", which resulted in 29,912,553 contigs, with a total length of 1.71×10^{11} bp, and an average of N50 of 46,532 bp, calculated with the "stats.sh" script (format = 5) from BBMap v38.22 (https://sourceforge.net/projects/bbmap/). Depth of coverage of each contig was calculated by mapping the raw reads back to their assemblies using BWA-MEM v0.7.17[42] with default options and then calculating the corresponding contig depth with SAMtools v1.10 and jgi_summarize_bam_contig_depths function from MetaBAT v2.12.1[23]. The gut MAGs from children were generated per sequencing run using three metagenomic binning tools (MetaBAT v2.12.1, MaxBin v2.2.6[24], and CONCOCT v1.0.0[25]) using metaWRAP v1.3.1[26] with default parameters. The minimal contig size for binning was set as default with 1000 bp for further processing, except for MetaBAT2, which required at least 1500 bp. Afterwards, the produced bins from each binning tool were integrated and refined with Bin_refinement module of metaWRAP with options "-c 50 -x 10", corresponding to the criterion of medium-quality draft MAGs[27]. The quality (estimated completeness and contamination) of bins was evaluated with CheckM v1.0.12 lineage workflow[43], implemented in metaWRAP with option "-quick". In order to control the genome quality strictly, the resulting 42,054 MAGs were checked by GUNC v1.0.5[28] to filter genomes potentially containing chimerism based on 'pass.GUNC' in the output file. The genome quality score was calculated as: completeness−5 × contamination. The ribosomal RNA (rRNA) genes were searched using Barrnap v0.9[44] with options "--reject 0.01 --evalue 1e-03", and transfer RNAs (tRNAs) of the standard 20 amino acids were identified with tRNAScan-SE v2.0.6[45] with options "-A -Q" for archaeal species and "-B -Q" for species belonging to bacterial lineages based on the annotation of GTDB.

Assessing the strain heterogeneity of MAGs

The CMSeq tool v1.0.3 was used to investigate the strain-level heterogeneity within each MAG[15]. Firstly, metagenomic reads from each sample used to generate MAGs were aligned to the assembled contigs

using BWA-MEM, and the aligned files were sorted and indexed with SAMtools. Secondly, the protein-coding genes of the assembled contigs were predicted with Prodigal v2.6.3 implemented in the Prokka v1.14.6 with the default settings, and the resulting GFF file was used for subsequent analysis. Finally, the "polymut.py" script from the CMSeq package was used to detect the nonsynonymous mutations from those positions mapped with the PHRED quality score of at least 30 and a depth of coverage of at least 10x. A position was considered non-polymorphic if the dominant allele frequency was >80%. The level of strain heterogeneity of each MAG was calculated by the proportion of the total number of nonsynonymous mutations in the total number of considered positions.

Reference genomes from public databases

The NCBI RefSeq database (accessed on 25th September 2020) that contains bacterial genomes and archaeal genomes were downloaded (https://ftp.ncbi.nlm.nih.gov/genomes/refseq/). In addition, the isolate genomes from the Human Gastrointestinal Bacteria Culture Collection (HBC, http://ftp.ebi.ac.uk/pub/databases/metagenomics/genome_sets/hbc_genomes.tar.gz)[11], and Culturable Genome Reference (CGR)[12] were also gathered. The completeness and contamination of all reference genomes were evaluated using CheckM lineage workflow with default settings. The genomes not having >50% completeness, <5% contamination, genome quality score <50, and failing to pass the chimerism detection by GUNC were then discarded, resulting in a total of 187,555 reference genomes including 184,392 bacterial genomes and 1039 archaeal genomes from RefSeq, 699 genomes from HBC, and 1425 genomes from CGR.

Species-level clustering of reconstructed MAGs and reference genomes

The total set of 219,832 genomes (32,277 MAGs and 187,555 reference genomes) were clustered at an estimated species level (ANI ≥ 95%; refereed as SGBs)[22] using dRep v2.6.2[46] with the following options: "-pa 0.9 -sa 0.95 -nc 0.30 -cm larger, -S_algorithm fastANI v1.33". In order to increase the computational efficiency to cluster the complete genome set, an iterative approach was applied where random chunks of 30,000 genomes were clustered independently, and then the selected representatives per cluster from each chunk were combined and subsequently clustered[22]. The genome with the higher score calculated based on the following formula was selected as the species representative in each iteration:

$$\mathrm{Score} = \mathrm{CMP} - 5 \times \mathrm{CNT} + 0.5 \times \log_{10}(\mathrm{N50})$$

where CMP and CNT represent the estimated completeness and contamination, respectively; and N50 is the minimum contig length in which 50% of the total genome is covered. In case of clusters that contained reference genomes and MAGs, the reference genomes were prioritized over MAGs and selected as the representative.

In addition, the pairwise distances for all conspecific genomes were calculated by using Mash v2.3[47] with default sketch size. Afterwards, the phylogenetic tree of each species was built with the "complete" hierarchical clustering method from 'fastcluster' R package[48], and then the number of sub-clusters were further obtained by setting a distance cutoff of 0.03 (97% ANI) and 0.01 (99% ANI) to investigate the within-species population diversity. The pairwise genome distances between 2172 representatives of ELGG and 4644 representatives of UHGG were calculated by Mash with default sketch size.

The obtained SGBs were subdivided into 3 groups: (i) cultured SGBs (cSGBs) containing at least one MAG and one reference genome (the uncultured score for a SGB was calculated as the proportion of MAGs in the total genomes belonging to that SGB); (ii) uncultured SGBs (uSGBs) containing exclusively MAGs; (iii) non-early-life SGBs that contained exclusively reference genomes.

Evaluation of MAGs reconstrued across metagenomic datasets

The MAGs reconstructed from a common set of 941 metagenomes that contained MAGs with >50% completeness and <5% contamination and without chimerism detection by GUNC from the current and two previous human gut MAG studies[13,15] were compared in terms of genomic and taxonomic features. The genome quality (completeness and contamination) from another two studies were re-estimated with CheckM lineage workflow with "-reduced_tree"[43] in line with the option "-quick" in metaWRAP and GUNC for chimerism detection. The resulted genomes were clustered to SGBs using dRep with "-pa 0.9 -sa 0.95 -nc 0.30 -cm larger, -S_algorithm fastANI". The similarity of SGBs of the same sample but in different studies was calculated as the percentage of shared SGBs in the smaller SGBs of the two studies. Conspecific genomes recovered in the same metagenomic samples but in different studies were also compared with the alignment fraction (AF) and ANI that were obtained from the output of dRep. Both the maximum AF and ANI for each pairwise comparison were considered.

Taxonomic annotation and phylogenetic analysis

Taxonomic annotation of each species representative was performed with GTDB-Tk v2.1.0 (reference database version R207) with "classify_wf" workflow using default parameters[49,50]. The NCBI taxonomy annotation was also generated for each species representative using the "gtdb_to_ncbi_majority_vote.py" script available in the GTDB-Tk repository.

The phylogenetic tree of 2171 bacterial representative genomes of SGBs was built using FastTree v2.1.11[51] with default settings using the protein sequence alignments generated by GTDB-Tk.

To estimate the increase in phylogenetic diversity (PD) contributed by the ELGG catalog, we computed the sum of branch length of the whole bacterial trees (PD_{all}) and the sum of branch length from 773 cSGBs (PD_{cSGBs}) implemented through the function 'pd' in the "picante" package[52]. The percentage gain in PD contributed exclusively by uSGBs was calculated as: $100 \times (PD_{all} - PD_{cSGBs})/ PD_{cSGBs}$.

Metagenomic read mapping

An additional number of 353 metagenomes with >50,000 sequencing paired reads that were randomly selected from child fecal samples but not used to generate ELGG or ELGP catologs (Supplementary Data 6) from five studies as cross-validation samples were retrieved from NCBI SRA with SRA Toolkit v2.11.2. These metagenomes were quality-filtered and host decontaminated (hg19 human reference genome) using KneadData with default parameters. Apart from the database reconstructed in the current study (ELGG, $n = 2172$), we built another two databases based on the representative genomes of ref. 15 (CIBIO, $n = 4930$) and the UHGG[22] ($n = 4644$) with Bowtie2 v2.4.5[53]. Moreover, 510 metagenomes from adult fecal samples with >50,000 sequencing paired reads from five studies were downloaded from NCBI SRA v2.11.2. All downloaded metagenomes were aligned to these three databases respectively using Bowtie2 in "end-to-end" mode with the option "--very-sensitive", and the generated mapping files were further filtered by removing alignments with an alignment score (AS:i tag) less than −20 that were likely to be spurious alignments in order to increase the reliability of mapping assessment.

Comparisons between early-life and adult gut microbiome

The UHGG collection was used for analysing assembled genomes from adults (≥18 years), mostly comprising non-redundant genomes from three large-scale human gut studies[13,15,54]. The age information was retrieved from each of the three studies. The species from the five most prevalent genera in either the UHGG or the ELGG catalog and containing >60 genomes (>90% completeness and <5% contamination, as well as free of chimerism detected by GUNC) were selected for downstream analyses. The quality and the taxonomic annotation of genomes from UHGG was re-estimated with CheckM and GTDB-Tk,

respectively, as performed for the ELGG. Finally, 12 species were retained for further analysis, i.e., *A. onderdonkii* (ELGG vs. UHGG: 82 vs. 725 MAGs), *Bacteroides caccae* (61 vs. 64 MAGs), *B. fragilis* (322 vs. 96 MAGs), *B. ovatus* (136 vs. 148 MAGs), *Bacteroides thetaiotaomicron* (80 vs. 63 MAGs), *B. uniformis* (330 vs. 1087 MAGs), *B. adolescentis* (213 vs. 569 MAGs), *B. bifidum* (629 vs. 162 MAGs), *B. longum* (969 vs. 559 MAGs), *B. pseudocatenulatum* (349 vs. 176 MAGs), *S. salivarius* (457 vs. 88 MAGs), *S. thermophilus* (136 vs. 143 MAGs). The corresponding genomes were then downloaded from the MGnify FTP site (http://ftp.ebi.ac.uk/pub/databases/metagenomics/mgnify_genomes/).

Gene diversity, pan-genome analysis and functional annotations

Protein clusters. The protein-coding sequences (CDS) of genomes were predicted and annotated with Prokka, which employed Prodigal with options "-c" (protein predictions with closed ends only), "-m" and "-p single". The 4,036,936 nonredundant ELGP clusters were generated from the set of 86,678,654 genes of 32,277 genomes by using CD-HIT v4.8.1[55] at 95% protein identity (-c 0.95 -n 5 -M 0 -d 0 -g 1). Comparisons between ELGP and UHGP were conducted by clustering the 4,036,936 nonredundant ELGP and 20,239,340 UHGP-95 clusters with CD-HIT at 95% protein identity.

Pan-genome analysis. The pan-genome of each species was analysed by Panaroo v1.2.10[56] with parameters "-c 0.90" for a minimum amino-acid identity of 90% for a positive match, "--core_threshold 0.90" and a family threshold (-f) of 50%, as well as option "--clean-mode strict" and "--merge_paralogs" by taking the GFF3 files created by Prokka. The core genes were defined as present in at least 90% of genomes. A single representative nucleotide sequence from each of the clusters was then annotated functionally by eggNOG-mapper v2.1.7 with database v5.0.2[57,58]. The COG[59], KEGG module, CAZy, GO, and EC annotations were derived from the eggNOG-mapper results.

Antimicrobial resistance genes. Predicted genes (the representative nucleotide sequence from each of clusters producd by Panaroo) were annotated with The Comprehensive Antibiotic Resistance Database (CARD v3.2.3) by using its accompanying Resistance Gene Identifier (RGI, v5.2.0) with default parameters[60], and only "Strict" and "Perfect" hits from RGI were retained for further analysis.

HMO gene homology analysis. The protein sequences of HMO gene cluster were obtained from strain *Bifidobacterium longum subsp. infantis* ATCC 15697 (GCF_000020425.1; BLON_RS12070-BLON_RS12215) from NCBI RefSeq database. Prediction of HMO gene cluster was performed by comparing the HMO protein sequences to the representative nucleotide sequence of each cluster in the pan-genome of a species from Panaroo using a tBLASTn v2.9.0 search. Specifically, identified hits were further filtered with identify percentage ≥70% and e-value <1e-10, and only the best hit per gene cluster was chosen.

Statistical analysis

When evaluating the dynamics of microbial features, we stratified the continuous age of children into nine categories, namely 0 month (0–1 day, $n = 326$), 1 month (2–30 days, $n = 2640$), 3 months (31–90 days, $n = 896$), 6 months (91–180 days, $n = 424$), 12 months (181–360 days, $n = 942$), 18 months (361–540 days, $n = 390$), 24 months (541–720 days, $n = 309$), 30 months (721–900 days, $n = 76$), and 36 months (900–1,162 days, $n = 44$).

Statistical significance was verified through Wilcoxon test either with or without a block factor if mentioned in the text implemented in the R package "coin" v1.3-1[61]. The produced p-values were adjusted for multiple testing using the Benjamini–Hochberg false-discovery rate (of 5%, FDR) as reported in the text. The proportion of explained variance (R^2) and significance of each clinical covariate was quantified by PERMANOVA calculated based on hamming distance of core genes or Jaccard distance of presence/absence of genes per genome, as implemented in the adonis2 function from R package "vegan" v2.5-7[62] with 1000 permutations. Genomes with missing metadata for the given covariate were excluded.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The 32,277 genome assemblies, 2172 representatives of ELGG, and protein catalog of ELGP reported in this paper have been deposited in the Zenodo repository under https://doi.org/10.5281/zenodo.6969520. The other data supporting the findings of this study are available within the paper and additional files. Source data are provided with this paper.

Code availability

All mentioned tools used for the data analysis in this study are publicly available, and the version and parameters used have been indicated. No custom code or pipeline was generated in this manuscript.

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Author contributions
S.Z., D.M., R.P.R., C.S., and S.W. conceived the study. S.Z., D.P., and S.W. performed the analyses. A.A. and Z.Z. contributed to the interpretation of the results. S.Z. and S.W. wrote the manuscript. All authors read, edited and approved the manuscript.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to Shaopu Wang.

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