Comparative Genomic Analysis of *Klebsiella* and *E. coli* Diversity: Assessing the Impacts of Travel, Animal Husbandry Practices and Lifestyle

by

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Abstract

Antimicrobial resistance is a significant public health problem which is further exacerbated by the increasing prevalence of resistant pathogens, particularly of bacterial origin, which cannot be effectively treated with existing antimicrobials. In East Africa this is a critical problem due to limited resources available to combat it. Currently there is very limited whole-genome sequencing (WGS) data addressing antimicrobial resistance genes (AMR) patterns and the underlying mechanisms of transfer of resistance genes. In the present study we employ WGS to shed light on the complex dynamics of AMR in Gram-negative opportunistic pathogens (*Escherichia coli* and *Klebsiella pneumoniae*) in East Africa. Here, AMR patterns were investigated before and after travel, in the context of animal husbandry, as well as in distinct communities with varying lifestyles. Consequently, this study provides insights into the AMR patterns in these three situations at genomic level while also contributing to the relatively scarce WGS data existing literature in East Africa.

Short-term colonisation of different *K. pneumoniae* species were observed pre- and post-travel, which lead us to suggest that travel may have an impact on composition of gut microbiota. Moreover, acquisition of AMR was mostly seen in multidrug-resistant (MDR) *Klebsiella variicola* strains both pre- and post-travel. Resistance genes in these strains were mainly associated with plasmids, giving weight to the important role of mobile genetic elements in the spread of AMR. Our study did not find evidence for widespread acquisition of ESBL-producing strains but found that colonization by MDR strains was common. Furthermore, the majority of *K. variicola* strains were closely related, suggesting a common source and/or transmission between individuals in the study population.

In a second study, significant proportion of *E. coli* strains from livestock exhibiting resistance to antimicrobials were observed, that were commonly employed in animal husbandry. Further analysis of the data revealed a higher prevalence of MDR in poultry compared to pigs. This was

attributed to varying antibiotic use between the two animal husbandry systems and context-specific variation in diseases prevalence. Upon further exploration of the data, sharing of strains between animal groups were seen, revealing evidence of different AMR reservoirs being shared between poultry and pigs. These findings emphasise the importance of adopting a One Health approach that considers the well-being of different animal groups, humans, and the environment.

Further to this, *E. coli* MAGs from previously published studies were reconstructed to conduct a comparative study so that we can understand the difference in gut *E. coli* diversity between the Hadza and the general population in Tanzania. Three *E. coli* MAGs were successfully reconstructed following metagenomic analysis of the sequences. No evidence of resistance was found and virulence genes in the identified MAGs. However, this may have been attributed to quality of the data. Further observation of the data revealed variation in genes linked aromatic amino acid metabolism between *E. coli* MAGs derived from the two communities, notably the absence of *proC*, *aroA* and *trpD* genes in the Hadza.

In summary, our study emphasizes the importance of considering local conditions in AMR research. Additionally, it sheds light on the transmission of resistant bacteria between different animal species, as well as the identification of genetic elements that contribute to the spread of AMR. Our findings underscore the importance of monitoring and targeted interventions that are adapted to environments. Moreover, our study supports the adoption of a One Health approach that integrates local practices, animal care and human behaviour to combat the growing problem of AMR, in Sub Saharan Africa.

For Samson and Perpetua

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Table of Contents

LIST OF FIGURES

Introduction

Chapter 2

Chapter 3

Chapter 4

LIST OF TABLES

Chapter 2

Chapter 3

Chapter 4

LIST OF ABBREVIATIONS

0°C	degrees Celsius
bp	base pairs
%	percentage
µl	microlitre
Mg	microgram
ng	nanogram
IS	Insertion sequence
SCAi	Simmons citrate agar with inositol
h	hour
GC	Guanine Cytosine
ST	Sequence type
BLAST	Basic Local Alignment Search Tool
MDR	Multidrug resistance
X2	Chi-squared
df	degree of freedom
PCR	Polymerase chain reaction
WGS	Whole genome Sequencing
AMR	Antimicrobial Resistance
n	number
MLST	Multi Locus Sequence Typing
cgMLST	core genome Multi Locus Sequence Typing
SNP	Single Nucleotide Polymorphisms
ICE	Integrative Conjugative elements
ESBL	Extended Spectrum beta-lactamase
CDC	Centres for Diseases Control and prevention
DALY	Disability Adjusted Life Year
EU	European union
LMICs	Low-income countries
AMRSNET	Africa to establish an AMR Surveillance Network
EUCAST	European Committee on Antimicrobial Susceptibility Testing
CLSI	Clinical and Laboratory Standards Institute
SSA	Sub-Saharan Africa
GBD	Global burden of disease
RNA	Ribonucleic Acid
DNA	deoxyribonucleic acid
EPS	extracellular polymeric substance
T3SS	Type III secretion system
T4SS	Type IV secretion system.
EPEC	Enteropathogenic *Escherichia coli*

EIEC	Enteroinvasive *Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
DAEC	Diffusely Adherent *Escherichia coli*
EAEC	Enteroaggregative *Escherichia coli*
AIEC	Adherent-Invasive *Escherichia coli*
STEC	Shiga Toxin-producing *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
UPEC	Uropathogenic *Escherichia coli*
NMEC	Neonatal Meningitis *Escherichia coli*
APEC	Avian Pathogenic *Escherichia coli*
SEPEC	Sepsis-Associated *Escherichia coli*
EXPEC	Extraintestinal Pathogenic *Escherichia coli*
OIE	Office International des Epizooties
LT	Heat-labile enterotoxin
CFA	colonization factors
TTP	thrombotic thrombocytopenia purpura
LEE	pathogenicity
LPS	lipopolysaccharide
BIGSdb	Bacterial Isolate Genome Sequence database
WHO	World Health Organisation
HGT	Horizontal gene transfer
ICEs	Integrative conjugative elements
TE	transposable elements
Tn	Transposons
oriV	origin of replication
NDM	New Delhi Metallo-beta-lactamase
NGS	Next generation Sequencing
ONT	Oxford Nanopore Technologies
MODREC	Ministry of Defence Research Ethics Committee
CUHAS	Catholic University of Health and Allied Sciences
CREC	CUHAS Research Ethics and Review Committee
BMC	BioMed Central
LB broth	Lysogeny broth

Chapter 1

Introduction

1.1. Global burden of antimicrobial resistance

Antimicrobial resistance (AMR) describes the phenomenon when microorganisms (bacteria, viruses, fungi, or parasites) become resistant to the actions of antimicrobials that were once effective in treating infections caused by these microbes (Gelband *et al.,* 2015; Grundmann *et al.,* 2011). As a result of AMR, these microorganisms become less susceptible, or completely immune to treatment resulting in potentially life-threatening infections (Grundmann *et al.,* 2011). AMR has become a growing public health concern in the 21st century (Grundmann *et al.,* 2011). It has both clinical and economic consequences. The clinical impact of AMR includes severe infections, increased morbidity, treatment failure, and mortality (Murray *et al.,* 2022). The number of deaths associated with AMR bacteria in 2019 alone were estimated to be 4.78 million (Murray *et al.,* 2022). By 2050, AMR may cause more than 10 million deaths annually (O'Neill, 2016). It could result in a $100 billion economic burden if it is not adequately addressed (O'Neill, 2016).

Globally, antibiotic-resistant bacteria are becoming increasingly prevalent (Figure 1.1) (Grundmann *et al.,* 2011; Gelband *et al.,* 2015). Based on 2014 estimates, 28.9 million to 50.1 million acute infections and 3.7 million to 6.4 million bloodstream infections were caused by third generation cephalosporin-resistant bacteria, including *Escherichia coli* and *Klebsiella pneumoniae* across 193 countries (Temkin *et al.,* 2018; Yam *et al.,* 2019). Moreover, in the same year, carbapenem-resistant bacteria caused an estimate of 2.7–3.1 million severe infections worldwide and 0.4–0.5 million bloodstream infections (Temkin *et al.,* 2018; Yam *et al.,* 2019). According to 2019 global estimates, deaths directly attributable to AMR were 929 000 (660 000–1 270 000) whilst 3.57 million (2.62–4.78 million) deaths were associated with AMR (Murray *et al.,* 2022). Amongst pathogens recorded to be the leading cause of deaths associated with resistance were *E. coli* and *K. pneumoniae* (Murray *et al.,* 2022). According to a 2013 CDC report, the aforementioned bacteria caused 44,000 deaths (CDC, 2013) in the United States alone. However, these estimates declined in the revised 2019 CDC report, with an estimate of approximately 2.8 million infections and 35,000 fatalities (CDC, 2019). Since 2013, the USA has made progress, in addressing the threat of resistance through effective interventions and stewardship programs like the National Action Plan to combat resistant bacteria. (CDC, 2019). In the European Union (EU), AMR is estimated to cause 671,689 new infections each year, of which 426,277 (63.5%) are related

to healthcare (Grundmann *et al.,* 2011) The estimates correspond to an incidence of 131 infections per 100,000 population and an attributable mortality rate of 6.44 deaths per 100,000 (Giacomini *et al.,* 2021). The clinical burden indicated in the European Union (EU) and European Economic Area (EEA) are 170 Disability-Adjusted Life Years (DALY) rates per 100,000 population, which is similar to the combined burden of HIV, influenza, and tuberculosis (Cassini *et al.,* 2019).

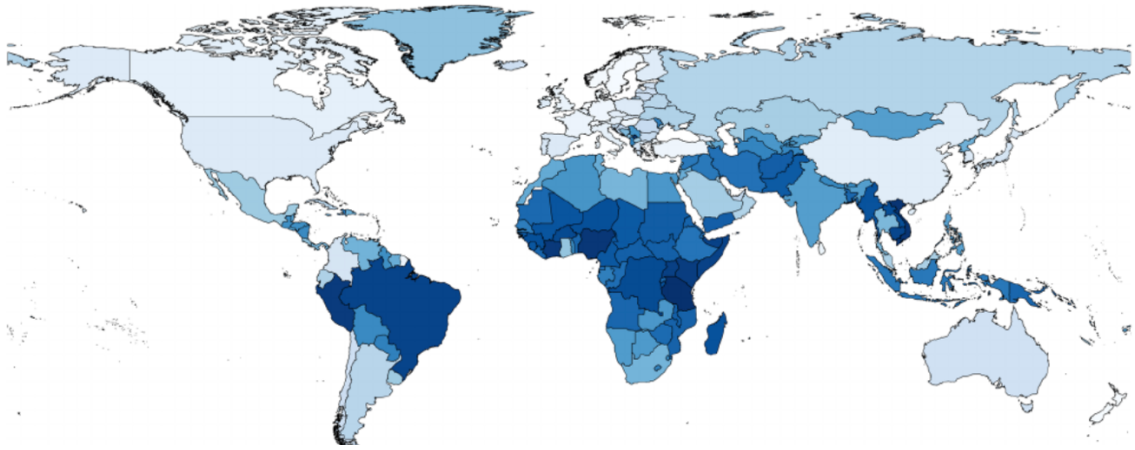

Figure 1.1: Global predictions of the abundance of AMR across different countries and territories around the world. The map is coloured to indicate the predicted level of AMR, ranging from light blue (low AMR abundance) to dark blue (high AMR abundance). The figure is adapted from (Hendriksen *et al.,* 2019)

In low- and middle-income countries (LMICs), AMR surveillance has not been implemented adequately despite attempts to consolidate consistent reporting. Relatively recently, an initiative has been launched in Africa to establish an AMR Surveillance Network (AMRSNET) through the Africa CDC framework for AMR, in line with the World Health Organization's objectives (Gulumbe *et al.,* 2022; Varma *et al.,* 2018). Since these initiatives are still in progress, the existing evidence regarding AMR in LMICs heavily depends on gathering information from limited prevalence studies. However, these studies vary significantly in terms of their design, sample size, methodology and interpretation schemes. For example, the use of protocols recommended by the Clinical Laboratory Standards Institutes (CLSI) and those recommended by the European Committee, on Antimicrobial Susceptibility Testing (EUCAST) (Murray *et al.,* 2022). Despite

these limitations, reports from Asia, particularly South Asia, have documented AMR mortality rates to range between 21.5 and 23.5 per 100,000 (Kariuki *et al.,* 2022; UK Health Security Agency, 2021; Murray *et al.,* 2022). The mortality rate due to AMR is highest in the region of Sub-Saharan Africa (SSA) (Figure 1.2), affecting approximately 27.3 out of every 100,000 people (Murray *et al.,* 2022). The rates in Eastern SSA, are 19.4, per 100,000 (Kariuki *et al.,* 2022; Murray *et al.,* 2022).

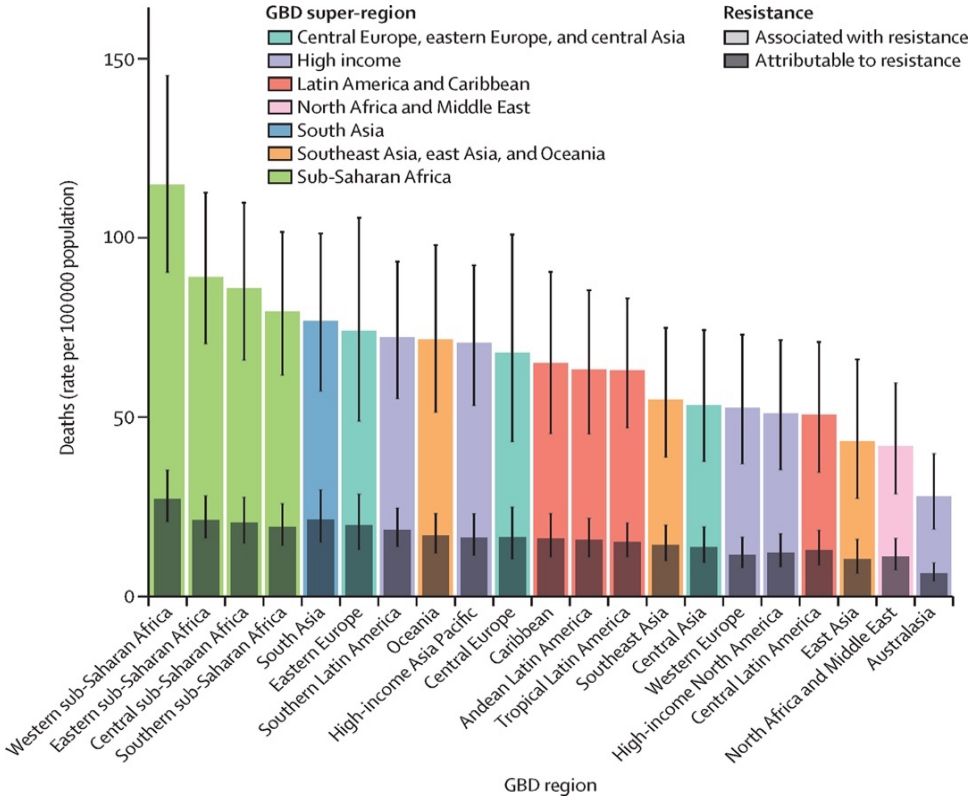

Figure 1.2: Deaths attributable to, and associated with, bacterial AMR in 2019 by GBD region. The figure is adapted from (Murray *et al.,* 2022)

1.2. Mechanisms of AMR

There are various mechanisms of antibiotic resistance that have been reported in the existing literature. These mechanisms include the use of efflux pumps (Compagne *et al.,* 2023), target site alteration (Darby *et al.,* 2023), inactivation of the antibiotic (Gauba and Rahman, 2023) and antibiotic modification (Kapoor *et al.,* 2017). Others include structural alterations such as changes in cell permeability (Cesur and Demiröz, 2013), and biofilm formation (Donlan, 2001; Tang and Zhao, 2023). Among the mechanisms listed above, inactivation of the antimicrobials is one of the mechanisms associated with resistance to some of the last resort antimicrobials (Soares *et al.,* 2023). The most pivotal ones include the production of beta-lactamases and carbapenemases (Moglad and Altayb, 2023; Harding-Crooks *et al.,* 2023). Bacteria producing these enzymes have been listed as one of the WHO's top priorities and are considered to be one of the most clinically relevant (Mancuso *et al.,* 2021). Their primary mechanism is to inactivate the antimicrobial agent through enzymatic degradation of the beta-lactam ring in penicillins, monobactams, cephalosporins and carbapenems, and subsequently causing the antibiotic to lose its activity (Barreiro *et al.,* 2023; Reygaert, 2018). Other enzymatic mechanisms causing resistance include aminoglycoside-modifying enzymes (Ramirez and Tolmasky, 2010), and chloramphenicol acetyltransferases (Denis *et al.,* 2010).

1.2.1. Beta-lactamases

Beta-lactam antibiotics have a common feature known as the beta-lactam ring which is important for their antibacterial activity (Sykes and Papich, 2014; Zabiszak *et al.,* 2023). The ring binds and inhibits the activity of enzymes involved in bacterial cell wall synthesis known as penicillin-binding proteins (PBPs) (Srivastava *et al.,* 2023). Beta-lactam antibiotics include penicillin, cephalosporins, monobactams and carbapenems (Reygaert, 2018). They can be neutralised by beta-lactamases which operate by breaking down the beta-lactam ring (Reygaert, 2018). Beta-lactamases on the other hand, can be categorised into various groups according to their genetic and

functional characteristics (Balsalobre *et al.,* 2019). These include the Ambler molecular classification and the Bush-Jacoby-Medeiros classification (Singh *et al.,* 2022; Bush, 2023). The most widely used scheme is the Ambler classification which groups the enzymes into four main classes; Class A, Class B, Class C, and Class D (Philippon *et al.,* 2015; Owusu *et al.,* 2023). Class A comprises of penicillinases and extended-spectrum beta-lactamases (ESBLs) which are inhibited by clavulanic acid (Naas *et al.,* 2016). Members of this group include TEM-1, SHV-1 and CTX enzymes. Bacteria that carry genes of this group are resistant to penicillins (e.g. ampicillin and amoxicillin), early-generation (e.g., cefalexin) and third-generation cephalosporins (cefotaxime and ceftazidime) (Shaikh *et al.,* 2015). They are frequently present in Gram-negative bacteria, including *Klebsiella* and *E. coli*. CTX-M-15, in particular, has recently become widespread globally (Koirala *et al.,* 2021; Emeraud *et al.,* 2021; Castanheira *et al.,* 2021).

1.2.2. Carbapenamases

Class B beta-lactamases, also known as metallo-beta-Lactamases include Imipenemases (IMP), Verona Integron encoded metallo-beta-lactamases (VIM), New Delhi Metallo-beta-lactamases (NDM), and São Paulo Metallo-beta-lactamase (SPM). They require divalent metal ions (i.e., zinc) for their catalytic activity (Bonomo, 2017; Wu *et al.,* 2019; Boyd *et al.,* 2020). Strains that carry enzymes of this class are resistant to carbapenems, such as imipenem and meropenem, which are considered as last line of defence against multidrug-resistant Gram-negative bacteria (Boyd *et al.,* 2020). Besides in *E. coli* and *Klebsiella*, Class B betalactmases are also often found in *Pseudomonas* and *Acinetobacter* species (Tanriverdi Cayci *et al.,* 2022). Class C beta-lactamases, also known as AmpC, include CMY, DHA, and ACT β-lactamases (Philippon *et al.,* 2022). Members of this group are susceptible to beta-lactamase inhibitors and are found in Gram-negative bacteria (Bush and Bradford, 2016). Class D beta-lactamases (also known as OXA-enzymes) include the important resistance determinant OXA-48 (Bush, 2023). Members of this group are associated with carbapenem resistance, and they have been identified in Gram-negative bacteria, including *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Codjoe and Donkor, 2017).

1.2.3. Other antibiotic resistance mechanisms

Other mechanisms that lead to antibiotic resistance among *Enterobacteriaceae* include the alteration of cell membrane permeability (Delcour, 2009; Reygaert, 2018). Microorganisms can alter the composition of macromolecules (lipids and porins) in their cell membranes, to regulate the entry of substances in their cells (Delcour, 2009). Hydrophilic antibiotics can easily penetrate through porins, but highly lipidated bacterial membranes prohibit them from passing (Nikaido, 2003). Hydrophobic antibiotics can easily pass through the lipid bilayer (Moravej *et al.,* 2018). Given Gram-negative bacteria have double cell membranes, which comprise of phospholipids, lipopolysaccharides, and lipoproteins, it is difficult for most antimicrobials to penetrate (Theuretzbacher *et al.,* 2023). Quinolones are an exception as they exhibit hydrophobic properties and can thus penetrate both outer and inner membranes of Gram-negative bacteria (Bryan and Bedard, 1991; Fedorowicz *et al.,* 2023; Tang and Zhao, 2023).

Another resistance strategy involves the modification of the antimicrobial target sites within bacterial cells to inhibit binding of antimicrobials to the target site, which renders them ineffective (Gauba and Rahman, 2023). Examples of AMR associated with these mechanisms include quinolone resistance, which is attributed to mutations in the DNA gyrase, and rifamycin resistance caused by mutations in the gene encoding the RNA polymerase β subunit (Redgrave *et al.,* 2014; Feng *et al.,* 2023). Bacteria are also capable of reducing intracellular concentrations of antimicrobials within cells using efflux pumps (Poole, 2007; Lorusso *et al.,* 2022). The expression of efflux pumps can vary according to environmental stimuli, including the presence of antimicrobials (Poole, 2007). Occasionally, the difference in expression may be due to spontaneous mutations, conferring competitive advantages in environments with antimicrobials (Li *et al.,* 2015). Basal efflux pump expression is thought to cause intrinsic resistance, while increased expression due to mutations results in higher minimum inhibitory concentrations (MIC) (Sandoval-Motta and Aldana, 2016; Annunziato, 2019). Efflux pumps are known to have different specificities for antimicrobials. Some are known to be broad spectrum, contributing to the efflux of multiple classes of antibiotics (Figure 1.3) (Piddock, 2006). For instance, the AcrAB-TolC system in *E. coli* is known to pump a wide range of antibiotics such as tetracycline, beta-lactams and fluoroquinolones (Pérez *et al.,* 2012; Bay *et al.,* 2017). In contrast, the Tet(A) and Tet(B) efflux

systems in *E. coli* are narrow spectrum efflux pumps and export as tetracyclines (Kareem and Atiyea, 2023).

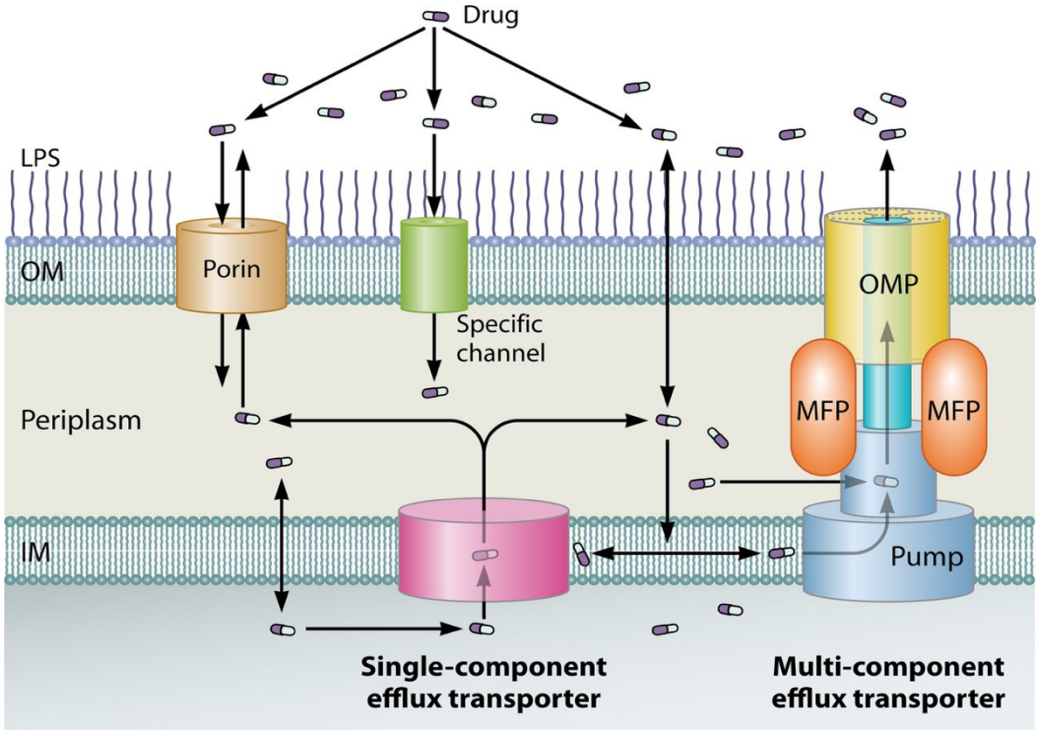

Figure 1.3: Antimicrobial efflux pumps in Gram-negative bacteria. Antimicrobials enter through lipid bilayers and porin channels, move to the inner membrane, and are expelled by transporters. Single pumps eject internal drugs via porins, while multi-component exporters release them externally. The balance between influx and efflux determines drug concentration. Image sourced from (Li *et al.,* 2015) .

Another effective strategy used by bacteria to increase their resistance to antimicrobials is the formation of biofilms (Donlan, 2000; Liu *et al.,* 2023). Biofilms are communities of microorganisms that are surrounded by a matrix made up of substances, also known as extracellular polymeric substance (EPS) (Flemming *et al.,* 2023). This EPS matrix acts as a barrier preventing agents from diffusing and reaching the bacterial cells within the biofilm (Flemming *et al.,* 2023; Ali *et al.,* 2024). Additionally, the reduced metabolic activity of cells within biofilms makes them less vulnerable to antibiotics that specifically target growing bacteria (Shree *et al.,* 2023). Within these biofilms there are also persister cells, which exhibit tolerance to antibiotics

and can survive treatment (Shree *et al.,* 2023; Busi, 2023). The cells possess the ability to regenerate once antimicrobial pressure is removed and cause infections (Busi, 2023).

1.3. *Escherichia coli*

Escherichia coli is a Gram-negative facultative anaerobic bacterium in the *Enterobacteriaceae* family. It inhabits various environments, including animal and human intestines, making it a useful marker for faecal contamination (Lim *et al.,* 2010). *E. coli* belongs to the family *Enterobacteriaceae*, which comprises other pathogens, such as *Klebsiella*, *Salmonella*, *Yersinia*, and *Shigella* (Martin and Bachman, 2018a). *E. coli*, as a commensal bacterium, aids digestion and prevent harmful microbes from colonizing the gut of humans and animals (Dobrindt *et al.,* 2003; Lim *et al.,* 2010). However, they can also function as pathogenic bacteria, causing various diseases (Lim *et al.,* 2010). Currently, *E. coli* is a major contributor of both healthcare-associated and community-acquired invasive bacterial infections. In Eastern sub-Saharan Africa (which includes East Africa), it is estimated to cause 24.7 deaths per 100,000 people (Ikuta *et al.,* 2022). *E. coli* is capable of causing a range of diseases, with various pathotypes that have distinct effects on health (Figure 1.4) (Allocati *et al.,* 2013). For instance, diarrhoea-causing enteropathogenic (EPEC) strains, employ intimin (*eae*), the bundle-forming pilus (*bfp*), and components of the Type III secretion system (T3SS) (Saldaña *et al.,* 2009; Denamur *et al.,* 2021). These allow EPEC to attach to intestinal cells, creating "attachment pedestals" (Caprioli *et al.,* 2005). *E. coli* can also cause traveller's diarrhoea which is due to enterotoxigenic *E. coli* (ETEC) which produces heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT), along with colonization factors (CFA/I, CFA/II, CS1-CS7), contributing to colonisation of intestines and watery diarrhoea (Qadri *et al.,* 2005; Lim *et al.,* 2010). Enteroinvasive *E. coli* (EIEC), on the other hand, causes dysentery-like illness characterised by inflammatory diarrhoea and cell death, through the use of invasion plasmid antigens (*Ipa*) and T3SS components to invade intestinal cells (Schroeder and Hilbi, 2008). Pathotypes such as Enterohemorrhagic *E. coli* (EHEC) cause haemorrhagic colitis, haemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP) through the production of Shiga toxins, which are encoded by the *stx1* and *stx2* genes, along with LEE pathogenicity

islands, which carry the virulence genes *eae, tir, espA, espB, espF,* and *map* (Kang *et al.,* 2014; Joseph *et al.,* 2020). Other pathotypes such as the Diffusely Adherent *E. coli* (DAEC) cause diarrhoea in both children and travellers (Jafari *et al.,* 2012). They adhere to intestinal cells diffusely and alter the structure of these cells using the Afa/Dr adhesins *(afa*, *daa*) (Servin, 2014). Enteroaggregative *E. coli* (EAEC) form biofilms on intestinal cells, causing prolonged episodes of diarrhoea in children (Kaur et al., 2010). They use aggregative adherence fimbriae and toxins such as heat stable enterotoxin to cause diarrhoea (Zamboni et al., 2004). Uropathogenic *E. coli* (UPEC) is responsible for urinary tract infection (UTIs). UPEC strains adhere to and infect the epithelium of the urinary tract by using fimbriae (*fim, pap, dra*), hemolysin (*hlyA*), and cytotoxic necrotizing factor (*cnf)* (Hilbert, 2011) (Terlizzi *et al.,* 2017). Neonatal Meningitis *E. coli* (NMEC) causes meningitis in newborns by invading the bloodstream and causing systemic infections (Wijetunge *et al.,* 2015). They cause meningitis using K1 antigen capsule synthesis genes (*kps*) and outer membrane proteins (encoded by *ompA*, *ompT*). Adherent-invasive *E. coli* (AIEC) is associated with Crohn's Disease as it attaches to and invades the epithelial lining of the intestines (Saitz *et al.,* 2022). Extraintestinal Pathogenic *E. coli* (ExPEC) encompasses a range of pathotypes causing infections (Sarowska *et al.,* 2019; Santos *et al.,* 2023). To infect birds, Avian Pathogenic *E. coli* (APEC) produces colicins (*col*), siderophores (*iuc, iro*), hemolysins (*hlyF*), and temperature-sensitive hemagglutinins (*tsh*) (Kathayat *et al.,* 2021). Molecular tools such as PCR use the *uidA* gene as a broad marker to detect *E. coli* whereas *stx* is used for the detection of Shiga-toxin-producing *E. coli* (STEC and EHEC), *lt* and *st* (*sta* and *stb*) are markers for the detection of enterotoxigenic *E. coli* (ETEC), *eae* for the detection of enteropathogenic *E. coli* (EPEC) and the *ipaH* gene for the detection of enteroinvasive *E. coli* (EIEC) (Müller *et al.,* 2007).

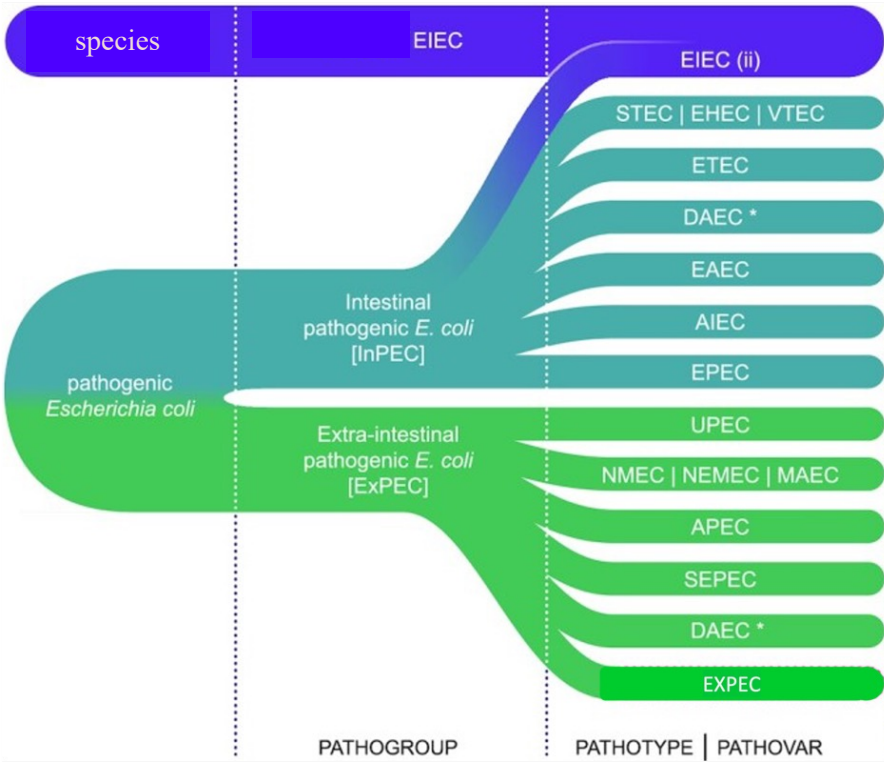

Figure 1.4: Classification of *E. coli* pathotypes. The symbol * indicates pathotypes linked to both intestinal and extraintestinal disease. The image have been adapted from (Geurtsen *et al.,* 2022).

The identification of *E. coli* encompasses a wide range of techniques, including both phenotypic and molecular methods (Müller *et al.,* 2007). Phenotypically *E. coli* colonies can be smooth or rough (Hasman *et al.,* 2000). Rough *E. coli* colonies are usually an irregular shape, with a rough texture whereas smooth colonies are circular raised and have a smooth texture (Hasman *et al.,* 2000). Phenotypically, preliminary identification of certain serogroups associated with *E. coli* infections can be accomplished through serological detection (Bahgat *et al.,* 2023). During serological detection, antibodies (antisera) interact with the antigens of the bacteria. Markers used to classify strains into distinct serogroups and serotypes include O, H, and K antigens, aiding precise strain differentiation (Fratamico *et al.,* 2016). H antigens comprise bacterial flagella, while O antigens are the external polysaccharide moieties of lipopolysaccharide (LPS) (Fratamico *et al.,* 2016). Meanwhile, K antigens make up the capsule of bacteria (Scheutz *et al.,* 2004).

MLST (Multi-Locus Sequence Typing) is widely used to genetically type bacterial pathogens, (Raveendran *et al.,* 2023). Achtman's 7-locus scheme for *E. coli* is the most consistent with whole-genome phylogenies (Chaudhuri and Henderson, 2012). It utilises seven housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA, recA*) which are used to compare strains and group organisms into different sequence types (STs) (Maiden *et al.,* 1998; Gai *et al.,* 2015). MLST has been extended to core-genome MLST (cgMLST), a scheme with over 2500 genes which is available in Enterobase and which differentiates species using a wider set of core genes). To date, *E. coli* are divided into eight phylogroups groups which are named A, B1, B2, C, D, E, F and G (Geurtsen *et al.,* 2022). Phylogroups A and B1 have been consistently associated with commensalism and less virulent pathotypes, whereas phylogroup B2 is associated with more virulent and invasive strains (Basu *et al.,* 2013; Iranpour *et al.,* 2015).

1.4. *Klebsiella*

Klebsiella is a genus of Gram-negative, non-motile, enveloped bacteria that are commonly found in the environment, including soil, surface water, medical devices, plants, and humans. Like *E. coli*, it is assigned to the family *Enterobacteriaceae* (Ronald, 2002; Mmatli *et al.,* 2023). *Klebsiella* strains have the intrinsic capacity to hydrolyse penicillins, with certain strains exhibiting additional resistance to cephalosporins, monobactams, carbapenems, and β-lactamase inhibitors (Merino *et al.,* 1992; Karami-Zarandi *et al.,* 2023). *K. pneumoniae* possess various virulence genes which contribute to its pathogenicity of the bacteria (Figure 1.5) (Elemary et al., 2023). For instance, they produce siderophores such as yersiniabactin, enterobactin and aerobactin which play a critical role in iron acquisition, especially in iron limited environments. They also possess capsular polysaccharides which help the bacteria to evade immune response (Bengoechea and Sa Pessoa, 2019; Elemary et al., 2023). Other factors such as fimbriae and lipopolysaccharides (LPS) help the bacteria attach to its host cells and trigger inflammatory response (Paczosa and Mecsas, 2016). Human mucosal surfaces such as the oropharynx and the gastrointestinal (GI) tract can easily be colonised by the bacterium, where its effect is mostly benign (Bagley, 1985; Dao *et al.,* 2014). However, *Klebsiella* can cause severe human infections upon entry to other body sites, such as the

lungs, bladder, brain, liver, eyes, blood, and wounds (Martínez *et al.,* 1991; Wang *et al.,* 2013; Tsai *et al.,* 2010). *Klebsiella* is responsible for a wide range of diseases, including pneumonia, urinary tract infections, bacteraemia and liver abscesses (Fang *et al.,* 2004; Shakya *et al.,* 2017; Zarkotou *et al.,* 2011) and is known for its ability to evade the immune system (Bachman *et al.,* 2011; Doorduijn *et al.,* 2016). In humans, nosocomial infections are caused by highly diverse *Klebsiella* strains that are considered to be opportunistic pathogens, as they mostly affect immunocompromised patients (Podschun and Ullmann, 1998; Martin and Bachman, 2018). *K. pneumoniae* and other closely related *Klebsiella* species form the *K. pneumoniae* complex. The *K. pneumoniae* complex is composed of seven phylogroups (Kp1 to Kp7) that match with seven closely related species of *K. pneumoniae,* including *K. quasipneumoniae* subsp *quasipneumoniae*, *K quasipneumoniae* subsp *similipneumoniae*, *K variicola* subsp *variicola* (generally known as *K. variicola*), *K. variicola* subsp *tropicalensis* and *K. africanensis* (Rodrigues *et al.,* 2019). Kp1, Kp2, and Kp3 are among the most prevalent clinically relevant phylogroups which correspond to *K. pneumoniae*, *K. quasipneumoniae* and *K. variicola* , respectively (Gómez *et al.,* 2021a). Due to their similar molecular and physiological characteristics, conventional approaches have proven to be ineffective at accurately differentiating members of the *K. pneumoniae* complex (Holt *et al.,* 2015; Lam *et al.,* 2021). Recent research has revealed that members of the *K. pneumoniae* complex, such as *K. variicola* and *K. quasipneumoniae*, are frequently misidentified as *K. pneumoniae* sensu stricto (Gómez *et al.,* 2021b). Consequently, the epidemiological significance of species in the complex that are not *K. pneumoniae* remains poorly understood (Gómez *et al.,* 2021b). In addition to the *K. pneumoniae* complex species, *Klebsiella oxytoca* is the second-most common *Klebsiella* species known to colonize the human gut (Singh *et al.,* 2016). *K. oxytoca* belongs to the *K. oxytoca* species complex (KoSC), which combines species with similar phenotypic characteristics (Singh *et al.,* 2016). The KoSC comprises nine phylogroups (Ko1-Ko9), which correspond to six species (Merla *et al.,* 2019). This complex includes *K. oxytoca*, *Klebsiella pasteurii*, *Klebsiella spallanzanii*, *Klebsiella grimontii*, *Klebsiella huaxiensis*, *Klebsiella michiganensis*, and three novel unnamed species (Singh *et al.,* 2016; Yang, 2022). Each major phylogroup (except Ko5, Ko7, and Ko9) represents a unique species and carry specific beta-lactamase gene variants (blaOXY–1 to blaOXY–9) (Fevre, 2005; Merla *et al.,* 2019). *K. oxytoca* and *K. michiganensis* are the two primary species of the complex that are linked to extraintestinal infections in humans (Yang, 2022).

To date, phylogenetic analysis is the most reliable method for identifying strains within the *K. pnuemoniae* complex (Rosenblueth *et al.,* 2004; Martínez-Romero *et al.,* 2018). *K. pneumoniae* complex members are believed to share 95-96% average nucleotide identity (Wyres *et al.,* 2020a). The primary marker used in phylogenetic analysis to distinguish *K. pneumoniae* from other members of the *K. pneumoniae* complex is the *rpoB* gene, which encodes the beta-subunit of RNA polymerase (Gómez *et al.,* 2021b), but whole genome sequencing remains the most accurate method for identifying *Klebsiella* species, as it allows an in-depth examination of the entire genome. The Institut Pasteur MLST scheme, which targets seven housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*) of the *K. pneumoniae* complex is widely used to genotype these bacteria (Jasim, 2020). The scheme results in an allele profile that can be used to identify related strains. As for *E. coli*, MLST has been extended to cgMLST which incorporates the core genes of the genus (Deurenberg *et al.,* 2017). There are various cgMLST schemes that have been developed to enable analysis of the genetic diversity and relationship among *Klebsiella* strains, such as BIGSdb-Kp, which targets 2,365 core genes (Miro *et al.,* 2020). SeqSphere+ offers several cgMLST schemes tailored to *Klebsiella* with varying core gene numbers to effectively capture genetic diversity (Uelze *et al.,* 2020).

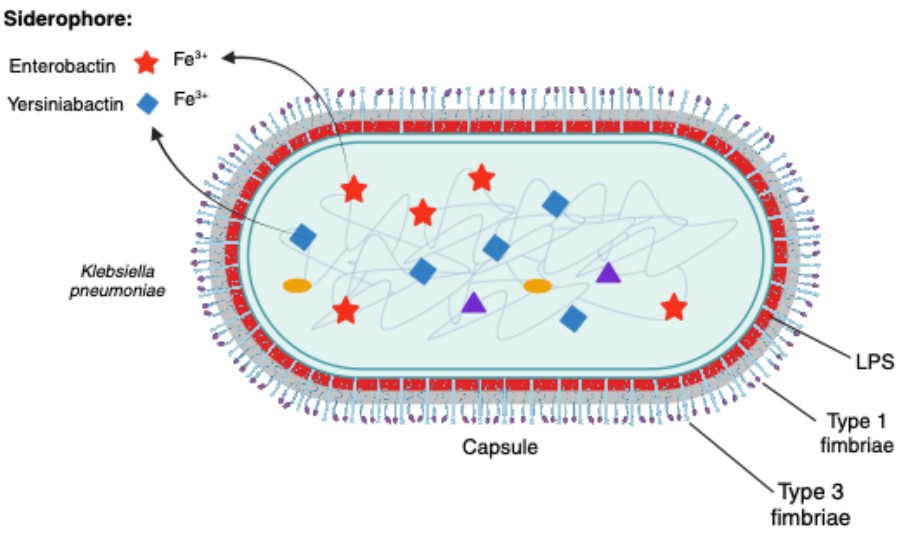

Figure 1.5: *K. pneumoniae* strains have extracellular membrane depicted in grey, which plays a significant role in pathogenicity of the bacteria. *K. pnuemoniae* also produces membrane adhesive structures, fimbriae (type 1 and type 3) and iron scavenging siderophores. Amongst *K. pneumoniae* strains enterobactin is the most commonly produced followed by yersiniabactin. Image adapted from (Paczosa and Mecsas, 2016)

1.5. Prevalence of ESBL-producing and carbapenem resistant *E. coli* and *Klebsiella* in sub-Saharan Africa.

Compared to high-income countries in North America and Europe there are significant gaps in data characterising AMR in SSA (Kariuki *et al.,* 2022). The majority of studies available in literature predominantly report on the prevalence of AMR in urban areas in Africa even though there is a large part of the population (70%) living in rural areas (Satterthwaite, 2017). However, based on the existing reports, AMR rates and carriage vary widely across different countries and populations in SSA (Tompkins *et al.,* 2021). Both *Klebsiella* and *E. coli* are among the leading causes of healthcare-related infections i.e., septicaemia, bacteraemia, urinary tract, and other enteric infections (Okomo *et al.,* 2019; Tompkins *et al.,* 2021; Kariuki *et al.,* 2022). Strains producing Extended Spectrum Beta-lactamases (ESBLs) and carbapenemases are two of the most serious antibiotic-resistant threats according to the World Health Organisation's (WHO) priority

list (Shaikh *et al.,* 2015; Wilson and Török, 2018; Sawa *et al.,* 2020). Data in SSA is still underreported and very fragmented. However, according to the existing data, ESBL-producing *Enterobacteriaceae* is becoming a growing threat in SSA. Existing data has consistently shown community level carriage of ESBL *Enterobacteriaceae* to be widespread (Valverde *et al.,* 2004; Woerther *et al.,* 2013; Otter *et al.,* 2019). For instance, in Tanzania intestinal carriage of ESBL-producing *Enterobacteriaceae* among healthy two-year-olds has been estimated at 11.6% whilst carriage amongst street children has been reported to be 31.8% (Tellevik *et al.,* 2016; Moremi *et al.,* 2017). Similarly, based on another study conducted at the community level in Tanzania by Mshana and colleagues, carriage of ESBL-producing *Enterobacteriaceae* was estimated to be 16.5% irrespective of age (Mshana *et al.,* 201). Regionally and within healthcare settings the burden of ESBL-producing *E. coli* and *Klebsiella* varies considerably (Sammarro *et al.,* 2023; Akenten *et al.,* 2023). In many studies in subs sub-Sarahan Africa, ESBL-producing *E. coli* has been associated with community-acquired urinary tract infections and bloodstream infections in adults and children (Larramendy *et al.,* 2020; Raphael *et al.,* 2023). In terms of health-related infections, a recent review looking at bloodstream infections in 12 sentinel locations in SSA using data collected between 2010 and 2014, found ESBL-producing *E. coli* was responsible for 5.7% for blood stream infections (Toy *et al.,* 2019). Conversely, observations in a smaller study conducted on children in Tanzania, revealed faecal carriage of ESBL-producing *E. coli* was widespread in healthy subjects whereas clinical infections rates were found to be rare (Letara *et al.,* 2021). Out of 350 children, only one case of blood stream infection was found, whereas 76 (22%) of children were colonised with ESBL *E. coli* (Letara *et al.,* 2021). However, this observation is not consistent with other studies. Based on another previous study conducted in Mwanza at Bugando Medical Centre, 28% out of 97 patients admitted carried ESBL-producing *E. coli* (Seni *et al.,* 2016). *Klebsiella*, on the other hand, has been primarily linked to outbreaks among neonates. It is one of the leading causes of infant fatalities in sub-Saharan Africa, with infection rates believed to be 3–20 times higher than in high-income countries (Kariuki *et al.,* 2022). A recent systematic review conducted on African countries, revealed the prevalence of ESBL-producing *Klebsiella* varied considerably between different countries with prevalence rates ranging from 5% to 100% (Tansarli *et al.,* 2014). These estimates are corroborated by data from other studies conducted in subsequent years in individual countries including Tanzania (Mohammed *et al.,* 2016; Founou *et al.,* 2019; Hertz *et al.,* 2019; Müller-Schulte *et al.,* 2020;

Letara *et al.,* 2021; Geuther *et al.,* 2023). In the case of carbapenem resistance in sub-Saharan Africa, data from a recent review revealed it is becoming a threat in public health, even though existing data is still limited (Kariuki *et al.,* 2022). Based on reviews carbapenem resistance is generally higher in *K. pneumoniae* than *E. coli* (Codjoe and Donkor, 2017; Murray *et al.,* 2022; Venne *et al.,* 2023). In a 2023 review, which examined 40 countries in Africa, resistance rates between different countries varied considerably, with three countries (i.e. Egypt, Mali and Sudan) having high resistance rates exceeding 5% (Venne *et al.,* 2023). However, in 14 countries, including Kenya and Tanzania, low resistance rates (<1%) were observed (Venne *et al.,* 2023). On the other hand, countries like Benin, Malawi, Mauritius, Morocco, Nigeria, Mauritania, Rwanda, and Uganda showed moderate rates, ranging between 1-5% (Mitgang *et al.,* 2018). Regarding *K. pneumoniae*, recent research that examined resistance rates in 15 counties classified the median rates as high (>5%) in 2 countries (Uganda and Madagascar), moderate (1–5%) in 3 countries (Kenya, Cameroon and South Africa), and less than 1% in 10 countries, including Tanzania (Mitgang *et al.,* 2018) .

1.6. Horizontal gene transfer

There are multiple mechanisms that allow the spread and distribution of genes responsible for AMR within and between bacterial populations (Sultan *et al.,* 2018). This process, termed Horizontal Gene Transfer (HGT), enables bacteria to acquire and share resistance genes, leading to the proliferation of resistant strains. The three main mechanisms of HGT are transduction, conjugation and transformation (Figure 1.6) (Villa *et al.,* 2019; Kilb *et al.,* 2023). Transduction involves the transfer of DNA by bacteriophages whilst conjugation involves pilus-mediated transfer of genes between cells. Transformation involves uptake of DNA from the environment (Zhu *et al.,* 2023). In transduction, there are three major mechanisms used by bacteriophages to mediate the transfer of DNA, including AMR genes (Sousa *et al.,* 2023). These mechanisms are generalised transduction, specialised transduction and lateral transduction (Michaelis and Grohmann, 2023). In the case of conjugation, DNA transfer will happen through a pilus-like structure which connects the donor and recipient during conjugation. This pilus structure is created by a Type IV secretion system (T4SS) (Christie, 2016; Michaelis and Grohmann, 2023). For transduction and conjugation, mobile genetic elements play a critically important role (Johansson *et al.,* 2021).

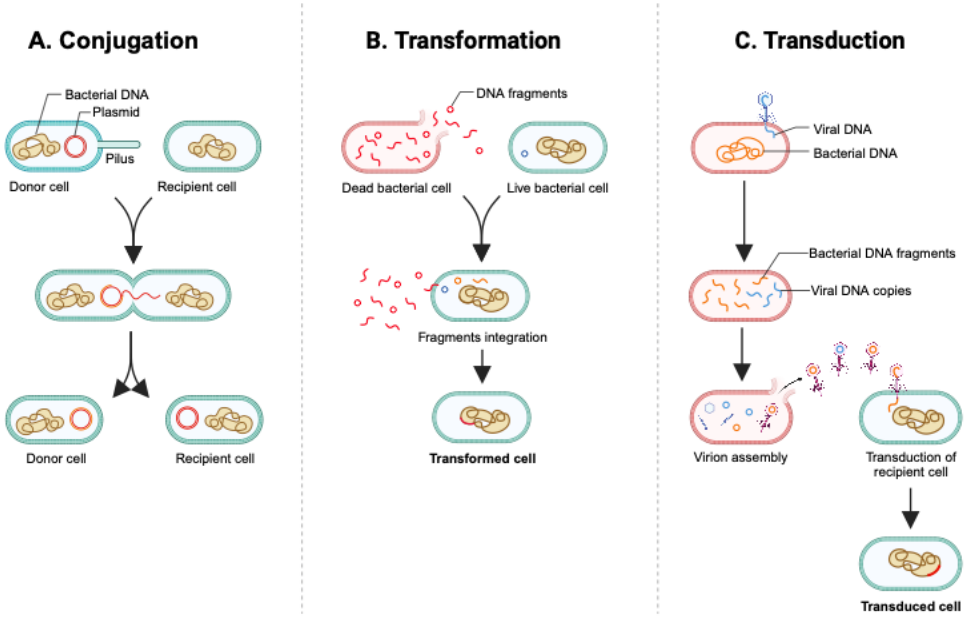

Figure 1.6: Mechanisms of HGT comprising of conjugation, transformation, and transduction. Image created in BioRender.com.

1.7. Mobile genetic elements

1.7.1. Translocatable genetic elements

Mobile genetic elements (MGEs) include transposons, insertion sequences (IS elements), integrative conjugative elements (ICEs) and plasmids (Johnson and Grossman, 2015; Tansirichaiya *et al.,* 2019; Carr *et al.,* 2023). Insertion sequences (IS) are short segments of DNA ranging between 700bp to 2500bp comprising of essential genes required for their transposition (Figure 1.7) (Sun *et al.,* 2023; Calvo *et al.,* 2023). They are thought to move by a cut-and-paste mechanism, meaning they can move from one DNA molecule to another (Siguier *et al.,* 2014; Bello-Morales, 2021). IS carry transposase enzymes which recognize specific sequences at the ends of IS elements and catalyse their removal from one site in the genome and insertion into another site (Siguier *et al.,* 2014). IS play a significant role in the mobilization and activation of resistance genes in microbial genomes (Siguier *et al.,* 2006; Chandler and Mahillon, 2007; Siguier

et al., 2014). IS can activate resistance genes by introducing strong promoters when they insert near a resistance gene, which subsequently increases the expression of the resistance genes (Wang *et al.,* 2023). This could lead to increased MICs to specific antibiotics (Wagner *et al.,* 2023). Moreover, since IS elements can insert randomly within the genome, they can disrupt resistance genes if they insert in them, thus inactivating the function of resistance genes (Fordham *et al.,* 2022; Greenrod *et al.,* 2023). When they are in close proximity to other mobile genetic elements, they can lead to recombination events between them and the adjacent element, resulting in the formation of composite transposons (Kaur and Talekar, 2022). Some antibiotic resistance genes whose relevance has been impacted by IS include the *ampC* β-lactam resistance gene. IS*1* and IS*2* are known to insert into the promoter regions of *ampC* β-lactamase genes in some bacteria, creating string of promoters leading to increased expression of the *ampC* gene and enhanced resistance to β-lactam antibiotics (Jaurin and Normark, 1983; Furmanek-Blaszk *et al.,* 2023). IS*10* and IS*26* have been found adjacent to tetracycline resistance genes (*tet* genes) (Anantham *et al.,* 2015; Partridge *et al.,* 2018). They are known to facilitate the movement of these genes and enhancing their expression through acquisition of strong promoters (Partridge *et al.,* 2018). IS*26* elements have also been associated with mobilisation of trimethoprim resistance genes (*dfr*) and promote their HGT between different bacteria (Sköld, 2001; Barbu *et al.,* 2018). Transposons (TE) are larger and more complex than insertion sequences (Kidwell, 2005; Almojil *et al.,* 2021). Like IS they can move within a genome, causing mutations, duplications or rearrangements of genetic material (Almojil *et al.,* 2021; Chu *et al.,* 2021). Similar to insertion sequences they also apply the cut and paste mechanism, where a copy of the TE is excised and inserted elsewhere (Serrato-Capuchina and Matute, 2018). Aside from dissemination of AMR genes they can carry genes associated with other functions (Ellabaan *et al.,* 2021). Transposons also contain inverted repeats like IS at their ends which are detected by transposase enzyme to facilitate movement (Cui *et al.,* 2002; Liao *et al.,* 2023). They also have the ability to disrupt gene function and subsequent regulation, leading to silencing or the expression of certain resistance genes when they integrate upstream of pre-existing resistance genes introducing novel promoter sequences or disrupting existing promoters (Negi *et al.,* 2016). Transposons that have been well documented in *E. coli*, include Tn3, Tn5 and Tn10 (Hickman and Dyda, 2016; Haniford and Ellis, 2015). These transposons are 4,957 base pairs (bp) 5,346 bp and 9,993 bp in size, respectively (Wishart *et al.,* 1985; Chang *et al.,* 2023). Genes encoding beta-lactamases (*bla*$_{\text{TEM}}$) and aminoglycosides

modifying enzymes (*aacA4*) have been detected in Tn3 whereas genes responsible for tetracycline resistance (*tetA* and *tetR*) have been detected in Tn10 and genes conferring resistance to kanamycin/neomycin have been found in Tn5 (Iyer *et al.,* 2013). Tn4401 has been reported in *Klebsiella* and it has been associated with carbapenemase genes, such as bla_{KPC} (Mathers *et al.,* 2017; Halat and Moubareck, 2020; Reyes *et al.,* 2021). ICEs are larger and more complex than transposons (Johnson and Grossman, 2015). Unlike transposons and insertion sequences, ICEs have a site-specific integration and excision mechanism (Burrus *et al.,* 2002; Johnson and Grossman, 2015). Known attachment sites in the host genome include *attB* (Gonçalves *et al.,* 2022). Upon integration into the host genome, they can become a stable component of it, allowing for long-term inheritance of acquired genetic material (Burrus *et al.,* 2006; Delavat *et al.,* 2017). Moreover, ICEs can integrate into extrachomosomal elements of the recipient (Johnson and Grossman, 2015; Carraro *et al.,* 2015). ICEs can also undergo conjugation which means they can transfer themselves and adjacent genes to recipient cells (Johnson and Grossman, 2015). Through these transfer mechanisms, ICEs are known to transfer resistance genes between different bacteria (Carraro and Burrus, 2015; Burrus, 2017). Resistance genes associated with ICEs include carbapenem resistance genes (e.g bla_{KPC} and bla_{NDM}) (Botelho *et al.,* 2018; Botelho *et al.,* 2020), ESBL genes (e.g $bla_{CTX\text{-}M}$) (Saiz-Escobedo *et al.,* 2023), aminoglycoside resistance genes (e.g., *aac*, *ant*) (Michael *et al.,* 2012), tetracycline resistance genes (e.g., *tetA* and *tetB*) (Rodríguez-Blanco *et al.,* 2012), quinolone resistance genes (e.g *qnr*), (Marin *et al.,* 2014), chloramphenicol genes (e.g., *catA* and *catB*) (Michael *et al.,* 2012) and sulphonamide resistance genes (e.g., *sul1* and *sul2*) (Eidam *et al.,* 2015).

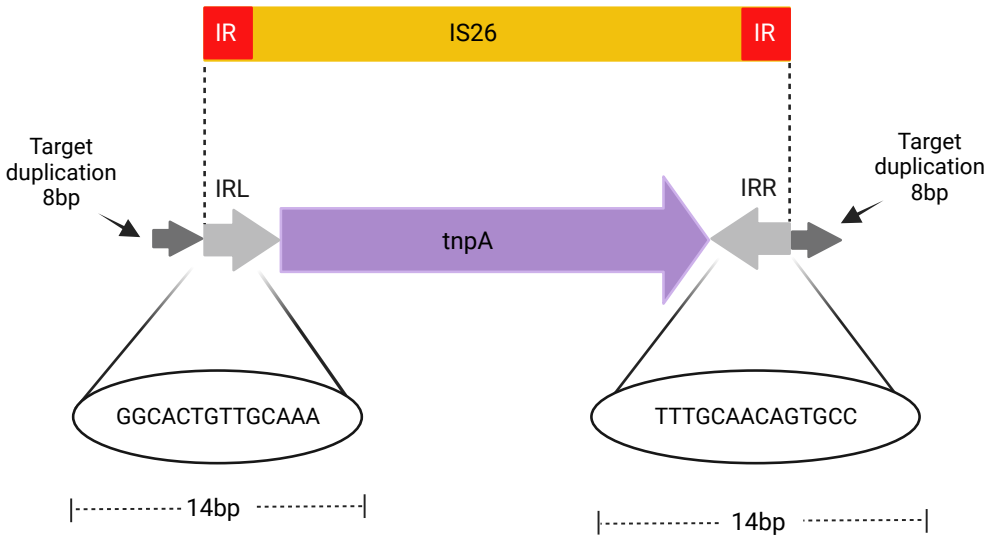

Figure 1.7: Structure of IS*26*. The IS is represented in yellow. Light grey arrows and red section represent inverted repeats (IRs) which are 14bp in size, with their DNA sequences shown below. Target repeats are displayed in dark grey. The purple arrow denotes the reading open reading frame of the transposase, with its orientation shown by the arrow. Image adapted from (Varani *et al.,* 2021)

1.8. Plasmids

Plasmids are DNA molecules that can replicate independently from the chromosome (Wang *et al.,* 2023). They are capable of transferring AMR genes and contain genetic elements that enable gene transfer (Tao *et al.,* 2022). Plasmids can vary widely in size ranging from approximately one thousand base pairs to hundreds of thousands of base pairs (Zhao *et al.,* 2023). They can be found in a wide range of organisms including bacteria, archaea, yeasts and, in rare cases, in plants (Hall *et al.,* 2021). Plasmids contain specific DNA sequences, known as replication origins (oriV), which serve as starting point for replication (Bingle and Thomas, 2001). They also contain *rep* genes which encode initiator proteins that recognise and bind to oriV, to initiate the process of replication (Schlüter *et al.,* 2007; Dewan and Uecker, 2023; Cervantes-Rivera *et al.,* 2011). Iterons are used to control the binding of Rep proteins to oriV, with iterons being present in multiple copies leading to lower plasmid copy numbers (Chattoraj, 2000). Separate to this, plasmids have another regulatory mechanism involving repressor proteins which bind to the operators or repressor

binding sites within the replicon control region near oriV, thereby inhibiting the unwinding of DNA and the replication machinery (Bingle and Thomas, 2001; Meyer, 2009). Plasmids may play a role in the survival, adaptation and competitiveness of host cells by carrying genes associated with virulence, metabolism, conjugation, resistance to metals, antitoxins, bacteriocins and stress response (Clewell *et al.,* 2014). Plasmids can be categorized as either conjugative or non-conjugative (Smillie *et al.,* 2010; Branger *et al.,* 2019; Coluzzi *et al.,* 2022). Conjugative plasmids are equipped with elements that facilitate their transfer to cells through pilus transfer origins, transfer proteins, auxiliary proteins and type IV section systems (T4SS) (Virolle *et al.,* 2020; Álvarez-Rodríguez *et al.,* 2020). These plasmids may also contain *tra* genes which create pores in recipient cells, membrane associated genes and DNA binding proteins that protect the transfer DNA strand (Hormaeche *et al.,* 2002). Non-conjugative plasmids use mechanisms such as transformation, transduction and specialized mechanisms that do not rely on the formation of a conjugative pilus (Ramsay *et al.,* 2016; Michaelis and Grohmann, 2023). In contrast, there is another set of plasmids (i.e., mobilizable plasmids) that are incapable of initiating conjugation independently, but possess DNA sequences that facilitate their transfer (i.e., oriT) when conjugative plasmids are present (Ramsay and Firth, 2017), thus allowing mobilisable plasmids to hitchhike with conjugative plasmids, facilitating their dissemination (Ramsay and Firth, 2017).

The classification of plasmids primarily relies on their inability to coexist within cells (Garcillán-Barcia *et al.,* 2023). Plasmids with similar partitioning and replication systems compete for resources such as replication machinery and regulatory factors, and those that lose this competition, experience reduced efficiency in replication (Ghosh *et al.,* 2006; Salje, 2010; Bouet and Funnell, 2019; Birge, 2006; Partridge *et al.,* 2018). This phenomenon can result in changes in copy number, ultimately leading to plasmid loss (Partridge *et al.,* 2018). In situations where plasmids share partitioning systems they may also compete for ParA and ParB proteins during cell division (Livny *et al.,* 2007; Ietswaart *et al.,* 2014). If these plasmids do not secure resources during division, they are likely not to be distributed properly between both daughter cells (Figure 1.8)(Gordon and Wright, 2000; Partridge *et al.,* 2018). As a consequence, some daughter cells end up with fewer or no copies of the plasmid. To overcome this compatibility issue and ensure survival and coexistence within the cell some plasmids carry multiple replicons belonging to different incompatibility groups as a backup strategy (Field and Summers, 2011).

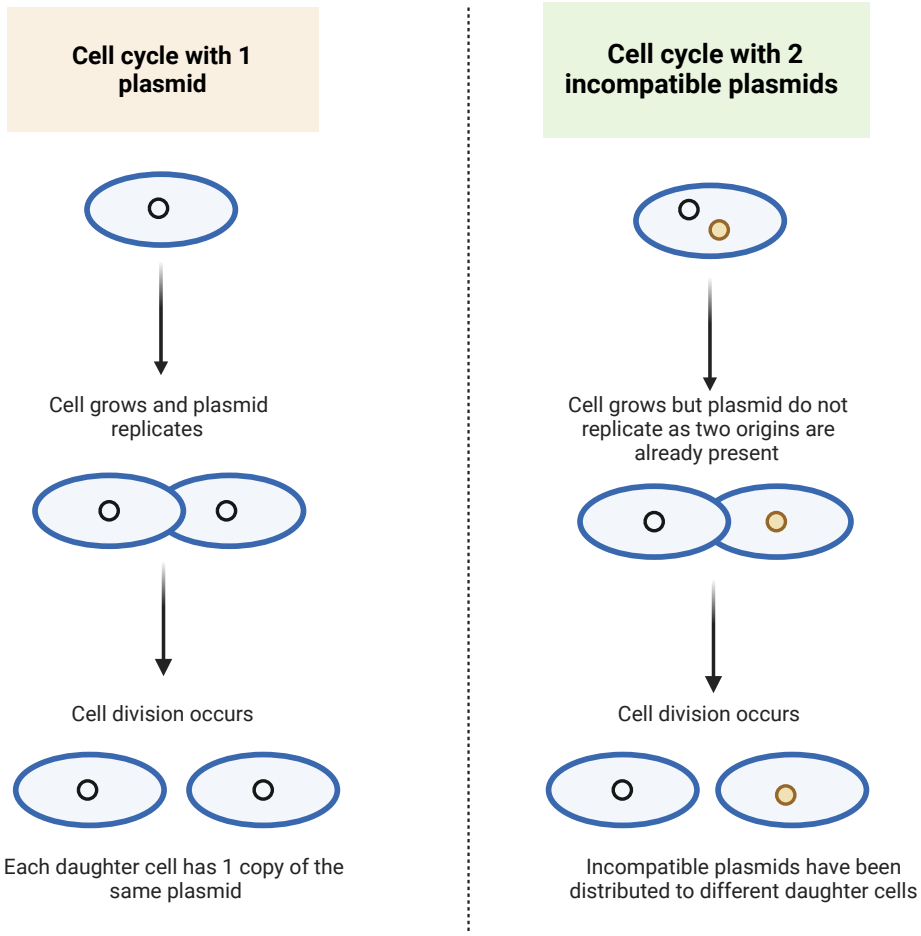

Figure 1.8: Plasmid distribution among daughter cells and plasmid incompatibility. At replication initiation, incompatible plasmids cannot be distinguished from each other and are therefore distributed to different daughter cells.

Some incompatibility groups, including IncA/C plasmids, are self-conjugative (Islam *et al.,* 2023). Plasmids belonging to this incompatibility group can be found in *Enterobacteriaceae*. Another group is the IncHI plasmids, which have a low copy number and are often associated with Gram-negative fish pathogens (Phan and Wain, 2008). Another group includes, the I plasmid Complex (I, K, B, Z) which involves plasmids sharing pili traits, and which are frequently found in *Enterobacteriaceae* (Foley *et al.,* 2021; Lian *et al.,* 2023). IncF plasmids are commonly found in *E. coli*, and are often self-transferable (Fernandez-Lopez *et al.,* 2016). IncP and IncL/M plasmids are thought to have wider host ranges (Shintani *et al.,* 2010; Yano *et al.,* 2013). They can replicate

in a wide range of bacterial species (Yano *et al.,* 2012). On the other hand, IncN plasmids have a narrower host range and are typically linked to *Enterobacteriaceae* like *E. coli*, *Salmonella,* and *Klebsiella* (Humphrey *et al.,* 2012; Wang *et al.,* 2023; Ma *et al.,* 2023).

1.9. The impact of travel, animal husbandry and lifestyle on the transmission of resistance

1.9.1. Travel

In addition to the use of antibiotics, travel has been implicated to be a major driver of AMR (Holmes *et al.,* 2016; Worby *et al.,* 2023). Studies have shown travelling to regions with high prevalence of resistance can increase the risk of AMR acquisition (Bokhary, 2021). In multiple studies, *E. coli* has been used as an indicator to determine travel-related acquisition of AMR (Kennedy and Collignon, 2010; Tängdén *et al.,* 2010). There are relatively few reports on *Klebsiella* acquisition related to travel (Reuland *et al.,* 2016), but travel to low-income countries is suspected of being a main source of gut colonization by *Klebsiella* (Dortet *et al.,* 2008; Leverstein-van Hall *et al.,* 2010; Hashimoto *et al.,* 2014). To date, there is growing evidence that carbapenem, quinolone and ESBL producing *Enterobacteriaceae* can be acquired during travel (Bokhary, 2021; Worby *et al.,* 2023). For instance, a recent study revealed there is increased risk of being colonised by ciprofloxacin-resistant and contracting ESBL producing *Enterobacteriaceae* when travelling to Southeast Asia and the Indian subcontinent (Reuland *et al.,* 2016a). In another study, multidrug *Enterobacteriaceae* were detected in individuals returning to the UK from Sri Lanka and India (Bevan *et al.,* 2018; Furuya-Kanamori *et al.,* 2020). Further, ESBL genes, like $bla_{\text{CTX-M}}$, have been found in the toilet waste of planes arriving in Copenhagen from low- and middle-income countries in South Asia, indicating widespread distribution of ESBL-producing *Enterobacteriaceae* among travellers (Vassallo *et al.,* 2022). Travelers returning from China, the Middle East, Southeast Asia, North Africa, Central America, and South America have also been found to carry higher levels of antibiotic-resistant bacteria (Sridhar *et al.,* 2021).

1.9.2. Animal husbandry

Animal husbandry has consistently been identified as a major contributor to the spread of resistance (Furuya-Kanamori *et al.,* 2020; Wu *et al.,* 2023). The growing demand for food of animal origin has resulted in an increase in livestock production and the widespread use of antibiotics (Mulchandani *et al.,* 2023). Since 2000 meat production in Africa has increased by 68% (Pinto Jimenez *et al.,* 2023). By 2030 low-income countries are expected to experience a 67% surge in antimicrobial use, due to intensive agriculture (Mulchandani *et al.,* 2023). Livestock farming plays a significant role in the socio-economic well-being of many African communities and supports the livelihoods of approximately 250 to 300 million people who rely on it as their primary or secondary source of income (Erdaw, 2023). Notably, 80% of farms in Africa use antimicrobials in livestock production (Ben Said *et al.,* 2015; Schar *et al.,* 2018). In animal production, antimicrobials are used for treatment and prevention of bacterial infections, and as growth promoters (Okey, 2023; Redwan Haque *et al.,* 2023). Although antibiotics are widely used in livestock production, they are largely unregulated in Africa (Davies and Davies, 2010; Eltayb *et al.,* 2012). For instance, practically all Tanzanian farmers use antimicrobials to raise animals (Azabo *et al.,* 2022). A study conducted in Zanzibar (Tanzania), showed that most poultry farmers (87%) rely on antimicrobials, like oxytetracycline to treat and prevent poultry diseases, while other farmers make their diagnoses without consulting veterinary authorities (Azabo *et al.,* 2022). Tetracycline resistance has been shown to be the most prevalent resistance class in Africa in animal production (Kimera *et al.,* 2020b). For instance, a recent assessment by (Van Boeckel *et al.* 2015) revealed that tetracycline resistance rates in isolates derived from chicken in Africa exceeded estimates from Asia and the Americas. Resistance to other antibiotic classes, such as ampicillin and sulfamethoxazole/trimethoprim, has also been demonstrated to be more common in animal production in Africa than in other continents (Mshana *et al.,* 2021). Despite the fact that the majority of farms use antimicrobials frequently, research has shown that the majority of African farmers lack a basic awareness of the consequences of using antibiotics (Manyi-Loh *et al.,* 2018; Ducrot *et al.,* 2021). Most farmers use over-the-counter antibiotics, and that access to veterinary care for most farmers is constrained by cost (Redding *et al.,* 2013; Gemeda *et al.,* 2020). The

burden of AMR is believed to be exacerbated by the quantity of substandard over-the-counter antibiotics that farmers can purchase (Acharya and Wilson, 2019; Adebowale *et al.,* 2020; Gulumbe *et al.,* 2022). Although it is evident that unregulated use of antimicrobials problems affect all livestock production systems in Africa, pigs and poultry have been identified as one of the main contributors of resistance dissemination (Chantziaras *et al.,* 2014; Van *et al.,* 2020). This is because these are two of the animal production systems that have experienced the fastest growth per capita in Africa (Mulchandani *et al.,* 2023). This trend is predicted to continue as countries transition from subsistence farming to intensive farming, which would result in an increased demand for antibiotics (Mulchandani *et al.,* 2023). Given these trends, it is crucial to monitor the transmission of resistance in these agricultural systems.

1.9.3. Gut microbiome and impact of diet

The gut microbiome is a complex ecosystem comprised of both commensal and pathogenic bacteria (Rinninella *et al.,* 2019; Dey and Ray Chaudhuri, 2023). It consists of both bacteria, fungi and viruses which collectively contribute to the diversity of this microbial ecosystem. Commensal bacteria are symbiotic and live in harmony with the mammalian host whilst a few are pathogenic (Rinninella *et al.,* 2019; Dey and Ray Chaudhuri, 2023). A healthy gut may consist of both pathogenic and commensal bacteria coexisting in the same environment (Leonard and Toro, 2023). The gut microbiome has a great potential to influence the physiology in both health and disease (de Steenhuijsen Piters *et al.,* 2015; Elzayat *et al.,* 2023). They contributes towards the development of immunity, prevent diseases and metabolic function (Wang *et al.,* 2023). Moreover, gut microbiome can extract nutrients from substances that our bodies are unable to digest (Fang *et al.,* 2023). In healthy individuals gut microbiome are generally stable, but can be sensitive to disruptions (Shade *et al.,* 2012; Ostrem Loss *et al.,* 2023). Factors such as underlying diseases and the use of antimicrobial drugs can significantly change the composition of gut microbiome (Gomaa, 2020). Research suggests that the gut microbiome has the potential to return to its original state in terms of diversity once the cause of disturbance has disappeared or diminished (Gomaa, 2020). However, this process can sometimes take a considerable amount of time. In certain cases,

these disruptions may be permanent and cannot be reversed (Fishbein *et al.,* 2023). Other factors that influence the diversity of the microbial ecosystem in the human gut is lifestyle (Conlon and Bird, 2014). Lifestyle includes variables such as environmental exposure to different microbes, physical activities, diet, and climate, and these are thought to have direct or indirect influence on the composition and function of microbiome (Li, 2015; Tasnim *et al.,* 2017; Jain *et al.,* 2020; de Franchis *et al.,* 2022). Food choices can induce perturbation in the gut, giving some species in the gut a competitive edge over others (Heiman and Greenway, 2016; Fassarella *et al.,* 2021). For instance, fibre-rich diets are thought to be associated with growth of a range of microorganisms including *Prevotella* and certain species of *Firmicutes* which are associated with improved metabolic and immune health (De Filippo *et al.,* 2017; Cronin *et al.,* 2021). These bacteria are known to convert complex plant fibres into short chain fatty acids, providing energy and promoting gut health (Simpson and Campbell, 2015; Cronin *et al.,* 2021). On the contrary, a diet rich in processed foods, which is high in sugar, fat and preservatives, can promote growth of certain pathogenic bacteria, inflammation in the gut or lead to the increment of certain species, including *E. coli* (Statovci *et al.,* 2017; Raoul *et al.,* 2022). Diets rich in sugar and processed foods are quite common in industrialised communities compared to those living in pristine environments, whose dietary habits are believed to be similar to the ancestral populations (Schnorr *et al.,* 2014; Dehingia *et al.,* 2015). Numerous studies have postulated that individuals living in industrialised communities are predisposed to suffer from chronic conditions such as colorectal cancer and autoimmune diseases such Crohn's Disease (Zuo *et al.,* 2018; Quaglio *et al.,* 2022). This has been hypothesised to be linked to the decreased diversity of beneficial bacteria that are thought to have protective functions in the gut (Makki *et al.,* 2018; Quaglio *et al.,* 2022). Industrialised communities tend to have high exposure levels to antimicrobials compared to communities living a hunter-gatherer lifestyle (Schnorr *et al.,* 2014a). Frequent antimicrobial exposure can lead reduced microbial diversity whose impact could be long-lasting (Prestinaci *et al.,* 2015; Serwecińska, 2020). In some cases, the alteration can persist for years (De La Cochetière *et al.,* 2005; Jernberg *et al.,* 2007; Dethlefsen *et al.,* 2008; Dethlefsen and Relman, 2011). In previous research, broad spectrum antibiotics were shown to impact at least 30% of the bacteria in the gut, reducing the taxonomic richness, diversity, and evenness of the gut (Dethlefsen *et al.,* 2008; Dethlefsen and Relman, 2011). Antibiotics can also alter gene expression of certain species of bacteria, protein activity, and general metabolisms (Ferrer *et al.,* 2017). These changes are thought

to happen more promptly than the replacement of intestinal taxa (Pérez-Cobas *et al.,* 2013). Microbiota exposed to β-lactam antibiotics have been shown to have enhanced enzyme activities linked to the breakdown of carbohydrates, leading to imbalanced sugar metabolisms similar to those observed in obese individuals (Hernández *et al.,* 2013).

1.9.4. One Health concept

The One Health concept describes an integrative approach involving multiple disciplines and sectors aimed at to improving health outcomes (Mackenzie and Jeggo, 2019). The concept originated from a meeting coordinated by the Wildlife Conservation Society, in New York in 2004 (Wildlife Conservation Society, 2004; Mackenzie and Jeggo, 2019). The fundamental goal of the meeting was to develop guidelines for overseeing and preventing epidemics and diseases that affect humans and animals in order to maintain the wellbeing and the integrity of the ecosystem (Mackenzie *et al.,* 2014). The Food and Agriculture Organisation (FAO), WHO and the Office International des Epizooties (OIE) have since embraced this idea to address health concerns, including food safety, zoonotic infections and AMR (Zinsstag *et al.,* 2023; Elnaiem *et al.,* 2023). Pathogen spill-over and antimicrobial exposure between humans, animals and the environment is expected to rise due to rapid population growth and the spread of human activities and settlements in wildlife habitats, and environments previously thought to be pristine (Arnold *et al.,* 2016; Dolejska, 2020; Vicente *et al.,* 2021). Humans are increasingly in close proximity to wild animals, livestock, and pets, resulting in the transmission of pathogens through direct and indirectly pathways (Figure 1.9) (Ellwanger and Chies, 2021; Vicente *et al.,* 2021; Tomori and Oluwayelu, 2023). Pathogen transmission can happen through direct interactions between humans and animals whilst an indirect pathway may involve transmission through the food chain or the environment (e.g., water, air, manure, sludge-fertilised soils) (Marshall and Levy, 2011; Silva *et al.,* 2014; Founou *et al.,* 2016; Jinadasa, 2023). In the context of environmental conditions, climate change has been reported to enhance the emergence and spread of different pathogens (Piret and Boivin, 2021; Usman Qamar, 2023; Darbandi *et al.,* 2023). Moreover, movement of humans and animals

to different geographical areas has been shown to facilitate disease spread (Gass Jr *et al.,* 2023; Ekwem *et al.,* 2023; Tardy *et al.,* 2023).

One of the major problems that is increasingly viewed and assessed through the One Health lens is AMR. It is still unknown how each of the three domains—human, animal, and environmental—contributes to the increasing burden and dissemination of resistance (Aslam *et al.,* 2021; Allel *et al.,* 2023). Some of the factors that have been reported to contribute towards the spread of resistance and proliferation of resistant bacteria, include irresponsible and excessive antibiotic use in agriculture, animal husbandry, and human medicine, as well as unregulated, over-the-counter availability of antimicrobials (Martin *et al.,* 2015; Ayukekbong *et al.,* 2017; Serwecińska, 2020). Furthermore, besides international travel and trade, reservoirs of AMR have been associated with bird migration, refugee movement, inadequate sanitation, hygiene, agricultural waste, and antibiotic residues in the environment (Lakhundi and Zhang, 2018; Serwecińska, 2020; Apreja *et al.,* 2022; Ibekwe *et al.,* 2023; Yusuff *et al.,* 2023). A good example highlighting the importance of One Health in addressing AMR is the spread of the *bla*$_{\text{NDM-1}}$ gene (Yong *et al.,* 2009; White and Hughes, 2019). This gene is thought have originally been discovered in India and spread to other countries, including the UK (Yong *et al.,* 2009). The gene is believed to have spread through medical tourism after its first detection in 2008 (Yong *et al.,* 2009). In subsequent years, *bla*$_{\text{NDM-1}}$ genes were detected in Bangladeshi surface, tap water and the environment, suggesting that this gene and its variants may have spread to this region and disseminated in the environment (Walsh *et al.,* 2011; Islam *et al.,* 2017; Khan *et al.,* 2017). Recently, researchers discovered the *bla*$_{\text{NDM-1}}$ gene in the highly nutrient-rich Arctic soil of the Norwegian archipelago, which is thought to have been transferred by migrating birds (McCann *et al.,* 2019). These genes have thus now spread globally. Another example of a widely disseminated antibiotic resistance gene is *mcr-1*(mobilized colistin resistance-1), which was originally discovered in Chinese pigs (Liu *et al.,* 2016). It has now spread to other countries (Marston *et al.,* 2016; Wang *et al.,* 2018). The *mcr-1* gene was recently detected in *E. coli* derived from migratory birds and human isolates originating from Pakistan (Lv *et al.,* 2018).

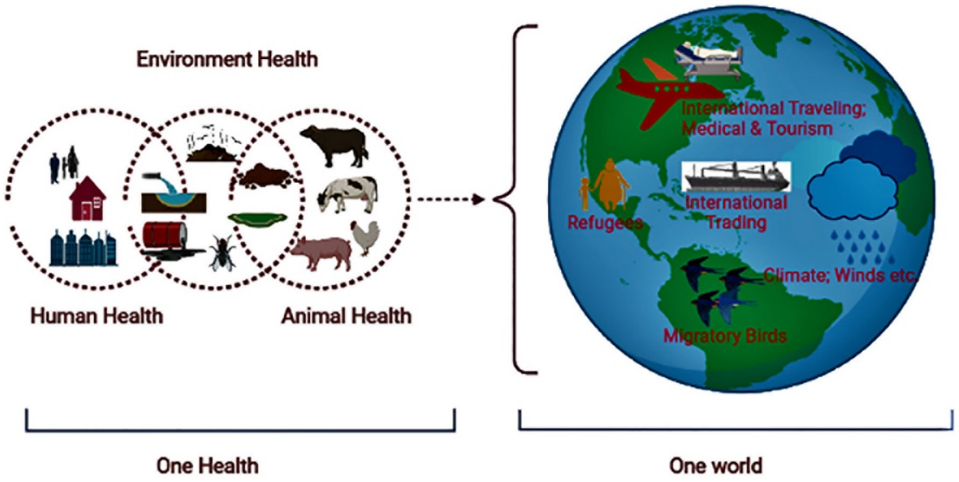

Figure 1.9: The One Health concept of AMR. Schematic showing how humans, animal and environment interact closely, as well as factors that facilitate AMR and disease spread in the one health continuum. Image adapted from (Aslam *et al.,* 2021)

1.10. Microbial genomics

Microbial genomics is a branch of genomics that focuses on studying the genetic makeup of microorganisms including viruses, bacteria, archaea and fungi (Foley *et al.,* 2021). The purpose is to understand their characteristics, including their diversity, evolution, functional capabilities and genetic structure (Zhang *et al.,* 2021). The advancement of microbial genomics began more than 50 years ago, when researchers made advancements in the field of DNA sequencing techniques (Heather and Chain, 2016). The pioneering efforts led by Frederick Sanger and his colleagues during the 1970s laid the groundwork for whole-genome sequencing (Sanger *et al.,* 1977; Crossley *et al.,* 2020). During that era their inventive work resulted in what is commonly referred to as Sanger sequencing (Sanger *et al.,* 1977; Crossley *et al.,* 2020). The approach allowed screening of DNA sequences through a chain termination process, allowing for higher accuracy and precision (Smith *et al.,* 2023). Over time, Sanger sequencing gained popularity and in 1987 Applied Biosystems introduced automation to this process, which marked a major milestone (Halloran *et al.,* 1993; Shendure *et al.,* 2017). For a long time, Sanger sequencing was the gold standard in DNA sequencing (Pareek *et al.,* 2011). However, next Generation Sequencing (NGS) revolutionized the field (Pareek *et al.,* 2011; Wang, 2023). NGS brought about a breakthrough as

it generates large amount of sequence data at a greatly reduced cost per base per single run, compared to Sanger sequencing (Ansorge, 2009; Satam *et al.,* 2023). With NGS, low-cost whole-genome sequencing of bacterial genomes became possible. To fully harness the power of these ground-breaking techniques, the use of state-of-the-art of bioinformatics tools became necessary for the processing and analysis of sequencing data (Dolled-Filhart *et al.,* 2013; Klasberg *et al.,* 2019; Pereira *et al.,* 2020). Illumina sequencing has played a significant role in the NGS revolution (Hu *et al.,* 2021). Illumina sequencing employs an approach known as sequencing by synthesis (Kchouk *et al.,* 2017); (Hu *et al.,* 2021). The process starts with generating DNA fragments, which are then combined with patterned glass plates to form clusters (Su *et al.,* 2011; Slatko *et al.,* 2018). By measuring fluorescence levels of terminator nucleotides incorporated into the developing DNA strand, the sequences of the target DNA are then determined (Figure 1.10; Slatko *et al.,* 2018). Illumina sequencing platforms include iSeq, MiniSeq, MiSeq, NextSeq, HiSeq, and NovaSeq, and they cover a wide range of capacities and applications (Kchouk *et al.,* 2017; Slatko *et al.,* 2018). Notably, Illumina sequencing platforms produce shorter reads ranging between 50 to 300 bp in length compared to Sanger sequencing which produces 500 bp to 800 bp reads (Holm *et al.,* 2019; Crossley *et al.,* 2020).

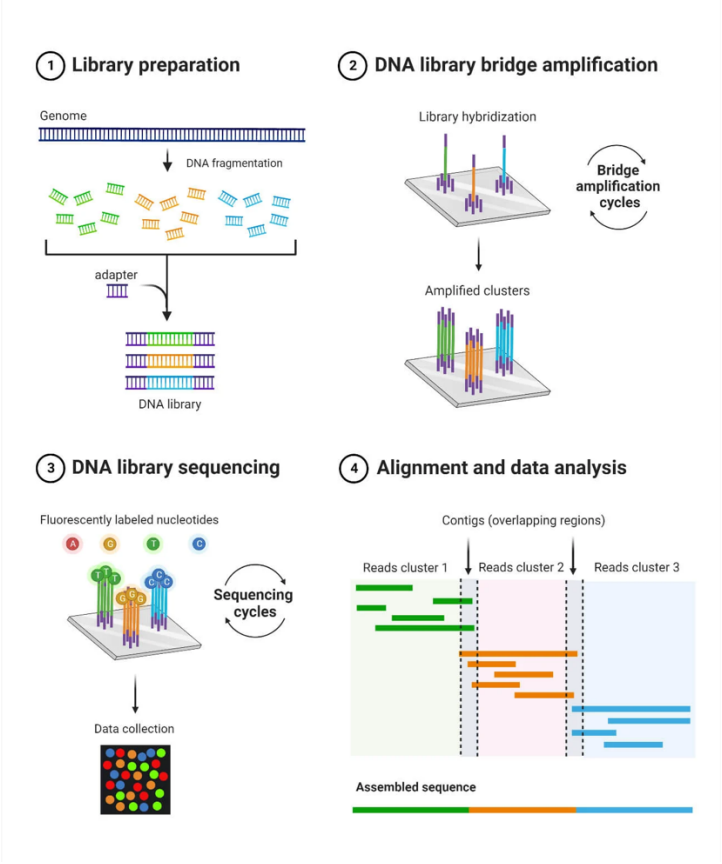

Figure 1.10: Basic principle of Illumina sequencing. The image has been adapted from (Aryal, 2022).

New sequencing technologies, sometimes termed third-generation sequencing, were developed to address the limitations of short-read sequencing. of the most prominent platforms are those developed by Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT) which offer longer read lengths, in comparison to second-generation methods like Illumina (Goodwin *et al.,* 2016; Satam *et al.,* 2023). Longer reads span genomic repeats, thus improving the assemblies of microbial genomes (Rhoads and Au, 2015; Cuber *et al.,* 2023). PacBio uses a Single Molecule Real Time (SMRT) Sequencing approach, in which circular DNA templates are amplified and sequenced in real time (Amarasinghe *et al.,* 2020; Satam *et al.,* 2023). This method produces sequencing reads often spanning thousands of base pairs to a hundreds of kilobases (Rhoads and Au, 2015). ONT uses fluctuations in a current when a strand of DNA passes through a pore to

sequence DNA (Figure 1.11). ONT has revolutionised sequencing by shrinking the technology into devices the size of smartphones (Cuber *et al.,* 2023; Wick *et al.,* 2023).

Researchers often combine long-read sequencing data with short-read Illumina data to enhance assembly precision and decode complex genomic architecture (Wick *et al.,* 2023; Warburton and Sebra, 2023; Di Marco *et al.,* 2023). For instance, while long reads are effective in filling gaps in the genome, it has a higher per base error rate (Gladman *et al.,* 2023; Schmeing and Robinson, 2023). Therefore, short read data is used to make up for this, since Illumina data has a very low error rate compare to long read data (Gladman *et al.,* 2023).

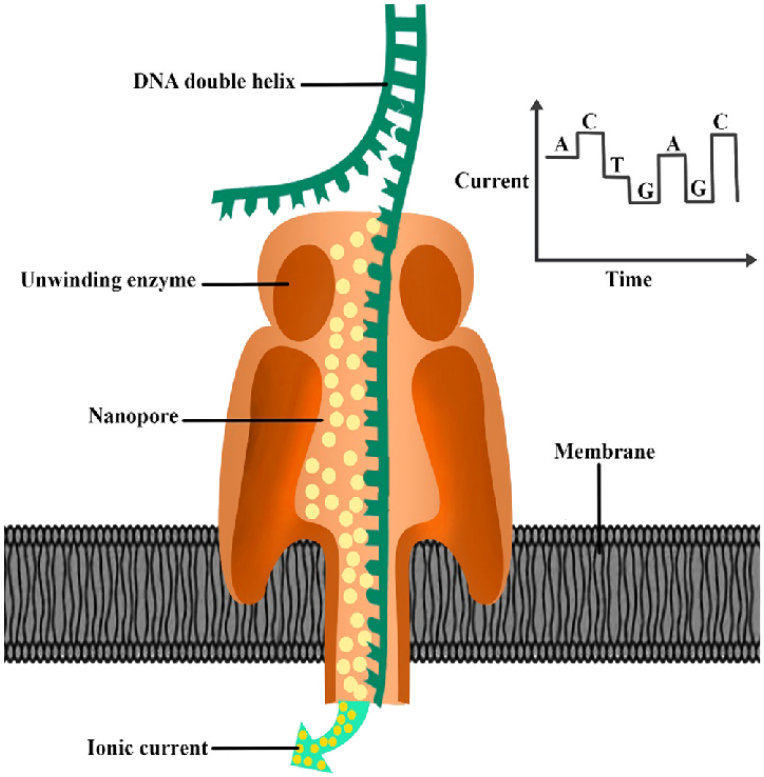

Figure 1.11: Fundamental concept of Oxford Nanopore sequencing. The MinION Oxford Nanopore Sequencer operates by unfolding DNA, then passing single-stranded DNA through a solid-state pore. Nucleic acid sequences can be determined by the distinct interruption in electrical current resulting from the unique geometry of each DNA base. Image adapted from (Shivashakarappa *et al.,* 2022)

1.11. Research aims and objectives.

The aim of my PhD research is to explore factors that contribute to acquisition of AMR such as travel, animal husbandry and lifestyle, and conduct comparative analyses amongst distinct populations to discern patterns of microbial diversity. By analysing genomic diversity of *Klebsiella* species in soldiers deployed to Kenya, this study unveils how geographical factors can influence the diversity of bacteria that are carried by healthy humans upon international travel (Chapter 2). In Chapter 3, I examine the genomic diversity of *E. coli* in two distinct husbandry systems in Tanzania. Here, the aim is to gain a better understanding of how each of these animal husbandry systems contribute towards resistance dissemination, as well as the genetic elements involved. In Chapter 4, metagenomic data of the Hadza community is used and the broader Tanzanian population, to reconstruct *E. coli* genomes and assess their relatedness. Through this comparative analysis, the objective to gain a better understanding of the diversity of *E. coli* among these two communities.

Chapter 2

Genomic Diversity of *Klebsiella* Species Colonising the Gut of British Soldiers in Kenya

2.1. BACKGROUND

In recent years, global travel has increased across various regions of the world raising concerns about the spread of antimicrobial resistance (Bokhary *et al.,* 2021). *Klebsiella* species have become one of the primary contributors to the dissemination of antimicrobial resistance genes among *Enterobacteriaceae* (Holt *et al.,* 2015; Wyres and Holt 2016). Through movement of individuals, resistance genes and microorganisms can spread across borders. Factors like travel, medical tourism and migration have all been reported contribute to the dissemination of these strains (Schaumburg *et al.,* 2019; Frost *et al.,* 2019; Riddle 2020; Tufic-Garutti *et al.,* 2021).

Klebsiella pneumoniae is notorious for its ability to acquire and disseminate resistance genes (Holt *et al.,* 2015; Wyres and Holt 2016; Padilla *et al.,* 2020). Due to its association with healthcare-related infections and the development of resistance mechanisms associated with production of extended spectrum beta lactamases (ESBLs) and carbapenemases, it is deemed to be of public health concern (Ullah, Malik, and Ahmed 2009; Mrowiec *et al.,* 2019; Jiang *et al.,* 2020). The acquisition of antibiotic-resistant *Klebsiella* strains during travel can occur through different means, including direct contact with colonised individuals, exposure to hospitals or contact with contaminated environments (Chong *et al.,* 2018; Schwartz and Morris, 2018). Additionally inadequate hand hygiene practices, access to healthcare services and inadequate sanitation facilities can also contribute to the spread of *Klebsiella* species during travel (Svenningsen *et al.,* 1984; Tsioutis *et al.,* 2021). Furthermore, certain geographical regions may experience a high prevalence of resistant strains leading to an increased risk of asymptomatic carriage and transmission (Tsioutis *et al.,* 2021). Food has also been attributed to the acquisition of resistant *Enterobacteriaceae* (Ruppé *et al.,* 2018; Davies *et al.,* 2022). Raw or undercooked meat and produce may harbour antibiotic-resistant strains, including *Klebsiella* that originate from livestock or agricultural settings (Guo *et al.,*. 2016; Hartantyo *et al.,* 2020). The consumption of such contaminated food can result in colonization and subsequent transmission (Guo *et al.,* 2016; Hartantyo *et al.,* 2020).

In recent years, different strategies and actions have been put in place to reduce the spread of antimicrobial resistant *Enterobacteriaceae* and their resulting implications. One common approach

to treating gastrointestinal infections contracted during travel is by using rifaximin (DuPont *et al.,* 2005). This drug specifically targets pathogens such as *Enterobacteriaceae* while having minimal impact on other beneficial gut bacteria (Layer and Andresen, 2010). Rifaximin inhibits the spread of resistant pathogens and limits their transmission by lowering their abundance in the gut (DuPont *et al.,* 2005); (Robins and Wellington 2005). When used appropriately in travel medicine, rifaximin can effectively mitigate *Enterobacteriaceae* infections, potentially including those caused by *Klebsiella* strains. This could greatly reduce the impact of infections associated with *Enterobacteriaceae* and reduce the risk of their spread (Koo and DuPont, 2010).

Rifaximin primarily targets RNA polymerase, the enzyme responsible for synthesizing bacterial RNA (Ojetti *et al.,* 2009; Koo and DuPont, 2010). It inhibits RNA synthesis by binding to the beta subunit of RNA polymerase (Ojetti *et al.,* 2009; Riddle, 2020). Due, to the disruption of the RNA polymerase activity, which is necessary for transcribing DNA into messenger RNA, leading to an inability to produce proteins for their growth and survival (Ojetti., 2009; Kane, and Ford, 2016). Mutations in *rpoB*, which encodes the beta subunit of bacterial RNA polymerase are a significant cause of rifaximin resistance (Koo *et al.,* 2009; Koo and DuPont, 2010). Additionally, rifaximin is known to have limited absorption, meaning it primarily affects the gastrointestinal tract and thus has localized effects within the gut (Layer and Andresen, 2010).

The primary objective of this study is to explore the variations, within *Klebsiella* species among soldiers deployed in Kenya. Samples collected from a placebo-controlled trial were used, where participants were given either a twice daily or once daily prophylactic dosage of rifaximin at 550mg concentration. Our main aim is to compare the distribution of *Klebsiella* species before and after the administration of prophylactic rifaximin treatment. Furthermore, the impact of rifaximin on the presence or absence of *Klebsiella* species will be assessed during the course of the study. To gain insights into the diversity of *Klebsiella* advanced whole genome sequencing techniques will be employed. By employing this approach, both the prevalence and genetic relatedness among treatment groups for *Klebsiella* species will be investigated. Additionally, our goal is to examine whether these isolates carry any resistance genes and determine which genetic elements play a significant role in this phenomenon. Through this analysis our objective is to understand underlaying mechanisms associated with resistance, as well as genetic elements driving spread of and persistence of resistance in *Klebsiella* species.

2.2. METHODS

2.2.1. Design of the study

Samples assessed in the current study were obtained from a randomised double-blind clinical study conducted among soldiers deployed from the UK to Nanyuki, Kenya. Ethical approval was granted by Ministry of Defence Research Ethics Committee (MODREC) 770/MODREC/16 UK. A total of 121 participants were included and divided into three subgroups which received different dosages of rifaximin to prevent traveller's diarrhoea. Rifaximin was administered to two groups with varying dosages. One group received a daily dose of 550 mg while another received 550 mg twice per day. The placebo group did not receive any treatment. Since each individual had a sample taken before and after rifaximin prophylaxis, a total of 242 samples were collected from the 121 individuals. Stool samples were taken before prophylaxis and after 6 weeks, and were stored at -80°C.

2.2.2. Isolation of presumptive *Klebsiella* isolates

Enrichment for *Klebsiella* was performed by adding 10µl of faecal material to 10 ml of Lysogeny broth (LB) with 10 mg/L ampicillin and incubating at 37°C ± 1°C for 24h ± 1h. Simmons Citrate Agar (Oxoid) supplemented with 1% inositol (SCAi) was used for the differential selection of *Klebsiella* colonies based on colour morphology, specifically targeting colonies exhibiting a yellow colour. SCAi was prepared as described previously (Van Kregten *et al.,* 1984). Using a 10-µl loop a portion of each enrichment culture was streaked on SCAi, which was incubated for 48 h ± 1h at 37°C ± 1°C. Individual isolates (a single isolate per plate) with a distinctive yellow appearance were selected from SCAi plates and re-streaked to purity on SCAi (Oxoid, Canada) followed by incubation for 48h ± 1h at 37°C ± 1°C.

2.2.3. Identification and storage of isolates

A single colony from each of the SCAi plates with purified colonies was streaked onto the surface of a non-selective agar medium (L Broth Agar; Sigma Aldrich) and incubated at 37°C for 24h ± 1h. Presumptive *Klebsiella* isolates were then stored in Brain Heart Infusion broth (Difco) + 15% (v/v) glycerol and stored at -80°C. *E. coli* (*Escherichia coli* strain K-12 substrain MG1655) and *K. pneumoniae* KP209 were used as negative and positive controls for SCAi medium and were obtained from the Van Schaik laboratory collection, and Janssen *et al.,* 2020, respectively.

2.2.4. DNA extraction

Prior to genomic DNA isolation, bacteria were cultured for 16 hours at 37°C with agitation (200 RPM) in LB broth. Cells were then pelleted by centrifugation at 16,000 x g for 2 minutes, and the supernatant was discarded. The Wizard® Genomic DNA Purification Kit (Promega) was then used to extract chromosomal DNA. Cells were resuspended in 480µl of 50 mM EDTA (Invitrogen), followed by 120µl of 10mg/ml lysozyme (Sigma-Aldrich). After incubation at 37°C for 60 minutes, the cells were pelleted again, and the supernatant was removed. Next, 600µl of nuclei lysis solution (which was used to break down bacteria cells to release intracellular components, including DNA) was added and mixed by pipetting up and down. Incubation of the cell suspension was done at 80°C for 5 minutes, then cooled to room temperature. Subsequently, 3µl of RNase solution was added and incubated at 37°C for 60 minutes before cooling again. 200µl of protein precipitation solution was added, and the cells were vortexed for 20 seconds for precipitation of the proteins in the sample. After incubating on ice for 5 minutes, the cells were centrifuged at 16,000 x g for 3 minutes to pellet the protein. To precipitate the DNA, the supernatant was mixed with 600µl of room-temperature isopropanol by inverting 10 times. The DNA was then pelleted by centrifugation, the supernatant was discarded, and 600µl of 70% ethanol was added to the pellet. After another round of centrifugation, the ethanol was removed, and the pellet was air-dried for 10

minutes. After resuspension of the pellet in EB buffer (Qiagen) and rehydration at 65°C for 1 hour, the DNA pellet was stored at -80°C until further use.

2.2.5. Determining DNA concentrations

For the measurement of DNA concentrations, two kits were employed: the QubitTM dsDNA HS Assay Kit (Invitrogen) for concentrations ranging from 0.2 to 100 ng, and the QubitTM dsDNA BR Assay Kit (Invitrogen) for concentrations between 2 and 1000 ng. By diluting Qubit® dsDNA BR/HS Reagent 1 in Qubit® dsDNA BR/HS Buffer at a 1:200 ratio, a working solution was prepared. For Standards 1 and 2, 190ml of the working solution was combined with 10ml of each of Qubit® dsDNA BR/HS Standard #1 and Qubit® dsDNA BR/HS Standard #2. DNA was measured by mixing 2µl of the DNA sample with 198µl of working solution in a separate QubitTM Assay Tube. At room temperature, the mixed solutions were vortexed briefly and incubated for 2 minutes. Using an Invitrogen Qubit 4 fluorometer, the assays were then read.

2.2.6. Illumina sequencing, analysis of sequence quality and phylogenetic analysis

Genome sequencing was performed by MicrobesNG (Birmingham, United Kingdom) on DNA samples of individual isolates, using the Nextera XT Library Prep kit and 2 x 250-bp paired-end sequencing runs on the Illumina HiSeq 2500 platform. FastQC v0.11.9 (Shi, H. and Xu, 2016) was used to evaluate paired-end raw reads, and a summary of read quality was calculated with MultiQC v1.8 (Ewels *et al.,* 2016). MultiQC and FastQC were both run with their default settings. Base sequence quality score (phred score) of 30 or above warranted inclusion in the analysis. Paired-end raw reads were trimmed to remove low-quality bases using Trimmomatic v0.39 (Bolger *et al.,* 2014) and *de novo* assembled with SPAdes v3.11.1 (Prjibelski *et al.,* 2020). The quality of the assemblies was evaluated using QUAST v5.0.0, (Gurevich *et al.,* 2013). The contiguity and quality

of each assembly were evaluated using the mean genome length, and the contig N50. To identify single nucleotide polymorphisms (SNPs) and generate a core-genome alignment, Snippy v4.3.2 was employed, a command line tool (https://github.com/tseemann/snippy), which aligns the reference genome with the input reads. The core genome alignment was further created using snippy-core option (Seemann, 2015), and subsequent cleaning of the core full alignment was conducted using snippy-clean_full_aln function (https://github.com/tseemann/snippy) to remove non-standard characters and regions with excessive missing data. Considering the potential impact of recombination on phylogenetic signals in *Klebsiella* genomes, recombination regions were identified and masked using Gubbins v2.3.4 (Croucher *et al.,* 2015). *K. pneumoniae* subsp *pneumoniae*, strain HS11286 add GenBank accession code, was used as a reference. A maximum-likelihood phylogeny was reconstructed from the core-genome alignment using RAxML v8.2.4 (Stamatakis, 2006), incorporating 1,000 bootstrap replicates and a general time-reversible nucleotide substitution model. The resulting tree was visualized and annotated in iTOL v5.0.0 (Letunic and Bork, 2021).

2.2.7. Characterisation of the *Klebsiella* genome

A mash tree was generated using all the genome sequences obtained in this investigation, with (*K. pneumoniae* subsp. *pneumoniae*, strain HS11286, accession GCA_000240185.2 (Table 2.1) as reference sequence for *K. pneumoniae* strains (Liu *et al.,* 2012). Other reference strains included in the construction of the Mash tree are listed in Table 2.1. Min-hash was used to generate a neighbour joining tree (Saitou and Nei 1987), as implemented in the Mash package (Ondov *et al.,* 2016) by Mashtree. Kleborate v2.1.0 (Holt, 2020) was used to analyse and characterize genome sequences for the determination of antimicrobial resistance gene profiles, species types, virulence genes, and Multi-Locus Sequence Types (STs).

Table 2.1: Reference genome sequences used in the Mash tree.

Isolate name	Accession Number	References
K. pneumoniae subsp. *pneumoniae*	GCA_000240185.2	(Liu, P *et al.,* 2012)
K. ornithinolytica	GCA_002806725.1	(Medina-Cordoba *et al.,* 2018)
K. oxytoca	GCF_001809025.1	**
K. michiganensis	GCA 000240325.1	(Shin *et al.,* 2012)
K. quasipneumoniae	GCA 000523395.1	(Hazen *et al.,* 2014)
K. variicola	GCA_009648975.1	CFSAN*, 2019

* CFSAN: Centre for Food Safety and Applied Nutrition
** no publication was associated with this strain, but it was submitted by Genome Institute at Washington University

2.2.8. Phylogenetic analyses

To generate a core genome alignment for *K. pneumoniae* and *K. variicola,* Snippy v4.0.2 (https://github.com/tseemann/snippy) was used to map short reads of the strains to their respective reference strains. The *K. pneumoniae*, strain HS11286 (accession GCA_000240185.2) as described by (Liu *et al.,* 2012)), previously used in the mash tree analysis, served as a reference strain for constructing a *K. pneumoniae*-specific tree. To address the uniqueness of *Klebsiella variicola* strains in the present study, a long-read hybrid assembly of isolate 139V2 was used. The methodology on nanopore sequencing is described in section 2.29 below. Genome alignments were generated for *K. pneumoniae* and *K. variicola* independently. The alignments were cleaned using Snippy-clean_full_aln, and DNA sequences subject to recombination were removed using Gubbins v2.3.4 (Croucher *et al.,* 2015). Variable positions in all strains were identified using SNP-Sites v2.4.0 (Page *et al.,* 2016). Maximum-likelihood trees were constructed for *K. pneumoniae* and *K. variicola* using FastTree (Price *et al.,* 2009) under RAxML-NG v0.9.0. Phylogenetic trees were annotated and visualized using iTOL (Letunic and Bork, 2021).

2.2.9. Nanopore sequencing

To evaluate genetic elements that were involved in the transmission of sulphonamide and trimethoprim resistance, 4 isolates were further sequenced using Oxford Nanopore Technologies (ONT) Minion flow cell (R9.4). DNA was extracted from overnight cultures using Wizard® Genomic DNA Purification Kit (Promega) as described below. The Ligation Sequencing Kit (SQK-LSK109) from Oxford Nanopore was used for library preparation.

To generate the libraries, DNA was first end-repaired by diluting 1 µl of DNA in 48 µl of nuclease-free water, followed by flicking and mixing the tube. This was followed by mixing 48µl of DNA with 3.5µl NEBNext FFPE DNA Repair Buffer, 3.5µl Ultra II End-prep Reaction Buffer, 3µl Ultra II End-prep Enzyme Mix and 2µl NEBNext FFPE DNA Repair Mix. The reaction was mixed thoroughly and incubated using a thermocycler at 20^0C for 5 mins, then 65^0C for 5 mins. It was then transferred to a clean Eppendorf DNA LoBind tube where 60µl of AMPure XP beads (Beckman Coulter) were added and mixed for purification. This was followed by incubating the mixture on a Hula mixer (Invitrogen) at room temperature for 5 mins. The beads were then pelleted using a magnet, supernatant removed and washed with 200µl of 70% ethanol. Washing was conducted twice with 70% ethanol, removing it after each wash. Afterwards pellets were dried for approximately 30 secs. The tube containing the mixture was then removed from the magnetic rack, 25µl of nuclease free water was added to resuspend the pellet and was then incubated for 2 mins at room temperature. Beads were removed using a magnet and 25µl of clear eluate was transferred to a new tube.

Barcodes were ligated to the end-repaired DNA, by adding 500ng end-repaired DNA to 22.5µl with nuclease free water. Each DNA sample was then mixed with 2.5µl of Native Barcode and 25µl Blunt/TA Ligase Master Mix. Mixing was conducted by flicking the tube and incubating at room temperature for 10 mins. Purification was conducted as done in the above section however with slight adjustment involving addition of 50µl of AMPure XP beads to each reaction and elution of the DNA library in 26µl in nuclease free water in the end.

Adapters were ligated by taking 65µl of barcoded DNA (700ng) and mixing it sequentially with 5µl AMII, 20µl Quick Ligation Reaction Buffer and 10µl T4 DNA Ligase. This was followed by mixing thoroughly by spinning up and down and 10mins incubation at room temperature. The DNA was cleaned and purified using AMPure beads as described above but with slight modification involving the use of 250µl of Long Fragment Buffer (Oxford Nanopore). Following that, the DNA was eluted with 15µl of elution buffer.

To prepare the DNA library for loading, 35µl of sequencing buffer and 25.5µl of loading beads were added in a tube and mixed. This was followed by addition of 12µl of DNA library to the mixture. Mixing was done by pipetting up and down the contents in the tube. After that, 75µl of the prepared sequencing library was loaded onto the pre-primed R.9.4.1 flowcell (Oxford Nanopore), followed by 72hrs of sequencing.

2.2.10. Hybrid assembly and plasmid analysis

Nanopore fastq sequences were, trimmed and quality checked using trimmomatic (version 0.32) (Bolger *et al.,* 2014) and filtlong v.0.2.0 (Steinig and Coin, 2022), respectively. The quality of short reads, which were used to generate a hybrid assembly were quality checked using fastp v0.23.1 (Pearson, 1990):(Shifu Chen *et al.,* 2018), and trimmed and assembled using trimmomatic and SPAdes, respectively. Hybrid assembly using Nanopore and Illumina reads was conducted using Unicycler v0.4.7 (Wick *et al.,* 2017). The output was then visualised using bandage (Wick *et al.,* 2015). Plasmids sequences were identified using plasmid finder PlasmidFinder v2.0.1, annotated using bakta v1.5.1 (Schwengers *et al.,* 2021) and visualised using Ugene (Okonechnikov *et al.,* 2012). To identify plasmid replicons present, plasmid sequences were blasted against PlasmidFinder database (Jolley *et al.,* 2018). Through Ugene visualisation, resistance genes and adjacent features, such as class 1 integron (*intl1*), associated with *dfr* genes and *sul* genes, were identified. GenBank annotation files (gbk) were used as input files for alignment of plasmid sequences with MUMmer 3.0 (Kurtz *et al.,* 2004) using the pgv-mummer script version 0.3.2 (Kurtz *et al.,* 2004). PyGenomeViz 0.3 (Sydow *et al.,* 2022) was used for visualization.

2.2.11. Determining the presence of insertion sequences

To identify insertion sequences (ISs) associated with trimethoprim and sulphonamide resistance genes in *Klebsiella* isolates, the ISFinder (Siguier *et al.,* 2006) BLASTn function was used with default parameters. The ISFinder database was queried using a hybrid-assembled whole-genome sequence of *K. variicola,* resulting in the detection of ISs. Queries were performed using signature sequences that spanned the junctions between IS and adjacent DNA. These signature sequences were then used to search the draft genomes of *K. variicola*. To facilitate this process, a local BLASTn database was created, incorporating all contigs from the draft genomes of the isolates included in this study. The database was then mapped to a 100bp signature sequence, which included an additional 50bp upstream and 50bp downstream of the IS element.

2.2.12. Jaccard distance analysis

Jaccard distance analysis was conducted to assess dissimilarity between individual strains. A matrix was therefore constructed where each gene was represented in a row and sample in each column. Following this, the Jaccard similarity coefficient was computed between pairs of strains by comparing the gene presence absence patterns. The Jaccard coefficient was derived by dividing the number of shared genes (intersection) by the total number of genes present in each sample (union) as shown in the formular below. The resulting coefficient ranged between 0 to 1, with zero representing absence of shared genes whilst 1 indicated an overlap in the gene content. The Jaccard distance was computed by subtracting the Jaccard similarity coefficient from 1 to quantify the dissimilarity between samples. Jaccard distance was employed to provide quantitative measure of how gene content varies between samples. A Jaccard distance of 0 indicates maximum similarity, while a distance of 1 represents dissimilarity.

Formula

Jaccard Similarity Coefficient:

$J(A, B) = |A \cap B| / |A \cup B|$

Jaccard Distance:

$D(A, B) = 1 - J(A, B)$

Where:

A and B are two samples (sets) being compared,

$|A \cap B|$ represents the number of genes present in both samples (intersection),

$|A \cup B|$ represents the total number of unique genes across both samples (union),

J (A, B) represents the Jaccard similarity coefficient, and

D (A, B) represents the Jaccard distance.

2.2.13. Statistical analyses

Statistical analyses were performed with R (version 3.6.1). To determine the effect of rifaximin on the occurrence of *Klebsiella* species Kruskal-Wallis test was employed to compare occurrence across different treatment groups against the placebo. Dunn's correction was employed to estimate adjusted p-values to provide corrections in the pairwise comparison of each treatment group against placebo. The Chi-square test was employed to compare between different categorical groups. Mann Whitney U test was used in the comparison of the prevalence of resistant strains between the two time points (i.e., 48 hours after arrival in Kenya and 6 weeks following initial collection). A P-value of <0.05 was used to indicate statistical significance.

2.3. RESULTS

2.3.1. Identification of *Klebsiella* species in stool samples

Of the 242 human faecal specimens, 81 tested positive for *Klebsiella* species indicated by the appearance of yellow colonies on SCAi medium (Figure 2.1). Due to the possibility of picking false positives, colonies that were pale yellow (n=7) were not assessed in the current study.

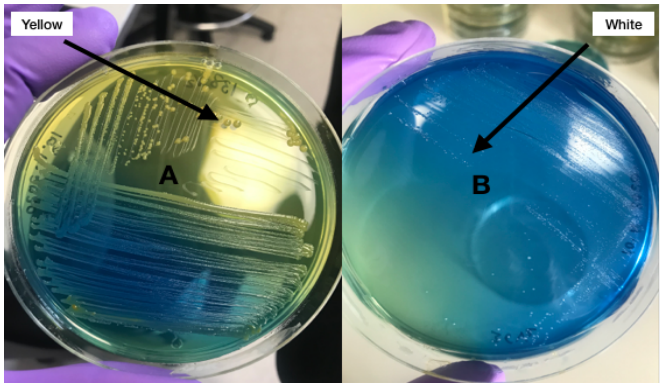

Figure 2.1: Phenotypes as observed on the SCAi media. Presumptive *Klebsiella* isolates were identified as yellow colonies (plate A), while non-*Klebsiella* isolates were identified as white colonies (plate B).

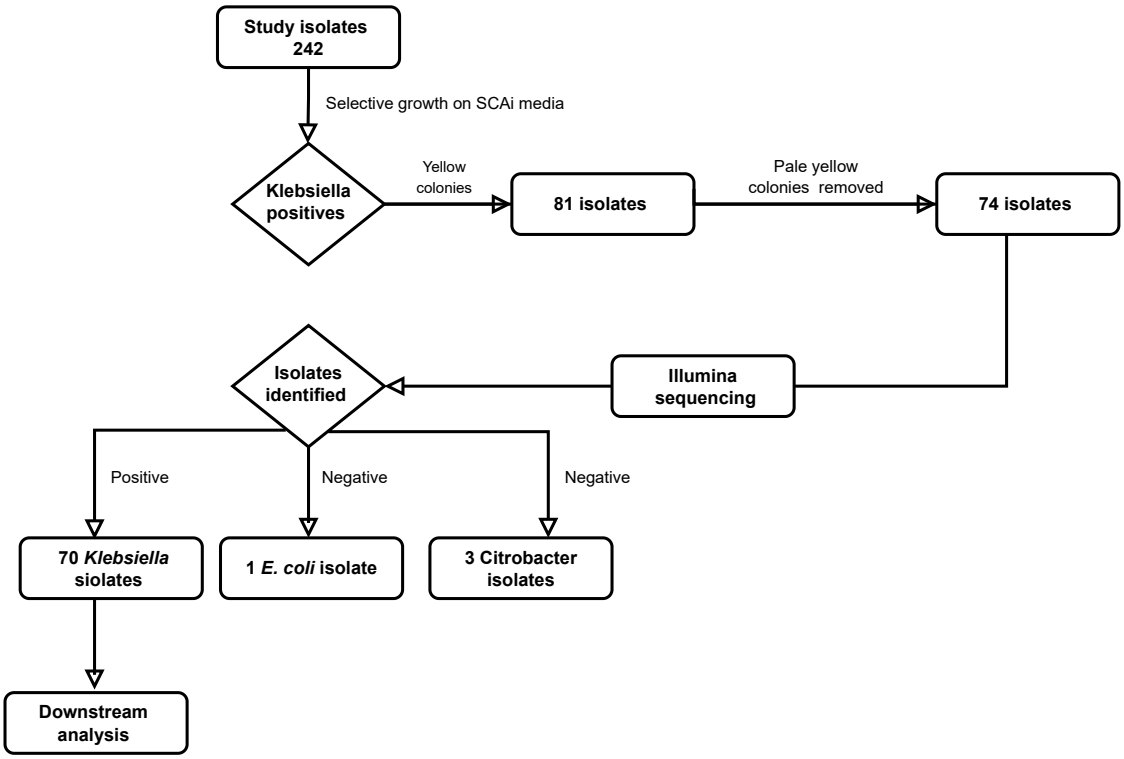

Figure 2.2: A comprehensive overview of total number of samples included in the current study and a sequence through which samples were processed. The flowchart also highlights how some samples were excluded in the initial step during laboratory analysis.

Whole-genome sequencing was thus conducted on 74 of the 81 presumptive *Klebsiella* isolates. Out of the 74 isolates, 70 isolates were classified as *Klebsiella* species, with a mean GC content of 56.88% and a mean contig N50 of 477,424 bp. Three sequences with significantly lower GC-contents (53.72%) were identified as *Citrobacter* (Figure 2.3). One isolate could not be identified by Kleborate but was identified as *E. coli* by MicrobesNG, using their Kraken pipeline. *Citrobacter* isolates and the *E. coli* isolate were removed from further analysis. *K. pneumoniae (*35/70, 50%) and *K. variicola (*22/70, 31.4%), were the most prevalent *Klebsiella* isolates (Figure 2.2), while the remaining isolates were identified as *K. michiganensis*, K. *grimontii*, *K. ornithinolytica*, *K. quasivariicola*, *K. pasteurii*, or *K. oxytoca*.

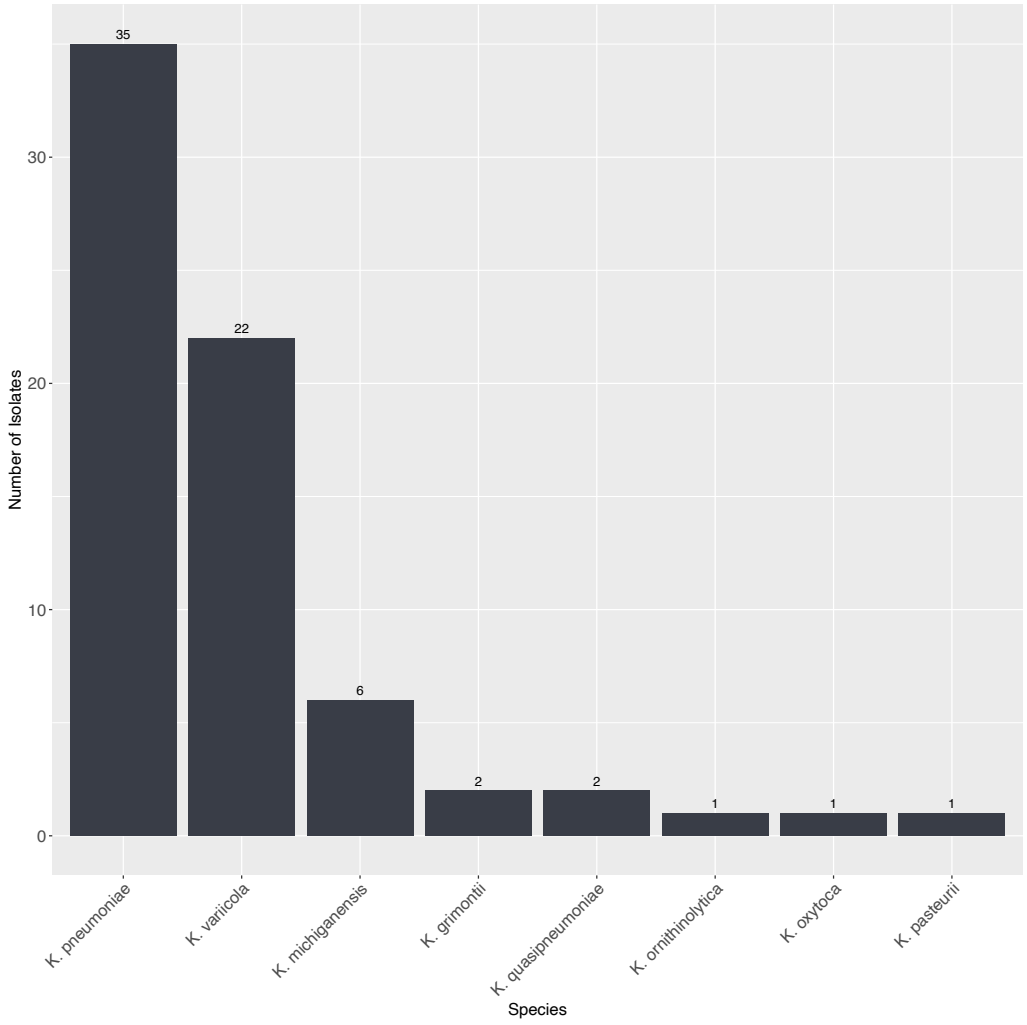

Figure 2.3: The proportion of *Klebsiella* species identified from sequence data. The bars represent the proportion of species in relation to the total number of sequences analysed. The X axis represents the species, while the Y axis indicates the number of strains.

2.3.2. Determining treatment effect on colonisation of *Klebsiella* species

To determine whether *Klebsiella* colonisation was influenced by rifaximin regimen the occurrence of colonisation was compared between treatment group against the baseline established by the placebo group. In our study, total of 25 *Klebsiella* positive samples were identified collected in the first time point (i.e., within 48 hours after the study participants arrived in Kenya) and in a subsequent collection after 6 weeks of arrival, 45 *Klebsiella* isolates were detected. The number of samples that tested positive in each treatment group and the placebo in the initial collection and the second, is shown in Table 2.2. Our analysis revealed there was no significant difference in the colonisation of *Klebsiella* species across the treatment groups and the placebo (Kruskall Wallis = 2.57, P = 0.4). Further pairwise comparison of each treatment group against placebo resulted in adjusted p-values according to Dunn's multiple comparisons test indicating there was no significant difference between placebo and rifaximin daily with a Z-value of 1.604 and a p-value of 0.2176 whilst comparison of the placebo and rifaximin twice daily yielded an adjusted p-value of 0.8454 and a Z-value of 0.8018. Analysed the data was further conducted to compare the colonisation of *Klebsiella* species between the first collection and the second time points. No significant difference in *Klebsiella* colonisation was found between the first and the second time points, Mann Whitney U test (Mann Whitney U=1, p=0.2).

Table 2.2: Detection of *Klebsiella* species in samples collected 48hrs and 6 weeks after arrival in Nanyuki, Kenya.

Treatment group	1st collection	2nd collection	Total	Proportion of *Klebsiella* positive samples per group (in %)	Kruskal Wallis	P value+
Placebo	5	12	17	25.7 (17/70)	2.57	0.4
Rifaximin daily*	13	17	30	42.8 (30/70)		
Rifaximin twice daily**	6	16	22	31.4 (22/70)		
Total	25	45	70			

*Represents rifaximin administered at a dose of 550mg once per day
** Represents rifaximin administered at a dose of 550mg twice daily
+Comparison between the first and the second collection across the groups using simple Kruskal Wallis test.

2.3.3. Colonisation patterns across individuals between the two sampling time points

Patterns of carriage of *Klebsiella* species between individuals were further analysed. Our observation revealed 38 out of 54 individuals carried *Klebsiella* species only once either during the first collection or second collection time point. Additionally, 16 out of 54 individuals were colonised by *Klebsiella* strains in both the first and the second time points (Table 2.3). Among the participants who tested positive for *Klebsiella* species in the first and the second collection time points (16 out of 54 individuals), 9 out of 24 individuals were observed in the rifaximin daily group, 4 out of 17 individuals came from the rifaximin twice daily group and 3 out of 13 individuals were from the placebo group. Among individuals who were colonized by *Klebsiella* species only once, a total of 29 individuals acquired *Klebsiella* species in the second collection. On the other hand, 9 individuals who were initially colonized did not test positive for *Klebsiella* species colonisation in the second collection.

Table 2.3: Distribution of individuals who harboured *Klebsiella* species in the first and second sample collection time points.

Treatment group	Both positive *	V1 positive only **	V2 positive only ***	Grand total+
Placebo (n = 13)	3	2	8	
Rifaximin 550mg daily (n = 24)	9	5	10	
Rifaximin 550mg twice daily (n = 17)	4	2	11	
Total	16	9	29	54

* Individuals who tested positive for *Klebsiella* isolates in the first and second collection, V1 and V2
** Individuals who tested positive for *Klebsiella* isolates in the first collection only, V1
*** Individuals who tested positive for *Klebsiella* isolates in the first collection only, V2
+ is the grand total found after adding the sums of all columns.

To understand whether there are individuals who carried same types of species between the first time point and the second time point, further analysis of the colonisation pattern was conducted in individuals. There were 7 individuals who tested positive for different types of *Klebsiella* species in the first and the second collection, while 9 individuals exhibited the same species colonisations in both collections (Table 2.4). Notably, among the latter individuals with detection of the same species, one individual showed colonisation of *K. michiganensis*, 3 individuals showed *K. pneumoniae* colonization while five individuals showed *K. variicola* colonisation in both collection points (Figure 2.4). Conversely, among individuals who displayed colonization by different *Klebsiella* species, three individuals were initially colonized by *K. pneumoniae*, but in the subsequent collection, they exhibited colonization by *K. variicola*. In contrast, two individuals revealed a patten initially presenting colonization by *K. variicola,* and subsequent colonisation by *K. pneumoniae*. In the remaining two individuals, variable colonisation between the two collection time points involving *K. variicola* to *K. ornithinolytica* in one individual and *K. quasineumoniae* to *K. pneumoniae* in another individual was observed.

Table 2.4: Types of *Klebsiella* species found in colonised individuals six weeks and 48 hours after arriving in Nanyuki, Kenya.

Sample	Collection 1	Collection 2	Number of individuals
Same *Klebsiella* species*			
	K. variicola	*K. variicola*	5
	K. pneumoniae	*K. pneumoniae*	3
	K. michiganensis	*K. michiganensis*	1
	Total		**9**
Different *Klebsiella* species+			
	K. pneumoniae	*K. variicola*	3
	K. variicola	*K. pneumoniae*	2
	K. variicola	*K. ornithinolytica*	1
	K. quasipneumoniae	*K. pneumoniae*	1
	Total		**7**
Individuals colonised only once			
Acquired *Klebsiella* species in the second collection	29		
Lost *Klebsiella* species in the second collection	9		
Total	38		

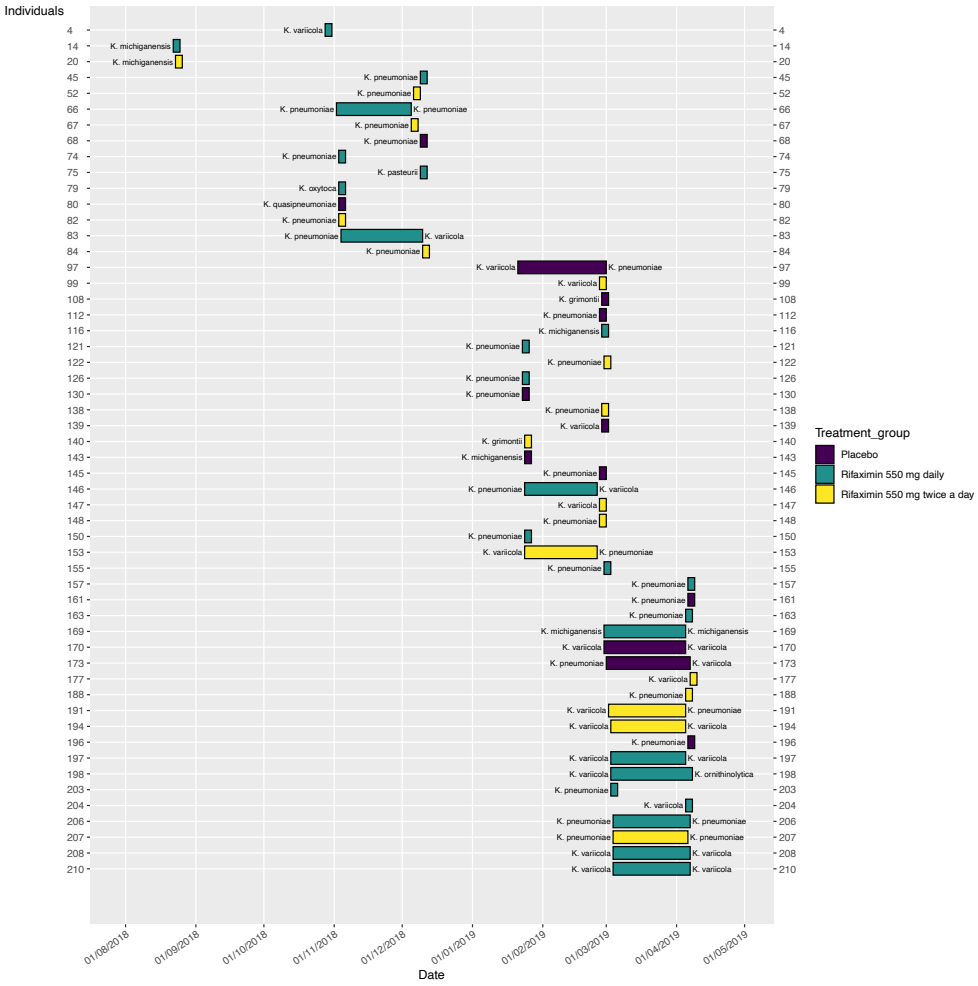

Figure 2.4**:** Prevalence of *Klebsiella* species in each treatment group per individual between the two sampling time points. Soldiers arrived at various times throughout the year, as shown on the Gantt chart. The first sample was taken 48 hours after the soldier arrived, and the second sample was taken 6 weeks later, depending on the time of year. Each square represents a single person, and each rectangle represents a person who tested positive for *Klebsiella* in both collections. The vertical axis represents proportions, while the X axis represents the two sampling times.

The data was further analysed to assess the distribution of sequence Types (STs) across individuals that tested positive for *Klebsiella* between the two time points. The majority (n=47) of individuals did not maintain the same ST (Figure 2.5). However, six (n=6) individuals exhibited the same ST in both collection points.

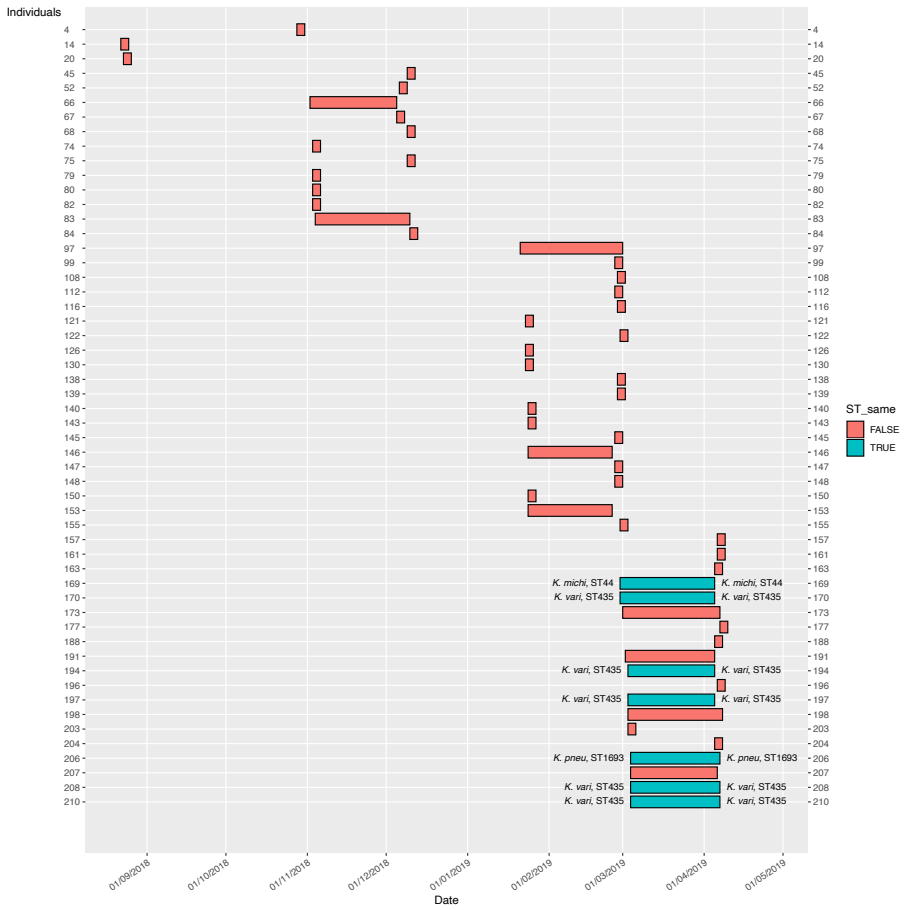

Figure 2.5: The presence or absence of the same ST detection between the first and second collections. The Gantt chart shows that soldiers arrived at different times. The first sample was taken 48 hours after the soldier arrived, and the second sample was taken 6 weeks later, depending on the time of year. Each square represents a single person, and each rectangle represents an individual who tested positive for *Klebsiella* in both collections. The vertical axis represents individual soldiers, while the X axis represents individuals carrying the same ST in both detections and those without. Each square represents a single individual, and a rectangle represents an individual who tested positive for *Klebsiella* in both collections. The X axis represents the two sampling times, while the vertical axis represents individual soldiers. Individuals with the ST who were retained are shown with a bar of the same colour at the beginning and end. *Klebsiella* colonisation was prevalent in samples collected between March and April of 2019.

2.3.4. Distribution of virulence and resistance genes across *K. variicola* and *K. pneumoniae* isolates.

Analysis of the data was conducted to identify the presence of virulence and antimicrobial resistance genes in the isolates. Yersiniabactin (*ybt)* and colibactin (*clb*) virulence genes in *K. pneumoniae* were found. Yersiniabactin was discovered in 3 out of 35 isolates while colibactin was found in 1 out of 35 *Klebsiella* isolates, respectively. The prevalence of resistance in *K. variicola* and *K. pneumoniae* across various antimicrobial classes is presented in more detail in Table 2.5, whereas Figure 2.6 demonstrates the prevalence of each gene for per antimicrobial class across *K. pneumoniae* and *K. variicola* isolates. Sulphonamide resistance, tetracycline trimethoprim resistance, and streptomycin resistance were the three most common resistance genes. Overall, the prevalence of sulphonamide resistance was significantly higher compared to other antimicrobial classes (X^2=182.4, df=11, P<0.0001) (Table 2.5). Moreover, in *K. variicola,* resistance was limited to four antimicrobial classes, namely tetracycline, sulphonamide, trimethoprim, and streptomycin. Detailed analysis revealed there was no significant difference in prevalence of resistant isolates between the two time points (48 hours after arrival and 6 weeks after the first collection) (Fisher's test, 2 tailed, p=0.9704).

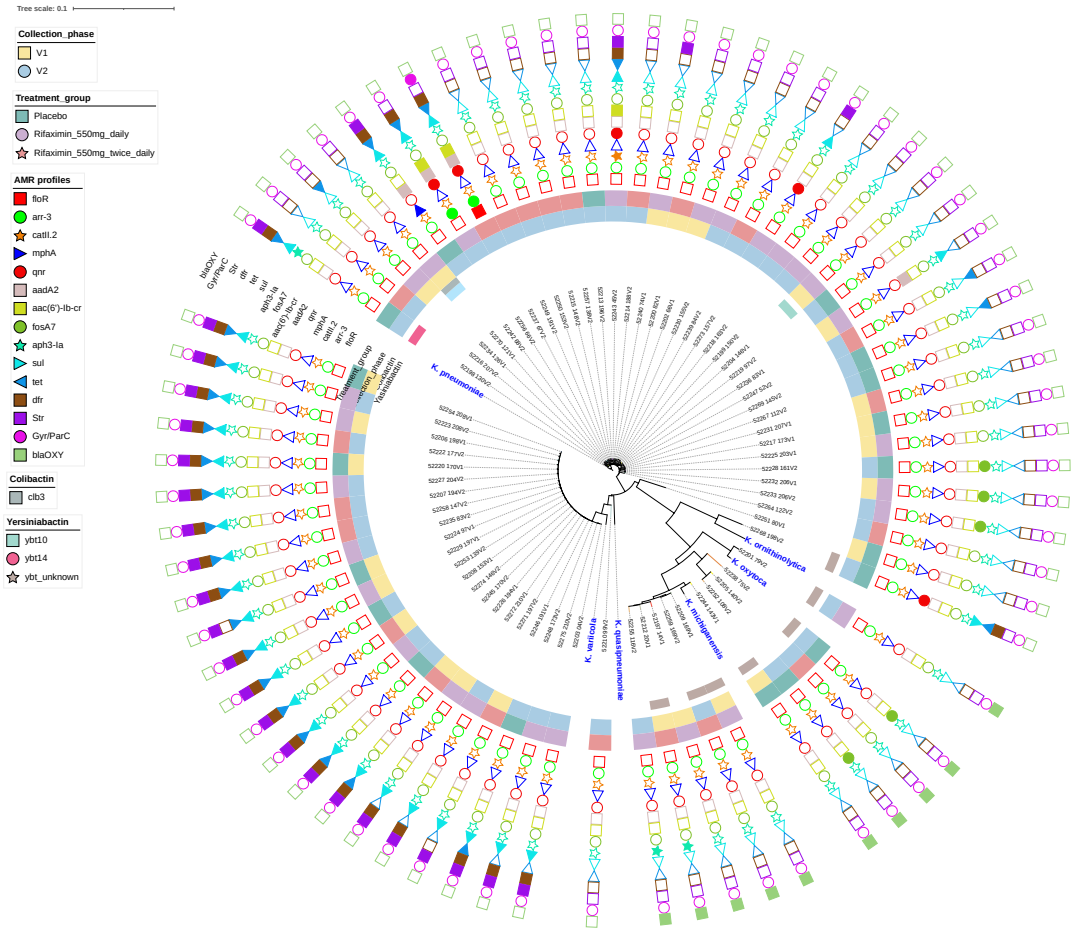

Figure 2.6: *Klebsiella* species observed across different individuals. Each isolate corresponds to a single individual with one isolate selected per sample. Scale bar represents 0.1 substitutions per site. iTOL (https://itol.embl.de/) was used to visualise resistance genes, virulence genes, and corresponding treatment groups associated with the individuals. Using all 70 sequences, the MinHash algorithm was used to calculate the distances between pairs and to create a neighbour joining tree that showed the relationships between the species using the Mash software package.

Table 2.5: Distribution of resistance profiles across individual isolates

Antimicrobial	*K. pneu* (%) [+] N=35	*K. varii* (%) [+] N=22	Total
Trimethoprim	5(20)	20(80)	25
Tetracycline	8(32)	17(68)	25
Sulphonamide	7(28)	22(76)	29
Fluoroquinolone	4(16)	0(0)	4
Florfenicol	1(4)	0(0)	1
Fosfomycin	3(13	0(0)	3
Streptomycin	5(20)	22(81)	27
Macrolide	1(4)	0(0)	1
Chloramphenicol	1(4)	0(0)	1
Rifampicin	2(8)	0(0)	2
Aminoglycoside	6(24)	0(0)	6
blaOXY	0(0)	0(0)	0

[+] Indicates the percentage of isolates with resistance to each animal class. Percentages are shown in brackets.
[a]Total #isolates, indicates the total number of isolates found per species.

Presence of strains that exhibited multidrug resistance profiles was also examined. Isolates with three or more resistance genes were considered to be multidrug-resistant (MDR). In our analysis, significantly higher prevalence of MDR strains (48.6%) was observed compared to the prevalence of isolates with single resistant genes (20%) and those without resistance genes (31.4%) (X^2 = 14.57, df=2, P<0.05). *K. variicola strains* exhibited a higher prevalence of multidrug resistance (X^2 =35.8764, df=1, P<0.05) compared to *K. pneumoniae,* with prevalence estimated at 100% (22/22) and 20.0% (7/35), respectively. Additionally, more variable range of resistance genes were observed in *K. variicola strains* in comparison to *K. pneumoniae* strains. For instance, more diverse genes associated with trimethoprim, sulphonamide, tetracycline and streptomycin were seen in *K. pneemoniae* compared to *K. variicola* (Table 2.6). The absence of resistance genes associated with aminoglycoside, quinolone, fluoroquinolone, fosfomycin, florfenicol, chloramphenicol, and rifampicin was observed in *K. variicola*.

On the other hand, the current study found the occurrence of spurious hits of *sul1* gene in 21 of the 22 *K. variicola* isolates annotated as *sul1*? 76%, indicating truncated sequences with 76% of the expected resistance protein, leading to their classification as spurious hits. Meaning, the sequences exhibit less than 100% coverage, suggesting incomplete matches even at 100% identity. Moreover, this study detected the *aph3 Ia.v1* in one *K. pasteurii* isolate and *fosA7* genes in one *K.*

quasipneumoniae isolate, denoted as *aph3 Ia.v1*? 0% and *fosA7*? 0% respectively, but with 0% truncated sequences. These findings indicate low-confidence spurious hits due to very low coverage and identity. These uncertain outputs were removed from the dataset from further analysis and were not included in the analysis in Table 2.5 and Table 2.6.

Table 2.6: Distribution of resistance genes amongst *Klebsiella* species

Resistance profiles	Resistance genes	*K. pneumoniae*	*K. variicola*
Tetracycline	*tet(D)*	11.4% (4/35)	77.3% (17/22)
	tet(A)	8.6% (3/35)	-
	tet(B)	2.9% (1/35)	-
Sulphonamide	*sul1*	5.7% (2/35)	-
	sul2	2.9% (1/35)	100% (22/22)
	sul1(two copies)	5.7% (2/35)	-
	sul1 and *sul2*	5.7% (2/35)	-
Trimethroprim	*dfrA7*	2.9% (1/35)	-
	dfrA5	-	90.9% (20/22)
	dfrA27	5.7% (2/35)	-
	dfrA12	2.9% (1/35)	-
	dfrA14	2.9% (1/35)	-
Streptomycin	*strA*	14.3% (5/35)	100% (22/22)
	strB	14.3% (5/35)	100% (22/22)
Aminoglycoside	*aadA2*	11.4% (4/35)	
	aac(6')-Ib-cr.	8.6% (3/35)	
	aph3-Ia.	2.9% (1/35)	
Quinolone	*qnrB4*	2.9% (1/35)	
	*qnrB6^*	5.7% (2/35)	
	qnrS1	2.9% (1/35)	
Fluroquinolone	*Gyr/ParC*	2.9% (1/35)	
Fosfomycin	*FosA7*	8.6% (3/35)	
Florfenicol	*floR*	2.9% (1/35)	
Chloramphenicol	*catII.2*	2.9% (1/35)	
Rifampicin	*arr-3*	5.7% (2/35)	

2.3.5. Temporal links of AMR patterns

To investigate any temporal links between resistance patterns and dates by which individual samples were collected the occurrence of certain resistance patterns were examined linked to the dates of sample collection (Figure 2.7). Overall, our data revealed occurrence of resistant strains

irrespective of the month in which the samples were collected. Each month had at least one sample that harboured resistance genes, suggesting occurrence happened throughout the time of sample collection. Moreover, across the dataset resistant strains were detected in April (n=12) followed by February (n= 8), March (n= 8), December (n= 6), Jan (n= 6) and Nov (n= 2). A recurrent pattern of resistance genes involving *sul2*, *tetD*, *dfrA5*, *strA*, and *strB* genes was present in the majority (n=16) of *K. variicola* strains that were collected across different months including January, February, March, April, and December. Another pattern of resistance genes, specifically *dfrA5, strA*, *strB*, *sul2* in four strains (n= 4) of *K. variicola* was observed. This pattern was identified in strains that were sourced in March, April and August. On the contrary, the distribution of *K. pneumoniae* was random, lacking temporal patterns with a notable exception of *fosA7* gene. This gene was observed in three isolates two of which were collected in the same month (April) and one in March.

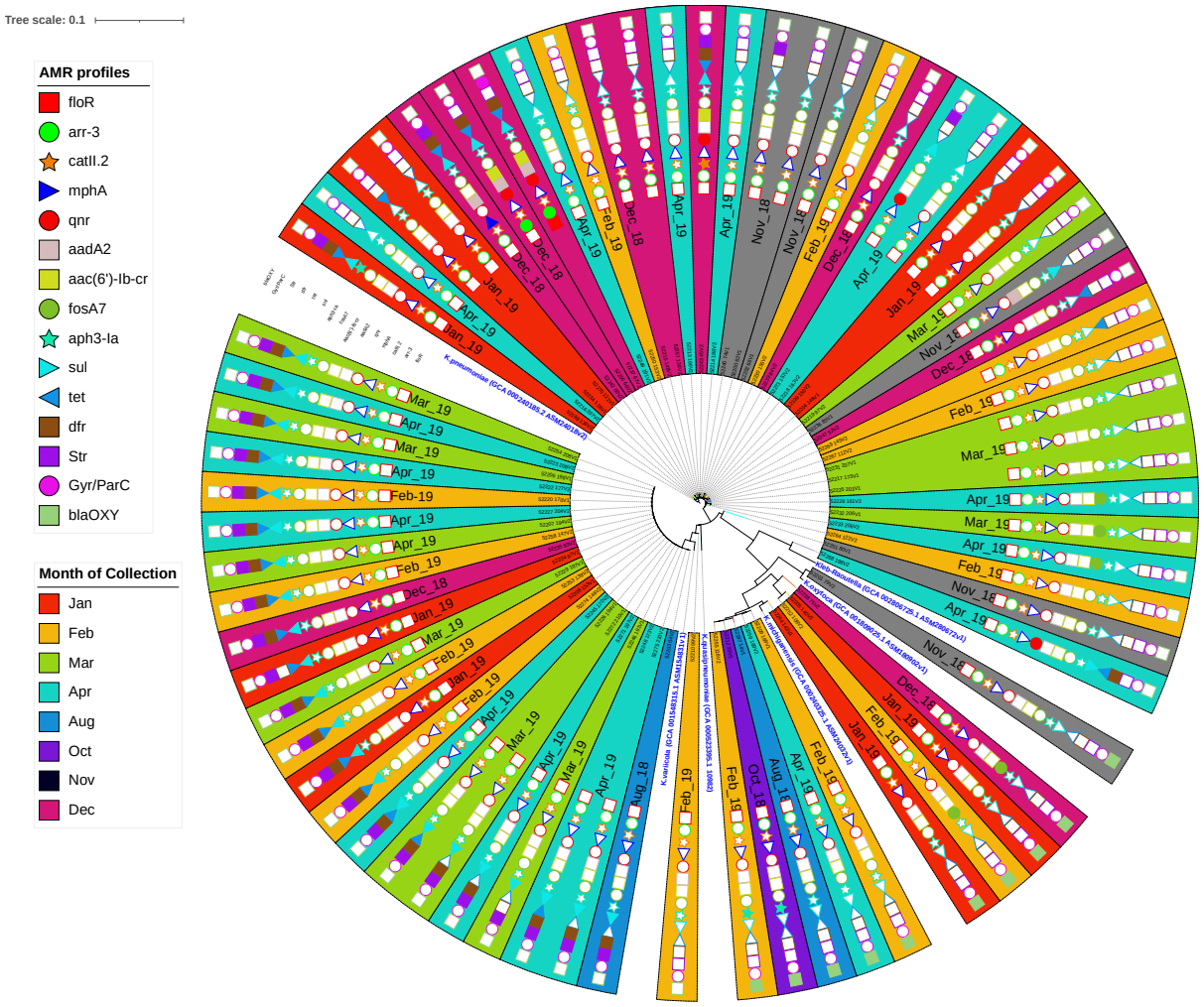

Figure 2.7: Resistance gene patterns of various isolates in relation to the time they were collected. Each isolate corresponds to a single individual with one isolate being selected per sample. Scale bar represents 0.1 substitutions per site. iTOL (https://itol.embl.de/). Each collected date is indicated by a colour code as indicated in the legend. The collection spanned the months of November 2018 (Nov), December 2018 (Dec), January 2019 (Jan), February 2019 (Feb), March 2019 (Mar), and April 2019 (Apr).

2.3.6. Phylogenetic relationship between *K. pneumoniae* and *K. variicola strains* and their genomic diversity

Phylogenetic analysis was conducted to determine the relationships of different lineages of *K. pneumoniae* and *K. variicola* species. Using the seven-gene MLST scheme, a total of 29 STs of *K. pneumoniae* and *K. variicola* were identified (Zurfluh *et al.,* 2015). Notably, 27 *K. pneumoniae* STs were detected (Figure 2.8) whilst *K. variicola* strains were limited to just two STs: (ST925

(n=1) and ST435 (n=21), (Figure 2.10). Amongst *K. pneumoniae* strains, ST17 and ST1693 were the most common, accounting for 8% (3/35) each. *K. pneumoniae* strains varied widely, with SNP distances ranging between 1 and 5591 SNPs across pairs of strains (Figure 2.9). There was significant genetic diversity between the V1 and V2 isolates. The majority of strains (25/35) from both before (V1) and after travel (V2) among individuals showed SNP distances greater than 200. Thus, suggesting that most individuals acquired new and genetically distinct strains after 6 weeks. This highlights the dynamic nature of bacterial population in response to environmental changes. The figure also shows there were some strains (10/35) with very close genetic relationship between paired individuals indicating they had undergone minimal genetic changes, were likely acquired from a common source, or have been transmitted between different individuals.

Contrary to observation seen among *K. pneumoniae* strains, *K. variicola* strains displayed SNP distances of up to 1772 SNPs, however most strains (n=14) had SNP distances between 1 and 10 (Figure 2.11), indicating minimal genetic diversity and suggesting many individuals shared closely related strains. Despite the predominance of closely related strains, a few (n=3) isolates displayed SNP distances greater than 100 from every other isolate, highlighting instances of significant genetic diversity and the occasional acquisition of new, genetically distinct strains. Recurrence of the same strain (as defined by the presence of 0 core genome SNPs) in a single individual carrying *K. pneumoniae* was very infrequent, with only one individual (206) having the same strain before and after prophylaxis. Sharing of *K. pneumoniae* STs between different individuals, was mostly observed in the second collection (13 individuals).

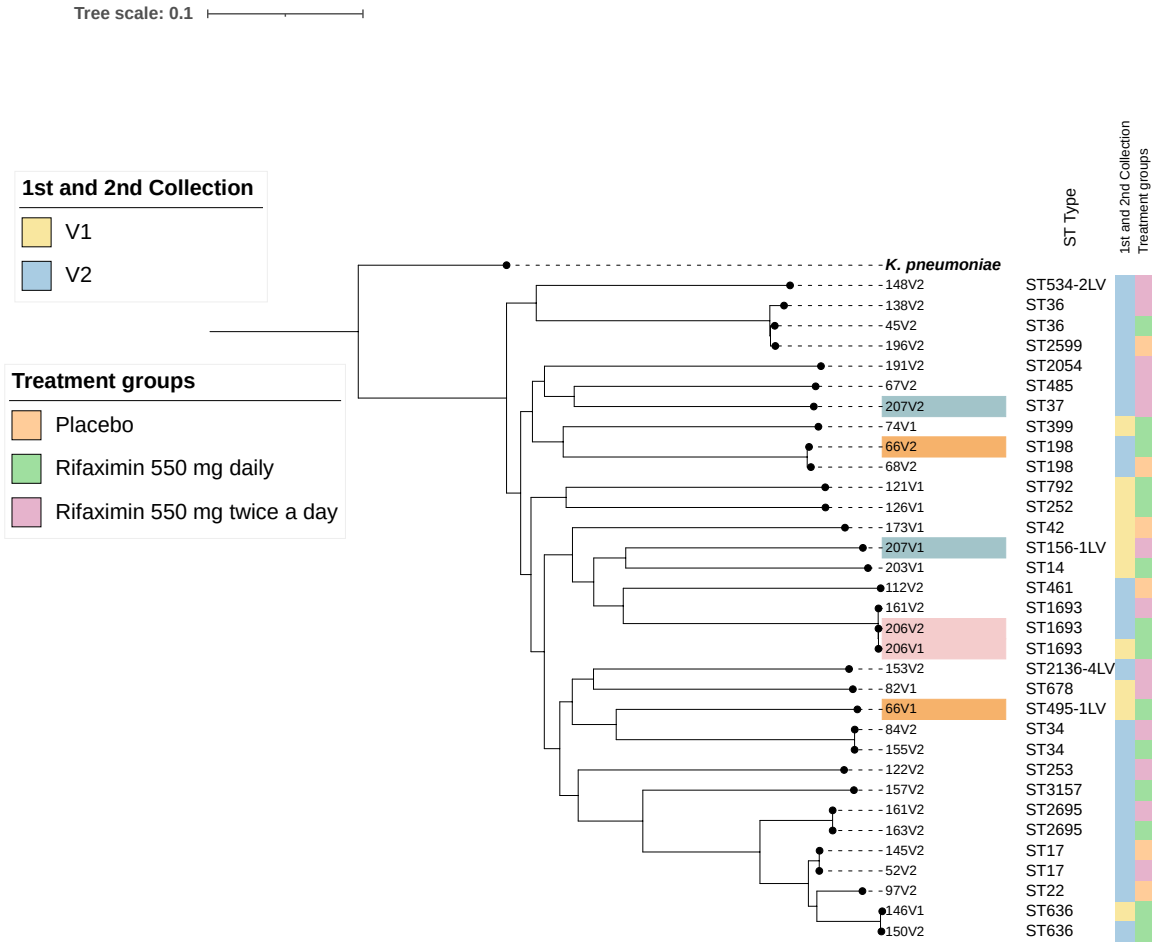

Figure 2.8: The diversity of *K. pneumoniae strains* across treatment groups, STs and sampling phases depicted on a maximum likelihood phylogenetic tree based on a core genome alignment. The scale bar indicates the number of substitutions per site per unit. The yellow (V1) and green (V2) bars denote the sampling phases at the start and end of the study, respectively. Orange, green, and pink bars represent the placebo, rifaximin daily, and rifaximin twice daily groups, respectively. The matching colour scheme, i.e., green for strain 207, orange for strain 66, and pink for strain 206, identifies individuals who were colonised with *K. pneumoniae* species in both the first and second collection timepoints. *K. pneumoniae* strain ATCC 13883 was used as a reference.

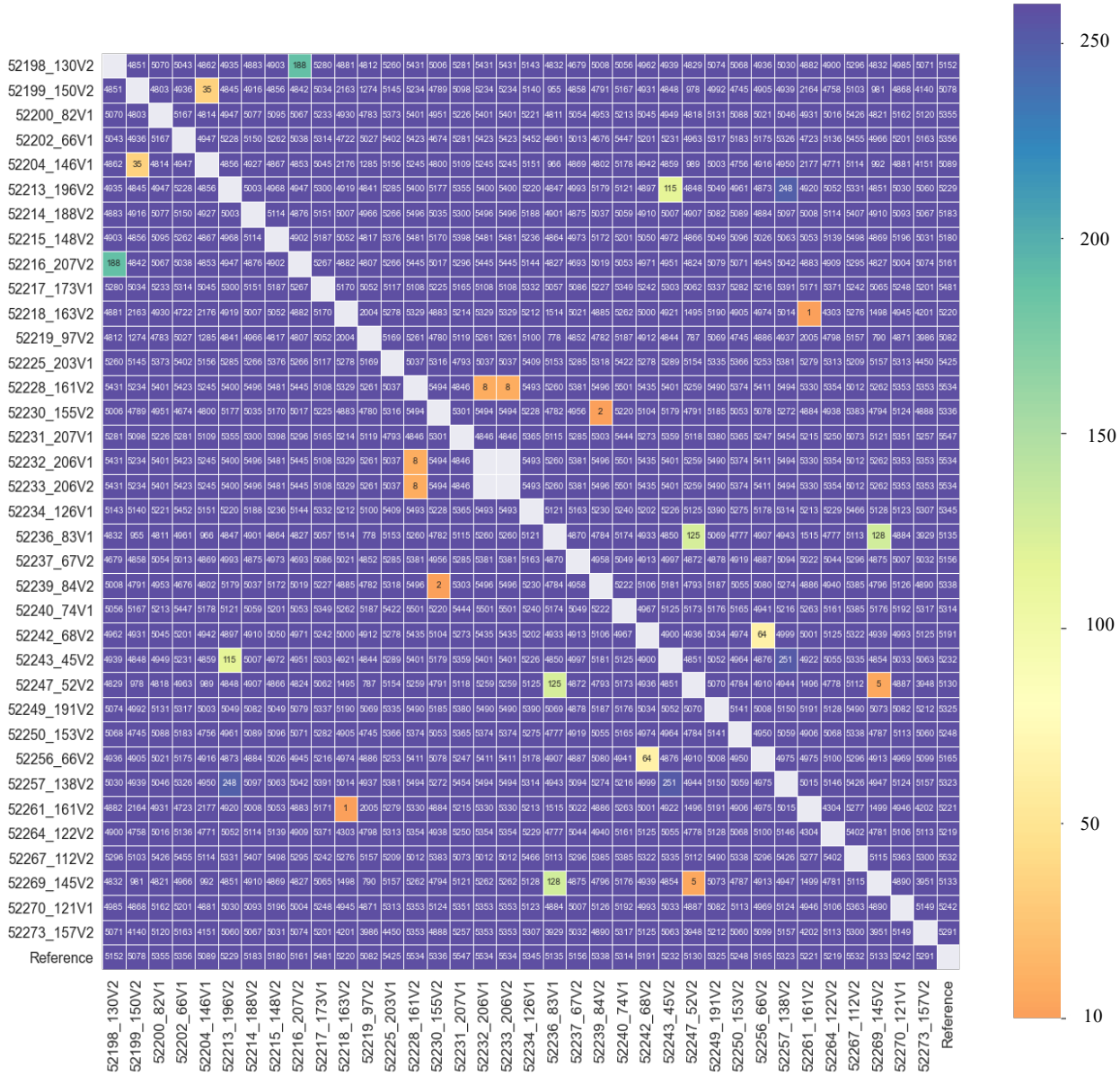

Figure 2.9: SNP distances between strains of *K. pnuemoniae*. The scale bar represents the number substitutions per unit per site. Strains with no SNPs are shown in light grey. Dark blue indicates >250 SNPs difference. iTOL (https://itol.embl.de) was used to visualise the tree. Isolates with no SNPs (in grey) were considered identical. *K. pneumoniae* subsp. *pneumoniae*, strain HS11286, was used as a reference for comparison with other *K. pneumoniae*.

Figure 2.10: The diversity of *K. variicola strains* across treatment groups, STs, and sampling phases depicted on a maximum likelihood phylogenetic tree based on a core genome alignment. The scale bar shows 0.1 substitutions per site. The yellow (V1) and green (V2) bars represent the sampling phases before and after the participants received prophylactic rifaximin for six weeks, respectively. The placebo, rifaximin daily, and rifaximin twice daily groups are represented by orange, green, and pink bars, respectively. The matching colour scheme identifies individuals who have *K. variicola* strains in the first and the second collection, which is green for individual 210, yellow for individual 208, pink for individual 194, and blue for individual 170. A hybrid assembly of strain 52253_139V2 was used as a reference.

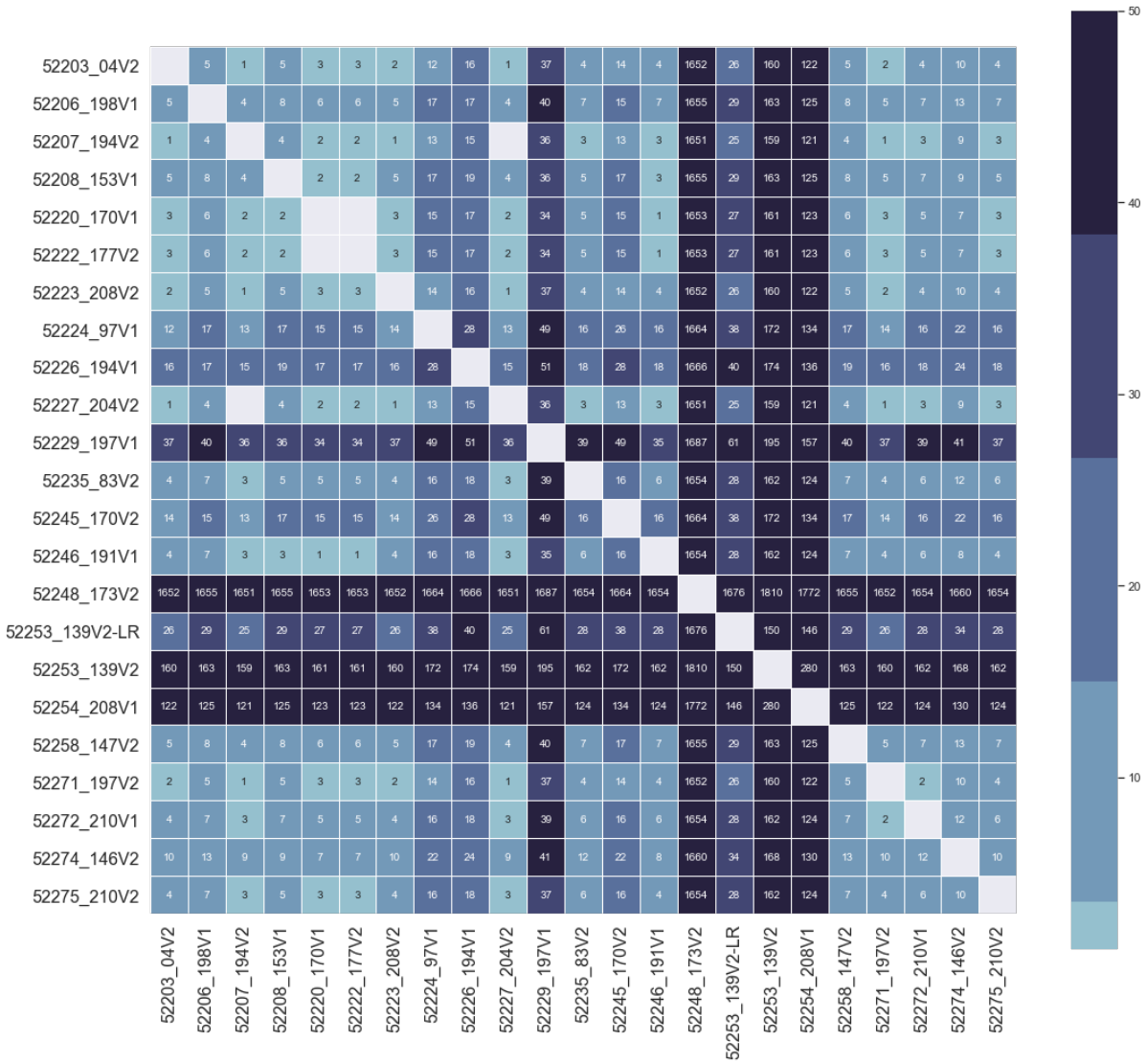

Figure 2.11: SNP distances between strains of *K. variicola*. The scale bar represents the number substitutions per unit per site. The tree displays the distribution of isolates across individuals in distinct treatment groups based on two stages of collection. iTOL (https://itol.embl.de) was used to visualise the tree. Isolates with no SNPs (in grey) were considered identical. A long-read hybrid assembly of sample, 52253_139V2_LR, was used as a reference for comparison with *K. variicola* to make more meaningful comparison since references from NCBI exhibited significant dissimilarity with *K. variicola* isolates in this study. Isolate 52253_139V2-LR represents a hybrid sequence derived from isolate 139V2, as denoted by its alternative name, 52253_139V2_Hi, in consecutive images below.

2.3.7. Diversity within *K. variicola* ST435

To better characterise the diversity of the dominant ST (ST435) in our collection of *K. variicola* genomes a pangenome matrix visualisation was employed (Figure 2.12). This matrix shows the distribution of various genes in the accessory genome and minor variance within the group, as well as a considerable proportion of genes that are shared by the isolates. Pangenome size was determined to be 5771 genes, with 5509 genes from the core genome and 262 coming from the accessory genes. To further evaluate the genetic variability among isolates, a Jaccard distance metric was employed, which provided insights into the structure of genetic diversity. Figure 2.13 illustrates the dissimilarity and similarity between samples based on the presence or absence of shared genes. Isolate 52248_173V2 exhibited the highest distance from its closest link, with a Jaccard distance of 0.7 in both directions, indicating a greater dissimilarity from its closest links and the rest of the isolates, highlighting its unique genetic composition. In contrast, isolates 52253_139V2_Hi (139V2) and 52246_191V1 (191V1) demonstrated identical distances of 0.4 from their closest links, suggesting a similar level of dissimilarity compared to their nearest neighbours. These isolates were observed to be positioned as the next furthest from the other isolates in the matrix. To better understand genes contributing to the observed variability, we annotated the accessory genes, as depicted in (Figure 2.14). The figure demonstrated clear distinctions between strains based on the functional profiles of their accessory genes, revealing distinctions that were not clear in (Figure 2.12). For instance, there were strains observed to lack resistance genes whilst other did. Notably other strains like 173V2, and 139V2_Hi exhibited unique profiles of additional accessory genes. Isolate 173V2 exhibited an additional set of genes associated with virulence, metabolism, regulation, translation and transporter further highlighting its unique genetic characteristics in terms of functions. There was a distinct difference in the accessory genome of 139V2_Hi compared to other strains. These differences were seen in a set of unique genes associated with metabolism, biosynthesis, transport, membrane function, regulations, stress response and genetic mobility. These detailed annotations reveal previously unseen genetic relationships and functional distinctions among the strains.

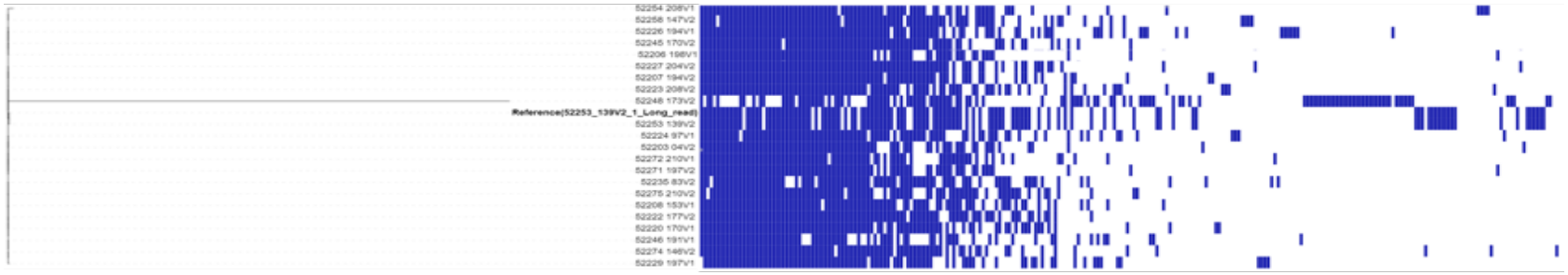

Figure 2.12: A pangenome matrix of accessory genes across *K. pneumoniae* strains. Phandango was used for visualisation of the tree and the gene presence-absence heatmap (https://itol.embl.de). Isolate 52253_139V2-LR (or 52253_139V2-Long_read) represents a hybrid sequence derived from isolate 139V2. It is denoted by its alternative name, 52253_139V2_Hi, in consecutive images below

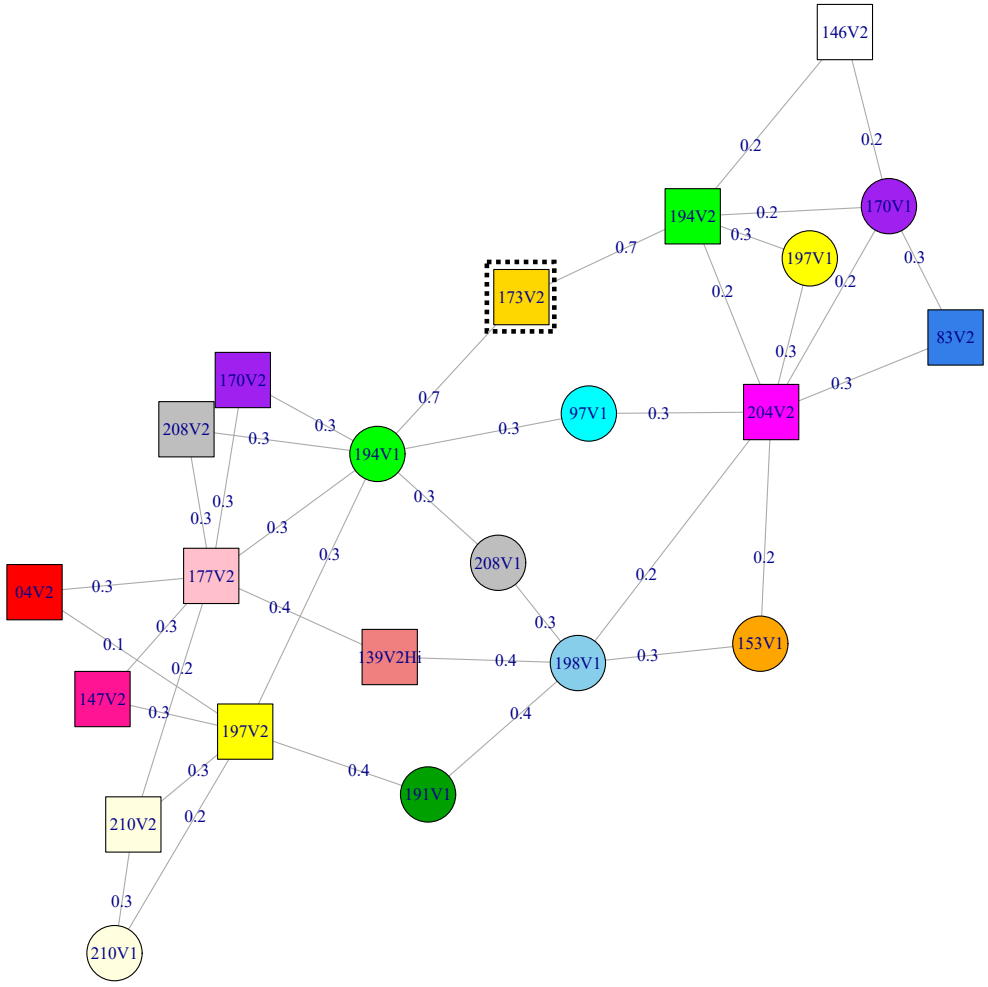

Figure 2.13: Jaccard distances between isolates in the accessory genome created using a presence-absence gene matrix. Each numerical value on the graph represents the Jaccard distance, indicating the dissimilarity between individual isolates. The highlighted isolate with dotted lines in the graph represents the isolate with the highest distance from its closest neighbours, highlighting its distinct genetic composition compared to the other isolates. Circles represent isolates that were collected 48hrs after arrival in Kenya whilst squares represent isolates collected 6 weeks after arrival. Furthermore, samples obtained from the same individual are shown in matching colours.

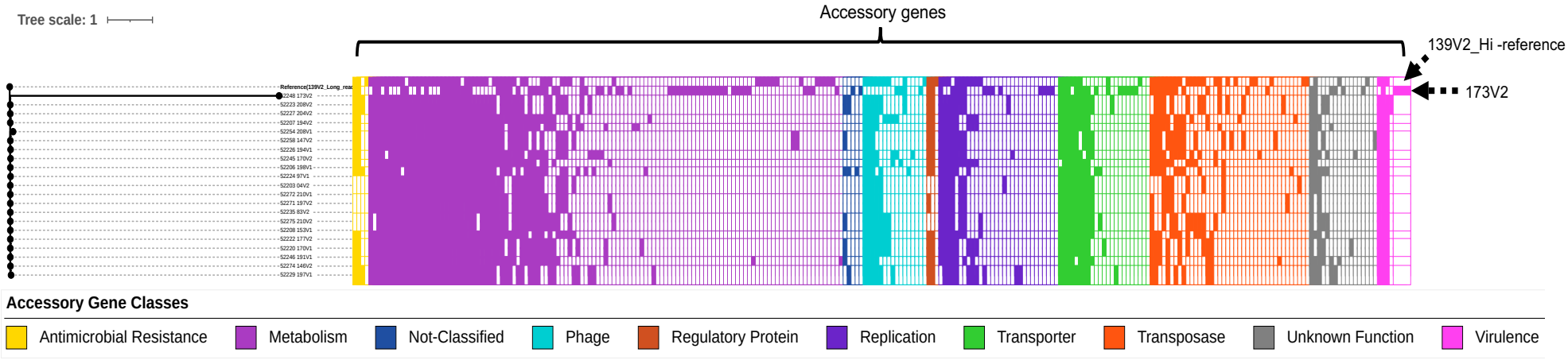

Figure 2.14: Accessory genome matrix showing the distributions of accessory genes categorized by their respective functions. Each colour code corresponds to the specific functions encoded by the genes. The matrix contains isolate 139V2_Hi used as a reference, while isolate 173V2 is represented in the second row.

2.3.8. Genetic elements involved in the transmission of trimethoprim and sulphonamide resistance.

To investigate genetic elements involved in the transfer of trimethoprim (*dfrA5*) and sulphonamide (*sul1*) resistance genes analysis of the underlying genetic elements was conducted. Trimethoprim (*dfrA5*), and sulphonamide (*sul1*) resistance genes were detected within class 1 integron (*intl1)*, which was found to be associated with IS*26* in *K. variicola* strains (n=22). In isolate 139V2, *intl1* was found in plasmid 1 (pBSKP5) at positions 114289 - 115302 bp, whereas in isolate 177V2, it was found in plasmid pBSKP3 at positions 13773 - 14786 bp. Upon further examination, three FIB-type replicons, namely FIA-2, FIIK-12, and FIK-4, were identified in isolate 139V2. (Figure 2.15). In contrast, pBSKP1 was identified in isolate 177V2 with replicons FIIK-4 and FIA21 and FIIK12. Our analysis revealed parts of plasmid pBSKP5 (247 kbp), bore resemblance with the plasmids pBSKP1 (154 kbp) and pBSKP3 (92 kbp), respectively (Figure 2.16). Like plasmid pBSKP5, plasmid pBSKP3 harboured sulphonamide (*sul1*, *sul2*), trimethoprim (*dfrA5*), and tetracycline (*tet(D*) resistance genes flanked by various insertion sequences, as demonstrated in Figure 2.15. There were no resistance genes detected in pBSKP1, pBSKP2, or pBSKP6. A comparative alignment of pBSKP1, pBSKP3, and pBSKP5, along with pBSKP2 and pBSKP6 sequences revealed significant similarities, with an average nucleotide identity of 99%, between pBSKP5 and pBSKP1, as well as pBSKP2 and pBSKP6, and an inverted similarity between pBSKP3 and pBSKP5. A comparison of similar regions of plasmids pBSKP5 and pBSKP3 revealed the presence of insertion sequences (IS*26*, IS*903*, IS*5075*, ISKpn*26*, ISLad*2*, IS*1663*, ISKpn*28*, ISEsa*2*, IS*1*A, ISPre*2*, and ISSn*4*). In pBSKP2, insertion sequences IS*26*, ISVsa*3*, ISPa*38*, and IS*4321*R were detected on segments showing inverted similarity to pBSKP5 and pBSKP3 (segments highlighted in green in Figure 2.16).

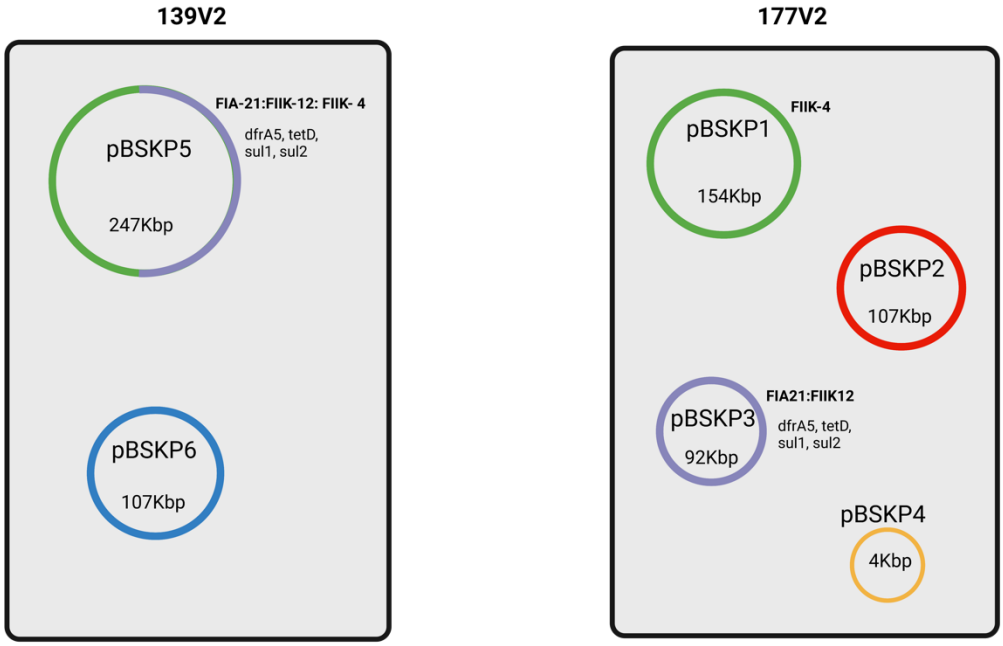

Figure 2.15: Differences were observed in plasmid composition between strains 139V2 and 177V2. Strain 139V2 carried a plasmid containing sulphonamide and trimethoprim resistance genes. In contrast, strain 177V2 possessed two separate plasmids that were cointegrates of plasmid pBSKP5 in strain 139V2.

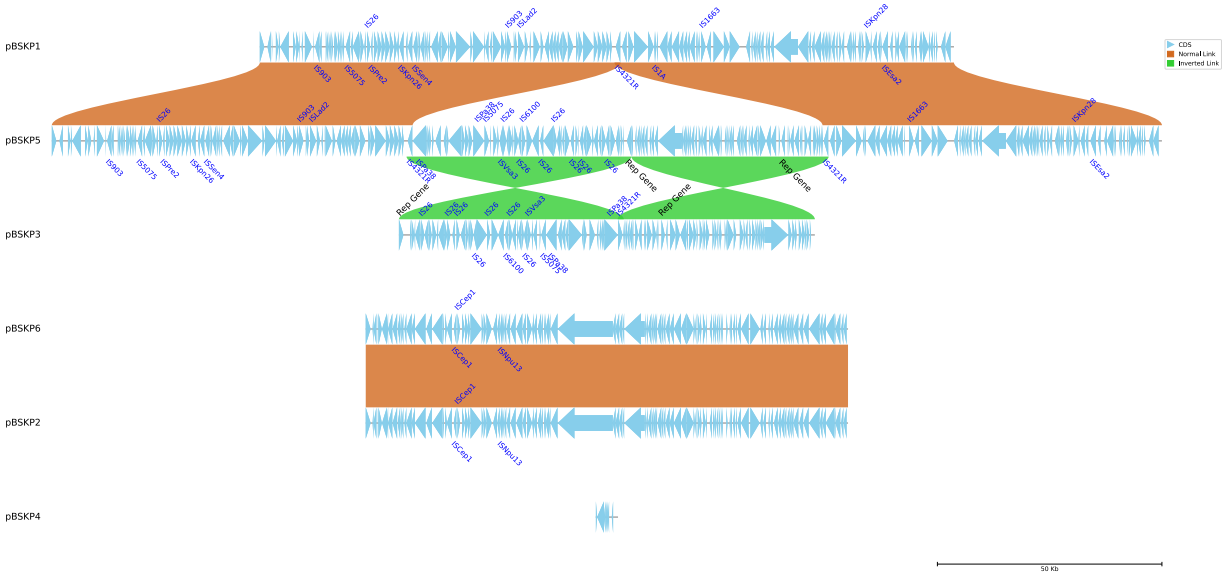

Figure 2.16: A Structure alignment of three plasmids. The orange highlight indicates regions that were similar between 177V1 plasmid 1(pBSKP1) and 139V2 plasmid 1 (pBSKP5). The comparison illustrated some parts of 117V2 plasmid 3 (pBSKP3) were reversed regions of pBSKP5. The illustration also depicts the locations of insertion sequences.

2.4. DISCUSSION

The aim of this study was to examine the diversity of *Klebsiella* species carried by British soldiers deployed to Kenya. *Klebsiella* is a major cause of extraintestinal infections and is a known reservoir of AMR genes. International travel has been documented to be an important determinant in the emergence and spread of these microbes in many geographical settings, and soldiers may play a role in the global dissemination of these bacteria (Doi, Iovleva, and Bonomo 2017; Arcilla *et al.,* 2017; ÖstholmBalkhed *et al.,* 2018). Various geographical variables have also been implicated in the epidemiology of these bacteria, including population structure and the prevalence of antimicrobial resistance and pathogenicity genes (Chakraborty *et al.,* 2016; Hilliquin *et al.,* 2018). There is, however, a significant knowledge gap regarding the diversity of *Klebsiella* species found in the human gut. Hence, the current study aims to bridge the gap by assessing the diversity of *Klebsiella* in a cohort of soldiers deployed to Kenya from United Kingdom. In all three groups, *Klebsiella* were found. In each group, *K. pneumoniae* strains were detected most frequently, followed by *Klebsiella variicola,* with less frequent identifications of other *Klebsiella* species. These findings are consistent with those of previous research, which demonstrated that *K. pneumoniae* is more prevalent than other species among various human populations (Neslihan *et al.,* 2011). However, detection rates of *K. pneumoniae* in the current study were lower than in previous hospital and community studies where prevalence ranged between 54% to 88% (Schwaber *et al.,* 2008; Høyland *et al.,* 2013; Niumsup *et al.,* 2018; Ita *et al.,* 2022). These observed differences could be attributed to a variety of factors, as *Klebsiella* colonization in the gut can vary by age, dietary habits, health status, antimicrobial use, geographical context, sample size, and study design (Boo and Lim, 2005; Mendelson *et al.,* 2005; Meatherall *et al.,* 2009; Chen *et al.,* 2023).

There was no significant difference in *Klebsiella* colonisation across different treatment groups. This implies that rifaximin had no impact on the presence of *Klebsiella*. These findings corroborate previous research that found rifaximin to have a limited influence on reducing bacterial load and diversity of the gut microbiota (Jiang *et al.,* 2000; Lagerbäck *et al.,* 2016). Counter to that, there is evidence suggesting that rifaximin may have an impact on the abundance of gut microbiota, despite the fact that the effect has only been proven to be transient at best (Hassouneh and Bajaj 202; Chang 2018).

Our findings also revealed that the majority (38 out of 54) of individuals did not retain *Klebsiella* colonisation between the initial and subsequent sampling time points. Gut microbiome diversity can fluctuate transiently and vary depending on diet, stress, illness or health conditions, probiotic supplementation, natural processes and antimicrobial use (Derrien and van Hylckama Vlieg 2015; Rogers *et al.,* 2016; Krupa-Kotara *et al.,* 2022; Radford-Smith and Anthony 2023). In the current study, information about the type of diet each individual took during the study period lacked, and antimicrobial intake during the study period, hence limiting our capacity to identify exact factors that may have influenced this observation. A total of 16 out of 54 individuals retained colonisation, and the observation was mostly concentrated in the rifaximin once daily group, with most isolates being identified as *K. variicola. K. variicola* strains exhibited a high prevalence of MDR, which may account for their dominant presence and persistence among individuals between the two sampling time points. This is also supported by existing literature since antimicrobial resistance has been linked to persistence of gut microbes (Singh *et al.,* 2018; Ernst *et al.,* 2020). However, because our study collected a single isolate per individual, some bacterial species may have been overlooked or, specifically, their prevalence may have been underestimated based on the approach used in the current study.

Our study also demonstrates that the majority (n=11) of individuals who maintained *Klebsiella* species colonisation were mostly collected between March and April 2019. In Kenya, March and April are part of a long rainy season (Igizeneza *et al.,* 2022). Rainy season can foster the risk of waterborne and foodborne diseases (Igizeneza *et al.,* 2022; Wekulo *et al.,* 2020). Poor sanitisation including contamination of water sources due to flooding can increase further the risk of illness or colonisation by multidrug-resistant bacteria (Wekulo et a., 2020; Egberongbe *et al.,* 2021). Food safety during this time is at most risk of being compromised i.e., spoilage of food, due to increased humidity which can promote the growth of bacteria. Foodborne pathogens and *Klebsiella* are known to thrive and persist for longer periods in the environment when there is high humidity (Moussé *et al.,* 2016; Bagumire and Karumuna, 2017; Wekulo etal., 2020). There is also a possibility that the *Klebsiella* species detected may have been circulating within the camp environment or in shared facilities.

Participants in the current study exhibited colonization by different STs of *Klebsiella*. Few individuals (6 out of 54) retained colonisation with the same ST. In contrast, more individuals (9 out of 54) exhibited different STs between the first and second collection. Individuals who retained colonization of the same ST in the first and the subsequent collection, harboured

ST435 *K. variicola*, ST1693 *K. pneumoniae* and ST44 *K. michiganensis*. In literature, ST44 and ST435 are underreported in *Klebsiella* isolates (Yang *et al.,* 2022). In our study, ST44 was identified as *K. michiganensis*, harbouring OXY genes, which are typically inherent in *K. oxytoca* and *K. michiganensis* strains (Yang *et al.,* 2022). Conversely ST435, represented a distinct, novel ST. On the other hand, ST1693, in past research has been linked to ESBL gene carriage and has been identified as originating from chickens, pigs, and cattle, suggesting that animals may act as reservoirs for this sequence type and contribute to propagation of antimicrobial resistance (Manges, 2015).

Among individuals who did not retain colonisation, the most prevalent lineages were ST17 and ST1693, followed by ST36, ST198, ST34, and ST2695. In previous studies, ST17, ST34, and ST198 were associated with ESBL and carriage of carbapenem resistance genes (Wyres *et al.,* 2020; Chen *et al.,* 2020; Lopes *et al.,* 2017). These STs, however, were not associated with resistance genes in the present study. Despite the absence of resistance genes in these strains, evidence suggests they play a significant role in AMR epidemiology in *Klebsiella* (Wu *et al.,* 2016). The lack of resistance genes in the present study does not preclude the potential of these strains acquiring resistance genes via horizontal gene transfer under selective conditions. ST198 strains have previously been recovered from plants, supporting its potential to circulate within the One Health triad, which links the environment with human and animal health (Lopes *et al.,* 2017).

In contrast to previous studies in which *K. pneumoniae* is known to be the major carrier of resistance genes (Klaper *et al.,* 2021; Hennequin and Robin 2016; Sanchez *et al.,* 2013), *K. variicola* isolates in this study carried more resistance genes than *K. pneumoniae.* Furthermore, 6 out of 35 of *K. pneumoniae* isolates had an AMR profile characterised by a single resistance gene, as opposed to *K. variicola* strains, which exhibited MDR profiles. On the other hand, in comparison to previous research in the same settings, the prevalence of resistance across all antimicrobials in the current study for all species was lower (Ogalo *et al.,* 2017; Taitt *et al.,* 2017; Muraya *et al.,* 2022). This disparity could be attributed to the demographic characteristics of the participants involved in the current study, as well as exposure levels to antimicrobials (Smith *et al.,* 2002; Pulingam *et al.,* 2022). Participants selected for the current study were healthy individuals. Notably, the majority (n=16) of *K. variicola strains*, especially those of strain *K. variicola* subsp. *tropica*, exhibited highly similar resistance gene profiles and were very closely related to each other based on core genome phylogenetic analyses. Among

the most prevalent resistance genes were those that conferred resistance to sulphonamides (*sul1* and *sul2*), trimethoprim *(dfrA5, dfrA27, dfrA14, dfrA12*, and *dfrA1*), and tetracyclines (*tetD, tetB*, *tetA*). Traditionally, trimethoprim and sulphonamide are used in synergy as a combination antimicrobial in the form of co-trimoxazole (Huovinen, 2001). Co-trimoxazole and tetracycline are low-cost broad-spectrum antimicrobials that are used for treating urinary tract infections, gastrointestinal disorders, and respiratory tract infections (Huovinen 200); Zhanel *et al.,* 2004). They are both employed in both human and veterinary medicine. In low-income countries resistance against these antimicrobials had been detected more frequently than in high-income countries (Gupta *et al.,* 2021). Additionally, different prescription behaviour in the human and veterinary sectors, as well as diversity in infection control programmes, greatly contribute to regional differences in the occurrence of *Klebsiella* resistance (Liu *et al.,* 2019). Moreover, extensive often indiscriminate use of co-trimoxazole and tetracycline in low-income countries has considerably contributed to the emergence of diminished susceptibility to these antimicrobials (Johnson *et al.,* 1995; Subha *et al.,* 2003). Strains resistant to these antimicrobials have been isolated from various environmental reservoirs, animal foods, and humans, suggesting their frequent circulation within the One Health continuum of the region (Adesoji *et al.,* 2015; Manyahi *et al.,* 2017; Gundogan and Avci, 2013; Ma *et al.,* 2021).

K. variicola is understudied, and there are significant gaps in our understanding of the species' evolution and population structure (Rodríguez-Medina *et al.,* 2019). In the present study, while *K. variicola* strains tightly clustered on the phylogenetic tree, significant diversity between several individual isolates was discovered. For example, the SNP distances of strains 139V2, 173V2, 191V1, and 208V2 were greater than 100 when compared to the rest of the *K. variicola* strains. Moreover, within the closely related ST435 strains, the presence of diversity within the *K. variicola* was further validated using Jaccard distance metrics. Our findings highlighted the distinctiveness of strain 52248_173V2 compared to the other strains, as it exhibited the highest Jaccard distance, demonstrating a greater genetic dissimilarity. This suggests that this strain may have experienced unique genetic modifications or acquired specific genetic elements not present in the other *K. variicola* strains of ST435 (Uruén *et al.,* 2020; Balasubramanian *et al.,* 2022). Further analysis of the data revealed the presence of additional genes in Isolate 52248_173V2, including metabolic-related genes, transporter genes, replication genes, translation genes, regulation genes and virulence genes, which were not observed in the other strains. In contrast, isolates 52253_139V2_Hi (139V2) and 52246_191V1 (191V1) exhibited comparable levels of dissimilarity from their closely related strains, indicating that they share

a comparable level of dissimilarity but have distinct genetic compositions from each other. These findings suggest the presence of multiple genetic subtypes or lineages within ST 435 each with its own unique genetic profile. These differences could be attributed to diverse evolutionary paths, the acquisition of specific genetic elements, or other factors such as horizontal gene transfer, mutation accumulation, environmental factors (e.g., host immunity and antimicrobial exposure), influencing their genetic makeup (Wiedenbeck and Cohan 2011; Oliveira *et al.,* 2017; Uruén *et al.,* 2020; Balasubramanian *et al.,* 2022).

A subset of strains within the *Klebsiella* population were found to harbour virulence genes, with the most prevalent ones being yersiniabactin (*ybt*) and colibactin (*clb*). Yersiniabactin plays a crucial role in the host's iron sequestration process, aiding in the acquisition of this essential nutrient (Wisgrill *et al.,* 2019; Lam *et al.,* 2018). On the other hand, colibactin is a genotoxic molecule that encodes a toxin responsible for disrupting the G2/M cell cycle, causing double-strand breaks in DNA, and inducing chromosome aberrations (Faïs *et al.,* 2018; Nougayrède *et al.,* 2021; Nougayrède *et al.,* 2006; Cuevas-Ramos *et al.,* 2010). These virulence genes have been frequently detected in the past in *Enterobacteriaceae*, including *K. pneumoniae.* The mobilisation of the *ybt* virulence genes in *K. pneumoniae* has been shown to depend on an integrative conjugative element (ICEKp) (Lam *et al.,* 2018). Furthermore, these genes can be transferred to plasmids carrying multiple resistance genes, facilitating co-transmission of virulence and resistance genes (Lam *et al.,* 2018; Gu *et al.,* 2018; Koczura and Kaznowski 2003). Although identifying the conditions that promote the spread of these virulence genes is outside the purview of this work, their presence highlights the importance of these genes since they can move into the plasmids of closely related organisms and mobilise resistance genes.

FII-K type replicons were found to be the most prevalent plasmid replicons in this study. This finding is consistent with past research that demonstrates that replicons associated with FII-K plasmids are widespread among *Klebsiella species* (Mohamed *et al.,* 2019). They are thought to be important reservoirs of antimicrobial resistance genes in clinical isolates of *Enterobacteriaceae* (Carattoli 2009; Mohamed *et al.,* 2019) and are frequently regarded in the literature as primary factors in the transmission of ESBL genes, notably the carbapenemase genes bla_{KPC} and $bla_{\text{CTX-M-15}}$ (Yao *et al.,* 2021) which were not found in the current investigation. Additionally, this study revealed two plasmids that may have recombined into a bigger plasmid (or *vice versa*), indicating that plasmid co-integration may have occurred. In

literature, this behaviour has been linked to IS*26* movement and is often associated with IS*26* duplication (Harmer *et al.,* 2014). IS*26* was associated with sequence inversion. This was particularly noticeable in strain 177V2_pBSKP3, which contained the same sequences as 139V2 plasmid1 but was inverted. Additionally, *K. variicola* strains had similar transmission mechanisms of sulphonamide genes. There was evidence of a copy of IS*26* that was adjacent to the *sul1* and *sul2* genes, comprising of a signature sequence shared with 22 *K. variicola* strains. This finding is consistent with previous research (Jiang *et al.,* 2019) linking transmission of *sul* genes with insertion sequences. Furthermore, evidence in the current study revealed IS*26* was also associated with trimethoprim genes which highlights its contribution in dissemination of these genes. However, despite this linkage under normal conditions mobilisations of IS*26* is relatively low (Harmer and Hall, 2021), although the frequency is reported to increase significantly under stress conditions. For instance, exposure to antibiotics, particularly those causing DNA damage, can induce the SOS response (Diaz-Diaz *et al.,* 2022), leading to a substantial increase in IS*26* activity. Similar increases in mobilization frequency can occur with exposure to heavy metals, extreme environmental conditions such as temperature extremes, nutrient limitation, and oxidative stress (Nagajyoti *et al.,* 2010; Sachdev *et al.,* 2021; Shin *et al.,* 2022). The increased occurrences, during these situations emphasize the risk of spreading of resistance genes with the help of IS*26* (He *et al.,* 2015; Oliva *et al.,* 2018). This emphasizes the significance of monitoring and managing environmental pressures to reduce the transmission of resistance. In our research IS*26* was detected in class 1 intergron situated on plasmids. pBSKP5 and pBSKP3 that carried these resistance genes. When IS*26* is present, on plasmids, which are genetic components it significantly boosts the potential for horizontal gene transfer (Weber *et al.,* 2019). Plasmids can move between bacteria through processes like conjugation or transformation aiding in the spread of resistance genes among bacterial populations and species (Smillie *et al.,* 2010; Millan, 2018).

The potential weakness of this study is its reliance on SCAi medium to select for *Klebsiella* species, which may generate false positives and false negatives (Van Kregten *et al.,* 1984). Various *Enterobacter*, *Serratia*, and *Citrobacter* species have been documented to mimic *Klebsiella* colonies on SCAi media, owing to their ability to utilize both inositol and citrate as carbon sources (Van Kregten *et al.,* 1984; Calderon-Gonzalez *et al.,* 2023; Raffelsberger *et al.,* 2023). Consistent with this, strains with *Klebsiella*-like colony morphology were observed, but which were identified as *Citrobacter* (n= 4). Conversely, slow-growing *Klebsiella* species that metabolise carbon sources slowly can form pale yellow colonies which can occasionally

be misinterpreted as white colonies that resemble to those of non-*Klebsiella* species, resulting in false negatives (Van Kregten *et al.,* 1984; Raffelsberger *et al.,* 2023).

In conclusion, this study has revealed that soldiers can be asymptomatically colonised by *Klebsiella* strains over the course of overseas deployment. Although colonisation with *Klebsiella* species *was* mostly transient, this knowledge is relevant as soldiers have a potential to be infected by these strains following injuries. It also suggests that *Klebsiella* clones can be spread across military bases to multiple individuals, as evidenced by the detection of very similar *K. variicola strains* that were found in multiple subjects.

Chapter 3

Genomic Analysis of Diverse *Escherichia coli* Isolates Derived from Poultry and Pigs Farms in Tanzania

3.1. BACKGROUND

Animal agriculture plays an important role in meeting the increasing demand for meat products (Agus and Widi, 2018). Livestock farming is a part of the industry making significant contributions to economic growth and ensuring food security (Ghose, 2014; Gandhi and Zhou, 2014). Pig and poultry farming are particularly important in animal protein production due to their high productivity rates (Cao and Li, 2013). Chickens, turkeys and ducks are commonly used for meat production because of their high production rates and ability to convert feed into meat efficiently (Kitalyi, 1998; Chowdhury *et al.,* 2023). Besides their growth pigs are also known for producing meat of quality (Garnett *et al.,* 2023). With the increasing demand for poultry and pig products, there has been a rise in commercial farming practices such as intensive farming and backyard farming (Mottet and Tempio, 2017; Castro *et al.,* 2023). These farming methods have also been linked to the emergence of resistance due to the frequent use of antibiotics (Mottet and Tempio, 2017). AMR poses a challenge to global health reducing the effectiveness of antibiotics (Hu *et al.,* 2020).Therefore, it is crucial to understand the causes of AMR in pig and poultry husbandry in order to implement strategies for mitigation.

There are several aspects of the pig and poultry husbandry practices that influence the development and spread of AMR (Ma *et al.,* 2021; Tian *et al.,* 2021; Peng *et al.,* 2022). Among them are high stocking densities, frequent use of antibiotics, and a lack of biosecurity measures in intensive farming systems which can foster the emergence and spread of AMR (Gilbert *et al.,* 2015; Murphy *et al.,* 2018). In intensive farming settings, AMR spread may also be facilitated by the limited genetic diversity of commercial breeds of pigs and poultry (Murphy *et al.,* 2018). Animals with limited genetic diversity are likely to have similar susceptibility to infections which may contribute to frequent use of antimicrobials. This in turn may create strong selective pressure which may lead to spread of resistant bacteria. Conversely, backyard farming presents its own unique risks, which contribute to the spread of AMR (Hedman *et al.,* 2019; Hedman *et al.,* 2020b). In backyard systems, animals may come into contact with humans, birds, rodents, insects and other animals that carry antibiotic-resistant bacteria (Mete *et al.,* 2013; Clothier *et al.,* 2018) and these interactions might help spread resistant strains within a backyard flock or herd. As a common practise in backyard systems, scavenging enhances animal exposure to reservoirs of AMR, since it exposes animals to a variety of food sources (i.e., kitchen scraps and agricultural waste), all of which can be sources of resistant

bacteria (Hamilton-West *et al.,* 2012; Correia-Gomes and Sparks, 2020). Moreover, contaminated feed associated with non-commercial feed, including homemade blends or regional feed sources, can introduce antibiotic resistant bacteria in backyard systems (Samanta *et al.,* 2018). For instance, in the preparation of homemade blends animal keepers usually use ingredients from various sources including plants that may be affected with microbial pathogens or food remains from the household which may be contaminated with various microorganisms (Crawshaw, 2012). These preparations often lack quality control measures, hence there is a chance of contamination during mixing or storage due to poor hygiene (Crawshaw, 2012). Arguably more importantly, the limited access to veterinary care and the use of over-the-counter antimicrobials in backyard poultry farming has a significant impact on the selection and maintenance of AMR (Tenzin *et al.,* 2017). Inadequate veterinary supervision, greatly increases the risk of inappropriate antibiotic use, which may involve underdosing or overuse of broad-spectrum antibiotics, both of which can promote the emergence and spread of AMR strains in backyard flocks (Cantón *et al.,* 2013).

The use of antimicrobials contributes significantly to the selection pressure in both domestic and industrial farming (Jibril *et al.,* 2021; Dias *et al.,* 2021). Transmission of resistant bacteria can also occur due to direct contact between animals. For instance, when pig and poultry farms are in close proximity to each other, there is a high possibility for animals to share resistant strains (Gilchrist *et al.,* 2007; Gržinić *et al.,* 2023). Other factors that are known to facilitate the emergence and spread of resistant bacteria are associated with geographical factors including climate and the environment, use of antimicrobials, as well as disease prevalence (Hao *et al.,* 2016; Tu *et al.,* 2023; Zhao *et al.,* 2021). Areas with high prevalence of diseases are prone to increased use of antimicrobials, hence risk the selection for resistant bacteria (Ikhimiukor and Okeke, 2023). In farm settings, the application of biosecurity measures remains one of the most efficient preventive ways to manage disease spread without the use of antimicrobials (Kariuki *et al.,* 2023). These measures may involve proper waste management, frequent animal checks and vaccination of animals, isolation of sick animals, proper waste management, sanitation and controlled farm access (Scollo *et al.,* 2023). However, major limiting factors known to affect farmers from maintaining biosecurity measures include socioeconomic constraints (i.e. some farmers cannot afford frequent veterinary services), inadequate infrastructure and cultural habits (Bastidas-Caldes *et al.,* 2023; Lambraki *et al.,* 2023). Apart from this limitation, awareness and level of knowledge is a

known influence impacting the effective adoption and adherence to biosecurity measures amongst farmers (Lambraki *et al.,* 2023; Bastidas-Caldes *et al.,* 2023; Mallioris *et al.,* 2023).

In conclusion, the interplay between farm practises and environmental factors can significantly affect the selection and spread of AMR (Scollo *et al.,* 2023; Kariuki *et al.,* 2023; Lambraki *et al.,* 2023). Implementing targeted, localised interventions and promoting sustainable farming methods that effectively address the development and spread of AMR requires a top-down understanding of regional variations in farming systems (Larson *et al.,* 2023).

This study focuses on several districts of Mwanza (Tanzania) where poultry and pig samples are reared in various farm types. Thus, the aim is to gain an understanding of AMR patterns within selected areas, potential transmission pathways, and underlying mechanisms of resistance. This will be achieved by examining AMR patterns of strains within different livestock populations, identifying lineages shared across animal groups and genes associated with each resistance. Additionally, interactions between resistance genes will be investigated within the target population to determine if there are any patterns or occurrences that contribute to co-selection. Furthermore, this study aims to uncover virulence factors by examining the role of plasmids, in transmitting AMR between reservoirs.

3.2. METHODS

The work described in this thesis, involves strains that were previously isolated by Mtemisika and colleagues (Mtemisika *et al.,* 2022). That study aimed to provide an overview of the phenotypic characteristics of *E. coli* strains isolated from pigs, broilers, and layers from the Mwanza region of Tanzania. The work associated with the design of the study, selection of study locations, farms and collection of samples was conducted prior to my involvement in the project. I did not participate in the initial isolation and identification of the *E. coli* strains. For the sake of completeness, I have outlined the methodology that was used by (Mtemisika *et al.,* 2022) in sections 3.2.1, 3.2.2, and 3.2.3 below. Information and samples in sections 3.2.4 - 3.2.6 was also collected by (Mtemisika *et al.,* 2022). Our work began with the extraction of DNA from selected *E. coli* isolates. This initial process was conducted in Tanzania at the Catholic University of Health and Allied Science (CUHAS) microbiology laboratory. DNA samples were prepared and shipped to the UK for whole genome sequencing and subsequent analyses.

3.2.1. Ethical approval

Isolates used in the current study were part of a study originally conducted by (Mtemisika *et al.,* 2022) which received ethical approval from the joint CUHAS/BMC Research Ethics and Review Committee, certificate number CREC/474/2021. As part of that study, farmers were asked to sign permission forms from Livestock and Fisheries authorities (Mtemisika *et al.,* 2022). Anonymised laboratory identification numbers were assigned and used consistently throughout the study.

3.2.2. Study design and locations

The cross-sectional study carried out by Mtemisika and colleagues was a conducted between June and August 2021. The study focused on domesticated poultry and pigs reared in 16 wards

located in three districts in the Mwanza region, Tanzania. Specifically, the study areas included the districts Nyamagana (specifically the towns Nyegezi, Buhongwa, Igoma, Busenga, Kilimahewa, Lumala, Mahina alliance, Malimbe, Mkolani, Nyamongolo, and Mabatini), Ilemela (Buswelu, Kiseke PPF, Nyamanoro, and Pasiansi), and Misungwi (Nyashishi) (Figure 3.1).

3.2.3. Selection of farms

Mtemisika *et al.* (2022) selected farms from a representative list of pig and poultry farms provided by livestock authorities. Poultry farms with a minimum of 100 birds and pig farms housing animals aged 20 weeks or older were included in the sampling framework. A total of 29 farms were enrolled, consisting of 9 pig farms, 9 broiler farms, and 11-layer farms. The current study focused on a distinct subset associated with a total 111 isolates from distinct animals comprising of 37 broilers, 35 layers and 39 pigs, which were selected for further downstream analysis using whole genome sequencing.

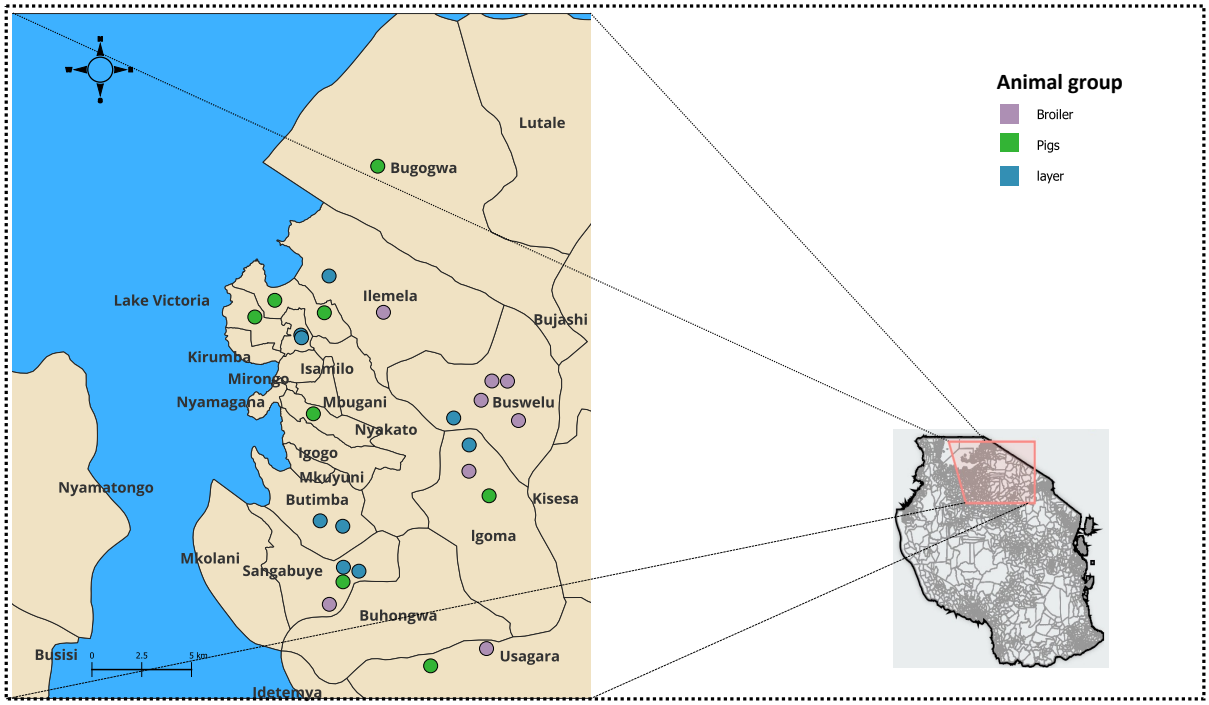

Figure 3.1: Locations of the selected farms in the Mwanza region where poultry and pig samples were collected. Broiler farms labelled in purple, pig farms in green and layer farms in blue.

Table 3.1: Characteristics of farm where isolates used in the present study were derived, as provided by (Mtemisika *et al.,* 2022)

FARM CHARACTERISTICS	BROILER	LAYERS	PIGS
ANTIBIOTIC USE			
Therapeutic	22	36	41
Prophylactic + therapeutic	15	0	0
FREQUENCY OF ANTIBIOTIC USE			
Once a week	4	0	0
Once in 1-2 months	0	34	4
Once in 2-4 weeks	33	2	0
Once in 3 months	0	0	27
FARM TYPE			
Backyard	14	33	20
Structured farm	23	3	21
LOCATION			
Ilemela	15	2	13
Misungwi	0	2	5
SOURCE OF CHICKS			
Amadolly	3	4	
Interchick	4	4	0
Kenbreed		3	
Silverlands	29	25	
Malawi	1		
FARM TRAINING			
Yes	8	18	5
No	29	18	35
WITHDRAWAL			
Yes	8	25	5
No	29	11	36
USE OF LAB RESULTS*			
Frequently	8		
Yes rarely	25	25	5
Never	4	11	36
*In directing antimicrobial use			

***** The statement "**Use of lab results**" in table represents those farms that relied on laboratory results before use of antimicrobials

3.2.4. Farm characteristics

The information presented in Table 3.1 gives an overview of the characteristics of farms as described by Mtemisika *et al.* (2022). The table provides includes characteristics of the farms involved, the reasons behind antimicrobial usage on each farm, frequency of use and source of chicks (i.e. hatcheries). Data on farmers' training on biosecurity measures and proper antimicrobial usage are also included.

3.2.5. Use of antimicrobials across farms

A breakdown of antimicrobial use in in different farms across the three animal populations is illustrated in the image below. Data used for constructing the image was sourced from (Mtemisika *et al.,* 2022). The data indicates that all three types of farms used oxytetracycline. As for other antimicrobials, use varied across different farms (Figure 3.2).

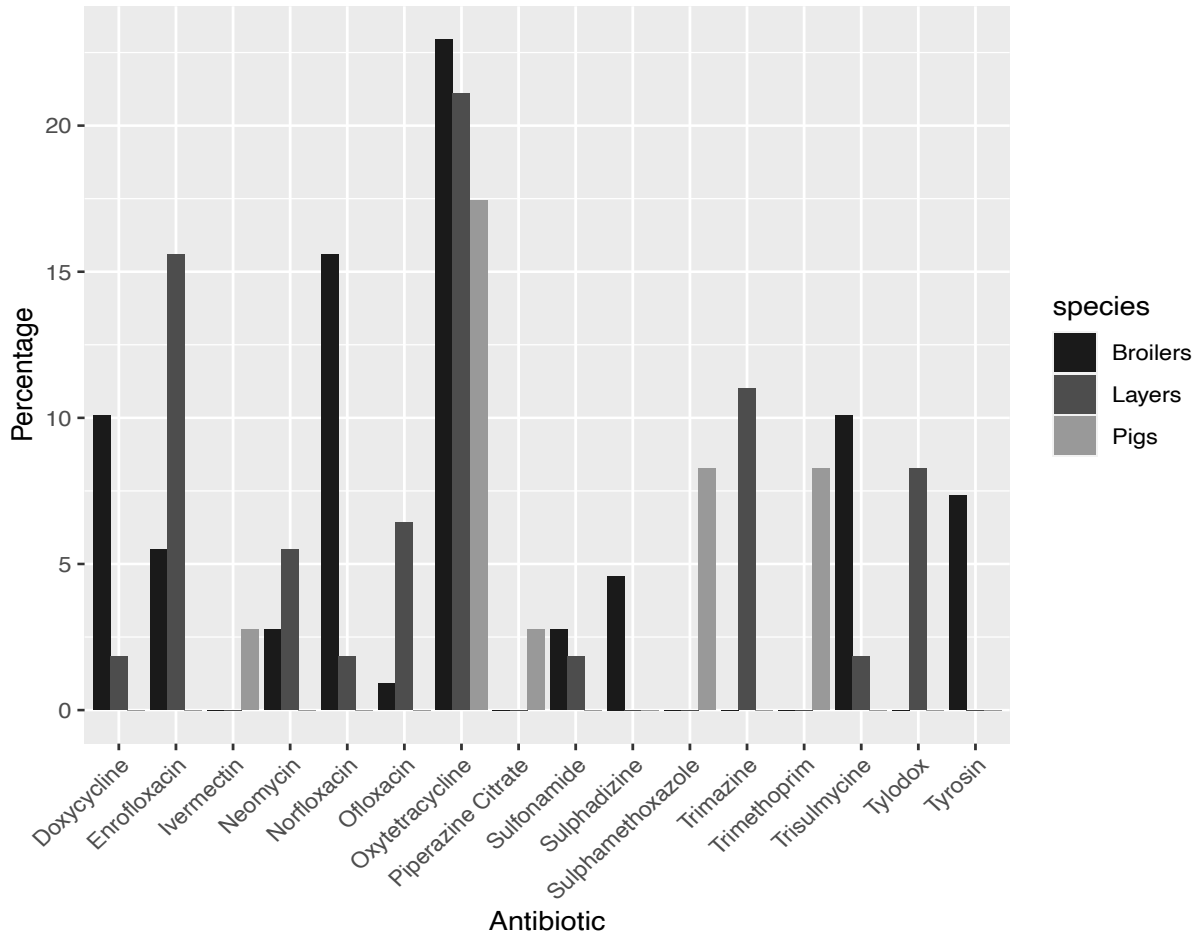

Figure 3.2: Antimicrobial used per animal population according to the self-reported use by farm owners. Some of the antimicrobials listed in the graph including Trimazine, Trisulmycine and Tylodox are combination drugs. Trimazine and Trisulmycine are both combination drugs that contain trimethoprim and sulphadiazine. Tylodox, on the other hand, is a combination drug that contains both tylosin and doxycycline.

3.2.6. Farms

Samples were collected from farms associated with three different animal groups (i.e. broilers, layers, and pigs). Samples were collected randomly from animals of different ages (Figure 3.3).

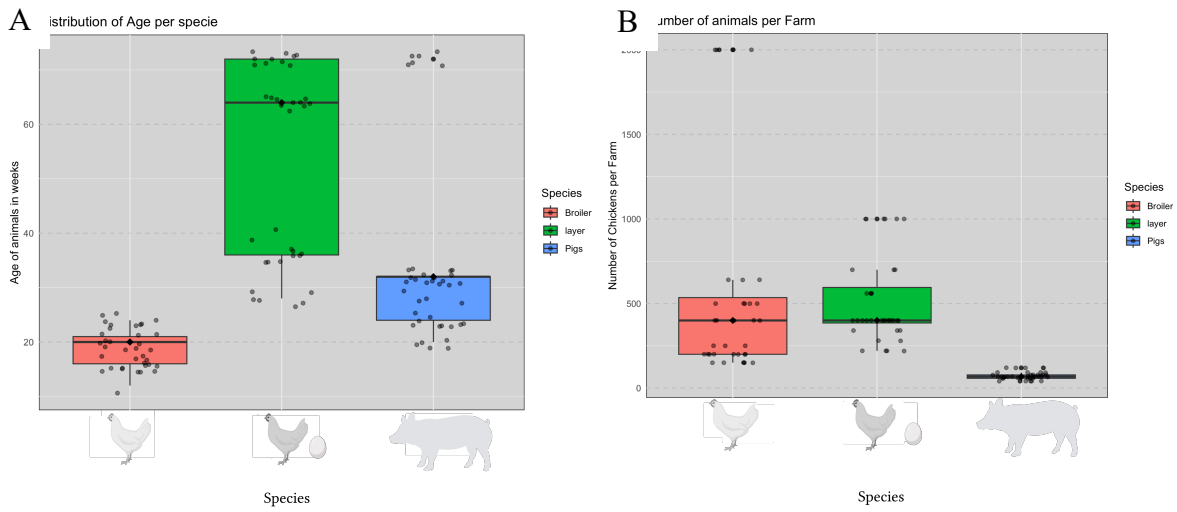

Figure 3.3: Data on animal age and size of sampled farms. Panel A: age distribution of animals, Panel B: size of farms. Data used to generate the image was obtained from (Mtemisika *et al.,* 2022).

3.2.7. Sample collection

Using sterile cotton swabs, faecal swabs were taken from the rectums of pigs and the cloaca of chickens. A gentle insertion of a sterile swab into the respective area followed by rotation, ensured adequate sample collection. The samples were collected and within eight hours they were shipped to the microbiology lab at the Catholic University of Health and Allied Sciences (CUHAS) in Mwanza, Tanzania, using Stuart transport media in a sterile, cold box.

3.2.8. Laboratory procedure

The entire *E. coli* isolation process described in the section below was carried out by (Mtemisika *et al.,* 2022). As previously mentioned, DNA extraction, subsequent steps in preparation for sequencing and bioinformatic analyses of sequence data was conducted in the present study.

3.2.9. Isolation of *E. coli* isolates

Briefly, the protocol involved placing of fresh faecal swabs directly onto plain MacConkey agar (MCA, HiMedia, India) and incubation of samples aerobically at 37°C for a duration of 18-24 hours. Colonies that appeared pinkish, round, medium-sized, and flat were chosen and subcultured on MacConkey agar to isolate pure cultures after incubation at 37°C for 16-20 hours. To confirm the presence of pure growth resembling *E. coli*, presumptive *E. coli* colonies were subjected to in-house biochemical identification tests (Mtemisika *et al.,* 2022). Pure cultures of presumptive *E. coli* isolates were archived in -80°C in a mixture of 15% glycerol and LB for long term storage and for further analysis.

3.2.10. DNA extraction and genome sequencing

DNA extraction and concentration determination was conducted according to section 2.2.4 and section 2.2.5, respectively. Illumina sequencing was conducted based on section 2.2.6 and *E. coli* str. K12 substr. MG1655 (NCBI accession: NC_000913.3) was used as a reference strain in phylogenetic analysis.

3.2.11. Multi-locus sequence typing and Clermont typing.

The Achtman scheme (Wirth *et al.,* 2006) was employed to perform *in silico* multi-locus sequence typing (MLST). Phylogroups were determined using ClermontTyper and EzClermont tools (Clermont *et al.,* 2013; Clermont, Gordon and Denamur, 2015). To ensure taxonomic identification and identify potential contaminants (non-target sequences), Centrifuge, a specialised tool designed for taxonomic classification, was used (Kim *et al.,* 2016).

3.2.12. Determination of accessory gene content

For the prediction of virulence factors, acquired antimicrobial resistance (AMR) genes, and plasmid replicons, we employed ABRicate v0.9.8 (https://github.com/tseemann/abricate) and PlasmidFinder v2.0.1 (Khezri *et al.,* 2021). The contigs were scanned against the VFDB, ResFinder, and PlasmidFinder databases, utilizing an identity threshold of ≥ 90% and a coverage of ≥ 70%. The identified virulence factors and AMR genes were visualized alongside the phylogenetic tree using Phandango (Hadfield et al., 2018).

3.2.13. Analysis of the average nucleotide identity

Average nucleotide identity (ANI) was calculated using FastANI v.1.32 (Jain *et al.,* 2018) to determine the genomic similarity between *E. coli* isolates and the reference strain *E. coli* str. K12 substr. MG1655. The ANI value was computed in percentage.

3.2.14. Plotting geographical data

Using QGIS (v3.32.1; Open Source), the geographical distribution of farms, as well as patterns of antimicrobial use, and plasmid replicons were plotted. For reasons of confidentiality, farm points were randomly shifted within a 5 km radius of their original locations and plotted on the maps.

3.2.15. Statistical analysis

The statistical analysis was performed with R (version 3.6.1). To compare between different categorical variables, we conducted Chi-square (χ^2) test. Pearson's correlation was employed to determine the correlation between the relative abundances of genes associated with different antimicrobial classes. To determine the correlation pattern between virulence and resistance genes, linear regression was used. The strength of the relationship between virulence and resistance genes was evaluated using the coefficient of determination (R^2 value), which quantified the extent to which variability in virulence genes can be explained by changes in antimicrobial resistance genes.

3.3. RESULTS

A total 114 *E. coli* genomes were successfully sequenced and assembled. These genomic assemblies obtained from short read Illumina sequencing were analysed, revealing a range of assembly sizes from 4,375,796 bp to 6,380,587 bp, with an average length of 4,970,221 bp (Figure 3.4). The assemblies had a GC content of 50.12%-53.3%, with a mean of 50.7%. The mean N50 value, which reflects assembly contiguity, was 213,221 bp, with the lowest N50 being 6,378 bp and the highest 678,534 bp. Using *Escherichia coli* strain K12 substr. MG1655 as the reference, an Average Nucleotide Identity analysis was carried out to assess how similar the genomes of the *E. coli* in our dataset were to the *E. coli* reference (Figure 3.5). The mean ANI value of the dataset was 98%. Outliers in the genome distributions were identified. Notably, three genomes in the dataset were found to have ANI values estimated to be less than 95% from the reference *E. coli* which were further examined for contamination, completeness, summarised in Table 3.2. Following the Clermont typing and Achtman's pubMLST analysis these sequences were removed from the dataset as their genomes were of poor quality and their identification could not be accurately determined. However further analysis of the centrifuge output revealed dominance of species other than *E. coli* in these sequences demonstrating they could be originating from other species. L96 contained a higher abundance of *Pantoea ananatis* at an estimated level of 0.468, whilst P118 exhibited an abundance of 0.78 of Citrobacter and P52 had an abundance 0.46 of *Ralstonia pickettii*. Thus, a total of 111 *E. coli* strains remained and were used for further analysis.

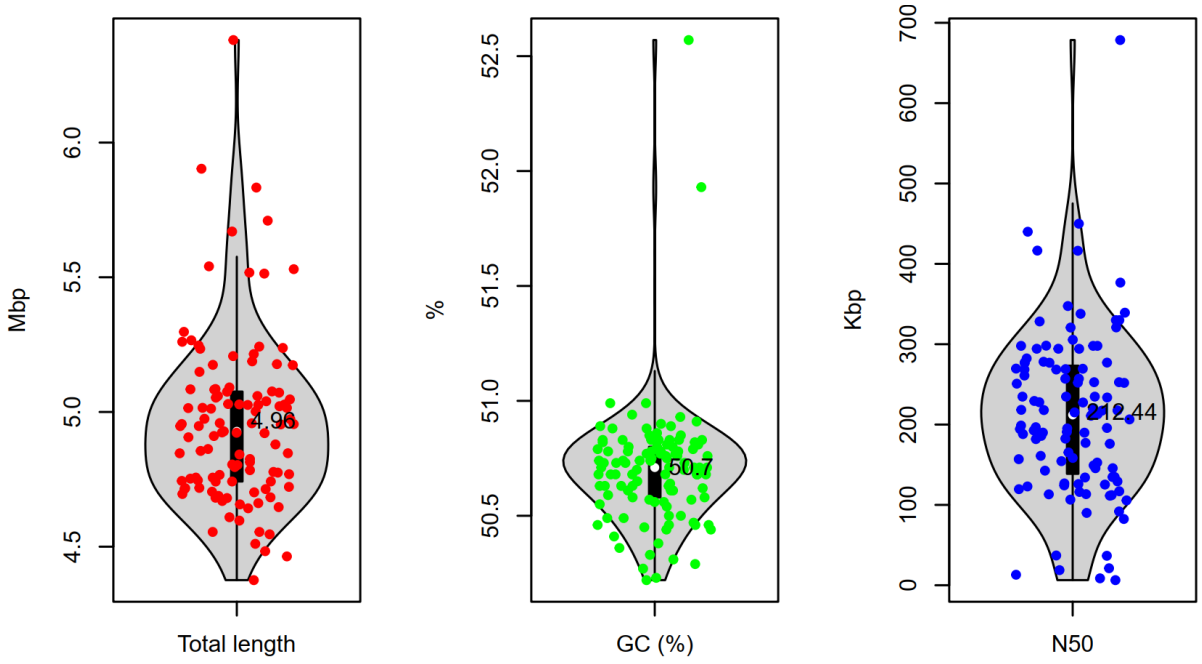

Figure 3.4: Distribution of the median sequence length for the assemblies in the dataset, the median %GC content, and median N50 value for each isolate, along with their respective values.

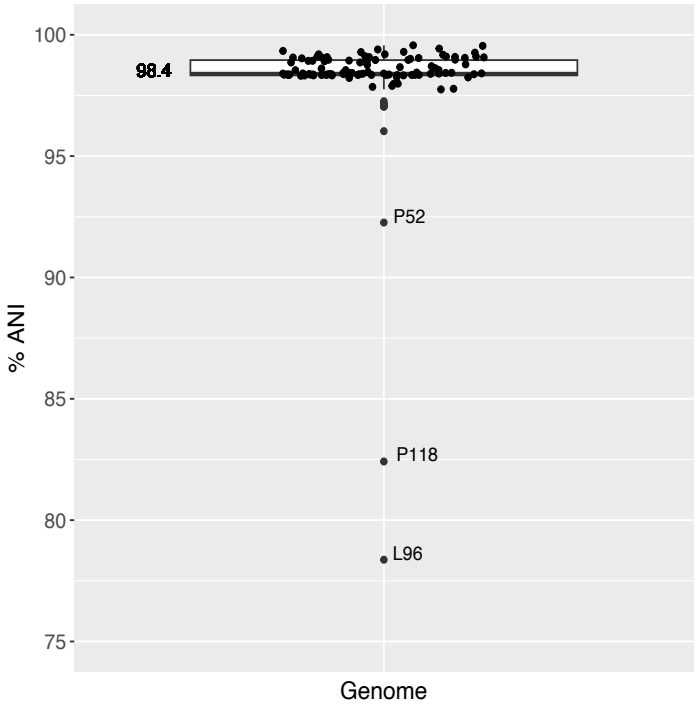

Figure 3.5: Distribution of Average Nucleotide Identity (ANI) values of the genomes in reference to *Escherichia coli* strain K12 substr. MG1655.

Table 3.2: Quality of *E. coli* genomes excluded from the dataset.

Assembly	# contigs	GC (%)	N50[1]	L50[2]	Contamination (%)	Completeness (%)	pubMLST output[3]
L96	19	53.33	345842	3	7.43	78.82	ND
P118	23	51.93	439987	5	6.61	93.02	ND
P52	42	50.59	347508	5	1.25	96.71	ND

[1]N50 - shortest contig length in a set of long contigs which combined cover 50% of the overall genome.
[2]L50 - count of contigs required to cover 50% of the overall genome.
[3]pubMLST output –STs identified based on Achtman's scheme.

3.3.1. Distribution of phylogenetic groups across *E. coli* strains

Phylogroup B1 was the most prevalent phylogroup (47/111) in the dataset followed by phylogroup A, D, F, G, and E (Table 3.3). Phylogroup A and B1 were represented in all three subgroups of animals. Phylogroup D strains were mostly found in broilers, whereas phylogroup C strains were found in both broilers and layers. Strains in phylogroup E were exclusively associated with layers and pigs, while those in phylogroup G have exclusively been linked to broilers.

Table 3.3: Distribution of animals according to phylogenetic groups.

PHYLOGENETIC GROUP	ANIMAL TYPE	NUMBER	PROPORTION PER PHYLOGROUP (%)
A (N=45)	Boiler	13	29
	Layer	14	31
	Pigs	18	40
B1 (N=47)	Broiler	12	26
	Layer	16	34
	Pigs	19	40
C (N=1)	Broiler	1	100
D (N=6)	Broiler	5	83
	Layer	1	17
E (N=3)	Layer	1	33
	Pigs	2	67
F (N=5)	Broiler	2	40
	Layer	3	60
G (N=4)	Broiler	4	100

3.3.2. Distribution of STs across animal categories

The distribution of STs among our study strains was examined to determine predominant lineages and their diversity within the *E. coli* population (Figure 3.6). ST1196 was the most prevalent ST (14% (16/111)), followed by ST155 (8% (9/111)), ST48 (5% (5/111)), ST4516 (4% (4/111)), and ST711 (4% (4/111)) (Figure 3.6). Compared to layers and pigs, layers demonstrated a higher occurrence (n=8) of ST1196 compared to broilers (n=6) and pigs (n=1).

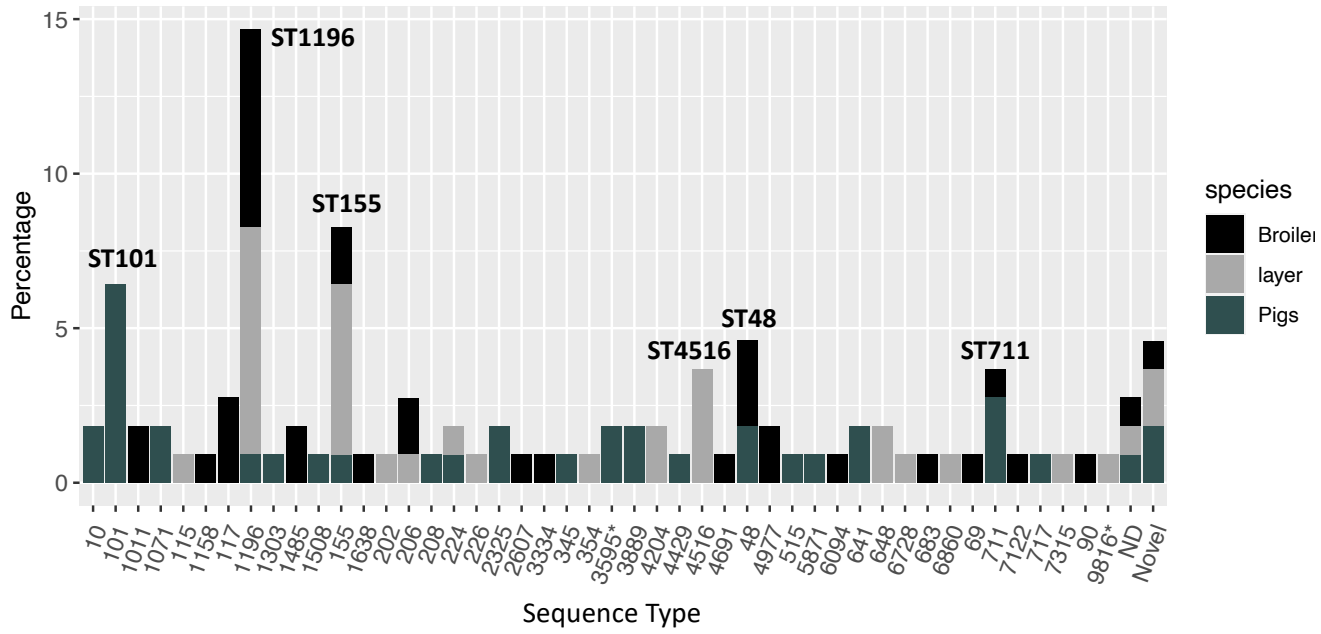

Figure 3.6: Overview of *E. coli* sequence types (STs) identified in this study. The proportion of different *E. coli* sequence types (STs) (n=49), with each distribution split based on the distribution of pigs, broilers, and layers. The Y axis shows the percentage of STs, and the X axis shows sequence types.

3.3.3. Distribution of STs across different locations

Our data revealed distinct associations between locations and STs. Several STs that were shared across multiple locations, while others were location-specific (Figure 3.7). The STs found in multiple locations included ST206, ST4516, ST48, ST155, ST1011, ST1196, ST3595, ST4204, ST101, ST224, ST641, and ST711.

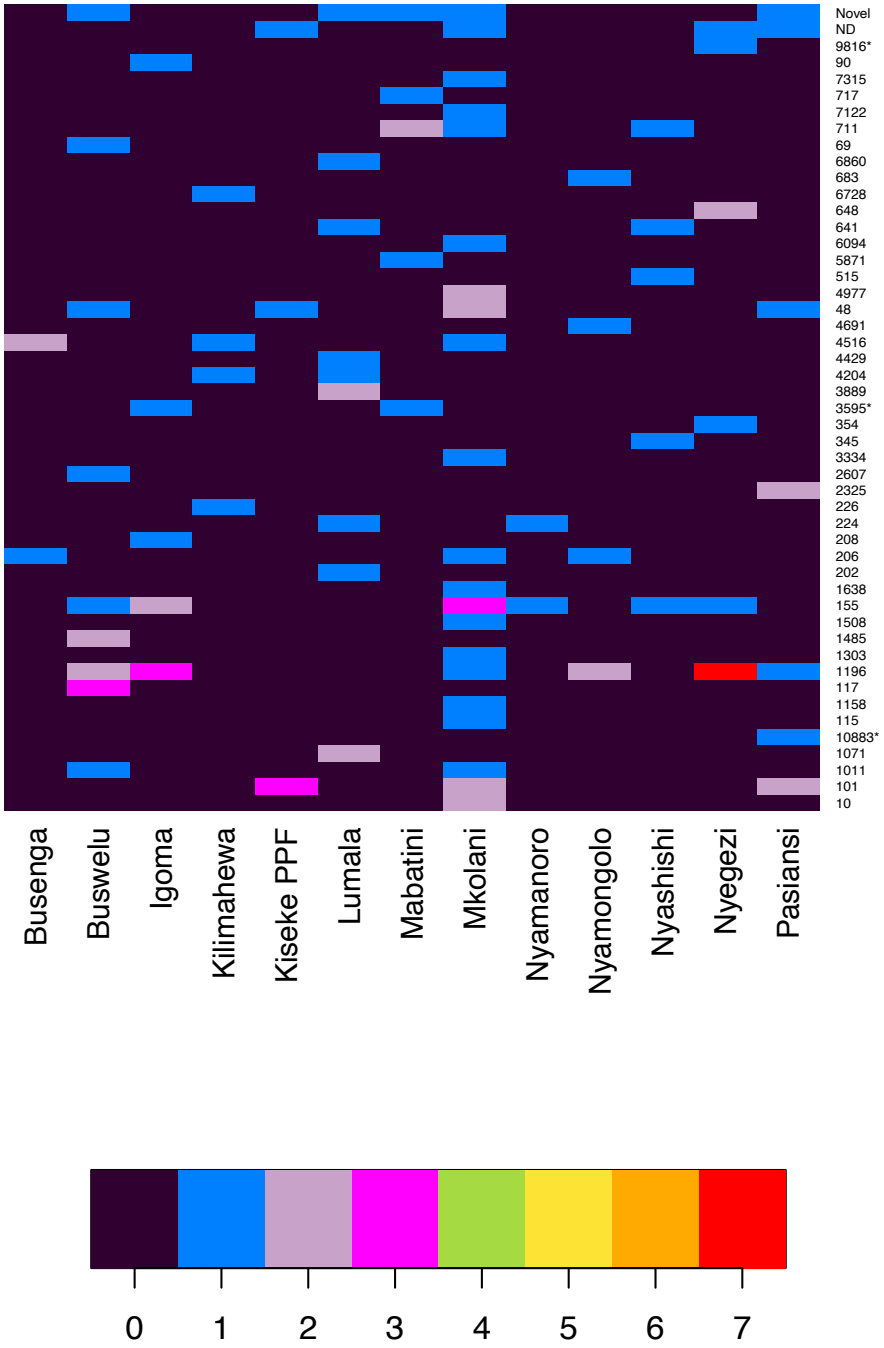

Figure 3.7: Distribution of different STs across various study locations. In this illustration STs were grouped based on their commonalities across the locations, demonstrating STs that were frequently found. Each colouring represents frequency or occurrence of STs in different locations. The clustering of each ST in each row helps identify the ST which is most prevalent across locations.

3.3.4. Cross-reservoir sharing of strains between broiler, layers, and pigs.

Cross reservoir analysis was conducted, to identify reservoirs shared amongst multiple populations. Our goal was to gain insight into potential strain transmission between different animal species and identify the genetic lineages shared across the three animal populations. Our observation revealed the presence of certain bacterial lineages that were shared across the three animal groups. There were four distinct lineages (ST48, ST224, ST711 and ST206) that were shared between two animal groups. ST224 was exclusively shared between layers and pigs indicating a link between these two populations. On the other hand, broilers and pigs shared lineages that belonged to ST711 and ST48 while layers and broilers shared ST206. Interestingly, ST1196 and ST155 emerged as the only STs shared across all three animal populations. These findings suggested potential linkage and transmission between different animal hosts. Further analysis revealed that ST48 and ST206 belonged to phylogroup A, the rest of lineages mentioned belonged to phylogroup B1. Figure 3.8 shows the lineages that were unique to each population, as well as the ones that overlap between them. Pigs displayed a higher frequency of STs shared within their population than other groups.

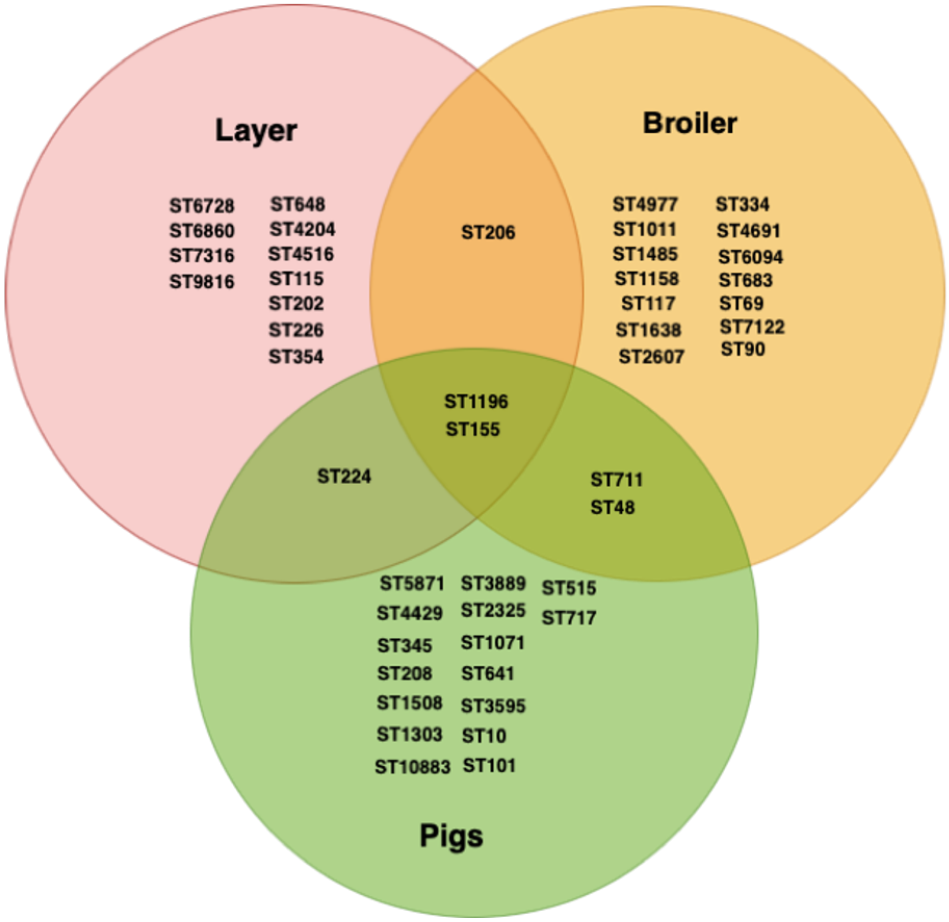

Figure 3.8: Distribution of STs across animal groups. Two STs are observed to circulate across all animal groups including ST1196 and ST155. The majority (n=41) of strains were clustered within distinct animal groups, four STs were shared by two animal groups.

3.3.5. Clonal lineages shared within and across different animal species and districts.

To understand the transmission dynamics and patterns of strains shared across different reservoirs or host populations, cross-reservoir analysis was carried out to identify links (geographical links, lineages, animal groups, and type of farms) between strains that were identical i.e., had identical

(0 SNPs) core genomes (Figure 3.9). Similar analysis was carried out on genomes that differed from one another by small numbers of SNPs (1-20 SNPs). A threshold of 20 SNPs has been adopted in this study as a threshold to determine closely related strains, in accordance with recent literature in microbial genomics (Szarvas *et al.,* 2021; Chomkatekaew *et al.,* 2023). This threshold has also been used in the past to define clones or strains that have a recent common ancestor in the context of short term outbreak investigations (Sherry *et al.,* 2021).

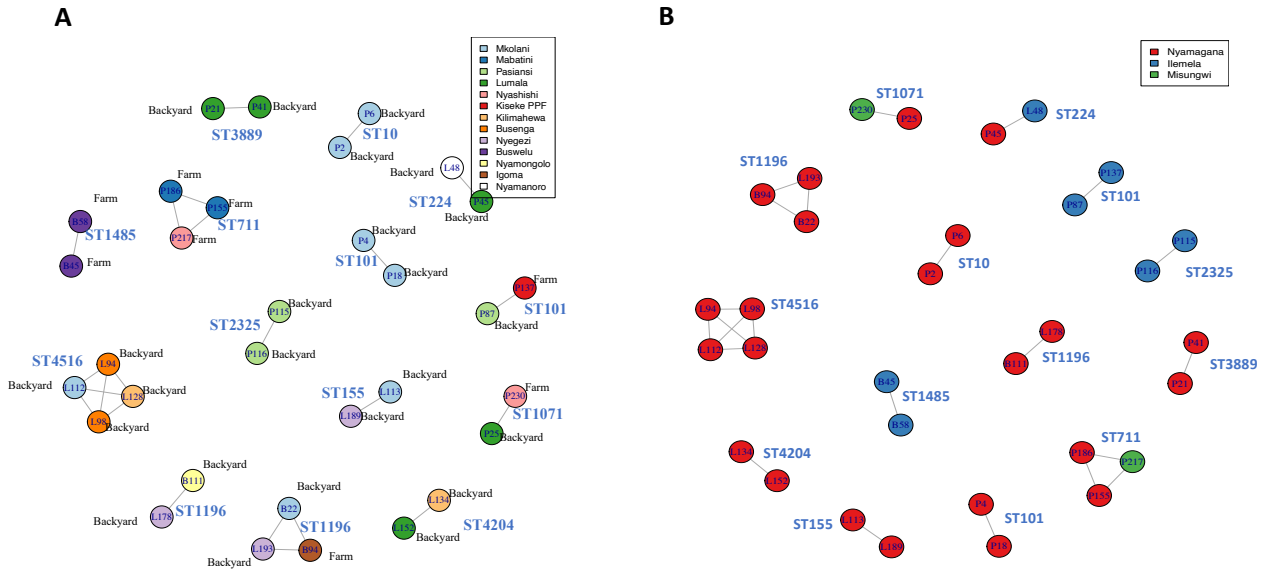

Figure 3.9: Distribution of strains with identical core genomes (0 SNPs). Strains clustered together are identical based on their core genome (0 SNPs). Locations are indicated by a different colour within the circle and are explained in both legends. Each sample comes from a different farm type, designated as either a backyard (small scale - household level) or farm (large scale). Animal types are denoted by the letters: "P" is for pig, "L" is for layers and "B" is for broilers. Panel A shows distribution across sites and panel B shows the distributions at district level.

Multiple strains with identical core genomes were detected across reservoirs in diverse locations. Sharing of strains with 0 SNPs was evident both within and between the different animals studied here. Transfer was more frequent within the same animal group (11 sharing events) than across animals (3 sharing events). Sharing events within animal groups were observed for ST10, ST101, ST1071, ST2325, ST1196, ST1485, ST155, ST3889, ST4516, ST711 and ST4204. Conversely,

across different animal populations sharing events were observed only for STs 1196 and 224. Most shared strains originated from backyard farms rather than structured farms.

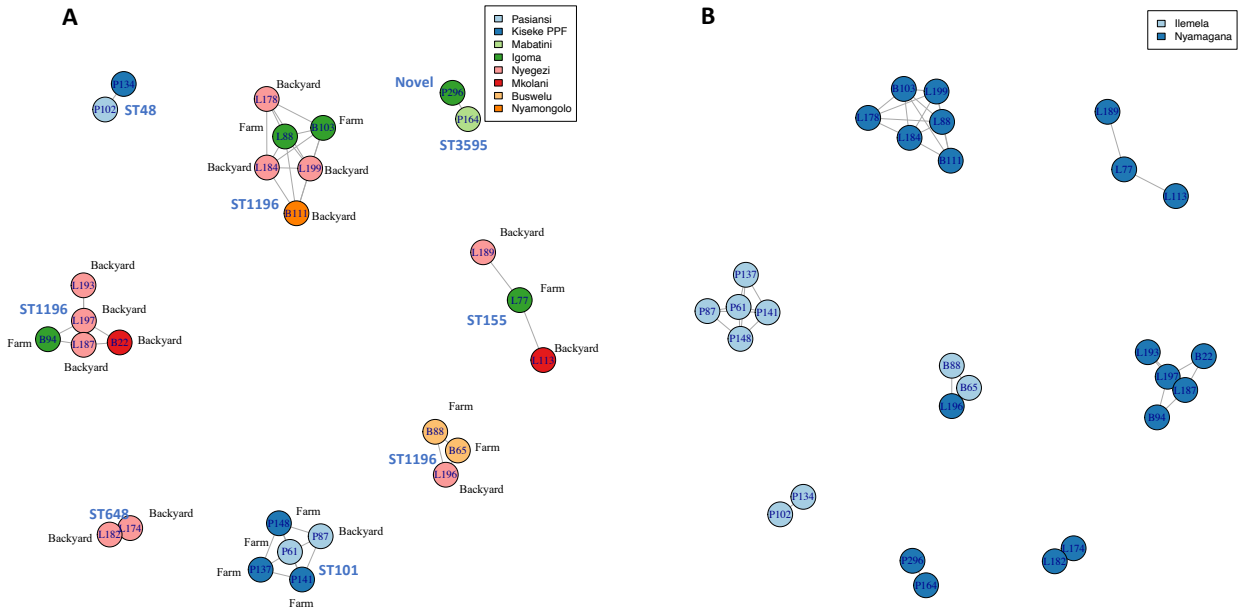

Figure 3.10: Distribution of closely related strains (≤20 SNPs in core genome). Origin of strains are indicated in different colours as depicted in the legends. Types of farms are represented in black labels, while orange labels indicate the hatcheries from where the poultry originate (in both panels A and B). The origin of strains is displayed using various colours as shown in the legends. Animal types are denoted by the letters: "P" is for pig, "L" is for layers and "B" is for broilers. Panel A shows distribution across sites and panel B shows the distributions at district level.

Different animal populations shared strains with low level-SNP distances (Figure 3.10). This phenomenon was more widespread among poultry than pigs, manifesting both in joint broiler-layer sharing events and separately within each group (i.e., within layers and broilers). In both animal groups, sharing events were observed in at least two animals for both poultry and pigs irrespective of farm sites. Occurrence of strains with low-level SNP distance sharing among poultry involved 6 animals, followed by pigs involving 5 animals. Furthermore, most of the animals which shared strains (both poultry and pigs) came from backyard farms, rather than structured farms. In our observations, sharing events of strains with low level SNP distances was more widespread in Nyamagana.

3.3.6. Phylogenetic relationship between *E. coli* strains and resistance gene distribution

Phylogenetic analysis was conducted to explore the relationship between different strains. Genes associated with AMR in the genomes were identified. Our findings demonstrated variable distribution of resistance genes, with considerable diversity seen across *E. coli* strains in the dataset. The presence of resistance genes was detected in all strains, phylogroups, and lineages (STs). There were 40 unique resistance genes found across 111 strains present in our dataset. Genes carried corresponded to 12 different antibiotic resistance classes including genes conferring resistance to tetracycline (*tetA*, *tetB* and *tetM*), sulphonamide (*sul1* and *sul2*), trimethoprim (*dfrA1*, *dfrA7*, *dfrA12*, *dfrA14*, *dfrA15* and *dfrA17*), aminoglycoside (*aadA2*, *aadA5*, *aadA8*, *aph(6)-Id*, *aph(3')-IIa*, *aph(3')-Ia*, *aph(3")-Ib*), quinolone (*qnrB9, qnrS1*, *qnrS11*, *qnrS13* and *qnrS2*), florfenicol (*flor*), phenicol (*cat*, *catA1*, and *catA2*), streptothricin (*sat*, *cmA-1* and *cmA-5*), fosfomycin (*fos1*), rifamycin (*arr-2*), macrolide (*mphA* and *mphB*) and β-lactamase genes (i.e. $bla_{\text{TEM-150}}$, $bla_{\text{TEM-235}}$, bla_{CMY} and bla_{OXA}). The presence of strains carrying multiple resistance genes was also discovered within the dataset. MDR strains were defined as strains with three or more resistance genes. The distribution MDR strains per animal group is shown in Figure 3.11. There were 10 strains that were devoid of resistance genes, which were associated with ST101 (n=7), ST2325 (n=2) and ST345 (n=1) strains from pigs.

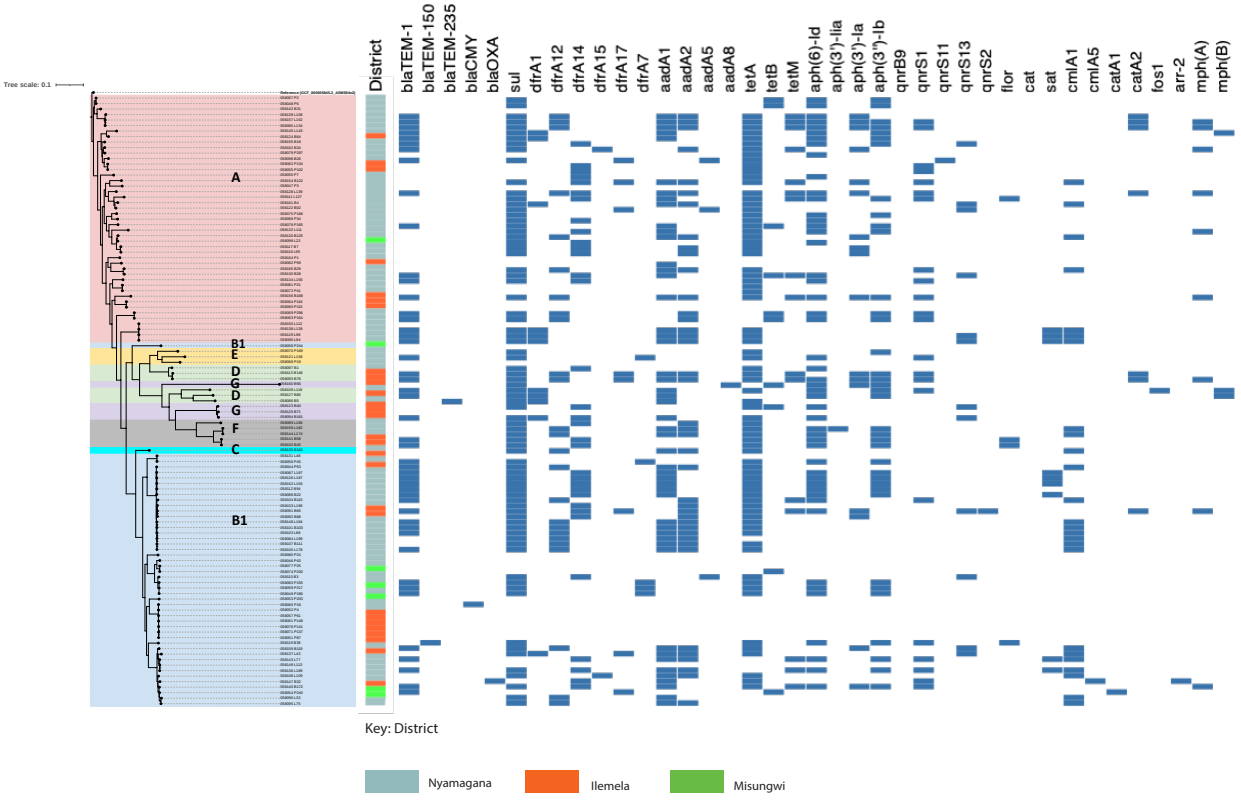

Figure 3.11: Presence of antibiotic resistance genes in *E. coli* genomes. A maximum likelihood (RAXML) phylogenetic tree of the *E. coli* core genome (n=111) with a heatmap showing the presence and absence of resistance genes. *E. coli* phylogroups are indicated by different colours.

3.3.7. Prevalence of predicted antimicrobial resistance patterns across animal species.

Our findings revealed variations in predicted resistance patterns between layers, broilers, and pigs. Across all animal groups, resistance genes to tetracycline, sulphonamide, quinolone, and aminoglycoside showed higher prevalence in broilers compared to the rest of the animal groups (Figure 3.12). By contrast trimethoprim, streptothricin and phenicol resistance genes were more prevalent in layers compared to the rest of the animal groups. Across all the antimicrobial classes, tetracycline resistance genes had the highest prevalence, and was detected in 80.2% (89/111) animals, followed by aminoglycoside resistance genes with a prevalence of 79.3% (88/111), sulphonamide resistance genes with a prevalence of 73.9% (82/111), and trimethoprim resistance

genes with a prevalence of 72.3% (81/111). Prevalence rates of the remaining classes are presented in Table 3.4. Chi-square analysis revealed there was a significant difference in prevalence rates of resistance genes across antimicrobial classes (X^2=592.3, df=11, p<0.0001). The table also illustrates comparison of resistance across the animal populations per antimicrobial class. The p-values in the table indicate whether the tested animal populations have statistically significant difference in their carriage of resistance genes of the indicated antibiotic classes.

Table 3.4: Distribution of predicted antimicrobial resistance across animal species.

Antimicrobial Class	Broiler(n=37) (%)	Layer (n=35) (%)	Pigs (n=39) (%)	Total	Chi-square (χ^2), (df)	P value
Tetracycline	35 (39.3)	32 (36.0)	22 (24.7)	89	20.02 (2)	**0.0001**
Trimethoprim	34 (42.0)	35 (43.2)	12 (14.8)	81	53.51 (2)	**0.0001**
Sulphonamide	35 (42.7)	34 (41.5)	13 (15.9)	82	49.84 (2)	**0.0001**
Quinolone	20 (52.6)	14 (36.8)	4 (10.5)	38	16.38 (2)	**0.0003**
Aminoglycoside	36 (40.9)	35 (39.8)	17 (19.3)	88	45.20 (2)	**0.0001**
Streptothricin	1 (9.1)	10 (90.9)	0 (0.0)	11	19.82 (2)	**0.0001**
Phenicol	6(50.0)	5 (41.7)	1 (8.3)	12	4.16 (2)	0.1248
Fosfomycin	0 (0.0)	1 (100.0)	0 (0.0)	1	2.163 (2)	0.3390
Rifamycin	1 (100.0)	0 (0.0)	0 (0.0)	1	1.991 (2)	0.3695
Macrolide	6 (50.0)	6 (50.0)	0 (0.0)	12	7.125 (2)	0.0284
ESBLs	3(75.0)	0 (0.0)	1(25.0)	4	3.542 (2)	0.1702
Bla-TEM1	20 (39.2)	22 (43.1)	9(17.6)	51	12.57 (2)	**0.0019**

(%), represents percentage values in brackets.
n, total number of samples per animal group
df, degrees of freedom
The **p-value** in this table compares the difference in the predicted resistance between the animal groups. Each row demonstrates the comparison of a single antimicrobial class across animal hosts (i.e. broilers, layers, and pigs)
Significant **P** values are bolded

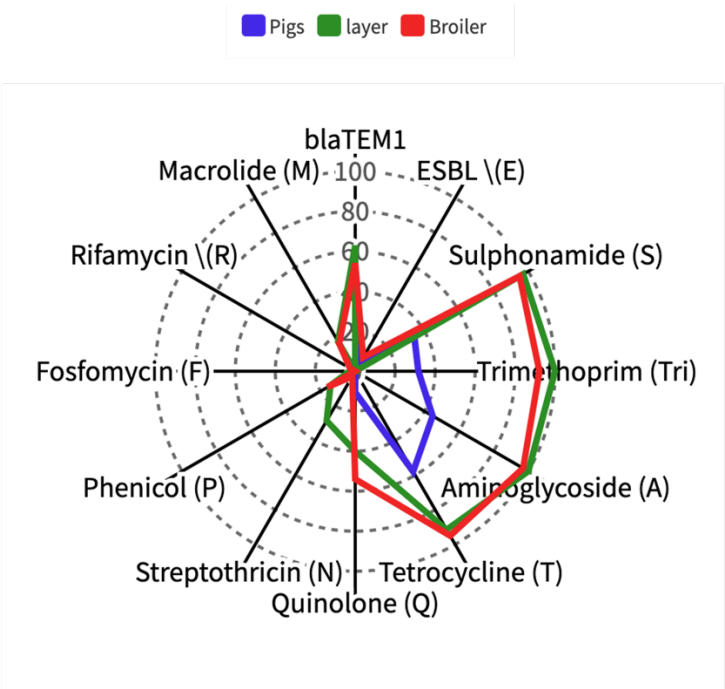

Figure 3.12: Distributions of predicted resistance profiles across animal groups. Red represents broilers, green represents layers, and blue represents pigs.

3.3.8. Patterns of multidrug resistance profile distribution in broiler, layer, and pig isolates

The distribution of predicted multidrug resistance (MDR) profiles was further studied the across the three animal populations (Table 3.5). MDR strains were defined as strains showing resistance to three or more resistance classes. Overall, our findings show there was no difference in MDR status between layers (100% (35/35) of strains are MDR) and broilers (97.3% (36/37) MDR) (Fisher's exact, p=0.2462). In comparison to both broilers and layers, the pig population exhibited a significantly lower number of MDR strains compared to broiler and layers (χ^2=125.6, df=2, p<0.0001). Among MDR strains, the highest number of antibiotic resistance gene classes in a single isolate was eight, which we found in two strains from broilers and nine strains from layers, but not in pig strains.

Table 3.5: Distribution of multidrug resistant strains across different animal groups

# of antimicrobial classes individual strains are resistant to	# of resistant strains			
	Broiler	Layer	Pigs	Total
Non-MDR				
0	1	0	15	16
1	0	0	6	6
2	0	0	1	1
Total Non-MDR	1	0	22	23
MDR				
3	1	3	7	11
4	6	7	2	15
5	11	6	6	23
6	13	10	2	25
7	3	5	0	8
8	2	4	0	6
Total MDR	36	35	17	88
Total	37	35	39	111

#, represents number of antimicrobial classes to which strains were predicted to exhibit. The numbers in the first column indicate the number of classes of resistance genes in a genome.

3.3.9. Correlation patterns of resistance genes

Pearson's correlation was used to identify classes of resistance genes that exhibit a pattern of co-occurrence. While most resistance classes had weak correlations ($r<0.5$), associations between tetracycline, aminoglycoside, trimethoprim, and sulphonamide resistance genes are significant with Pearson's correlations ranging from 0.5 to 0.8, demonstrating a pattern of co-occurrence between these resistance classes (Figure 3.13).

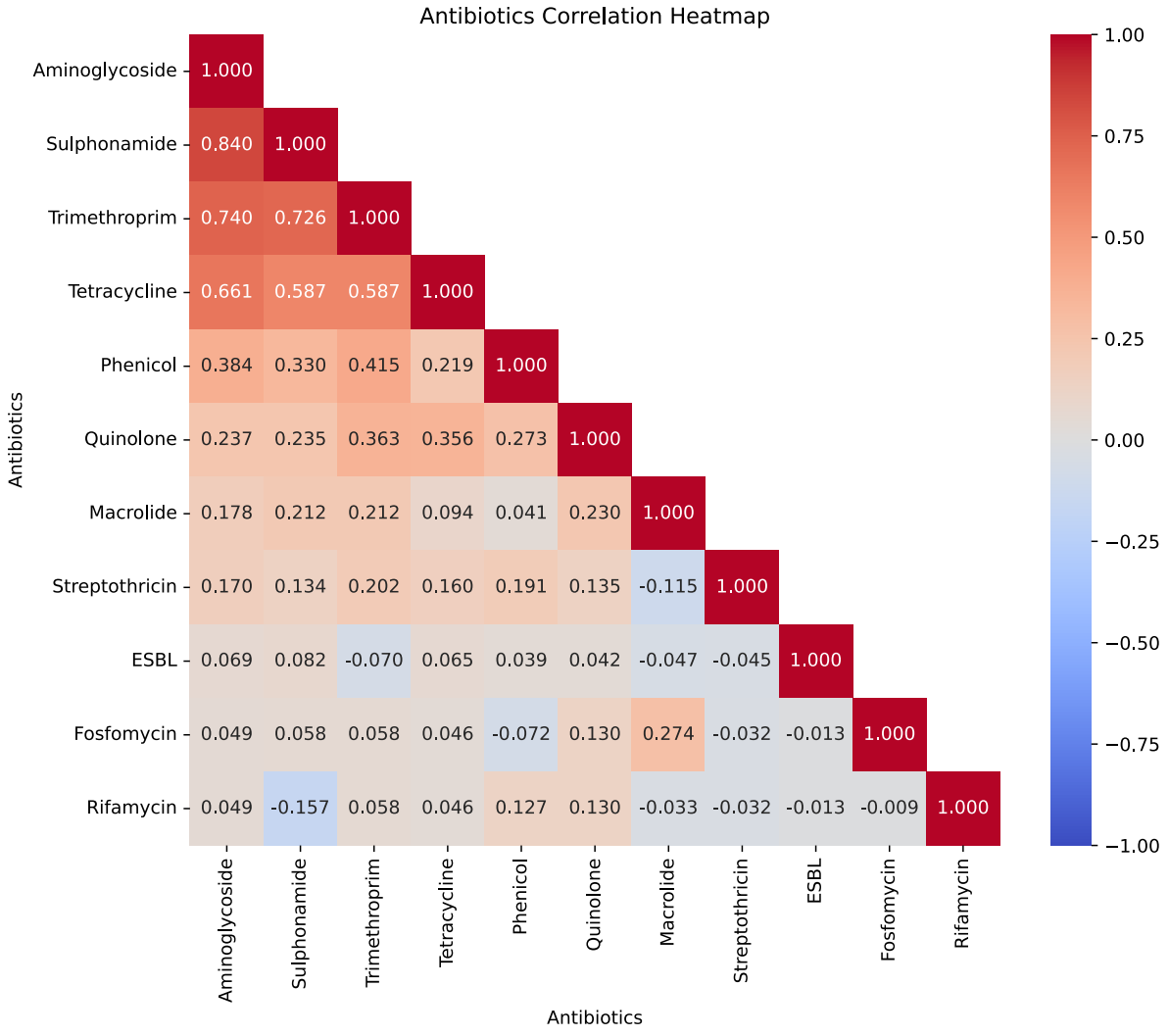

Figure 3.13: Correlation matrix of antibiotic resistance gene classes in *E. coli* strains. Pearson's correlations (r) between various genes conferring resistance to different antimicrobial classes in *E. coli* strains.

3.3.10. Diversity of plasmid replicons that co-occurred with resistance genes.

The objective of our analysis was to determine whether plasmid replicons and resistance genes co-occurred within genomes in our dataset. To achieve this, PlasmidFinder was first used to screen for the presence of plasmid replicons across genomes (n=111). Our initial screening revealed 106 genomes which contained plasmid replicons. Notably, 18 distinct types of replicons were identified. From the latter subset of genomes (n=106), contigs that carried both resistance genes and plasmid replicons were identified. ResFinder was used to screen contigs which were initially identified to have plasmids replicons to determine whether they also carried resistance genes. Our observation revealed there were 54 contigs (across 20 genomes), which contained both replicons and resistance genes. There were 14 distinct replicon types which co-occurred on the same contigs with resistance genes (Figure 3.14). These were: X1, HI2A, Col, Q1, FIB, FII, X2, I1-I, FIC, p0111, Y, N, HI2, FIA. The most frequently identified replicons co-occurring with resistance genes were FII followed by FIB.

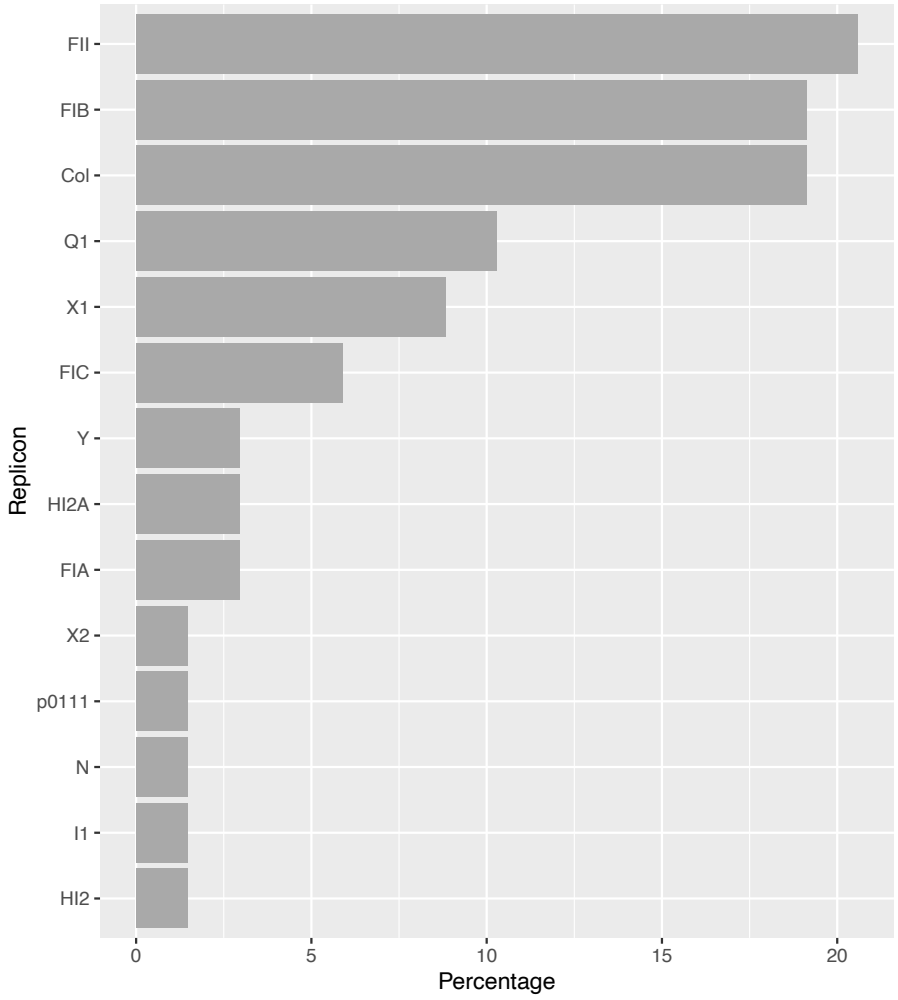

Figure 3.14: Plasmid diversity across *E. coli* strains associated with resistance genes. Col replicons were the most prevalent followed by FII. Other replicons are indicated in the image. The Y axis depicts the replicon types found whilst the X axis shows their percentage.

3.3.11. Identification of plasmid replicons associated with resistance gene profiles that are common to multiple strains.

To gain insight into the mechanism underlying transmission of resistance genes shared across multiple strains, strains in the collection that possessed common resistance patterns were examined. Amongst these, strains with common resistance patterns, strains that had the same plasmid replicons were identified and contigs that contained both ARGs and plasmid replicons were screen. This approach was chosen due to the constraints of Illumina sequencing, which does not allow the reconstruction of complete sequences for large, complex plasmids. Contigs that contained plasmid replicons and ARGs represented segments of antimicrobial resistance plasmids but were not considered complete plasmid sequences unless they were shown to be circular. Where putative plasmid sequences with the same combinations of replicons and ARGs were found in different host strains, they were compared to determine whether they were likely the same plasmid.

A total of 11 contigs with the same combinations of ARGs and plasmid replicons were found across 10 genomes, which represented 10 different STs. Five putative plasmids were found (Table 3.6). Of these, three putative plasmids that were expected to confer a multidrug resistant (MDR) phenotype (containing >3 resistance genes, conferring resistance to at least three different classes) were identified, as well as two that contained a single resistance gene each. The putative plasmids consisted of X1, FIB/FII, and Col(pHAD28) replicons. One of the MDR plasmids was shared between three different isolates whilst the ones associated with X1 and FIB/FII replicons were each shared between two strains, respectively. The GenBank non-redundant nucleotide database was queried with each of these contigs to identify the complete plasmid sequences that they were most closely related to. This revealed that all putative plasmids matched a GenBank plasmid with coverage of 77-100% and identity of 99.92-100%.

The three putative plasmids bearing multiple resistance genes (with Col(pHAD28), X1 and FIB/FII replicons) matched different GenBank plasmids (pCF_20.1 MDR, pDAX4A and pME16, respectively). The Col(pHAD28) and X1 plasmids were detected in strains belonging to different STs, with the Col(pHAD28) plasmids being associated with ST1103, ST4429 and ST9816 and the

X1 plasmids with ST3595 and a strain with a novel ST, indicating that these putative plasmids were present in multiple hosts. The FII/FIB plasmid was detected in two strains of ST48.

Two other putative plasmids associated with the Col(pHAD28) replicon bearing single resistance genes were found. One was linked to *tet*(A), whereas the other was linked to *qnrB19*. When queried in GenBank database they were found to be closely related to different plasmids, with each being shared in different host lineages on the basis of their STs. In both cases, coverage was 100% and identity was 99%.

To determine whether any of the putative plasmid contigs represented complete plasmid sequences, the first and last 1000 bp from each contig were compared to one another using BLASTn. Only contigs representing one putative plasmid, containing a Col(pHAD28) replicon and *strAB*, *sul2*, *dfrA14* and *tet*(A) resistance genes (highlighted purple in Table 3.6), featured a 127 bp overlap and could thus be circularised, indicating that they represented complete plasmid sequences of 9074 bp, 9107 bp and 9096 bp. The differences in the sizes of these plasmids were accounted for by the number of copies of an 11 bp repeat sequence that were present in each. Together, these data indicate that closely related small plasmids were carried by *E. coli* from three different STs, consistent with horizontal transmission.

Table 3.6: ARG and plasmid replicon-containing contigs found in multiple *E. coli* hosts in this collection.

	Replicon	ARG(s)	Host				Contig size (bp)	Closest GenBank match			
			ID*	Animal group	Location	ST		Plasmid	Accession	Coverage (%)	ID (%)
Plas1	Col(pHAD28)	*strAB*, *sul2*, *dfrA14*, *tet*(A)	P7	Pig	Nyamagana	1103	9074	pCF_20.1 MDR	MW115421	100	99.95
			P34	Pig	Nyamagana	4429	9107	pCF_20.1 MDR	MW115421	100	99.99
			L22	Layer	Misungwi	9816*	9096	pCF_20.1 MDR	MW115421	100	99.95
Plas2	X1	*strAB*, *aadA2*, *bla*$_{TEM}$, *qnrB1*, *sul3*, *dfrA12*	P164	Pig	Nyamagana	novel*	26618	pDAX4A	CP126344	96	100
			P296	Pig	Nyamagana	3595*	26630	pDAX4A	CP126344	96	100
Plas3	FIB, FII	*qnrS1*, *tet*(A), *dfrA14*	P102	Pig	Ilemela	48	153323	pME16	MT868891	77	99.92
			P134	Pig	Ilemela	48	153366	pME16	MT868891	77	99.92
Plas4	Col(pHAD28)	*qnrB19*	L109	Layer	Nyamagana	155	3116	pFP119	KX452393	100	99.97
			B29	Broiler	Nyamagana	4977	2826	pQNR14-AB01030	CP081670	100	100
Plas5	Col(pHAD28)	*tet*(A)	L109	Layer	Nyamagana	155	4636	pLAO84	OP242239	100	99.98
			L152	Layer	Nyamagana	4204	8157	pEF7-18-51_3	CP063490	100	99.96

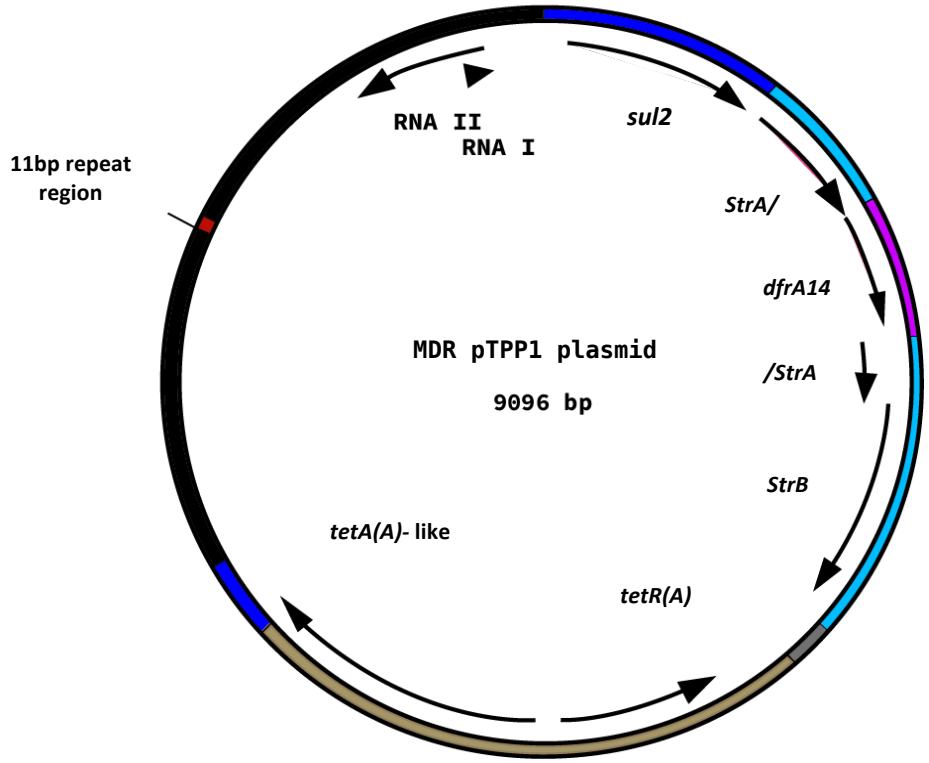

Figure 3.15: A plasmid map of pTPP1 which is a Col plasmid carried by L22, P34 and P7. The plasmid matched GenBank plasmid pCF_20.1 and is associated with multidrug resistance. The size of the plasmid (9096 bp) is indicated at the centre of the plasmid. The red coloured region (labelled 11bp repeat region) illustrates a variable region with repeated sequences, denoting variations between variants of this plasmid harboured by the hosts. Specifically, the L22 variant consists of 5 sets of 11 repeats while the P7 variant has 3 sets of 11 repeats and the P34 variant has 6 sets of 11 repeats. The plasmid harbours the *sul2* sulphonamide resistance gene, the trimethoprim resistance gene *dfrA14*, and the streptomycin resistance genes *strA* and *strB*. Locations and directions of resistance genes are indicated on the circular map of the plasmid.

3.3.12. Distribution and diversity of virulence genes across *E. coli* isolates

Virulence gene profiles of all strains were examined to gain a better understanding of the underlying patterns of the distribution of virulence genes among the different isolates. Most of the strains (n=111 and n=109, respectively) in the dataset were found to contain the *csg* gene cluster (*csgABC*) and *cgs* gene clusters (*cgsDEFG*), which are all components of the machinery linked to

curli fibre and biofilm formation, known for adherence and survival in the environment (Figure 3.16) (Shu *et al.,* 2012; Nicastro *et al.,* 2022; Sleutel *et al.,* 2023). A variable distribution of genes associated with the type III secretion system (T3SS) was also detected, including *espX* genes (*espX1245*), *espL1*, and *espR1*, in the dataset (Lu *et al.,* 2011; Sanchez-Garrido *et al.,* 2021). A ubiquitous presence of *fdeC* was identified which is associated with iron acquisition and metabolism (Nesta *et al.,* 2012). Other genes that were preserved across most isolates included the *fim* genes (*fimABCDEFGHI*) which encode type 1 fimbriae, which have a role in adhesion and attachment of *E. coli* strains on host cells (Schwan, 2011; Hasan and Alsammak, 2023). Other genes that were present in a majority (n=105) of strains included the *allB* genes which are associated with AI-2 transporter system, which is important for quorum sensing (Escobar-Muciño *et al.,* 2022). Further analysis of the pattern of virulence gene absence and presence in the dataset showed no discernible pattern associated with clustering to specific lineages. Most of the genes in the dataset were dispersed randomly over various STs and phylogenetic groupings, and there were no patterns demonstrating links between various sets. These genes include *hly* genes (*hlyABCD*), *iuc* genes (*iucBCD*), *irp* genes (*irp12*), *iro* genes (*iroBCDEN*), *ybt* genes (*ybtAEPQSTUX*), *rfbK1*, *asLA* and *east1* genes.

Figure 3.16: Distribution of virulence genes across different *E. coli* strains. Coloured blocks indicate the presence of genes. The MDR strains and non-MDR strains are also indicated.

Carriage rates of virulence genes among the three animal groups were further examined by comparing their distributions. To achieve this, counts of virulence genes were aggregated within each animal group per cluster based on their functions (e.g. summing counts of *csg*, *hly*, *cgs*, *ybt*, *fim* genes etc., in each group) to summarise the data. Following this, the data was normalised to the mean virulence carriage rate per host to account for varying population size. The normalised relative abundance for each of virulence genes per host population was then calculated. The resulting percentage mean values for relative abundance of virulence genes in broilers, layers and pigs were 35.78%, 32.58% and 31.64% as illustrated in Figure 3.17. Kruskal Wallis was then used to determine whether the distributions varied significantly between the animal groups. Pairwise comparisons between these animal groups was conducted and adjusted for multiple comparison using Bonferroni correction. The percentage relative abundance of virulence genes across animal groups was determined not to be significantly different, with the adjusted p values of the comparisons being >0.05.

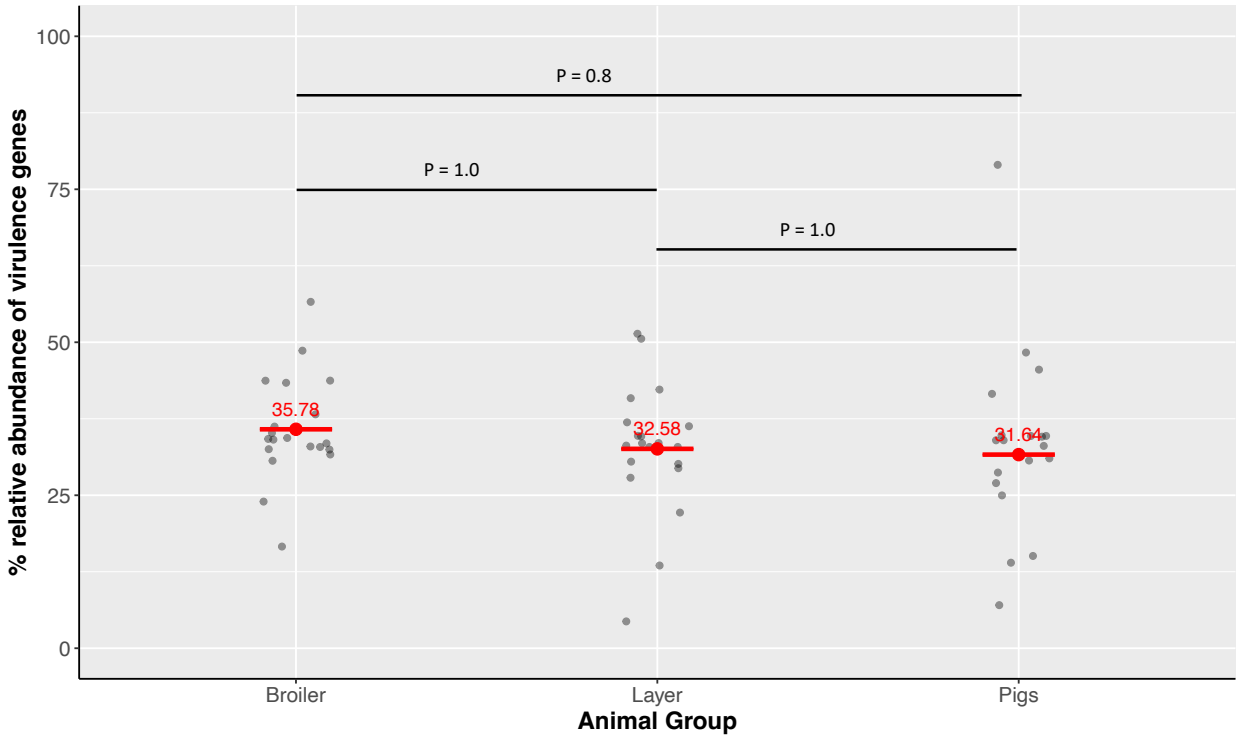

Figure 3.17: Distribution of the relative abundance of virulence genes across different animal groups. The red lines depict the mean relative abundance per animal group whilst the black lines denote the adjusted p values of the groups being compared.

3.3.13. The link between resistance and virulence genes across *E. coli* strains

Linear regression was conducted a to determine the correlation pattern between the number of virulence genes and the number of resistance genes in each genome. Our findings reveal a weak, but statistically significant, relationship between virulence and resistance genes (R^2=0.055, P< 0.05) (Figure 3.18).

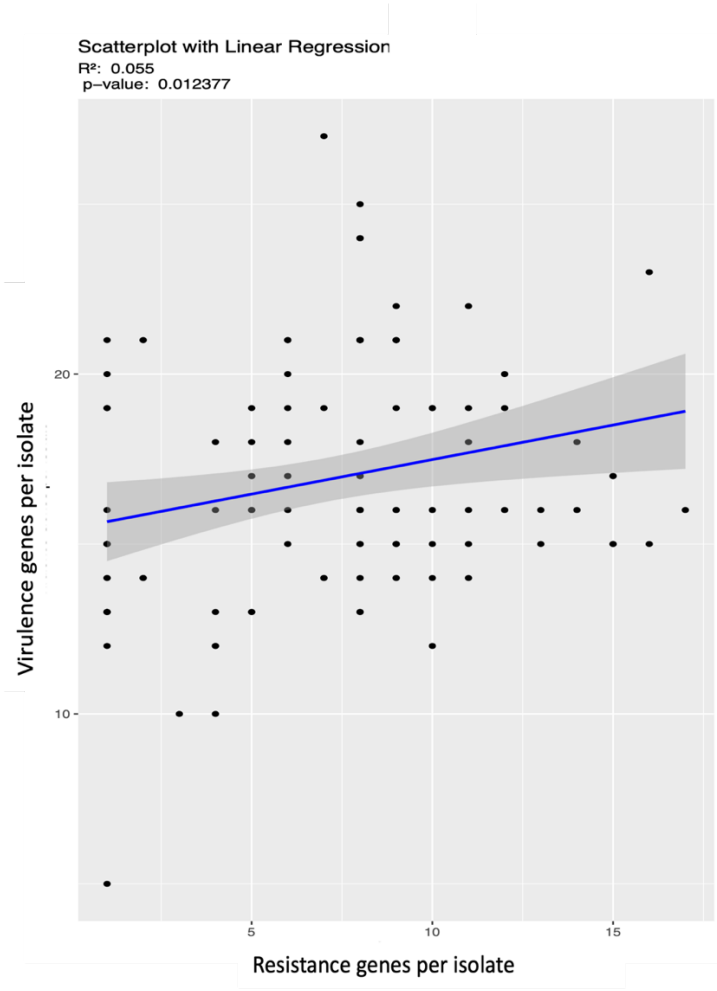

Figure 3.18: Correlation between the number of resistance genes and virulence genes in the *E. coli* genomes. Linear regression showing a weak relationship between virulence and resistance genes across *E. coli* strains within the dataset. The shading on either side of each line represents the 95% confidence interval.

3.4. DISCUSSION

This study aimed to determine the diversity of *E. coli*, and their antibiotic resistance and virulence genes, in broiler, layer, and pig populations in the Mwanza region in Tanzania. Our primary objective was to identify reservoirs, genetic lineages, and characteristic markers implicated in resistance transmission. Our genomic analysis enabled us to explore patterns of clonal expansion, horizontal gene transfer, and possible cross-species resistance transmission by delineating relationships among resistant isolates. Here the current study contextualises our primary findings regarding genetic lineages, and genetic determinants implicated in resistance and virulence. Broader implications of our findings on animal health, public health, and antimicrobial stewardship are also highlighted.

Our study revealed high prevalence of resistance to tetracycline, aminoglycoside, sulphonamide, and trimethoprim, with prevalence rates exceeding 70% for each antimicrobial class. These findings are consistent with previous research conducted in Tanzania, highlighting resistance to tetracycline is consistently more common compared to other antimicrobial classes (Zuhura *et al.,* 2021; Sonola *et al.,* 2021; Katakweba *et al.,* 2018). Distinct patterns of resistance were also observed between animal populations. For instance, the occurrence of streptothricin was more prevalent among layers compared to broilers and pigs. Additionally, prevalence of aminoglycoside, sulphonamide and trimethoprim resistance was higher in poultry compared to pigs, despite both sectors using these antimicrobials (Kimera *et al.,* 2020a). These variations between poultry and pigs, along with the similarities within the poultry population, could be attributed to various factors. Among them are varying immunity levels to pathogens (Koenen *et al.,* 2002), which can influence the types of antimicrobials that are applied (Düpjan and Dawkins, 2022). Moreover, farming practices for poultry and pigs differ significantly (Andretta *et al.,* 2021). In pigs, the use of antimicrobials is less frequent and more targeted, which may explain the lower prevalence rates compared to poultry (Kimera *et al.,* 2020; Kimera *et al.,* 2021; Luiken *et al.,* 2022). In the context of poultry farming, layers and broilers often receive similar types of antimicrobials (Chowdhury *et al.,* 2022). The similarity in antimicrobial resistance patterns reflects the broad-spectrum approach to disease prevention and treatment. Individual differences can be shaped by disease prevalence, farm management practices, antimicrobial stewardship, and biosecurity measures

(Jibril *et al.,* 2021). High prevalence of disease in one region may foster the need for more frequent antimicrobial use. In addition, some farms have higher stocking densities and less stricter biosecurity measures which may affect the health of the birds hence the need for antimicrobials. Moreover, some farms may observe more responsible practices for the use of antimicrobials.

Compared to pigs, poultry showed higher rates of multidrug resistance. This difference may have been caused by a variety of factors. Firstly, the unique dynamics of poultry production, which are characterised by relatively shorter production cycles (Ma *et al.,* 2021), lasting on average around 6 weeks (Wilkinson, 2011). This allows for the successive raising of multiple generations of birds in a period of time comparable to a single pig production cycle lasting around 6 months (Li, 2017). Factors such as breed, confined environments, and high livestock densities which are typical in commercial-scale chicken farming, create an ideal environment for rapid disease transmission (Hedman *et al.,* 2020; Sonola *et al.,* 2021). Due to this increased disease pressure, antimicrobials are administered relatively frequently in poultry farming, both as preventive and therapeutic measures to safeguard the flock's health (Dhaka *et al.,* 2023). Poultry farming has consistently been associated with higher levels of non-therapeutic antibiotic use compared to pigs (Carrique-Mas *et al.,* 2015). Although antibiotics may occasionally be administered to the entire herd in pig production for preventive purposes, pigs have longer lifespans and body-mass making this method more costly in pigs and hence targeted treatment of specific sick animals is preferred over extensive prophylactic antibiotic use (Kimera *et al.,* 2020; Li, 2017). Higher stock densities and quicker cycle of production turnover rates, both of which are traits of poultry farming, are a factor in the increased use of antibiotics (Gržinić *et al.,* 2023; Imam *et al.,* 2021). Additionally, chickens are usually given antibiotics from the beginning of their lives, from hatching through maturity, resulting in substantial and protracted exposure (Hofacre *et al.,* 2002; Moreno *et al.,* 2019), and this may be another factor in the prevalence of multidrug resistance in poultry populations when compared to pigs. It should however be noted that a significant number of poultry in our study came from a single hatchery, which may account for the comparable rates of multi-drug resistance seen in the isolates. In conclusion, this finding indicates that there is widespread use of antimicrobials in both poultry and pig farming systems and that differences between operations maybe due to varying farming practices.

Correlation analysis found a strong link between the resistance profiles to tetracycline, sulphonamide, aminoglycoside and trimethoprim, pointing to the co-occurrence of resistance genes. These associations are most likely selected for by the specific antibiotic usage in farming practices (Muloi *et al.,* 2019). According to Mtemisika *et al.* (2022) who collected the samples, broad-spectrum drugs including oxytetracycline, trimazine (a combination drug which includes trimethoprim and sulphonamide), and trimethoprim, which target a variety of Gram-negative bacteria, were being used by farms in this study. These antibiotics appear to widely use in agricultural settings in Tanzania (Nonga *et al.,* 2010; Paintsil *et al.,* 2021). The widespread use of combination drugs containing both sulphonamide and trimethoprim, as well as tetracycline, in broiler and layer farms raises the possibility that long term exposure to both antimicrobial classes may selectively favour bacteria that carry trimethroprim, sulphonamide and tetracycline resistance genes (Muloi *et al.,* 2019). Tetracycline, sulphonamide, and trimethoprim resistance genes were found to be co-located on certain contigs with FIB, FII, and Col (pHAD28*)* replicons, suggesting these resistance genes may be disseminated in bacterial populations through the same plasmids. The co-occurrence of these genes on the same plasmid suggests a potential for co-selection, where the use of any of these antibiotics could lead to the selection of the plasmid thereby promoting and expanding resistance to multiple antibiotics simultaneously (Holmes *et al.,* 2016; San Millan and MacLean, 2017; Engin *et al.,* 2023). The presence of these genes on plasmids may facilitate their horizontal transfer, leading to rapid dissemination across diverse bacterial species and environments (Rodríguez-Beltrán *et al.,* 2021; Engin *et al.,* 2023). Additionally, the genomic context around these genes including their arrangement and regulatory elements plays a crucial role, in their expression and mobilisation, influencing the efficiency of co-selection and transfer (Smillie *et al.,* 2010; Maddamsetti *et al.,*

2024). This underscores the importance of monitoring antimicrobial use and stressors to control the proliferation of multidrug resistant bacteria. The genetic context underlying these resistance patterns could, in future work, be further investigated through in-depth analyses of plasmids, using long-read sequencing approaches. However, despite the evidence offered in the current study suggesting a strong correlation between AMR genes, it does not definitively prove if the use of antimicrobials in these settings directly leads to the presence of these genes. Therefore, drawing conclusion on these observations requires in-depth analysis of data on farming practices information that is lacking in the current study.

Cross-reservoir sharing of genetic lineages among the three animal groups was also explored with the aim to gain insight into transmission dynamics and to identify sources. Our study revealed distinct *E. coli* phylogroup and STs distributions, where phylogroups C and D were exclusively found in broilers, and D and F were found in poultry (broilers and layers). On the other and STs including ST101, ST1071, ST1303, ST10, ST1508, ST208, ST2325, ST3595, ST3889, ST345, ST4429, ST515, ST5871, ST641 and ST717 were exclusively found in pigs. This exclusivity likely results from a combination of factors unique to each animal group and biology. Various factors including physiological differences in the gastrointestinal tract (Martinez, 2009; Borda-Molina *et al.,* 2018), coupled with environmental conditions (Lin *et al.,* 2024), diet (Castillo *et al.,* 2007); (Zentek *et al.,* 2024), and immune system variations (Koboziev *et al.,* 2014; Zhang *et al.,* 2017; Wang *et al.,* 2024) between poultry and pigs, can influence these patterns. For instance, the complex and anaerobic nature of the large intestine of pigs likely fosters a more diverse microbial community adapted to anaerobic conditions (Khalil *et al.,* 2021; Lin *et al.,* 2024), preventing the dominance of specific phylogroups. This observation underscores the importance of considering host-specific factors (Maritan *et al.,* 2024). Moreover, reduced fermentation capacity in the ceca of chickens may restrict the diversity of microbial taxa present in this area (Svihus *et al.,* 2013; Rychlik, 2020). This, in turn, can impact colonisation patterns and type of bacteria that can thrive in their respective gastrointestinal tract of each of the species. The varied diets of chickens and pigs play a crucial role, in shaping their gut microbiomes (Patangia *et al.,* 2022; Kogut, 2022). Chickens mainly eat plant-based foods like grains, seeds, fruits and insects while pigs being omnivores have a diet that includes grains, vegetables, fruits and animal proteins (Pond *et al.,* 2004; King and Pluske, 2003). These dietary differences foster distinct microbial communities in their digestive tracts, each adapted to efficiently process the respective diets (Pereira and Berry,

2017). Furthermore, variations in management practices, including litter management, biosecurity measures, and housing conditions, also impact the persistence and exchange of specific bacterial species between these animal groups (Sibanda *et al.,* 2018; Adegbeye *et al.,* 2024). On the other hand, chickens and pigs have different immune response to microbial colonization (Song *et al.,* 2022). Variations in innate and adaptive immune factors can selectively modulate the diversity and abundance of bacterial strains within the gut microbiota, affecting species shared (Bouwens and Savelkoul, 2019) Such disparities may influence the selection and maintenance of distinct microbial taxa within the gut microbiota of animals. Other studies suggest that host-specific adaptations, and antimicrobial use may also contribute to these variations (Maritan *et al.,* 2024); Zentek *et al.,* 2024).

Our data also revealed the presence of microbial lineages that were shared across all animal populations. These included ST1196 and ST155, which were widespread across the three animal populations. Furthermore, we found STs that were shared between two animal populations, including ST206 which was shared between layers and broilers, ST224 which was shared between layers and pigs, as well as ST711 and ST48 which were shared between pigs and broilers. This sharing demonstrates certain lineages within our study population may have been generalists that can cross a broad range of hosts in the region. This mobility of strains across hosts has been reported in past studies and contributes to create optimal conditions for genetic exchange between distinct lineages (Wetzel and LeJeune, 2006; Massé *et al.,* 2023). Further research and surveillance efforts are necessary to understand the specific drivers behind the cross-reservoir sharing of STs in these diverse animal populations to effectively combat antimicrobial resistance.

Distinctive patterns of virulence genes across *E. coli* strains were not found. Random distribution of virulence genes was observed, with certain genes (e.g. *cgs* and *csg*) existing in multiple strains. Conserved virulence genes were linked to functions essential for bacterial survival and colonisation. A small number of genes was discovered, in the strains that were previously linked to pathogenic *E. coli* strains. For instance, in some strains *east1* genes which have been linked to enterohemorrhagic (EHEC) were found, enterotoxigenic (ETEC), enteropathogenic (EPEC) and enteroaggregative (EAEC) *E. coli* strains (Silva *et al.,* 2014; Ellis *et al.,* 2020). The *east1* gene is known for encoding a heat-stable enterotoxin which causes watery diarrhoea (Savarino *et al.,* 1996; Ruan *et al.,* 2012). In previous studies *east1* genes have been associated with outbreaks of

diarrhoea in humans and animals (Dubreuil *et al.,* 2016; Dubreuil, 2019). However, the link between *east1* gene and diarrhoeal disease is yet to be fully established and some studies suggest it may need to interact with other virulence genes, such as heat labile toxin and heat stable toxins including type I (*STa*) and type II (*STb*), to actively cause diarrhoeal diseases (Ruan *et al.,* 2012). Other genes that were found in our *E. coli* dataset include the *iro*, *ybt*, *iuc*, and *irp* genes which are linked to the synthesis of siderophores, which facilitate the acquisition of iron in bacteria, enabling bacteria to thrive in resource-limited host environments (Gao *et al.,* 2012; De Serrano *et al.,* 2016; Wareth *et al.,* 2022). Similarly, the presence of genes such as the *fim* and *esp* gene clusters were found to be broadly conserved. These genes are associated with synthesis of components essential for colonisation, survival, and interaction with other bacteria (Ogasawara *et al.,* 2020; Taglialegna *et al.,* 2020; Ou *et al.,* 2023).

Our data offers insights into the role that plasmids play in spreading resistance genes in animal populations. Plasmids associated with MDR linked to X1 and FIB/FII replicons were identified. These replicons are known for their adaptability and are frequently distributed among *Enterobacteriaceae* (Rozwandowicz *et al.,* 2018). In our study, X1 plasmids that were shared between different STs and the FII/FIB plasmids within STs in the pig population were observed. This underscores the significance of these plasmids in shaping the diversity of antimicrobial resistance within bacterial lineages. Small plasmids with single AMR genes and some with MDR profiles in our data were identified. For instance, Col(pHAD28) plasmids were identified, together with a variant of pCF_20.1 which were associated with multidrug resistance (MDR). These findings highlight the importance of conducting, in depth analysis using long read data to enhance understanding of underlying AMR mechanisms, within this context.

Plasmids identified in the current work have been documented in previous research in geographically distant locations, except for pTPP1 which was closely related to pCF_20.1, which was previously identified in Tanzania (at the coast of Zanzibar within plastic waste) (Rasool *et al.,* 2021). The pTPP1 plasmid carried multiple resistance genes, conferring resistance to streptomycin, beta-lactams, quinolones, sulphonamides, and trimethoprim. The remarkable resemblance, between pTPP1 and pCF_20.1 raises concerns about the role of waste as a reservoir, for genes and plasmids associated with resistance. It also highlights the possibility of environmental resistance plasmids transferring to animal populations or *vice versa*. Plasmids that

showed similarity to variants in distant locations included plasmid FII/FIB plasmids, which were found to be similar to pME16, which was previously documented in France (Camiade *et al.,* 2020). Within the subset of small plasmids harbouring single gene profiles, those with *qnrB19* variants were related to pFP119, which was previously found in Turkey (Cunha *et al.,* 2019), and pQNR14-AB01030 from Germany (Cunha *et al.,* 2019; Juraschek *et al.,* 2022). In regards to plasmid variants containing *tet(A)* resistance genes, a plasmid that exhibited similarities with pLAO84 was identified (Snaith *et al.,* 2023) and another with pEF7-18-51_3 (Moser *et al.,* 2021). These observations underscore the remarkable adaptability of these plasmids and showcase their capacity to disseminate and flourish across diverse hosts and geographical locations.

Although this study provides insights into the diversity of *E. coli* among different livestock in Tanzania, the limited size of our samples restricts our ability to make conclusions that can be generalised to larger populations without additional investigation. Our research has illuminated aspects related to the dissemination of resistance. Unfortunately, due to the use of Illumina sequencing in this study the distribution of plasmids could not be assessed. The short Illumina read data was only capable of resolving small, less complex plasmids. However, because of the fragmented nature of the sequences large plasmids could not be reconstruct for in-depth analysis. Future analyses using long-read sequencing will deepen our understanding of how resistance genes spread between *E. coli* clones.

Chapter 4

Comparative Analysis of Metagenome-Assembled *E. coli* Genomes from the Hadza and the Wider Population of Tanzania

4.1. BACKGROUND

Metagenomics is the study of genetic material recovered from environmental and clinical samples through sequencing (Thomas *et al.,* 2012; Zhang *et al.,* 2021). The term "metagenomics" was first coined by J. Handelman in 1998. It describes a culture-independent approach that involves collective analysis of a set of genomes (metagenomes) present in samples (e.g. water, air, soil, or stool) (Gilbert, 2013; Mineta and Gojobori, 2016). Soon after its inception, metagenomic analyses became prominent in studying environmental samples but metagenomics has since extended to clinical and medical research, particularly in the study of the human microbiome (Chiu and Miller, 2019; Bharucha *et al.,* 2020; Batool and Galloway-Peña, 2023). Early studies of human gut microbiome (Laetsch and Blaxter, 2017) predominantly focused on amplifying taxonomic markers and subsequent sequence analysis of these amplicons (Ahmad and Raza, 2019; Satam *et al.,* 2023). However, the advancements in sequencing technologies and computational methods have allowed for deeper analysis of genetic makeup of diverse microbial communities through the use of shotgun metagenomic sequencing (Ahmad and Raza, 2019; Satam *et al.,* 2023). One of the early studies which involved the reconstruction of nearly complete genomes from metagenomic data was conducted in 2004 by Gene Tyson and colleagues at the University of California, Berkeley, and the Joint Genome Institute (Tyson *et al.,* 2004). The study resulted in reconstruction of several bacteria and archaea from an acid mine drainage system previously believed to be unculturable (Tyson *et al.,* 2004; Liu *et al.,* 2022). This became a pivotal study which demonstrated the potential for shotgun sequencing being used to uncover and assemble whole genomes from environmental samples (Liu *et al.,* 2022). Moreover, it became clearer with time that culture-based methods could only capture less than 1% of bacterial and archaeal species in environmental samples (Grice and Segre, 2012; Duan *et al.,* 2021) and 70% in stool samples (Walker and Hoyles, 2023). A significant portion of microbial diversity was thus being missed by traditional, culture-dependent methods (Duan *et al.,* 2021).

In the present study the diversity of microorganisms was investigate using metagenome-assembled genomes (MAGs) from two human populations with different lifestyles and exposure levels to antimicrobials (Schnorr *et al.,* 2014; Smits *et al.,* 2017; Marlowe, 2010). By reconstructing MAGs from the Hadza and the general population in Tanzania (referred to as urban throughout this

chapter) the aim is to gain insight into the diversity of gut microbiome and prevalence of resistance gene determinants. The Hadza community represents a baseline community from which the human microbiome can be studied in the context of minimal use of antibiotics and their traditional diet which lacks processed foods (Schnorr *et al.,* 2014; Smits *et al.,* 2017). This will help us understand how differences in lifestyle in the general population can affect the diversity of microbial communities.

The Hadza community presents a unique community of hunter-gatherers in the Central Rift Valley of Tanzania who live a traditional lifestyle with a unique diet that is centred around gathering and hunting small animals (Schnorr *et al.,* 2014; Smits *et al.,* 2017; Marlowe, 2010). Their activities are largely focused around food acquisition (Smits *et al.,* 2017). They live in camps with approximately 5 to 30 people per camp, with numbers varying depending on season and available resources (Jones *et al.,* 1999; Smits *et al.,* 2017). They rely on natural, unprocessed foods that have an abundance of plant fibres, complex carbohydrates, and diverse nutrients which serve as a rich source for a multitude of microorganisms, thus leading to a highly diverse microbiota (Marlowe and Berbesque, 2009; Marlowe *et al.,* 2014). In addition to meat, the main foods consumed by the Hadza are berries, honey, baobab, and tubers (Marlowe *et al.,* 2014; Schnorr *et al.,* 2014). The activities of the Hadza are usually affected by the local environment and are subject to two distinct seasons, wet (November to April) and dry (May to October; Smits *et al.,* 2017). Hunting is usually most successful during the dry season (Smits *et al.,* 2017). Berry foraging and consumption of honey is usually favoured during the wet season (Smits *et al.,* 2017). Tubers form an integral part of the Hadza diet serving as their primary source of sustenance (Marlowe and Berbesque, 2009; (Schnorr *et al.,* 2014a) . Besides their carbohydrate-rich diet, previous studies have found a significant lack of aromatic amino acids in their foods (Schnorr *et al.,* 2014a). Honey, for instance, which the Hadza consume all year round, lacks these essential aromatic amino acids (Rampelli *et al.,* 2015; Schnorr *et al.,* 2014). This deficiency in aromatic amino acids is thought to be compensated for by the gut microbiota of the Hadza population (Rampelli *et al.,* 2015; Schnorr *et al.,* 2014). Aromatic amino acids contribute to the synthesis of neurotransmitters like serotonin, which controls appetite, digestion, sleep, and mood, and dopamine, which controls motor coordination, focus, attention, and mood (Rampelli *et al.,* 2015). Conversely, the urban Tanzanian population often consumes diets characterised by high levels of processed foods, added sugars, and saturated fats (Smits *et al.,* 2017; Schnorr *et al.,* 2014). Such diets are linked with reduced

microbial diversity, causing the proliferation of certain bacterial species associated with inflammation and metabolic disorders (Schnorr *et al.,* 2014a).

In the current chapter, diversity of *E. coli* MAGs will be explored between two distinct human populations in Tanzania, living in different ecological contexts, presumed to have varying levels of antimicrobial exposure. The hypothesis is that modern practices associated with use of antimicrobials and diet in the general population could influence diversity of *E. coli.* Our objective is therefore to compare the diversity of the MAGs between the two populations, paying special attention to on *E. coli*. The aim is to identify and compare the virulence and antimicrobial resistance determinants linked to the identified MAGs.

4.2. METHODS

4.2.1. Reconstruction of metagenome assembled genomes (MAGs)

Hadza metagenomic data used in the current study was obtained from a previously published article by (Rampelli *et al.,* 2015) For the wider community data was obtained from previous study by Stražar *et al.* (2021). Our analysis was based on a MAG reconstruction pipeline developed by Dr Matthew Davies as part of his PhD research at the University of Birmingham. The analysis pipeline is outlined as described below.

Raw reads were trimmed using Trimmomatic (Bolger *et al.,* 2014) and then assembled using metaSPades (Nurk *et al.,* 2017). Reads were mapped back to their assemblies to calculate the contig coverage using BWA MEM (Li, 2013). This helped us identify any contigs that had low length (<2500 bp) to remove them from our dataset. As a preliminary step, assemblies were also mapped to the *E. coli* K-12 MG1655 genome using CoverM (https://github.com/wwood/CoverM) to predict metagenomic assemblies that contain *E. coli.* To generate MAGs, assembled contigs were binned using MetaBat2 (v2.12.1) (Kang *et al.,* 2019), with a minimum contig size of 2000 bp and using the parameters: minimum completeness value (minCV) of 1.0, minimum cumulative completeness sum (minCVSums) of 1.0, maximum probability (maxP) of 95%, minimum score (minS) of 60, and a maximum edge number (maxEdges) of 200. Using dRep (v3.0.0) (Olm *et al.,* 2017) which includes checkM (v1.0.7), a default minimum contamination and a maximum completeness was assessed, using a threshold of 10%. To identify and consolidate highly similar MAGs dRep was used, which subsequently retained the most representative quality MAGs (i.e., which are more complete and have low contamination). The process helped to eliminate redundant incomplete MAGs, hence improving the efficiency and accuracy of our analysis (Figure 4.1).

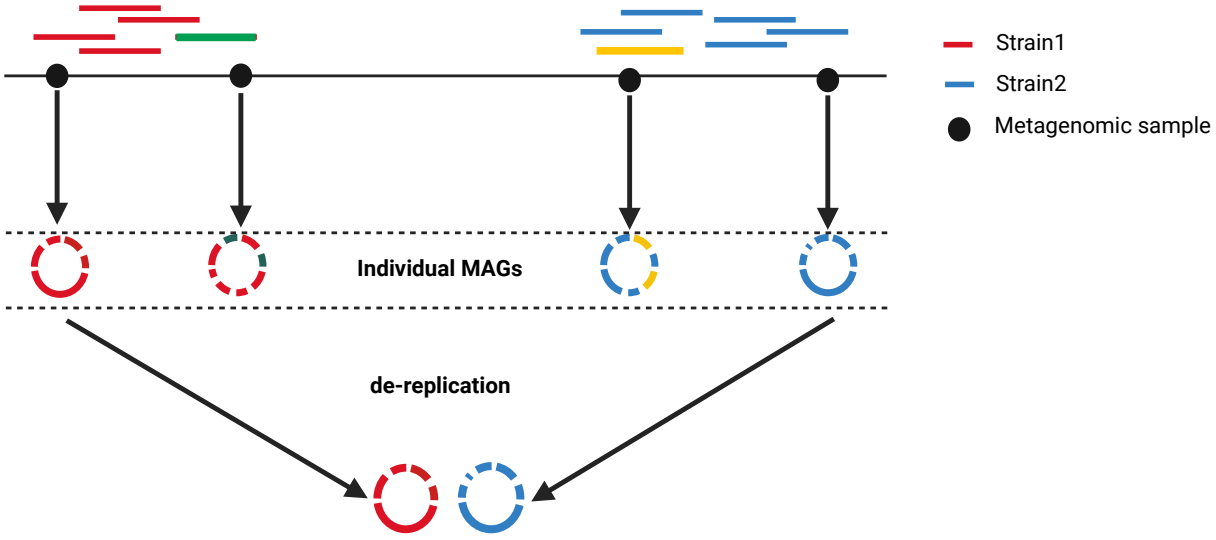

Figure 4.1: The dereplication process conducted to remove incomplete and redundant MAGs. In this process highly similar MAGs are put together, and representative quality MAGs (i.e. more complete and less contaminated) are retained.

4.2.2. Taxonomic assignment of MAGs

Dereplicated MAGS were screened through GTDB-Tk database (Chaumeil *et al.,* 2022) for taxonomic identification.

4.2.3. Phylogenetic analysis of *E. coli* MAGs

To place the *E. coli* MAGs in the context of the global *E. coli* population, core phylogenetic tree was generated using the reconstructed *E. coli* MAGs and reference genomes. *E. coli* sequences, previously used by (Janssen *et al.,* 2020) were used as reference, encompassing the phylogenetic diversity of *E. coli*. The downloaded reference sequence types which were involved in the analysis entailed 166 genome sequences. Sequences were first annotated using Prokka (v1.13) (Seemann, 2014) which ultimately converted the fasta files into gff files. Core genes of the reconstructed

MAGs were then identified and aligned with core genes of the reference sequences using Panaroo (v1.1.2) (Tonkin-Hill *et al.,* 2020). The resulting core gene alignment was used to conduct a maximum likelihood phylogenetic analysis using IQ-Tree (Nguyen *et al.,* 2015). The tree was then visualised and annotated in iTOL. Clermont typing was carried out using EzClermont (v0.6.3) (Waters *et al.,* 2020) to assign MAGs and reference sequences to different phylogroups. Multilocus sequence typing was performed using MLST (v2.23.0) (Maiden *et al.,* 2013) with the pubMLST database.

4.2.4. Identifying the presence of resistance and virulence genes

Abricate (v1.0.1) (Abdelrazik *et al.,* 2021) was used with the ResFinder database (Bortolaia *et al.,* 2020) to query for antimicrobial resistance genes. The presence of virulence genes was also screened using Abricate (v1.0.1) (Abdelrazik *et al.,* 2021) in conjunction with the Virulence Factor Database (VFDB) (Chen, 2004).

4.2.5. Identifying metabolic pathways of interest

Using Ecocyc.org, metabolic pathways of interest were identified and obtained the genes linked to the glycolysis pathway and aromatic amino acid metabolism. These genes were subsequently screened against the MAGs from the Hadza and urban communities using Abricate with customised database comprised of the target genes. Details of the genes in each pathway are given in Figure 4.4 and Figure 4.5.

4.3. RESULTS

Out of 693 MAGs identified, 509 originated from the wider Tanzanian community while the Hadza community contributed 184 MAGs. From the initial 2547 input bins (982 from the Hadza and 1565 from urban), 1530 (60%) of the bins passed the checkM filtering (981 Hadza, 549 urban), and these were subsequently dereplicated into 693 individual MAGs (509 Hadza, 184 Urban) (Figure 4.2). Patterns indicating the dominance of specific classes of bacteria within each community (Table 4.1) were observed. In the wider community, Clostridia MAGs were the most frequent compared to other classes, followed by *Bacteroidia* MAGs. Conversely, within the Hadza community, the most frequent class was Bacilli, followed by Clostridia.

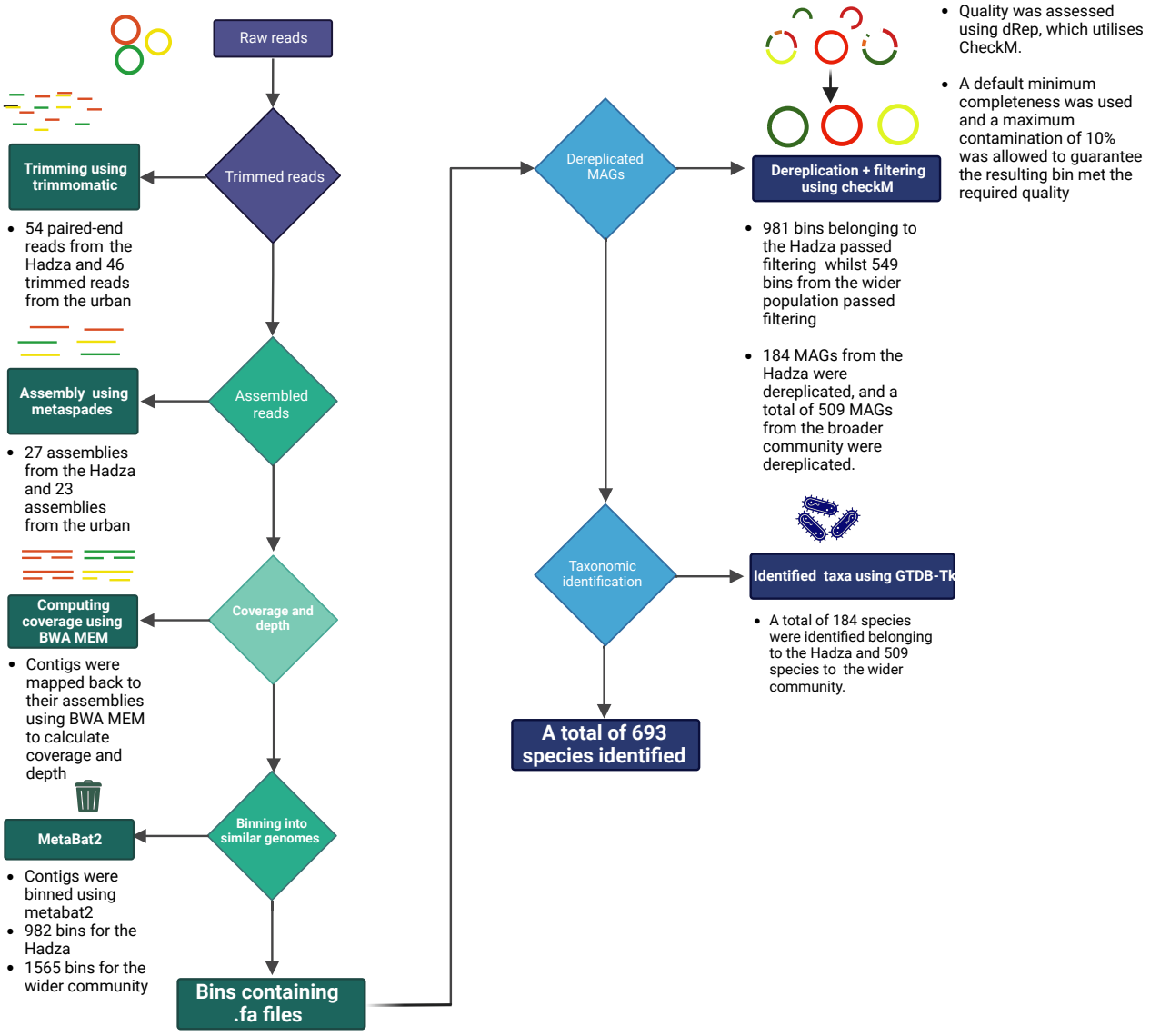

Figure 4.2: MAG analysis workflow. The diagram indicates different steps involved in the MAG generation pipeline, including the tools that were used in each step, as well as the number of MAGs that were ultimately reconstructed.

Table 4.1: Distribution of classes of bacteria identified in dereplicated MAGs.

Class	Hadza (%)	Wider community (%)
Epsilonproteobacteria	3.8	-
Actinomycetia	-	1.4
Alphaproteobacteria	0.5	0.6
Bacilli	39.7	2.2
Bacteroidia	1.1	12.8
Clostridia	35.9	70.5
Elusimicrobia	1.6	0.2
Gammaproteobacteria	3.8	3.1
Lentisphaeria	1.1	0.4
Spirochaetia	3.3	1.0
Vampirovibrionia	7.6	0.4
Negativicutes	-	3.7
Xenobia	1.1	-
Verrucomicrobiae	-	1.0
Coriobacteria	-	2.8

4.3.1. Identification and phylogenetic relationship of the *E. coli* MAGs and reference strains

Based on our preliminary analysis in which 50 assemblies were mapped to the *E. coli* K-12 MG1655 reference genome using coverM as described in the method section above to predict the presence of *E. coli*, we discovered 39 assemblies did not map to the reference genome, whereas 5 assemblies from the Hadza community and 6 assemblies from the urban community mapped, as shown in Table 4.2. In contrast to these findings, subsequent analysis revealed presence of only three *E. coli* Metagenome-Assembled Genomes (MAGs), as depicted in (Figure 4.3). Through phylogenetic analysis, two of the detected *E. coli* MAGs were linked to members of phylogroup group A and one to phylogroup B1. This link was subsequently confirmed through Clermont typing, affirming the classification of the identified strains to these phylogroups. MLST typing identified MAGs, SRR13348582 and SRR1929408 as ST216 and ST10, respectively, and strain

SRR1930122 could not be classified effectively. Considering the challenge of classifying SRR1930122, its allele profile in pubMLST (Jolley *et al.,* 2018) was further examined and found it had an allelic profile that differed from ST4946 by four alleles due to the absence of *fumC*, *icd*, *mdh*, and *recA* alleles. To further validate the sequence quality of *E. coli* MAGs, additional CheckM analysis was conducted to determine the level of contamination and completeness of the MAGs. SRR1930122 was found to have the lowest completeness (91.7%) and a contamination level of 49.8% (Table 4.3). By contrast, SRR1929408 had a completeness level of 97.28% with contamination at 24.5%, while SRR13348582 had a completeness level of 97.9% with minimal contamination 6.4%. Virulence or resistance genes in the MAGs could not be identified after querying them against VFDB and ResFinder databases using Abricate.

Table 4.2: Percentage of metagenomic assembly which maps to *E. coli* K-12 MG1655 reference sequence.

Assembly	Unmapped (%)	Genome mapped (%)
Hadza		
SRR1929408*	98.13	1.9
SRR1930122*	95.49	4.5
SRR1929484	95.45	4.5
SRR1930133	91.66	8.3
SRR1929485	85.93	14.0
Urban		
SRR13348582*	98.7	1.2
SRR13348589	98.5	1.5
SRR13348581	98.2	1.8
SRR13348579	97.3	2.7
SRR13348576	97.0	2.9
SRR13348578	96.3	3.7

*Metagenomes shaded in grey are those for which *E. coli* MAGs have been assembled.
The table only comprises of assemblies that mapped to the reference (others were 0%).
Note: 39 out of 50 assemblies which did not map to the *E. coli* reference are not included in the table.

Table 4.3: Quality of *E. coli* MAGs.

Assembly	# contigs [1]	Largest contig	Total length [2]	GC (%)[3]	N50[4]	L50[5]	Contamination (%)	Completeness (%)
SRR13348582	123	136603	4342688	51.25	50430	27	6.4	97.9
SRR1929408	120	216316	4308024	51.07	70833	19	24.5	97.3
SRR1930122	419	53581	4071421	50.57	13143	93	49.8	91.7

1 # contigs represents total number of contigs in a MAG.
2 Total length represents a sum of all contigs lengths in a MAG.
3 GC is the proportion of bases (i.e. guanine G and cytosine C) that make up the DNA.
4 N50 represents the shortest contig length in a set of long contigs which when combined cover 50% of the overall genome.
5 L50 is the count of contigs required to cover 50% of the overall genome.

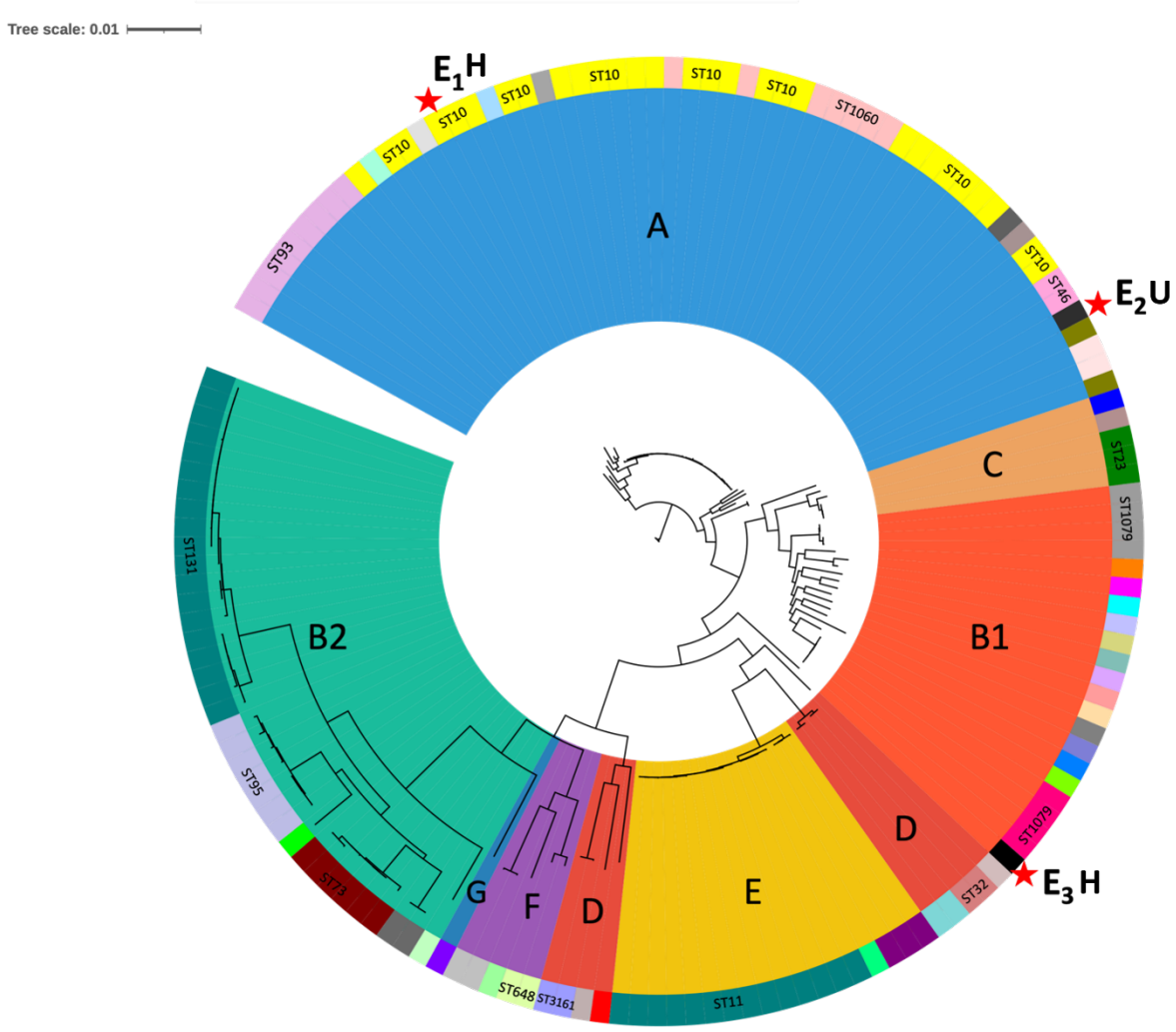

Figure 4.3: A core genome phylogenetic tree of three *E. coli* MAGs and reference sequences. IQ-Tree was employed to visualise a phylogenetic tree based on a core genome alignment. Different coloured backgrounds depict phylogroups groups that have been identified through Clermont typing. Dark orange stands for phylogroup B1, green for B2, light orange for C, red for D, yellow for E, purple for F, dark blue for G and blue for phylogroup A. Sequence types are illustrated in the circular strip. SRR1930122, SRR13348582, and SRR1929408 are represented by E1, E2, and E3, respectively. SRR13348582, originates from the wider community whereas SRR1930122 and SRR1929408 are Hadza-derived. SRR13348582 in the present study has been identified as ST216 whilst SRR1929408 has been identified as ST10. The abbreviations in the diagram H represents Hadza and U, urban.

4.3.2. Comparative analysis of the aromatic amino acid and glycolysis pathways of *E. coli* MAGs from Hadza derived and urban population

To gain a baseline understanding of the variability of the functional characteristics of *E. coli* MAGs of the Hadza and the general urban population, this study focused on the aromatic amino acid pathway for comparative analysis due to its significant role in microbial metabolism and potential impact in host physiology (Schnorr *et al.,* 2014; Rampelli *et al.,* 2015; Swer *et al.,* 2023). The aromatic amino acid pathway plays a role in the production of essential compounds that bacteria cannot acquire from their host (Liu *et al.,* 2020). This pathway plays a significant role in the adaptability and survival of bacteria in their host environment (Liu *et al.,* 2020). Hence our goal was to analyse the genes related to this pathway in both populations to identify any *E. coli* traits influenced by the dietary and environmental conditions of the Hadza community, compared to the general population. It is hypothesised that the Hadza, due to their unique dietary practices, which is characterised by the consumption of low levels of aromatic amino acids, could rely on their gut microbiome to synthesize these essential compounds and make up for this dietary deficiency (Rampelli *et al.,* 2015). In our analysis, most of the essential aromatic amino acid synthesis genes were therefore expected to be found in the Hadza *E. coli* compared to the *E. coli* from the urban population. Subsequent to this, genomes that are likely complete were explored on the basis of the analysis of glycolysis pathway genes. Thus, the glycolysis pathway was used as a control to check for completeness of the assemblies. The choice of the glycolysis pathway as a control is grounded in its essential role in bacterial metabolism (Chandel, 2021; Bao *et al.,* 2022). Therefore, we screened for genes associated with the glycolysis metabolic pathway, since bacteria were expected to have all genes necessary for glycolysis. *E. coli* K-12 MG1655 genome was included as a control.

Distinct patterns in genes associated with aromatic amino acid biosynthesis were found (Figure 4.4). Each MAG lacked specific genes for aromatic amino acid biosynthesis. The *aroC* gene was absent from both Hadza MAGs. In contrast to the other two strains, SRR1930122, which originates from the Hadza community, lacked both the *aroE* and *trpD* genes. Additionally, *aroD* gene was absent in the genome of the strain originating from the wider community. Apart from these variations, all strains contained *aroF, aroH, aroG, arok, aroL, aroA, tyrA, aspC, tyrB, pheA, ilvE, trpE, trpC, trpA,* and *trpB.* To further explore whether these metabolic routes were truly incomplete

in the strains, and not an artefact of the metagenomic assembly approach used here, genes of the glycolysis pathway were determined. This pathway is essential for *E. coli* and its gene typically are conserved (Figure 4.5). All reconstructed genomes lacked the *kduI* gene. In addition, the *bglF* gene was absent from the SRR13348582 genome and the *glpX* gene from the SRR1930122 genome.

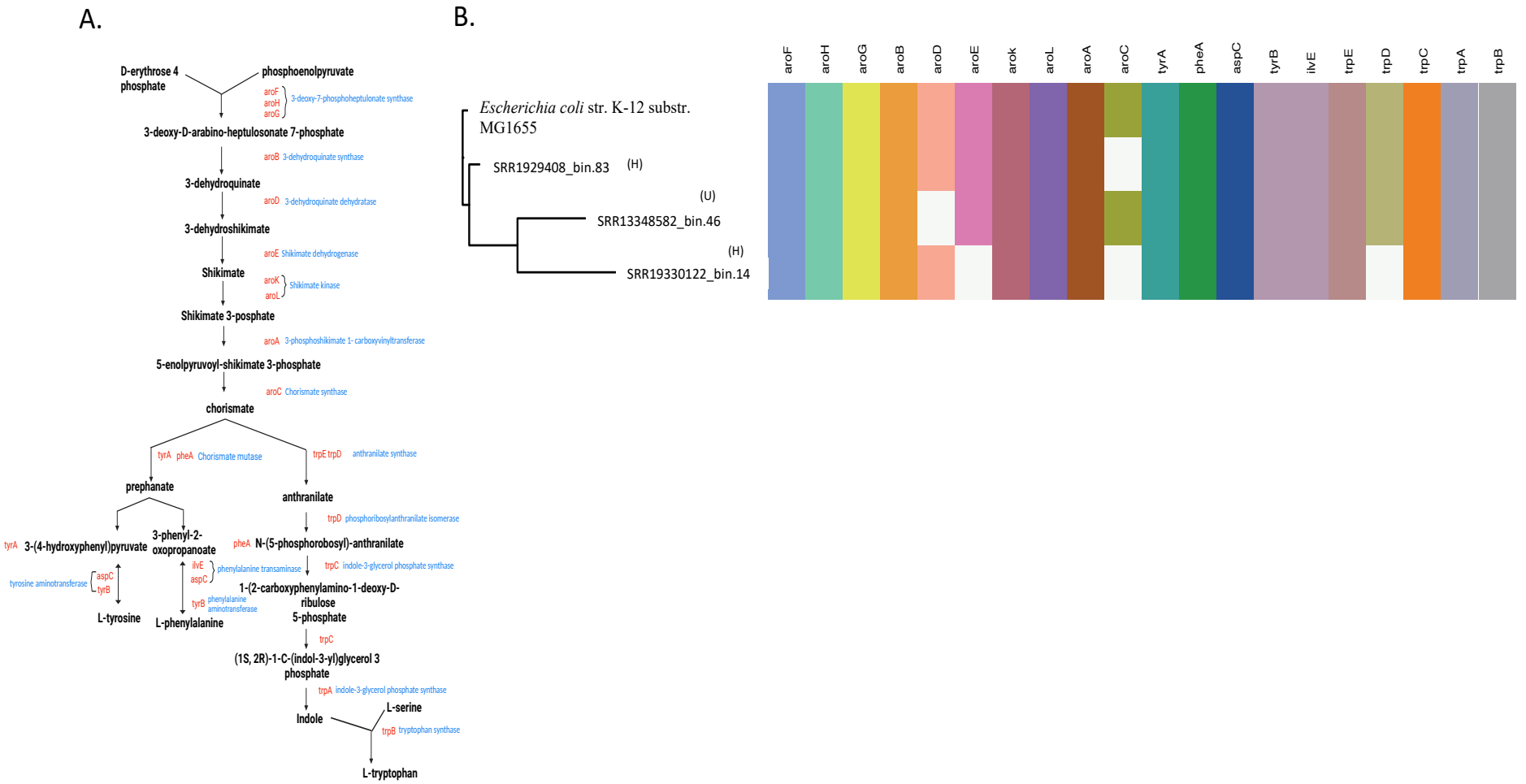

Figure 4.4: Pathway for the synthesis of aromatic amino acids and a heatmap showing gene presence/absence in the MAGs. The genomes of the three MAGs were compared to a bespoke database built using Abricate which contained the genes of the aromatic biosynthesis pathways. *Escherichia coli* str. K-12 substr. MG1655 was used a reference isolate. White spaces in the heatmap indicate missing genes. (H) represents Hadza-derived *E. coli* genomes and (U) represents the urban population-derived *E. coli* genome.

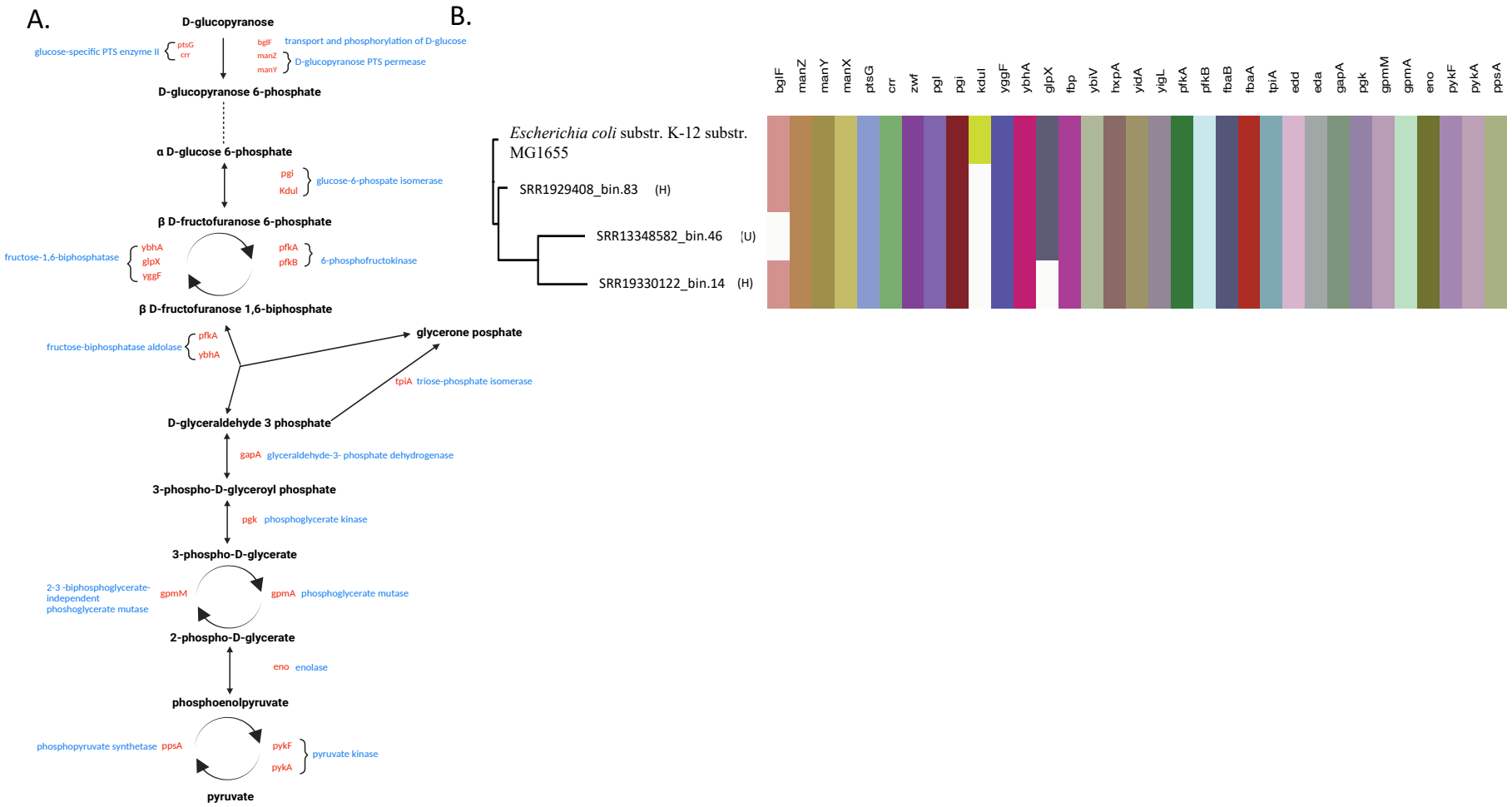

Figure 4.5: The glycolysis metabolic pathway along with a heat map showing which genes are present and absent in the MAGs. Genomes of the three MAGs were compared to a bespoke database built using Abricate that contained the genes of interest of the glycolysis pathway. *Escherichia coli* str. K-12 substr. MG1655 was the reference isolate used. White spaces in the genome indicate missing genes. H in brackets represents Hadza derived isolates and U in brackets represent urban population derived isolate.

4.4. DISCUSSION

In the analysis, 693 Metagenome-Assembled Genomes (MAGs) representing different bacterial species were successfully reconstructed. Although most of my study was devoted to *E. coli* genomes, I discovered unique distributions of other species at the class level, with MAGs assigned to Bacilli being most frequent amongst Hadza, while MAGs from Clostridia were common in the wider community. This finding was intriguing, and it could be explained by the intricate interaction between multiple variables that affect the composition of the gut microbiota. However, these observations were interpreted with caution since data was collected from two separate studies using different methods, including DNA isolation protocols, and sample collection times, which may have impacted the reconstruction of community composition on the basis of MAGs.

Dietary habits have consistently been identified as a major contributor to the divergence in gut microbiota composition between industrialized and Hadza populations (Schnorr *et al.,* 2014; Fragiadakis *et al.,* 2019). Both Clostridia and Bacilli consume polysaccharides and amino acids as carbon sources (Tiffany *et al.,* 2021) but the type of substrates that are metabolized can vary between the two communities and individual species. Presence of polysaccharides and amino acids in the diet of both communities might explain why both classes of bacteria are unequally distributed among the two communities (Benson *et al.,* 2010). Potentially, seasonal differences in eating habits may cause the observed differences (Tiffany *et al.,* 2021; Smits *et al.,* 2017). Changes in food availability are thought to affect Hadza dietary patterns (Fragiadakis *et al.,* 2019). These shifts in food sources may impact the presence and development of specific bacterial groups like Bacilli and Clostridia. Adaptability of bacteria to nutrient availability and dietary composition may also play a role in gut microbiota dynamics (De Filippo *et al.,* 2010; Koren *et al.,* 2012; Fan *et al.,* 2014; Mahdavinia *et al.,* 2019). Host-related factors, as well as hygiene, and bacterial interactions in the gut are also reported to contribute to variation in the composition of the gut microbiota (Spor *et al.,* 2011; Benson *et al.,* 2010). Diets high in fibre are known to select for bacteria that are adept at breaking down fibrous foods, such as Bacteroides (David et al., 2014). This may help to explain why Bacilli were more commonly observed in the Hadza population, versus the urban population, as the Hadza diet is relatively rich in fibre. On the contrary, diet in the urban population

incorporates processed foods, fats, sugar and low in fibre which is known to favour the growth of other bacteria, like Clostridia.

Out of the entire set of reconstructed assembled genomes, only three *E. coli* genomes were identified. This finding could be partially explained by the inherent quality of the data (De Simone *et al.,* 2020). The construction of MAGs from Illumina shotgun metagenomic data has limitations, which may be associated with sequencing depth, contamination and other challenges in the reconstruction of MAGs (Wooley and Ye, 2010; Royalty and Steen, 2018; De Simone *et al.,* 2020). For instance, low sequencing depths may lead to an underrepresentation of low-abundance species, whereas contamination (presence of foreign DNA in the MAGs) can prevent successful reconstruction of low abundance species (Wooley and Ye, 2010; Royalty and Steen, 2018; De Simone *et al.,* 2020). Other challenges may be associated with the inability to recover complete genomes of MAGs. Furthermore, the lack of optimal balance between sensitivity and specificity during dereplications may affect the preservation of species diversity (Evans and Denef, 2020). Aggressive dereplication thresholds may result in losing species, whereas insufficient dereplication can lead to an overrepresentation of dominant species in the MAG dataset (Evans and Denef, 2020). In our study we used checkM to determine the quality of the *E. coli* genomes. Despite SRR1930122 having 91.7% completeness, the contamination level in this MAG was remarkably high at 49.8%. This implies that the genetic content in this MAG likely originates from organisms other than *E. coli* or that it lacks essential core genes that would allow accurate determination of the sequence types. This is likely the reason the genome could not be assigned a sequence type (ST). On the contrary, both SRR1929408 and SRR13348582 had high levels of completeness (97.3% and 97.9%, respectively) suggesting that a significant portion of the target genome is present in each of them. Furthermore, the contamination levels for the two MAGs were relatively low, estimated at 24.5% for SRR1929408 and 6.4% for SRR13348582, suggesting minimal presence of genetic material from contaminants.

E. coli genomes that were successfully reconstructed belonged to phylogroup A and one from phylogroup B1. Phylogroup A strains are typically identified as commensal or non-pathogenetic *E. coli* strains. They are generally part of the normal gut microbiota and may coexist harmlessly with their host (Russo and Johnson, 2000; Derakhshandeh *et al.,* 2013). The identified *E. coli* genomes belonged to different ST types. Two of the Hadza strains were ST10 and ST216, while

the third had an allelic profile which was four alleles distant from ST4946. ST10 is ubiquitous and has been found in various environments and the human gut (Schaufler *et al.,* 2019; Tarabai *et al.,* 2021). ST216 strains have previously been linked to avian hosts i.e., poultry (dos Santos *et al.,* 2023) and this study suggests that strains of this ST can also be present in humans, suggesting transmission between human and livestock through direct and indirect transmission routes. Both of these STs have also been linked to virulence and resistance in previous studies (Schaufler *et al.,* 2019; Tarabai *et al.,* 2021). Since reconstructed MAGs are largely composed of chromosomal genomes (Steward and Rappé, 2007; Hugenholtz and Tyson, 2008), virulence and resistance genes can be easily missed as they are commonly carried on plasmids (Wooley and Ye, 2010).

Aromatic amino acid biosynthesis genes were identified in *E. coli* genomes to gain insight into their metabolic potential. An absence of *aroC* gene in two strains originating from Hadza individuals was found. This gene is critical for the synthesis of chorismate which is an intermediate compound of the Shikimate pathway in aromatic amino acid biosynthesis (Charles *et al.,* 1990); Chandel, 2021). The *E. coli* strains thus do not appear to be able to synthesise aromatic amino acids *de novo* and it is possible that other members of the gut microbiota, that have not been analysed here, might have this function. Moreover, evidence shows microorganism can still synthesize aromatic amino acids even in the absence of chorismate but these alternative pathways are still poorly understood (Winter *et al.,* 2014). In the *E. coli* genome (SRR13348582) reconstructed from an urban sample, *aroD* was found missing. The *aroD* protein functions as a 3-deydroquinate dehydratase, which is also a crucial step in the biosynthesis of aromatic amino acids and a key intermediate in the shikimate pathway (Peek *et al.,* 2011; Peek, 2014). The absence of *aroE* and *trpD* genes in MAG SRR1930122 was also observed. However, the analysis of this MAG is taken with some caution as this MAG exhibited incompleteness. The *aroE* gene in the shikimate pathway is known to encode the enzyme shikimate dehydrogenase synthase which catalyses the conversion of 3-dehydroshikimate to shikimate, which is an intermediate product in aromatic amino acid pathway (Peek *et al.,* 2011) whilst *trpD* plays a significant role in tryptophan biosynthesis (Zimmer *et al.,* 1991; Balderas-Hernández *et al.,* 2009). The *trpD* encodes for the enzyme (anthranilate synthase) which catalyses the synthesis of anthranilate from chorismate (Balderas-Hernández *et al.,* 2009). The absence of these genes in MAG SRR1930122 suggests that the strain lacks the intrinsic capability to synthesize phenylalanine and tryptophan (Parthasarathy *et al.,* 2018). However, *E. coli* can survive without this gene as it is non-essential (Goodall *et al.,*

2018). The absence of genes in the aromatic amino acid biosynthesis pathway could mean the strain relies on external sources for essential aromatic amino acids. This is consistent with previous research which purported that strains, including *E. coli*, originating from industrialised or urbanised populations are heavily reliant on food as sources of aromatic amino acids (Rampelli *et al.,* 2015)

The absence of the *aroC, aroE, trpD,* and *aroD* genes in the MAGs may also indicate presence of unique adaptive strategies employed by these bacterial strains. The absence of these genes may be compensated through mechanisms such as horizontal gene transfer (HGT) (Tasse *et al.,* 2010; Hehemann *et al.,* 2010) or metabolic cooperation (Kost *et al.,* 2023; Zachar and Boza, 2022). For instance, bacteria within the gut microbiota may absorb amino acids produced by other bacteria that contain the complete biosynthetic pathway (Ashniev *et al.,* 2022). This absorption eliminates the need for having internal synthesis, thereby reducing the necessity for maintaining complete biosynthesis pathways. More specifically for *aroD* gene which encodes 3-dehydroquinate dehydratase, there certain bacteria and archaea that have isoenzymes or similar enzymes that can carry out comparable reactions to *aroD* (Nunes *et al.,* 2020; Schoner and Herrmann, 1976). Both Type I and Type II 3 dehydroquinate dehydratase enzymes despite their differences, facilitate the same reaction (Liu *et al.,* 2015; Millán-Pacheco *et al.,* 2023). *E. coli* could potentially obtain these genes through horizontal gene transfer (HGT) creating functional redundancy (Tasse *et al.,* 2010). However, the presence of these acquired genes depend on HGT occurrence and selective pressures in the environment, so they may not be widely distributed or universally found in *E. coli* populations (Acar Kirit *et al.,* 2022). However further research is required with a much larger dataset for a more comprehensive analysis.

Nearly all the genes associated with the glycolysis pathway were present in the *E. coli* isolates. Interestingly, the *kdul* gene was missing in all isolates but the *pgi* gene was found which performs a similar function to *kdul.* The enzymes encodes by these genes both catalyse the conversion of glucose-6-phosphate to fructofuranose-6-phosphate, which is a crucial step in the breakdown of glucose during glycolysis (Teplyakov *et al.,* 1999; Chandel, 2021). This suggests that these strains might have adopted a redundancy reduction strategy (diCenzo and Finan, 2015) where strains use just one gene for this metabolic process. The absence of *kdul* could be attributed to changes in the

environment affecting nutrient availability, making the strains exclusively use the *pgi* catabolic pathway for their metabolism. The *kdul* has also been documented to be a non-essential gene whose absence does not affect the growth of *E. coli* (Goodall *et al.,* 2018). The absence of the *bglF* gene was also observed in both MAG SRR13348582, originating from the wider community, and SRR1930122 originating from the Hadza community. The *bgIF* gene codes for a protein that functions as glucose transporter. This loss of this gene might be due to the absence of selective pressure to retain it, particularly in environments lacking sugars and substrates that require *bglF* (Amster-Choder, 2005). This means that, under certain conditions, these genes may be silenced by regulatory controls or mutations in the regulatory region. It is a non-essential gene in *E. coli* (Goodall *et al.,* 2018). Diets that are either low in simple carbohydrates, or completely exclude them, may suppress the necessity of *bgIF* gene expression for effective metabolism (Amster-Choder, 2005). However, this has not been thoroughly established outside laboratory conditions.

One significant drawback of this study is the low number (n=3) of Metagenome Assembled Genomes (MAGs) that could be reconstructed from *E. coli*. The interpretation of one of the three *E. coli* MAGs was significantly impacted due to its poor quality. Moreover, the samples used in this study were derived from two research studies that employed different approach for sample processing prior to metagenomic analysis. Consequently, the disparities observed in the composition of gut microbiota between the two communities may largely be attributed to variations in methodology including protocols for DNA isolation and differences in the timing of these studies. However, despite these limitations, with additional time, various approaches could have been employed to improve the quality of the data in this study to enhance the accuracy and reliability of identifying *E. coli* MAGs. One effective approach would involve cross validating the MAGs using advanced genome assembly algorithms like MEGAHIT (Li *et al.,* 2015) and binning tools such as CONCOCT (Alneberg *et al.,* 2014), MaxBin) (Wu *et al.,* 2016) which helps in confirming MAGs and minimizing positives originating from contamination. Another useful approach involves manual curation of MAGs by examining genomic features like gene content, and phylogenetic markers to assess and address contamination. By using markers like 16S rRNA genes or conserved single copy genes to crosscheck the taxonomic identity of MAGs against reference sequences in databases, discrepancies that indicate contamination can be detected. For example, if a purported *E. coli* MAG contains markers from unrelated taxa it will indicate

contamination. Additionally, while the GTDB Tk (Parks *et al.,* 2020) database serves as a tool for taxonomic classification, conducting screenings against other databases like NCBI NT/NR (Sayers *et al.,* 2021) or UniProt (The UniProt Consortium, 2021) could assist, in identifying and eliminating contaminant sequences not adequately represented in GTDB Tk. In addition, adjusting the assembly settings by experimenting with different k-mer sizes, and alternative assembly modes could improve the assembly process. Specific assembly modes, such as --meta-sensitive or --meta-large in MEGAHIT, are designed to handle data complexities effectively by adjusting algorithms to better deal with highly complex or large datasets (Li *et al.,* 2015). For example, the --meta-sensitive mode enhances sensitivity to low-abundance sequences, improving the detection and assembly of rare genomes within a metagenomic sample, while the --meta-large mode optimizes the assembly process for large datasets by balancing computational load and assembly accuracy Li *et al.,* 2015. Employing evaluation tools such as QUAST (Mikheenko *et al.,* 2018), which also offers metrics like N50, and total assembly size would offer comparative data output for cross validation with checkM. Additionally, the use BUSCO (Manni *et al.,* 2021) which assesses completeness using conserved single copy orthologous genes could also further elevate data quality. Integrating these assessments with those from dRep and checkM (Parks *et al.,* 2020) coupled with visualizing composition through BlobTools (Laetsch and Blaxter, 2017) aids, in pinpointing and eliminating contaminants. By implementing these approaches, the data quality for identifying *E. coli* MAGs can be significantly improved, leading to more accurate and reliable results. To effectively address these limitations future research endeavours should incorporate large datasets to facilitate reanalysis and conduct a thorough examination of variations, between the broader Tanzanian community and Hadza communities.

Chapter 5
General Discussion

5.1. General Discussion

With the advent of whole genome sequencing (WGS), the identification and characterisation of opportunistic pathogens has become significantly easier. This breakthrough has allowed tracking of mutations and genes that contribute to antimicrobial resistance and pathogenicity. Public health initiatives are increasingly using WGS to understand and implement control strategies to control disease outbreaks. The current study focused on identifying reservoirs of AMR amongst British travellers to Kenya and in different animal hosts, specifically in two animal husbandry systems (poultry and pigs), to understand the dynamics of AMR spread and acquisition of opportunistic pathogens, *Klebsiella* and *E. coli*. The diversity of reconstructed *E. coli* MAGs in two communities with varying levels of exposure to antimicrobials and different lifestyles was examined to gain a deeper understanding of the dynamics of AMR and virulence.

The analysis presented in Chapter 2 is building on previous work which indicates that visiting areas with high rates of antibiotic usage and disease prevalence, increases the likelihood of contracting antibiotic-resistant bacteria (Wiklund *et al.,* 2015; Schwartz, 2018). This seeks to gain insight into the possible acquisition of resistance bacteria during the deployment of British soldiers to Kenya. *Klebsiella* species diversity was evaluated and screened for potential antimicrobial resistance genes, virulence genes, and mobile genetic elements associated with the resistance genes. One of the primary conclusions in this chapter was that colonisation of soldiers by *Klebsiella* species was mostly transient, as different *Klebsiella* strains were found in the first and second sampling timepoints. Relatively few individuals were found who retained the same strain between the first and second timepoints, suggesting the persistence of certain strains within the gut microbiota. Considering that the majority of strains were obtained from individuals whose samples were collected during the rainy season, it is evident that acquisition of these strains frequently occurred during this time. In addition, multidrug-resistant *K. variicola* strains were identified with minimal genetic distance (less than 20 SNPs), that were detected in most individuals in the second time point. This result prompted us to speculate about the potential existence of a point source that served as a reservoir for the *K. variicola* clone that was identified in multiple individuals. Despite not being the focus of our study, this research thus also suggests the potential presence of alternative sources of AMR reservoirs other than human hosts within military deployment settings

that likely contributed to the spread of resistant bacteria. Sampling of these reservoirs in future studies in addition to the stool samples of individual soldiers, is recommended to determine transmission pathways among military personnel. Contrary to the existing literature which follows the acquisition of Extended-Spectrum Beta-Lactamase (ESBL)-producing bacteria as a significant concern during travel (Tängdén *et al.,* 2010; Lübbert *et al.,* 2015; Worby *et al.,* 2023), this study found acquisition of ESBL-producing *Klebsiella* was relatively rare, compared to MDR strains. Therefore, this study emphasizes the importance of considering other resistance profiles beyond acquisition of ESBL-producing bacteria during travel.

In chapter 2 the potential mechanisms by which MDR could be transferred between *K. variicola* strains and between hosts was investigated. The genomic context of the *dfr* and *sul* genes in all the MDR *K. variicola* strains was found to be linked to the same signature sequence, which was associated with IS*26*. This strongly implied that there was a mobile genetic element mediating the transfer of these resistance genes between strains. To further examine this, long-read sequencing of two of the MDR *K. variicola* strains was conducted, which led to the discovery of a complete plasmid containing the *dfr* and *sul* genes, along with IS*26* from which the signature sequence was identified in one of the isolates. Moreover, two individual plasmids were identified in one strain that cointegrated into one plasmid in the other strain during horizontal gene transfer between bacterial cells. This discovery affirmed that multidrug resistance was being conferred by these plasmids and subsequent cointegrates across the rest of the isolates. This chapter adds to the existing evidence demonstrating the importance of mobile genetic elements in the transmission and sharing of AMR genes during travel and highlights the importance of further study in this area to aid in understanding the future spread of AMR. The limitation that only single isolates were examined in this study is acknowledged. Therefore, the use of multiple isolates in the future is suggested or metagenomic data to gain a more complete understanding of transmission dynamics and the AMR landscape. On the other hand, conducting a longitudinal study that tracks *Klebsiella* colonization and resistance levels over a period of time and observing how strains persist and evolve before during and, after travel could help to get a comprehensive overview of bacterial acquisition and AMR development during travel. Additionally, delving into the mechanisms behind resistance acquisition by conducting in depth sequencing and analysis along with examining resistance islands could offer valuable insights into the genetic elements contributing to resistance development. The other approach is exploring host related factors like responses and

microbiome composition which may shed light on how they impact *Klebsiella* colonization and the transfer of resistance genes. Similarly, conducting sampling and ecological investigations could help pinpoint sources of *Klebsiella* bacteria and resistance genes in various settings shedding light on the environmental factors facilitate the spread of resistance. Moreover, comparative genomics studies involving surveillance of different geographical locations and phylogenetic analysis could also help map out transmission pathway and distribution of resistance genes. This additional analysis would provide a comprehensive understanding of the factors influencing *Klebsiella* colonization and resistance, aiding in the development of better strategies to combat antibiotic resistance.

In chapter 3, role of different animal husbandry systems in the dissemination of antimicrobial resistance was studied. There were contrasting resistance patterns between the three animal groups (pigs, broilers, and layers). One major finding was that layers exhibited higher prevalence of resistance genes to phenicols and streptothricin whereas broilers and pigs showed higher prevalence of resistance genes to trimethoprim, sulphonamide, and aminoglycosides. These differences suggest the presence of varying factors that influence patterns of antimicrobial resistance in *E. coli* across animal sectors. Research has shown that the dynamics of antimicrobial resistance are shaped by the use of antibiotics (Baran *et al.,* 2023), environmental factors (Yang *et al.,* 2022; Fu *et al.,* 2022), agricultural practices (Van Boeckel *et al.,* 2019), and regulatory practices (Bavestrello *et al.,* 2002; Joshi *et al.,* 2023). Poultry-derived isolates were found to be more frequently resistant to multiple antimicrobial classes compared to pig isolates, which suggests that poultry farming fosters the development and spread of resistance to a greater extent than pig farming. In a broader context, these results highlight the essence of tailoring effective strategies which consider unique requirements and difficulties encountered by different livestock industries. Our findings also contribute to the existing body of work which demonstrates the poultry sector is linked to a high prevalence of antibiotic resistance. The complete assembly of plasmids in this chapter and the identification of resistance genes on these plasmids, shed light on the mechanisms behind the dissemination of antibiotic resistance. It is evident from the data gathered in this chapter that plasmids played a critical role in the spread of antibiotic resistance among bacterial populations associated with livestock, and this may pose issues in the design of effective efforts to contain the global spread of antibiotic resistance genes among opportunistic pathogens.

To gain a comprehensive overview of transmission dynamics in these animal populations, AMR spread, and genetic elements involved, future studies using long-read sequencing technologies such as Oxford Nanopore or PacBio should be conducted. These technologies are known to address the constraints of Illumina sequencing by offering providing more complete and contiguous genome assemblies, which enhance the resolution of plasmids, mobile genetic elements, and the architecture of AMR genes. Similarly, validating the functions of the identified AMR genes through cloning and expression in *E. coli* strains followed by antimicrobial susceptibility testing may help confirm phenotypic resistance traits associated with these genes. Moreover, analysing plasmids and exploring gene transfer mechanisms such as conjugation and transformation through experiments under controlled conditions in laboratory settings could offer insights on how HGT events occur in *E. coli* and other bacterial species. By analysing the regulation and expression of resistance genes using RNA sequencing techniques and investigating the response of promoters to stress could help reveal pathways influencing the expression of resistance related genes. Exploring the gut microbiota, in pigs and poultry through such analysis may also provide insight into how microbial communities interact and influence the behaviour of *E. coli* strains in terms of survival, colonization and resistance. On the other hand, studies on environmental sources such, as water, soil and feed in the farms can offer valuable insights into on environmental reservoirs of *E. coli* and how it spreads between different species. Furthermore, in depth investigations that integrate spatial analysis and monitoring across different farm locations can pinpoint transmission pathways which may aid in the prevention of resistant strain dissemination. These additional investigations will enhance our understanding of factors influencing the colonisation and resistance dynamics in *E. coli* along, with the evolutionary context associated with the spread of antimicrobial resistance these animal populations.

Our objective in chapter 4 was to understand how different ecological contexts associated with varying levels of antimicrobial exposure may impact the diversity of *E. coli* within the human gut. This was assessed by constructing *E. coli* genomes from publicly available shotgun metagenomic data of the gut microbiome of the Hadza (hunter-gatherers in Tanzania) (Rampelli *et al.,* 2015) and the general urban population in Tanzania (Stražar *et al.,* 2021). Hadza were used as a baseline community with minimal exposure to antimicrobials, as well as unique diet to understand how diversity of gut microbiome varied compared to the general urban population. Out of the

reconstructed MAGs three *E. coli* MAGs were successfully identified, two of which belonged to phylogenetic group A and one in phylogenetic group B1. The presence of phylogroup A *E. coli* strains in both communities highlights the importance of this lineage as commensals of the human gut (Stoppe *et al.,* 2017; Aswal *et al.,* 2023). In the case of other taxa identified, there was considerable variations between the Hadza and the general urban population. For instance, Bacilli were prominent in the Hadza community whilst Clostridia was more prevalent in the wider community. The differences between the two communities were hypothesized to have resulted from variations in lifestyles due to dietary and antibiotic exposure. Variations in diet, lifestyle and antimicrobial exposure have been reported in the past to promote varying biotic profiles (Schnorr *et al.,* 2014; Arora *et al.,* 2021). Since we did not find any resistance or virulence genes in the metagenomes, we decided to acquire an understanding of the variability of functional characteristics with particular focus on aromatic amino acid biosynthesis in the two communities. The absence of *aroC*, *aroE* and *trpD* was found in the *E. coli* MAGs in the Hadza population compared to the urban population. This could be partly attributed to the quality of the data as MAG SRR1930122, which belonged to Hadza, was found to have a high level of contamination. On the contrary, this might suggest that there are differing capacities for aromatic amino acid synthesis, almost certainly due to differing diets between the host populations. Data used in this chapter had limitations due to its diverse sources, covering studies conducted at various times throughout the year. Seasonal variations in the hunter-gatherer diet practiced by the Hadza people were also not accounted for and may have a significant impact on the findings if repeated. For instance, it is known that the Hadza people experience limited food availability during the cold and dry seasons (Marlowe *et al.,* 2014; Shadrack and Mwalilino, 2022), which could impact the composition of their gut microbiota.

To further support and expand on the findings in this chapter, several follow-up experiments could be valuable. One of them includes conducting long read metagenomics to increase the quality of the MAGs. The other is conducting in-depth functional analyses of the *E. coli* MAGs to help verify the metabolic capabilities and adaptation strategies of these bacteria in different communities. Expanding sampling and metagenomic sequencing to include more diverse populations and conducting longitudinal studies to observe the temporal dynamics of *E. coli* populations would enhance the understanding of microbial community resilience and adaptation to environmental or lifestyle changes. Additionally, comparative genomics with other bacterial species could reveal if

similar genomic adaptation patterns are present across the gut microbiome. Finally, host-microbe interaction studies focusing on immune response and nutrient absorption, along with ecological studies, could provide insights into the interactions between microbial populations within the gut ecosystem under different dietary and environmental pressures. These approaches would not only deepen our understanding of *E. coli* adaptations but also inform broader ecological and evolutionary theories of the human microbiome

This thesis sheds new light on the dynamics of antimicrobial resistance in different contexts in sub-Saharan Africa. This work highlights the need for an integrated One Health approach to tackling AMR spread and management. This study demonstrates that *Klebsiella* acquired during travel can include strains with antimicrobial resistance genes, and thus highlight the importance of AMR surveillance amongst travellers before and after they return home. This will allow facilitation of refined control strategies which consider travel as a contributing factor in the dissemination of MDR bacteria. Furthermore, this study shows how the dynamics of AMR differ between different *E. coli* hosts in animal production context, showing the need for context specific AMR stewardship initiatives in different agricultural sectors. Since pork and poultry are commonly used as primary dietary protein sources in many regions of the world including sub-Saharan Africa, the presence of MDR *E. coli* strains in these parts of the food chain is concerning as this offers a route for these pathogens to reach humans. The study also shows the pivotal role that mobile genetic elements such as plasmids play in the dynamics of resistance and the consequent risk for rapid dissemination and spread of AMR genes through and between human and animal hosts. This finding gives insight into potential reasons for the AMR problem in sub-Saharan Africa and gives more weight to the importance of adopting a One Health approach to antimicrobial governance. The study also adds some much-needed WGS data to the otherwise sparce literature around the question of AMR patterns in sub-Saharan Africa. In conclusion, taking a One Health approach and implementing targeted, context-specific interventions that are sensitive to local culture and practices are vital to stem the rising tide of AMR in sub-Sahran Africa.

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7. Appendix

Table A1 Functions of unique genes identified in strain 173V2.

DISTINCT FUNCTIONS OF UNIQUE GENES	GROUP FUNCTION
Selenocysteine-specific elongation factor	**Translation factors:**
Nickel/cobalt efflux system Putative transporter ATP-binding component Putative heme utilization/adhesion exoprotein Proline/betaine transporter	**Transporters and efflux Systems**
Acyl-CoA dehydrogenase Mannose-1-phosphate guanylyltransferase Putative nitrate reductase ribonucleoside-diphosphate reductase Long-chain-alcohol dehydrogenase Putative iron-containing alcohol dehydrogenase NAD(P)H azoreductase Myo-inositol 2-dehydrogenase Phosphoenolpyruvate carboxylase Malonyl-[acyl-carrier protein] O-methyltransferase Quinone oxidoreductase Putative lavin-dependent oxidoreductase Oxaloacetate decarboxylase (Na(+) extruding) Glycosyltransferase family 32 protein Deoxyribodipyrimidine photo-lyase	**Metabolism**
Hemolysin	**Virulence factors and toxins**
Filamentous hemagglutinin Enterobactin synthase subunit F transcription factor or regulatory protein.	**Regulatory proteins**
Cytochrome c-type biogenesis protein CcmF ATP-dependent dethiobiotin synthetase BioD 1	**RNA processing enzymes**

Table A2 Functions of unique genes identified in 139V2_Hi (a hybrid sequence derived from isolate 139V2)

DINSTINCT FUNCTIONS OF UNIQUE GENES	GROUP FUNCTION
Putative inner membrane permease of ABC transporter Transporter protein	**Transport and membrane function**
Mannose-1-phosphate guanylyltransferase Phosphomannomutase Transketolase 3-methylmercaptopropionyl-CoA ligase 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1- 3D-(3,5/4)-trihydroxycyclohexane-1,2-dione hydrolase Acyl-CoA dehydrogenase family protein	**Metabolism and biosynthesis**
Nitrogen fixation regulatory protein Stress-induced acidophilic repeat motif-containing protein	**Regulation and stress response**
Transposase IS110 family transposase IS4321R IS481 family transposase ISKpn28	**Genetic mobility**