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**METAGENOMICS-ENABLED INVESTIGATION OF
MULTIDRUG RESISTANT ORGANISMS AND
ANTIMICROBIAL RESISTANCE GENES IN URBAN
BUILT ENVIRONMENTS**

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PhD

The Hong Kong Polytechnic University

2026

The Hong Kong Polytechnic University

Department of Health Technology and Informatics

**Metagenomics-Enabled Investigation of Multidrug
Resistant Organisms and Antimicrobial Resistance Genes
in Urban Built Environments**

LAM, Alison Yee Ting

A thesis submitted in partial fulfilment of the requirements for the
degree of Doctor of Philosophy

November 2025

Certificate of originality

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(LAM, Alison Yee Ting)

Abstract

Background: The urban built environments (BE) contain a diverse set of microorganisms. However, their health impacts remain poorly understood. In this thesis, large-scale, city-wide surveillance for multidrug-resistant organisms (MDROs) and antimicrobial resistance genes (ARGs) was conducted in two urban BE, the Hong Kong subway system and public washrooms.

Methods: Surface samples were collected from high-touch surfaces from both environments, which were subjected to both culture-dependent and culture-independent methods. Chromogenic agars were used to isolate MDROs from direct surface samples, followed by whole-genome sequencing (WGS) to obtain the full genome for molecular typing and comprehensive ARG profiles. For culture-independent approach, unbiased shotgun metagenomics was performed, followed by taxonomic classification and resistome profiling. For a subset of samples, target enrichment by oligonucleotide probe was performed on metagenomic libraries to enhance the detection of low-abundance and clinically important taxa and ARGs.

Results: Using culture-dependent methods, MDROs were detected in 4.11% of subway samples and 7.33% of washroom samples, with methicillin-resistant *Staphylococcus aureus* (MRSA) being the most detected MDRO in both environments. Whole genome sequencing (WGS) identified clinically significant sequence types (STs), highlighting the presence of high-risk strains in both environments. Metagenomic profiling revealed the ubiquitous existence of high-risk opportunistic pathogens, such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *S. aureus* in high-contact surfaces. Furthermore, clinically relevant ARGs were identified, with *mecA*, the hallmark of MRSA resistance, dominating both environments. Comparative analysis demonstrated that target probe capture enrichment significantly improved the

detection for rare taxa and ARGs by several folds compared to conventional shotgun metagenomics, highlighting its efficacy in detecting low-abundance targets.

Conclusions: These findings contextualize urban BE as reservoirs for MDROs and ARGs, particularly MRSA and the *mecA* gene, posing potential health risks to their occupants. Furthermore, this study demonstrates the utility of metagenomics and target probe capture enrichment in public health surveillance by enabling a sensitive detection of rare but high-risk resistance determinants in low-biomass environmental samples. This study offers a comprehensive and nuanced assessment of the microbial and resistome landscape of urban BE, offering a revolutionary approach for future environmental surveillance.

Publications and presentations

Publications arisen from this study

Alison Yee Ting LAM, Chun Hei LAU, Wing Yin TAM, Chloe Toi Mei CHAN, Tsun Ming LOK, Lam Kwong LEE, Elaine Yin Ying YEUNG, Tsz Kei LAM, Wai Kam CHEUNG, Man Wa CHUI, Ho sing SOONG, Franklin Wang Ngai CHOW, Simon Ching LAM, Sony Nai Yeung SO, Sam Kit San YUEN, Lorna Kwai Ping SUEN, Gilman Kit Hang SIU. (2025). Targeted Probe Capture Metagenomics-Enabled Surveillance of Multidrug-Resistant Organisms and Antimicrobial Resistance Genes in Post-Handwashing Areas of Public Washrooms. *Environmental Microbiome*, 20(1), 143.

Presentations

Alison Yee Ting LAM, Chloe Toi Mei CHAN, Wing Yin TAM, Jasper Shue Kei CHAN, Elaine Yin Ying YEUNG, Jasmine Ho Ching TONG, Ivan Tak Fai WONG, Tsun Ming LOK, Iain Chi Fung NG, Sally Choi Ying WONG, Chun Hei LAU, Patrick Pak Tin HAU, Franklin Wang Ngai CHOW, Gilman Kit Hang SIU. Urban Metagenomics in Action: A Metagenomic-Enabled Surveillance of Pathogens and Antimicrobial Resistance in the Hong Kong Subway System, *The 8th Annual Conference of the Metagenomics and Metadesign of Subways and Urban Biomes (MetaSUB) International Consortium*. 2024, Tokyo.

Awards obtained during PhD study

Alison Yee Ting LAM and Gilman Kit Hang SIU. Urban Metagenomics in Action: A Metagenomic-Enabled Surveillance of Pathogens and Antimicrobial Resistance in the Hong Kong Subway System. *PolyU Research Student Conference 2024 Best Presentation Runner-up*

Acknowledgement

I would like to express my special thanks of gratitude to my supervisor and mentor, Prof. Gilman Siu, who gave me the golden opportunity to work on this project. His guidance, expertise, and passion for science have inspired me throughout my PhD study. The door to Prof. Siu's office was always open whenever I ran into trouble or had a question about my research. He was there to steer me to the right direction. It is an honor to be part of his research team and take part in the urban metagenomics study.

I would also like to thank Dr. Franklin Chow, Dr. Bear Xiong and Dr. Annie Lee, for their invaluable advice and bioinformatic support in my study. Thank you to all past and present members of Gilman's Team for your comments and suggestions on my data and presentations, and for creating a nourishing environment for research. A special thanks to Chloe Chan, who provided a solid foundation for this project and support at the early stage of urban metagenomics study. Thank you, Eddie Lee and Jake Leung, for coaching my bioinformatic skills. Thanks also to Wing Tam and Cyrus Lau for their effort and technical support in sample processing and analysis. I would also like to extend my sincere gratitude to Hiu Yin, Ivan, Iain, Sally, Jasper, Jasmine Tong, Elaine Yeung, Connie, Kei, Forrest and Nicky for their help in sample collection and technical support.

Finally, thank you to my family, parents Helen and Alfred, and my brother, Dayton, for your love and moral support over my PhD journey. My friends, Kitty, Vivian, Irene, KitLing, Tiffany, and Rachel, whom we have spent precious moments and created laughter together. Finally, a special thanks to my husband Ming for supporting my decision to pursue my PhD. I certainly would not have come this far without your love and encouragement.

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List of Abbreviations

ARB	Antibiotic-resistant bacteria
ARG	Antibiotic resistance genes
AMR	Antimicrobial resistance
BE	Built environments
BHI	Braine heart infusion
BPW	Buffered peptone water
CARD	Comprehensive Antibiotic Resistance Database
CDC	Centers for Disease Control and Prevention
CLSI	Clinical & Laboratory Stan
CRAB	Carbapenem-resistant <i>Acinetobacter baumannii</i>
CRE	Carbapenem-resistant <i>Enterobacterales</i>
CRPA	Carbapenem-resistant <i>Pseudomonas aeruginosa</i>
ESBL-E	Extended spectrum β -lactamases (ESBL)-producing <i>Enterobacterales</i>
ESKAPE-E	<i>Enterococcus faecium</i> , <i>Staphylococcus aureus</i> , <i>Klebsiella pneumoniae</i> , <i>Acinetobacter baumannii</i> , <i>Pseudomonas aeruginosa</i> , <i>Enterobacter spp.</i> , and <i>Escherichia coli</i>
HAIs	Hospital acquired infections
HGT	Horizontal gene transfer
HMMs	Hidden Markov Models
ITS	Internal transcribed spacer
MAG	Metagenome-assembled genomes
MALDI-ToF	Matrix-assisted laser desorption/ionization time-of-flight
MDRA	Multidrug-resistant <i>Acinetobacter</i>

MDRO	Multidrug resistant organism
MHA	Mueller Hinton agar
MLST	Multi-locus serotyping
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MTS	Mass transit system
NCBI	National Center for Biotechnology Information
PBS	Phosphate buffered saline
PCoA	Principal Coordinates Analysis
pDST	Phenotypic drug susceptibility testing
PDR	Pandrug-resistant
QACs	Quaternary ammonium compounds
RGI	Resistance Gene Identifier
RPKM	Reads per kilobase million
SNP	Single Nucleotide Polymorphism
ST	Sequence type
VAP	Ventilator-associated pneumonia
VRE	Vancomycin-resistant <i>Enterococcus</i>
WGS	Whole genome sequencing
WHO	World Health Organization
XDR	Extensively drug-resistant

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[Supplementary Table ST10](#) Antibigram and molecular typing results of CRPA isolates from the post-handwashing areas of public washrooms.

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Chapter 1.

Introduction

1.1. Overview

The urban microbiome significantly influences human health. As of 2025, over half (55%) of the world's population, approximately 4.4 billion people, resides in urban areas. This number is projected to reach 6 billion, around 70% of the world's population, by 2050 (UNFPA, 2024; United Nations, 2018). Urban built environments (BE), such as homes, workplaces, public infrastructure, and transportation systems, play a vital role in shaping human health, making it crucial to understand the urban microbiome (Gilbert & Hartmann, 2024). Urban dwellers spend approximately 90% of their time indoors, where they interact with complex microbial communities through activities like skin contact, respiration, and shedding (Klepeis et al., 2001; Stephens, 2016). These interactions shape the urban microbiome and resistome, known as the collection of microbial species and drug resistance determinants, respectively, in urban settings, playing a critical yet understudied role in public health (King, 2014; Bosch et al., 2024). Indeed, urban BE have been implicated in the transmissions and disseminations of various pathogens and antimicrobial determinants (Gilbert & Hartmann, 2024). Consequently, exposure to microbes in BE can therefore result in infectious diseases, highlighting the need to understand microbial communities for monitoring emerging pathogens and ensuring community well-being. In view of their importance in public health, this thesis investigates the microbiome and resistome compositions of two urban BE in Hong Kong, the mass transit system and public washrooms, focusing on multidrug-resistant organisms (MDROs) and antimicrobial resistance genes (ARGs).

Antimicrobial resistance (AMR) is one of the top 10 critical global health threats facing by humankind in the 21st Century, posing significant challenges to healthcare and public health (WHO, 2023; EClinicalMedicine, 2021). It is estimated that AMR contributed directly to 1.27 million deaths per year, and this annual figure is alarmingly projected to increase to 1.91 million by 2050 (Murray et al., 2022; GBD 2021 Antimicrobial Resistance Collaborators,

2024). The surveillance of AMR from the environment is an integral part of the One Health Approach, as the emergence and dissemination of AMR are inextricably linked between human, animal, and the environment (Djordjevic et al., 2024; Hernando-Amado et al., 2019). Among them, MDROs are the top priority of healthcare practitioners worldwide, as they have a fatality rate of 45% among infected patients (Ciapponi et al., 2023). Currently, most AMR surveillance has been primarily focused on healthcare and agricultural settings (Diallo et al., 2020), while efforts to examine MDRO and AMR prevalence in urban BE remain limited (Essack, 2018; Cave et al., 2021).

Urban BE are critical interfaces for human-microbe interactions, harboring a wide array of microbial communities influenced by human activities, building design, and connection to outdoor environments (Meadow et al., 2014; Leung & Lee, 2016; Kembel et al., 2014). The influence of the urban microbiome is further amplified in high-traffic environments, resulting from frequent microbial exchange between occupants and BE. In a metropolitan city, the mass transit system (MTS) is indispensable to its citizens, connecting people from all corners of the city. With an annual ridership of approximately 1.6 billion, the Hong Kong's subway system is the most popular transportation mode among its citizens (Wong, 2015) and was ranked the 10th busiest metro system globally (UITP, 2018). Given its high passenger volume and intense usage, an extensive microbial exchange between passengers and the environment in the urban city's transportation system is consequential (Fernández-Iriarte et al., 2021; Passi et al., 2021). The subway microbiome offers a dynamic environment for studying the dissemination of MDROs and ARGs within the community, as it aggregates microbial contributions from local populations, enabling the identification of transmission patterns. There is a plethora of studies showing that MTS harbors a wide array of pathogens and antimicrobial determinants, which may disseminate AMR within the community (Kang et al., 2018; Danko et al., 2021). However, the role of MTS in the dissemination of clinically important MDROs and ARGs remains

understudied. In Chapter 3 of this thesis, the microbiome and resistome of HK subway system were characterized, focusing on MDROs and clinically important ARGs.

Public washrooms, particularly the post-handwashing areas (e.g., faucets, exit door handles), are often overlooked as potential reservoirs for MDROs and ARGs (Mkrtchyan et al., 2013; Suen et al., 2019). Public washrooms pose elevated infection risks compared to private washrooms due to their greater microbial diversity and high foot traffic (Flores et al., 2011). While handwashing is the cornerstone of personal hygiene, the re-contamination by direct skin contact with these post-handwashing facilities is poorly characterized. Post-handwashing surfaces, including faucets, paper dispensers, hand dryers, and exit door handles, serve as critical junctions for microbial exchange (Vardoulakis et al., 2022). Outbreaks of enteric pathogens such as norovirus have been traced to contaminated public washrooms in cruise ships and international flights (Ho et al., 1989a; Holmes & Simmons, 2009). Chapter 4 delineates the microbiomes and resistomes of post-handwashing sites across different public washrooms in Hong Kong, including healthcare, commercial, and recreational facilities, focusing on MDROs and clinically important ARGs identified in post-handwashing surfaces.

Traditionally, pathogen surveillance of BE was accomplished by culture-based methods, which have a limited scope and are inefficient in identifying new emerging pathogens. With the advent of high-throughput sequencing (HTS), the study of microbial communities in BE was revolutionized (Forbes et al., 2017). With the advent of high-throughput sequencing (HTS), whole genome sequencing (WGS) of isolates provides comprehensive genomic profiles, which enable phylogenetic studies and a full resistance profile. Recently, the applications of metagenomics, which explores all genetic materials, have demonstrated its ability in public health surveillance (Ko et al., 2022). In 2015, the founding of the Metagenomics and Metadesign of the Subways and Urban Biomes (MetaSUB) International Consortium further highlighted the utility of metagenomics in studying the BE microbiome, including the detection

of emerging infectious disease outbreaks, antimicrobial resistance mapping, and the identification of novel pathogens (Mason et al., 2016a).

Therefore, understanding the prevalence of MDROs and AMR determinants within the context of the urban BE comprehensively using novel genomic tools, which is the aim of this study, is essential for safeguarding both personal and public health. Unraveling the composition of urban microbiome could contribute to the development of novel public health surveillance strategies and guide the formulation of public health policies in the long run.

1.2. Antimicrobial Resistance (AMR)

1.2.1. The Rise of AMR as a Major Public Health Threat

Antimicrobial resistance (AMR), defined as the ability of microorganisms to counteract the effects of antimicrobial drugs and thus render them ineffective, has emerged as a major threat to human health in recent years. It is an ancient and naturally occurring phenomenon that is widespread in the environment (D’Costa et al., 2011). According to the global bacterial AMR burden study conducted by Antimicrobial Resistance Collaborators, it was estimated that 1.27 million and 4.95 million deaths were directly and indirectly caused by AMR in 2019, respectively (Murray et al., 2022). It has been projected that, if no counter-measures are taken, the death toll caused by AMR will rise to 1.91 million annually by 2050 (GBD 2021 Antimicrobial Resistance Collaborators, 2024). In view of the aggravating severity of AMR, WHO has declared AMR as one of the top 10 global public health threats encountered by humanity (EClinicalMedicine, 2021; WHO, 2023).

The rise of AMR can be primarily attributed to the widespread misuse and inadequate stewardship of antimicrobials across various sectors, including healthcare, animal husbandry and, agriculture (Ferri et al., 2017; Heuer et al., 2011). Antibiotics are unnecessarily and inappropriately prescribed to patient with non-bacterial infection, such as viral infections,

which contributes significantly to the problem. According to the Centers for Disease Control and Prevention (CDC), up to 50% of all outpatient antibiotic use was deemed inappropriate, including unnecessary use and inappropriate selection, dosing, and duration (Pichichero, 2002; Shapiro et al., 2014). Moreover, antibiotics have been extensively used in livestock farming and agriculture for treatment, control of diseases, and growth promoters in livestock and crop production (Witte, 2000). Further exacerbating the situation, the dense environments in which food animals are raised facilitate the exchange of drug-resistant organisms and antimicrobial determinants, creating a positive selection pressure for resistance (Manyi-Loh et al., 2018).

While historically AMR has been predominantly associated with bacterial infections, the emergence of antifungal resistance has been a concern lately. Although not as severe as bacterial resistance, nosocomial antifungal resistance has been increasingly reported worldwide (Fisher et al., 2022). Examples include the increasing identification of azole-resistant variants of *Aspergillus fumigatus* in patients and the newly emerging fungal pathogen, *Candidozyma auris* (basonym: *Candida auris*), which is resistant to multiple antifungal drugs with a reported mortality rate of 30-50% in infected persons (Benedict et al., 2023; Egger et al., 2022; Ortiz-Roa et al., 2023; Rivelli Zea & Toyotome, 2022).

The burden of AMR on healthcare systems and society is substantial as it renders bacterial infections more difficult to treat (Roberts et al., 2009). Therefore, it is imperative to enhance AMR surveillance and improve the identification of ARGs circulating within the community. Events of AMR spill-over from hospitals and agriculture into urban BE, such as public transportation systems, nursing homes, parks, and food supply chains, are well-documented (Conceição et al., 2013; Lutz et al., 2014; Mody et al., 2025; Roberts et al., 2009; Santos et al., 2020).

Having a comprehensive understanding of the AMR is therefore crucial in the formulation of public health policies and targeted disinfection strategies to mitigate the

dissemination of antimicrobial determinants present in the environment. The study of resistome is a promising way to trace the dissemination of ARGs within the environment (D'Costa et al., 2006; Djordjevic et al., 2024). The metagenomic approach is ideal for AMR surveillance as it enables the examination of all ARGs present within the sample, allowing a comprehensive evaluation of AMR burden (Wheeler et al., 2023). Identifying clinically important ARGs thus serves as an early indication of the presence of resistant strains within the community and a potential outbreak.

1.2.2. Origins of Antimicrobial Resistance

Antibiotics are found in bacteria and fungi as natural defense mechanisms (D'Costa et al., 2011). Bacteria become resistant to antibiotics by two mechanisms: intrinsic resistance and acquired resistance.

1.2.2.1. Intrinsic Resistance: The Natural Defense against Antibiotics

Intrinsic resistance, also known as natural resistance, refers to the inherent ability of bacterial species to resist specific antibiotics. It is a natural characteristic independent of prior antibiotic exposure (Cox & Wright, 2013; Martinez, 2014). The genes responsible for intrinsic resistance are present in the bacterial core genome, universally shared within a species, and are consistently expressed (Reygaert, 2018). Since the genes reside on the bacterial chromosome, they cannot be easily transferred between different species of bacteria.

Common mechanisms of intrinsic resistance include a lack of affinity of the drug to the bacterial target, reduced permeability of the outer membrane, extrusion of antibiotics by efflux pump, and the presence of an antibiotic-degrading enzyme. [Table 1.1](#) summarizes some examples of common environmental bacteria with intrinsic resistance. For example, *Pseudomonas aeruginosa*, a commensal in soil and water, is naturally resistant to

aminoglycosides, penicillins, and celphalosporins due to its low permeability of the cell wall (Lambert, 2002). While efflux pumps were common in soil bacteria, such as *Pseudomonas*, *Stenotrophomonas*, *Sphingobacterium*, and *Chryseobacterium*, making them intrinsically resistant to a wide array of drugs (Armalytè et al., 2019).

Table 1.1. Examples of microorganisms with intrinsic drug resistance.

Organism	Intrinsic resistance	Reference
<i>Acinetobacter</i> spp.	Ampicillin Glycopeptides Aztreonam Ertapenem Chloramphenicol Fosfomycin	CLSI 2022
<i>Pseudomonas aeruginosa</i>	Sulfonamides Ampicillin 1st and 2nd generation Cephalosporins cefotaxime ceftriaxone Chloramphenicol Tetracycline	CLSI 2022
<i>Bacteroides</i> spp.	Aminoglycosides Penicillin Ampicillin	CLSI 2022
<i>Klebsiella pneumoniae</i>	Ampicillin Ticarcillin	CLSI 2022
<i>Enterococci</i>	Cephalosporins Aminoglycosides Clindamycin Fusidic acid Trimethoprim	CLSI 2022
All gram positives	Aztreonam	Reygaert, 2018
All gram negatives	Glycopeptides Lipopeptides	Reygaert, 2018
<i>Candida auris</i>	Fluconazole Voriconazole Amphotericin B Echinocandins	Gómez-Gaviria et al., 2023

CLSI, Performance Standards for Antimicrobial Susceptibility Testing, CLSI M100 (32nd ed)

1.2.2.2. Acquired Resistance: How Bacteria Learn from Each Other

Unlike intrinsic resistance, which is specific to the entire bacterial species, acquired resistance arises from individual strains due to alterations of their existing genetic composition. There are two main mechanisms, spontaneous mutation and horizontal gene transfer (HGT) (Djordjevic et al., 2024).

Although spontaneous mutation occurs at a rate of 1 event per 10^7 to 10^{10} , which is relatively rare, the large population and fast growth rate of bacteria still enable the emergence of AMR mutants over time (Michael et al., 2014; Rosenblatt-Farrell, 2009). Ciprofloxacin resistance resulting from mutations in DNA topoisomerase IV was identified in *Mycobacterium tuberculosis* and *Staphylococcus aureus* (Gao et al., 2015; Hjort et al., 2020). Additionally, random mutations in the RNA polymerase β -subunit (rpoB) confer resistance to rifampin in *M. tuberculosis* (Hjort et al., 2020).

HGT, however, is more concerning as it mediates the spread of ARGs between bacteria, even across different species. Mobile genetic elements (MGEs), such as composite transposons, plasmids, and bacteriophages, act as vehicles that mobilize genetic elements from one bacterium to another. There are three main mechanisms of HGT: conjugation, transduction, and transformation. In conjugation, direct physical contact between neighboring bacterial cells is required as the ARG-carrying plasmid is injected into recipient cells from the donor cell via sex pili. In comparison, transduction involves a bacteriophage, which first infects the donor cell. During the replication of the donor cell, ARG is incorporated into the bacteriophage genome, which then infects the recipient cell and thus transmits the ARG into the recipient genome. Lastly, transformation involved the uptake of extracellular DNA from the surrounding environment. It can occur naturally and has been described in at least 60 species of bacteria, such as *Streptococcus pneumoniae*, *Helicobacter pylori*, *Neisseria gonorrhoeae*, and *Acinetobacter baylyi* (Domingues et al., 2012).

1.2.3. Mechanisms of Antibiotic Resistance

Bacterial AMR, also known as antibiotic resistance, arises through diverse mechanisms that undermine the efficacy of antimicrobial agents. These mechanisms can be broadly classified into five categories: (1) limiting drug uptake, (2) active drug efflux, (3) alteration of drug targets, (4) inactivation of drugs, and (5) target bypass. A summary of antibiotic modes of action and corresponding resistance mechanisms is provided in [Table 1.2](#).

Limiting drug uptake

Bacteria can reduce antibiotic uptake by altering outer membrane permeability, primarily through modifications to porin proteins. Porins, which facilitate the passive diffusion of antibiotics across the outer membrane of Gram-negative bacteria and some Gram-positive bacteria, can be downregulated, functionally impaired, or replaced with less permeable variants (Vergalli et al., 2020; Prajapati et al., 2021). For instance, the *oprD* mutation in *Pseudomonas aeruginosa* results in the production of aberrant porins, significantly reducing drug uptake and thereby rendering resistance to carbapenems (Quale et al., 2006). Contrastingly, *Mycobacteria* adopt a distinct strategy via the production of mycolic acid layer and a capsule-like coat of polysaccharide and proteins, creating a robust permeability barrier and limiting entry of antibiotics (Draper, 1998; Grzegorzewicz et al., 2012; Kalscheuer et al., 2019). Recent studies also highlight the role of biofilm formation in further limiting drug diffusion to individual bacterial cells, further enhancing drug resistance (Mirghani et al., 2022).

Active drug efflux

In contrast to porins, efflux pumps are energy-dependent transmembrane proteins that actively extrude antibiotics out of bacterial cells, thus reducing intracellular drug concentrations and conferring broad-spectrum resistance. There are six families of efflux

pumps, including the resistance nodulation division (RND) family, ATP-binding cassette (ABC) transporter family, multidrug and toxin extrusion (MATE) family, major facilitator superfamily (MFS), small multidrug resistance (SMR) family, and proteobacterial antimicrobial compound efflux (PACE) family (Zack et al., 2024). Overexpression of efflux pumps is a key driver of multidrug resistance in various pathogens, including *P. aeruginosa* (Lomovskaya et al., 2001), *M. tuberculosis* (Long et al., 2024), *S. aureus* (Yu et al., 2022), and *E. coli* (Bohnert et al., 2007; Keeney et al., 2008; Yasufuku et al., 2011). Research also highlights the synergistic roles of efflux pumps with other resistance mechanisms, such as biofilm formation and target modification, in high-level resistance (Singh et al., 2012; Luo et al., 2023).

Alterations of drug targets

To exert its effect, antibiotics rely on high-affinity binding to specific cellular targets. This binding is dependent on the target's structure or composition. Bacteria can reduce this affinity through genetic mutations or post-transcriptional modifications, which alter the structure of the cellular target. Mutations in penicillin-binding proteins (PBPs), the targets of beta-lactam drugs, are a hallmark example. For instance, a mutation in PBP3 in *E. coli* results in lower affinity to monobactams, thereby preserving cell wall synthesis (Periasamy et al., 2020).

Modifications of the drug target are another critical resistance mechanism. Ribosomal methylation of 23S rRNA by *erm*-encoded methyltransferases prevents macrolide binding (Bhujbalrao & Anand, 2019). Likewise, 16S rRNA methylation, mediated by enzymes like ArmA or RmtB, confers aminoglycoside resistance in *Enterobacteriaceae*, *P. aeruginosa*, and *Acinetobacter spp.* (Doi & Arakawa, 2007; Sharkey et al., 2016). Resistance to colistin, a last-resort antibiotic treating multidrug-resistant Gram-negative bacteria infections, arises from the

addition of phosphoethanolamine (pEtN) to lipopolysaccharide (LPS) by MCR-1, thus lowering lipopolysaccharides' affinity to colistin (Gao et al., 2016).

In addition to target modification, protein-mediated target protection is also common. Recent studies have identified the ATP-binding cassette proteins of the F-subtype (ABC-F) in Gram-positive bacteria protecting ribosomes from antibiotics like pleuromutilin, lincosamide, and streptogramin by binding to the peptidyltransferase center, which is critical in protein synthesis (Crowe-McAuliffe et al., 2021). Protection of the drug target is also exemplified by the plasmid-encoded Qnr proteins in Gram-negative species, which shield DNA gyrase and topoisomerase IV from quinolones, thus increasing the number of enzymatically active enzymes available for DNA replication (Aldred et al., 2014).

Inactivation and modification of drugs

Besides modifying drug targets, modifications can also be made on the drug. This is achieved by the production of degrading enzymes, which neutralize or counteract the effect of the drug. Beta-lactamases, which hydrolyze the amide bond of the beta-lactam ring, are a key example (Tooke et al., 2019). To date, a total of 338 types and over 7,000 subtypes of beta-lactamases have been reported (Zdarska et al., 2024). Among them, carbapenemases, such as KPC, NDM, and OXA variants, are of particular concern owing to their ability to inactivate last-resort carbapenems.

Tetracycline resistance, on the other hand, is mediated by hydrolases encoded by the *tet(X)* family, which has been reported in many bacterial species. Variants *tet(X3)*, *tet(X4)*, and *tet(X5)* are prevalent among *Enterobacterales* and *Acinetobacter* isolates (Li et al., 2021; Yao et al., 2025). Another class of enzymes, aminoglycoside-modifying enzymes (AMEs), including acetyltransferases, phosphotransferases, and nucleotidyltransferases, add chemical

moieties to aminoglycosides, reducing their ribosomal binding affinity (Ramirez & Tolmasky, 2010; Szychowski et al., 2011).

Target bypass

Target bypass utilizes alternative pathways or enzymes to evade actions of the antibiotics, thereby sustaining essential cellular pathways for cell survival. For instance, methicillin-resistant *S. aureus* (MRSA) acquires the exogenous *mecA* gene located in the staphylococcal cassette chromosome *mec* (SCC*mec*), which encodes for PBP2a, an alternative transpeptidase with reduced affinity to beta-lactams. This allows MRSA to maintain peptidoglycan synthesis, while preserving cell wall integrity regardless of the presence of beta-lactams (Peacock & Paterson, 2015).

Similarly, vancomycin resistance in *Enterococci* is mediated by the *van* gene cluster, with *vanA* and *vanB* being the most potent and prevalent. In susceptible bacteria, vancomycin binds to the D-alanine-D-alanine terminal of peptidoglycan precursors, inhibiting peptidoglycan crosslinking. While expression of *van* genes in the resistant strain results in peptidoglycan precursors ending with D-alanine-D-lactate or D-alanine-D-serine, which exhibit lower affinity to vancomycin (Cetinkaya et al., 2000).

Table 1.2 Summary of different antibiotic classes, modes of action and mechanisms of drug resistance.

Antibiotic class	Antibiotic name	Targets	Mode of action	Mechanism of resistance
Aminoglycosides	Gentamicin Streptomycin Kanamycin	30S ribosomal subunit of 16S rRNA	(1) Bind to 30S ribosomal subunit of 16S rRNA, thus inhibiting protein synthesis. (2) Positively charged drugs attach to outer membrane, causing depolarization and loss of membrane integrity	- Modification of drug by acetyltransferase, phosphotransferase, nucleotidyltransferase - Methylation in 16S rRNA - Mutation of 16S rRNA gene - Decrease influx - Increase efflux
Beta-lactams	Penicillins Cephalosporins Cephamecins Carbapenems Monobactams	Penicillin-binding proteins (PBPs)	Bind to PBPs, thus inhibiting crosslinking of peptide chain in peptidoglycan production, impairing cell wall integrity and leading to cell lysis	- Beta-lactamases - Modification of PBPs - Reduce cell membrane permeability - Increase influx
Glycopeptides	Vancomycin	D-alanyl-D-alanine in the peptide chain	Bind to D-alanyl-D-alanine in the peptide chain, thus inhibiting crosslinking of peptidoglycan	- Impermeable outer membrane (intrinsic resistance in Gram-negatives) - Mutation in D-alanyl-D-alanine
Fluoroquinolones and Quinolones	Ciprofloxacin	DNA gyrase, topoisomerase IV	Bind to DNA gyrase and topoisomerase IV, thus inhibiting DNA replication	- Mutation in topoisomerases - Protection of topoisomerases - Increase efflux
Lincosamides	Clindamycin	23S rRNA of the 50S ribosomal subunit	Bind to 23S rRNA of the 50S ribosomal subunit, thus inhibiting protein translation.	- Methylation of 23S rRNA - Inactivation of drug - Increase efflux
Lipopeptides	Daptomycin	Cell membrane	Insert in cell membrane, causing depolarization and ion leakage, which inhibit normal cellular processes like DNA replication, protein translation, cell wall synthesis, and cell death	Thickening and increasing the positive charge of cell wall to reduce the depolarization
Macrolides	Azithromycin Erythromycin	23S rRNA of the 50S ribosomal subunit	Bind to 23S rRNA of the 50S ribosomal subunit, thus inhibiting protein translation.	- Methylation of 23S rRNA - Mutation in ribosome - Modification of drug by phosphotransferase and esterase - Ribosome protection by ATP-binding cassette F (ABC-F) - Increase efflux
Oxazolidinones	Linezolid	23S rRNA of the 50S ribosomal subunit	Bind to 23S rRNA of the 50S ribosomal subunit, thus inhibiting protein translation.	- Methylation of 23S rRNA - Ribosome protection by ATP-binding cassette F (ABC-F)
Phenicol	Chloramphenicol	50S ribosomal subunit	Bind to 50S ribosomal subunit, thus inhibiting protein translation.	- Methylation of 23S rRNA - Inactivation of drug by acetylation - Increase efflux
Polymyxin	Colistin	Lipid A in lipopolysaccharide	Bind to lipid A in lipopolysaccharide, creating pores in membrane, leading to cell lysis.	Modification or removal of lipid A
Pyrimidines	Trimethoprim	Dihydrofolate reductase	Inhibit dihydrofolate reductase, thus blocking folic acid production.	- Acquire new dihydrofolate reductase gene - Increase efflux
Rifamycins	Rifampicin	RNA polymerase	Bind to RNA polymerase, thus inhibiting transcription.	- Mutation in drug target (rpoB) - Ribosylation of drug - Inactivation of drug
Streptogramins	Dalfopristin	23S rRNA of the 50S ribosomal subunit	Bind to peptidyl-transferase of 23S rRNA of the 50S ribosomal subunit, thus inhibiting protein translation.	- Mutations in 23S rRNA - Modification of drug by acetylation - Increase efflux
Sulfonamide	Sulfamethizole	Dihydropteroate synthase	Inhibit dihydropteroate synthase, thus blocking folic acid production	- Mutation in dihydropteroate synthase gene - Acquire new dihydropteroate synthase via <i>sul1</i> and <i>sul2</i>
Tetracyclines	Tigecycline Tetracycline	16S rRNA of the 30S ribosomal subunit	Bind to 16S rRNA of the 30S ribosomal subunit, thus inhibiting protein translation.	- Mutation of ribosome - Inactivation of drug - Ribosome protection - Increase efflux

1.2.4. Last Resort Antibiotics and Associated Genetic Determinants

The escalating crisis of AMR, coupled with the limited economic incentives for developing new antibiotics, has significantly diminished the existing global pool of effective antibiotics over the past decades, jeopardizing the treatment of infectious diseases (Cook & Wright, 2022). To preserve the effectiveness of the current antibiotic repertoire, the WHO had outlined the “AWaRe” Classification to guide the appropriate use of antibiotics in 2017 (Zanichelli et al., 2023).

Under this framework, antibiotics are categorized into three groups: the first tier “Access” group antibiotics, which are those having low resistance potential and are recommended for empirical treatment, the second tier “Watch” antibiotics that are those with higher resistance potential, and the last-resort “Reserve” antibiotics, which should only be used to treat severe infections caused by MDROs, such as carbapenem-resistant *Enterobacterales* (CRE) or MRSA. [Table 1.3](#) provides a list of last resort antibiotics, their drug classes, and associated ARGs.

Table 1.3. Last-resort antibiotics and associated ARGs.

Class	Last-resort antibiotic	Corresponding ARGs
Aminoglycosides	Plazomicin	grdA
Carbapenems	Imipenem/cilastatin/relebactam	blaNDM, blaVIM, blaIMP, blaOXA-48
	Meropenem/vaborbactam	
Fifth-generation cephalosporins	Ceftaroline-fosamil	mecA
	Ceftobiprole-medocaril	mecA
	Ceftolozane/tazobactam	blaNDM, blaVIM, blaIMP, blaAmpC, blaCTX-M
Glycopeptides	Dalbavancin	vanA, vanB
	Oritavancin	
	Telavancin	
	Tigecycline	AcrAB-TolC, tet(X), tet(M), AcrR, MarA, SoxS, RamA, RarA
Lipopeptides	Daptomycin	pgsA, liaFSR, dltABCD, mprF, yycG, cls, gdpD, liaSR, yycFG, drc
Monobactams	Aztreonam	blaNDM, blaVIM, blaIMP, blaAmpC, blaCTX-M
	Carumonam	
Other-cephalosporins	Cefiderocol	bla NDM, bla VIM, bla IMP
Oxazolidinones	Linezolid	cfr, oprA, poxtA
	Tedizolid	
Penems	Faropenem	ompC, envZ
Phosphonics	Fosfomycin	fosA, fosB, murA
Pleuromutilin	Lefamulin	not applicable
Polymyxins	Colistin	mcr-1, mcr-2, mcr-3
	Polymyxin-B	
Streptogramins	Dalfopristin/quinupristin	ermB, vgb vat(E), vat(D)
Tetracyclines	Eravacycline	tet genes
	Minocycline	
	Omadacycline	
Third-generation Cephalosporins	Ceftazidime/avibactam	bla NDM, bla VIM, bla IMP
Trimethoprim-derivatives	Iclaprim	not applicable

1.3. Multidrug-resistant organisms (MDROs) in Built Environments

MDROs are defined as microorganisms resistant to at least three or more antimicrobial categories (Magiorakos et al., 2012). MDRO, predominantly bacteria, is a global threat to human health. Recently, the scope has expanded to include clinically isolated fungal species that exhibit a broad spectrum of drug resistance. Clinically significant MDROs were discussed below.

1.3.1. Multidrug-resistant Bacteria: ESKAPE-E organisms

The ESKAPE-E organisms, comprising seven highly virulent and antibiotic resistant bacterial pathogens – Gram-positive *Enterococcus faecium/faecalis* and *S. aureus*, and the Gram-negative *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp., and *Escherichia coli* – are the leading cause of most nosocomial infections worldwide (De Oliveira et al., 2020; Denissen et al., 2022). Together, they accounted for 70%-97% of the total hospital-acquired infections (HAIs) (Arbune et al., 2021). Most of them are multidrug resistant isolates and often carry transmissible antimicrobial resistance (Botelho et al., 2023).

In view of the aggravating AMR in ESKAPE-E organisms, the WHO had published the Bacterial Priority Pathogen List (BPPL) in 2024 to guide research and development, and public health strategies against AMR (WHO, 2024). MDROs were classified into three priority groups: “Priority 1: Critical”, “Priority 2: High”, and “Priority 3: Medium”.

Methicillin-resistant *Staphylococcus aureus* (MRSA)

S. aureus is a ubiquitous bacterium commonly found on human skin and the nasal cavity as part of the normal flora. While it usually resides asymptotically in healthy individuals, it can sometimes cause a wide range of infections, including skin and soft tissue infections

(SSTIs), wound infections, urinary tract infections (UTI), pneumonia, and bloodstream infection, particularly in immunocompromised individuals. The emergence of MRSA is characterized by resistance to beta-lactam drugs, such as penicillins and cephalosporins. MRSA was first reported in the UK in 1961, shortly after the introduction of methicillin (Jevons, 1961). Over time, MRSA has become one of the most frequent causes of infections in both hospital and community settings (Lee et al., 2018), resulting in more than 100,000 deaths worldwide in 2019 (Murray et al., 2022). MRSA was therefore considered a “Priority 2: High” pathogen in the WHO BPPL.

MRSA arose when *S. aureus* acquired the *mecA* gene, which encodes a modified penicillin-binding protein (PBP2a) with a low affinity to beta-lactam drugs. MRSA infections are categorized into two types: hospital-acquired (HA-MRSA) and community-acquired (CA-MRSA), distinguished based on the staphylococcal cassette chromosome *mec* (SCC*mec*) type and the presence of Panton-Valentine leucocidin (PVL) gene. HA-MRSA strains typically carry SCC*mec* type I, II, or III, whereas CA-MRSA strains predominantly carry type IV or V. Moreover, the PVL gene is also exclusively found in CA-MRSA strains but not in HA-MRSA (Vandenesch et al., 2003). These molecular classifications guide clinical management and infection control strategies.

MRSA has been identified as the most prevalent pathogen in urban BE (Cave et al., 2021; Denissen et al., 2022). Its presence is well-documented in hospital wards (Creamer et al., 2014; French et al., 2004; Yuen et al., 2015), athletic facilities (Montgomery et al., 2010), and public spaces. Despite rigorous disinfection protocols, healthcare settings remain susceptible to MRSA contamination, emphasizing the need for ongoing monitoring in hospital environments (Coia et al., 2006). Additionally, MRSA is the most frequently detected MDRO in public transport systems (Ly et al., 2024). In the US, both the hospital-associated SCC*mec* type II and community-associated SCC*mec* type IV MRSA strains were detected in buses

serving both hospital and community routes (Lutz et al., 2014). Culture-based studies have also detected identical MRSA strains in various public transport systems and their passengers, indicating a potential transmission linkage between passengers and their surroundings (Conceição et al., 2013; Lin et al., 2017; Lutz et al., 2014; Mendes et al., 2015; Otter & French, 2009; Peng et al., 2015). Notably, more than half of the MRSA strains isolated from public transport exhibit multidrug resistance (Lutz et al., 2014; Stepanović et al., 2008). MRSA is also common in washroom environments, with one study reporting MRSA found in 3.3% of hospital toilet seats (Giannini et al., 2009). These findings highlight the widespread distribution of MRSA in urban BE.

Extended spectrum β -lactamases (ESBL)-producing *Enterobacterales* (ESBL-E) and Carbapenem-resistant *Enterobacterales* (CRE)

Enterobacterales, a diverse family of Gram-negative bacteria, are primarily associated with the human intestinal tract, but are also prevalent in environmental reservoirs such as foods, waters, and urban surfaces (Davin-Regli et al., 2019). Their presence usually indicates a sign of fecal contamination (Boehm & Sassoubre, 2014). Clinically significant members such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter spp.* cause infections ranging from urinary tract infections to life-threatening bacteremia.

Extended spectrum β -lactamases (ESBLs) are enzymes that hydrolyze beta-lactam antibiotics, including penicillins, first-, second-, and third-generation cephalosporins, and monobactams (Paterson & Bonomo, 2005). They are mainly plasmid-borne and are classified into three main groups: Ambler class A ESBL (ESBL_A), miscellaneous ESBL (ESBL_M), and ESBL degrading carbapenems (ESBL_{CARBA}) (Ur Rahman et al., 2018). Most ESBLs belong to the ESBL_A group, which includes sulfhydryl reagent variable β -lactamases (*bla*_{SHV}), Temoniera β -lactamases (*bla*_{TEM}), and cefotaxime-M β -lactamases (*bla*_{CTX-M}) (Husna et al.,

2023). ESBLs are commonly found in *Klebsiella spp.*, *Escherichia coli*, *Enterobacter*, *Serratia*, *Citrobacter*, *Proteus*, and *Salmonella* (Pitout & Laupland, 2008). First identified in 1983 as the culprit of drug-resistant hospital-acquired *Enterobacterales* infections (Kliebe et al., 1985), ESBL-E have disseminated globally (Cantón et al., 2008; Coque et al., 2008), with a marked increase in community-onset infections (Pitout et al., 2005). To date, the most predominant ESBL family is *bla*_{CTX-M}, with the *bla*_{CTX-M-15} variant dominating globally (Castanheira et al., 2021), followed by the *bla*_{SHV} family (Paulitsch-Fuchs et al., 2022).

Treating infections caused by *Enterobacterales* is increasingly challenging due to multidrug resistance (MDR) (Iredell et al., 2016). The emergence of MDR *Enterobacterales*, particularly carbapenem-resistant *Enterobacterales* (CRE), has been a major global health threat due to their resistance to last-resort antibiotics, namely carbapenems and colistin (Codjoe & Donkor, 2017). Owing to their widespread antibiotic resistance among *Enterobacterales*, third-generation cephalosporin-resistant and carbapenem-resistant *Enterobacterales* were listed as “Priority 1: Critical” pathogens in the WHO BPPL (Sati et al., 2025).

CRE are important pathogens in urban BE, where they were frequently reported in diverse settings, including hospitals, waterways, natural environments, companion animals, food animals, wildlife, and agricultural environments (Mills & Lee, 2019). Carbapenem resistance arises from carbapenemases, which are enzymes that hydrolyze carbapenems. Carbapenemases are further classified into four groups based on their molecular structures by the Ambler classification system, three of which (A, B, and D) are associated with *Enterobacterales* (Hall & Barlow, 2005; Queenan & Bush, 2007). Class A carbapenemases include the *K. pneumoniae* carbapenemase (KPC), non-metallo-carbapenemase type A (NMC-A) and, *Serratia marcescens* enzyme (SME), While Class B carbapenemases are associated with metallo- β -lactamases (MBL), including the New Delhi metallo- β -lactamases (NDM), imipenemase (IMP), and the Verona integron–encoded metallo- β -lactamases (VIM). Class D

oxacillinases are mainly OXA-48-like carbapenemases. These enzymes are often encoded in mobile genetic elements (MGEs) such as plasmids, facilitating their dissemination among bacterial populations. In Hong Kong, the five most prevalent carbapenemases are NDM-1, IMP-1, KPC, OXA-48-like family, and VIM-1 (Ho & Wu, 2017).

Environmental dissemination of drug-resistant *Enterobacterales* is increasingly documented in urban BE. Though less frequently reported than MRSA, CRE and ESBL-E have been reported in public transport systems. In Beijing China, 11 carbapenem-resistant *K. pneumoniae* (CRKP) isolates carrying the *bla*NDM-5 gene were identified from the city's subway system (Cao et al., 2020). In another study by Shen et al. (2018), *mcr-1*-producing strains of *E. coli*, which possess colistin resistance, were identified in public transportation in Guangzhou, China (Shen et al., 2018). Notably, a high proportion (76.2%) of *mcr-1* positive isolates were recovered from areas with high density of hospitals or heavy traffic, suggesting a potential spillover of the *mcr-1* gene from hospital to the community.

Vancomycin-resistant *Enterococcus* (VRE)

Enterococci are commensals of the human intestines and female genital tracts. As a “Priority 2: High” pathogen, VRE has spread worldwide and posed a significant threat to healthcare systems since its initial identification in Europe in 1986 (O’Driscoll & Crank, 2015). It arises due to the use of glycopeptides in livestock and becomes prevalent in clinical setting in consequence of increased vancomycin prescriptions (Levitus et al., 2024). While a majority of isolates are *E. faecalis*, VRE isolates are predominantly *E. faecium* (Ahmed & Baptiste, 2018).

Vancomycin, a glycopeptide, is the last line of defense against serious infections caused by Gram-positive bacteria. Resistance to vancomycin is encoded by vancomycin resistance genes identified from *vanA* to *vanG*. Among them, the plasmid-associated *vanA* and *vanB* are

the most common and potent phenotypes. Furthermore, VRE also acts as a reservoir of mobile resistance genes as they can disseminate resistance genes to other species, namely *S. aureus*, through the action of transposons (Gardete & Tomasz, 2014).

VRE was infrequently detected in urban BE. Although *E. faecium* was frequently detected in the subway of New York City, no vancomycin resistance genes were detected simultaneously (Afshinneko et al., 2015), suggesting the absence of VRE. Despite this, VRE had been identified from shared bicycles in Chengdu, China (Gu et al., 2020).

Multidrug-resistant *Acinetobacter baumannii* (MDRAB), Carbapenem-resistant *Acinetobacter baumannii* (CRAB) and Carbapenemase-producing *Acinetobacter baumannii* (CPAB)

Acinetobacter is a genus of environmental bacteria commonly found in soil and water. Among them, *Acinetobacter baumannii* is the most clinically concerning species, causing opportunistic infections in the bloodstream, urinary tract, lungs, and wounds. Notably, *A. baumannii* harbors a highly adaptable genome, enabling it to rapidly develop antibiotic resistance and virulence factors that enhance its survival under adversity, such as desiccation, disinfectants, and oxidative stress (Harding et al., 2018).

Carbapenems are a crucial class of antibiotics for treating *A. baumannii* infections. Therefore, the emergence of carbapenem-resistant strains, namely multidrug-resistant *Acinetobacter baumannii* (MDRA), Carbapenem-resistant *Acinetobacter baumannii* (CRAB), and Carbapenemase-producing *Acinetobacter baumannii* (CPAB), is clinically important and are being closely monitored in clinical isolates. The growing prevalence of CRAB has led WHO to designate it as one of the “Priority 1: Critical” pathogens (WHO, 2017). Resistance to carbapenems arises from multiple mechanisms, including enzymatic degradation of antibiotics, overexpression of efflux pumps, and production of class D β -lactamases (i.e., OXA,

oxacillinase). Class D β -lactamase are the most common type of resistant mechanism exhibited by *A. baumannii*, with OXA-23, OXA-24, and OXA-58 dominating clinical strains globally (Walther-Rasmussen & Høiby, 2006).

Despite being a well-documented MDRO in hospital environments, MDRA are rarely reported in non-healthcare BE (Abbo et al., 2005).

Multidrug-resistant *Pseudomonas aeruginosa* (MDRPA) and Carbapenem-resistant *Pseudomonas aeruginosa* (CRPA)

Pseudomonas, like *Acinetobacter*, is commonly found in the environment, including soil and water. Unlike many environmental bacteria, *P. aeruginosa* is a significant opportunistic pathogen associated with nosocomial infections and ventilator-associated pneumonia (VAP) (Livermore, 2002). These infections are linked to high mortality rates and have shown increasing resistance to carbapenems (Reyes et al., 2023). Consequently, carbapenem-resistant *P. aeruginosa* (CRPA) was also designated as one of “Priority 2: High” pathogens (WHO, 2017).

The resistance mechanisms of *P. aeruginosa* can be classified into intrinsic, acquired, and adaptive (Pang et al., 2019). The intrinsic resistance involves low outer membrane permeability, expression of efflux pumps, and production of inactivating enzymes. The acquired resistance in *P. aeruginosa* is achieved by HGT or spontaneous mutations that give rise to ARGs (Breidenstein et al., 2011). Adaptive resistance involves biofilm formation in the lungs of infected patients, where the biofilm serves as a diffusion barrier to limit antibiotic access to the bacteria (Drenkard, 2003). By far, the most common resistance mechanism is the overproduction of the efflux pump systems for aminoglycosides, β -lactams, and quinolones (Henrichfreise et al., 2007).

Despite its clinical significance, multidrug-resistant *P. aeruginosa* is seldom reported from urban BE, suggesting healthcare settings are its primary reservoir.

1.3.2. Multidrug-resistant Fungi

Multidrug-resistant fungi, though less clinically critical than some bacterial pathogens, have emerged as significant health threats. The isolation of multidrug-resistant fungi from clinical samples is gradually increasing around the globe, posing substantial challenges to healthcare systems. In response, the WHO had published the first fungal priority pathogens list in 2022, further highlighting the threats of emerging fungal diseases (Fisher & Denning, 2023).

Candida spp. and Candidozyma auris

Candida spp. are a group of yeast-like fungi commonly found in the environment and on the skin and mucous membranes of humans (Talapko et al., 2021). Although *Candida* are considered non-pathogenic, they are important causes of severe infections with high morbidity and mortality among immunosuppressed patients (Evren et al., 2022). Clinically important *Candida* spp., including *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and the closely related *Candidozyma auris* (Fisher & Denning, 2023).

Candidozyma auris (basonym: *Candida auris*), an MDR yeast first isolated from the external ear canal of a patient in Japan in 2009, has emerged as a significant global health threat recently. Since then, it has rapidly spread to over 50 countries and caused serious infections globally. Consequently, the WHO had recognized *C. auris* as one of the fungal priority pathogens (WHO, 2022b). The clinical significance of *C. auris* could be attributed to its intrinsic resistance to multiple antifungal drug classes, including azoles, echinocandins, and amphotericin B. It is isolated not only from immunocompromised patients but also from

otherwise healthy persons (Pekard-Amenitsch et al., 2018). Clinically, *C. auris* infections can be manifested as bloodstream infection, wound infection and otitis media.

Currently, *C. auris* infections are primarily reported in healthcare settings, with no confirmed community-acquired cases (Sabino et al., 2020). However, its detection in wastewater suggests its circulation in an urban city (Barber et al., 2023), highlighting the need for perpetual surveillance and screening to preclude its spread in the community.

1.4. Urban Built Environment Microbiomes in Health and Disease

The urban microbiomes comprise diverse microbial communities within urban environments, harbor unique assemblages of microorganisms that significantly influence both personal and public health (Bosch et al., 2024; National Academies of Sciences et al., 2017). They are often shaped by anthropogenic activities, building design, and environmental factors. Among all urban microbiomes, the BE microbiomes interact with urban dwellers in complex ways, offering both benefits and challenges to public health. Despite increasing recognition of their importance, substantial knowledge gaps remain regarding the roles of BE microbiomes in health and disease. As urban residents spend most of their time indoors, continuous exposure to indoor microbiomes escalates the risk of microbial infections, highlighting the need to understand their composition and health implications (Chawla et al., 2023).

1.4.1. Factors Shaping Composition of Built Environment Microbiomes

The urban BE microbiomes, comprising diverse microbial communities in man-made establishments, are distinct from natural ecosystems. The BE microbiomes are shaped by various factors, including but not limited to human activities, building design, finishing materials, air circulation, and connectivity with the outdoor environment. Several key factors determining the composition and dynamics of the BE microbiome are discussed below.

Apparently, the size and type of occupants contribute significantly to BE microbiomes. Human occupancy is a primary determinant of the BE microbiome composition (Leung & Lee, 2016). Everyday activities such as talking, breathing, coughing, and sneezing release over 10^6 biological particles per hour, introducing skin and mucosal taxa, like *Cutibacterium*, *Corynebacterium*, *Staphylococcus*, *Streptococcus*, and *Propionibacterium*, into indoor environments (Lax et al., 2014; Meadow, 2015; Hospodsky et al., 2012; Leung & Lee, 2016). New buildings become rapidly colonized by human-associated genera, including *Escherichia*, *Pseudomonas*, and *Klebsiella* spp., after occupants move in (Young et al., 2023). Similarly, Klassert et al. (2021) observed that high-touch surfaces in a newly opened hospital were dominated by skin-associated species (Klassert et al., 2021). In addition to humans, the presence of pet animals further modifies indoor microbiomes, contributing to increased species richness and diversity (Sitarik et al., 2018; Mäki et al., 2021).

The outdoor environment also plays a critical role in shaping BE microbiome. Urban buildings are not isolated from their surroundings; they constantly exchange air, dust, and microorganisms with external environments. This exchange is governed by the building's porosity, determined by ventilation systems, window usage, and building envelope design, which mediate the flow of microbial matter. Studies have shown that houses located near green spaces exhibit higher bacterial and fungal diversity in their indoor microbiomes (Dockx et al., 2021). Additionally, buildings with more windows tend to have indoor microbiome compositions that closely resemble that of the outside environment, primarily driven by increased airflow (Stephens, 2016).

The mode of ventilation significantly affects microbiome composition by controlling air exchange and consequently the dispersal of microorganisms. Buildings with mechanical ventilation tend to harbor a lower microbial diversity and favor the colonization of human-associated bacteria, including potential pathogens, whereas natural ventilation results in an

indoor microbiome more akin to outdoor air, with enhanced microbial diversity (Kembel et al., 2012, 2014; Meadow et al., 2014). Households that frequently open windows have reduced presence of Gram-negative bacteria, thereby reducing their chance of causing infections (Sharpe et al., 2020).

Building design and architecture, such as the type and locations of rooms and building materials, can also influence the type of microorganisms inhabiting the indoor environments. For instance, Kembel et al. (2012) observed differences in microbial communities between interconnected rooms and those that are more isolated (Kembel et al., 2012). The choice of finishing materials also shapes the indoor microbiome, as different materials support varying rates of microbial growth and survival (Hu et al., 2019; Mhuireach et al., 2021). Indoor lighting also correlates positively with bacterial abundance and diversity (Fahimipour et al., 2018; Lam et al., 2024).

1.4.2. Microbial Composition of Urban Built Environments

Bacteria make up a significant part of indoor microbiomes. Among them, human-associated taxa dominate. Common skin commensals, such as *Staphylococcus*, *Micrococcus*, *Corynebacterium*, and *Cutibacterium*, are consistently detected in diverse settings, such as classrooms (Meadow et al., 2014), offices (Hewitt et al., 2012; Li et al., 2022), and sports facilities (Wood et al., 2015). Notably, *Cutibacterium acne* exhibits a stable presence in high-touch environments like mass transit systems (Danko et al., 2021). Public washroom surfaces, frequently contacted by hands, are dominated by *Propionibacteriaceae*, highlighting the significant role of human contact in shaping the washroom microbiomes (Flores et al., 2011). Beyond human-associated taxa, microbes of environmental origins also contribute to the BE microbiomes. Soil dwellers like *Sphingobium*, *Blastomonas*, *Arthrobacter*, and *Psychrobacter* are commonly found in the air of mass transit systems, likely introduced through ventilation

systems or connections with outdoor environments (Leung et al., 2014; Robertson et al., 2013). This combination of human and environmental origins results in a dynamic bacterial profile unique to each BE, potentially influencing occupant health.

Fungi are ubiquitous in indoor environments, especially in damp and poorly ventilated environments, where they contribute to allergic reactions, infections, and other health issues (Portnoy et al., 2005). The presence of fungi in BE is heavily influenced by outdoor environments, with air exchange facilitating the introduction and dispersal of fungal spores (Adams et al., 2013a). Household drains are often enriched with *Exophiala*, *Candida*, and *Fusarium*, while *Cladosporium* and *Cryptococcus* were abundant on window sills (Adams et al., 2013b). In hospital settings, fungi pose a significant risk by contributing to nosocomial infections, particularly among immunocompromised patients. *Aspergillus*, especially *A. fumigatus*, and *Penicillium* are among the most frequently detected fungal pathogens in hospitals, alongside *Fusarium* and *Candida* (Perdelli et al., 2006; Tong et al., 2017; Ghodsi et al., 2025). These findings warrant the need for effective ventilation and maintenance strategies to control fungal proliferation in high-risk environments.

Viruses, although less characterized than bacteria and fungi, are significant contributors to the overall microbial dynamics, impacting both human and environmental health (Prussin et al., 2015). Their abundance is comparable to that of bacteria, contributing approximately 10^5 viral particles per cubic meter of indoor air (Prussin et al., 2015). The indoor virome originates from diverse sources, including humans, pets, plants, and outdoor air (Prussin & Marr, 2015), resulting in a viral assemblage unique to each type of BE. For instance, in washroom environments, viruses such as enterophages, human papillomavirus (HPV), and herpesviruses are predominant (Gibbons et al., 2015). The indoor virome also exhibits seasonal fluctuations, as observed by Prussin et al. (2019) in a daycare center study that human-associated viruses were much more diverse and dominant during winter than summer (Prussin et al., 2019).

Intriguingly, the viral-host interactions in BE also play a critical role in facilitating the dissemination of ARGs, thereby enhancing bacterial survival and posing potential health risks (Du et al., 2023).

1.4.3. Role of Built Environments in Pathogen Transmission

Urban BE are critical hubs for microbial exposure and pathogen dissemination, primarily through airborne and surface-mediated transmission pathways. The composition and dynamics of indoor microbiomes, impacted by occupant density, turnover rates, and environmental conditions (e.g., temperature, relative humidity), significantly contribute to the spread of infectious agents. Importantly, the compactness of a BE correlates positively with the frequency of microbial exchange and, consequently, pathogen transmission (Indriyani et al., 2022). Understanding these transmission pathways is therefore critical for developing strategies to mitigate the spread of infectious diseases and AMR in urban settings.

Respiratory pathogens are consistently documented across diverse BE. SARS-CoV-2, for example, has been detected in the air and surfaces within schools (Cantú et al., 2022), public buses, and subway systems (Moreno et al., 2021), demonstrating its environmental pervasiveness. Similarly, *Mycobacterium tuberculosis*, a highly transmissible airborne pathogen, was found in indoor air of classrooms, public transportation, and other buildings (Bunyasi et al., 2022; Edelson & Phypers, 2011). Seasonal influenza viruses are also common, as evidenced by the detection of Influenza virus A and B in 16.9% and 4.5% of air samples from a university campus, respectively (Xie et al., 2020). Bioaerosol sampling in an airport also revealed 17% of samples were positive for influenza D virus and adenovirus (Bailey et al., 2018). During the 2009 H1N1 Influenza A pandemic, an outbreak was identified among train passengers in China, highlighting the role of compact BE in viral infection (Cui et al., 2011). Furthermore, adenovirus and respiratory syncytial virus type A (RSVA) were positive in 10%

and 4.5% of air samples, respectively, from Singapore's subway system (Coleman et al., 2018). Collectively, these findings highlight the role of BE, especially those densely occupied and high in traffic, as significant hubs for the airborne transmission of epidemic-associated viruses and bacteria.

Direct or indirect contact with contaminated surfaces or fomites represents another critical pathway for pathogen transmission within BE. Opportunistic pathogens, notably *P. aeruginosa* and *A. baumannii*, are prevalent in urban BE and associated with both nosocomial and community-acquired infections. High-touch hospital surfaces, such as bedrails, bedside tables, call bells, and door handles, are frequently contaminated with carbapenem-resistant strains (Chia et al., 2020). Similarly, there is a plethora of evidence showing MRSA heavily colonizes high-touch hospital surfaces, with bedrails identified as key reservoirs (Boyce, 2007; Yuen et al., 2015). Furthermore, this contamination extends beyond healthcare settings. A study of public transportation systems revealed that MRSA was detected in 63% of buses, with two-thirds of isolates recovered from routes serving hospitals, indicating a potential spillover of healthcare-associated MRSA into the community (Lutz et al., 2014). Moreover, drug-resistant *staphylococci* were recovered from post-handwashing facilities such as paper dispensers, hand dryers, and exit door handles of public washrooms (Suen et al., 2019). Drug-resistant *Staphylococcus epidermidis* has also been found on computer touch screens in offices (Gerba et al., 2016). Collectively, the detections of pathogens in BE surfaces emphasize the role, particularly the high-touch area, as environmental reservoirs for diverse pathogens.

1.5. Environmental Surveillance for Pathogens and Antimicrobial Resistance in Urban Built Environments

MDRO and AMR pose significant global health threats, with urban BE serving as critical hubs for microbial transmission. Non-healthcare BE, such as public transportation,

schools, offices, and public washrooms, are increasingly recognized as reservoirs for MDROs and ARGs. However, surveillance efforts in these settings remain limited compared to healthcare and agricultural environments (Essack, 2018). These high-traffic areas facilitate the spread of pathogens and ARGs through air, surfaces, and human interactions, necessitating robust environmental surveillance to inform public health strategies.

This section reviews current methods for pathogen and AMR surveillance in BE, emphasizing traditional culture-based approaches and emerging molecular and genomic techniques. A comparative overview of various public health surveillance techniques was provided in [Table 1.4](#).

Table 1.4 Comparison of methods for pathogen and AMR detection in public health surveillance.

Methods	Applications	Advantages	Disadvantages
Conventional culture-based methods: • Selective and differential culture • pDST • MALDI-TOF	- Species-level identification of cultured isolates* - Phenotypic drug sensitivity of the organisms	✓ Low cost, resources and expertise ✓ Viability of target pathogen tested ✓ Phenotypic AMR of pathogen tested ✓ Whole genome sequence for phylogenetic analysis ✓ Isolates can be biobanked	✗ Low speed, throughput, sensitivity, specificity and scalability ✗ Not all species are culturable in laboratory conditions
Quantitative PCR (qPCR) Reverse transcription qPCR (RT-qPCR) QPCR array	Sensitive and specific detection of single to multiple targets of pathogens or ARGs in environmental samples	✓ No isolation of organisms; direct identification of pathogens ✓ Low cost ✓ Short turnaround time ✓ Highly sensitive	✗ Low throughput ✗ Limited number of targets ✗ Does not confirm pathogen viability and phenotypic resistance ✗ Signal dropout due to mutation(s) at primer binding site
Whole-genome sequencing	- Characterization of unknown species - Molecular typing of organisms and phylogenetic analysis	✓ Detailed investigation of microbial genome; Comprehensive profiling of ARGs, virulence factors, and MOBs ✓ High-resolution spatiotemporal comparison of different isolates, facilitating outbreak investigation	✗ Organism must be culturable and isolated from sample before sequencing
16S rRNA gene sequencing	Species-level identification of bacterial isolates, including closed-related species	✓ High throughput ✓ Can be performed on direct sample; no isolation of organisms is needed ✓ Low interference from non-bacterial DNA	✗ Limited to bacterial pathogens only ✗ Cannot differentiate closely related species with highly similar 16S sequence ✗ No information on AMR
Amplicon sequencing	Hypothesis-driven identification low-abundance bacterial, fungal and viral pathogens in environmental samples by PCR amplification	✓ Higher sensitivity due to higher coverage, allowing for more accurate species identification	✗ High cost ✗ Cannot differentiate between viable and non-viable organisms ✗ Uneven amplification of targets due to primer bias leads to inaccurate relative abundance of organisms present ✗ PCR introduces chimeras and errors
Shotgun metagenomic sequencing	Unbiased and hypothesis-free simultaneous identification of pathogen and antimicrobial determinants	✓ No priori knowledge of organisms required ✓ Random primers reduce primer bias ✓ Interrogation of all genetic materials – culturables, unculturables, bacterial, viruses, fung ✓ Capture other genetic information like ARGs and functional genes ✓ Identification of novel species	✗ Broad specificity may lead to decreased sensitivity for low-abundance organisms ✗ Cannot differentiate between viable and non-viable organisms
Targeted probe capture metagenomics (TCM)	Enrichment of low-abundance targets for sequencing at low depth	✓ Low interference from non-targeted reads (e.g. Human, insects, plants) ✓ Allow detection of low-abundance reads at low sequencing depth ✓ Require less computational requirement ✓ Recovery of whole genome from direct metagenomic sample ✓ Tolerate 20-30% mismatches with probe, can identify mutated strains of novel species	✗ High cost (additional cost for capture probe set) ✗ Labor-intensive workflow ✗ Require expertise in probe panel design ✗ Cannot differentiate between viable and non-viable organisms

1.5.1. Microbial Culture: Cornerstone for In-depth Genomic and Phenotypic Analysis

Public health surveillance programs are essential for identifying disease-causing microorganisms and drug resistance. Traditionally, these programs target a pre-determined list of clinical and public health-significant pathogens, focusing primarily on healthcare settings and the food industry. In healthcare environments, routine surveillance of pathogens prevents nosocomial outbreaks by monitoring pathogens like VRE, MRSA, ESBL-E, CRE, CRPA, and MDRA (Khan et al., 2017). This is mainly accomplished by sampling air, water, and surface samples, followed by identification of target pathogens using culture-based methods. Similarly, foodborne pathogen surveillance was performed regularly to prevent foodborne illnesses. Important foodborne pathogens, namely *Salmonella* and *Listeria*, were screened in ready-to-eat foods to prevent outbreaks (Health Canada, 2023; WHO, 2004).

Traditionally, pathogen surveillance relies heavily on culture-based methods, where microorganisms are cultivated on selective media, followed by phenotypic characterization using biochemical tests, MALDI-ToF mass spectrometry, and antimicrobial susceptibility testing (AST). Culture-based methods offer several key advantages for pathogen and AMR surveillance in urban BE. First, cultivation confirms pathogen viability, providing insights into their potential infectivity. Second, these methods enable phenotypic AMR assessment, which is essential given the often inconsistent correlation between AMR genotypes and phenotypes (Yee et al., 2021). Third, cultured isolates can undergo WGS, facilitating phylogenomic analyses that are a critical prerequisite to outbreak investigation and transmission linkage studies (Gilchrist et al., 2015; Quainoo et al., 2017). Despite these strengths, culture-based methods have notable limitations. They are time-consuming, with turnaround times often spanning days, and their low throughput restricts scalability. Additionally, culture-based methods are hypothesis-driven, meaning that they rely on prior knowledge of the target

pathogen to select appropriate culture media, limiting their utility for broad-spectrum screening. A significant constraint is their bias toward culturable organisms, as over 99% of environmental strains are unculturable using standard culturing techniques in normal laboratory conditions (Rappé & Giovannoni, 2003). This leads to potential false negatives and incomplete representation of overall microbial diversity in BE. Consequently, while culture-based approaches remain invaluable for targeted, in-depth investigations of specific pathogens, they are less suitable for comprehensive screening aimed at detecting a broad range of pathogens and ARGs.

1.5.2. qPCR Assays and Arrays: Optimal Strategies for Sensitive and Specific Pathogen Detection

Since its emergence in the 1990s, quantitative polymerase chain reaction (qPCR) has revolutionized pathogen detection by overcoming many limitations of culture-based methods, and has become an indispensable tool in modern microbiology laboratories (Deepak et al., 2007). Its critical role was exemplified during the COVID-19 pandemic, where qPCR became the gold standard for detecting SARS-CoV-2 owing to its speed and accuracy (Kevadiya et al., 2021). Similarly, qPCR assays targeting *Clostridioides difficile* (basonym: *Clostridium*) toxin genes *tcdA* and *tcdB* have largely replaced traditional tissue culture cytotoxicity assays, which require up to 48 hours to complete (de Jong et al., 2012). These examples highlight qPCR's rapid turnaround time and its pivotal role in clinical and public health settings.

qPCR offers several key advantages. One major advantage is that it is highly specific and sensitive, enabling the detection of a few copies of the target nucleic acid sequence in complex clinical or environmental samples. This makes it particularly effective for identifying low-abundance pathogens. Additionally, qPCR is highly scalable, allowing the detection of diverse targets, including pathogen-specific genes, ARGs, and virulence genes. Through

multiplexing, qPCR assays can simultaneously amplify and detect multiple targets within a single reaction. For instance, FilmArray, an automated PCR system enabling the simultaneous interrogation of multiple pathogens, making it ideal for broad-spectrum screening for respiratory tract infection (Poritz et al., 2011). Unlike culture-based methods, qPCR assays have a fast turnaround time. For instance, foodborne pathogen *Listeria monocytogenes* requires at least 5 days to culture, however, DNA extraction and qPCR only take several hours (Law et al., 2014). Furthermore, qPCR also detects the presence of pathogens regardless of their viability status, bypassing the need to isolate the pathogens. This is particularly valuable for viral pathogens, which are often challenging and time-consuming to culture.

Despite its strength, qPCR has limitations, including its hypothesis-driven nature, which requires prior knowledge of target sequences, restricting its ability to detect novel or emerging pathogens. For instance, the lack of conserved regions and frequent mutations in viral genomes will lead to signal dropout, requiring frequent updating of primer sequences (Bozidis et al., 2022). While multiplexing enhances throughput, multiplex PCR still remains limited to a pre-determined set of targets. Nevertheless, qPCR assays and arrays remain an effective, sensitive, and specific approach for detecting low-abundance targets in clinical and environmental samples. Their rapid turnaround time and high scalability make them indispensable tools in modern surveillance programs, though complementary methods are necessary for a comprehensive surveillance.

1.5.3. Genomic and Metagenomic Sequencing: Comprehensive Insights into Microbial Composition and Resistance Determinants

High-throughput sequencing (HTS) has revolutionized public health surveillance by offering a hypothesis-free, high-throughput methodology to study the microbiome. Unlike conventional approaches, HTS enables simultaneous detection and characterization of diverse microorganisms without prior knowledge, bypassing the cultivation while providing a more comprehensive microbial profile (Ko et al., 2022; Suminda et al., 2022). This section explores HTS applications in pathogen and AMR surveillance within urban BE, focusing on WGS, 16S rRNA sequencing, amplicon sequencing, and metagenomics, and evaluates their strengths and limitations.

HTS offers two approaches for studying the environmental microbiome, which are WGS of individual microbial isolates and metagenomics of the whole microbial community ([Figure 1.1](#)). WGS involves cultivating target pathogens followed by sequencing of their entire genomes, offering high-resolution insights into individual strains. This method not only generates a comprehensive genotypic profile of ARGs, virulence factors, MGEs, and other functional genes, but also enables phylogenetic analyses to trace outbreak origins and transmission dynamics. For instance, WGS is particularly effective in characterizing MDROs like MRSA and CRE during nosocomial outbreaks (Madigan et al., 2018; David et al., 2019). However, its dependence on culturable isolates limits its applicability in BE surveillance, where most environmentally recovered strains are unculturable using standard cultivation techniques (Rappé & Giovannoni, 2003). This constraint restricts WGS to targeted investigations rather than broad environmental screening in urban BE.

In contrast, 16S rRNA sequencing utilizes the conserved region of the 16S ribosomal RNA gene to identify bacterial species, offering an alternative solution to identify unculturable strains or when conventional methods fail. For instance, sequencing of the full-length 16S

rRNA gene on the long-read sequencing platform, such as the Oxford Nanopore, can resolve bacterial identities when MALDI-ToF failed (Lao et al., 2022). This technique has been applied to direct environmental samples from urban BE, which identified potential human pathogens (Kembel et al., 2012). While it is effective for broad speciation, it struggles with resolution problems as 16S rRNA sequencing fails to differentiate closely related species with highly similar 16S rRNA sequences (Janda & Abbott, 2007). For example, *Bacillus globisporus* and *Bacillus psychrophilus* share over 99.5% sequence similarity of their 16S rRNA genes but exhibit only 50% relatedness in reciprocal hybridization, indicating that 16S rRNA is not always a reliable marker for species differentiation (Fox et al., 1992). Additionally, its focus on bacteria precludes fungi, viruses, and parasites from analysis, limiting its scope for comprehensive microbial surveillance.

Amplicon sequencing, another culture-independent method, employs sets of primers targeting specific genes of interest, amplifying their signals through PCR to enhance sensitivity and resolution. This technique has been used to detect enteroviruses and *Salmonella typhi* in sewage, demonstrating its utility in environmental surveillance (Saingam et al., 2018; Troman et al., 2025). However, amplicon sequencing is prone to PCR bias, which can distort microbial diversity and composition representation (Hugerth & Andersson, 2017). Additionally, its reliance on predefined primers limits its ability to detect novel species outside the targeted panel.

In contrast, shotgun metagenomics analyzes all genetic materials within a sample, providing an unbiased, holistic view of microbial diversity, resistance profiles, and functional dynamics. By detecting both culturable and unculturable organisms as well as species across all kingdoms, metagenomics provides a comprehensive snapshot of the microbiome, including AMR determinants and functional genes, and facilitates the discovery of novel genes or species (Bharti & Grimm, 2021). Despite these advantages, there are still some conceptual and

technical challenges presented with metagenomics-based environmental surveillance. First, metagenomics, like most molecular methods, cannot differentiate signals from viable or dead cells, which may overestimate pathogen load and thus infection risk. Also, some metagenomic studies have low and insufficient sequencing depth, which may hinder the discrimination between pathogens and closely-related non-pathogenic strains, potentially leading to false positives (Pereira-Marques et al., 2019). Additionally, environmental samples generally have low biomass, making them prone to contamination and complicating the analysis (Fierer et al., 2025).

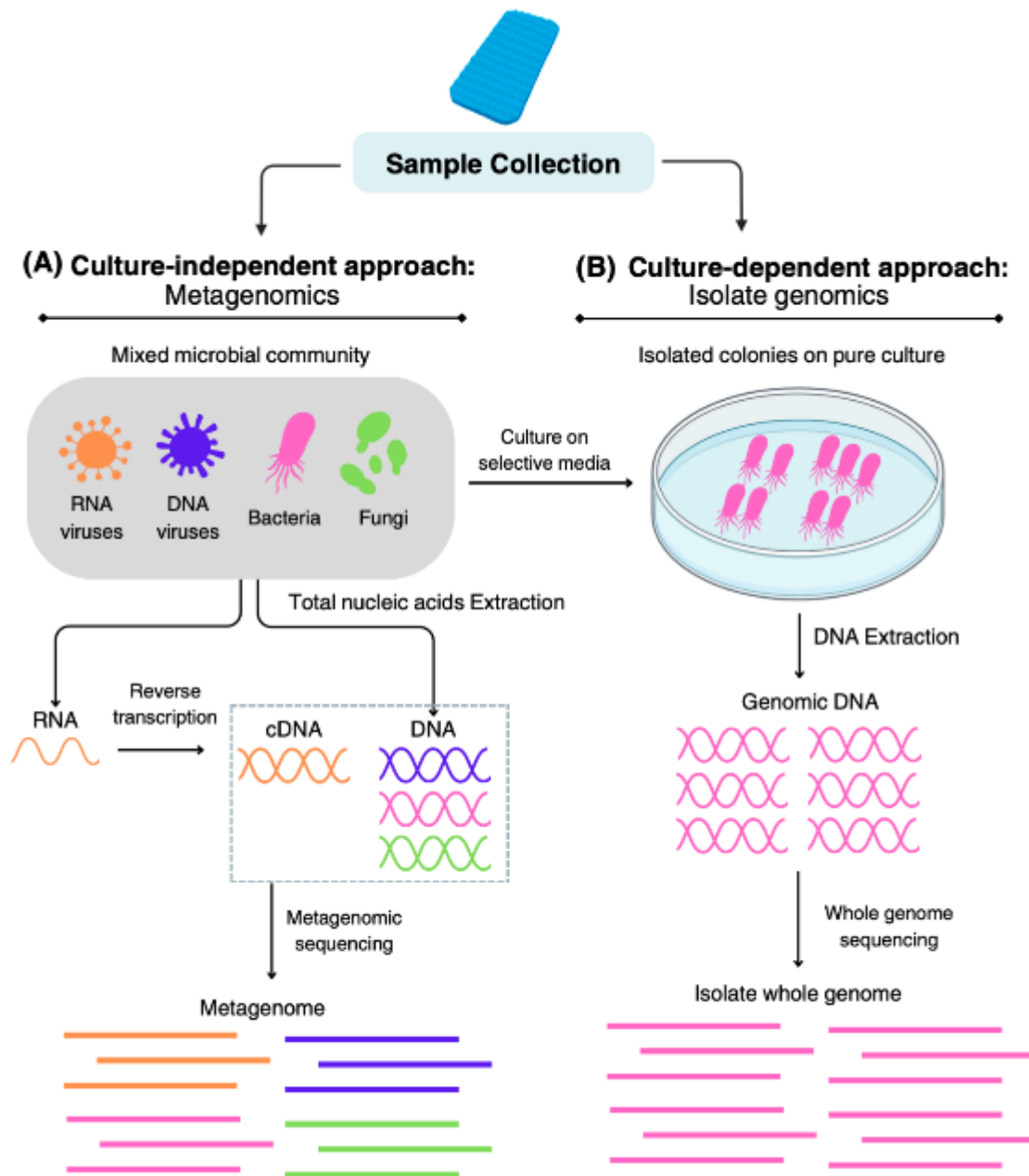


Figure 1.1 Schematic representation of two high-throughput sequencing approaches in studying microbial community.

(A) Culture-independent method (Metagenomics). Environmental samples were extracted, and sequencing was performed on metagenomic DNA. The metagenome of a microbial community composed of mixed species is obtained.

(B) Culture-dependent method. Environmental samples were cultured on selective media to isolate single colonies. Genomic DNA was extracted from isolates and sequencing was performed on genomic DNA. Genome of a single isolate is obtained.

1.5.4. Target Enrichment by Capture Probe Hybridization: Enhancing Detection of the Minority Taxa and Genes in Microbial Community

In environmental metagenomic samples, low-abundance pathogens and ARGs are often overwhelmed by high background DNA, such as host or commensal DNA, obscuring their detection. The poor detection of low-abundance targets is exacerbated by both low microbial load and poor DNA quality, which are typical of environmental samples. To overcome these challenges, target enrichment of pathogen and ARG sequences prior to sequencing is essential. While marker-based enrichment techniques, such as PCR, 16S rRNA sequencing, and amplicon sequencing, are commonly used, they are limited by low target coverage, primer bias, and variable amplification efficiency. Alternatively, target probe capture (hereafter abbreviated as TC) circumvents these problems encountered by amplicon-based and shotgun metagenomic sequencing.

The TC workflow ([Figure 1.2](#)) utilizes biotinylated oligonucleotide probes (or referred to as ‘baits’ or ‘probes’) containing sequences complementary to target genes of interest (i.e., pathogen or ARG reads). Following the hybridization (binding), unbound non-target DNA is removed by washing, retaining bound sequences to be included in subsequent sequencing. Unlike PCR, which relies on short oligonucleotide primers, a TC probe panel usually contains hundreds to thousands of probes, ranging from 75 to 140 nucleotides in length, designed based on target reference sequences. This approach enables precise and flexible enrichment of rare DNA targets directly from complex environmental matrices.

TC has been successfully applied in diverse fields, including cancer gene detection (Pritchard et al., 2014), genetic disorder diagnosis (Choi et al., 2009), studying pathogens from ancient clinical samples (Bos et al., 2019; Spyrou et al., 2019), and pathogen identification from clinical samples (Gaston et al., 2022; Gaudin & Desnues, 2018; Yin et al., 2024). Its application in environmental surveillance remains less common but is emerging, as evident by

the successful enrichment and characterization of circulating SARS-CoV-2 strains in wastewater surveillance for community-level outbreak tracing (Tisza et al., 2023).

TC offers several advantages over amplicon-based and shotgun metagenomic sequencing for environmental surveillance. First, the flexibility of probe design accommodates diverse targets, including clinically relevant ARGs and emerging pathogens. Unlike PCR, TC probe binding can tolerate up to 20-30% sequence mismatches, making it exceptionally useful for enriching for novel or mutated variants (Alanko et al., 2022). Additionally, when compared to shotgun metagenomics, TC successfully increases relative abundances of viral targets in wastewater by several orders of magnitude (Crits-Christoph et al., 2021; Rothman et al., 2021; Jiang et al., 2024). Using TC, the number of respiratory pathogen reads in clinical samples increased by more than 30-fold (Jia et al., 2024), thereby improving their detection. Third, by enriching the target DNA, the required sequencing depth can be significantly reduced, thereby lowering flowcell usage, computational intensity, and data storage requirements. Furthermore, capturing the whole genomes directly from a metagenomic sample is feasible. For example, whole-genome enrichment of *M. tuberculosis* was achieved by capture probe, enabling lineage identification and drug-resistance characterization (Sundararaman et al., 2023). Similarly, the TC probe applied to aquatic samples captures whole genomes of *Vibrio* spp., thereby enabling phylogenetic comparison among *Vibrio* isolates (Brumfield et al., 2025).

Despite the advantages of current TC workflows, a few limitations still need to be addressed before their widespread application in environmental surveillance. First, the technique is labor-intensive, requiring extra time and procedures for setting up hybridization. Moreover, designing effective probe panels demands specialized bioinformatic expertise to ensure broad coverage and high specificity (Kantor & Jiang, 2024). Finally, the high cost of customized probe sets limits their utility for large-scale surveillance.

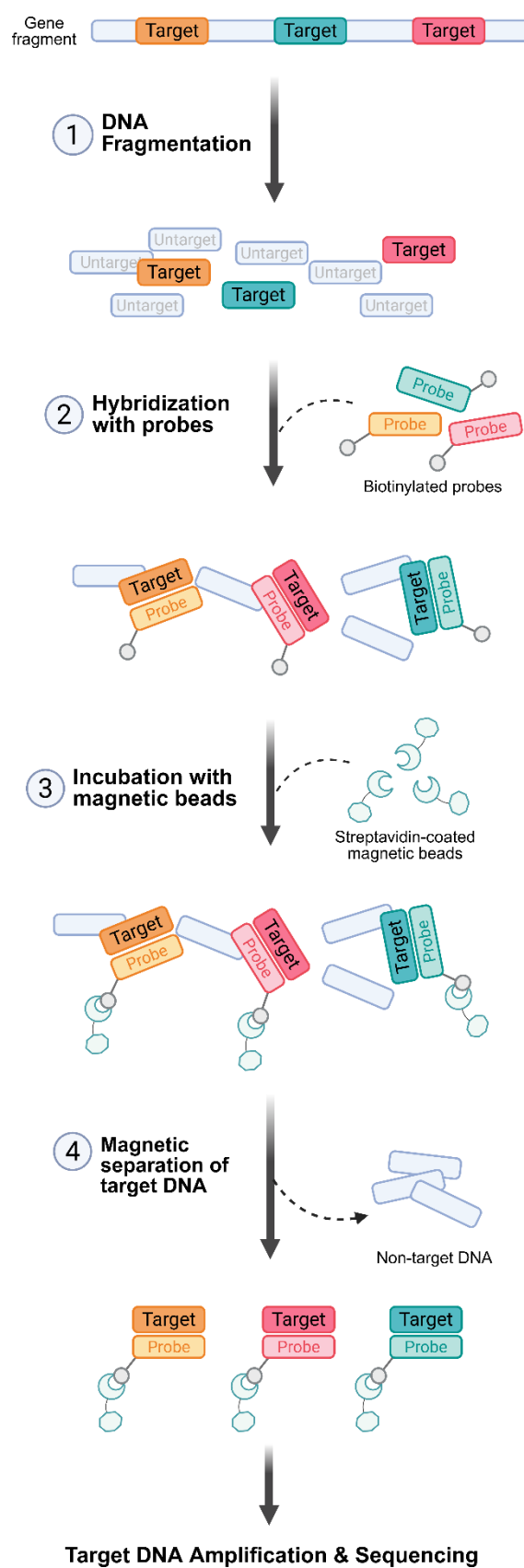


Figure 1.2. Principle of target enrichment by capture probe hybridization.
(See legend description next page)

Diagram illustrating the principle of target enrichment by hybrid probe capture. **Step 1:** Genomic DNA is fragmented into smaller segments to prepare a DNA library. **Step 2:** Biotinylated oligonucleotide probes, which are complementary to target gene sequences, hybridize specifically to the designated DNA fragments. **Step 3:** The DNA-probe hybrids are incubated with streptavidin-coated magnetic beads, which bind to the biotinylated probes. **Step 4:** A magnet is used to separate the bead-bound DNA-probe hybrids, while unbound, non-target sequences are washed away, thus enriching the target signal for downstream analysis.

1.5.5. The One-Health Approach: A Holistic Framework for Public Health Surveillance

The One Health concept, which integrates human, animal, and environmental health, is pivotal in contemporary public health surveillance ([Figure 1.3](#)). This holistic framework addresses the transmission and spread of emerging pathogens and AMR across these domains. Indeed, more than 60% of globally reported emerging or re-emerging infectious diseases over the past two decades were vector-borne or zoonotic (Morse et al., 2012), and approximately 75% of the new human pathogens originated in animals (Mackenzie et al., 2013). Human health is also closely tied to the environment we live in, with both natural and built environments playing important roles in shaping our health (Gilbert & Stephens, 2018). Human, animal, and environmental health are therefore inherently inter-connected, especially in the context of infectious diseases and AMR (Lappan et al., 2024). Pathogens and AMR circulate and transmit at the interfaces of these three domains via hosts, vectors, and environmental pathways, highlighting the need for an integrated surveillance to prevent their spread within the One Health triad. Recognizing this interconnectedness, WHO has recently launched the One Health joint plan of action, promoting an integrated and holistic approach to sustain the health and well-being of humans, animals, and the environment (WHO, 2022a).

AMR is one of the most pressing global health issues addressed by the One-health approach (Djordjevic et al., 2024; McEwen & Collignon, 2018). The interconnected nature at the human-animal-environment interface facilitates the spread of emerging pathogens and antimicrobial determinants across sectors ([Figure 1.3](#)). For example, the use of antibiotics at sub-therapeutic doses in food animals can lead to the emergence of antibiotic-resistant bacteria, which will then be transmitted to humans, posing significant health risks. This is well exemplified by the increasing proportion of clinical *E. coli* isolates carrying *mcr-I*, a colistin resistance gene, which was first identified from food animals in China and then gradually

dominated in clinical samples (Liu et al., 2016). This example underscores the need for cross-sectoral surveillance to track AMR transmission.

Currently, AMR surveillance primarily focuses on clinical isolates from humans and food animals, leaving the environmental domain underexplored (Essack, 2018). Yet, environmental bacteria, due to their abundance and diversity, serve as vast reservoirs of AMR that can eventually be acquired by human and animal pathogens. In addition to being one of the three main domains of the One-Health triad, it interacts intricately with the human and animal domains as it exists in all three domains. Environmental transmission is also a route of zoonoses, serving as a vector for human-animal transmission. Therefore, to effectively combat AMR and detect emerging threats, environmental surveillance should be warranted, particularly within indoor environments, which are integral in contemporary urban life.

Given the increasing human activities and overlapping interactions among stakeholders in One Health, effective disease prevention and control require multisectoral collaboration at the interfaces of humans, animals, and the environment. Surveillance programs for emerging pathogens strategically placed at these interfaces warrant the detection of early signs of emerging threats. For instance, monitoring food animals for foodborne pathogens is crucial to prevent contamination throughout the food supply chain (Newell et al., 2010). The COVID-19 pandemic further demonstrated the value of environmental genomic surveillance, such as wastewater analysis, in identifying hidden transmission links in the community (Shah et al., 2022).

In conclusion, an integrated surveillance system that consolidates data from all sectors of the One Health system enables the early detection of outbreaks and supports rapid responses, thereby strengthening pandemic preparedness. By providing a timely and comprehensive framework for disease outbreak prevention and control, adopting the One Health approach in

future environmental surveillance holds immense potential to mitigate global health threats from infectious diseases (Hayman et al., 2023).

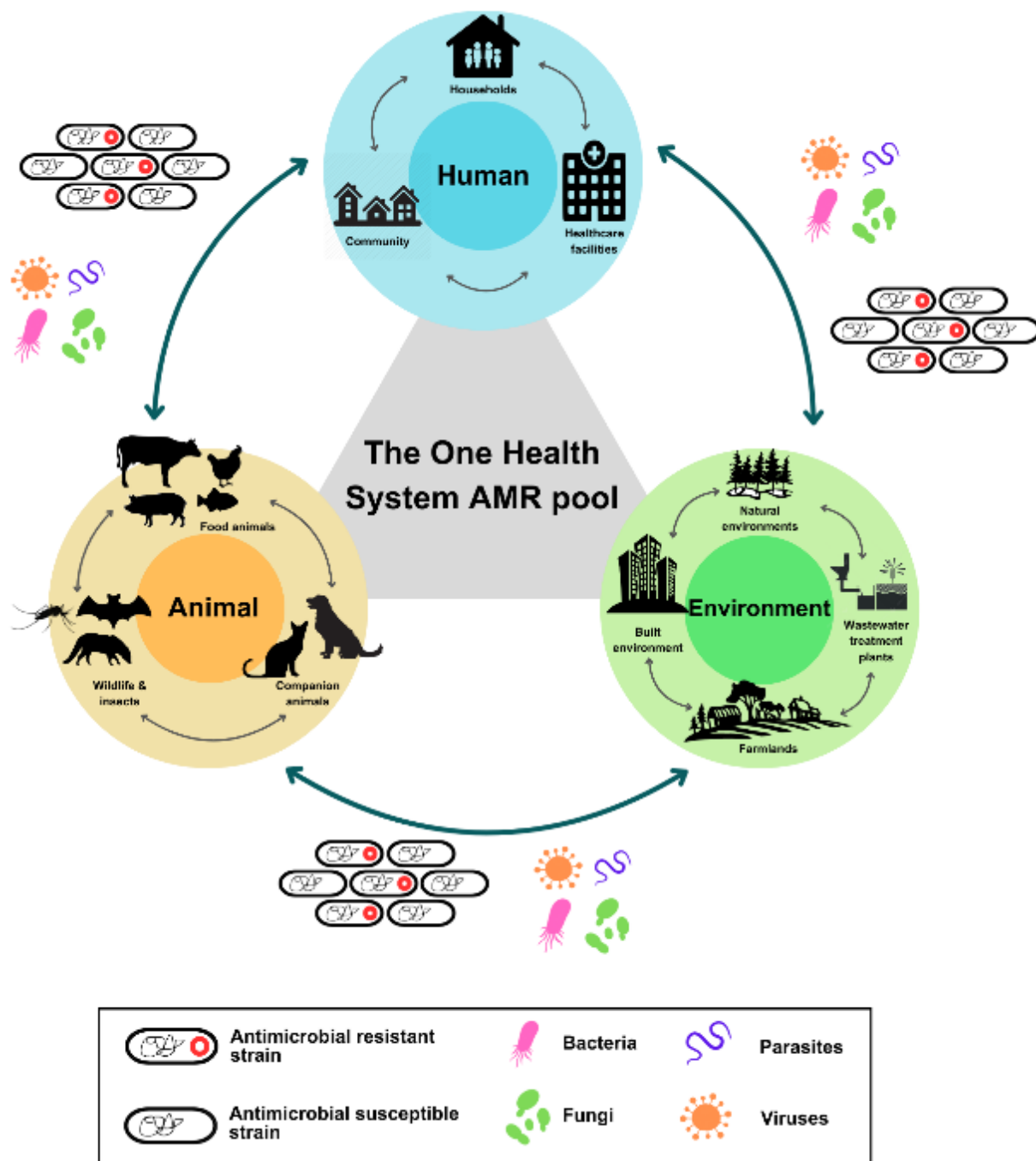


Figure 1.3. The One Health system and AMR transmission.

The One Health System is a holistic approach emphasizing the interconnectedness between human, animal, and environmental health. Pathogens and AMR determinants can be transmitted within the interlinkages of the system, affecting all stakeholders of the One Health system.

1.6. Essential Concepts in Microbiome Studies

1.6.1. Inference of Microbial Taxonomy and ARGs in Microbiome

The characterization of the taxonomic composition and ARGs in metagenomic samples is a cornerstone of microbiome research. This is accomplished by a bioinformatic pipeline, a collection of computational tools. The choice of bioinformatic tools that employ distinct algorithms can influence the identification of microbial taxa and ARGs. These algorithms can be broadly categorized into four major approaches: alignment-based, k -mer-based, marker-gene-based, and machine learning-based.

Alignment-based tools, such as BLAST (Altschul et al., 1990) and DIAMOND (Buchfink et al., 2015), offer high accuracy through base-by-base sequence comparisons against reference databases. However, their computational intensity renders them less feasible for large metagenomic datasets (Bharti & Grimm, 2021). Contrastingly, k -mer-based approaches break sequences into smaller substrings of length k (k -mers) and compare their frequency or presence against reference databases, enabling rapid and scalable target classification. While less accurate than alignment-based methods, k -mer approaches are suitable for large datasets and can rapidly classify most taxa present (Breitwieser et al., 2018). Commonly used k -mer-based taxonomic classifiers include Kraken2 (Wood et al., 2019) and CLARK (Ounit et al., 2015). Marker gene-based approaches, on the other hand, leverage specific and conserved genes (e.g., 16S rRNA for bacteria) to infer taxonomy. Tools like QIIME2 (Bolyen et al., 2019) and MetaPhlAn4 (Blanco-Míguez et al., 2023) are widely used due to their specificity in targeting these markers, providing robust taxonomic inference. Machine learning and statistical models assign taxonomy based on sequence features. These tools learn patterns from training datasets and classify new sequences accordingly. DeepMicrobes, for instance, employs a Deep Neural Network (DNN) to classify taxa (Liang

et al., 2020), and its performance is comparable to that of alignment-based tools (Mathieu et al., 2022).

In addition to classification algorithms, the choice of taxonomic reference database also significantly influences profiling accuracy (Pereira-Marques et al., 2019; Smith et al., 2022; Van Uffelen et al., 2024). Context-specific databases, such as those built from the gut microbiome, may fail to recall environmental taxa from non-clinical samples (Shen et al., 2021). Thus, selecting a comprehensive and relevant database is paramount for accurate taxonomic inference (Ye et al., 2019; Smith et al., 2022; Yunlong Liu et al., 2024).

Identifying ARGs in metagenomes is inherently more challenging than taxonomic classification due to their low abundance and the complexity introduced by MGEs, such as plasmids, transposons, and phages (Erkorkmaz et al., 2025). These elements can fragment assemblies, hindering ARG detection. Consequently, a sufficiently high sequencing depth of at least 80 million reads is usually required for comprehensive ARG identification (Gweon et al., 2019). ARG profiling employs similar approaches to taxonomic classification, including alignment-based tools such as AMRFinderPlus (Feldgarden et al., 2021) and ResFinder (Florensa et al., 2022), as well as machine learning-based tools like DeepARG (Arango-Argoty et al., 2018).

There are several hurdles associated with using these ARG profiling tools to classify ARGs. First, curated databases used by many of the ARG profiling tools often fail to detect novel ARGs, thereby limiting their ability to uncover emerging resistance determinants (Olatunji et al., 2024; Wu et al., 2023). Additionally, linking ARGs to their host species remains underexplored but is critical for understanding AMR evolution in microbiomes. Recent advances in long-read sequencing have enabled the identification of species-resolved ARGs (Chen et al., 2025). Furthermore, while environmental resistomes harbor diverse ARGs, their clinical relevance and public health implications remain underexplored. Recent efforts have

been devoted to classifying environmental ARGs by their clinical relevance (Xu et al., 2024). Environmental samples are prone to low biomass and other confounding factors that can compromise the accuracy of results. First, the presence of non-microbial reads (e.g., human or plant DNA) and other contaminants can obscure the detection of rare taxa and ARGs, particularly when sequencing depth is insufficient. Increasing sequencing depth and coverage can enhance detection (Bengtsson-Palme et al., 2017), but this approach is costly and impractical.

Alternatively, TC probe enrichment can improve the detection of rare taxa and ARGs from the metagenome (Guiton et al., 2019). Moreover, benchmarking different taxonomic classifiers and ARG profiling tools, preferably with microbial community standards, is also essential for selecting optimal tools and databases (Ye et al., 2019). Additionally, rarefaction analysis (i.e., subsampling of reads) can determine the minimum sequencing depth required to capture the greatest microbial diversity and ARG profiles. Given the complexity and variability of environmental microbiomes, broad-spectrum reference databases are preferred to enhance the identification of diverse taxa and ARGs present.

1.6.2. Alpha and Beta Diversity: Quantifying Microbial Community Variations

Microbiome analysis often involves characterizing microbial diversity using alpha and beta diversity.

According to the hallmark study by Whittaker in 1960, community diversity can be described by alpha diversity and beta diversity (Whittaker, 1960, 1972). Alpha diversity refers to the species richness and evenness within a given sample. It increases with both the number of species present (i.e. richness) and the evenness of their distribution. Over the years, several algorithms have been developed to quantify alpha diversity. Among these, the most widely

used alpha diversity metrics are species richness (Fisher et al., 1943), Shannon diversity index (Shannon, 1948), and Simpson's diversity index (Simpson, 1949).

Beta diversity, on the other hand, measures the degree of similarity between microbiomes from different samples. Visualization of beta diversity is typically performed using Principal Coordinate Analysis (PCoA) (Lahti et al., 2021), which, unlike Principal Component Analysis (PCA), that only relies on Euclidean distances, accommodates various distance metrics, such as Bray-Curtis dissimilarity, Jaccard index, and UniFrac distance. This measurement flexibility is particularly advantageous in capturing the unique characteristics of microbial communities through non-Euclidean measures.

1.7. Aims of Study

This study aims to address knowledge gaps in the surveillance and characterization of MDROs and ARGs in urban BE. This study focuses on the surveillance in high-contact public spaces in HK, specifically the mass transport system (MTS) and public washrooms, to provide insights into the prevalence, distribution, and dynamics of AMR. There are three aims of this study:

Aim 1: Characterize the distribution and abundance of MDROs in urban built environments

This study characterizes the distribution and abundance of MDROs in two key urban BE: the MTS and public washrooms. These environments are high-traffic areas with frequent human contact, making them potential hotspots for MDRO transmission. Surface samples were collected from high-contact sites to quantify the prevalence and diversity of MDROs. Conventional microbiological techniques, including cultivation on chromogenic agars, MALDI-ToF, and pDST, were employed to isolate and identify MDROs. Additionally,

phylogenetic analysis was conducted to elucidate the genetic relationships among MDRO isolates, providing insights into their clonal spread and evolutionary dynamics within these urban settings. This aim will establish a foundational understanding of MDRO prevalence in these environments.

Aim 2: Quantify the AMR burden through detection and analysis of ARGs in urban built environments

The second aim focuses on assessing the AMR burden in urban BE by characterizing ARGs using metagenomic approaches. By analyzing environmental DNA extracted from high-contact surface samples, this study will identify the diversity and abundance of ARGs. Dominant and clinically important ARGs among these environments were determined. Understanding the ARGs present in these environments is critical for assessing the risk of ARG transfer to human pathogens and for developing evidence-based strategies to mitigate AMR dissemination in urban settings.

Aim 3: Evaluate the utility of target probe capture (TC) enrichment in environmental surveillance

This study also aims to evaluate the efficacy of TC enrichment as a novel tool for detecting low-abundance MDROs and ARGs in complex environmental samples from urban BE. The performance of TC will be compared against standard shotgun metagenomics to assess its ability to enhance the detection of pathogens and clinically relevant ARGs and cost-effectiveness. This aim will contribute to the advancement of methods in pathogen and AMR surveillance in the urban BE.

Chapter 2.

Materials and Methods

2.1. Sample Collection and Processing

For the subway microbiome study ([Chapter 3](#)), sample collection was conducted from summer 2022 until winter 2025. A total of 37 stations of the Hong Kong subway system were included in the study ([Supplementary Table ST1](#)). At each subway station, four high-contact surfaces, including the automated teller machine (ATM), ticket vending machine, escalator handrails, and buttons and handles inside and outside of an elevator, were sampled ($n = 730$). For capturing bacteria, Polywipe™ (Medical Wire & Equipment, UK, cat.# MW722), a pre-moistened cotton pad, was used to wipe down all surfaces. Each surface was wiped with vertical, diagonal, and horizontal “S”-strokes for 3 minutes. For capturing viruses, two sterile swabs were immersed in 400 µL of a transport and storage medium suitable for both DNA and RNA (DNA/RNA Shield, Zymo Research, cat.# R1100) and sampled using the same method as Polywipe. For each round of sampling, the metadata describing the sample collection dates, daily temperature, relative humidity, and weather conditions were recorded in [Supplementary Table ST2](#).

For the washroom microbiome study ([Chapter 4](#)), samples were collected from public washrooms located in hospitals, catering outlets, transportation systems, campus, hotels, wet markets, and recreational facilities in Hong Kong between July and October 2024. A summary of surface samples collected ($n = 232$) was provided in [Supplementary Table ST7](#). At each washroom, sampling was performed on four post-handwashing surfaces, including faucets, paper dispensers, hand dryers, and exit door handles. The same surface sampling procedures described above were applied to post-handwashing surfaces. Only Polywipe was adopted in the washroom microbiome study. For each round of sampling, the metadata describing the

sample collection dates, daily temperature, relative humidity, and weather conditions were recorded in [Supplementary Table ST8](#).

After sample collection, Polywipe and swabs were stored at 4°C before returning to the laboratory for processing on the same day of sampling. For Polywipe, 45 mL of 1% peptone water-Tween 80 was added aseptically to the stomacher bag containing Polywipe. The mixture was then put into a stomacher and run for 5 minutes, set at 3.8 rpm/s, followed by centrifugation at 4347 rpm for 15 minutes to separate microorganisms. After that, the pellet was resuspended with 1 mL sterile PBS. For the identification of antimicrobial-resistant bacteria, 100 µL resuspension was cultured in buffered peptone water (BPW; Thermo Fisher Scientific, cat.# CM0009) at 37°C overnight. For *Candida* spp. and *Candidozyma auris* detection, the protocol from the Centers for Disease Control (CDC) was followed (CDC, 2024a). In brief, 100 µL resuspension was cultured onto Salt Sabouraud Dulcitol (SSD) broth supplemented with gentamicin (50 mg/L) and chloramphenicol (50 mg/L) at 40°C for 5 days, followed by subculture onto chromogenic agars ([Appendix 1](#)). The remaining resuspension was pelleted and stored at -80°C and subsequently processed as a direct sample using metagenomic approach. Swab samples were stored at -80°C before undergoing RNA extraction.

Two negative controls, air and surface control, were prepared by holding the Polywipe and swab moistened with DNA/RNA shield in air and wiping down the laboratory bench surface for 3 minutes, respectively. They were then processed alongside environmental samples.

2.2. Culture-dependent methods

2.2.1. Bioburden

For the washroom microbiome study, total bacterial counts were determined by the aerobic plate count (APC) method. A total of 100 µL of the resuspension from Polywipe was cultured onto brain heart infusion (BHI) agar (Becton Dickinson, USA, cat.# 211065) and

spread evenly using a sterile L-shaped disposable spreader. All plates were incubated aerobically at 37 °C for 48 h. Total bacterial counts were enumerated using DigiCounter™ of the Biomic V3 Microbiology System (Giles Scientific Inc., Santa Barbara, CA, USA). Colony-forming units per mL (CFU/mL) were subsequently determined.

2.2.2. MDRO Identification using Conventional Culture

For bacterial isolation, after overnight incubation, 100 µL incubated BPW from section 2.1 was subcultured into BHI broth (Becton Dickinson, USA, cat.# 211059) supplemented with antibiotics to select antimicrobial-resistant bacteria according to [Appendix 2](#), followed by incubation at 37°C overnight. Subsequently, the incubated antibiotic-BHI broth was plated out on designated chromogenic agars for further selection ([Appendix 3](#)). All streaked plates were then incubated at 37°C for 2 days, followed by examination for the colors and morphology of the colonies as instructed by the manufacturer. MALDI-TOF was performed to confirm the identity of the colonies using the MALDI Biotyper sirius system (Bruker Daltonics, Bremen, Germany) and MBT Compass HT software (v5.1.300) with MBT Compass Library (v12.0.0.0). Isolates with a score > 2.00 were selected and subjected to subsequent antibiotic susceptibility testing.

For *Candida* spp. isolation, the SSD broth was checked everyday for turbidity up to 5 days. If the SSD broth becomes turbid, it will be plated out on chromogenic agar and incubated at 37°C for another 5 days for further selection. Subcultured plates were observed daily for growth of colonies. On the fifth day, all clear broth was also subcultured onto chromogenic agar to avoid false negatives. DNA extraction of yeast colonies was then performed as described in section 2.2.4. ITS PCR followed by Sanger sequencing was performed for species identification according to the CDC's protocol (CDC, 2024b). In brief, ITS1 and ITS4 were

used to amplify the ITS region of the yeast species, which was subsequently subjected to BLAST analysis for species assignment.

2.2.3. Phenotypic Drug Susceptibility Testing (pDST)

pDST was performed on isolated bacterial colonies to confirm their drug resistance status according to the Kirby-Bauer Disk Diffusion Susceptibility Test Protocol (Hudzicki, 2009). For each bacterial species, antibiotic discs were placed on Mueller-Hinton agar (MHA; Becton Dickinson, USA, cat# 211438). Antibiotic discs used to test for the drug resistance in *S. aureus* ([Appendix 4](#)), *Enterobacterales* ([Appendix 5](#)), *Acintebacter baumannii* ([Appendix 6](#)) and *Pseudomonas aeruginosa* ([Appendix 7](#)) were provided. Zones of inhibition were measured by the Biomic V3 Microbiology System (Giles Scientific Inc., Santa Barbara, CA). The status of resistance or susceptibility was determined according to Performance Standards for Antimicrobial Susceptibility Testing, CLSI M100 (35th ed) (CLSI, 2025). Specifically, MRSA was identified by resistance to ceftazidime (30 µg), with an inhibition zone diameter ≤ 21 mm. Carbapenem-resistant *P. aeruginosa* (CRPA) was defined by resistance to at least one carbapenem drug (imipenem, doripenem, or meropenem), with an inhibition zone diameter ≤ 15 mm. Extended-spectrum beta-lactamase (ESBL) production was confirmed using the combined disk method, testing ceftazidime (CTX, 30 µg) alone and with clavulanic acid (CTX/CLA, 30/10 µg), as well as cefturoxime (CXM, 30 µg) alone and with clavulanic acid (CXM/CLA, 30/10 µg). An isolate was classified as ESBL-positive if the inhibition zone diameter of either combined disk (CTX/CLA or CXM/CLA) was ≥ 5 mm larger than that of the corresponding single disk (CTX or CXM). Bacterial isolates were also classified into three drug resistance levels, namely multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR) (Magiorakos et al., 2012).

2.2.4. DNA extraction from MDRO isolates

DNA was extracted from a pure culture plate using QIAamp BiOstic Bacteremia DNA Kit (QIAGEN, Hilden, Germany, cat.# 12240-50) according to the manufacturer's protocol. In brief, DNA was released after bacterial cell lysis via both chemical and mechanical homogenization. DNA was subsequently purified by column-based method and collected by elution buffer. The DNA concentration was determined by Qubit 4 fluorometer (Thermo Fisher Scientific, USA) with Qubit 1X dsDNA HS Assay Kit (Thermo Fisher Scientific, USA, cat.# Q33231).

2.2.5. Whole Genome Sequencing of MDRO isolate by Oxford Nanopore

For cultured MDRO isolates, libraries were constructed using the Rapid Barcoding Kit (Oxford Nanopore Technologies, UK, cat.# SQK-RBK114.96) according to the manufacturer's protocol. In brief, 200 ng of extracted DNA were fragmented and barcoded simultaneously by transposonase. A total of 16 barcoded libraries were pooled in equal volumes, cleaned with AMPure XP beads (Beckman Coulter, Inc., Brea, CA, USA, cat.# A63881), and followed by adapter ligation. Subsequently, sequencing was performed using R10.4.1 flow cells on GridION for 72 hours and basecalled using the super accurate (SUP) model. Raw Nanopore reads were then *de novo* assembled using Hybracter (v0.11.2) (Bouras et al., 2024). The assembly was then processed by Bactopia (v3.2.0) (Petit & Read, 2020) for the complete analysis of bacterial genome. Species identification by GTDB-Tk (Parks et al., 2021; Chaumeil et al., 2022), *in silico* multilocus sequence typing (MLST) by mlst (Seemann, n.d.; Jolley et al., 2018), and ARG profiles by AMRFinderPlus (Feldgarden et al., 2021) were obtained based on the assembly.

2.3. Culture-independent methods

2.3.1. Nucleic Acid Extraction from Direct Environmental Samples

DNA extraction was performed on the pellet obtained from section 2.1 using QIAamp BiOstic Bacteremia DNA Kit (QIAGEN, Hilden, Germany, cat.# 12240-50) according to the manufacturer's protocol.

RNA extraction was performed on swab samples using QIAamp Viral RNA Mini kit (QIAGEN, Hilden, Germany, cat.# 52904). Reverse transcription was immediately performed to prevent RNA degradation.

2.3.2. Reverse Transcription and Second Strand Synthesis

Reverse transcription was performed immediately after RNA extraction from swab samples using LunaScript® RT SuperMix Kit (NEB, UK, cat.# E3010L) according to the manufacturer's protocol.

Subsequently, the second strand was synthesized using the NEBNext® Ultra™ II Non-Directional RNA Second Strand Synthesis Module (NEB, UK, cat.# E6111L) according to the manufacturer's protocol.

2.3.3. Library Construction for Metagenomics by Illumina

For the subway microbiome study, nucleic acids from the four high-contact surfaces (i.e., ATM, ticket vending machine, escalator handrails, and elevator surfaces) of the same subway station were pooled in equal volumes prior to library construction.

For the washroom microbiome study, nucleic acids from each post-handwashing surface sample undergo metagenomic library construction.

In brief, metagenomic libraries were constructed using the NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina (NEB, UK, cat.# E7805L) according to the manufacturer's protocol, with modification of adaptor concentration adjusted to 0.3 μ M to avoid adaptor

dimers. Quantification of library concentration was performed using Qubit® fluorometer 4.0 with Qubit 1X dsDNA HS Assay Kit. Quality of libraries was assessed using Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA) in combination with Agilent High Sensitivity DNA Kit (Agilent Technologies, Palo Alto, CA, USA, cat # 5067-4626). Shotgun metagenomic libraries were then sequenced on Illumina Novaseq 6000 (Illumina, San Diego, CA, USA) using paired-end 150-bp reads. Approximately 7 GB data per library was generated.

2.3.4. Targeted Probe Capture Enrichment of Metagenomic Libraries

Targeted probe capture enrichment was performed using the Respiratory Pathogen ID/AMR Enrichment Panel (RPIP) Kit (Illumina, San Diego, CA, USA, cat.# 20047050) on metagenomic libraries obtained from section 2.3.3. Overnight capture was performed following the manufacturer's protocol. The captured metagenomic libraries were then sequenced on Illumina Novaseq 6000 (Illumina, San Diego, CA, USA) using paired-end 150-bp reads. Two GB of data per captured metagenomic library was generated.

Taxonomic classification and ARG profiling on captured metagenomic libraries were performed using Explify® RPIP Data Analysis (v2.1.1). Raw sequencing reads were analyzed by Explify RPIP Data Analysis (v2.1.1) following the manufacturer's instructions. Microbial targets detected were classified into three phenotypic groups: 'Phenotypic Group 1' with microorganisms that frequently considered part of the normal flora, but may be associated with disease in certain settings, 'Phenotypic Group 2' are microorganisms that may represent normal flora but are frequently associated with disease, and 'Phenotypic Group 3' are microorganisms that are not generally considered part of the normal flora and are generally considered to be associated with disease. For ARG detection, the confidence of detection was classified into three categories according to protein sequence coverage and percent identity (PID). Sequences

considered ‘High’ indicate a 100% sequence coverage and PI, and ‘Medium’ indicate $\geq 90\%$ sequence coverage and PID, were included in subsequent analysis.

2.3.5. Illumina Short-read Pre-processing

Raw sequencing reads were filtered for low-quality bases with Phred score < 33 and trimmed for adaptors using `fastp` (S. Chen et al., 2018). Human reads removal was then performed by mapping filtered and adaptor-trimmed reads to human reference genome assembly GRCh38 (accession GCA_000001405.29) using `bowtie2` (v2.5.3) (Langmead & Salzberg, 2012) and `samtools` (v1.17) (Danecek et al., 2021); all mapped reads were removed. Two assemblers, `megahit` (v1.2.9) (Li et al., 2015) and `metaSPAdes` (v3.15.5) (Nurk et al., 2017) were used.

2.3.6. Taxonomic Classification with Illumina Shotgun Metagenomic Data

Non-assembled and assembled reads were then subjected to taxonomic classification by `Kraken2` (v2.1.3), which utilizes a k -mer-based approach to assign sequences to taxon (Wood et al., 2019). The PlusPFP-16 database, comprising of RefSeq archaea, bacteria, viral, plasmid, protozoa, and plant, was utilized (database version: 20231009). `Bracken` was then used to calculate the proportion of reads assigned to a taxon with k -mer uniqueness-based scaling. `MetaPhlan4` (v4.1.1), another taxonomic classifier, was also used as a comparison (Truong et al., 2015). Species with a relative abundance of less than 0.01% were excluded from analysis.

2.3.7. Antimicrobial Resistance Profiling of Metagenomic Reads

Identification of ARGs from metagenomic data was accomplished by three standalone bioinformatic tools: AMRfinderPlus (v 3.11.14), ResFinder (v 4.5.0), and DeepARG (v1.0.4-0)

AMRfinderPlus utilizes the National Center for Biotechnology Information (NCBI)'s curated reference gene database (database version: 2024-01-31.1) and the curated collection of Hidden Markov Models (HMMs) (Feldgarden et al., 2021). By comparing the metagenomic data against HMMs, the presence of ARGs was identified based on sequence similarity. For processing results from AMRfinderPlus, ARG hits with (1) sequence identity $\geq 90\%$ and (2) coverage of reference sequence $\geq 60\%$ were included in subsequent analysis (Mahfouz et al., 2020; Whittle et al., 2022).

ResFinder utilizes the Comprehensive Antibiotic Resistance Database (CARD) as a reference for identifying ARGs. ResFinder enables identification of ARGs from both raw metagenomics reads (Bortolaia et al., 2020; Clausen et al., 2018) and assemblies (Bortolaia et al., 2020; Camacho et al., 2009). For metagenomic reads, it first constructs *k*-mer alignment (KMA) and then compares against the CARD database. While for assemblies, BLAST was utilized to search for ARGs based on sequence similarity. The default parameters, sequence with $\geq 90\%$ sequence identity and $\geq 60\%$ coverage of reference sequence, were adopted to ensure specificity and avoid ambiguous hits (Lal Gupta et al., 2020).

DeepARG employs a Deep Neural Network (DNN) to identify ARGs from metagenomic reads by referencing a curated ARG database from CARD, Antibiotic Resistance Genes Database (ARDB), and UniProt (Arango-Argoty et al., 2018). Only ARG hits with a minimum probability $\geq 80\%$ and sequence coverage $\geq 80\%$ were included.

2.3.8. Removal of contaminating reads

All contaminating taxa and ARGs were statistically identified by the R package “decontam” (v1.22.0) (Davis et al., 2018) by comparing environmental surface samples and the two negative controls (i.e., laboratory air and surface control). Identified species and ARGs were then excluded from subsequent analysis.

2.3.9. Sequencing Reads Subsampling

Sequencing reads were repeatedly subsampled ($n = 3$) from metagenomic data using `seqtk` (v1.4-r122) into the following percentages of the original sequencing depth: 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% and 90%. Taxonomic classification and ARG profiling were then performed on subsampled reads as described in sections 2.3.6 and 2.3.7, respectively.

2.3.10. Calculation of MASH distances between metagenomic samples

A total of 40 human commensal microbiome samples of the gut (Liu et al., 2022), skin (Wilkins et al., 2021), respiratory tract (Kim et al., 2024), and urinary tract (Moustafa et al., 2018), 10 samples per site, were retrieved from the NCBI Sequence Read Archive (SRA) database. MASH sketches and MASH distances of all metagenomes were constructed and calculated using MASH (v2.3) (Ondov et al., 2016). In brief, MASH uses MinHash sketching to estimate the similarity between metagenomes by approximating the proportion of shared k -mers. The resulting MASH distance ranged from 0 to 1, with higher values indicating greater dissimilarity.

2.3.11. Alpha and Beta diversity

Three alpha diversity indices, including species richness (i.e., number of species), Shannon diversity index, and Inverse Simpson's diversity index, were calculated using the *vegan* package in R (Oksanen et al., 2024).

For beta diversity, the Bray-Curtis dissimilarity matrix was determined using the *vegdist* function in the *vegan* package in R (Bray & Curtis, 1957) and visualized using principal coordinate analysis (PCoA) by the *prcomp* package (Holland, 2008).

2.4. Statistical analysis, Graphs, and Taxonomic Tree Construction

Bar plots and heatmaps were constructed using RStudio with the *ggplot2* package (Wickham, 2016). An area proportional Venn diagram was constructed using the *eulerr* package (Larsson et al., 2024). A phylogenetic tree was constructed using *Mashtree* (v1.4.6) (Katz et al., 2019) and visualized using *iTOL* (v6.9.1) (Letunic & Bork, 2019). The Kruskal-

Wallis test was carried out for all non-parametric data. For all statistical comparisons, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****) were used as the significance levels.

2.5. Data availability

For the subway microbiome study, raw sequence data will be made available on request.

For the washroom microbiome study, raw sequence data generated are available in the NCBI Sequence Read Archive (SRA) database under BioProject accession PRJNA1250189.

Chapter 3.

Surveillance of MDRO and AMR in Mass Transit System

3.1. Introduction

The mass transit system (MTS) is a city's hub, where passengers from different parts of the city constantly meet and leave for different destinations, spending relatively short durations (from minutes to hours). Therefore, unlike other BE such as households or offices, subway systems foster a unique microbiome shaped by both human occupants and external environmental factors (Gilbert & Stephens, 2018; Leung & Lee, 2016). Humans, the primary microbial contributors, emit approximately 10^6 biological particles per hour, forming individualized "microbial clouds" that disperse into the surrounding environment (Meadow, 2015). Outdoor environments also play a role in shaping the subway microbiota (Leung & Lee, 2016). A few studies revealed that the microbiome composition of indoor and outdoor air of subway stations resembled each other, driven by frequent air exchange (Gohli et al., 2019; Leung et al., 2014). These microbial signatures subsequently integrate into the subway microbiome, reflecting the enrichment of human and soil commensals.

Despite its small geographic area, Hong Kong (HK) holds the world's 4th highest population density with an average of 7140 inhabitants per km² (World Population Review, 2024). Its highly developed and extensive subway system accounts for 44% of all trips made on public transport, accommodating approximately 5 million passenger journeys daily (*Hong Kong: The Facts - Transport*, 2023). Owing to its high passenger traffic, the HK subway system was ranked the 10th busiest metro system globally in 2018 (UITP, 2018). Given such high population density and passenger flow, significant microbial exchange through frequent contact with high-touch surfaces like handrails and buttons is therefore consequential, and surveillance at these sites is therefore pivotal in preventing their spread within the subway system (Fernández-Iriarte et al., 2021; Passi et al., 2021).

The subway microbiome is dominated by commensals of human skin, soil, plants, and sewage (Peimbert & Alcaraz, 2023a; Ly et al., 2024). Skin commensals, including

Staphylococcus, *Streptococcus*, *Cutibacterium* (basonym: *Propionibacterium*), *Micrococcus*, *Corynebacterium* and *Kocuria*, were commonly identified from the subway's microbiota (Peimbert & Alcaraz, 2023b; Ly et al., 2024). Soil-associated bacteria, including *Methylobacterium*, *Bradyrhizobium*, *Paracoccus*, *Arthrobacter*, and *Rubellimicrobium*, were most enriched in urban subway (Grydaki et al., 2021). Thus, the subway serves not only as a transportation network but also as an epitome of an urban city's microbiome.

MTS have been implicated in the pathogen transmission, including airborne infections such as COVID-19 and Influenza, which are associated with public transportation (Shen et al., 2020; Thomas et al., 2022; Gubert et al., 2023). Furthermore, a plethora of studies reported identification of pathogens, especially drug-resistant strains, from MTS around the globe (see [Table 3.1A](#) and [Table 3.1B](#)). The subway environments, therefore, play a pivotal role in pathogen and AMR dissemination. Despite this, the transmission dynamics of pathogens and AMR within MTS remain poorly understood. This study employs both conventional culture-based methods and metagenomic approaches to provide a comprehensive and nuanced view of the phylogeographic distribution of MDROs and AMR within the HK's subway system. By elucidating this relationship, it is aimed to inform evidence-based public health interventions to mitigate the spread of MDRO and AMR in subway environments.

3.2. Literature Review

Microbiological investigation of the MTS began in the late 1990s, initially relying on culture-dependent methods targeting one to two specific bacterial or fungal species. These early studies established the role of MTS as potential reservoirs for pathogen transmission. However, the constraints of culture-based approaches limit our understanding of the overall microbial diversity and dynamics, highlighting the need for more comprehensive methodologies.

Since the early 2010s, advancements in high-throughput sequencing (HTS) and decreasing sequencing costs have revolutionized subway microbiome research. HTS, namely 16S rRNA sequencing and shotgun metagenomics, has enabled the broad, culture-independent profiling of microbial communities in MTS locally and worldwide. In addition to identifying the microbial diversity present in air and surface samples, these post-HTS studies revealed the presence of antimicrobial determinants, offering an in-depth understanding of the subway microbiome. This section reviews pre- and post-HTS studies of the subway environments, highlighting their findings on microbial diversity, pathogen prevalence, and AMR in MTS.

3.2.1. Culture-Dependent Studies of the Subway Microbiomes

Culture-dependent studies of the subway microbiomes have been conducted since the late 1990s to characterize the microbial communities of subway environments. These investigations primarily focused on two key areas, subway air ($n = 12$; [Table 3.1A](#)) (Gilleberg et al., 1998; Awad, 2002; Seino et al., 2005; Cho et al., 2006; Bogomolova & Kirtsideli, 2009; Hwang et al., 2010; Kawasaki et al., 2010; Kim et al., 2011; Dybwad et al., 2012; Zhou & Wang, 2012; Hoseini et al., 2013; Hernández-Castillo et al., 2014) and high-touch surfaces ($n = 15$; [Table 3.1B](#)) (Stepanović et al., 2008; Yeh et al., 2011; Iwao, Yabe, et al., 2012; Conceição et al., 2013; Lutz et al., 2014; Peng et al., 2015; Mendes et al., 2015; Lin et al., 2017; Angbuhang, 2018; Shen et al., 2018; Zou et al., 2019; Kahsay et al., 2019; Cao et al., 2020; Gu et al., 2020; Smelikova et al., 2025). These studies usually employed selective media to isolate certain bacterial or fungal species, followed by identification using biochemical tests or MALDI-ToF mass spectrometry, or, in later studies, 16S rRNA sequencing. For drug-resistant isolates, molecular typing techniques such as multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), or species-specific typing (e.g., *SCCmec*, *spa*, *agr*, *coa* for *S.*

aureus) were adopted to further characterize strains. Recent studies also incorporated WGS to determine the full resistance profile and detailed molecular typing of the drug-resistant isolates.

These investigations primarily targeted bacterial taxa, revealing a predominance of human-associated taxa, such as *Staphylococci* and *Micrococcus*, in subway environments. (Awad, 2002; Seino et al., 2005; Kim et al., 2011; Dybwad et al., 2012; Zhou & Wang, 2012; Stepanović et al., 2008; Conceição et al., 2013; Lutz et al., 2014; Peng et al., 2015; Lin et al., 2017; Angbuhang, 2018; Gu et al., 2020; Smelikova et al., 2025). Comparative studies revealed higher bacterial and fungal load in underground stations than above-ground stations, with indoor stations exhibiting a higher bacterial load than outdoor stations (Awad, 2002; Bogomolova & Kirtsideli, 2009; Gilleberg et al., 1998; Hoseini et al., 2013; Kawasaki et al., 2010; Kim et al., 2011). Among MDROs, MRSA was the most frequently identified MDRO in the subway environments, with CRE occasionally detected in public transportation (Lutz et al., 2014; Shen et al., 2018; Cao et al., 2020; Gu et al., 2020).

Despite their limited scope, culture-dependent studies have provided critical evidence of viable, drug-resistant strains in subway environments, highlighting the potential of MTS as reservoirs for pathogen transmission. Furthermore, detailed molecular characterization of these isolates has facilitated cross-study comparisons. Culture-dependent studies are thus dedicated to the understanding of the BE microbiome by providing a foundational understanding of the subway microbiome, paving the way for culture-independent approaches, such as metagenomics, which offer a more comprehensive exploration of microbial diversity and AMR dynamics in MTS.

Table 3.1A. List of culture-dependent studies of subway air.

City	Year	Sampling sites	Method	Findings	Reference
Culture-dependent study (Air; n = 12)					
London, UK	1998	Underground station air	Enrichment medium for fungi; genus level identification by microscopy	- <i>Aspergillus versicolor</i> were the most abundant fungi - Fungal count of outdoor air is lower than underground air	Gilleberg et al., 1998
Cairo, Egypt	2002	Air from one underground and above-ground station	Selective medium for <i>Staphylococci</i> (Baird Parker agar); Selective medium for <i>Candida</i> ; Selective medium for fungi (malt extract agar); Selective medium of <i>Actinomyces</i> (starch nitrate agar)	- The concentrations of <i>Staphylococci</i> were higher in underground station. - Spore forming bacteria, <i>Candida</i> spp., fungi and <i>Actinomyces</i> were found at higher levels in the surface station than in the underground station. - <i>Cladosporium</i> , <i>Penicillium</i> and <i>Aspergillus</i> were the dominant fungi isolates	Awad, 2002
Tokyo, Japan	2005	Air in an underground concourse	Enrichment medium; species identification by microscopy and biochemical tests	<i>Staphylococcus epidermidis</i> and <i>Micrococcus</i> spp. dominant	Seino et al., 2005
Seoul, Korea	2006	Air sample from 5 subway stations	Selective medium for fungi: malt extract agar (MEA) with chloramphenicol; identification by microscopy	- <i>Penicillium</i> spp., <i>Penicillium janthinellum</i> , <i>Aspergillus</i> spp., <i>Aspergillus niger</i> , <i>Aspergillus nidulans</i> , <i>Cladosporium</i> spp., <i>Cladosporium cladosporioides</i> , and <i>Mucor</i> spp. were identified from the air samples. - Airborne fungi concentrations during morning and evening commute hours were significantly higher than those during non-commute hours.	Cho et al., 2006
St. Petersburg, Russia	2009	Air in 4 types of subway stations: open-type deep underground, open-type above-ground, closed-type deep underground, large closed deep underground transfer station	Czapek agar, malt agar, meat-peptone agar (MPA) for bacterial count; identification by microscopy	- 50 fungal species were found in underground station, mostly were detritogenic fungi. - The most abundant genera were <i>Acremonium</i> , <i>Aspergillus</i> , <i>Cladosporium</i> and <i>Penicillium</i> .	Bogomolova and Kirtsideli, 2009
Seoul, Korea	2010	Air from 25 stations	Selective medium for Gram-negative bacteria; enumeration of colonies	Airborne bacteria were detected at 23 stations (92%) and Gram-negative bacteria were detected at two stations (8%).	Hwang et al., 2010
Tokyo, Japan	2010	Air from one underground and above-ground station	Selective medium for fungi (Dichloran-glycerol agar); genus level identification by microscopy	- Most abundant fungi is <i>Aspergillus</i> (<i>Aspergillus versicolor</i>), <i>Cladosporium</i> , <i>Penicillium</i> - Distribution and composition of fungi differ depending on the structure of station	Kawasaki et al., 2010
Seoul, Korea	2011	22 randomly-selected stations; 8 above-ground stations and 14 underground stations	Selective medium for bacteria: Trypticase Soy Agar with cycloheximide; species identification by biochemical tests Selective media for fungi: Malt Extract Agar with chloramphenicol; species identification by microscopy	- <i>Staphylococcus</i> and <i>Micrococcus</i> comprised over 50% of the total airborne bacteria - <i>Penicillium</i> and <i>Cladosporium</i> comprised over 60% of the total airborne fungi	Kim et al., 2011
Oslo, Norway	2012	Underground station air	Selective medium for bacteria (Trypticase soy agar with cycloheximide); species identification by 16S rRNA sequencing and MALDI-ToF	- A total of 37 different genera were identified in the air. - Majority of the airborne bacteria belonged to the genera <i>Bacillus</i> , <i>Micrococcus</i> , and <i>Staphylococcus</i>	Dybwad et al., 2012
Shanghai, China	2012	Air inside subway carriages and station	Nutrient agar plates and Czapek's medium plates; species identification by microscopy and biochemical tests	Detected mostly Gram-positive coagulase-negative <i>Staphylococcus</i> strains (CNS)	Zhou and Wang, 2012
Tehran, Iran	2012	Air from one underground and above-ground station	Selective medium for fungi: Sabouraud Dextrose Agar (SDA) with chloramphenicol; species identification by microscopy	- Most dominant fungi identified in indoor and outdoor air were <i>Penicillium</i> spp. and <i>Alternaria</i> spp., respectively. - Indoor air quality in subway is worse than the outdoor air.	Hoseini et al., 2012
Mexico City	2014	Air sample from the busiest and least used station	Selective culture for fungi (Sabouraud Dextrose Agar) and bacteria (m-Endo-Agar Les); species identification by colony morphology	- Fungi and bacteria indoor concentrations in the subway were 8 times higher than outdoor - Most abundant bacteria is nonsporulating short <i>Bacillus</i> - Most abundant fungi were <i>Aspergillus</i> , <i>Penicillium</i> , and <i>Alternaria</i>	Hernández-Castillo et al., 2014

Table 3.1B. List of culture-dependent studies of subway surface.

City	Year	Sampling sites	Method	Findings	Reference
Culture-dependent study (Surface; n = 15)					
Belgrade, Serbia	2008	Handrails from 55 vehicles (trolleybuses, trams and buses)	Selective medium for MRSA; PCR for <i>mecA</i> ; pDST by disc diffusion	- 30.1% samples were positive for methicillin-resistant coagulase-negative <i>staphylococci</i> (MRCoNS) - Nearly 50% of MRCoNS isolates resistant to beta-lactams and ≥ 2 other classes of antimicrobials - No MRSA was detected	Stepanović et al., 2008
Portland, USA	2011	Train floors, cloth seats, handrails, underside of seats, windows, vinyl seats.	Enrichment medium; 16S rRNA sequencing; pDST by disc diffusion	- Of the 14 isolates sequenced, 11 were <i>staphylococci</i> , of these, 5 were resistant to penicillin and ampicillin	Yeh et al., 2011
Tokyo, Japan	2011	Surfaces of the straps and handrails of 349 trains with cotton swabs	Culture; pDST by agar dilution; molecular typing: <i>spa</i> , <i>agr</i> , <i>coa</i> , <i>SCCmec</i> ; strain typing by PFGE	- Of 349 trains examined, eight (2.3%) were positive for MRSA. - MRSA clones belonged to ST5, 8, 88, and 89	Iwao et al., 2011
Lisbon, Portugal	2013	Handrails, seat rails, handgrips, stop buttons and the surface of the validation tickets machine of public buses	Selective medium for MRSA; molecular typing: MLST, <i>spa</i> , <i>SCCmec</i> ; strain typing by PFGE	- 36.2% MRSA clones were from bus route serving more than three hospitals - MRSA clones detected on bus surfaces were also detected from hands of passengers	Conceição et al., 2013
USA	2014	Stanchions, seats, seat rails, back door, driver's area, heating, ventilating, and air conditioning return vent	Enrichment medium; species identification by colony morphology and biochemical tests; pDST by disc diffusion; strain typing by PFGE	- 68% samples were contaminated with <i>S. aureus</i> , and 63% were contaminated with MRSA - Seats and seat rails were most frequently contaminated, followed by the back door and stanchions - 62.9% of the MRSA isolates were classified as CA-MRSA clones (<i>SCCmec</i> type IV), and 22.9% were HA-MRSA clones (<i>SCCmec</i> type II) - Of the MRSA strains, 65% were multidrug resistant	Lutz et al., 2014
Guangzhou, China	2015	Handrails, seats, stanchions, Ticket Vending Machines (TVMs), and escalators	Enrichment medium; species identification by colony morphology and biochemical test; PCR for <i>mecA</i> , MLST, <i>SCCmec</i> ; pDST by disc diffusion	- 75.6% samples were contaminated with <i>Staphylococci</i> - The <i>Staphylococci</i> isolates, especially the methicillin resistant isolates, were resistant to most of the antibiotics, with 79.8% classified as multidrug resistant - 8 strains of MRSA carried a range of <i>SCCmec</i> types [I (1), II (3), III (2) and NT (2)].	Peng et al., 2015
Porto, Portugal	2015	Handrails and on-board stop buttons inside trains	Selective medium for MRSA; PCR for <i>mecA</i> ; molecular typing: <i>spa</i> , <i>SCCmec</i> ; strain typing by PFGE	The MRSA detected in 8.9% train samples	Mendes et al., 2015
Guangzhou, China	2017	Ticket Vending Machines (TVMs), elevators buttons, toilet faucets, booking desks and canteen desks.	Selective medium for <i>Staphylococci</i> ; species identification by colony morphology, microscopy, biochemical tests; PCR for <i>mecA</i> and <i>qac</i> ; pDST by disc diffusion > Molecular typing: <i>PVL</i> , <i>SCCmec</i> , MLST	- 39.21% of all samples were <i>Staphylococci</i> isolates - 1.58% of <i>Staphylococci</i> isolates were MRSA isolates - The proportion of multidrug resistant among 149 <i>Staphylococci</i> isolates was 75.84%.	Lin et al., 2017
Kathmandu Valley, Nepal	2018	Handle and seat surface of each vehicle	Selective medium for <i>Staphylococci</i> ; species identification by colony morphology, microscopy, biochemical tests; pDST by disc diffusion	Out of 56 samples, 25.9% identified <i>S. aureus</i> and 31.4% of them being MRSA	Angbuhang et al., 2018
Guangzhou, China	2018	Handrails of vehicles, elevators, escalators, and the screens and buttons of ticket vending machines	PCR of <i>mcr-1</i> performed on direct samples; subculture of <i>mcr-1</i> -positive samples on non-selective agar; species identification by MALDI-ToF; pDST by MIC	- Of the 737 samples with bacterial growth, 26 isolates were positive for <i>mcr-1</i> , including 23 <i>E. coli</i> and 3 <i>Klebsiella pneumoniae</i> isolates. - 76.9% of the <i>mcr-1</i> -positive samples were distributed in areas with either a high density of hospitals or heavy traffic	Shen et al., 2018
Beijing, China	2019	Handlebars and brakes of the bikes	Selective medium for drug-resistant <i>Enterobacteriaceae</i> ; species identification by MALDI-ToF and 16S rRNA sequencing; pDST by agar dilution	- Identified 444 <i>Enterobacteriaceae</i> isolates - The isolates exhibited low rates of resistance (0.5%–6.3%) to all antimicrobial agents except sulfamethoxazole-trimethoprim (31.5%). - Three ceftazidime-resistant <i>E. coli</i> isolates were positive for blaCTX-M-199 and two of them were positive for blaNDM-5.	Zou et al., 2019
Mekelle city, Tigray, Ethiopia	2019	Handles in buses	Selective medium for Gram-negatives; species identification by colony morphology, microscopy and biochemical tests; pDST by disc diffusion	- Detected <i>E. coli</i> , <i>Enterobacter</i> spp. and <i>S. aureus</i> in 4%, 1.3% and 18% samples, respectively. - MRSA detected in 5.7% samples and 31.5% of the <i>S. aureus</i> isolates. - <i>E. coli</i> and <i>Enterobacter</i> spp. showed resistance for ampicillin and 75% displayed resistance for chloramphenicol	Kashay et al., 2019
Beijing, China	2020	Subway handrails, seats, doorknobs, ticket machines, bathroom door handles, toilet vanities and escalator handrails	Selective and differential agar for CRE; Molecular typing by WGS; pDST by broth microdilution	- Identified 11 carbapenem-resistant <i>K. pneumoniae</i> isolates from the Beijing subway environment. - 9 isolates classified as ST726 and containing a blaNDM-5-carrying IncX3 plasmid. - <i>Staphylococci</i> (n = 241) and <i>Enterococci</i> (n = 69) were widely distributed across sampling locations at a frequency of 2.3%–12.9%, and 0.08%–5.5%, respectively. - Bicycle or rider-borne Gram-positive bacteria were frequently resistant to clinically important antibiotics, indicating multidrug resistance. - 52.6% of <i>S. aureus</i> isolates were MRSA	Cao et al., 2020
Chengdu, China	2020	Handlebar surfaces of bicycles; palms and fingers of riders	Selective medium for <i>Staphylococci</i> and <i>Enterococci</i> species identification by 16S rRNA Sanger sequencing; pDST by disc diffusion; Molecular typing: MLST and SNP analysis; WGS for ARG profile	- <i>S. aureus</i> was the most frequent opportunistic bacterial pathogen found (3.7%) followed by <i>Enterobacteriales</i> (1.8%). - No MRSA, ESBL or carbapenemase-producing bacteria were detected. - One <i>S. aureus</i> isolate was resistant to vancomycin	Gu et al., 2020
Czech	2025	In metro trains and ground transport vehicles: handrails and stop buttons; In stations: escalators handrails, ticket machines, lift buttons, disinfection dispensers	Selective medium for Enterobacterales, Pseudomonas, Staphylococci; species identification by MALDI-ToF; pDST by broth microdilution; WGS for MLST and SNP analysis	- <i>S. aureus</i> was the most frequent opportunistic bacterial pathogen found (3.7%) followed by <i>Enterobacteriales</i> (1.8%). - No MRSA, ESBL or carbapenemase-producing bacteria were detected. - One <i>S. aureus</i> isolate was resistant to vancomycin	Smelikova et al., 2025

3.2.2. Culture-Independent Studies of the Subway Microbiomes

3.2.2.1. Culture-Independent Studies of Subway Microbiomes around the Globe

Culture-independent studies have been conducted in subway systems worldwide ([Table 3.2A](#)). Most of these studies employed sequencing of the 16S rRNA and ITS regions, which limited metagenomic findings to bacterial and fungal taxa. The first culture-independent study, conducted in New York City's subway system, employed pyrosequencing of the 16S rRNA and ITS regions to identify dominant bacterial and fungal taxa (Robertson et al., 2013). Afshinnkoo et al. (2015) subsequently identified potential pathogens, namely *Bacillus anthracis* (causative agent for anthrax) and *Yersinia pestis* (causative agent for bubonic plague), from the NYC metro using a metagenomic approach (Afshinnkoo et al., 2015). However, this study was substantially criticized for its defective study design and unsupported claims concerning public health impacts (Ackelsberg et al., 2015). Despite these limitations, this work pioneered metagenomic applications in subway microbiome research and provided a foundation for future study. As sequencing technology advanced, unbiased shotgun metagenomics has increasingly become the mainstay of subsequent subway microbiome studies (Hsu et al., 2016; Gohli et al., 2019; Klimenko et al., 2020; Hernández et al., 2020; Vargas-Robles et al., 2020; Grydaki et al., 2021; Guevarra et al., 2022; Harnpicharnchai et al., 2023; Pochtovyi et al., 2022; Siriarchawatana et al., 2023; Yongping Liu et al., 2024; Zhang et al., 2024). Notably, a significant proportion of these studies focused on the air microbiome rather than surface-associated communities.

In contrast to bacteria, fewer studies have explored the mycobiome (i.e., fungal communities), though some have reported dominant fungal species within the subway system (Harnpicharnchai et al., 2023). Viruses, especially pathogenic viruses, are rarely reported in metagenomic studies. For example, viruses made up only 0.03% of the DNA in the New York

City subway study, let alone the detection of pathogenic ones (Afshinnkoo et al., 2015). This is likely due to their low abundance and the fact that most studies adopted DNA-based extraction protocols, which exclude RNA viruses from analysis. However, more sensitive methods like qPCR have successfully identified respiratory viruses like SARS-CoV-2 and Influenza viruses from subway environments (Coleman et al., 2018; Moreno et al., 2021).

Similar to culture-dependent studies, many studies concluded that humans are a major contributor to subway microbiomes and that anthropogenic activities play a role in shaping the subway microbiomes, as evident by the frequent detection of human skin taxa, including *Cutibacterium*, *Corynebacterium*, *Micrococcus*, *Staphylococcus*, *Streptococcus*, and *Kocuria* (Leung et al., 2014; Siriarchawatana et al., 2023; Park et al., 2025). Several studies have also compared the microbiome composition of indoor and outdoor stations, finding that indoor stations have a higher bacterial load but lower microbial diversity. Exposure to pathogens was also higher in indoor stations than in outdoor stations (Gohli et al., 2019; Grydaki et al., 2021). Similar to culture-dependent studies, MRSA was the most reported MDRO in subway environments. Afshinnkoo et al. detected the *mecA* gene alongside *S. aureus*, suggesting the presence of MRSA in the subway system (Afshinnkoo et al., 2015). Pathogenic *Staphylococcus* strains have been identified in air filters installed inside a subway station (Park et al., 2025). Together, these findings consolidate the role of subway systems as potential reservoirs for pathogen dissemination.

Leveraging HTS technologies, culture-independent studies have significantly expanded our understanding of subway microbiomes. Beyond species identification, these approaches provide quantitative analysis of the relative abundance of taxa present in the sample. More importantly, metagenomic analyses also identified ARGs, offering insights into resistance dynamics (Kang et al., 2018; Guevarra et al., 2022; Zhang et al., 2024). By integrating taxonomic, functional, and ecological data, culture-independent studies have provided

comprehensive investigations on microbial communities and their public health implications in subway environments.

Table 3.2A. List of culture-independent studies of single-city subway system.

City	Year	Sample	Sampling sites	Method	Dominant Kingdom/Phyla/Family/Genus	Dominant Species	Reference
Single city study							
New York City	2013	Air	-	Sanger sequencing (universal) and pyrosequencing (bacterial) for 16S and ITS	Phylum level: <i>Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes</i>	<i>Staphylococcus spp.</i>	Robertson et al., 2013
Hong Kong	2014	Air	-	Sequencing of V4 region of 16S rRNA gene on Illumina MiSeq	Phylum level: <i>Proteobacteria, Actinobacteria, Firmicutes, Deinococcus-Thermus</i> Genus level: <i>Micrococcus, Enhydrobacter, Cutibacterium, Staphylococcus, Corynebacterium</i>	-	Leung et al., 2014
New York City	2015	Surfaces	Turnstiles, Emergency exits, Metro Card Kiosks, Wooden and metal benches, Stair handrails, Trashcans	Metagenomic sequencing on HiSeq2500 Illumina	Phylum level: <i>Pseudomonas</i>	<i>Pseudomonas stutzeri</i> <i>Stenotrophomonas maltophilia</i> <i>Enterobacter cloacae</i> <i>Acinetobacter radioresistans</i> <i>Acinetobacter nosocomialis</i> <i>Lysinibacillus sphaericus</i> <i>Enterococcus casseliflavus</i> <i>Brevundimonas diminuta</i> <i>Acinetobacter lwoffii</i> <i>Bacillus cereus</i>	Afshinnekoo et al., 2015
Boston	2016	Surfaces	Seat, seat back, Horizontal pole, Vertical pole, Hanging grip, Wall, Touchscreen	Sequencing of V4 region of 16S rRNA gene on Illumina MiSeq	Genus level: <i>Cutibacterium, Corynebacterium, Staphylococcus, and Streptococcus</i>	<i>Cutibacterium acnes</i>	Hsu et al., 2016
Barcelona	2016	Air	-	Pyrosequencing of the 16S rRNA gene; QPCR (for RNA virus)	Genus level: <i>Methylobacterium, members of Chitinophagaceae, Bradyrhizobium, Paracoccus, and Sphingomonas</i>	-	Triadó-Margarit et al., 2017
Hong Kong	2018	Passenger's hands	Hands of individual after 30 minutes of contact with handrails	Metagenomic sequencing on Illumina HiSeq 1500	Phylum level: <i>Actinobacteria, Proteobacteria, Firmicutes, Bacteroidetes</i>	<i>Cutibacterium acnes</i> <i>Micrococcus luteus</i> <i>Cutibacterium modestum</i> <i>Acinetobacter baumannii</i> <i>Staphylococcus epidermidis</i> <i>Escherichia coli</i> <i>Staphylococcus aureus</i>	Kang et al., 2018
Oslo	2019	Air, Surfaces	Kiosks, Railings, Benches	Sequencing of V3-V4 region of 16S rRNA gene on Illumina MiSeq	Genus level (Air): <i>Micrococcus, Staphylococcus, Rubrobacter, Sphingomonas, Hymenobacter</i> Genus level (Surfaces): <i>Staphylococcus, Sphingomonas, Streptococcus, Hymenobacter, Corynebacterium</i>	-	Gohli et al., 2019
Moscow	2020	Surfaces	Floor, Railings near escalator, Information stand, Bench, Wall (at shoulder level)	Sequencing of V4 region of 16S rRNA gene on Illumina MiSeq	Phylum level: <i>Actinomycetaceae, Rhodobacteraceae, Enterobacteriaceae, Comamonadaceae, Clostridiaceae families and Gammaproteobacteri</i> Genus level: <i>Stenotrophomonas, Pseudomonas, Dietzia, Brevundimonas, unclassified genera from Intraspangiaceae family</i>	-	Klimenko et al., 2020
Mexico City	2020	Surfaces	Turnstiles, Vertical handrails inside train	Sequencing of V3-V4 region of 16S rRNA gene on Illumina MiSeq	Genus level: <i>Cutibacterium, Corynebacterium, Streptococcus, Staphylococcus</i>	<i>Cutibacterium acnes</i> <i>Staphylococcus epidermidis</i>	Hernández et al., 2020
Mexico City	2020	Surfaces, Passenger's hands	Turnstiles, Escalator handrails, Stair handrails, Platform floors, Poles, Train seats, Passenger's hands	Sequencing of V3-V4 region of 16S rRNA gene on Illumina MiSeq	Phylum level: <i>Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, Fusobacteria</i> Genus level: <i>Acinetobacter, Corynebacterium, Streptococcus, Staphylococcus, Cutibacterium</i>	<i>Acinetobacter sp.</i> <i>Staphylococcus sp.</i> <i>Cutibacterium acnes</i> <i>Streptococcus sp.</i> <i>Staphylococcus epidermidis</i>	Vargas-Robles et al., 2020
Athens	2021	Air	Aerosol samples of underground station	Sequencing of 16S rRNA and ITS gene on Illumina MiSeq	Genus level: <i>Paracoccus, Sphingomonas, Kocuria, Acinetobacter, Staphylococcus</i>	-	Grydaki et al., 2021
Moscow	2022	Air, Surfaces	Turnstiles, Checkout areas	Sequencing on 7 regions of the 16S rRNA gene (V2, V3, V4, V6, V7, V8, and V9) on Ion S5XL sequencer	Genus level: <i>Acinetobacter, Streptococcus, Staphylococcus, Ralstonia</i>	-	Pochtovyi et al., 2022
Seoul, South Korea	2022	Surfaces	Air-conditioning vents, Bells, Benches, Elevator buttons, Entrance doors, Escalator handrails, Floors, Garbage cans, Ground (sidewalk), Stairway handrails, Poles, Information boards, Seats, Ticket machines, Vending machines, Walls	Metagenomic sequencing on Illumina HiSeq X Ten System	Family level: <i>Enterobacteriaceae, Propionibacteriaceae, Alteromonadaceae, Moraxellaceae, Bradyrhizobiaceae</i> Phylum level: <i>Proteobacteria, Actinobacteria, Firmicutes</i>	<i>Salmonella enterica</i> <i>Cutibacterium acnes</i> <i>Alteromonas mediterranea</i>	Guevarra et al., 2022

Table 3.2A. (continued) List of culture-independent studies of single-city subway system

City	Year	Sample	Sampling sites	Method	Dominant Kingdom/Phyla/Family/Genus	Dominant Species	Reference
Single city study (continued)							
Seoul, South Korea	2022	Particulate matter samples	Air purifiers installed in the platform	Sequencing of V3-V4 region of 16S rRNA gene and ITS2 on Illumina MiSeq	Phylum level: Actinobacteria, Proteobacteria, Bacteroidetes, Firmicutes, Cyanobacteria, Deinococcus-Thermus and Choloroflexi Genus level: Cutibacterium, Micrococcus, Staphylococcus, Enhydrobacter, Corynebacterium	-	Hassan et al., 2022
Bangkok	2023	Air	Aerosol sample	Sequencing of V3-V4 region of 16S rRNA gene on Illumina	Phylum level: Proteobacteria, Actinobacteria, Firmicutes, Cyanobacteria, Bacteroidetes, Deinococcus Genus level: Acinetobacter, Moraxella, Sphingomonas, Stenotrophomonas, Agrobacterium	<i>Acinetobacter ursingii</i> , <i>Stenotrophomonas maltophilia</i> , <i>Moraxella osloensis</i> , <i>Sphingomonas ASV_3</i> , <i>Paracoccus ASV_22</i> , <i>Agrobacterium rubrifaciens</i> , <i>Cutibacterium acnes</i> , <i>Staphylococcus capitis</i> , <i>Pseudomonas stutzeri</i> , <i>Staphylococcus hominis</i>	Siriarchawatan et al., 2023
Bangkok	2023	Air	Aerosol sample	Sequencing of ITS gene on Illumina	Phylum level: Ascomycota, Basidiomycota Genus level: Yarrowia, Cladosporium, Hortaea, Cutaneotrichosporon, and Leptobacillum	-	Harnpicharnchai et al., 2023
Shanghai	2024	Air	Aerosol sample	Metagenomic sequencing on MGISEq-2000	Kingdom level: Bacteria, Fungi, Virus, Archaea	Bacteria: <i>Staphylococcus haemolyticus</i> , <i>Shewanella chilensis</i> , <i>Actinomyces erythropolis</i> , <i>Propionibacterium acnes</i> , <i>Staphylococcus hominis</i> , <i>Micrococcus luteus</i> , <i>Staphylococcus epidermidis</i> , <i>Leptospira borgpetersenii</i> , <i>Staphylococcus aureus</i> , <i>Escherichia coli</i> , <i>Agrobacterium. sp.DE0009</i> , <i>Staphylococcus pettenkoferi</i> , <i>Salmonella enterica</i> , <i>Pseudomonas fortis</i> . In the case of <i>Bacillus pumilus</i> , <i>Bacillus thuringiensis</i> Fungi: <i>Malassezia restricta</i> , <i>Malassezia globosa</i> , <i>Alternaria alternata</i> and several <i>Aspergillus</i> species Virus: Phages	Liu et al., 2024
Seoul	2024	Air	Aerosol sample	Sequencing of V3-V4 region of 16S rRNA gene on Illumina	Phylum level: Firmicutes, Proteobacteria, Bacteroidetes, Cyanobacteria, Actinobacteria Genus level: <i>Lysinibacillus</i> , <i>Acinetobacter</i> , <i>Enterobacter</i> , <i>Flavobacterium</i> , <i>Tissierella</i> , <i>Flaviflexus</i> , <i>Clostridium</i> , <i>Cytobacillus</i> , <i>Agrobacterium</i> , <i>Urmitella</i> , <i>Pseudomonas</i> , <i>Sporosarcina</i> , <i>Paenibacillus</i> , <i>Leucobacter</i> , <i>Schuerera</i> , <i>Brevundimonas</i> , <i>Bacillus</i>	-	Sharma et al., 2024
Shanghai	2024	Air	Aerosol sample	Metagenomic sequencing on MGISEq-2000	Phylum level: Pseudomonata, Euryarchaeota, Cyanobacteriota, Bacteroidota, Actinomycetota Genus level: <i>Pseudomonas</i> , <i>Ewingella</i> , <i>Halalkalicoccus</i>	unclassified <i>Pseudomonas</i> , <i>Ewingella americana</i> , <i>Halalkalicoccus subterraneus</i>	Zhang et al., 2024
Bangkok, Thailand	2024	Air, Surfaces	Aerosol samples handrail Floor	Metagenomic sequencing on Illumina MiSeq	Moraxella was abundant in the bioaerosol and handrail samples, and Staphylococcus, which was abundant in the aerosol samples	-	Harnpicharnchai et al., 2024
Beijing	2024	Air	Aerosol samples	- Trypticase soy agar bacteria and Sabouraud dextrose Agar for fungi. - 16S rRNA and ITS sequencing on 454 GS-FLX pyrosequencing platform	Genus level: <i>Aspergillus</i> , <i>Staphylococcus</i> , <i>Pseudomonas</i>	-	Fan et al., 2024
Busan, South Korea	2025	Air	Aerosol samples	Sequencing of V4 region of 16S rRNA gene on Illumina	Genus level: <i>Corynebacterium</i> , <i>Staphylococcus</i>	<i>Staphylococcus epidermidis</i> (pathogenic ST190, ST54, ST992, ST817)	Park et al., 2025

3.2.2.2. Culture-Independent Studies of the Hong Kong Subway

Microbiome

To date, two culture-independent metagenomic studies have been conducted on the air and surface microbiome of the Hong Kong (HK) subway system, providing a basic understanding of the HK subway system for this study.

The first study, conducted by Leung et al. (2014), examined the air microbiome inside various subway stations and train carriages (Leung et al., 2014). By sequencing the V4 region of the 16S rRNA gene, this study characterized the microbial composition of air samples. The study revealed that the HK's subway air microbiome was dominated by the phyla *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Deinococcus-Thermus*. Furthermore, it identified anthropogenic activities and the surrounding outdoor environment as key factors shaping the air microbiome of stations.

The second study, by Kang et al. (2018), investigated the skin microbiome on passengers' hands after making contact with high-touch surfaces in subway stations (Kang et al., 2018). Clinically important ARGs, notably *blaCTX-M*, were identified on passengers' skin. Additionally, they observed a time-specific enrichment of human-associated commensals and clinically important ARGs in the afternoon, most likely due to increased passenger flow and cumulative microbial deposition throughout the day.

The two studies highlight the complex interaction between human activity, environmental elements, and microbial communities in the HK subway system, emphasizing its potential as a reservoir for antimicrobial resistance and pathogen transmission.

3.2.4. Subway Microbiome Studies at a Global Scale: The MetaSUB Consortium

Culture-independent studies of the subway microbiome were conducted at a global scale, which involves cross-city investigation ([Table 3.2B](#)). In the study conducted by Leung et al (2021), air samples from MTS of 6 metropolitan cities across three continents were characterized. City-specific dominance of bacterial species was observed, showing distinction in geographical and climatic variations.

In response to the expanding body of culture-independent studies of the subway microbiomes, efforts have been made to standardize sample collection, processing, sequencing, and bioinformatic analysis to facilitate the cross-comparison of microbiome data from different mass transit systems (MTS) worldwide. Under this context, the Metagenomics and Metadesign of the Subways and Urban Biomes (MetaSUB) International Consortium was established in 2015 to optimize the workflow for urban microbiome study (Mason et al., 2016b). In its seminal work, 4,728 metagenomic samples were collected from MTS across 60 cities worldwide over three years, presenting the first systemic global atlas of urban microbiome (Danko et al., 2021). Key findings include the identification of 4,246 known taxa and a core set of 31 species present in $\geq 97\%$ of samples. Beyond these known taxa, a substantial number of novel bacteria and viruses were also identified. Variations in microbial compositions across cities were observed, driven mainly by climatic changes and geographical locations. Furthermore, ARG profiles of different MTS displayed significant variations in type and abundance across different cities, with many of them being plasmid-associated, indicating a potential for widespread dissemination. The comprehensive metagenomic atlas presented by the MetaSUB Consortium highlights the role of MTS as reservoirs for microbial diversity and AMR, with implications for public health and forensic applications. This work has also provided a standardized

workflow of sample collection, processing, and bioinformatic analysis in studying urban microbiomes, facilitating cross-city comparisons.

Table 3.2B. List of culture-independent studies of multi-cities subway systems.

City	Year	Sample	Sampling sites	Method	Dominant Kingdom/Phyla/Family/Genus	Dominant Species	Reference
Multi-city study							
Denver Hong Kong London New York City Oslo Stockholm	2021	Air	Aerosol sample	Metagenomic sequencing on Illumina HiSeq X Ten System	Kingdom level: Bacteria, Virus, Fungi, Archaea	<i>Cutibacterium acnes</i> <i>Micrococcus luteus</i> <i>Propionibacterium granulosum</i> <i>Staphylococcus hominis</i> <i>Kocuria rhizophila</i> <i>Enhydrobacter aerosaccus</i> (Hong Kong only)	Leung et al., 2021
Global study							
60 cities around the globe	2021	Surfaces	Door knob, Button, Bike kiosk, Pole, Handrails, Escalator, Touchscreen, Railing, Table, Kiosk, Seat, Infoboard, Bollard, Turnstile, Garbage, Floor, Window	Metagenomic sequencing on Illumina HiSeq X Ten System	Phylum level: Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidota	31 core taxa of >97% occurrence:	Danko et al., 2021
60 cities around the globe	2021	Surfaces	Door knob, Button, Bike kiosk, Pole, Handrails, Escalator, Touchscreen, Railing, Table, Kiosk, Seat, Infoboard, Bollard, Turnstile, Garbage, Floor, Window	Metagenomic sequencing on Illumina HiSeq X Ten System	Family level of novel OTUs: Microbacteriaceae, Sphingomonadaceae, Xanthomonadaceae, Pseudomonadaceae, Burkholderiaceae	1,448 high-quality MAG were assembled. 732 new species were found	Wu et al., 2021

3.2.6. Research gap

Despite significant progress in subway microbiome research through culture-independent methods, several critical research gaps remain. Notably, current studies have not adequately explored the public health or clinical implications of microbial communities within subway environments, especially regarding the presence of MDROs. There is a deficiency in knowledge regarding the prevalence of these resistant strains and their potential risks to public health. Although AMR is a major public health threat, few studies have investigated the dissemination of AMR within subway systems (Cave et al., 2021). Furthermore, no studies so far have integrated companion analysis involving culture-based methods, which could offer valuable insights into the viability and pathogenicity of the detected microbes. Addressing these gaps is essential for understanding the public health implications of subway microbiomes and informing strategies to mitigate associated risks.

3.4. The Design of the Hong Kong Subway Microbiome Study

A longitudinal study of the microbiome and resistome of the HK subway system was conducted from July 2022 to February 2025 ([Figure 3.1A](#)). Sampling was carried out bi-annually during the winter and summer to capture seasonal variations. A total of 730 Polywipe samples and 296 swab samples were collected across 36 subway stations (37 stations starting from Winter 2024), with two subway stations in each district of Hong Kong ($n = 18$) ([Figure 3.1B](#); [Supplementary Table ST1](#)). Stations were selected based on two criteria: (1) designation as an interchange station between different subway lines, and/or (2) proximity to hospital(s) within 1 km. At each station, four high-contact surfaces, including the automated teller machine (ATM), ticket vending machine, escalator handrails, and buttons and handles of the elevator, were sampled ([Figure 3.1C](#)). The average daily temperature of sampling days during winter and summer seasons was $18.53 \pm 2.58^{\circ}\text{C}$ and $30.01 \pm 0.94^{\circ}\text{C}$, respectively ([Supplementary Table ST2](#)).

Each surface sample was subjected to three analytical workflows to enable a comprehensive assessment of microbial communities and AMR ([Figure 3.2A](#)). First, chromogenic agars were used to detect the presence of MDROs and provide insights into the viability of drug-resistant clones. Second, culture-independent shotgun metagenomics was performed on all direct surface samples to characterize microbial diversity and resistome profiles. Third, a subset of samples, targeted probe capture (TC), was employed to enhance the detection of clinically relevant species and ARGs. This multi-modal approach was aimed to enable a robust and detailed interrogation of pathogen prevalence and antimicrobial resistance within the subway system.

Bioinformatic analyses of shotgun metagenomic data mainly achieve two objectives: taxonomic classification and ARG profiling. To optimize the sequencing depth and bioinformatic workflow, a subsampling experiment was performed on three subway

metagenomic samples, ensuring the majority of taxa and ARGs present in the samples were sufficiently identified ([Supplementary Data 1](#)). The final bioinformatic pipeline and tools selected were presented in [Figure 3.2B](#). For target probe captured datasets, analyses were performed using the companion bioinformatic analysis platform, Explify® RPIP (Illumina). This tool not only performs the two objectives above, but also categorizes microbial species into three phenotypic groups based on inferred clinical risks (see section 2.3.4) (Illumina, 2024). To ensure accuracy, results from Explify RPIP results were validated by applying the same bioinformatic pipeline used for shotgun metagenomics to target probe captured data ([Figure 3.2B](#)).

A



B



C



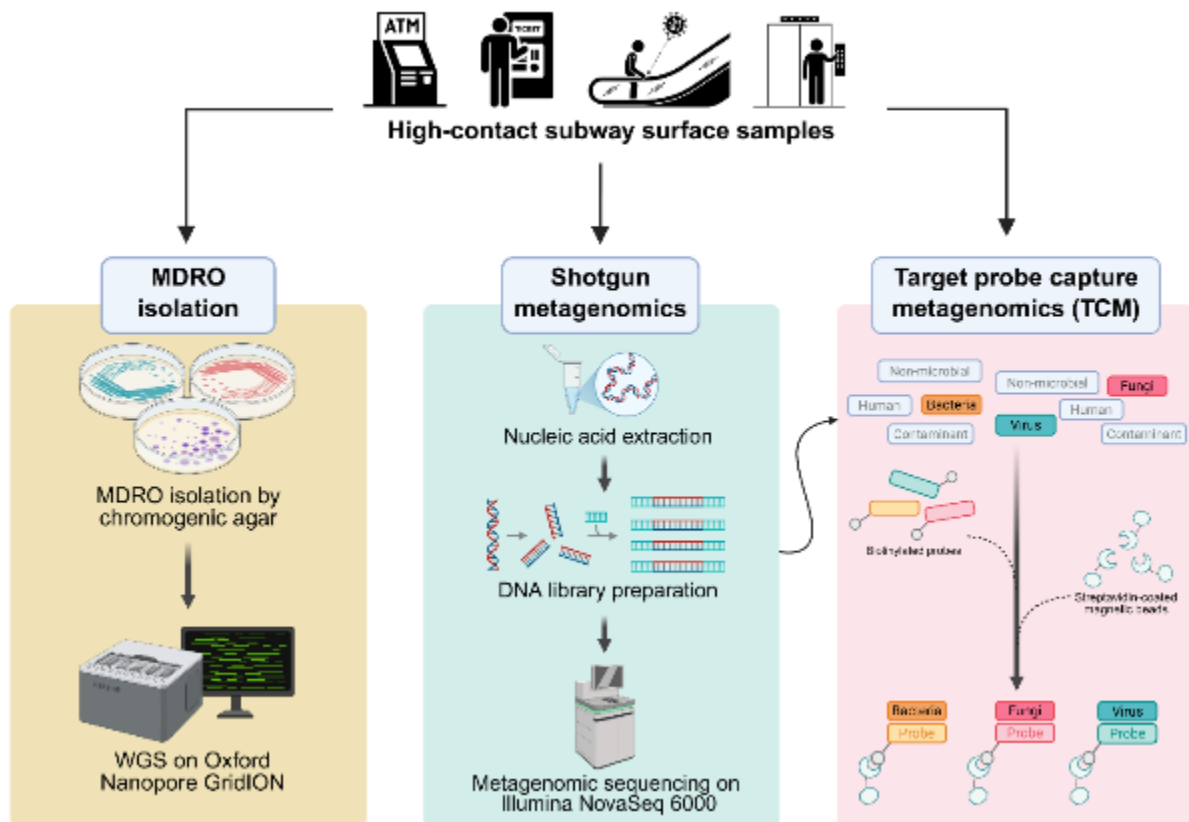
Figure 3.1. Overview of the subway microbiome study.

(A) Sampling timeline and the number of stations and samples collected in this study. A total of 730 Polywipe and 296 swab samples were collected from 36 subway stations (37 from winter 2024 onwards) across all districts of Hong Kong over 2.5 years, from summer 2022 to winter 2025.

(B) Map showing locations of 37 subway stations across 18 districts of Hong Kong selected for this study.

(C) Four high-contact sites were sampled at each subway station, including ATM (top-left), ticket vending machine (top-right), escalator handrails (bottom left), and elevator buttons and handles (bottom right).

A



B

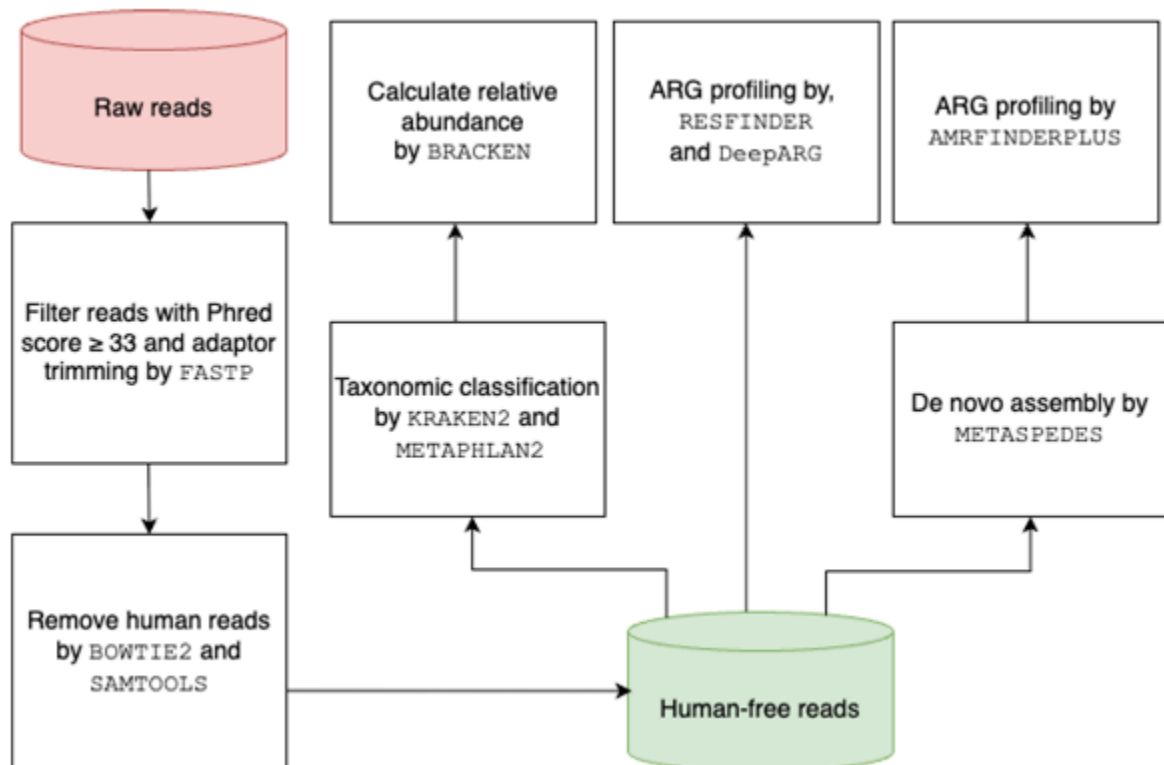


Figure 3.2. Study methodologies of the subway microbiome study.
(Figure description on next page.)

(A) Surface samples were subjected to three workflows. Left panel, MDRO isolates were recovered by chromogenic agars, followed by pDST and WGS (Top panel). Middle panel, shotgun metagenomics was performed on all direct surface samples, followed by taxonomic classification and ARG profiling. Right panel, a subset of metagenomic libraries ($n = 74$) was subjected to targeted enrichment for clinically important taxa and ARGs using gene-specific oligonucleotide probes.

(B) Bioinformatic pipeline of the subway microbiome study. Raw fastq reads were filtered by Phred score ≥ 33 and removed for human reads before taxonomic classification and ARG profiling using multiple tools for result comparison.

3.6. Culture-Based Surveillance of MDRO in the Hong Kong Mass Transit System

From the 730 subway surface samples collected, 51 drug-resistant isolates were identified, representing a prevalence of 6.99%. All drug-resistant isolates were classified into different levels of drug resistance (i.e., single-drug resistant, MDR, XDR, PDR), based on criteria established for clinical isolates (Magiorakos et al., 2012) ([Appendix 4-7](#)). Over half of drug-resistant isolates (59%, $n = 30$) were classified as MDR, while the remaining 41% ($n = 21$) were single-drug resistant ([Figure 3.3A, E](#)). No XDR or PDR isolates were recovered.

Among the MDR isolates, 77% ($n = 23$) were recovered during summer times, suggesting a potential seasonal influence on MDRO prevalence ([Figure 3.3B, E](#)). Consistent with prior studies, MRSA was the predominant MDRO, comprising 53% ($n = 16$) of total isolates ([Figure 3.3C, E](#)). ESBL-producing members of the *Enterobacterales* were the second most common, accounting for 34% ($n = 10$) of total isolates. MDRA and CRPA were infrequently identified, comprising only 10% ($n = 3$) and 3% ($n = 1$) of the isolates, respectively. No drug-resistant *Enterococcus* spp. and *Candidozyma auris* were detected in the HK subway system. The distribution of MDROs across high-contact surfaces revealed that elevator (37%, $n = 11$) and escalator handrails (33%, $n = 10$) were the primary reservoirs for MDROs ([Figure 3.3D, E](#)).

These findings demonstrate the effectiveness of culture-dependent methods in isolating MDROs from environmental samples and highlight the HK subway system as a potential reservoir for drug-resistant pathogens.

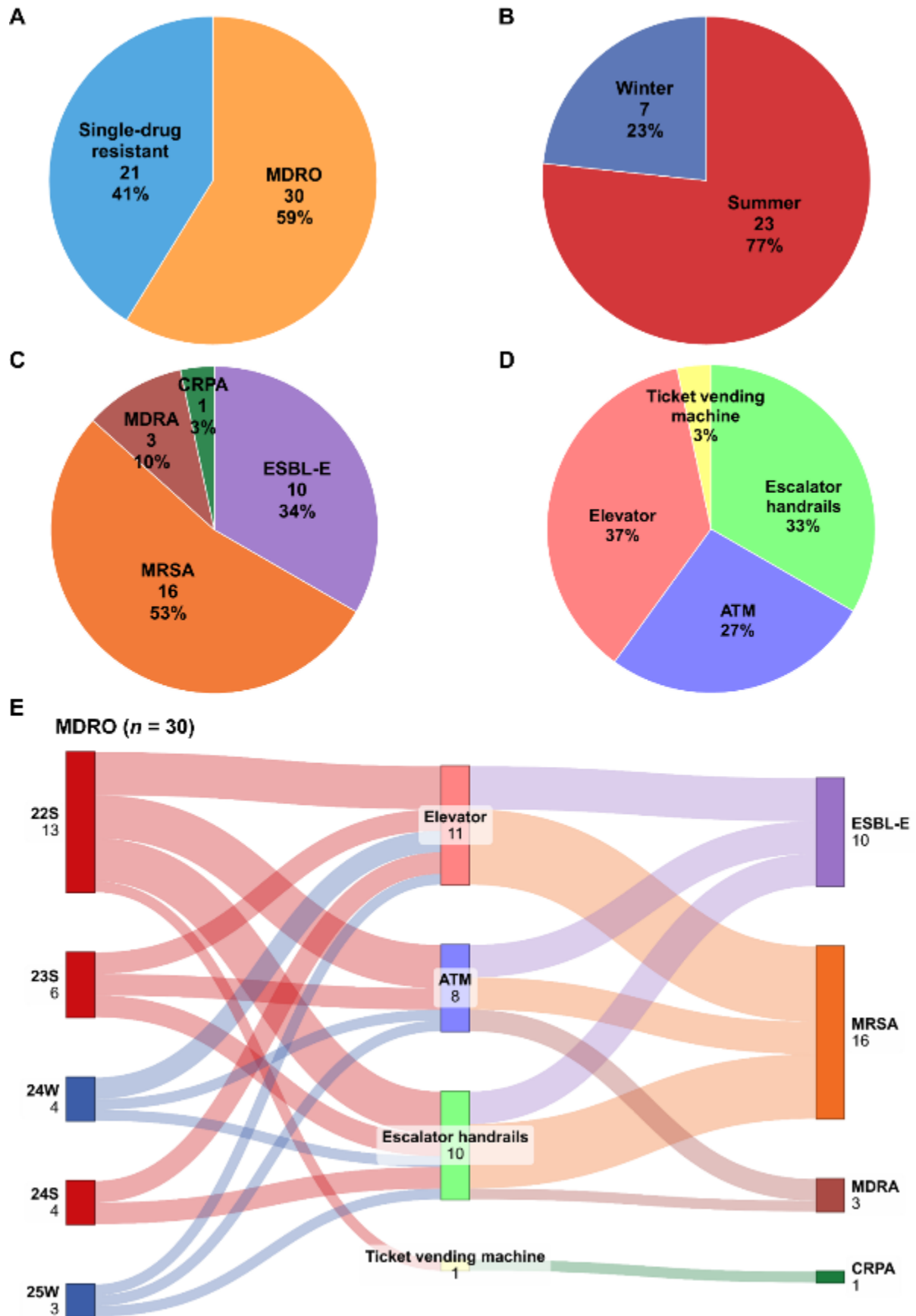


Figure 3.3. Prevalence and distribution of MDROs in the Hong Kong subway system.

(Figure description on next page.)

(A) Proportion of single-drug resistant and multidrug resistant (MDR) isolates among all isolates identified.

(B) Proportion of MDRO isolates identified during summer and winter times.

(C) Proportion of MDRO isolates by species.

(D) Distribution of MDRO isolates recovered from different high-contact sites.

(E) Sankey diagram showing the timeline of all MDRO isolates identified from the HK subway system at different sampling times and high-contact sites.

22S, Summer 2022; 23S, Summer 2023; 24W, Winter 2024; 24S, Summer 2024; 25W, Winter 2025.

3.7. Identification of Clinically Significant Strains from the Hong Kong Mass Transit System by Whole-Genome Sequencing

Next, WGS and *in silico* multilocus sequence typing (MLST) were employed to characterize MDRO isolates from the HK subway system (**Figure 3.4A**). Clinically significant sequence types (STs) were identified.

Out of 16 MRSA isolates, 15 harbored the *mecA* gene and were classified as community-associated SCC*mec* type IV (66.67%) and V (33.33%) (**Figure 3.4A**, **Supplementary Table ST3**). Eleven distinct STs were identified, with ST59 being the most common ($n = 4$; 25%), followed by ST1047 ($n = 2$; 12.5%). Most isolates belonged to *agr*-type I ($n = 13$; 81.25%). Spa typing identified 10 *spa* types, with t437 ($n = 3$; 23.08%) and t304 ($n = 2$; 15.38%) being the most common. Six STs were previously reported as human pathogens. ST59, found on escalator handrails and elevator surfaces, is a dominant CA-MRSA lineage in East Asia, frequently causing skin and soft tissue infections (SSTIs) (Chen et al., 2013; Pang et al., 2020). It accounts for 56% of pediatric CA-MRSA infections in Taiwan (Huang et al., 2008; Chuang & Huang, 2013) and is increasingly prevalent in China (Li et al., 2018; Liang et al., 2019). In Hong Kong, ST59 was responsible for an MRSA outbreak in a neonatal intensive care unit (Cheng et al., 2019). It was suggested that this clone possesses a fitness advantage over other clones, thereby posing a critical public health threat (Li et al., 2018). Two MRSA ST1047 clones were detected from elevator and ATM, respectively. Although no specific clinical infections have been documented, ST1047 has been reported to colonize residents, staff, and environmental surfaces of elderly homes in HK (Wong et al., 2022). ST121, isolated from ATM, is a globally disseminated and highly virulent clone associated with severe infections requiring prolonged hospitalization and extended antimicrobial treatment (Rao et al., 2015). An ST22 clone, recovered from escalator handrails, is another global lineage and a major hospital-associated clone transmitted between healthcare facilities, predominantly causing

human mastitis (Zhou et al., 2022; Zhao et al., 2023; Gu et al., 2024). One ST5 clone found on elevator surface is a prevalent hospital-associated clone, causing infections across multiple countries (Chen et al., 2024, p. 5). The clonal lineage ST6 SCCmec IV/t304, found on one elevator surface, was reported to be prevalent in clinical isolates in Bangladesh (Roy et al., 2024). This clone has also been linked to mastitis in cows, indicating a possible host-jumping event from humans to animals (Kløve et al., 2022). One ST8 SCCmecIV1 (CA-MRSA/J) lineage was recovered from escalator handrails. Originating from Japan, this lineage is causing a broad range of infections, including one fatal case owing to its strong pathogenicity (Iwao, Takano, et al., 2012; Hung et al., 2013; Ishitobi et al., 2018). ST8 SCCmecIV1 was also previously found on high-touch surfaces of subway trains in Japan (Iwao, Yabe, et al., 2012). This clone was potentially introduced into Hong Kong through a Japanese family, whose members were all infected or colonized (Iwao, Takano, et al., 2012; Ho et al., 2007). This is the first report of ST8 SCCmecIV1 in the HK subway system, indicating a potential transmission from hospital environments to public transportation. Phylogenetic analysis between clinical and subway isolates from Japan and HK will help delineate their geospatial and temporal transmission.

All 10 ESBL-E isolates belonged to the *Enterobacter cloacae* complex (**Figure 3.4A**, **Supplementary Table ST4**). Six isolates were typeable, spanning across four STs, including ST151 ($n = 2$), ST346 ($n = 2$), ST527 ($n = 1$), and ST809 ($n = 1$). Limited clinical information is available for these STs, suggesting their minimal known health impacts.

One MDR *A. baumannii* ST768 was identified. Although it is not commonly reported in clinical cases, it was reported to cause bloodstream infections (Wu et al., 2024). The remaining two MDRA isolates were identified as *A. pittii* ST93 and ST1457, detected from escalator handrails and ATM, respectively (**Figure 3.4A**, **Supplementary Table ST5**). The OXA-58-positive ST93 clone is rarely reported in clinical cases but has been found in

companion animals (Klotz et al., 2018). An NDM-1-producing ST457 was reported causing nosocomial infection in Turkey (Roca et al., 2014). However, the ST457 clone isolated from the HK subway system did not carry *bla**NDM-1*.

One CRPA isolate, identified as ST357, was recovered from ticket vending machines (**Figure 3.4A**, [Supplementary Table ST6](#)). This strain has emerged as an XDR lineage in Central Europe, the Netherlands, and Bangladesh, carrying high-risk ARGs including *bla**IMP* and *bla**NDM* (Hrabák et al., 2011; Rossel et al., 2024; Mondol et al., 2024). Despite this, the clone recovered from the HK subway system did not harbor these high-risk ARGs.

ARG profiles of all MDRO isolates were characterized. Resistance genes for beta-lactams were the most common, found in 96.7% of MDRO isolates, followed by fosfomycin (73.3%) and tetracyclines (63.3%) resistance genes (**Figure 3.4B**). These findings highlight the predominance of beta-lactam resistance in the subway environment, underscoring the need for continued surveillance of AMR dissemination in high-traffic public spaces.

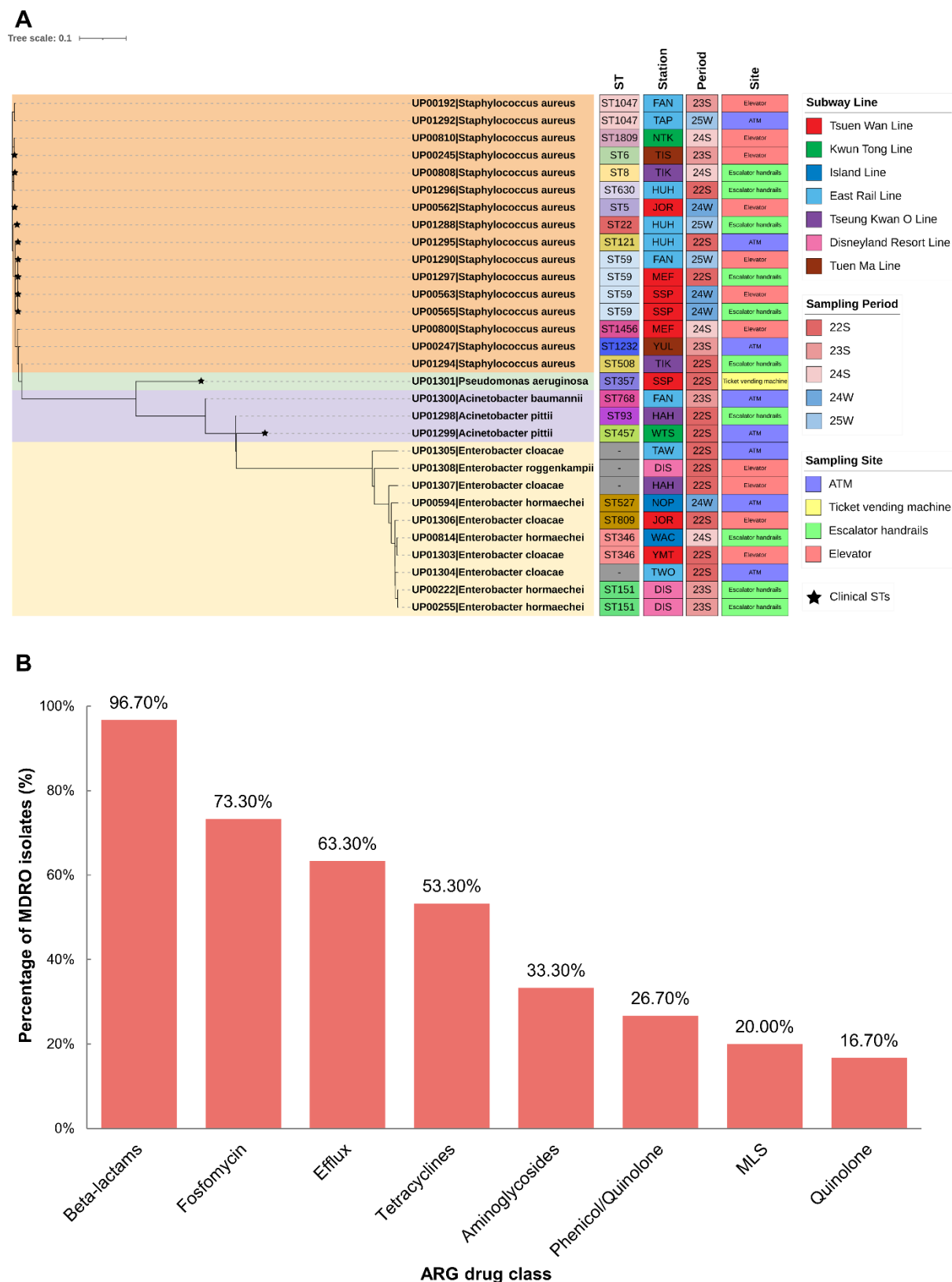


Figure 3.4. Whole-genome sequencing revealed clinically relevant STs and dominant ARG class among MDRO isolates from the Hong Kong subway system.
 (A) Phylogenetic tree of MDRO isolates from the Hong Kong subway system.
 (B) Prevalence of ARG drug class among MDRO isolates.

3.8. Sequencing Metrics of Subway Metagenomic Samples

Shotgun metagenomic (uncapture) sequencing on subway surface samples yielded an average of $46,541,157 \pm 9,987,959$ reads per sample, with microbial reads comprising $17.57\% \pm 6.57\%$ ($8,543,741 \pm 3,734,516$ reads) ([Figure 3.5A](#)). Proportions of human-derived reads ranged from 3.34% to 62.21%, with a mean value of $32.38\% \pm 11.69\%$, while unclassified reads accounted for $50.04\% \pm 9.29\%$ ($24,207,699 \pm 7,166,010$) of total reads.

Target probe capture (TC) was performed on a subset of subway surface samples from summer 2024 ($n = 37$) and winter 2025 ($n = 37$) to enrich for reads associated with pathogens and AMR of clinical and public health significance. This approach yielded an average of $18,038,025 \pm 6,040,35$ reads per sample, with microbial reads comprising $69.93\% \pm 10.10\%$ ([Figure 3.5A](#)). Human-derived reads and unclassified reads accounted for $28.11\% \pm 10.02\%$ (ranging from 13.31% to 52.68%) and $1.32\% \pm 0.25\%$ of total reads, respectively.

Taxonomic classification of microbial reads from shotgun metagenomic libraries revealed $92.52\% \pm 6.45\%$ ($7,810,527 \pm 3,242,915$) as bacteria, $7.16\% \pm 6.34\%$ ($703,147 \pm 844,474$) as fungi, and $0.14\% \pm 0.15\%$ ($14331 \pm 18,250$) as viruses ([Figure 3.5B](#)). Similar taxonomic distribution was observed for TC libraries, with bacteria comprising $95.17\% \pm 1.44\%$ ($11,778,392 \pm 3,623,250$), fungi $4.70\% \pm 1.42\%$ ($576,841 \pm 249,034$), and viruses $0.12\% \pm 0.07\%$ ($14,681 \pm 9,411$) ([Figure 3.5B](#)).

Compared to shotgun metagenomics, TC significantly reduced the proportion of human-derived and unclassified reads. A comparative analysis of sequencing metrics showed that, despite a 2.58-fold reduction in average reads for TC libraries ($18,038,025$ vs $46,541,157$), the proportion of microbial reads increased by 3.98-fold (69.93% vs 17.57% ; [Figure 3.5C](#)). Furthermore, proportions of human-derived reads decreased by 1.15-fold (28.11% vs 32.38%), and unclassified reads decreased by 37.91-fold (1.32% vs 50.04%). These results demonstrate that TC was able to enhance microbial read assignment at a lower sequencing depth while

reducing the proportions of human and unclassified reads, potentially improving the detection efficiency of low-abundance pathogens and ARGs in complex metagenomic samples.

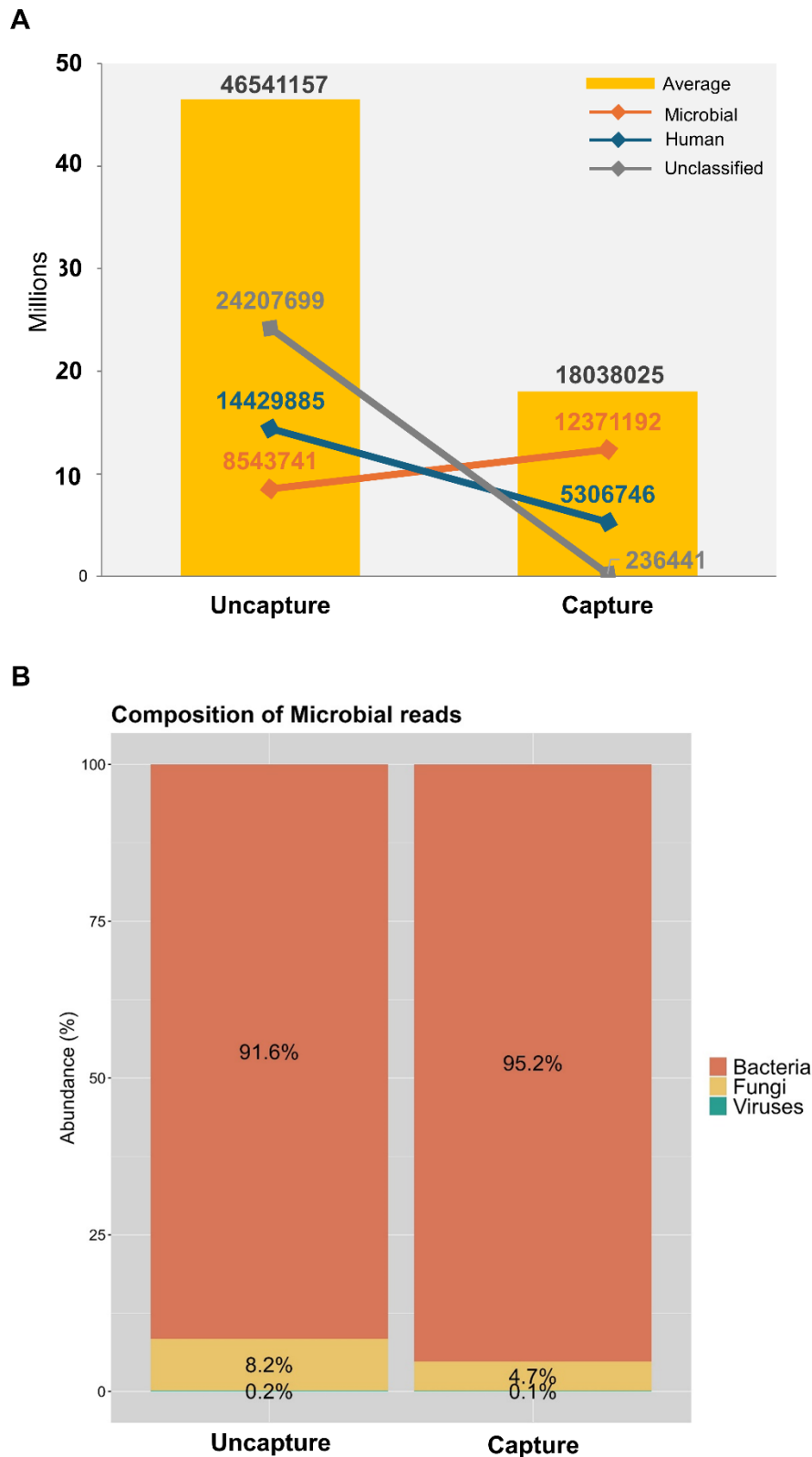


Figure 3.5. Sequencing metrics of subway metagenomic samples.

(A) Barplot showing the composition of sequencing reads yielded from shotgun metagenomic (uncapture) libraries ($n = 179$) and target probe captured (capture) libraries ($n = 74$). Orange, blue, grey lines indicate microbial, human, and unclassified reads, respectively.

(B) Taxonomic composition of overall microbial reads of uncaptured and captured libraries.

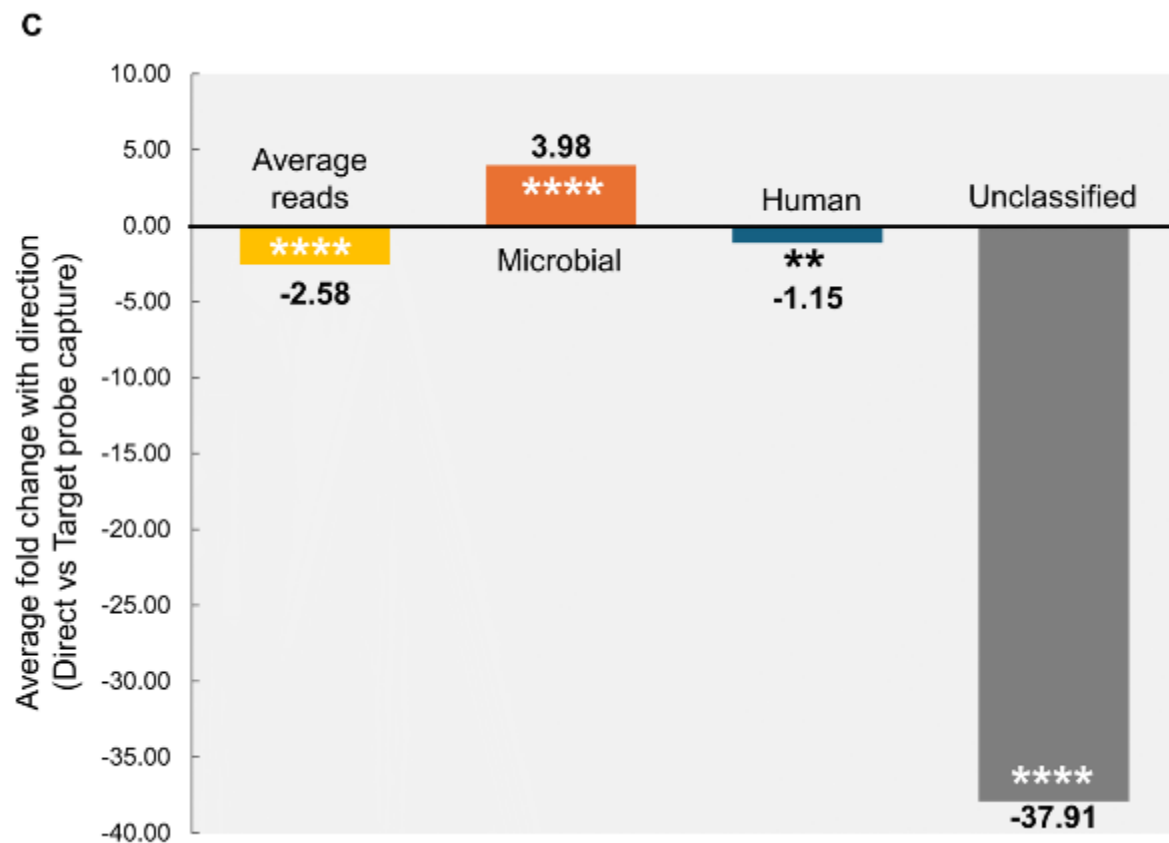


Figure 3.5. (continued) Sequencing metrics of subway metagenomic samples.

(C) Comparison of average reads, microbial reads, human-associated reads, and unclassified reads between captured and uncaptured libraries.

3.9. Taxonomic Profiling Reveals Human Pathogens in Dominating Species of the Hong Kong Mass Transit System

Taxonomic classification of metagenomic reads identified the dominant species across all sampled stations ([Figure 3.6A](#); [Supplementary Figure S4.1-40](#) for individual stations). Among the top 100 dominant species, bacteria made up 93.09% of the reads, followed by fungi at 6.91% ([Figure 3.6B](#)). No viral species were observed among the top 100 species. At the phylum level, *Actinomycetota* predominates, representing 43.95% of the top 100 species, followed by *Pseudomonadota* (37.65%), *Bacillota* (10.84%), and *Basidiomycota* (6.53%) ([Figure 3.6C](#)). Skin-associated bacteria were the most common, accounting for 50.72% of the reads, followed by environmental strains at 30.80% ([Figure 3.6D](#)). From the clinical perspective, five ESKAPE organisms, including *A. baumannii*, *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, and *E. coli*, were found but present at a low level (4.43%). Non-ESKAPE organisms (95.57%) still made up the majority of the top 100 dominant species ([Figure 3.6A, E](#)).

To identify persistent microbial taxa constituting the subway microbiome, the core microbiome, defined as species that were (i) detected across all five sampling periods (from summer 2022 to winter 2025), (ii) present in $\geq 97\%$ of stations, and (iii) reported by both Kraken2 and MetaPhlAn4. The core microbiome of the HK subway system included 22 species, with 21 (95.45%) bacteria and one (4.55%) fungal species ([Figure 3.7A, B](#)). The phylum distribution was similar to dominating taxa, with *Actinomycetota* (40.91%) dominating, followed by *Bacillota* (27.27%), *Pseudomonadota* (27.27%), and *Basidiomycota* (4.55%) ([Figure 3.7C](#)). Similarly, the majority of the taxa were skin-associated taxa (59.09%), followed by environmental strains (31.82%) ([Figure 3.7D](#)). Contrastingly, no ESKAPE organisms were identified as the core taxa of the HK subway microbiome.

A comparison with the MetaSUB Consortium's core taxa identified in $\geq 97\%$ subway stations across 60 cities (Danko et al., 2021) revealed 13 overlapping species (59.01%) with the HK subway core microbiome, including *Acinetobacter johnsonii*, *Cutibacterium acnes*, *Cutibacterium granulosum*, *Dermacoccus nishinomiyaensis*, *Janibacter indicus*, *Kocuria palustris*, *Kocuria rhizophila*, *Micrococcus luteus*, *Moraxella osloensis*, *Paracoccus yeei*, *Staphylococcus epidermidis*, *Staphylococcus hominis*, *Streptococcus mitis* ([Figure 3.7A, E](#)). While the remaining 9 taxa - *Acinetobacter junii*, *Kocuria indica*, *Kytococcus sedentarius*, *Malassezia restricta*, *Paracoccus sanguinis*, *Roseomonas mucosa*, *Staphylococcus capitis*, *Staphylococcus haemolyticus*, and *Staphylococcus saprophyticus* - were unique to the HK subway system.

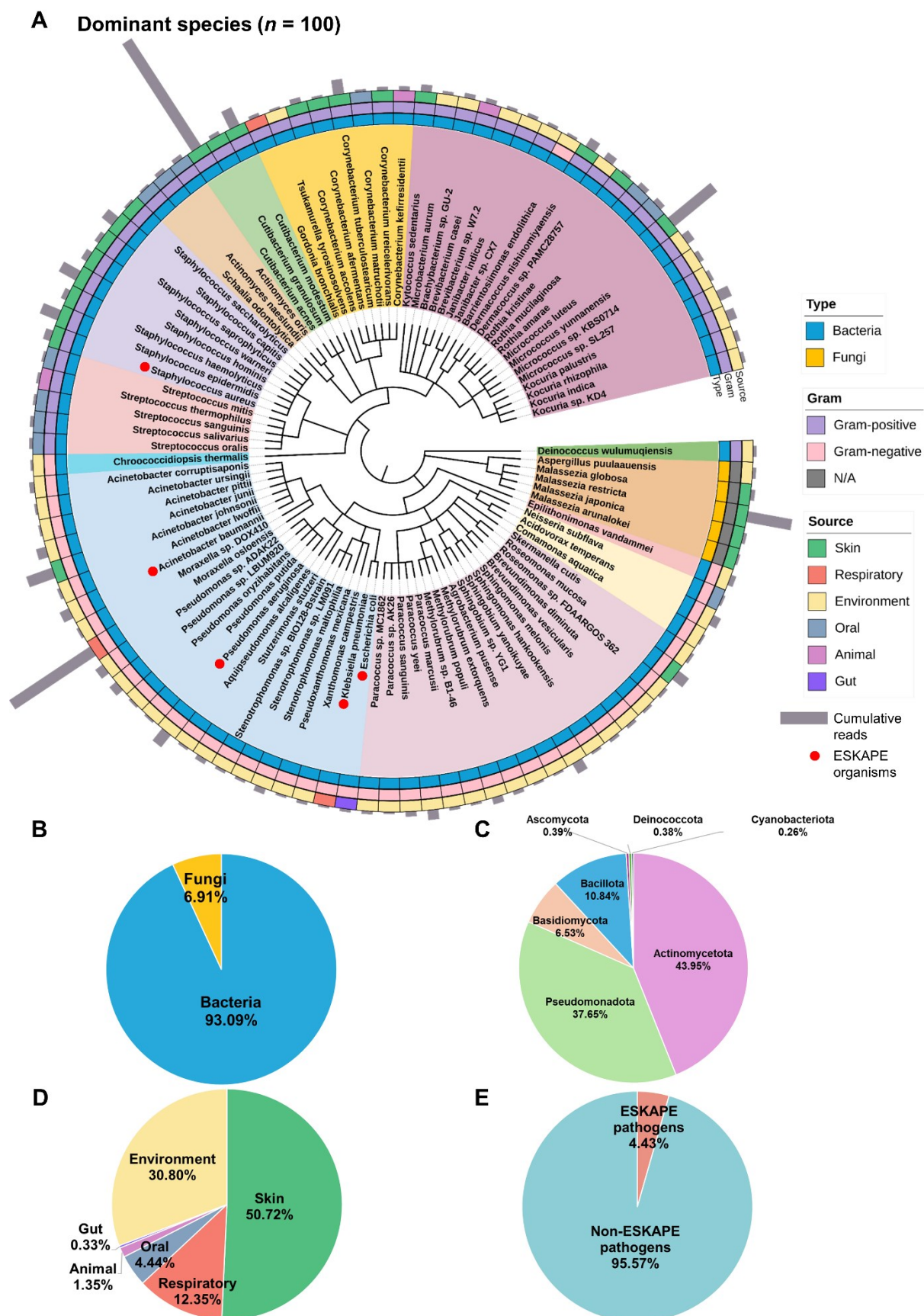


Figure 3.6. Dominant taxa of the Hong Kong subway microbiome.
(Figure description on next page.)

(A) Top 100 dominant species of the HK Subway System. Grey bars represent the cumulative relative abundance. Red dots indicate ESKAPE organisms.

Proportion of reads assigned to (B) bacterial and fungal taxa, (C) phyla, and (D) sources.

(E) Proportion of reads assigned to ESKAPE and non-ESKAPE pathogens

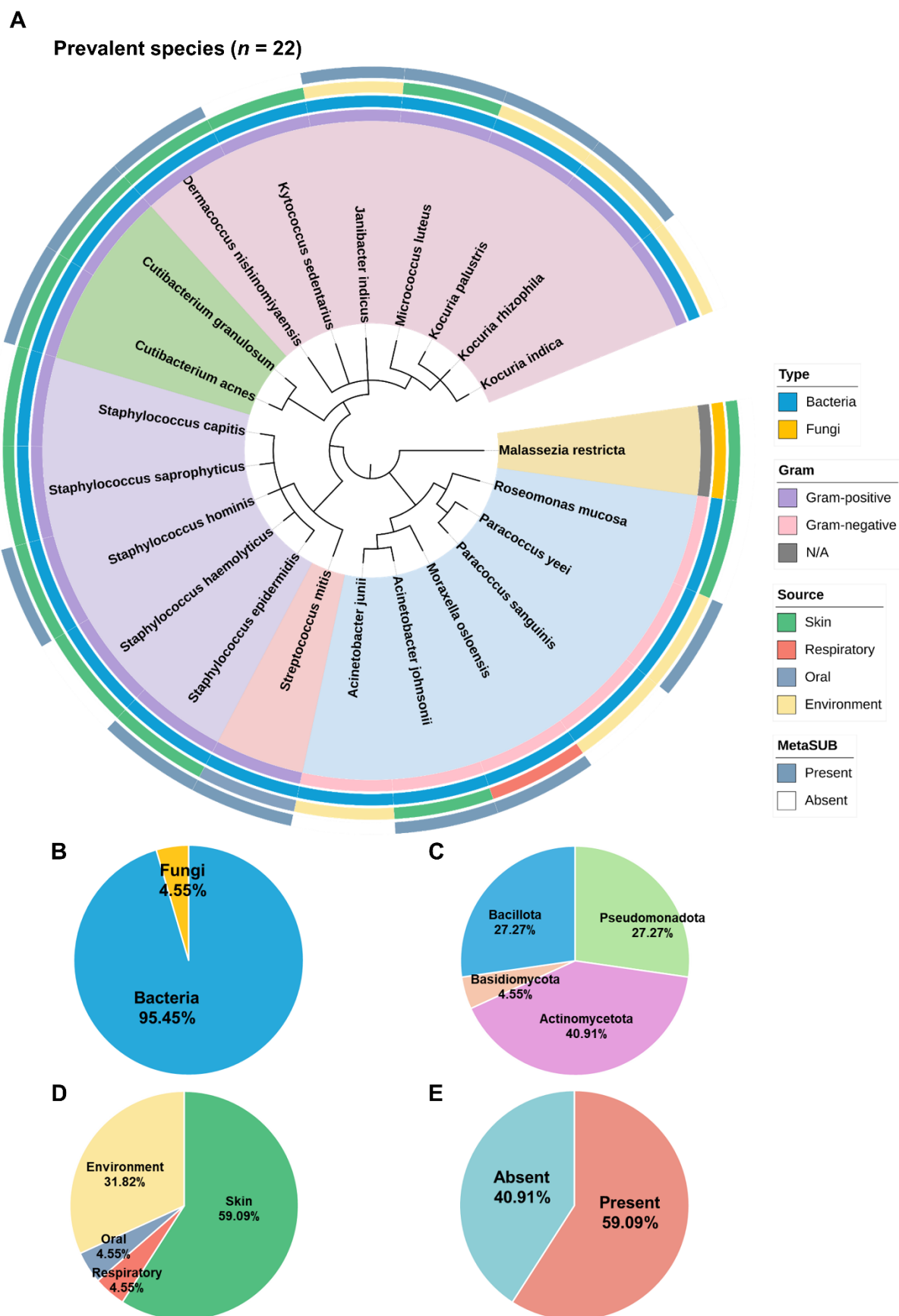


Figure 3.7. Core microbiome of the Hong Kong subway system.
(Figure description on next page.)

(A) Core microbiome of the HK Subway System, composed of species that are detected (i) in all five samplings, (ii) $\geq 97\%$ of stations, and (iii) by both taxonomic classifiers (i.e., Kraken2 and MetaPhlAn4), includes 22 species, with 21 bacterial taxa and one fungal taxon.

Proportion of (B) bacterial and fungal taxa, (C) phyla, (D) sources comprising the core microbiome

(E) Comparison of the core microbiome of HK subway system and the MetaSUB core microbiome (Danko et al., 2021).

3.10. Stations Near Hospitals Harbor Lower Microbial Diversity

Alpha diversity of microbiomes from subway stations was evaluated using three indices: species richness, Shannon diversity index, and inverse Simpson's index ([Figure 3.8A-C](#)). Stations were categorized into four quartiles, Q1-Q4, according to their rankings in each metric. The species richness and Shannon diversity index for air control (airctrl), surface control (surctrl), and no-template control (NTC) samples consistently fell into Q4, indicating a low microbial contamination from laboratory environments.

Concordant findings between the three alpha indices were observed. The average species richness (i.e., number of distinct species) across all subway stations was 807.83 ± 176.46 , ranging from 645 (KWT station) to 1024.2 (LAK station) ([Figure 3.8A](#)). Summer samples had significantly higher species richness than winter samples ($P = 0.000018^{****}$, Kruskal-Wallis test). While stations near hospitals (within 1 km) displayed lower species richness ($P = 0.00974^{**}$). No significant difference was observed between the species richness of interchange stations and non-interchange stations ($P = 0.8018$).

The average Shannon diversity index of all subway stations was 4.72 ± 0.56 . Consistently, LAK had the highest Shannon diversity index of 5.45 ± 0.26 , while KWT had the lowest at 3.80 ± 1.33 ([Figure 3.8B](#)). Similarly, summer samples displayed significantly higher Shannon diversity than winter samples ($P = 0.00000123^{****}$). Stations near hospitals exhibited lower Shannon diversity ($P = 0.00254^{**}$), while no significant difference was found between interchange and non-interchange stations ($P = 0.4252$).

Inverse Simpson's indices demonstrated consistent findings with the above two indices, with an average value of 30.52 ± 16.06 for all stations. Consistently, LAK station harbored the highest inverse Simpson's diversity of 67.86 ± 30.99 , but WAC was found to have the lowest at 16.89 ± 10.48 ([Figure 3.8C](#)). Again, summer samples had significantly higher inverse Simpson's diversity than winter samples ($P = 0.000005^{****}$). Stations near hospitals also

showed lower diversity ($P = 0.00762^{**}$), with no significant difference between interchange and non-interchange stations ($P = 0.2702$).

The results consistently show that proximity to hospital strongly correlates with reduced microbial diversity across all alpha diversity metrics, while seasonal changes favor a higher diversity during summer. The absence of differences between interchange and non-interchange stations suggests that station connectivity does not significantly impact microbial diversity.

Beta diversity was assessed to examine differences in microbiomes across stations, visualized using principal coordinate analysis (PCoA) plot ([Figure 3.9](#)). Microbiomes clustered more by sampling period than station location, suggesting microbiome composition changes over time regardless of station locations. The average cluster area of summer samples (0.0731 ± 0.0140) was larger than that of winter samples (0.0589 ± 0.0014), indicating a greater microbial diversity between stations during summer. These findings highlight the dynamic nature of the HK subway microbiome, with seasonal variations affecting its composition over time.

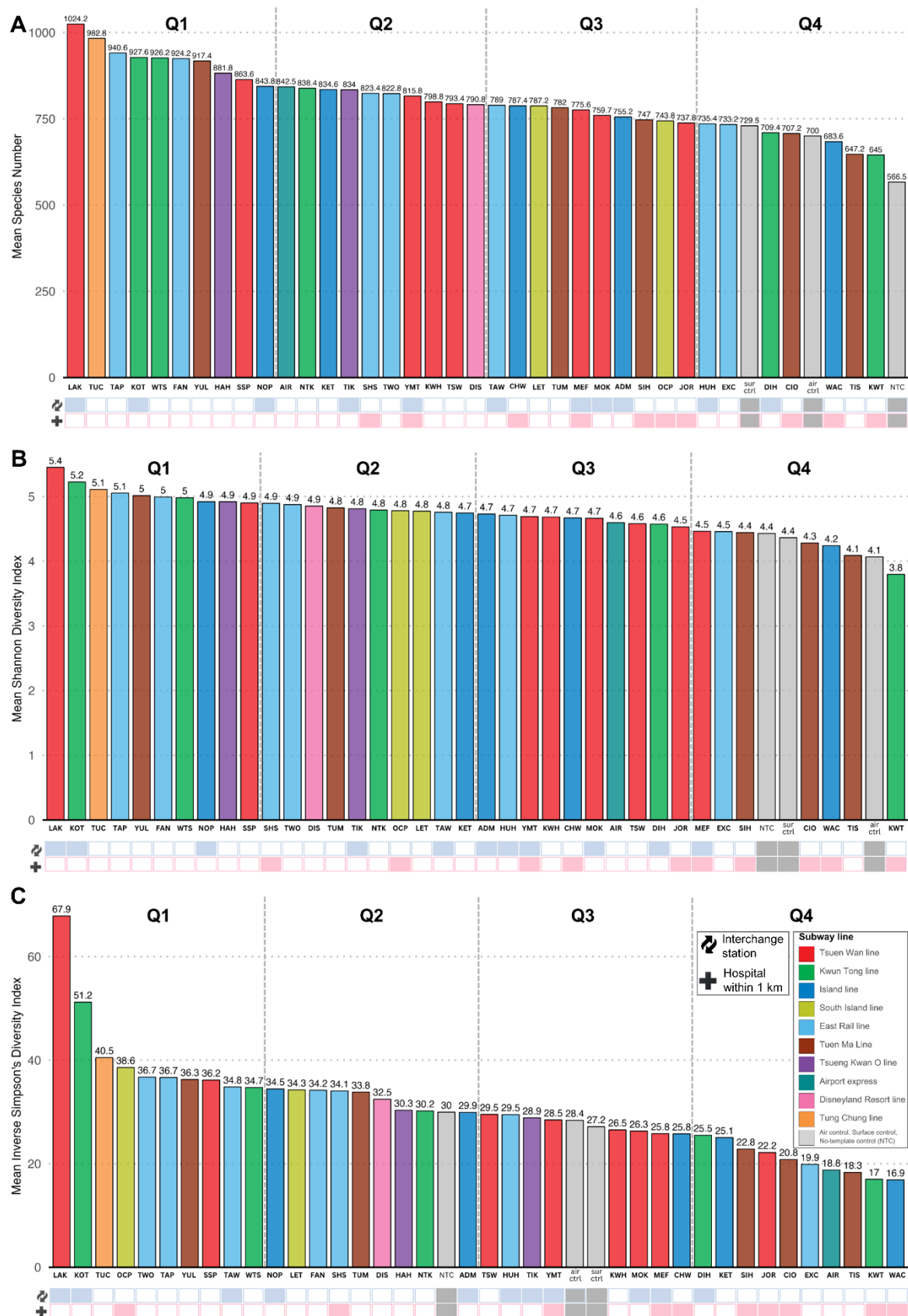


Figure 3.8. Alpha diversity of subway station surface samples.

Average alpha diversity indices, including (A) Species richness, (B) Shannon diversity index, and (C) Inverse Simpson's diversity index, of the 37 subway stations.

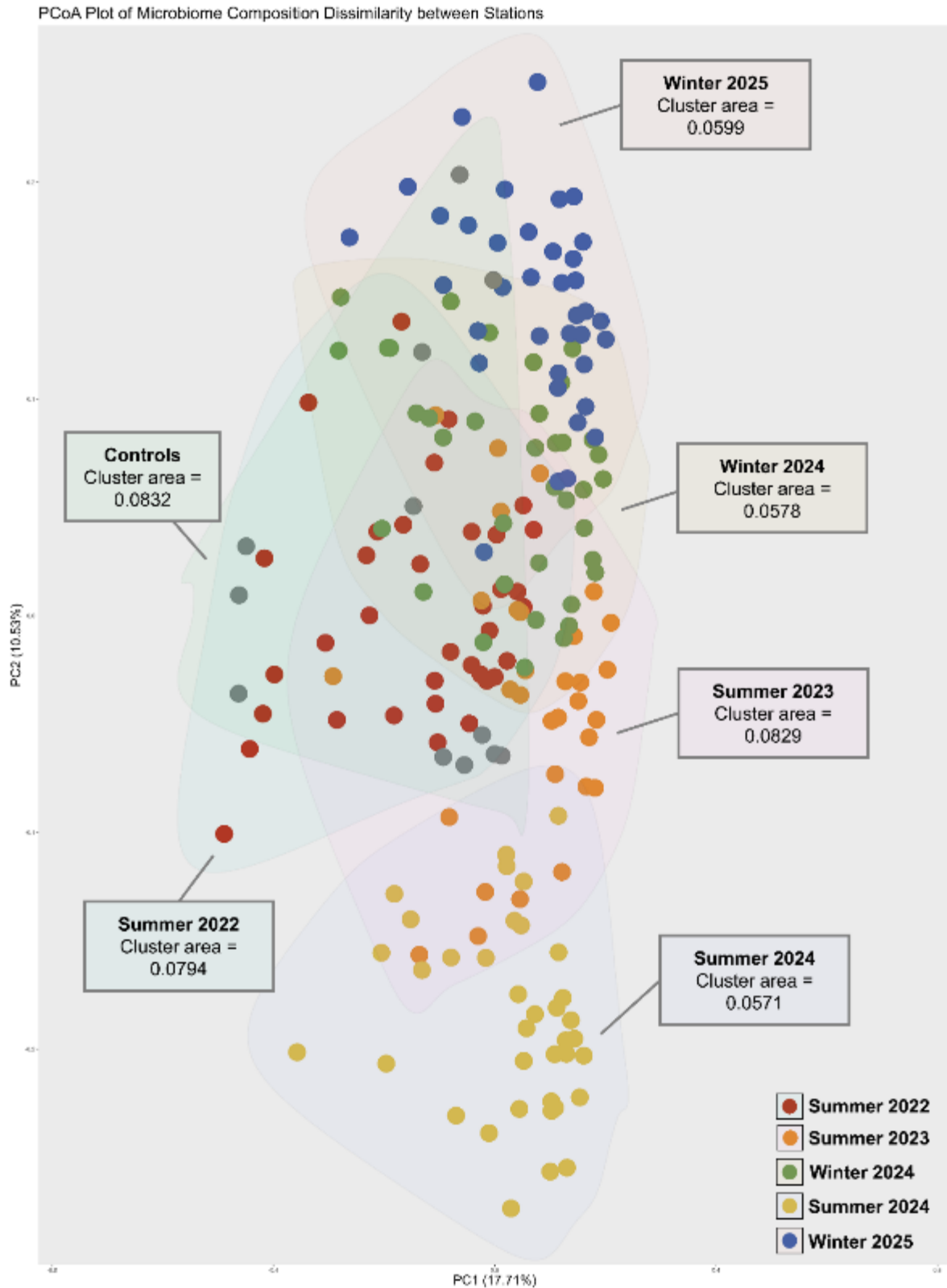


Figure 3.9. Beta diversity of subway station surface samples.

PCoA plot showing microbiome composition dissimilarity between subway surface samples collected during summers of 2022, 2023, and 2024 and winters of 2024 and 2025. Bray-Cutis dissimilarity index was employed.

3.11. Resistant Gene Profiling of the Hong Kong Mass Transit System

The average number of ARGs detected at each subway station was determined. Intriguingly, the four stations with the highest ARG counts were all interchange stations ([Figure 3.10A](#)). However, statistical analysis revealed no significant difference in ARG counts between interchange and non-interchange stations ($P = 0.121$). Furthermore, no significant difference in ARG counts was observed between stations located near hospitals and those that are not near ($P = 0.688$).

ARGs related to multidrug resistance were the most prevalent, followed by genes conferring resistance to macrolides, lincosamides, streptogramins (MLS), and tetracyclines, ranking as the second and third most frequently identified ARG classes, respectively ([Figure 3.10B](#)). These findings align with Danko et al. (2021), who identified MLS as a dominant ARG class in the global MTS resistome (Danko et al., 2021). Resistance genes for chloramphenicol (*floR*), tetracycline (*tetM*, *tetA*, *tetB*), and beta-lactams (*mecA*) were also reported by previous studies, confirming their widespread prevalence in subway systems (Afshinnikoo et al., 2015; Hsu et al., 2016; Kang et al., 2018).

The top 50 ARGs by number of hits, normalized to total reads per sample, are shown in [Figure 3.11A](#). Over half of the normalized hits (53.72%) corresponded to multidrug resistance ARGs, followed by MLS resistance genes (17.71%). The most frequently detected ARG in the HK subway system was *rpoB2*, which confers rifamycin resistance, followed by *MexF* (an efflux pump in *P. aeruginosa*) and *norA* (an efflux pump in *S. aureus*). Notably, *mecA*, ranked 33rd, was the only clinically significant ARG within the top 50.

To identify prevalent ARGs, a core resistome was constructed by ARGs that (i) are present in $\geq 80\%$ stations, (ii) are detected by at least two of the three ARG profiling tools, and (iii) are detected in three out of five sampling periods. The core resistome is predominantly comprised of ARGs conferring multidrug resistance (51.61%), followed by MLS (16.13%) and

aminoglycoside (16.13%) resistance genes. The presence of *mecA* in the core resistome highlights its pervasive and abundant existence in the HK subway system.

The dissimilarity between compositions of subway resistomes was compared by the Bray-Curtis dissimilarity index and then visualized by the PCoA plot ([Figure 3.12](#)). High overlapping between resistomes between all sampling timepoints (except summer 2022) was observed, suggesting that the composition of the subway resistome is stable across time.

To evaluate the clinical significance of the subway resistome, detected ARGs were compared against a database of high-risk environmental ARGs compiled by Xu et al. (2024), which ranks ARGs based on health risks associated with mobile ARGs in ESKAPE pathogens (Xu et al., 2024). Of the 50 Rank I ARG (highest health risk), 22 (44.0%) were identified in subway surface samples, including *aadB*, *bacA*, *blaZ*, *dfrA1*, *dfrA12*, *dfrA14*, *dfrA15*, *ermB*, *floR*, *lnuA*, *lnuB*, *mepA*, *mphA*, *tetM*, *tolC*, *aadE*, *ermC*, *ermT*, *fosB*, *mecA*, *msrA*, and *tetL*. This indicates a moderate presence of clinically relevant ARGs in the HK subway system, underscoring potential public health concerns.

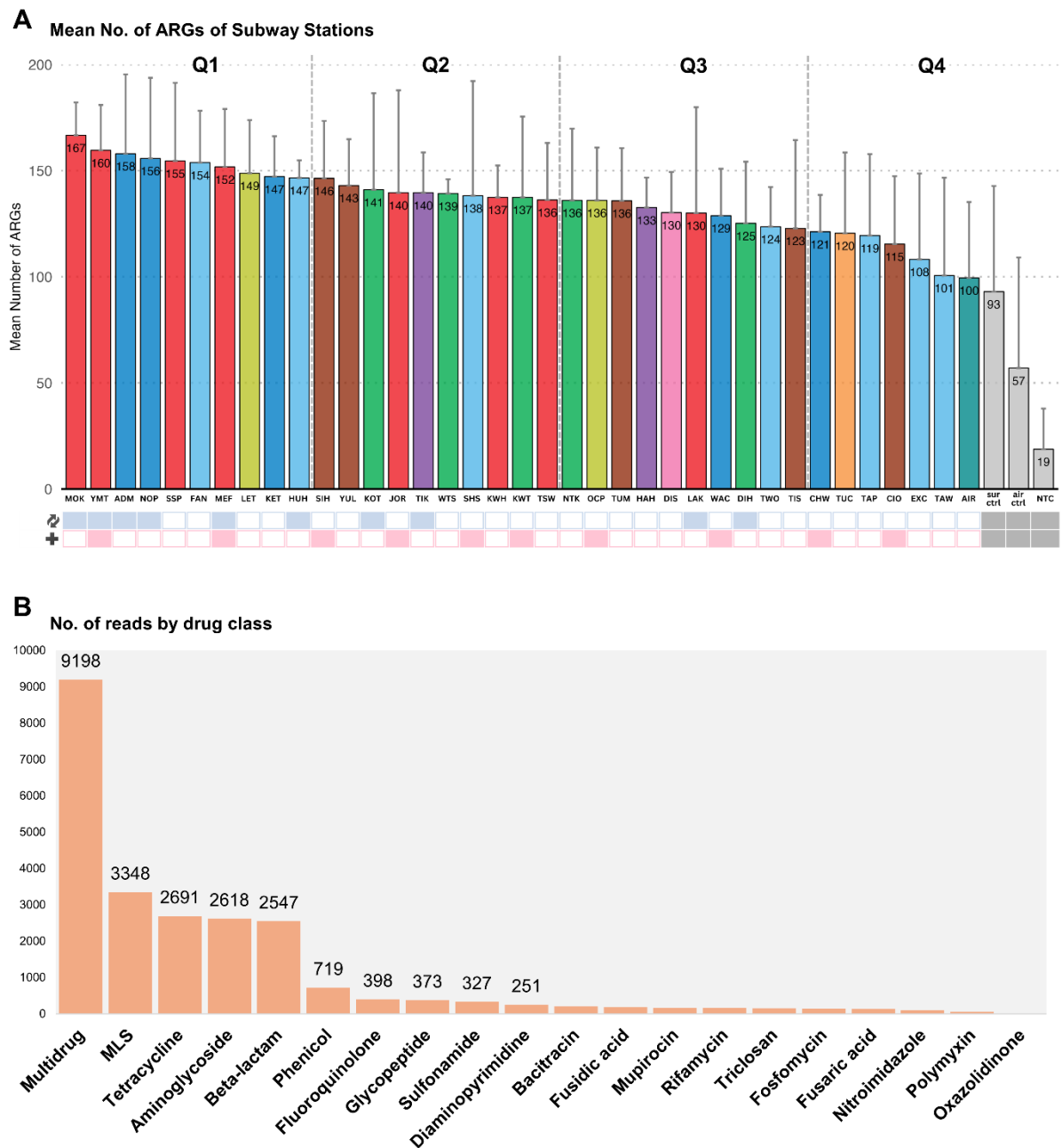


Figure 3.10. AMR burden of the Hong Kong subway system.

(A) Mean number of ARGs detected at each subway stations.

(B) Number of assigned ARG reads of different drug classes

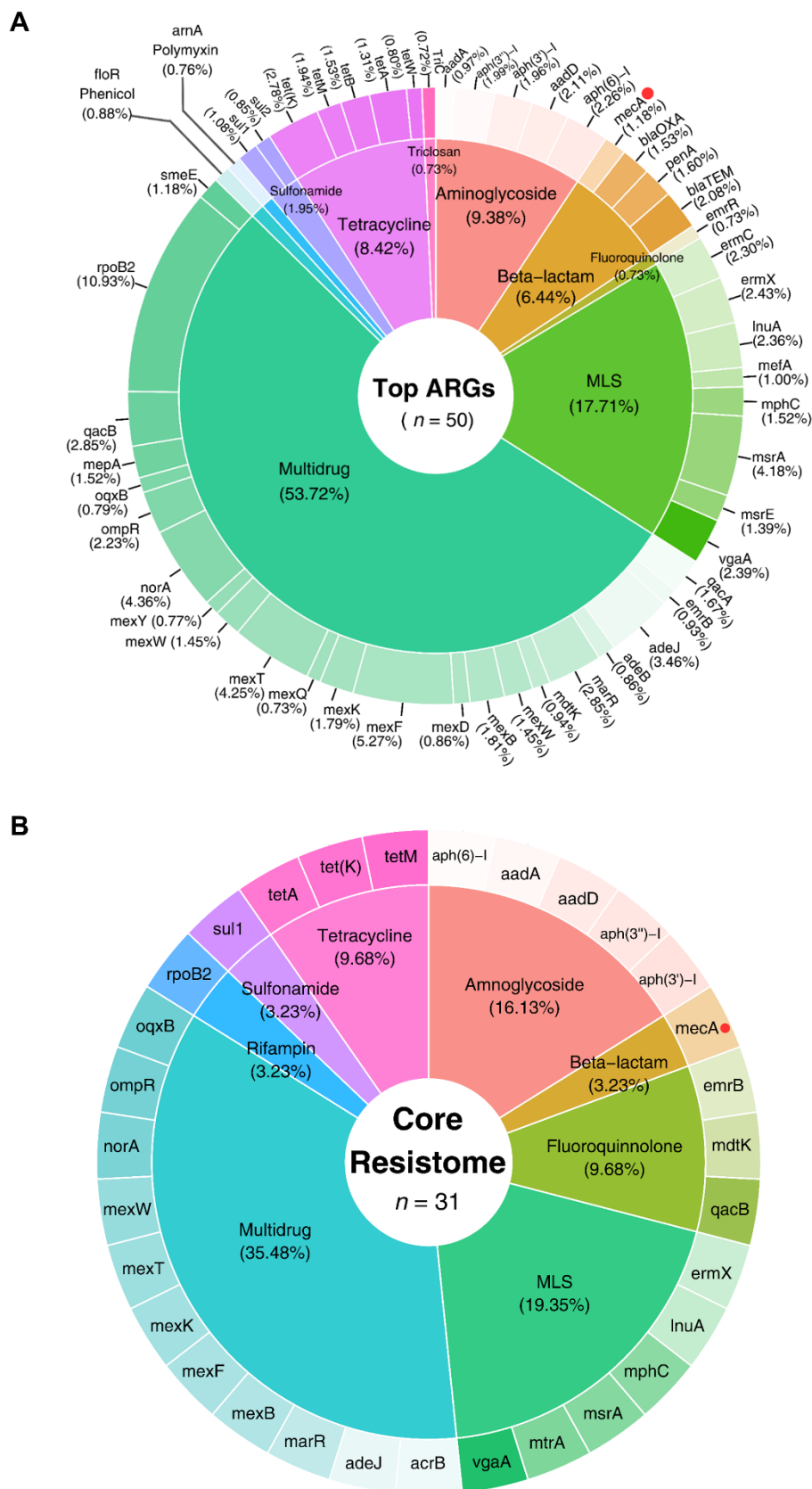


Figure 3.11. Characterization of dominant and prevalent ARGs in the Hong Kong subway system. (Figure description on next page.)

(A) Top 50 dominating ARGs of the HK subway system.

(B) Core resistome, defined by i) detection in $\geq 80\%$ stations, (ii) detected by any two of the three ARG profilers, and (iii) detected in three out of five sampling periods.

Red dot indicated clinically important ARG.

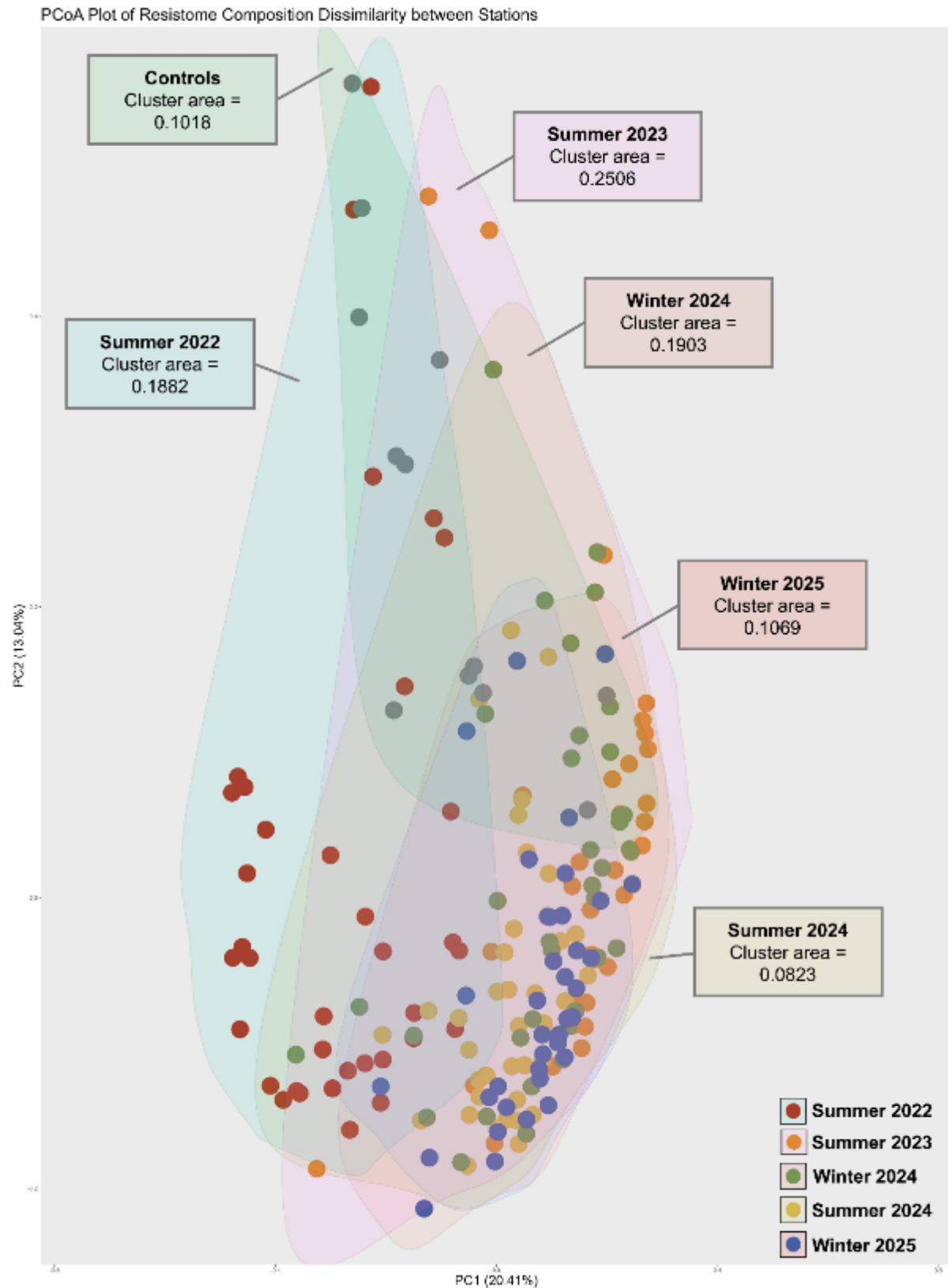


Figure 3.12. Dissimilarity between of ARG profiles of different subway stations.
 PCoA plot showing the dissimilarity between the ARG profiles of different subway stations.
 PCoA, Principal coordinates analysis.

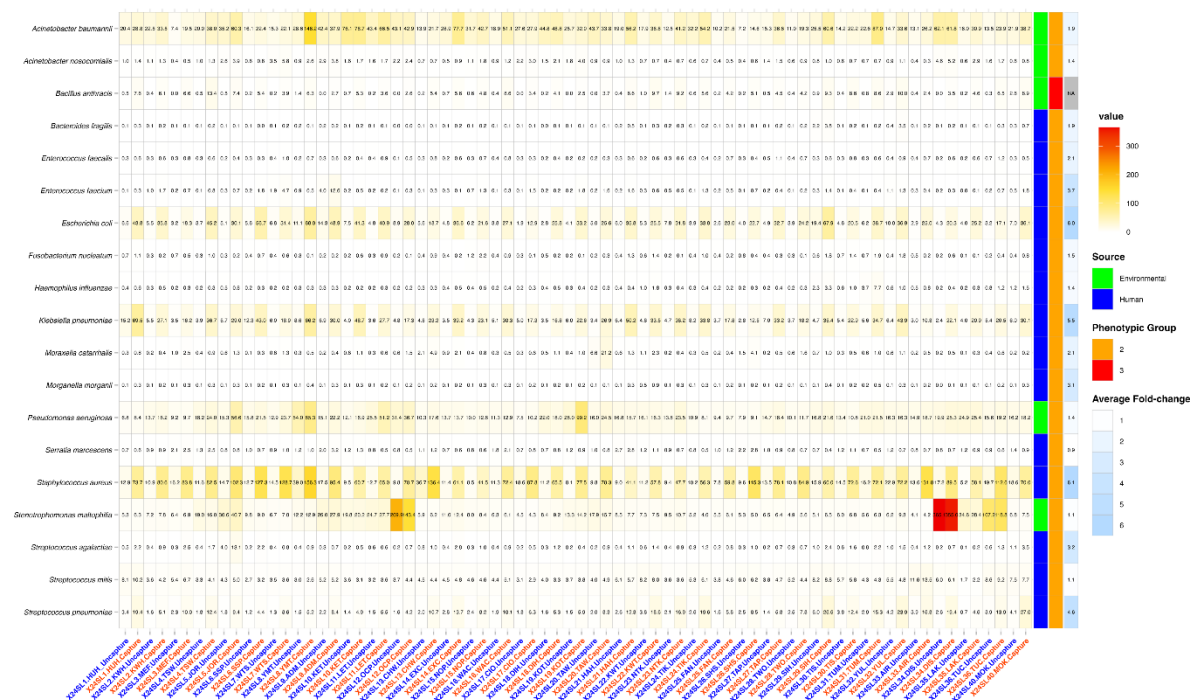
3.12. Target Probe Capture Enrichment Enhanced Detection of Low-Abundance Pathogens and ARGs

TC was additionally performed on metagenomic libraries from summer 2024 ($n = 37$) and winter 2025 ($n = 37$). Its performance in enriching clinically important taxa and ARGs was evaluated by comparing against shotgun metagenomic (uncaptured) libraries.

The normalized read counts of clinically important taxa and ARGs to total reads were compared between TC and uncaptured libraries ([Figure 3.13](#)). TC libraries exhibited a significant enrichment, with an average increase of 5.59-fold in read counts assigned to clinically important taxa. Furthermore, several clinically significant ARGs, including *blaCTX-M*, *blaGES*, *blaIMP*, *blaKPC*, *mecA*, *blaNDM*, *blaSHV*, *vanA*, *vanC*, *vanR*, *vanY*, *blaVIM*, which were non-detectable in uncaptured libraries, were successfully detected in TC libraries. The reads assigned to these clinically important ARGs showed a remarkable average increase of 98.5-fold.

In conclusion, TC significantly enhances the detection of clinically important taxa and ARGs in low-biomass environmental samples by substantially increasing their read counts compared with uncaptured libraries. This result demonstrated the ability of TC in identifying low-abundance pathogens and resistance genes, offering improved sensitivity over the conventional shotgun metagenomic workflow.

A



B

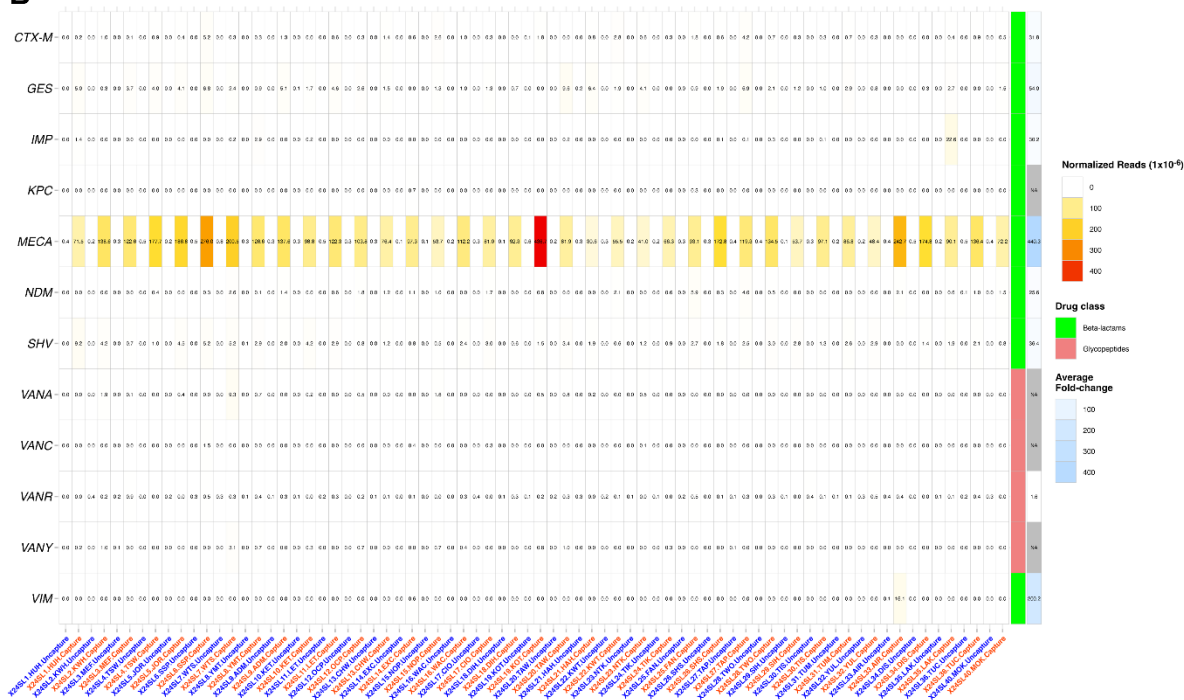


Figure 3.13. Target probe capture enrichment enhances the detection of clinical important taxa and ARGs.
Heatmap showing the normalized number of reads to total reads assigned to clinical important (A) taxa and (B) ARGs.

3.13. Discussions

Mass transportation is vital to urban life, facilitating millions of passengers moving from any corner of a metropolitan city. Owing to its high passenger density and foot traffic, the subway microbiome is a unique blend of microbial assemblages of both the passengers and the environment. Despite previous efforts in characterizing the subway microbiome and resistome, the potential health risks posed by pathogens and ARGs remain unexplored. In this study, a comprehensive 2.5-year city-wide surveillance of MDROs and AMR determinants within the HK subway system was conducted. High-contact surfaces were sampled in 37 stations across different districts of HK, examining the geographical and seasonal differences in MDRO prevalence and AMR landscape.

Previous subway microbiome studies, as reviewed in [section 3.2](#), primarily employed either culture-dependent or culture-independent methods, limiting the depth of pathogen and AMR characterization. In contrast, this study integrated both approaches, combining microbial culture for MDROs and metagenomics on direct surface samples. This dual methodology provided a comprehensive metagenomic overview alongside detailed strain-level insights into MDROs circulating within the HK subway system, offering a more holistic understanding of its microbial ecology.

Despite serving millions of passengers daily, the HK subway environment exhibits a low microbial burden. DNA yields from surface samples were consistently low (<1 ng/ μ L), aligning with Kang et al. (2018) while contrasting with the high yields (40-200 ng/ μ L) reported in Boston's subway system (Hsu et al., 2016; Kang et al., 2018). This suggests that the HK subway system is a relatively hygienic environment, likely due to the robust antimicrobial measures, including vaporized hydrogen peroxide robots, nano-silver titanium dioxide coatings (NSTDC) on high-contact surfaces, UV-illuminated escalator handrails, and touchless buttons (MTR Corporation, 2021). Additionally, sustained passenger behaviors post-COVID-19, such

as increased use of touchless interfaces, mask-wearing, and social distancing, likely further contributed to the reduced microbial load.

Despite these measures, viable MDROs were recovered from 4.11% of subway surface samples collected in this study, which is a relatively low prevalence compared to other BE, such as long-term care facilities (Lee et al., 2017; Rodríguez-Villodres et al., 2021), nursing homes (McKinnell et al., 2020), public beaches (Thapaliya, Hellwig, et al., 2017), children playgrounds (Thapaliya et al., 2019), and school campuses (Ababneh, Jaradat, et al., 2022; Thapaliya, Taha, et al., 2017). Using WGS, pathogenic strains of MRSA, MDRA, and CRPA were identified. Notably, MRSA ST8 SCC*mecIV*I was reported for the first time in the HK subway system. This strain was previously documented in Japan's subway trains and clinical cases in both Japan and HK (Iwao, Takano, et al., 2012; Iwao, Yabe, et al., 2012; Ho et al., 2007), suggesting a potential spillover of resistant strains from clinical to community settings and warranting further phylogenetic and epidemiological investigations to trace dissemination pathways.

Shotgun metagenomics revealed an enrichment of human skin-associated commensals in the HK subway environments, consistent with previous studies (Kang et al., 2018; Leung et al., 2014). Five ESKAPE pathogens were among the top 100 species, indicating their high abundance. Core microbiome analysis identified 22 prevalent species present in $\geq 97\%$ stations across all sampling periods, none of which were clinically important taxa. Alpha diversity analysis showed a higher microbial diversity in summer compared to winter, potentially driven by elevated temperature and relative humidity conducive to microbial growth (Kritzberg & Bååth, 2022). Surprisingly, stations located near hospitals exhibited significantly lower microbial diversity. This is possibly due to enhanced hygiene practices or antimicrobial interventions implemented at these stations, which may limit microbial survival and dispersal.

These findings highlight the influence of seasonal and geographical factors on station microbiome composition, with implications for public health and infection control.

Resistome profiling identified ARGs conferring resistance to multidrug, MLS, and aminoglycosides as dominating classes, consistent with local and global subway studies (Kang et al., 2018; Danko et al., 2021). The *mecA* gene, a hallmark of beta-lactam resistance in MRSA, was a prevalent and dominant ARG in the HK subway system. The concurrent detection of *S. aureus* and *mecA* further supports the predominance of MRSA in the subway environments. Other ARGs, including those against beta-lactams, chloramphenicol, lincosamide, and tetracycline, were also detected, aligning with prior studies (Afshinnkoo et al., 2015; Hsu et al., 2016; Kang et al., 2018; Zhang et al., 2024).

Enrichment of genomic targets by TC prior to sequencing significantly enhanced the detection of low-abundance clinically important taxa and ARGs in low-biomass environmental samples. Despite lower read depth compared to shotgun metagenomics ([Figure 3.5A](#)), TC increased reads assigned to clinically relevant species (e.g., *S. aureus*, *E. coli*, *K. pneumoniae*) and ARGs (e.g., *mecA*, *blaVIM*, *blaGES*, *blaCTX-M*) by several fold. Furthermore, TC also reduced the proportions of human and unclassified reads, thus optimizing flowcell usage and computational resources. Previous studies reported similar enrichments, with TC enhancing ARG read proportions by over three orders of magnitude, thereby doubling the detectable resistome richness and revealing numerous low-abundance ARGs (Lanza et al., 2018; Noyes et al., 2017). Nevertheless, TC libraries require a lower read depth at around 10 million reads while detecting more target pathogens and ARGs compared to the conventional metagenomic workflow, which normally requires ≥ 50 million reads to detect the majority of microbial taxa and ARGs in the microbiome (Zaheer et al., 2018).

Although this study detected pathogens and ARGs from the subway environments, there are some limitations. First, the lack of correlation with clinical and epidemiological data

weakens the public health implications of detecting MDRO in subway surfaces. Secondly, some hospitals are not located close to any subway stations, and they rely on other transportation modes (e.g., buses). Future surveillance for MDROs and ARGs of these transportations should therefore be warranted. Third, neither shotgun metagenomics nor TC indicates the viability of detected organisms, where the signals may come from dead cells, leading to overestimation of pathogen load and thus infection risks in these environments. Fourth, the current probe panel employed by the TC workflow cannot distinguish pathogenic from non-pathogenic strains among detected taxa, as it lacks strain-level resolution for virulence genes or pathogenic marker genes, thereby limiting clinical interpretability. Finally, neither shotgun metagenomics nor TC detected clinically significant fungi or viruses, suggesting their minimal presence. However, more sensitive methods, such as qPCR, should be employed to confirm their absence, as low-abundance pathogens may fall below the detection limit by metagenomic sequencing.

To conclude, the subway microbiome study provides a comprehensive analysis of the HK subway microbiome and resistome, revealing a low MDRO prevalence but persistent reservoirs of potential pathogens and ARGs. By integrating culture-based and sequencing techniques, this study offers a robust framework for characterizing subway microbiomes. The findings underscore the influence of environmental factors, such as seasonal variations and proximity to healthcare premises, on BE microbiomes. From a public health perspective, this study establishes a foundation for future metagenomic investigations of MTS and other BE, emphasizing their roles in monitoring and mitigating AMR dissemination in the urban community. These insights are critical for safeguarding community health in high-traffic public spaces and ultimately contributing to improved pandemic preparedness.

Chapter 4.

Surveillance of MDRO and AMR in

Public Washrooms

4.1. Introduction

Public washrooms, characterized by the high number of users and persistently humid environments, are underrecognized reservoirs for drug-resistant microorganisms (Mkrtchyan et al., 2013; Suen et al., 2019). While handwashing remains a cornerstone of personal hygiene, post-handwashing areas, where individuals may inadvertently re-contaminate cleansed hands, are much-neglected as potential reservoirs of MDROs and ARGs. Understanding the microbiological and AMR landscapes of post-handwashing areas is therefore crucial not only to evaluate the efficacy of current hygiene practices but also to develop effective infection control strategies.

Public washrooms harbor a unique microbiome shaped by high humidity, poor ventilation, and constant microbial shedding from users, making them potential hubs for pathogen transmission (Flores et al., 2011). Compared to private washrooms, public washrooms pose elevated infection risks due to greater microbial diversity and high foot traffic. Post-handwashing surfaces, including faucets, paper dispensers, hand dryers, and exit door handles, serve as critical junctions for microbial exchange (Suen et al., 2019; Vardoulakis et al., 2022). Studies demonstrate that damp hands transfer microorganisms to surfaces and become re-contaminated more readily compared to dry hands (Huang et al., 2012; Lopez et al., 2013), with high-touch surfaces acting as hubs for pathogen transmission (Harrison et al., 2003; Abney et al., 2021; Vardoulakis et al., 2022). Notably, drug-resistant and virulent strains, such as MRSA and *Legionella pneumophila*, are frequently isolated from washroom surfaces, potentially causing infections to washroom users (Suen et al., 2019; Mkrtchyan et al., 2013; Couturier et al., 2020; Giannini et al., 2009; Ibrahim et al., 2024; Valzano et al., 2024). Outbreaks of enteric pathogens such as norovirus have been traced to contaminated washrooms

in international flights (Holmes & Simmons, 2009) and cruise ships (Carling et al., 2009; Ho et al., 1989b), further indicating their role in pathogen dissemination.

Pathogen transmission in washrooms primarily occurs through fomite contact and aerosolization (Vardoulakis et al., 2022; Abney et al., 2021; Johnson et al., 2013). Fomite contamination arises from direct contact with high-touch surfaces, which are often dominated by skin-associated microorganisms (Boone & Gerba, 2007; P. Wang et al., 2021). Fecal pathogens, such as *E. coli*, *S. aureus*, and *Shigella spp.*, can persist on these surfaces for days to months (Abad et al., 1994; Kramer et al., 2006; Kramer & Assadian, 2014). The risk is further amplified, particularly by low-infection-dose pathogens, such as norovirus, rotavirus, and enterohemorrhagic *E. coli* (EHEC) (Gerhardts et al., 2012; Abney et al., 2024). Aerosolization, another significant transmission route, occurs during activities such as toilet flushing, handwashing, and the use of jet air dryers (Johnson et al., 2013; Verani et al., 2014), thereby posing a respiratory infection risk (Huang et al., 2012; Suen et al., 2019; Verani et al., 2014). For instance, fecal-derived aerosols in hospital washrooms have been shown to contain the most SARS-CoV-2 (Ding et al., 2021). Similarly, toilet flushing can disperse *Clostridioides difficile* (basonym: *Clostridium*) into the air, leading to surface contamination and contributing to fecal-oral transmission (Best et al., 2010, 2012; Wilson et al., 2020). Studies have also demonstrated that jet air dryers disperse more enteric bacteria compared to paper towels (Best & Redway, 2015; Wilcox et al., 2017). Inadequate ventilation in public washrooms further exacerbates these risks by allowing aerosols to persist in stagnant air, increasing the likelihood of cross-infection (Lee & Tham, 2021). Collectively, these findings highlight the combined risks of fomite- and aerosol-mediated transmission in washrooms, particularly in areas utilizing hand-drying facilities.

Conventional pathogen surveillance, relying on culture-based methods and quantitative PCR (qPCR), provides valuable insights into pathogen viability and target-specific detection,

respectively. However, their utility in environmental surveillance is constrained by a narrow analytical scope, such as a predefined set of limited targets, and dependence on a *priori* knowledge like primer sequences (Ko et al., 2022). Shotgun metagenomics, while offering a hypothesis-free approach to analyze all genetic materials in a sample and thus the simultaneous detection of bacterial, viral and fungal species, however, struggles to detect low-abundance pathogens and ARGs in environmental samples due to the overwhelming background DNA from various sources (e.g., human, insects, plants) (Zaheer et al., 2018; Gweon et al., 2019; Graham et al., 2019). Target probe capture prior to metagenomic sequencing (TCM) addresses these limitations by selectively enriching a broad range of microbial and AMR targets from complex and low-biomass samples. Leveraging the sequence-specific probe-DNA interaction, TCM isolates target DNA, thus minimizing interference from non-target sequences. More specifically, enrichments with probes increase the proportion of target genes by several orders of magnitude, thus enhancing the signal of low-abundance reads and improving their detection (Slizovski et al., 2022). This technique enables cost-effective, high-resolution profiling, even in low-biomass environments (e.g., air, surfaces) (Noyes et al., 2017; Lanza et al., 2018; Guiton et al., 2019). Although the utility of TCM in pathogen and AMR surveillance was demonstrated in complex matrices like foods (Flint et al., 2023; Shay et al., 2023), wastewater (Martínez-Puchol et al., 2021; Tisza et al., 2023), and wildlife (Kuchinski et al., 2022), its applications to urban BE, such as hospitals, workplaces, and public transport systems, remain unexplored, let alone public washrooms.

The aim of the washroom microbiome study is to characterize the prevalence of different MDROs and AMR determinants in public washrooms across diverse sectors in HK, including wet markets, school campuses, transportation hubs, healthcare facilities, recreational facilities, and catering outlets, with a particular focus on post-handwashing sites. By leveraging advanced metagenomic techniques, including TCM, this study provides a comprehensive understanding

of the washroom microbiome and resistome, guiding targeted infection control strategies to mitigate the spread of drug-resistant pathogens in high-traffic public spaces.

4.2. Literature Review

A plethora of studies have shown that public washrooms serve as major reservoirs for microbial contamination, including viruses and drug-resistant bacteria, posing substantial public health risks. Existing research on washroom microbiomes primarily relied on culture-based methods for bacterial pathogens and RT-PCR for viral pathogens, with only one study to date employing 16S rRNA sequencing to explore the bacterial diversity in these environments. [Table 4.1](#) summarizes key studies investigating bacterial and viral pathogens in the washroom environments.

4.2.1. Bacterial pathogen in washrooms

Bacterial pathogens, particularly drug-resistant strains, are frequently detected in washroom settings, with *Staphylococcus* being the most common genus. Gianni et al. (2009) identified MRSA on 3.3% toilet seats in hospital washrooms (Giannini et al., 2009). Mkrtchyan et al. (2013) investigated 18 public washrooms, sampling hand dryers, toilet seats, inner door surfaces, taps, soap dispensers, and found 19 *Staphylococcus* isolates, of which 37.8% displayed drug resistance (Mkrtchyan et al., 2013). Suen et al. (2019) studied 55 public washrooms across hospitals, hotels, restaurants, shopping centers, and other public spaces by sampling paper towels, paper towel dispensers, air dryer outlets, and exit door handles (Suen et al., 2019). Their culture-based methods showed that over 97% of *Staphylococcus* isolates were resistant to at least one first-line antibiotics, namely penicillin, cefoxitin, and erythromycin, and 22.6% exhibited co-resistance to three or more agents. Similarly, Ibrahim et al. (2024) sampled 32 washrooms in shopping malls using non-selective media followed by

AST, detecting multidrug-resistant *Staphylococcus* spp., *A. baumannii*, and *P. aeruginosa* (Ibrahim et al., 2024).

Enteric bacteria are also prevalent in washroom environments. Mohamed et al. (2015) sampled 56 public washrooms in fast-food outlets, gas stations, parks, supermarkets, and healthcare facilities, detecting *E. coli* in 2.2% of samples. Notably, extraintestinal pathogenic *E. coli* (ExPEC), which a major cause of urinary and bloodstream infections, was detected in 0.9% of samples (Mohamed et al., 2015).

To date, only one study employed culture-independent methods to characterize washroom microbiomes. Flores et al. (2011) sampled door handles, stall handles, faucet handles, soap dispensers, toilet seats, flush handles, and floors in 12 campus washrooms (Flores et al., 2011). Pyrosequencing of the V1-V2 region of 16S rRNA genes revealed that high-contact surfaces were dominated by skin-associated *Propionibacteriaceae*, while female washrooms showed a prevalence of vagina-associated *Lactobacillaceae*, highlighting the human origins of microbial communities in the washroom environments.

4.2.2. Viral pathogens in washrooms

Viral pathogens are frequently dispersed in washrooms, primarily through surface contamination and aerosolization. Norovirus, a leading cause of acute gastroenteritis, is commonly detected. Somura et al. (2017) detected norovirus in 43.6% toilet bowls and 14.5% toilet seats, with lower prevalence in doorknobs and floors (Somura et al., 2017). Similarly, Park et al. (2020) reported norovirus on 53.6% of toilet seats in cruise ship washrooms, highlighting public washrooms as potential hotspots for norovirus transmission (Park et al., 2020).

In addition to enteric viruses, respiratory viruses are also widespread in washroom surfaces. Verani et al. (2014) sampled toilet seats, covers, flushing handles, and internal door

handles in hospital and office washrooms, detecting human adenovirus and Torque teno virus in 70% and 31% of washroom surface samples, respectively (Verani et al., 2014). SARS-CoV-2 has also been frequently detected. Ding et al. (2021) sampled hospital washrooms following a COVID-19 outbreak, finding that washrooms were detected with the highest titre of SARS-CoV-2, highlighting washrooms as high-risk areas for SARS-CoV-2 transmission (Ding et al., 2021). Amoah et al. (2021) reported SARS-CoV-2 on 54-69% of contact surfaces in shared washrooms, emphasizing the role of fomite-mediated transmission (Amoah et al., 2021). These findings collectively highlight the critical role of washroom surfaces and aerosols in viral pathogen dissemination, necessitating routine surveillance and targeted infection control measures.

Table 4.1 List of microbiological studies of washroom environments

Country	Washroom specifics	Detection method(s)	Pathogen(s) detected	Reference
South Africa	Setting: Shared washrooms (n = 8) Site: Cistern handle, toilet seat, floor surface in front of the toilet, internal pull latch of cubicle door and tap in handwash basin	qPCR and droplet digital PCR	54–69% contact surfaces were contaminated by SARS-CoV-2	Amoah et al., 2021
China	Setting: Hospital ward washroom (n = 1) Site: Toilet seat, toilet seat cover, washbasin faucet lever, washroom ceiling-exhaust louvre	RT-PCR	SARS-CoV-2 detected in washroom door handle, toilet seat, washbasin faucet lever, washroom ceiling exhaust louver	Ding et al., 2021
Italy	Setting: Washrooms in hospital ward (n = 5) and office (n = 2) Site: Toilet seat and its cover, the flushing handle/button, the internal door handle	RT-PCR	Human adenovirus and Torque teno virus detected in 70% and 31% of washroom surface samples, respectively	Verani et al., 2014
Japan	Setting: Food production site (n = 1): Site: Toilet bowl, toilet seat, doorknob, floor	RT-PCR	Norovirus detected mostly in toilet bowl (43.6%), toilet seat (14.5%), and some doorknob and the floor.	Somura et al., 2017
USA	Setting: Cruise ship (n = 5): Site: Toilet seats, toilet door handles	RT-PCR	Norovirus detected on 56.3% toilet seats	Park et al., 2020
Qatar	Setting: Shopping mall (n = 32) Site: Toilet seat, water sprayer, tap, inner door handle, outdoor handle	Non-selective media (nutrient agar, macConkey agar) followed by antibiotic susceptibility testing	- Dominated by <i>Klebsiella pneumoniae</i> and <i>Pseudomonas aeruginosa</i>. - Presence of multidrug-resistant <i>Staphylococcus haemolyticus</i>, <i>Staphylococcus kloosii</i>, <i>Acinetobacter baumannii</i>, and <i>P. aeruginosa</i>.	Ibrahim et al., 2024
USA	Setting: Hospital public washrooms (n = 6) Site: Toilet seats	Selective media	MRSA was recovered from 3.3% of hospital toilet seats	Giannini et al., 2009
Hong Kong	Setting: Public washrooms in hospitals, hotels, restaurants, shopping centres, sports centres, tourist spots, public housing estates, food markets (n = 55) Site: Outlet of paper towel dispensers, air outlet of air dryers, exit door handles, paper towels	Non-selective media (Tryptone Soy Agar) followed by antibiotic susceptibility testing	- Over 97% of the pathogenic <i>Staphylococcus spp.</i> tested were resistant to at least one first-line antimicrobial therapeutic agent, including penicillin, cefoxitin, erythromycin, co-trimoxazole, clindamycin and gentamicin. 22.6% demonstrated co-resistance to at least three antimicrobial agents, with co-resistance to penicillin, erythromycin and clindamycin being the most common.	Suen et al., 2019
UK	Setting: Surfaces in public washrooms (n = 18) Site: Hand dryer systems, toilet seats, inner door surfaces, taps, soap dispensers and urinal floors.	Selective media followed by antibiotic susceptibility testing	Identified 19 isolates different <i>Staphylococcal spp.</i> and 37.8% of the isolates were drug resistant	Mkrtchyan et al., 2013
USA	Setting: Public washrooms in fast-food outlets, gas stations, parks, supermarkets, healthcare (n = 56) Site: toilet associated (e.g., floor surfaces within 2 ft of the toilet, toilet seat, toilet surfaces, toilet water, and in-stall sanitary napkin receptacle), non-toilet associated (e.g., stall latch, sink drain, cold water tap, and handle of the diaper changing table)	Selective broth and agar followed by antibiotic susceptibility testing	<i>E. coli</i> detected in 25 samples (2.2%), with 10 of them (0.9%) contained extraintestinal pathogenic <i>E. coli</i> .	Mohamed et al., 2015
USA	Setting: Campus washrooms (n = 12) Site: Door handles into and out of the restroom, handles into and out of a restroom stall, faucet handles, soap dispenser, toilet seat, toilet flush handle, floor around the toilet and floor around the sink	Pyrosequencing of V1-2 region of 16S rRNA gene	- Skin-associated bacteria, especially the <i>Propionibacteriaceae</i>, dominated surfaces routinely touched with our hands. - Vagina-associated <i>Lactobacillaceae</i> were widely distributed in female restrooms.	Flores et al., 2011

4.2.3. Research gap

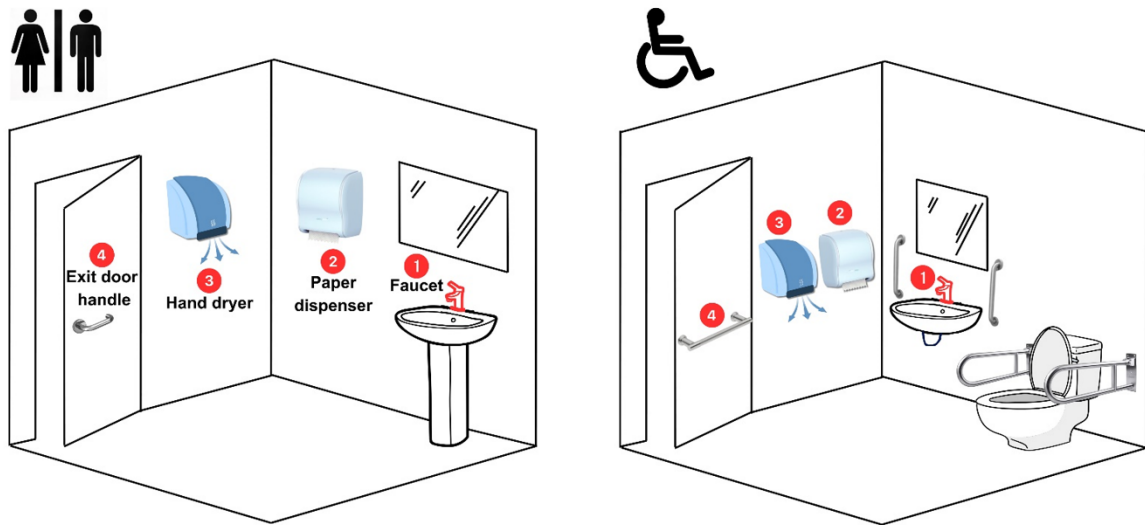
The current understanding of public washroom microbiomes is limited by a lack of comprehensive, culture-independent studies that reflect the full spectrum of microbial communities and their AMR profiles. Existing research relies on cultivation or marker-based methods (e.g. RT-PCR) to detect specific pathogens like *S. aureus*, *P. aeruginosa*, norovirus, and SARS-CoV-2. These approaches preclude the detection of non-culturable microbes or emerging pathogens and the full spectrum of drug resistance in washroom surfaces, thus hindering the scope of surveillance. The only culture-independent study of the washroom, which adopted 16S rRNA pyrosequencing, was restricted to bacteria and did not address viral or fungal pathogens or the presence of resistance genes (Flores et al., 2011).

4.3. The Design of the Public Washroom Microbiome Study

Twenty-eight public washrooms from hospitals, catering outlets, hotels, sports facilities, campus, recreational facilities, and subway stations in Hong Kong were sampled between September to October 2024. A total of 232 surface samples of high-contact post-handwashing sites were collected. For each washroom, four post-handwashing sites, including faucets ($n = 72$), paper dispensers ($n = 63$), hand dryers ($n = 55$), and exit door handles ($n = 42$) were sampled ([Figure 4.1A](#); [Supplementary Table ST7](#)). The average daily temperature of sampling days was $28.56 \pm 2.03^{\circ}\text{C}$ and $79.71 \pm 7.09\%$ humidity ([Supplementary Table ST8](#)).

Each surface sample was subjected to three analytical workflows ([Figure 4.1B](#)). First, total bacterial counts were enumerated to assess the bioburden level using aerobic plate count (APC). Second, the presence of multidrug-resistant organisms (MDROs) was determined using chromogenic agars. Third, taxonomic classification and ARG profiling of each sample were performed by adopting the targeted probe capture metagenomics (TCM). This multi-modal approach enabled a comprehensive interrogation of pathogen prevalence and antimicrobial resistance within the post-handwashing area. Metagenomic analyses were performed using the companion bioinformatic analysis platform, Explify® RPIP (Illumina). Additionally, categorization of microbial species into three phenotypic groups based on inferred clinical risks was also performed (see section 2.3.4) (Illumina, 2024). To validate Explify RPIP results, a collection of third-party bioinformatic tools was also employed ([Figure 4.1C](#)). Of the initial 232 samples, 28 were excluded from downstream analysis due to failed library construction, resulting in 204 samples for subsequent analysis.

A



B

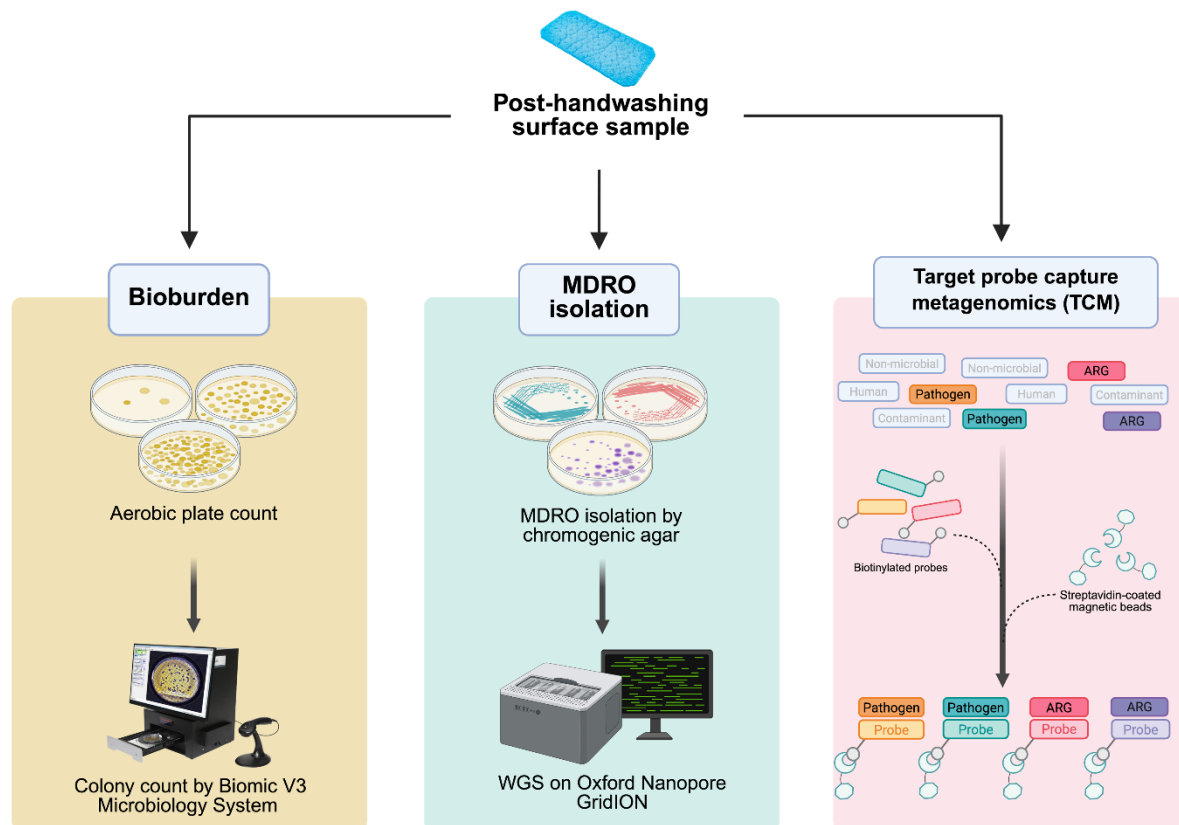


Figure 4.1. Study design and workflow of the MDRO and ARG surveillance of post-handwashing areas in public washrooms.
(Figure description on next page.)

C

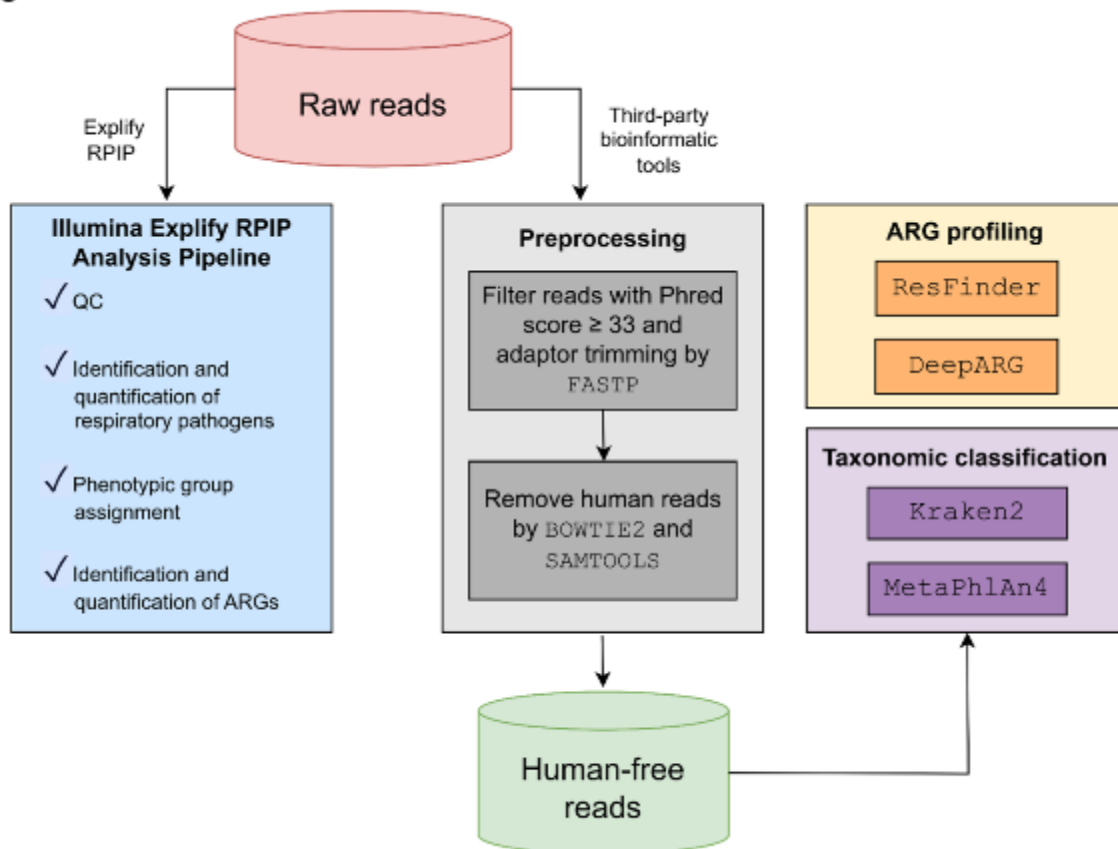


Figure 4.1. (continued) Study design and workflow of the MDRO and ARG surveillance of post-handwashing areas in public washrooms.

(A) Four post-handwashing sites in public washrooms, including faucets, paper dispensers, hand dryers, and exit door handles, with high risk of contamination by MDROs and ARGs were sampled.

(B) Surface samples were subjected to three workflows. Left, total bacterial count of each sample were enumerated using aerobic plate count. Middle, MDROs were isolated from surface samples using chromogenic agars. Right, target probe capture was performed on metagenomic libraries to enrich pathogen and ARG reads.

(C) Bioinformatic pipeline of the washroom microbiome study. Raw fastq reads were processed by Explify® RPIP (Illumina). Taxonomic classification and ARG profiling results were verified with two standalone bioinformatic tools, respectively.

MDRO, multidrug-resistant organisms; ARG, antimicrobial resistance gene; pDST, phenotypic drug sensitivity testing; WGS, whole genome sequencing.

4.4. Bioburden of Post-Handwashing Areas

To assess the bioburden of post-handwashing sites, total bacterial counts were determined (Figure 4.2). Aerobic plate count (APC) revealed significant differences in bacterial counts among the four sites ($p = 0.00107^{**}$, one-way ANOVA).

Faucets exhibited the highest microbial load, with a median colony-forming units per milliliter (CFU/mL) of 3.89×10^3 , possibly due to their moist microenvironments that promote microbial proliferation. Exit door handles ranked second (2.52×10^3 CFU/mL), followed by hand dryers (1.94×10^3 CFU/mL), while paper dispensers harbored the lowest bioburden (1.24×10^3 CFU/mL). These findings highlight faucets as critical re-contamination hotspots following handwashing.

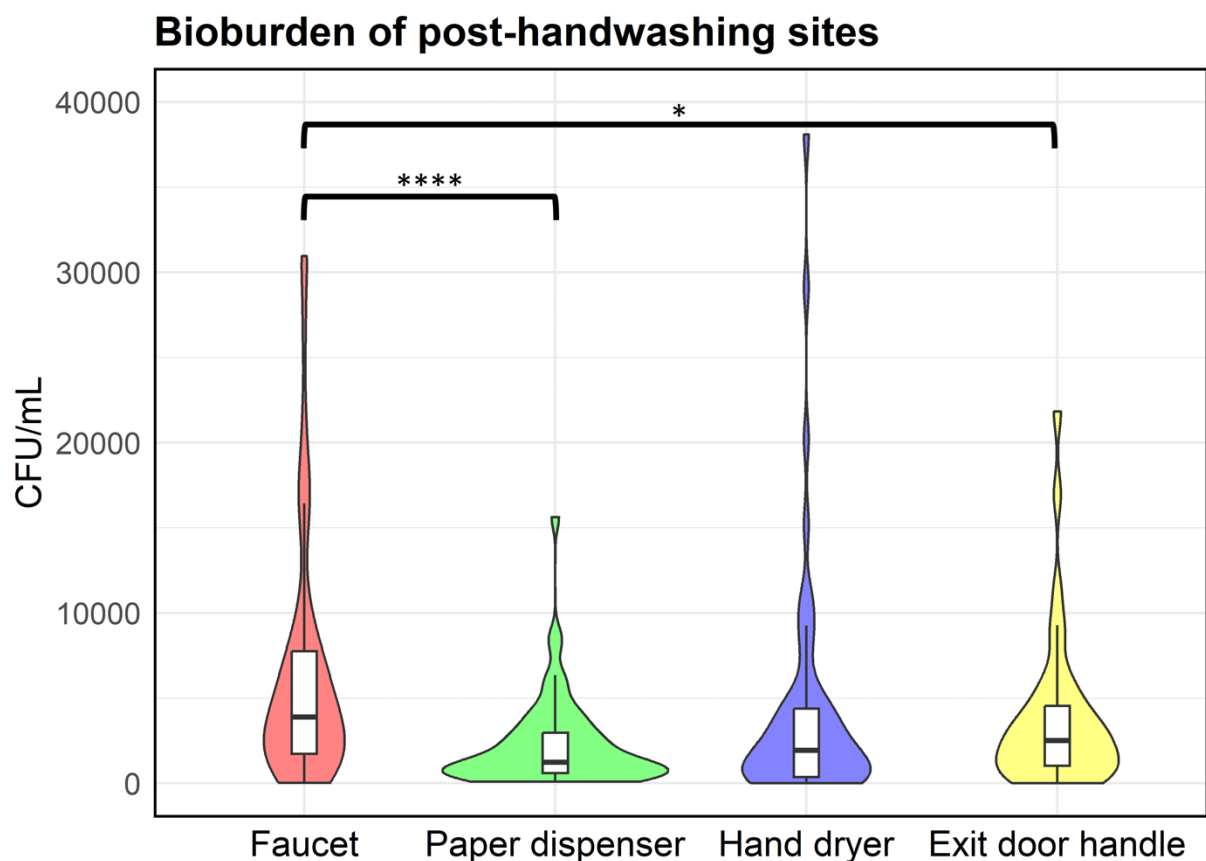


Figure 4.2. Bioburden of post-handwashing sites in public washrooms.

Bioburden of the four post-handwashing sites were assessed by aerobic plate count. Mean CFU/mL of each sites were determined. Faucet harbored the highest mean CFU/mL.

Statistical significance: * $P < 0.05$, **** $P < 0.0001$. Abbreviation: CFU, colony-forming unit

4.5. Isolation of Clinically Significant MDRO strains from Post-Handwashing Areas

To assess the viability and prevalence of MDROs, the presence of five clinically critical pathogens listed in the WHO Bacterial Priority Pathogen List 2024 (WHO, 2024) was determined using chromogenic agars. The five target MDROs were ESBL-E, CRE, VRE, CRPA, and MRSA. Among the 232 surface samples, 17 MDRO isolates (7.33%) were identified. ESBL-E was predominant, comprising 67% ($n = 10$) of isolates, followed by CRPA ($n = 5$, 33%) and MRSA ($n = 2$, 13%) ([Figure 4.3A, B](#)). No CRE or VRE was isolated. Among the post-handwashing sites, faucets represented the most contaminated sites by MDRO, harboring 53% ($n = 9$) of isolates, followed by hand dryers ($n = 4$, 24%), exit door handles ($n = 3$, 18%), and paper dispensers ($n = 1$, 6%) ([Figure 4.3C, D](#)).

Whole-genome sequencing (WGS) and *in silico* multilocus sequence typing (MLST) of MDRO isolates identified clinically significant sequence types (STs) and their resistance profiles. Two community-acquired MRSA (CA-MRSA) clones were isolated from post-handwashing sites. One clone recovered from paper dispenser, was assigned to ST338, carrying staphylococcal cassette chromosome *mec* (*SCCmec*) type Vb, *spa*-type t437, and *agr*-type I ([Supplementary Table ST9](#)). The other clone from hand dryer was assigned to ST1457, with *SCCmec* IV, *spa*-type t067, and *agr*-type II. Both clones possessed *mecA*, which is consistent with their resistance to cefoxitin. ST338 was previously reported as a multidrug-resistant, highly virulent CA-MRSA clone identified in Taiwan, China, and Poland (Chen et al., 2020; Chuang & Huang, 2013; Huh & Chung, 2016; Jin et al., 2021; Szymanek-Majchrzak & Młynarczyk, 2022). This clone was linked to severe bloodstream infections. Consistent to previous studies, this clone also carried *ermB*, which confers resistance to MLS antibiotics (Chen et al., 2020; Szymanek-Majchrzak & Młynarczyk, 2022). In contrast, ST1457 is poorly documented, suggesting its limited clinical relevance.

A total of five CRPA clones were recovered from faucets, spanning across four STs, including ST27, ST313, ST1182, and ST1341 ([Supplementary Table ST10](#)). Among them, ST313 is the most-well-documented and was reported as an intestinal colonizer of healthy individuals (Valenza et al., 2015) and is commonly associated with urinary tract infections (Valik et al., 2024). Consistent with our pDST results, ST313 exhibited resistance to carbapenems (Dong et al., 2022). ST27 and ST1182 have been detected in clinical samples and drinking water networks (Horikian et al., 2024; Shen et al., 2023; Wei et al., 2020), whereas ST1182 and ST1341 are less characterized.

Among the ten ESBL-E isolates, two high-risk STs were identified ([Supplementary Table ST11](#)). An *Enterobacter hormaechei* ST133 clone, recovered from a hand dryer, was reported as a virulent and multidrug-resistant strain linked to bloodstream infection (Huang et al., 2023; Zhou et al., 2023). However, this clone lacked the critical beta-lactamase genes (e.g., *blaCTX-M*, *blaNDM*) as previously reported (Huang et al., 2023; Zhou et al., 2023) and was tested sensitive to carbapenems. Additionally, a multidrug-resistant *Escherichia coli* ST1193 clone of serotype O75:H5 from a faucet sample was positive for *blaCTX-M-55*. This globally emerging clone was associated with community-onset UTIs and bloodstream infections (Pitout et al., 2022; Xia et al., 2017; Mazumder et al., 2021). Collectively, the isolation of these clinically important STs further highlights post-handwashing areas as potential hubs for disseminating virulent and drug-resistant strains.

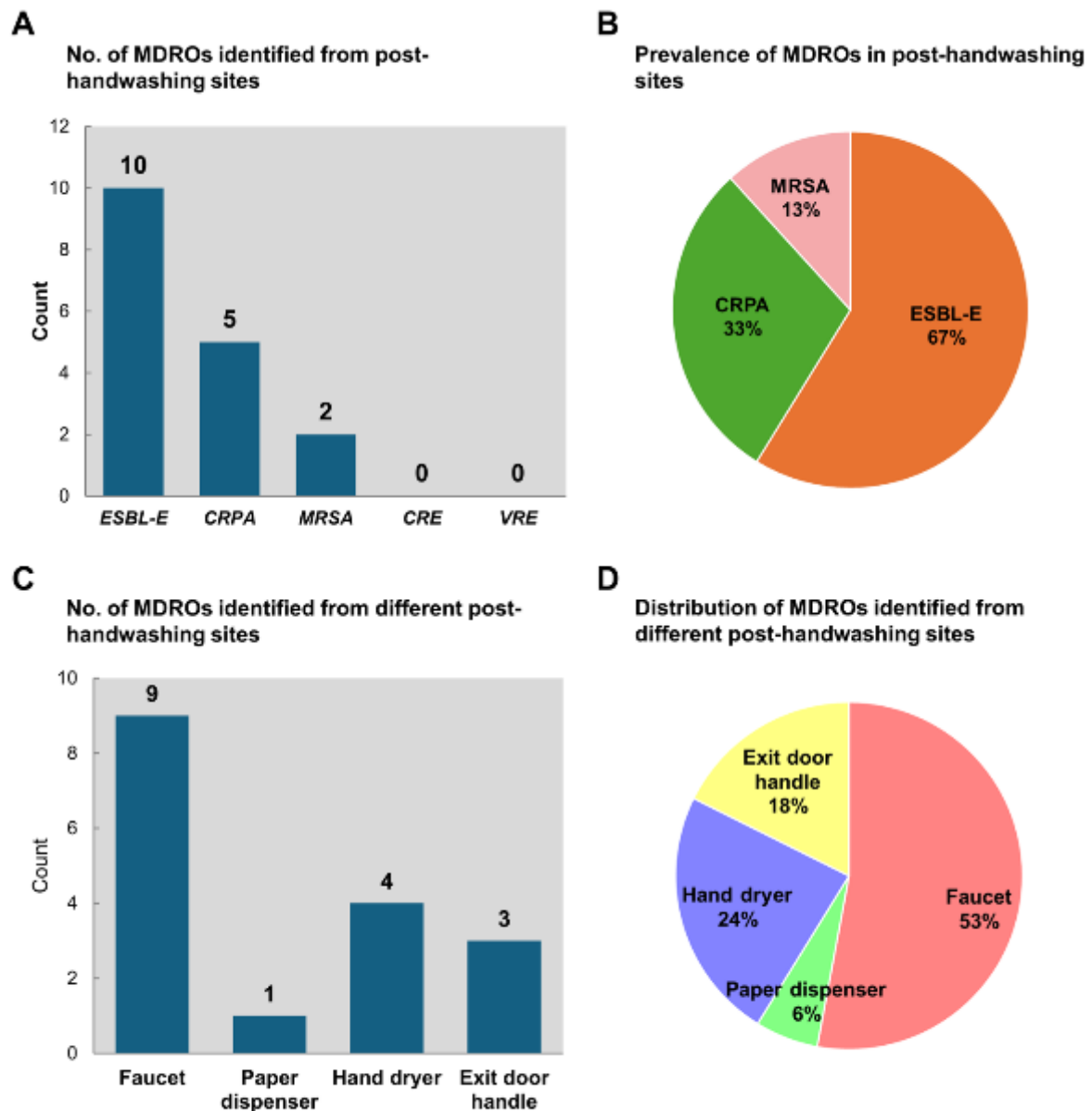


Figure 4.3. Prevalence and distribution of MDROs identified in post-handwashing area of public washrooms.

(A, B) ESBL-E is the most common MDRO identified from post-handwashing area.

(C, D) Over half of the MDRO isolates were identified from faucets. MDRO, multidrug-resistant organisms.

4.7. Taxonomic Profiling and the Core Microbiomes of Post-Handwashing Areas

High-throughput sequencing (HTS) of target-captured libraries generated an average of $18,443,657 \pm 10,066,472$ reads per sample, with microbial reads comprising $79.71\% \pm 2.36\%$ of total reads ([Figure 4.4A](#)). Human-derived reads and unclassified reads accounted for $7.25\% \pm 1.82\%$ and $7.21\% \pm 0.85\%$ of total reads, respectively. Taxonomic classification of microbial reads revealed $97.17\% \pm 0.22\%$ ($14,546,045 \pm 2,474,831$ reads) as bacteria, $2.30\% \pm 0.15\%$ ($341,272 \pm 35,932$ reads) as fungi, and $0.53\% \pm 0.09\%$ ($78,669 \pm 8,324$ reads) as viruses ([Figure 4.4B](#)).

The top 10 dominating species of each post-handwashing site, ranked by reads per kilobase million (RPKM), were identified ([Figure 4.4C](#)). Four bacterial species, including *Moraxella osloensis*, *Rothia mucilaginosa*, *Actinomyces naeslundii*, and *Pseudomonas aeruginosa*, were ubiquitous across all sites. Site-specific taxonomic patterns were observed. *Acinetobacter israelii* [RPKM = 258 ± 0], *Mycobacterium chelonae* [203 ± 385], *Pseudomonas stutzeri* [197 ± 407], *Corynebacterium striatum* [180 ± 0], and *Bacillus cereus* [179 ± 0] were exclusively detected in faucets, while *Trichosporon asahii* [411 ± 0], *Streptococcus agalactiae* [140 ± 0], *Tsukamurella pulmonis* [118 ± 0], and *Enterococcus faecium* [112 ± 0] were unique to paper dispensers. Hand dryers exclusively detected with *Delftia acidovorans* [419 ± 570], *Enterococcus faecalis* [94 ± 143], *Dolosigranulum pigrum* [85 ± 0], *Klebsiella pneumoniae* [75 ± 59], and *Fusarium solani* [65 ± 0], whereas *Moraxella catarrhalis* [225 ± 354], *Achromobacter xylosoxidans* [148 ± 0], *S. aureus* [117 ± 131], and *Kytococcus sedentarius* [80 ± 55] were only detected in exit door handles.

Alpha diversity metrics indicated hand dryers represented the most taxonomically diverse surfaces ([Figure 4.4D, E](#)), although the inter-site differences were not statistically significant ($p > 0.05$, ANOVA). Beta diversity, visualized via principal coordinates analysis (PCoA),

showed faucet microbiomes exhibiting the greatest dissimilarity (i.e. greatest cluster area of 0.471), followed by paper dispensers (cluster area = 0.428) ([Figure 4.4F](#)). Hand dryers displayed the smallest dissimilarity (cluster area = 0.324), indicating they share a similar microbiome composition despite being sampled from different washrooms.

To validate taxonomic assignments by Explify RPIP, taxonomic classification by two third-party taxonomic classifiers, Kraken2 and MetaPhlAn4, was performed. Concordance analysis revealed partial overlap ($42.41\% \pm 10.59\%$) between species detected by Explify RPIP and third-party classifiers. However, only 2 to 4 species overlapped out of the top 10 dominating species identified by different classifiers ([Supplementary Figure S5](#)). These discrepancies are likely due to differences in classification algorithms and reference databases used by different classifiers (Ye et al., 2019; Almeida et al., 2018; Portik et al., 2022; Smith et al., 2022).

To identify persistent microbial taxa constituting the microbiomes of different post-handwashing sites, core microbiomes, defined as the 10 most prevalent species, were characterized for each site ([Figure 4.5A-D](#)). Comparative analysis revealed high similarity among core microbiomes of different post-handwashing sites, which were predominantly comprising human-associated commensals and environmental strains ([Figure 4.5E, F](#)). Six bacterial species were found prevalent across all four sites, including *Acinetobacter baumannii*, *Actinomyces naeslundii*, *Moraxella osloensis*, *Pseudomonas aeruginosa*, *Rothia mucilaginosa*, and *S. aureus*. Four of these species, *A. baumannii*, *A. naeslundii*, *P. aeruginosa*, and *S. aureus*, were classified as phenotypic group 2 (opportunistic pathogens), while *M. osloensis* and *R. mucilaginosa* belonged to phenotypic group 1 (commensals). No phenotypic group 3 (high-risk pathogens) was included in any of the core microbiomes.

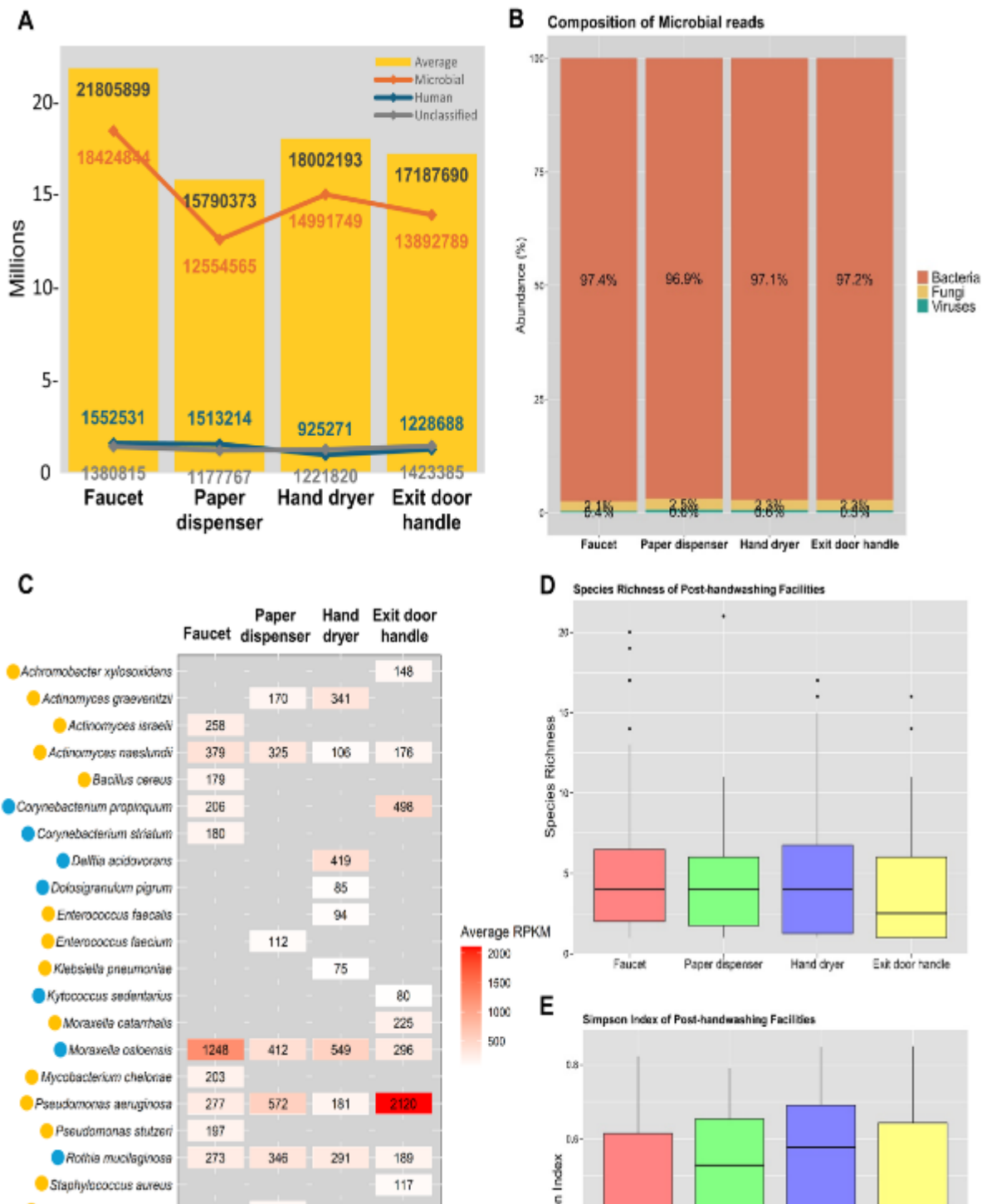


Figure 4.4. Microbiome analysis of post-handwashing sites in public washrooms.

(A) Barplot showing the composition of sequencing reads yielded from four post-handwashing sites, with orange, blue and grey lines indicating microbial, human, and unclassified reads, respectively.

(B) Taxonomic compositions of overall microbial reads of the four post-handwashing sites.

(C) Top 10 dominating species of post-handwashing sites ranked by RPKM. Blue and orange dots represent phenotypic group 1 and 2, respectively.

(D) Species Richness (i.e. number of species) and (E) Simpson index showed that hand dryers have the highest alpha diversity.

RPKM, reads per kilobase million.

F

PCA Plot of Microbiome Composition Dissimilarity between Post-Handwashing Facilities

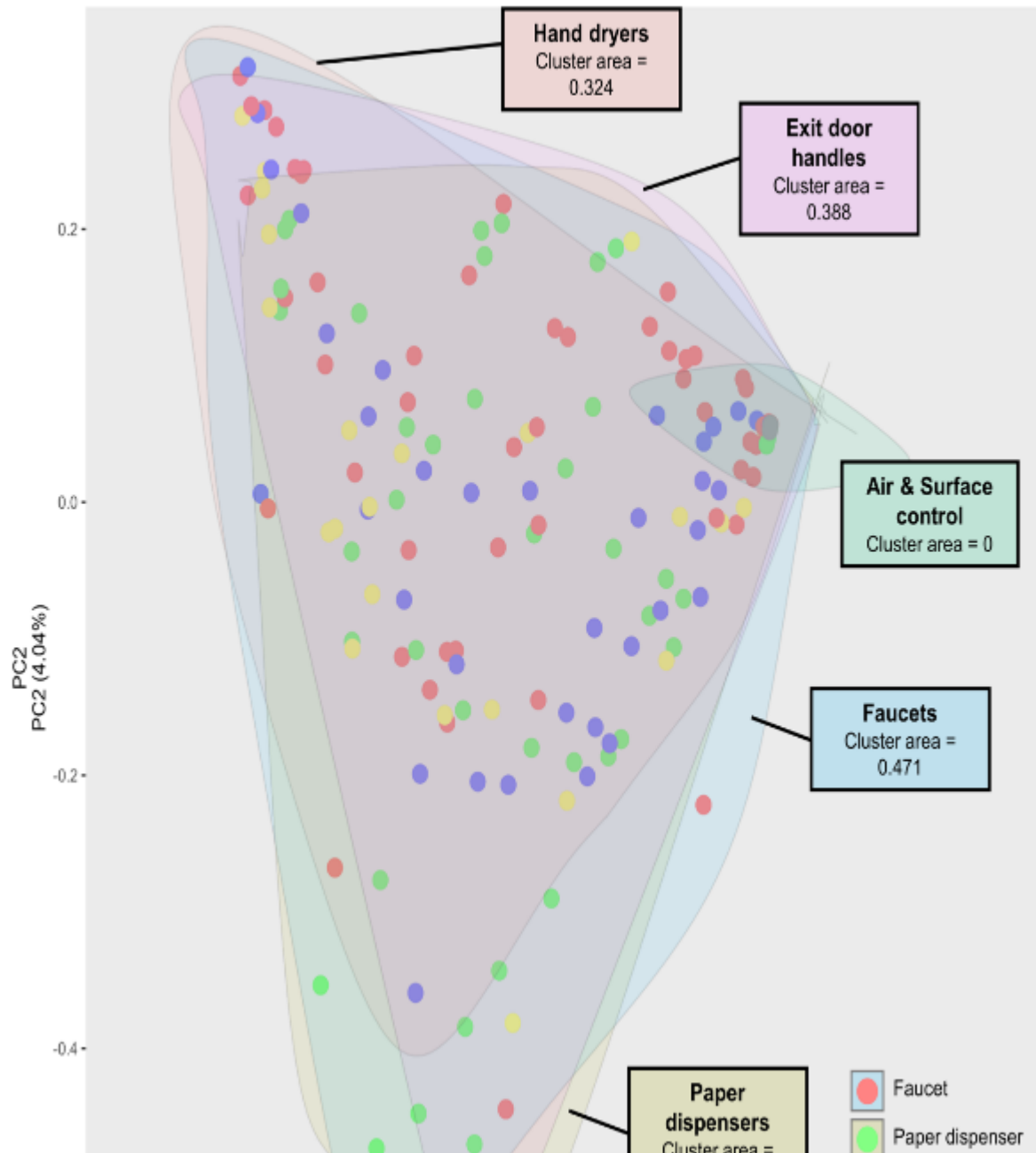


Figure 4.4. (continued) Microbiome analysis of post-handwashing sites in public washrooms

(F) Principal coordinates analysis (PCoA) showing microbiome composition dissimilarity of post-handwashing sites in different washrooms

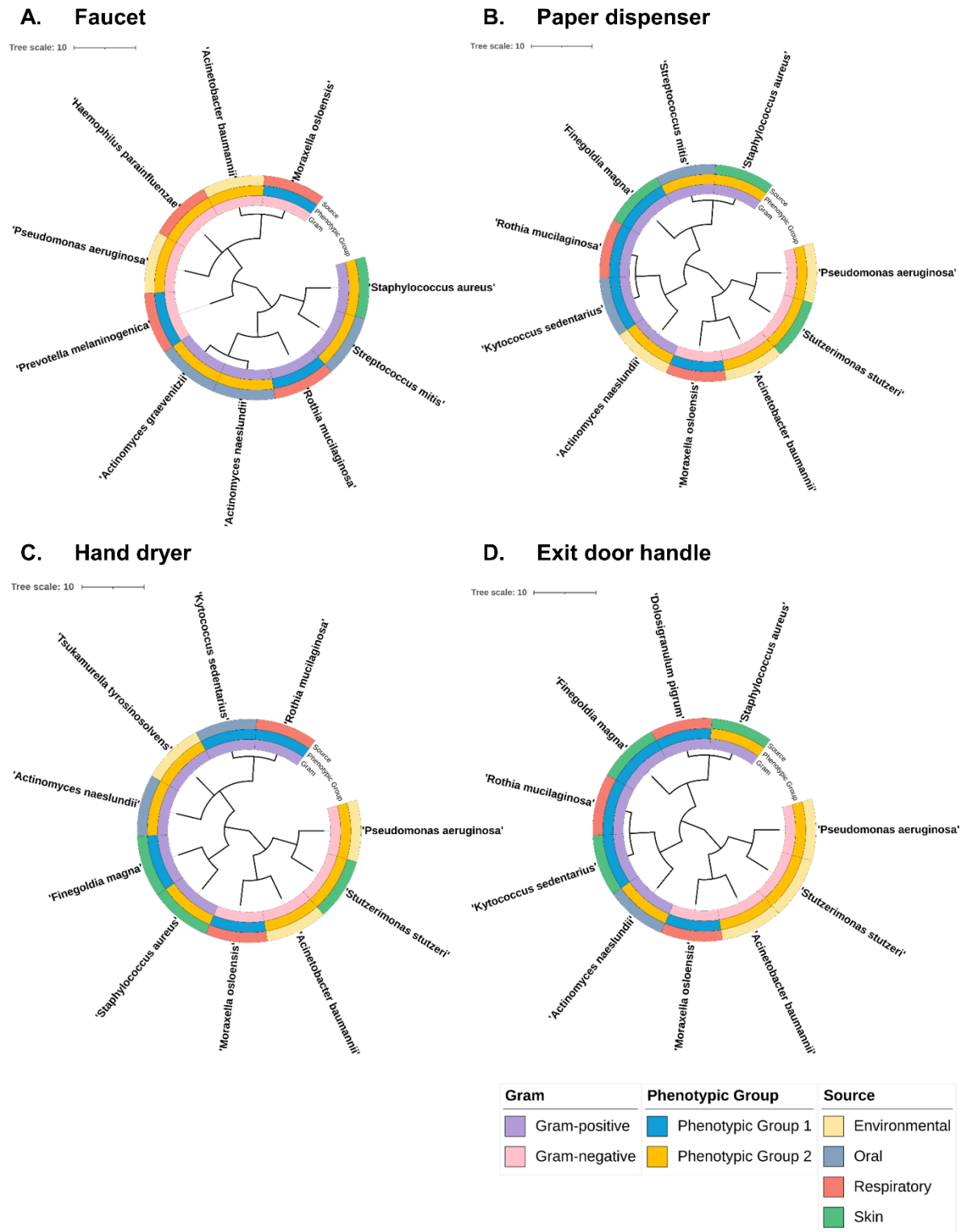


Figure 4.5. Core microbiomes of different post-handwashing sites in public washrooms.

Core microbiome was defined as 10 most prevalent species per site. Core microbiome of (A) faucets, (B) paper dispensers, (C) hand dryers, and (D) exit door handles.

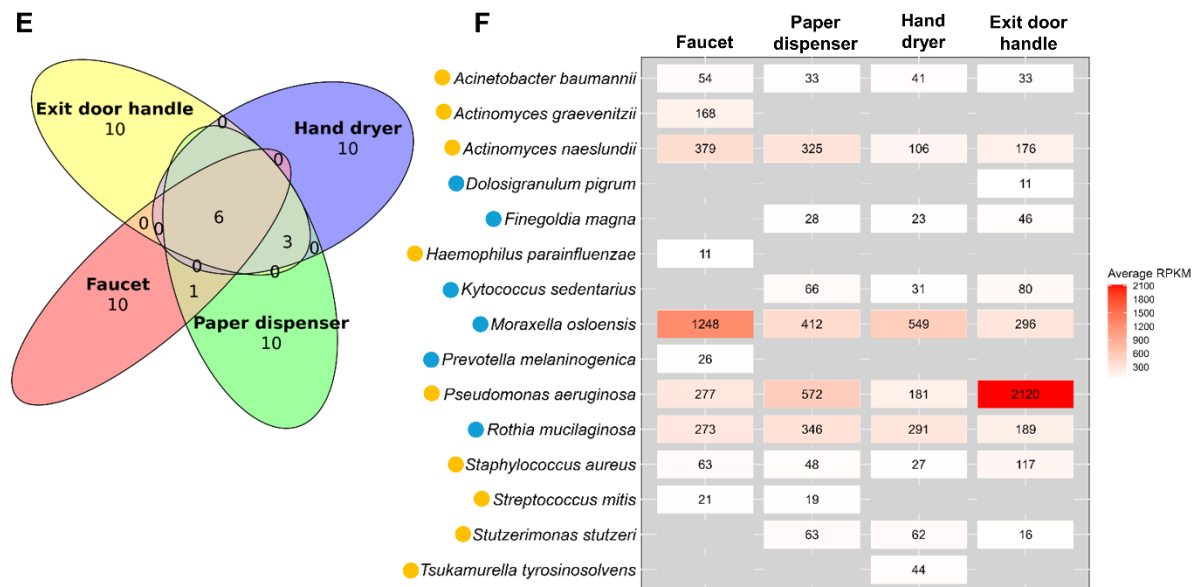


Figure 4.5. (continued). Core microbiomes of different post-handwashing sites in public washrooms.

(E) Venn diagram showing number of species in common between the core microbiomes of different post-handwashing sites.

(F) Heatmap showing the RPKM of the 10 core species of different sites. Blue and orange dots indicate phenotypic group 1 and 2, respectively. RPKM, reads per kilobase million.

4.8. Post-Handwashing Microbiomes Show Resemblance to Human Microbiota

As human-associated microbiota are major contributors to microbial communities of post-handwashing surfaces (Flores et al., 2011; Prussin & Marr, 2015), the similarity of post-handwashing microbiomes to commensal human microbiomes was assessed by determining the MASH (MinHash-based) distances, which approximate genomic dissimilarity between microbial communities. MASH distances between microbiomes of post-handwashing sites to 40 human commensal microbiome samples, including skin, gut, respiratory tract, and urinary tract (10 samples per site), were determined. Faucet microbiomes displayed the greatest genomic similarity to human skin and gut microbiomes (i.e., lowest MASH distance; [Figure 4.6A, B](#)). While hand dryer microbiomes showed the greatest similarity to respiratory tract microbiomes ([Figure 4.6C](#)). In contrast, no significant differences were observed in MASH distances between different post-handwashing sites and urinary tract microbiomes ($p = 0.266$, one-way ANOVA) ([Figure 4.6D](#)), suggesting their comparable similarity to this commensal microbiome.

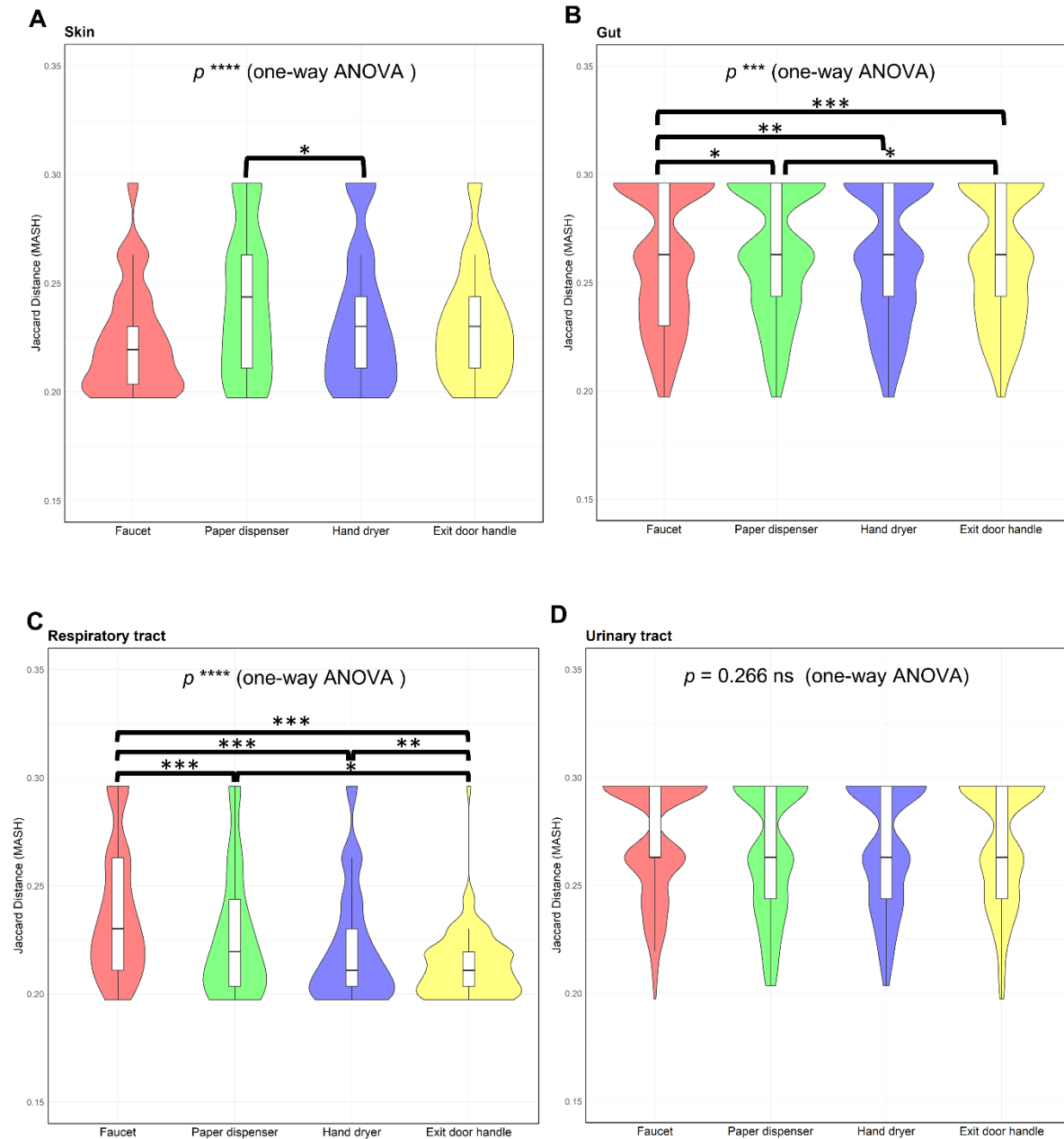


Figure 4.6. Jaccard similarity of MASH indices to human microbiomes.

MASH distance (k -mer-based) between various post-handwashing sites and human commensal microbiome of the (A) skin, (B) gut, (C) respiratory tract, and (D) urinary tract. Statistical significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

4.10. ARG Profiling and the Core Resistomes of Post-Handwashing Areas

To characterize the antimicrobial resistance dynamics of post-handwashing areas, ARG profiling for all surface samples was performed. ARGs from the drug class of macrolide-lincosamide-streptogramin (MLS) were found dominating the post-handwashing area resistome, accounting for 29.09% of total ARG hits, followed by aminoglycoside (28.13%) and beta-lactam (21.53%) resistance genes ([Figure 4.7A](#)). The top 20 most detected ARGs, ranked by average RPKM, were identified ([Figure 4.7B](#)). The most detected ARG, *erm(36)* (18.46%), confers MLS resistance in *Micrococcus luteus* (Liebl et al., 2002), while *ermX* (8.42%; macrolide resistance) (Rosato et al., 2001) and *ANT(4')-Ib* (5.33%; aminoglycoside resistance in *Staphylococcus* and *Bacilli*) (McDougal et al., 2010; Bozdogan et al., 2003) were the second and third most detected ARG, respectively. Despite exhibiting the highest ARG diversity ($n = 91$), faucets yielded the lowest average AMR-associated reads ([Figure 4.7C](#)). Hierarchical clustering of the top 20 ARGs per site demonstrated a closer clustering between paper dispensers and hand dryers than other sites ([Figure 4.7D](#)), suggesting their similarity in resistome compositions.

The identification of ARGs by Explify RPIP was validated by two third-party ARG profiling tools, ResFinder and DeepARG. Concordance analysis confirmed a substantial proportion of ARGs ($72.86\% \pm 4.88\%$) overlapped between them. Comparing the dominating ARGs, 3 to 5 out of the top 10 ARGs overlapped between different tools ([Supplementary Figure S6](#)).

Similar to core microbiomes, core resistomes, defined as the 10 most prevalent ARGs, of each four post-handwashing sites were characterized to identify persistent resistance determinants ([Figure 4.8A-D](#)). Our analysis revealed that MLS-associated ARGs dominated across all core resistomes ($57.77\% \pm 6.03\%$), followed by aminoglycosides ($19.96\% \pm 3.28\%$) and sulfonamide ($14.28\% \pm 4.69\%$) resistance genes. Intriguingly, eight out of the ten

ARGs – *ANT(4')-Ib*, *APH(3')-IIIa*, *blaZ*, *mecA*, *dfrC*, *Erm(36)*, *lnuA*, and *sulI* – were conserved across all core resistomes, reflecting the presence of a stable and persistent assemblage of ARGs within the post-handwashing area ([Figure 4.8E, F](#)). The *mecA* gene was the only high-risk ARG that met our core resistome threshold and was consistently present in core resistomes of all post-handwashing sites. Together with the prevalence of *S. aureus* in core microbiomes ([Figure 4.5A-D](#)), these findings imply that post-handwashing areas may serve as potential reservoirs for MRSA.

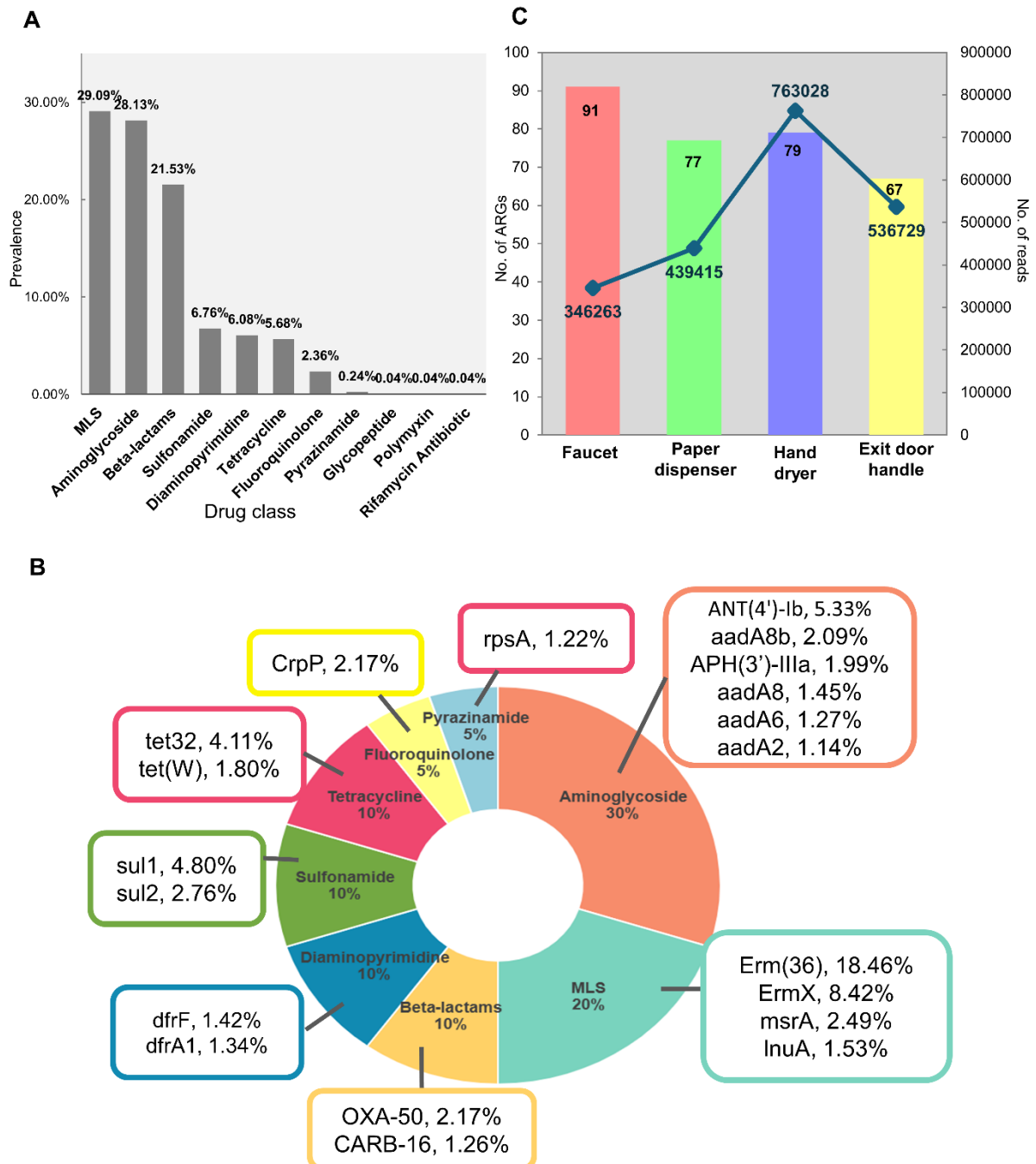


Figure 4.7. AMR burden of post-handwashing area in public washrooms.

(A) Total percentage of reads assigned to ARGs of difference drug classes. The most common ARG class is MLS.

(B) Pie-chart showing 20 most detected ARGs, ranked by percentage of total reads, from post-handwashing area and their drug class.

(C) Total number of ARGs (bars) and total reads (Blue line) assigned to ARGs of different post-handwashing facilities. ARG, antimicrobial resistance gene.

D

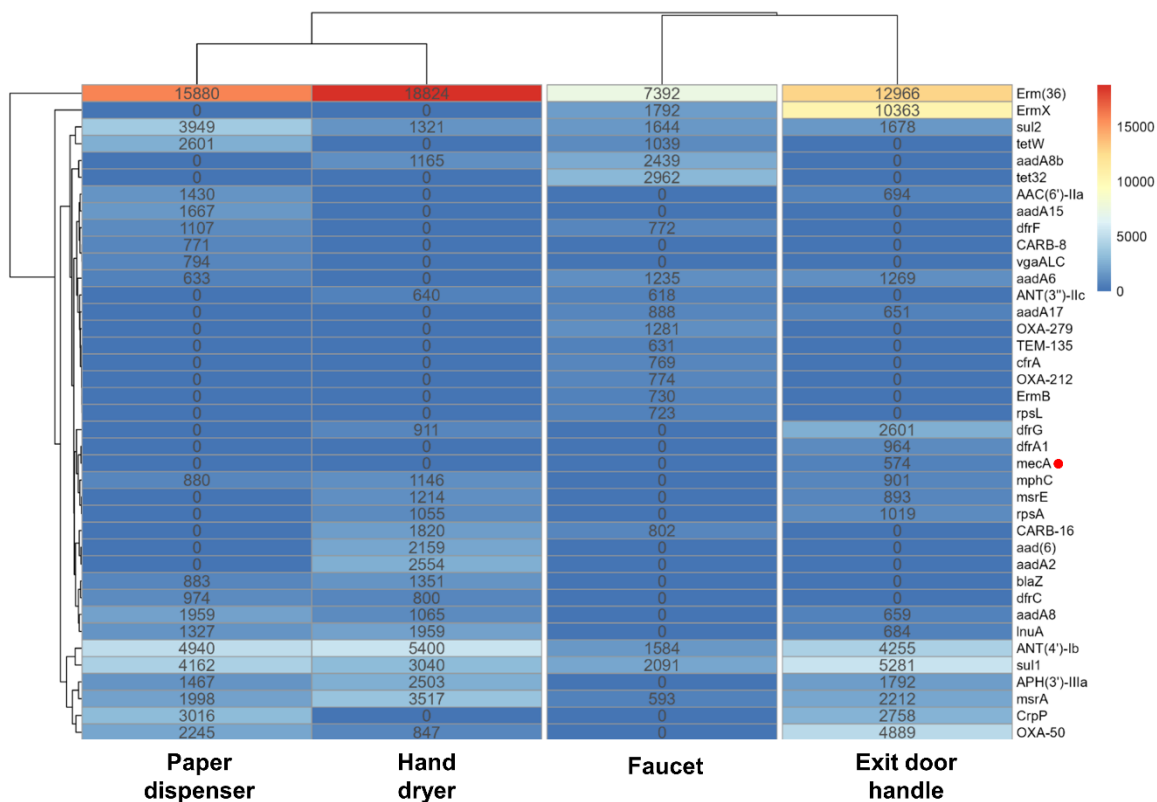


Figure 4.7. (continued). AMR burden of post-handwashing area in public washrooms. (D) Comparison of top 20 ARGs of each post-handwashing facilities. Red and blue fields represent high and low RPKM, respectively. Red dot indicates clinically important ARGs. ARG, antimicrobial resistance gene.

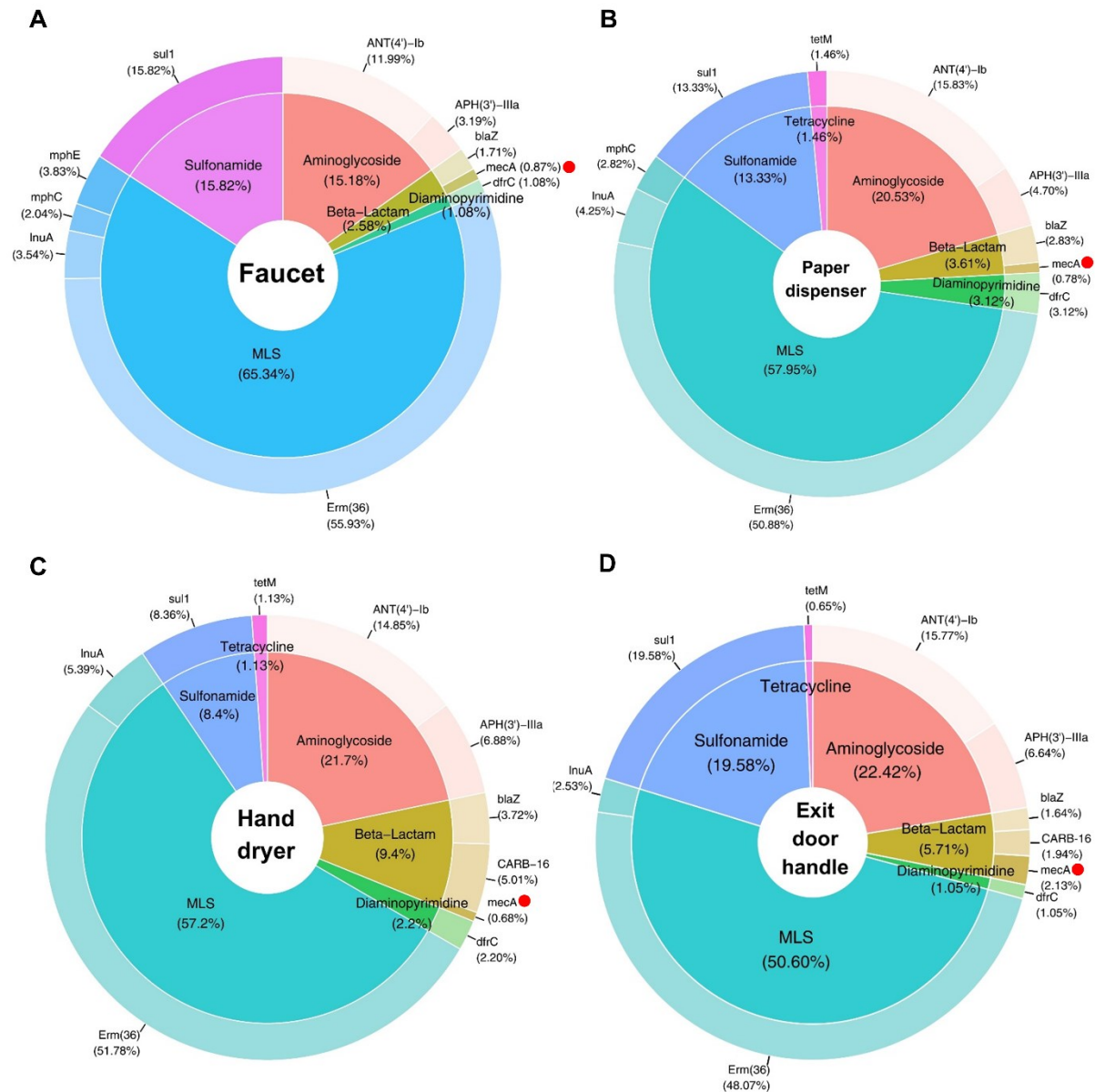


Figure 4.8. Core resistomes of post-handwashing sites in public washrooms. Core resistome, defined as 10 most prevalent ARGs per site, of (A) faucets, (B) paper dispensers, (C) hand dryers, and (D) exit door handles. Red dot indicates clinically important ARGs. ARG, antimicrobial resistance gene.

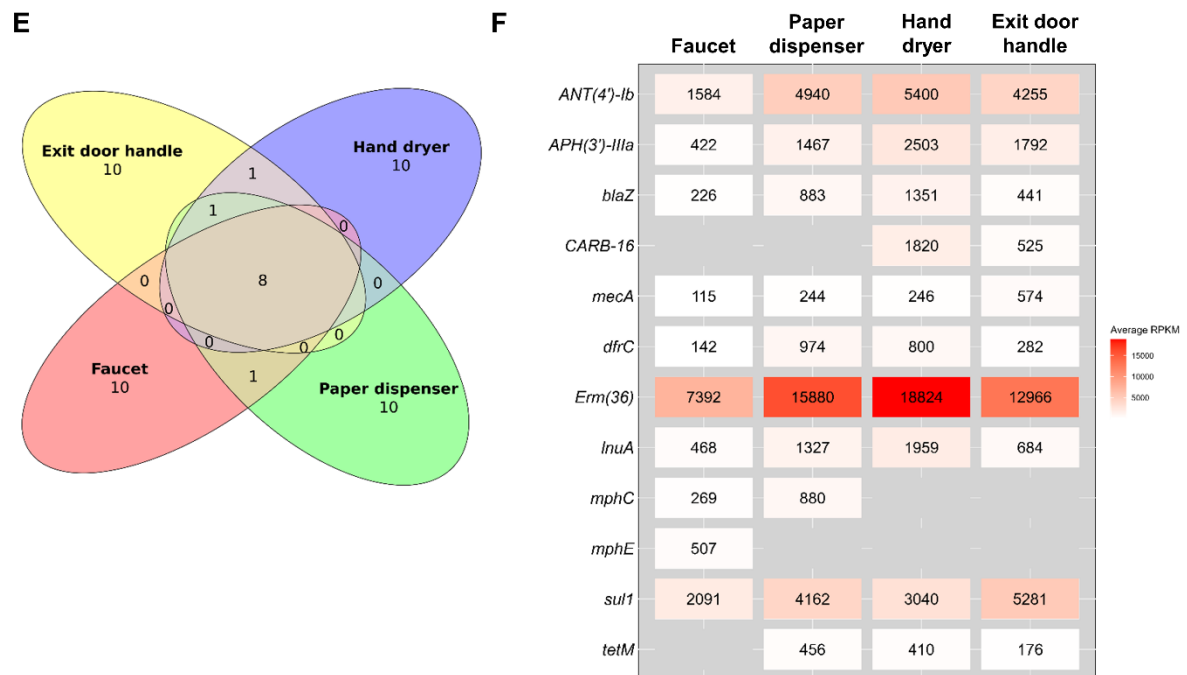


Figure 4.8 (continued). Core resistomes of post-handwashing sites in public washrooms. (E) Venn diagram showing number of ARGs common between the core resistomes of different post-handwashing sites. (F) Heatmap showing the RPKM of core ARGs of different post-handwashing sites. ARG, antimicrobial resistance gene; RPKM, reads per kilobase million.

4.11. Target Probe Capture Enrichment Identified Opportunistic Pathogens and Clinically Critical ARGs in Post-Handwashing Areas

Using TCM, a total of 46 species of opportunistic pathogens (phenotypic group 2 organisms) were identified across all post-handwashing sites ([Figure 4.9A](#)). TCM revealed that 65.20% of samples were detected with at least one opportunistic pathogen. Among the four sites, faucets harbored the greatest number of species ($n = 32$), followed by paper dispensers ($n = 28$), hand dryers ($n = 23$), and exit door handles ($n = 18$). Clinically important opportunistic pathogens were enriched on specific sites. *A. baumannii*, often associated with bloodstream infections, UTIs, lung infections, and open wounds, and *M. chelonae*, causing skin and soft tissue infections (SSTIs), were predominantly enriched in faucets (Ababneh, Abu Laila, et al., 2022; Akram et al., 2025). *E. faecalis* and *E. faecium*, two *Enterococcus spp.* of clinical importance associated with UTIs, wound infections, sepsis, and endocarditis (Said et al., 2025), were enriched in hand dryers and paper dispensers, respectively. Common agents of community-acquired pneumonia, namely *K. pneumoniae* and *Streptococcus pneumoniae*, were enriched in hand dryers, posing potential infection risks to users (Aliberti et al., 2021). In contrast, *H. influenzae* and *M. catarrhalis* were exclusively detected in paper dispensers and exit door handles, respectively. Important skin infection agents were also detected. *S. aureus* (Del Giudice, 2020), *P. aeruginosa* (Wu et al., 2011), and *E. coli* (Petkovsek et al., 2009) were enriched on exit door handles, alongside Herpes simplex virus 1 (HSV-1). Additionally, *S. agalactiae* (Group B *Streptococcus*), a critical pathogen in neonatal and immunocompromised infections (Raabe & Shane, 2019), as exclusively detected in paper dispensers. The presence of these opportunistic and clinically relevant pathogens implicates an elevated infection risk of microbial transmission and potential infection in post-handwashing areas, particularly for skin, respiratory, and systemic infections.

To assess the AMR burden of the post-handwashing area, we specifically looked for the presence of clinically critical ARGs. A total of 13 high-risk ARGs were detected ([Figure 4.9B](#)). At least one of these high-risk ARGs was detected in 63.24% of samples. Among the four sites, paper dispensers harbored the highest number of ARGs ($n = 8$). Strikingly, *mecA* was detected with the highest number of average reads [RPKM = 574 ± 144] on exit door handles. In addition, the beta-lactamase gene *blaGES-5* was also detected across all sites, with faucets harboring the highest average reads [149 ± 242]. Site-specific identification of high-risk ARGs was also observed. For instance, *blaSHV-104* and *vanC* were exclusively detected in faucets, while carbapenemases *blaIMP-26* and *blaNDM-5* were unique to hand dryers. Six high-risk beta-lactamase genes were exclusively found in paper dispensers, including *blaCTX-M-64*, *blaCTX-M-65*, *blaGES-15*, *blaNDM-5*, *blaSHV-I*, and *blaSHV-II*. All of the high-risk ARGs detected by Explify RPIP were concurrently detected by third-party ARG profiling tools, authenticating their presence on post-handwashing surfaces. These findings further demonstrate the persistence of clinically significant resistance determinants in high-contact washroom spaces, warranting the need for enhanced hygiene practices to mitigate AMR dissemination risks.



Figure 4.9. Health impact of pathogens and ARGs identified in post-handwashing area of public washrooms.

(A) Relative number of reads assigned to of phenotypic group 2 (opportunistic) organisms identified from different sites.

(B) Heatmap showing the average number of reads assigned to clinically important ARGs of site. ARG, antimicrobial resistance gene; RPKM, reads per kilobase million.

4.12. Discussions

Over the past decades, the rise of antimicrobial resistance has represented one of the most pressing public health threats, and it is exacerbated by urbanization and various anthropogenic activities (Danko et al., 2021; Czatzkowska et al., 2022; Chen et al., 2023; Mahnert et al., 2019). In response, the One-Health initiative (WHO, 2022a), a global collaborative framework addressing AMR from the human, animal, and environmental aspects, has been advocated. While most surveillance efforts have been focusing on healthcare and agriculture contexts, environmental surveillance, particularly BE, is often neglected when it comes to AMR surveillance (Cave et al., 2021; Larsson & Flach, 2022; Essack, 2018). This study therefore addresses this gap by characterizing MDROs and ARGs in public washrooms, which are high-traffic urban hubs implicated in pathogen and antimicrobial determinants transmission (Suen et al., 2019; Mkrtchyan et al., 2013; Flores et al., 2011). Particular attention is given to the post-handwashing area, a key junction where re-contamination of washed hands might occur, as reservoir for pathogen and AMR transmission. Herein, a total of 232 surface samples were collected from four high-contact post-handwashing sites (i.e., faucets, paper dispensers, hand dryers, exit door handles) of public washrooms across different sectors in Hong Kong ([Figure 4.1](#); [Supplementary Table S3](#)). By employing an integrated approach combining conventional culture-based methods and novel targeted probe capture metagenomics (TCM), the post-handwashing areas were revealed as reservoirs for opportunistic pathogens and clinically critical ARGs. Culture-based methods confirmed the presence of viable MDROs, including MRSA, ESBL-E and CRPA in the post-handwashing area ([Figure 4.3](#)), while TCM revealed the presence of low-abundance opportunistic pathogens and clinically critical ARGs ([Figure 4.9](#)).

The results suggest that the post-handwashing areas of public washrooms might be a potential hotspot of MRSA infection. Even though only two MRSA isolates were identified

from culture-based approach, taxonomic and ARG profiling revealed the concurrent enrichment of *S. aureus* and *mecA* reads in exit door handles of public washrooms, implicating that these sites could be an important reservoir of MRSA. This finding is consistent with the 2019 study by Suen et al., who also reported the enrichment of *S. aureus* on exit door handles, suggesting the persistence of *S. aureus* at this post-handwashing site (Suen et al., 2019). WGS of MRSA isolates identified a clinically significant clone, *ermB*-positive ST338-SCC*mec* Vb, which was linked to severe bloodstream infection cases (Huh & Chung, 2016; Chen et al., 2020). Taken together, it is proposed that exit door handles of public washrooms may serve as a critical hotspot for community MRSA dissemination via hand contact, warranting targeted disinfection procedures.

Taxonomic profiling revealed the dominance of human-associated commensals and environmental strains in post-handwashing. While none of these taxa are inherently pathogenic, their roles as opportunistic pathogens in healthcare and community settings were well characterized. For instance, *A. baumannii*, present in core microbiomes of all four post-handwashing sites ([Figure 4.5](#)), is a leading cause of ventilator-associated pneumonia and frequently harbors carbapenemase genes (Moubareck & Halat, 2020). Although absent from this study, carbapenem-resistant strains were reported in faucets, hand dryers and air samples of public washrooms (Huesca-Espitia et al., 2018; Lv et al., 2019). Similarly, other human pathogens like *E. coli* (Mohamed et al., 2015; Chang et al., 2022), *K. pneumoniae* (Buchan et al., 2019; Heireman et al., 2020; Nakamura et al., 2021), *P. aeruginosa* (Halabi et al., 2001; Breathnach et al., 2012), and *Enterococcus spp.* (Best et al., 2018) were identified, all of which have been previously reported to exhibit antimicrobial resistance in washroom environments. MRSA has also been extensively reported from public washrooms (Mkrtychyan et al., 2015; Roberts et al., 2011), with multiple studies reporting its isolation from various surfaces, including toilet seats (Giannini et al., 2009; Katsuse et al., 2017), exit door handles (Ababneh,

Jaradat, et al., 2022), and floors (Best et al., 2018). In addition to previously reported opportunistic pathogens, this study reveals a broader spectrum of opportunistic pathogens present in public washrooms, namely *H. influenzae*, *M. catarrhalis*, *S. agalactiae*, and *M. chelonae* ([Figure 4.9A](#)), posing heightened infection risks to immunocompromised individuals (Dropulic & Lederman, 2016). These findings highlight public washrooms as a critical reservoir for opportunistic pathogens and therefore the need for enhanced hygiene vigilance in public washrooms to prevent potential infections, especially for the immunocompromised cohorts.

This study illustrates a successful application of TCM in a large-scale pathogen and AMR surveillance of urban BE. This work highlights several key advantages of TCM in environmental surveillance. First, TCM was able to remove non-target reads from environmental samples. In this study, the proportion of human-associated reads was significantly lowered to 7.25% for all samples, whereas they can constitute up to 70% of total reads in a conventional metagenomics workflow (Danko et al., 2021). This reduction allows the detection of target reads at a much lower sequencing depth and thus faster turnaround time. Second, the proportion of unclassified reads was lowered to 7.21%, while it can constitute up to 40% in a conventional metagenomics workflow (Danko et al., 2021). Third, TCM enables a robust detection of rare pathogens and clinically important ARGs in low-biomass environmental samples, demonstrating its utility in uncovering hidden AMR threats in BE. While unbiased metagenomics workflows often require at least 200 million reads for comprehensive ARG profiling (Gweon et al., 2019), the TCM workflow achieved similar detection at an average read depth of 18 million reads. This represents an 11-fold reduction in sequencing depth requirements, further highlighting its cost-effectiveness for public health surveillance. These observations suggest that TCM has the potential to extend its applications beyond washroom environments to other BE, such as hospitals, elderly homes, and schools,

where pathogen surveillance is essential. Future efforts could focus on the design and optimization of a custom probe panel for washroom surveillance targeting enteric pathogens like norovirus and EHEC during outbreaks to deliver timely and robust surveillance (Metsky et al., 2019).

Despite the utility of TCM in uncovering hidden AMR transmission in public washrooms, this study has limitations. First, the nucleic acid extraction kit employed in this study was designed to capture only DNA, thus precluding the detection of RNA viruses from metagenomic analysis. RNA viruses, including influenza virus, coronaviruses, and norovirus, are etiological agents for numerous infectious diseases in humans and animals, posing significant public health problems globally. These pathogens are frequently detected in washroom environments, where their presence may contribute to disease transmission (Ding et al., 2021; Amoah et al., 2021; Leone et al., 2016). Second, this study lacks genomic comparisons with clinical isolates from local hospitals or complementary epidemiology investigations. Therefore, the public health and clinical impacts of the MDROs identified were unexamined. Such analyses are critical for determining whether the detected MDROs are genetically related to strains causing human infections or contribute to disease burden in the community. Third, the cross-sectional design of this study captures a single time point, which may not fully reflect the dynamic nature of microbial contamination. Transient pathogens introduced to the washroom environment may be removed by routine cleaning protocols, potentially leading to an underestimation of the true microbial load and diversity. To address these limitations, future research should adopt a longitudinal approach to monitor microbial persistence in post-handwashing areas over time. Additionally, employing total nucleic acid extraction methods to capture both DNA and RNA will provide a more holistic view of the microbial communities. Phylogenetic comparison with clinical isolates and epidemiological

data will further elucidate the public health significance of the detected pathogens, enhancing the translational impact of future studies.

In summary, the washroom microbiome study provides a comprehensive and nuanced characterization of the MDRO and AMR landscape of post-handwashing areas in public washrooms using an integrated approach. By leveraging TCM, a hidden infection risk of MRSA via exit door handles as well as the presence of a broad assemblage of opportunistic pathogens in public washrooms were uncovered, highlighting the need of targeted infection control measures in these high-traffic environments. This work demonstrates the utility of TCM in characterizing pathogen and AMR landscape from low-biomass environmental samples. As sequencing costs continue to decline and automation technologies advance (e.g., automated library preparation) (Ko et al., 2022; Miller et al., 2013; Gardy & Loman, 2018; Baker et al., 2023), it is envisaged that TCM would revolutionize the toolbox for MDRO and AMR surveillance, ultimately leading to a paradigm shift in surveillance strategies.

Chapter 5.

Discussions and Future Directions

5.1. Summary

The urban BE significantly impacts human health by harboring diverse microbial communities and AMR determinants (Perdue et al., 2003). In Hong Kong, surveillance for MDRO and AMR has primarily focused on human, animal, and food sectors, while urban BE has been seldom monitored (Cheng et al., 2025). In this thesis, two large-scale surveillances for MDRO and AMR were conducted in two urban BE, MTS and public washrooms, contributing to efforts in safeguarding public health while raising public awareness of potential infection risks in these two BE. Using an integrated approach of culture-dependent and culture-independent methods, these environments were shown to serve as potential reservoirs for pathogen and AMR transmission, thereby posing public health risks.

MTS, characterized by high occupant density and frequent passenger turnover, serves as an interface where microbial exchange between passengers and environments occurs, contributing to a dynamic transmission network. Particularly, pathogens and antimicrobial determinants deposited on surfaces can persist and spread, potentially contributing to community-acquired infections. Using selective culture, viable MDRO isolates were isolated from subway surface samples, including clinically important STs. Metagenomic sequencing further revealed high prevalences of clinically relevant taxa, such as *P. aeruginosa* (100%), *A. baumannii* (99.5%), and *S. aureus* (97.9%), in subway surface samples, highlighting MTS as reservoirs for pathogens. Moreover, 96.1% of surface samples were detected with at least one clinically important ARG, such as *mecA* (90.1%) and *blaOXA* (79.6%), revealing their widespread existence in the MTS. These findings contextualize subway environments as critical hotspots for pathogens and drug resistance, where routine surveillance for pathogens and ARGs should be warranted.

Public washrooms, often associated with high humidity and insufficient ventilation, are recognized as potential sites for pathogen transmission, particularly at post-handwashing areas,

where re-contamination of cleansed hands may inadvertently occur. Importantly, viable MDRO isolates, including those clinically reported strains, were recovered from these areas, emphasizing their role in pathogen and AMR transmission. Using target probe capture, at least one human pathogenic taxa was present in 65.2% of samples, including *P. aeruginosa* (78.4%), *Acinetobacter baumannii* (77.9%), and *S. aureus* (71.1%). While at least one clinically critical ARGs, such as *blaCTX-M* (2.0%), *blaNDM* (2.9%), *blaSHV* (3.4%), and *mecA* (62.3%), were detected in 63.7% of samples, indicating a potential transmission within the post-handwashing area. Notably, co-enrichment of *S. aureus* and the *mecA* gene was observed on exit door handles, identifying these sites as hotspots for MRSA transmission among washroom users. These results highlight the need for targeted disinfection measures in high-risk post-handwashing areas to reduce microbial burden and stop potential transmission.

Comparing the two BE, public washrooms exhibited slightly higher MDRO prevalence than MTS, likely due to differences in usage, humidity, and ventilation. The stringent antimicrobial measures implemented in the HK's subway system may contribute to the lower MDRO prevalence on subway surfaces. Notably, MRSA was found to be the most prevalent MDRO in both environments, indicating its pervasive distribution across urban BE.

In addition, a robust framework for MDRO and AMR surveillance in urban BE was established. First, a standardized sampling protocol was developed, ensuring consistent and reliable recovery of microbial data. Second, the culture-based methods adopted in this study successfully isolated viable MDROs from environmental samples, enabling WGS and the subsequent identification of clinically relevant strains and full ARG profiles. Third, metagenomic library preparation and target enrichment by capture probe were optimized for low-biomass environmental samples, enhancing the detection of low-abundance pathogens and ARGs. This integrated workflow offers a scalable, cost-effective framework for MDRO and ARG surveillance, potentially advancing current pathogen surveillance in urban BE.

Although metagenomic-based methods allow detection of rare pathogens and ARGs in complex environmental samples, offering a holistic view of the microbiome and resistome, several hurdles must be addressed before their broad application in public health surveillance, as elaborated below.

5.2. Challenges of Sequence-based Surveillance of Built Environment Microbiomes

Sequence-based surveillance of the BE microbiome is subjected to several challenges, which include issues concerning sample collection and processing, limitations in sequencing technology, and difficulties in interpreting the resulting data ([Figure 5.1](#)). These challenges can hinder the ability to accurately assess microbial diversity, track pathogen transmission, and understand the health impact of the BE microbiome on human health.

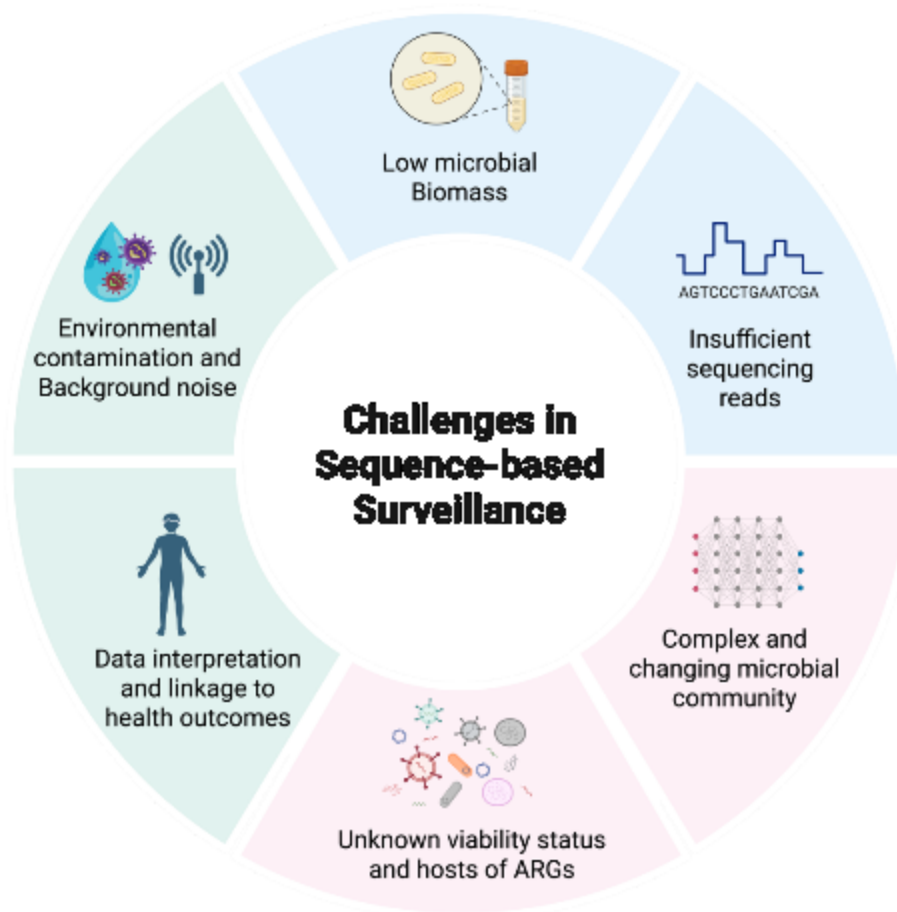


Figure 5.1. Challenges of sequence-based surveillance in built environment microbiomes.

5.2.1. Technical and Analytical Challenges

5.2.1.1. Low microbial biomass

Surfaces of BE often exhibit low microbial biomass due to the inhospitable conditions associated with these surfaces, making them a challenging type of sample for metagenomics (Gibbons, 2016). Low-biomass samples are defined as those containing only 10^2 to 10^4 cells/mL (Shen et al., 2021). To produce sufficient DNA for sequencing, PCR amplification is often necessary. Library preparation for low-biomass samples often involves PCR steps, which can introduce biases that distort microbial community representation (Silverman et al., 2021). Specifically, primer biases may lead to uneven amplification of the microbial community, resulting in the underrepresentation or loss of microbial diversity (Johnson et al., 2019). Despite these drawbacks, PCR amplification remains a cornerstone in sequence-based surveillance, especially for detecting low-abundance targets.

To balance the need for sufficient DNA and the preservation of microbial diversity, studies suggest limiting PCR cycles to less than 15 to minimize amplification bias (Kelly et al., 2019). Alternatively, target enrichment using oligonucleotide probes could also enhance signals from rare targets while reducing interfering signals from non-target DNA (Kantor & Jiang, 2024), thereby improving the sensitivity and accuracy of metagenomic profiling in low-biomass BE samples.

5.2.1.2. Contamination

Low-biomass samples are particularly prone to contamination owing to their low microbial content, which can be easily overwhelmed by extraneous DNA from environmental or laboratory sources (Shen et al., 2021). Contaminations in reagents and laboratory environment are ubiquitous and can critically impact sequence-based microbiome studies (Salter et al., 2014). They can be introduced to metagenomic samples at multiple stages,

including sample collection, nucleic acid extraction, and library preparation (Eisenhofer et al., 2019; Fierer et al., 2025). Specifically, these contaminants can distort the relative microbial abundances, confound epidemiological analyses, and hinder strain-level tracking efforts (Laurence et al., 2014; Eisenhofer et al., 2019). In low-biomass samples, even a minute amount of contaminants can significantly skew the results, particularly when the target signals approach the limit of detection (Eisenhofer et al., 2019).

To minimize contamination from surrounding air and surfaces, laboratory workbenches should be UV-irradiated or cleaned with DNA-degrading agents prior to sample processing. Incorporation of negative controls and no-template controls (NTC) alongside samples is essential to identify potential contamination sources. Furthermore, post-sequencing computational tools can be employed to filter out contaminating reads from metagenomic datasets, enhancing the accuracy of microbial and resistome profiling (Davis et al., 2018).

5.2.1.3. Sequencing limitations

Metagenomic sequencing, while a powerful tool for characterizing BE microbiomes, can introduce errors and biases to the data that lead to inaccurate representations of microbial composition and abundance (Bharti & Grimm, 2021). A critical factor influencing data quality is sequencing depth and coverage, which directly affect the ability to detect low-abundance taxa (Zaheer et al., 2018; Gweon et al., 2019). Currently, there is no consensus on the optimal sequencing depth for BE microbiome studies, owing to the diverse and complex microbial communities across different environments (Pereira-Marques et al., 2019). Indeed, microbiome studies often suffer from insufficient sequencing depth (Bharti & Grimm, 2021). This is particularly pernicious to public health surveillance aiming to distinguish pathogens from closely related non-pathogenic strains (Shen et al., 2021).

Given the high variability in BE microbiomes, establishing a universal sequencing depth cutoff is impractical. To address this, incorporating mock community standards during sample processing and analysis can effectively identify and correct biases introduced during sequencing and bioinformatic analysis (Kool et al., 2023). In addition, conducting rarefaction analysis during the pilot phase of the study can help determine the optimal sequencing depth required for comprehensive microbiome and resistome profiling (Zaheer et al., 2018; Gweon et al., 2019). These approaches will ensure sufficient coverage to capture low-abundance taxa or other targets while balancing sequencing and computational constraints.

5.2.1.4. Lack of viability status and ARG host identification

Metagenomic approaches provide a comprehensive overview of microbial communities and resistomes in BE. However, they often omit critical information in translating findings into public health insights.

Most metagenomic studies did not report on the viability status of detected pathogens, which is essential for accurately assessing infection risks. The inclusion of signals from non-viable cells can lead to overestimation of health threats. Viability-linked metagenomics using promidium monoazide (PMA) addresses this by selectively penetrating cells with compromised cell membranes (i.e., non-viable cells), binding to DNA, and thereby preventing PCR amplification of DNA from non-viable cells (Nocker et al., 2007). This method has been successfully applied to low-biomass environments, such as hospitals (Shen et al., 2022), the International Space Station (Singh et al., 2018), and clean room environments (Vaishampayan et al., 2013; Weinmaier et al., 2015). However, PMA-based metagenomic studies have limitations. Factors like matrix complexity (e.g., low number of viable cells or high debris content) and microbial diversity (e.g., spore-forming bacteria, variable cell membrane

thickness, or target gene lengths) can influence PMA efficiency in differentiating viable and non-viable reads, potentially leading to inaccurate viability assessment (Y. Wang et al., 2021).

Another critical gap is the missing linkage of ARGs detected to their bacterial hosts, which is essential in studying AMR evolution and transmission, and identifying important hotspots for ARG transmission (Cave et al., 2021). This limitation arises from short-read sequencing, where fragmented contigs (~100 to 300 bp) preclude the resolution for ARG-host assignment. Recent advances in long-read sequencing, however, enable species-resolved ARG detection, providing greater insights into the ecological and public health significance of ARGs (Arango-Argoty et al., 2019; Slizovski et al., 2022; Chen et al., 2025).

5.2.2. Complexity and Dynamics of the Built Environment Microbiomes

Unlike human or food microbiomes, which often exhibit relatively stable resident microbial communities, BE microbiomes are highly dynamic, characterized by transient taxa that vary spatially and temporally. This variability results in inconsistent microbial profiles, obscuring the detection of underlying correlations and meaningful patterns in microbial composition and ARG distribution.

As discussed in section 1.4.1, the variability in BE microbiomes arises from multiple factors, including geographical location, architectural design, surface material type, humidity levels, and the degree of human activities. These elements collectively create a complex and constantly evolving microbial landscape, posing challenges for standardizing sampling and analysis protocols. To address this variability, longitudinal sampling is critical to capture temporal fluctuations and spatial heterogeneity, enabling a more comprehensive understanding of the BE microbiome and resistome.

5.2.3. Difficulties in Result Interpretation and Linkage to Human Health

Connecting metagenomic findings from the BE microbiome to health outcomes is inherently challenging due to the multifaceted factors that shape environmental microbiomes. These variables contribute to dynamic microbial communities, making it difficult to establish consistent correlations between microbiome composition and health impacts. Moreover, establishing causality is complicated by the ecological complexity of BE, where multiple interacting factors obscure direct links to disease transmission. Furthermore, the absence of comprehensive clinical and epidemiological data, such as records of nosocomial or community outbreaks, further limits the ability to interpret BE microbiome data in a public health context (Fairchild et al., 2018; Harel et al., 2018).

Molecular epidemiology addresses part of these challenges by integrating genomic profiles of clinical and environmental isolates with epidemiological data. For example, a study in a hospital combined WGS of carbapenem-resistant *K. pneumoniae* isolates from patients and patient-transfer data to trace the transmission and identify high-touch facilities as key reservoirs (Snitkin et al., 2017). Likewise, during pandemics involving rapidly mutating viruses, SNP-based phylogenetic analyses can resolve strain-level transmission chains, enhancing the precision of outbreak tracing (Houwaart et al., 2022). Adopting such an integrative approach can bridge metagenomic findings with health outcomes, facilitating more precise identification of transmission risks in BEs.

5.3. The Interdisciplinary Study of the Built Environment Microbiome

Understanding the transmission of pathogens and antimicrobial determinants in BE requires more than identifying the types and quantities of microorganisms present. The BE microbiome is a complex and dynamic ecosystem that is intrinsically shaped by the interplay between physical conditions, human activities, and spatial dynamics (McAlister et al., 2025). Consequently, characterizing MDROs and ARGs within the BE context necessitates an interdisciplinary perspective, integrating insights from architecture, microbiology, cartography, and epidemiology.

5.3.1. Architectural Designs and the Built Environment Microbiome

Architectural design significantly impacts the BE microbiome by altering indoor environmental conditions, such as temperature, relative humidity, and air flow (Kembel et al., 2012). Specific design features, including space type, building layout, patterns of human activity and movement, and ventilation sources, substantially shape microbial communities (Kembel et al., 2014). Conventional architectural designs often minimize natural elements like soil and plants, thus reducing the influx of environmental microbes (Bosch et al., 2024).

Over the past two decades, biologically informed architectural design has emerged as a strategy to alter the indoor microbiome and is increasingly recognized as an integral component of the healthy building movement (Brown et al., 2016; Green, 2014). This approach, extending from the principles of evidence-based medicine, utilizes scientific evidence to inform design decisions, particularly in healthcare settings (Pati, 2011). For instance, maximizing natural ventilation and integrating daylight exposure in building design will harbor indoor microbiomes with a lower incidence of pathogens (Joseph et al., 2025). Ventilation experiments conducted in university classrooms have further corroborated the idea that

ventilation strategies directly impact the composition of indoor air microbiota (Meadow, et al., 2014).

Bio-informed design incorporates microbial communities into the planning of buildings and urban spaces, which support the establishment of beneficial microbial communities. Achieving this requires interdisciplinary collaboration among architects, microbiologists, anthropologists, and epidemiologists, who bring diverse perspectives on spatial, temporal, and scalar dynamics (Bosch et al., 2024).

5.3.2. Genomics and Metagenomics approaches

Contemporary genomic and metagenomic approaches offer unparalleled insights into the BE microbiome, enabling a comprehensive analysis of the microbiome communities and their resistome. WGS enables the acquisition of complete genomes from environmental strains, facilitating phylogenetic analysis with clinical isolates to signify potential community outbreaks. Metagenomics, meanwhile, delivers species-level identification of all microorganisms in a sample, but also the detection of important genes, namely ARGs, MGEs, virulence factors, functional genes, and phage-host interactions. These approaches provide an in-depth analysis of both the indoor microbiome and resistome.

Metagenomic studies have been instrumental across diverse indoor environments, including healthcare facilities, public transport, and school campuses (Zolfo et al., 2018). For instance, metagenomic profiling of hospital sinks and wastewater has revealed these sites as significant reservoirs of pathogens associated with hospital-acquired infections (HAIs) (Silvester et al., 2025). Similarly, metagenomics has revealed the widespread presence of ARGs in urban mass transit systems (Danko et al., 2021). These studies have demonstrated the effectiveness of metagenomics in detecting pathogens and ARGs within BE. These studies also highlight the ability of metagenomics to map spatial and temporal microbial dynamics,

geographic distribution patterns, and the influence of anthropogenic factors (e.g., occupancy, cleaning practices) on microbial communities. For example, metagenomic profiling of environmental samples from a hospital ward allowed the precise mapping of drug-resistant strains to specific locations and times, enabling actionable mitigation strategies (Chng et al., 2020).

Metagenomics also plays a pivotal role in early outbreak detection. For instance, viral metagenomics identifies fecal contamination in freshwaters, marine waters, foodstuff, soil, and air (Santiago-Rodriguez & Hollister, 2023) and novel viral pathogens potentially causing the next pandemic (Mohsin et al., 2021). Metagenomic profiling is recognized for its role in developing evidence-based strategies for managing urban microbiomes, which is crucial for public health, sustainability, and building science (Fierer et al., 2022). The study of two high-traffic indoor environments in this thesis further demonstrates the power of metagenomics in characterizing clinically important taxa and ARGs. Furthermore, its importance is recognized by a number of international initiatives aiming at improving indoor microbiomes. For instance, the Hospital Microbiome Project leverages metagenomics to optimize hospital designs for healthier microbial communities for both patients and staff (Smith et al., 2013), while the MetaSUB Consortium maps the city microbial landscapes and AMR dissemination using metagenomic approaches (Mason et al., 2016a).

As sequencing costs decline, metagenomics is poised to become the mainstay for pathogen and AMR surveillance (Ko et al., 2022). However, challenges concerning metagenomic studies as discussed in [section 5.2](#) must be addressed to unlock its full potential in pathogen and AMR surveillance.

5.3.3. Spatial and Temporal Dynamics of the Built Environment Microbiome

Understanding the spatial and temporal distribution of microbial communities in BE is critical for translating metagenomic findings into evidence-based public health interventions (McAlister et al., 2025; Shi et al., 2021). Despite its importance, research on the spatiotemporal relationships between microbes and their environments remains scarce. Indeed, such analyses could bring invaluable benefits like real-time assessment and prediction of infection risks to occupants of BE and through longitudinal monitoring of pathogens and ARGs in environments.

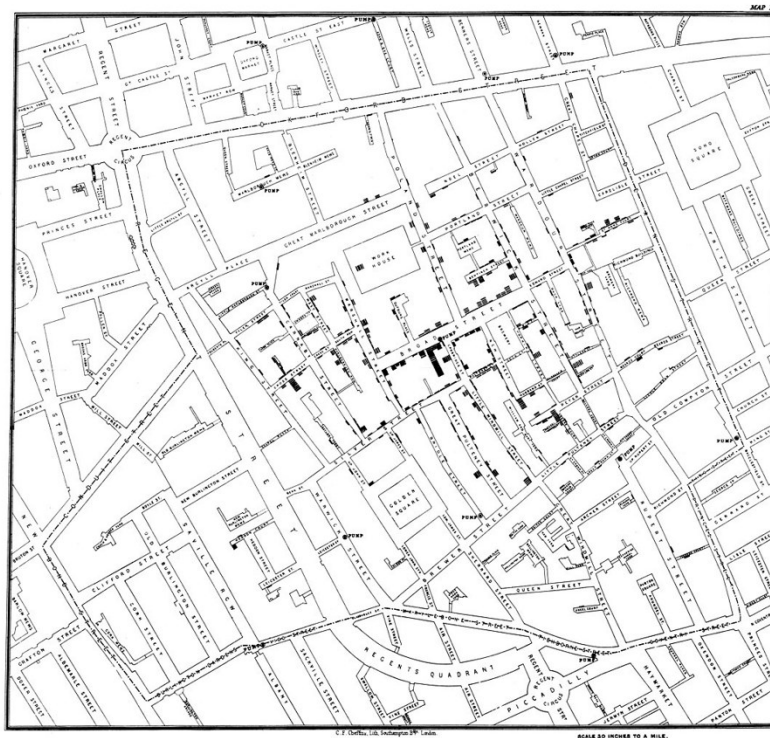
Spatial mapping has proven effective in elucidating microbiome distribution in BE. For instance, a hospital ward study identified environmental drug-resistant strains with high sequence identity to outbreak isolates, pinpointing multiple infection hotspots in a hospital ward and guiding targeted disinfection measures (Chng et al., 2020). This example highlights the value of spatial-temporal-phylogenetic analysis in outbreak investigation in the healthcare setting. Similarly, mapping of *mcr-1*-positive MDR *Enterobacteriaceae* clones from public transportation onto a city map revealed their concentration in hospital-dense areas (Shen et al., 2018). Similar patterns were observed for drug-resistant *Enterobacteriaceae* clones identified from shared bikes, which also showed clustering with hospitals (Zou et al., 2019). These studies have demonstrated the utility of spatial mapping in identifying potential transmission pathways in community settings.

Geographical Information Systems (GIS) offer transformative potential and analytical power for mapping pathogen dissemination and identifying disease outbreaks (Rogers & Randolph, 2003). In brief, GIS are spatial data management systems that integrate multiple layers of information in the same geographical area (Fradelos et al., 2014). A historical example is the seminal work by Dr. John Snow in 1854, who mapped cholera cases on the map of London's Soho district and revealed its waterborne transmission through water pumps (Snow, 1856) ([Figure 5.2](#)). More recently, GIS has been used to track COVID-19 spread (Al-Kindi et al., 2020) and monitor the spatial-temporal trends of antibiotic-resistant pathogens in

wastewater (Rahman et al., 2023). Recognizing their capabilities in identifying potential public health risks, many organizations, such as the CDC and WHO, leverage GIS to monitor disease spread, detect hidden transmission, and signify early outbreak signals (CDC, 2025; WHO, 2025).

In conclusion, GIS-based mapping of MDROs and ARGs in urban BE holds promise for identifying transmission hotspots and informing evidence-based building design and public health policies (Fradelos et al., 2014; Cromley, 2003; McLafferty, 2003). Despite this, challenges such as data standardization, computational scalability, and interdisciplinary expertise must be addressed to fully harness the potential of GIS (Fletcher-Lartey & Caprarelli, 2016). Spatiotemporally resolved microbiome and resistome analysis will therefore enhance future pathogen surveillance by enabling proactive interventions to mitigate public health risks in urban BE.

A



B

Explore the 1854 Cholera Outbreak in London

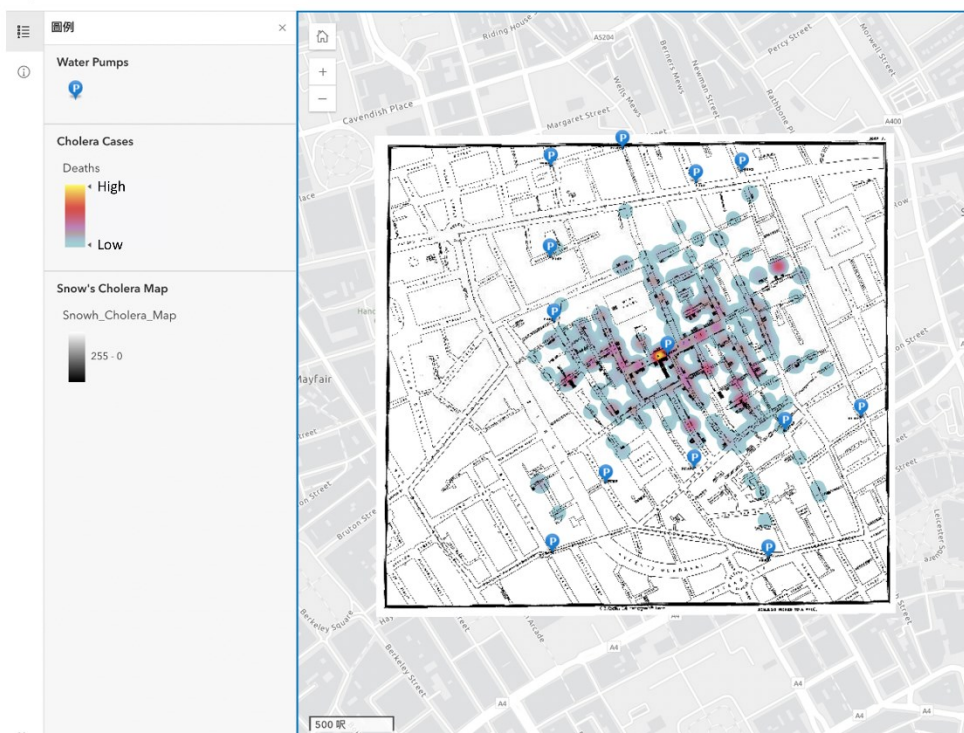


Figure 5.2. Example of GIS in outbreak investigation and public health: The 1854 cholera outbreak in the Soho district of London, UK.
(Figure description on next page.)

(A) The original map by Dr. John Snow mapping locations of cholera cases and water pumps, showing a strong correlation between cholera cases and the use of water from the pump, leading him to conclude that the disease was waterborne.

(B) Re-creation of Dr. John Snow's cholera map using modern GIS approach. Cholera cases are now represented using heatmap symbology for visualization of case density. Purple and yellow colors represent the lowest and highest case density, respectively. "P" represents locations of water pumps. The pump closest to the yellow zone with the highest case density is the Broad Street pump, identified by John Snow in his original map as the water pump responsible for the 1854 cholera outbreak. Adapted from:

<https://learn.arcgis.com/en/projects/map-a-historic-cholera-outbreak/>

5.3.4. One Health Approach in Pathogen and AMR Surveillance

The One Health framework is essential for the effective surveillance and management of AMR, as drug-resistant organisms can rapidly spread across healthcare settings, food systems, and environmental reservoirs (Velazquez-Meza et al., 2022). Therefore, the surveillance of AMR should be comprehensive and warrant multidisciplinary collaboration across the human, animal, and environmental sectors. This integrated framework will help elucidate how pathogens and AMR propagate across these interfaces. In view of its pivotal role in AMR surveillance, the WHO issued the Global Action Plan for AMR in 2015, emphasizing the importance of incorporating the One Health framework in surveillance programs (WHO, 2015).

Several studies have demonstrated the value of One Health surveillance. For example, a study in Canada employed a One Health approach to investigate *Enterococcus* isolates from clinical settings, urban wastewater, and food animals, identifying the prevalence of different species and ARGs from different sources, most likely driven by environmental adaptation (Zaheer et al., 2020). Another study in China examining *E. coli* and *S. aureus* from clinical and food animal sources found that some MDR strains emerged in food systems before appearing in human clinical samples, suggesting their transmission across the human-animal interface (Lv et al., 2024). These findings underscore the interconnected transmission of drug-resistant strains across different One Health sectors, highlighting the need for integrated surveillance.

5.4. Limitations of Study

5.4.1. Experimental limitations

As discussed in [section 5.2.1.1](#), PCR amplification is inevitable during library preparation, especially when working with low-biomass environmental samples. The trade-off will be the introduction of PCR biases into metagenomic data (Aird et al., 2011).

Such biases may result in the misclassification of taxonomy and the misassignment of ARGs, leading to an inaccurate reflection of the true microbiome and resistome. However, due to the low biomass of direct environmental samples, PCR amplification is indispensable to enhance signals from low-abundance gene targets. To mitigate PCR biases, the highest possible fidelity polymerases and the minimum number of PCR cycles should be used in microbiome studies (Sze & Schloss, 2019). Additionally, using a slower ramping rate would further mitigate the effect of PCR bias, especially on GC-rich regions (Aird et al., 2011). Likewise, mixed and imbalanced GC content of the microbial community also aggravates the problem. Therefore, extended denaturation times should be warranted to ensure complete denaturation of GC-rich regions (Aird et al., 2011). In conclusion, for future microbiome analysis, employing fewer than 15 PCR cycles, using a slower ramping rate, and ensuring prolonged denaturation times should be implemented to minimize PCR biases.

5.4.2. Computational limitations

Recent developments in sequencing technologies have led to an exponential increase in bioinformatic tools. However, several challenges in computational analysis persist.

In this study, microbial taxa were identified using two taxonomic classifiers, Kraken2 and MetaPhlAn4, did not correlate with each other, suggesting discrepancies between taxonomic classifiers. This observation could be attributed to the different algorithms and pre-

downloaded databases used by different tools. The choice of different taxonomic classifiers reduces the reproducibility and diminishes inter-study comparability. This issue could be partly addressed by evaluating bioinformatic protocols using a mock microbial community (i.e., a fixed and known percentage of mixed populations). Therefore, the choice of bioinformatic tools at each processing step should be made conscientiously.

Likewise, the accurate identification of ARG in metagenomic genomes relies not only on the performance of the bioinformatic tool used, but also the ARG database utilized for profiling. However, studies benchmarking different AMR prediction tools using various algorithms and databases yield very discrepant results (Feldgarden et al., 2019; Papp & Solymosi, 2022). This could be attributed to the lack of a consensus on the best practice for ARG identification from genomic data (Mahfouz et al., 2020), leading to discrepancies in ARG identified and misinformation in the literature (Djordjevic et al., 2024). Therefore, standardization of prediction algorithms and databases used for ARG predictions for future AMR surveillance is necessary.

To overcome the limitations of experimental and bioinformatic procedures, the evaluation and optimization of protocols using standard mock communities should be warranted. Inclusion of a mock community as a positive control when processing each batch of samples, just as is already done in clinical practice, should also be adopted to reduce batch effects and ensure experimental and bioinformatic procedures are effective and efficient.

5.4.3. Limitations of Metagenomics

Metagenomic studies are prone to several limitations that impact their ability to assess microbial risks in urban BE.

Firstly, an unbiased shotgun metagenomic workflow cannot distinguish viable and non-viable microorganisms, which is critical in assessing pathogen infectivity. Inclusion of non-viable cells may lead to overestimation of pathogen load and the infection risks.

Secondly, ARGs detected from metagenomic data cannot be traced back to their species of origin due to the short-read nature of the sequencing platform used in this study. Methods like the *in vivo* proximity-ligation by Hi-C, which physically links plasmids to chromosomes before nucleic acid extraction, can overcome this limitation (Stalder et al., 2019). Furthermore, long-read sequencing has successfully correlated resistome and mobilome to their host species, contributing to AMR evolution and source attribution (Slizovskiy et al., 2022)

Thirdly, metagenomic detection of human pathogenic taxa and clinically important ARGs alone does not provide direct evidence of immediate health risks to BE occupants. As mentioned above, the viability status of pathogens detected in metagenomic data was unknown, and there is also a lack of information on the host species of these clinically important ARGs. Whether these pathogenic determinants cause any measurable effects on passengers' health remains to be elucidated. Epidemiological studies should be warranted in future studies to understand the clinical relevance of exposure to urban BE microbiome.

Finally, neither shotgun metagenomics nor the target probe capture approach can differentiate between pathogenic and non-pathogenic strains within detected taxa, as it lacks strain-level resolution for virulence genes or pathogenic marker genes. This constraint reduces the clinical interpretability of metagenomic data.

5.5. Future Directions

5.5.1. Construction of Phylogeographic Database for MDROs and ARGs in Urban Built Environments

To translate metagenomic insights into actionable public health strategies, the development of a comprehensive phylogeographic database for MDROs and ARGs in urban BE is proposed. This database would serve as a centralized repository, integrating diverse datasets to facilitate advanced surveillance and predictive analysis. The database would incorporate phylogeographic elements, combining phylogenetic relationships with geospatial mapping to track the spatiotemporal dynamics of AMR in urban settings. Key information layers would include (i) genomic profiles of MDROs and ARGs (e.g., presence of high-risk ARGs), (ii) epidemiological data, such as outbreak incidence, infection clusters, and antimicrobial usage patterns, (iii) demographic information, including population density, socioeconomic factors, and (iv) geospatial information of healthcare-associated facilities, alongside the locations and identities of MDROs and ARGs detected.

Such a database would empower real-time monitoring of outbreaks by identifying the potential sources and transmission networks of circulating MDROs in the community through an interactive dashboard (e.g., ArcGIS), enabling public health practitioners to pinpoint high-risk hotspots. Additionally, longitudinal surveillance data could be leveraged to develop prediction models that identify potential outbreaks before infection cases emerge, allowing timely mitigation measures to be implemented.

5.5.2. Investigation of MDRO and AMR in Other Built Environments

Expanding the surveillance of MDRO and AMR to other BE is essential for comprehensive pathogen monitoring and safeguarding the public health.

Healthcare-associated settings are prone to MDRO colonization and dispersal, posing substantial health risks to their occupants. Other than hospitals, where routine environmental sampling is established, other healthcare-associated facilities, such as residential care homes for the elderly (RCHEs), nursing homes, and outpatient clinics are recognized hotspots for pathogen transmission. Surveillance of nursing homes in the U.S. has revealed that over half of the residents harbor MDROs of clinical and public health significance (McKinnell et al., 2020). In HK, more than 30% of RCHE residents were detected with MRSA (H. Chen et al., 2018). Alarming, the prevalence of MDROs in HK's RCHEs has surged from 2.8% to 48.7% between 2005 and 2021 (Cheng et al., 2025). Moreover, *C. auris* was found to persist on over 20% of room surfaces in nursing facilities, highlighting its environmental persistence (Sansom et al., 2024). Therefore, routine surveillance in these settings is essential to prevent pathogen spillover into the broader community, mitigating public health risks (Kanamori et al., 2023).

In non-healthcare settings, high-risk environments, such as schools, daycare centers, and recreational facilities, are also critical surveillance targets. Daycare centers, for example, are prone to frequent outbreaks of hand-foot-and-mouth disease (HFMD), influenza, and acute gastroenteritis caused by pathogens like norovirus and *Shigella* (Mattison et al., 2022, pp. 2009–2020). Given limited resources, prioritizing surveillance in high-risk BE, including RCHEs, nursing homes, schools, and community centers, should be warranted. The vulnerable populations, including children and the elderly with compromised immunity, may serve as entry points for pathogens and AMR into widespread circulation within the community (Kumar et al., 2024). Routine surveillance in these settings can identify potential hotspots for pathogen spillover from healthcare to community environments, enabling timely interventions to curb MDRO and AMR transmission.

5.6. Concluding remarks

From the Spanish Flu to COVID-19, the race between humanity and pathogens remains ongoing, with the silent pandemic of AMR increasingly undermining our antibiotic arsenal, rendering infectious diseases, especially those caused by MDROs, more difficult to treat. Urban BE, where the majority of the global population spends most of their time, thus becomes a quintessential interface where pathogens and AMR disseminate. Consequently, surveillance in these environments is of paramount importance to safeguard public health.

This study focuses on two high-risk urban BE in a metropolitan city, the subway system and public washrooms, offering a comprehensive framework for MDRO and ARG surveillance in urban environments. Subway systems, as high-traffic mass transit hubs, facilitate microbial exchange among passengers from diverse geographic and demographic backgrounds, making them widely regarded as potential hotspots for pathogen transmission. Public washrooms, particularly the post-handwashing surfaces, were identified as reservoirs of MRSA and may play a role in their spread. The detection of clinically significant MDROs and ARGs in both urban BE warrants the need for routine surface sampling to monitor and mitigate the emergence of potential outbreaks within the community.

By employing metagenomic approaches, this study advances pathogen surveillance in urban BE through a scalable and robust workflow for the spatially-resolved characterization of MDROs and ARGs, overcoming limitations like low microbial mass while enhancing the detection of low-abundance yet clinically relevant targets. For future studies, leveraging novel advancements and cross-disciplinary approaches, such as AI and GIS, will help put forward metagenomic findings. As sequencing costs decline, it is envisaged that metagenomics will revolutionize the toolbox for MDRO and AMR surveillance, ultimately leading to a paradigm shift in surveillance strategies to strengthen pandemic preparedness and protect public health in urban BE.

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Supplementary Materials

Supplementary Data

Supplementary Data 1. Impact of sequencing depth, human read removal and genome assembly on taxonomic classification and ARG profiling of metagenomic reads.

To determine the optimal sequencing depth and post-sequencing bioinformatic analysis algorithm for urban metagenomic data, a subsampling experiment was performed using metagenomic data from three subway stations: CIO, HUH, SSP. The proposed bioinformatic analysis was then performed on the subsampled metagenomic data as illustrated in **Supplementary Figure S1A**.

To study the effect of sequencing depth on taxonomic classification and ARG profiling of urban metagenomic data, sequencing reads from the three subway stations were randomly subsampled ($n = 3$) using seqtk to the following percentages of the original sequencing depth: 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% and 90%. The number of species identified were plotted against the percentages of sequencing depth in a rarefaction curve (**Supplementary Figure S1B, C**). None of the rarefaction curves reached a plateau, indicating that a higher sequencing depth would be required to identify all species present in the metagenome. However, rarefaction curves of route A (i.e. without genome assembly) showed the tendency to level off at around 65% of total reads, which is equivalent to approximately 11 million reads. This is much higher than the study by Gwoen et al., which reported that only 1 million reads were sufficient to achieve full taxonomic composition. Notably, taxonomic classification without genome assembly identified a greater number of species than rarefied reads with genome assembly (**Supplementary Figure S1B, C**). For rarefied reads undergoing genome assembly (i.e. Route B and C), two assemblers, MEGAHIT and metaSPAdes, were compared in terms of the total number of species classified. A higher number of species were identified from assemblies by metaSPAdes compared to that of MEGAHIT.

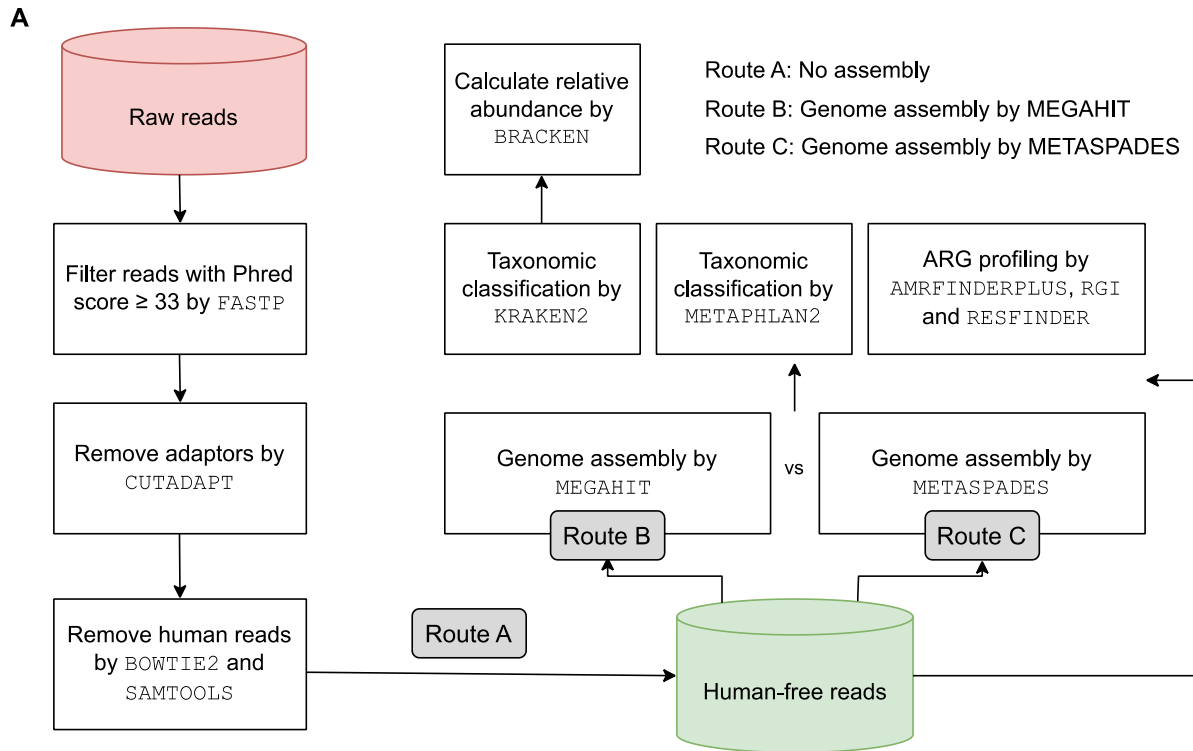
For ARG profiling, all rarefaction curves did not reach a plateau or show tendency to level off, suggesting that a much higher depth is required in order to capture all ARG present in metagenomic sample (**Supplementary Figure S1D**). It was shown in studies of other environmental samples that, at least 60-80 million reads per sample were required to recover the full richness of different AMR gene families (Gwoen et al., 2019; Zaheer et al., 2018). The much higher read depth for ARG profiling than taxonomic classification could be

attributed to the fact that ARG is present in few copies within a cell, whereas the entire genome of the cell can be utilized for taxonomic classification. The number of ARGs identified from assemblies obtained from the two assemblers, MEGAHIT and metaSPAdes, were comparable (**Supplementary Figure S1D**).

Next, the effect of human read removal on taxonomic classification and ARG profiling was evaluated (**Supplementary Figure S2A**). All rarefaction curves from metagenomic data with or without human read removal overlapped completely (**Supplementary Figure S2B-D**). This suggested that human read removal did not affect taxonomic classification and ARG profiling from metagenomic data.

Lastly, the choice of taxonomic classifiers was assessed by testing two classifiers, Kraken2 and metaPhlAn2. As shown in rarefaction curve, Kraken2 identified a greater number of species than metaPhlAn2. Despite this, metaPhlAn2 resulted in a higher proportion of classified reads than Kraken2 (**Supplementary Figure S3**). In view on this, two taxonomic classifiers were used simultaneously for side by side comparison in all metagenomic analyses.

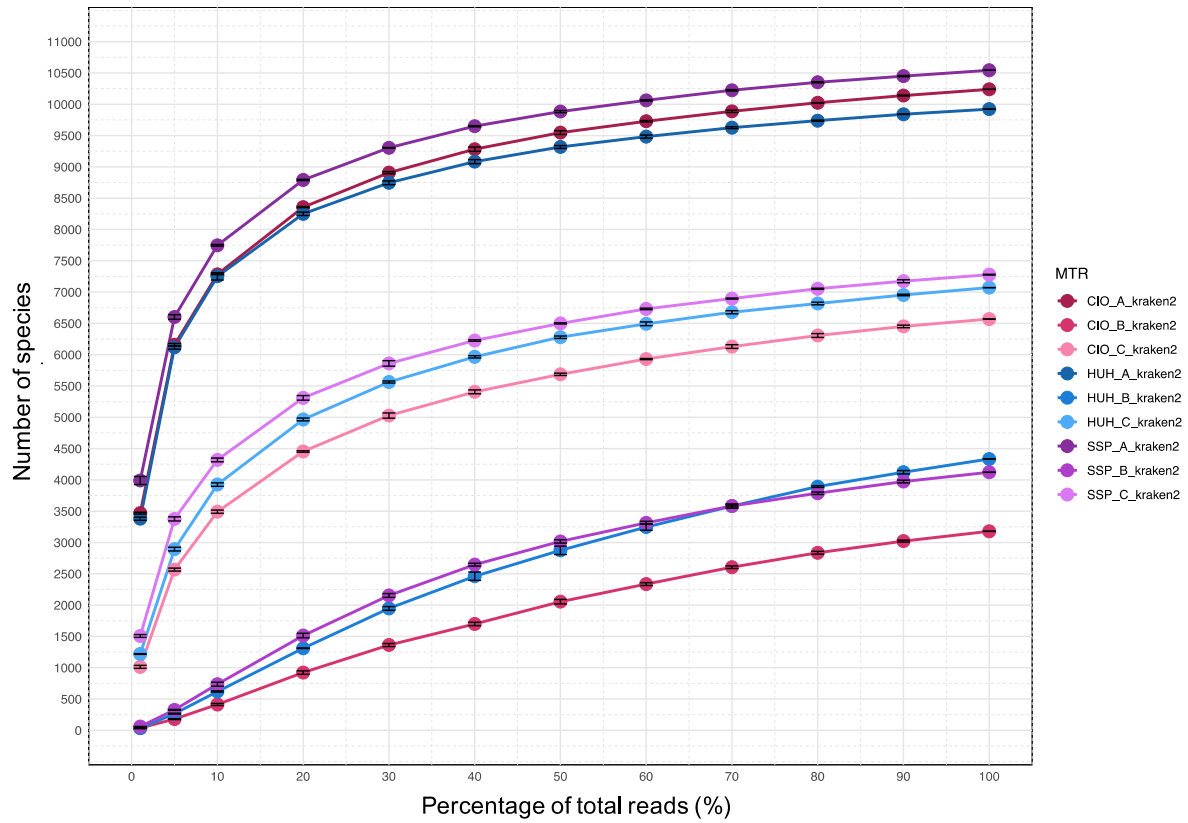
In conclusion of the subsampling experiment, taxonomic classification would be performed from raw metagenomic reads using both Kraken2 and metaPhlAn2 after the human read removal in subsequent analysis. While ARG profiling would be performed after genome assembly with metaSPAdes.



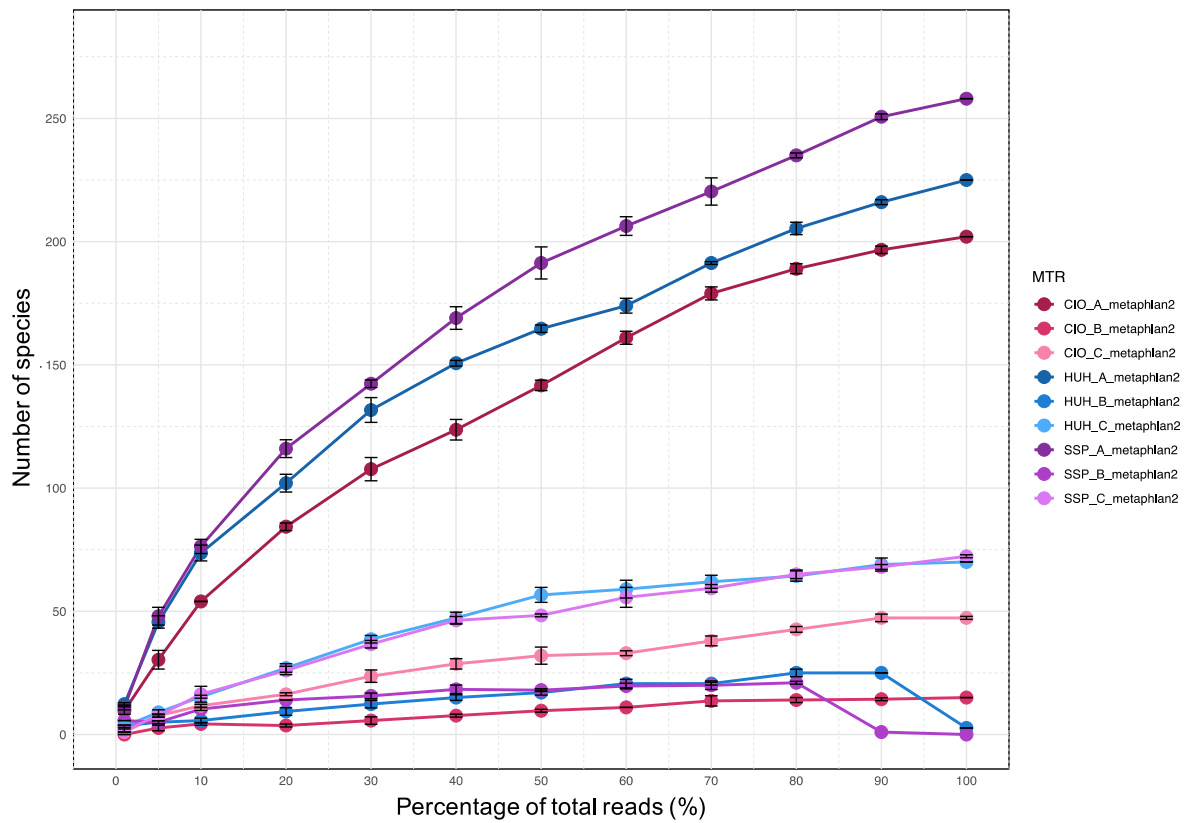
Supplementary Figure S1. Optimization of bioinformatic analysis workflow for metagenomic data analysis - Subsampling experiment determines the optimal sequencing depth and bioinformatic analysis pipeline (with human reads removal).

(A) The proposed bioinformatic analysis algorithm (with human reads removal). Analysis of the subsampled and human-read removed raw metagenomics reads from 3 stations, CIO, HUH and SSP, were processed following 3 different routes. Route A bypasses genome assembly and directly perform taxonomic classification on raw reads, while Route B and C undergo genome assembly by two assemblers, MEGAHIT and METASPADES, respectively before taxonomic classification and ARG profiling were performed.

B Number of species identified by KRAKEN2 at different percentages of reads subsampled

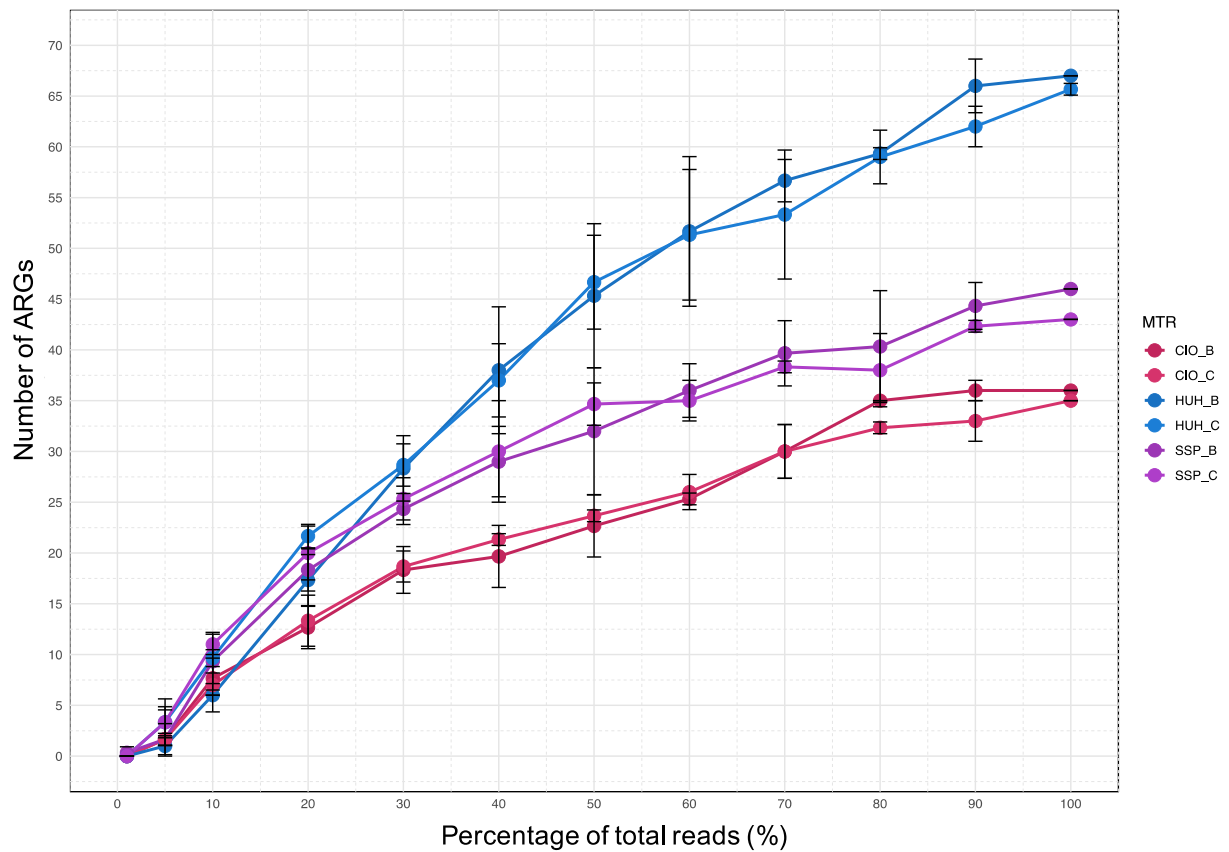


C Number of species identified by METAPHLAN2 at different percentages of reads subsampled



D

Number of ARGs identified different percentages of reads subsampled

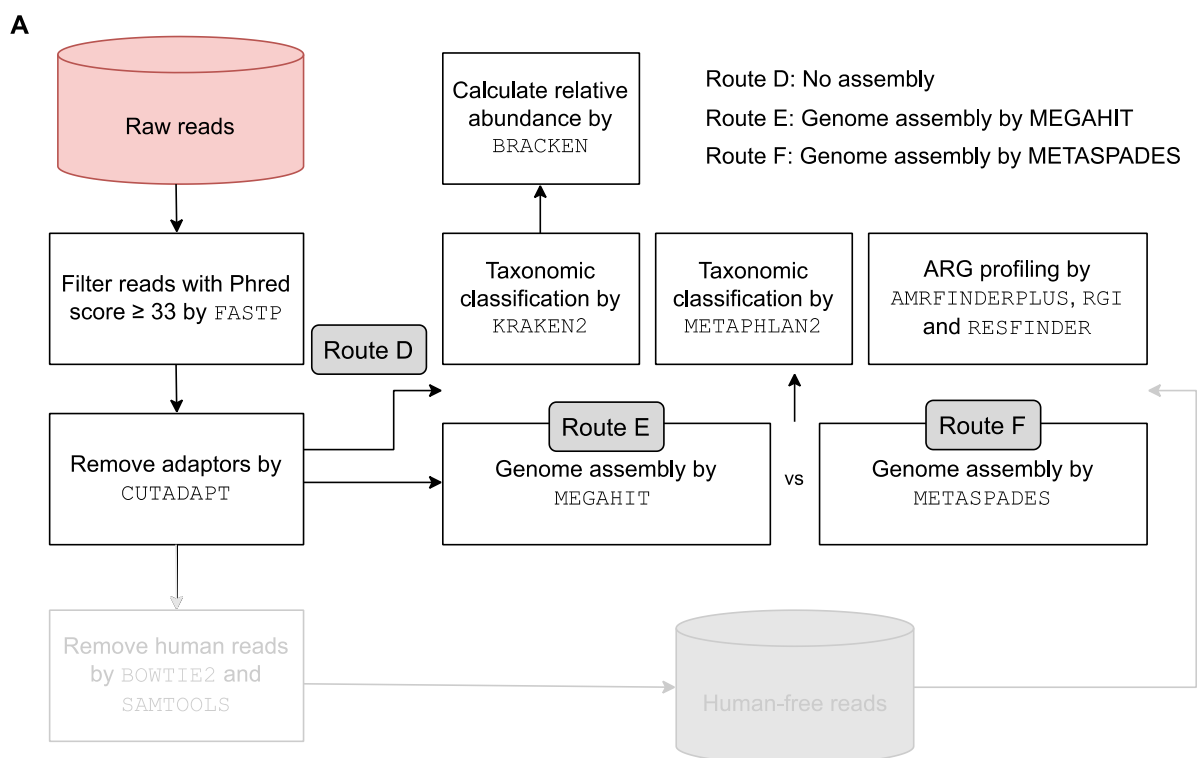


Supplementary Figure S1. (continued) Optimization of bioinformatic analysis workflow for metagenomic data analysis - Subsampling experiment determines the optimal sequencing depth and bioinformatic analysis pipeline (with human reads removal).

(B) Rarefaction curve plotting the number of species identified by Kraken2 against the percentage of total reads rarefied.

(C) Rarefaction curve plotting the number of species identified by MetaPhlAn4 against the percentage of total reads rarefied.

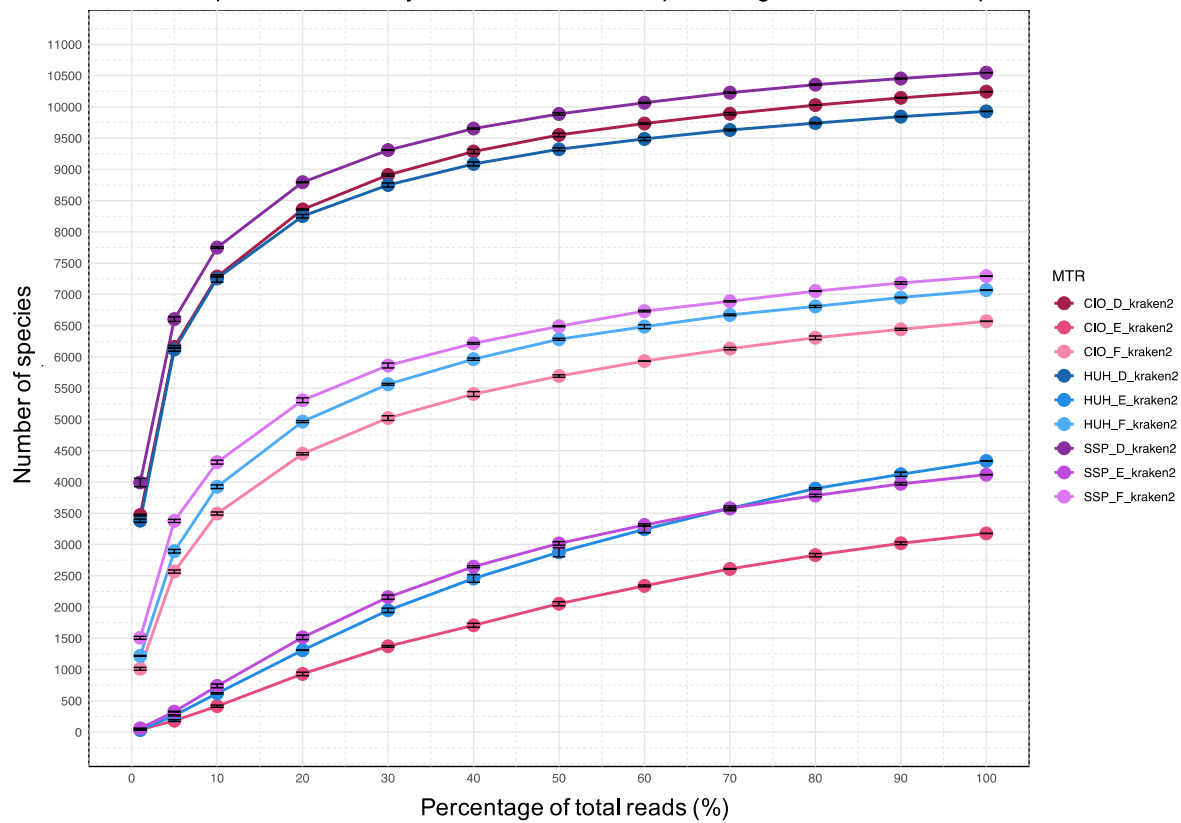
(D) Rarefaction curve plotting the number of ARGs identified against the percentage of total reads rarefied.



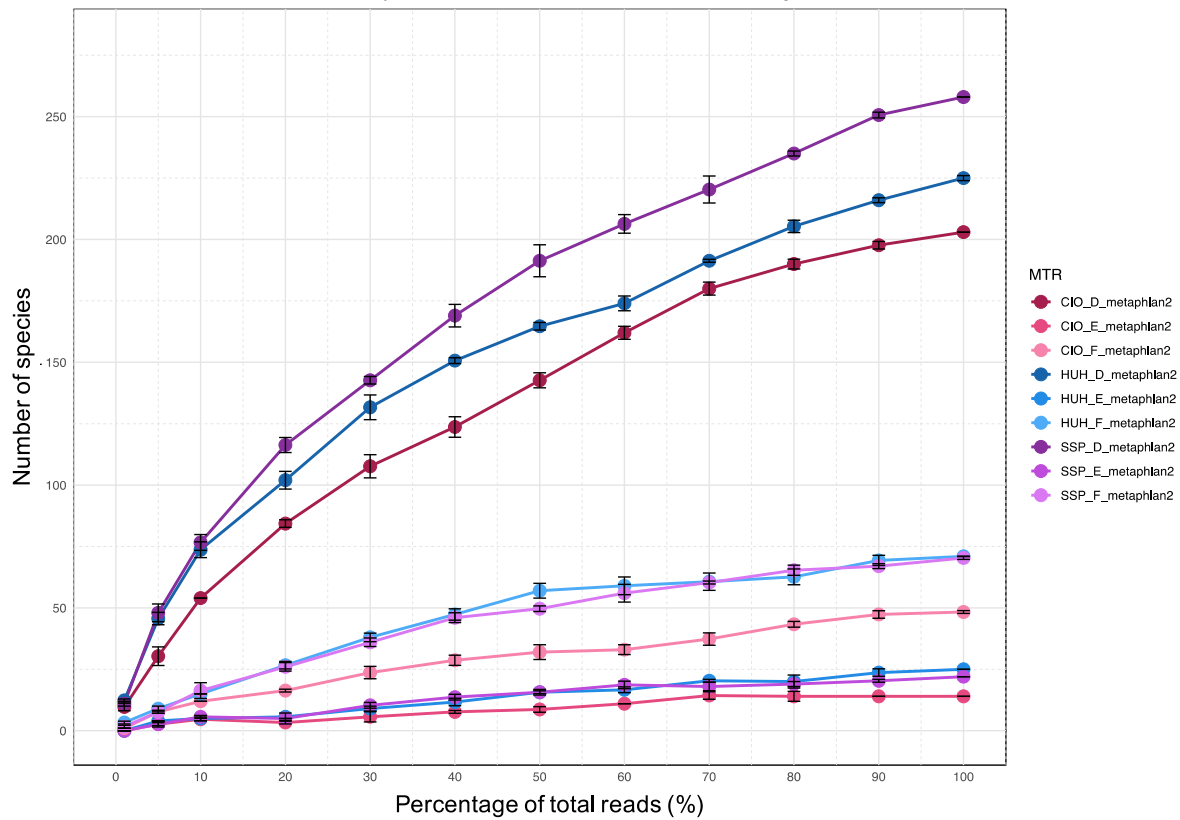
Supplementary Figure S2. Optimization of bioinformatic analysis workflow for metagenomic data analysis - Subsampling experiment determines the optimal sequencing depth and bioinformatic analysis pipeline (without human reads removal).

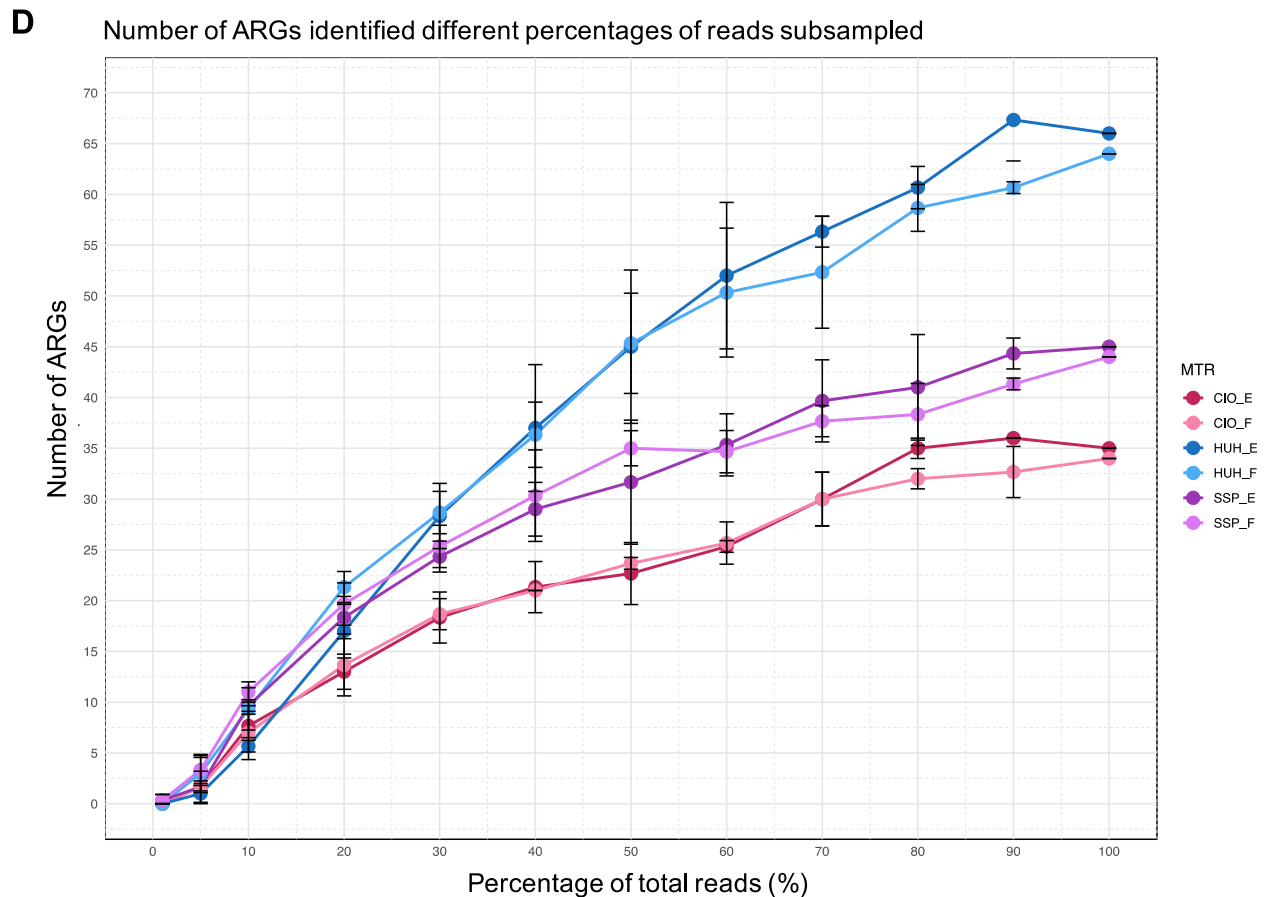
(A) The proposed bioinformatic analysis algorithm (without human reads removal). Analysis of the subsampled raw metagenomics reads from 3 stations, CIO, HUH and SSP, were processed following 3 different routes. Route D bypasses genome assembly and directly perform taxonomic classification on raw reads, while Route E and F undergo genome assembly by two assemblers, MEGAHIT and METASPADES, respectively before taxonomic classification and ARG identification were performed.

B Number of species identified by KRAKEN2 at different percentages of reads subsampled



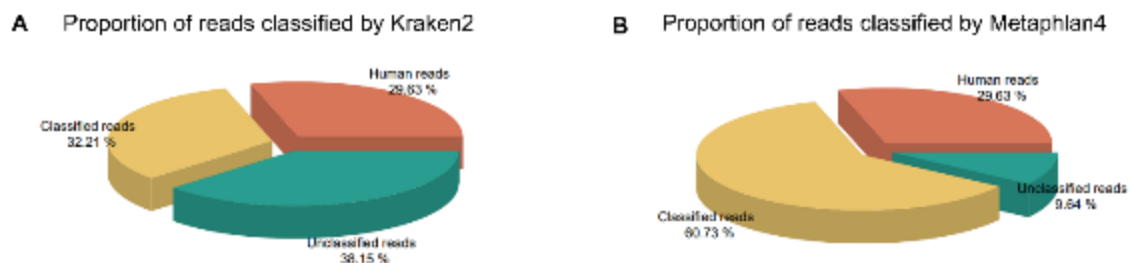
C Number of species identified by METAPHLAN2 at different percentages of reads subsampled





Supplementary Figure S2. (continued) Optimization of bioinformatic analysis workflow for metagenomic data analysis - Subsampling experiment determines the optimal sequencing depth and bioinformatic analysis pipeline (without human reads removal).

(B) Rarefaction curve plotting the number of species identified by Kraken2 against the percentage of total reads rarefied. (C) Rarefaction curve plotting the number of species identified by metaPhlAn2 against the percentage of total reads rarefied. (D) Rarefaction curve plotting the number of ARGs identified against the percentage of total reads rarefied.



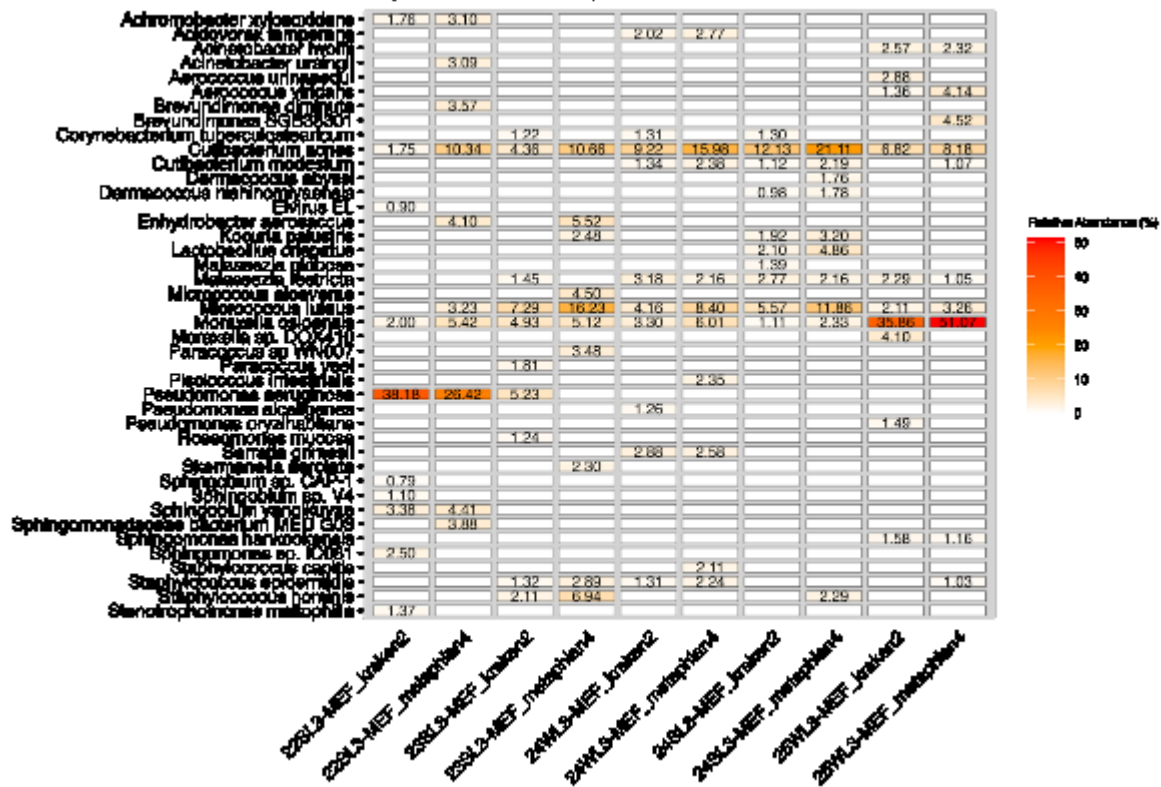
Supplementary Figure S3. Proportion of human reads, classified reads and unclassified reads by KRAKEN2 and METAPHLAN2 of metagenomic reads from Hong Kong Subway Stations

Average human reads, classified reads and unclassified reads by (A) Kraken2 and (B) MetaPhlAn4.

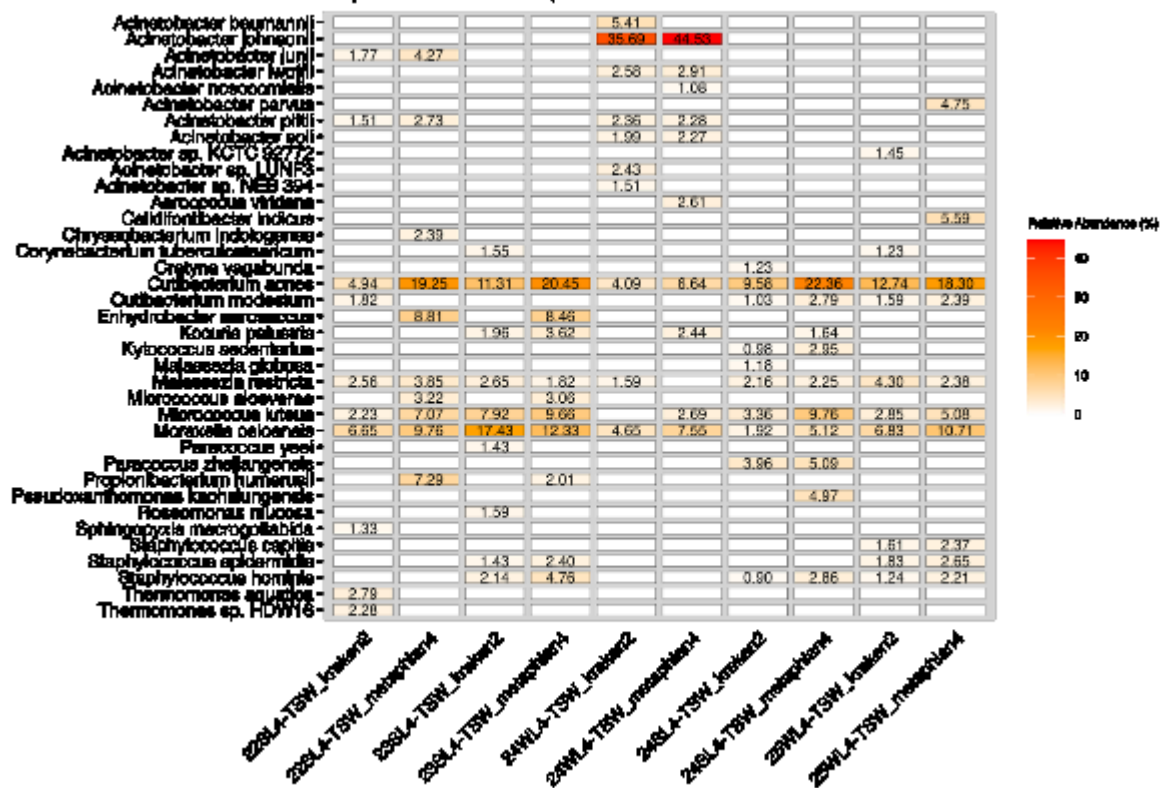
Supplementary Data 2. Top 10 dominant species of individual subway station.
(Supplementary Figure S4.1-40)



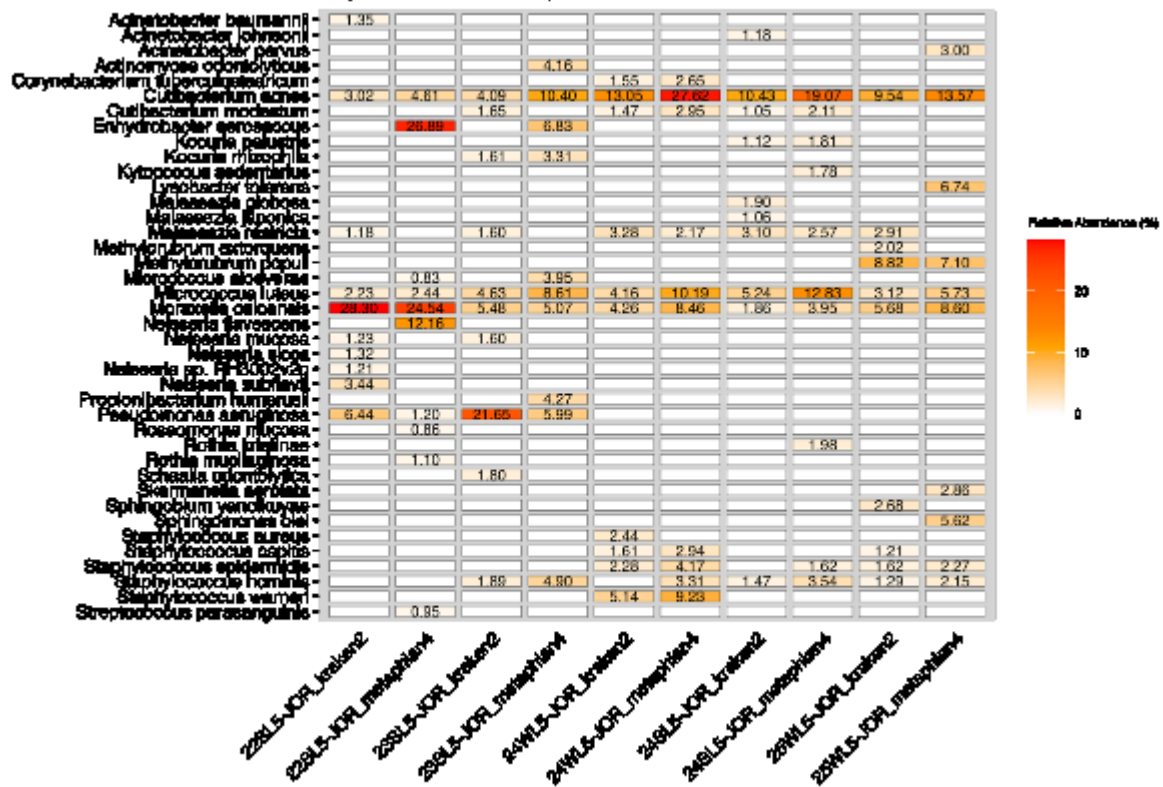
Top 10 Dominant Species of L3-MEF



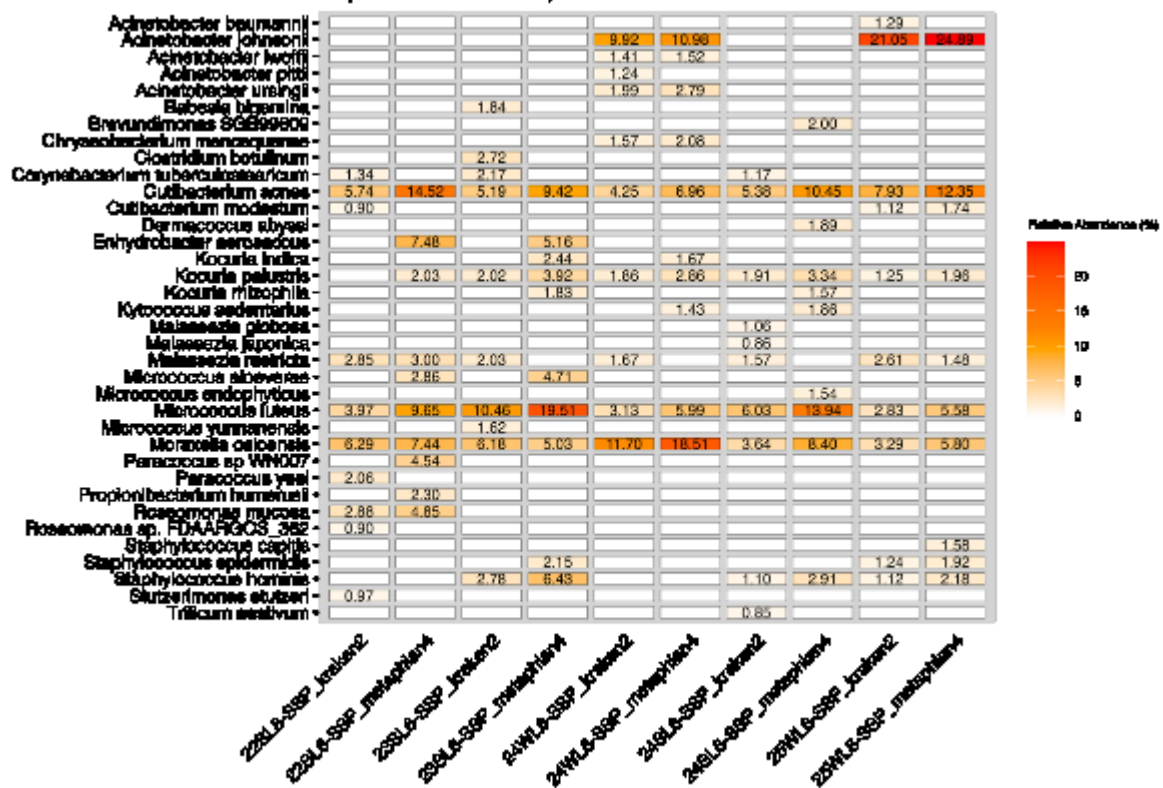
Top 10 Dominant Species of L4-TSW



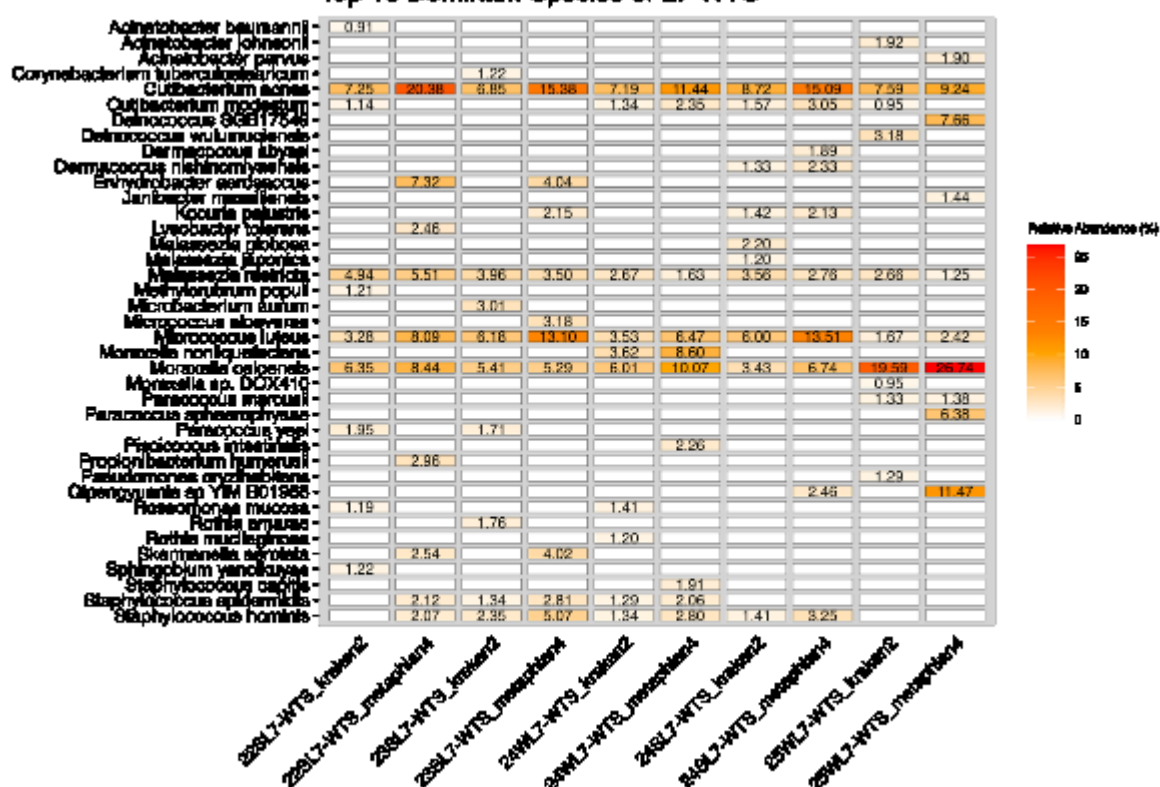
Top 10 Dominant Species of L5-JOR



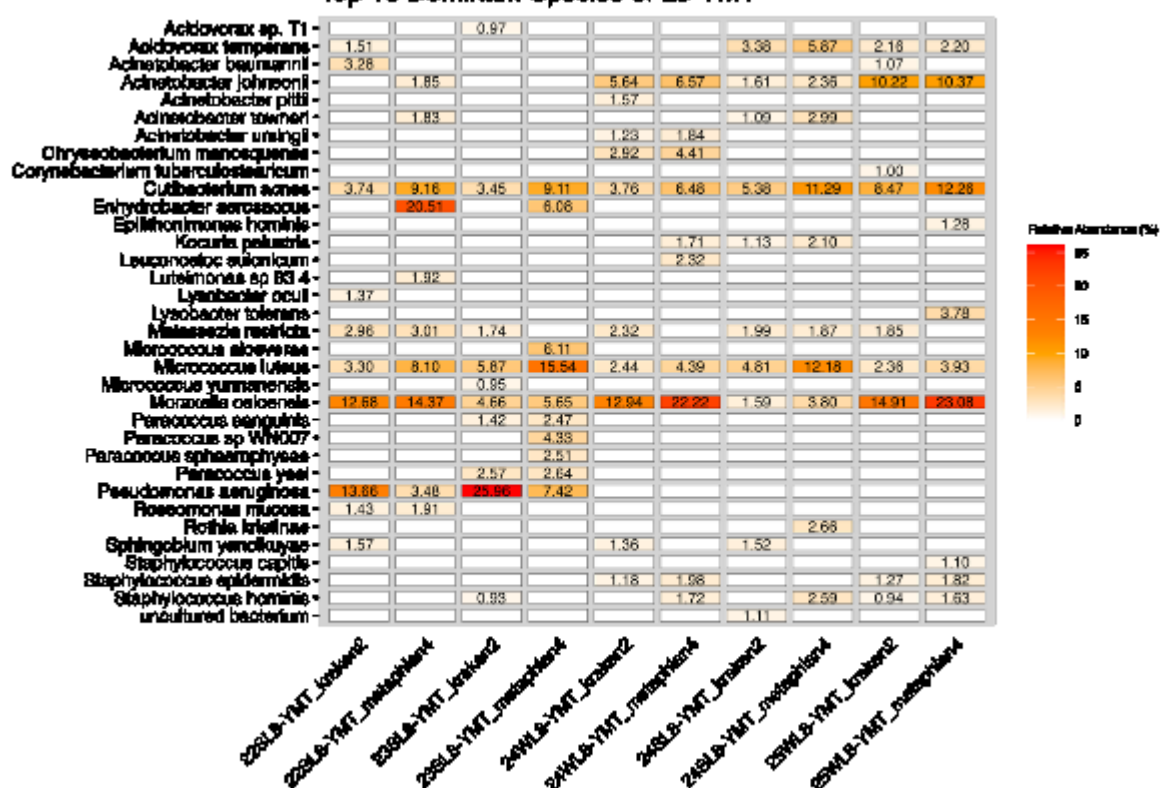
Top 10 Dominant Species of L6-SSP



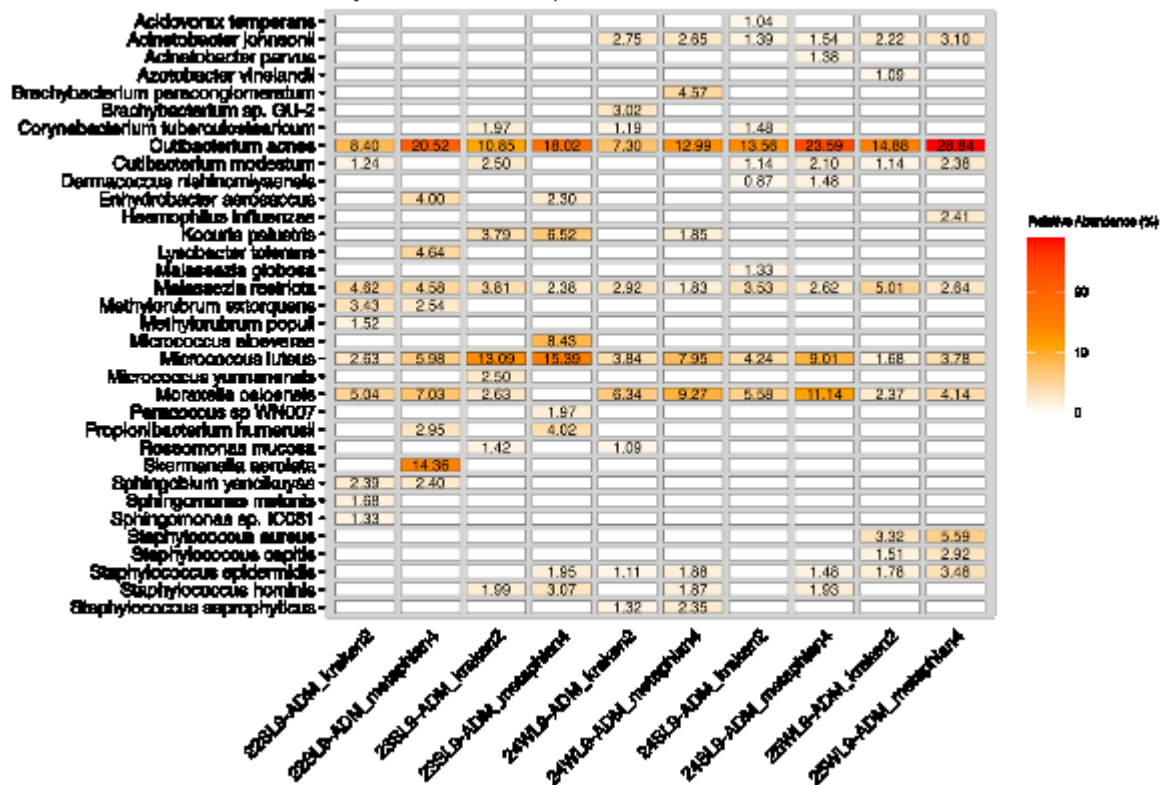
Top 10 Dominant Species of L7-WTS



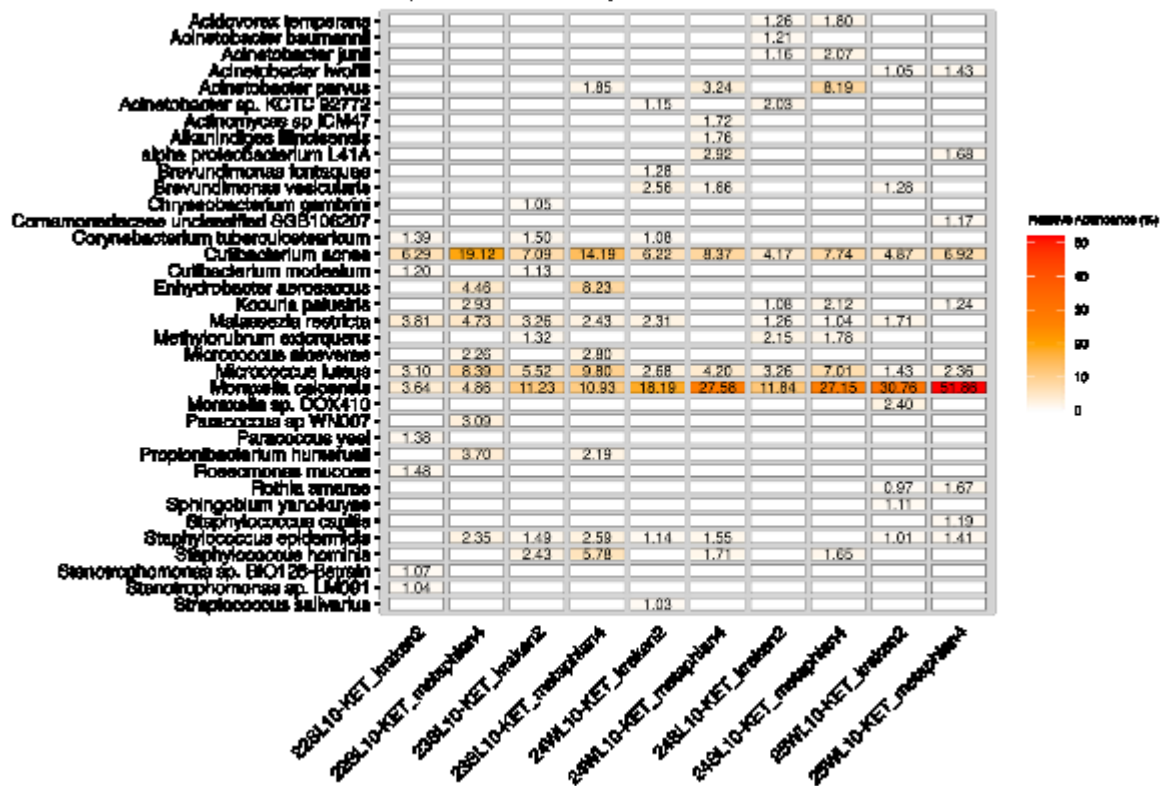
Top 10 Dominant Species of L8-YMT



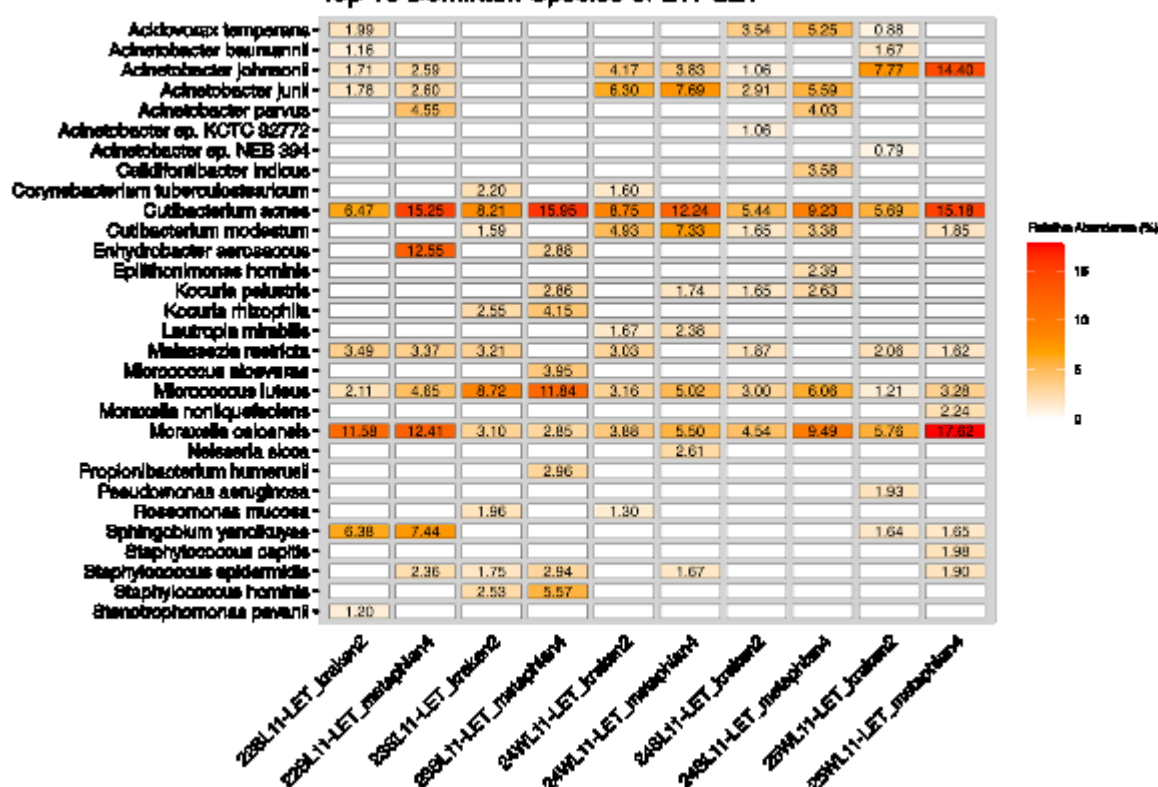
Top 10 Dominant Species of L9-ADM



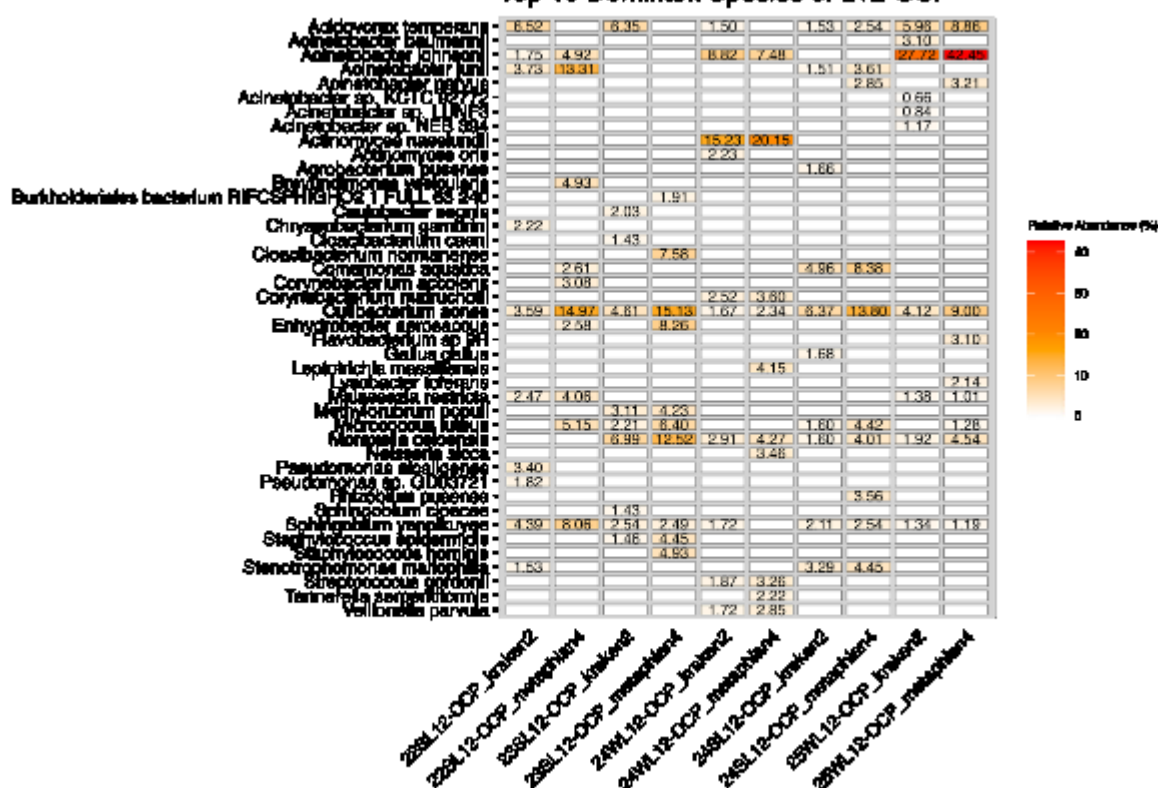
Top 10 Dominant Species of L10-KET



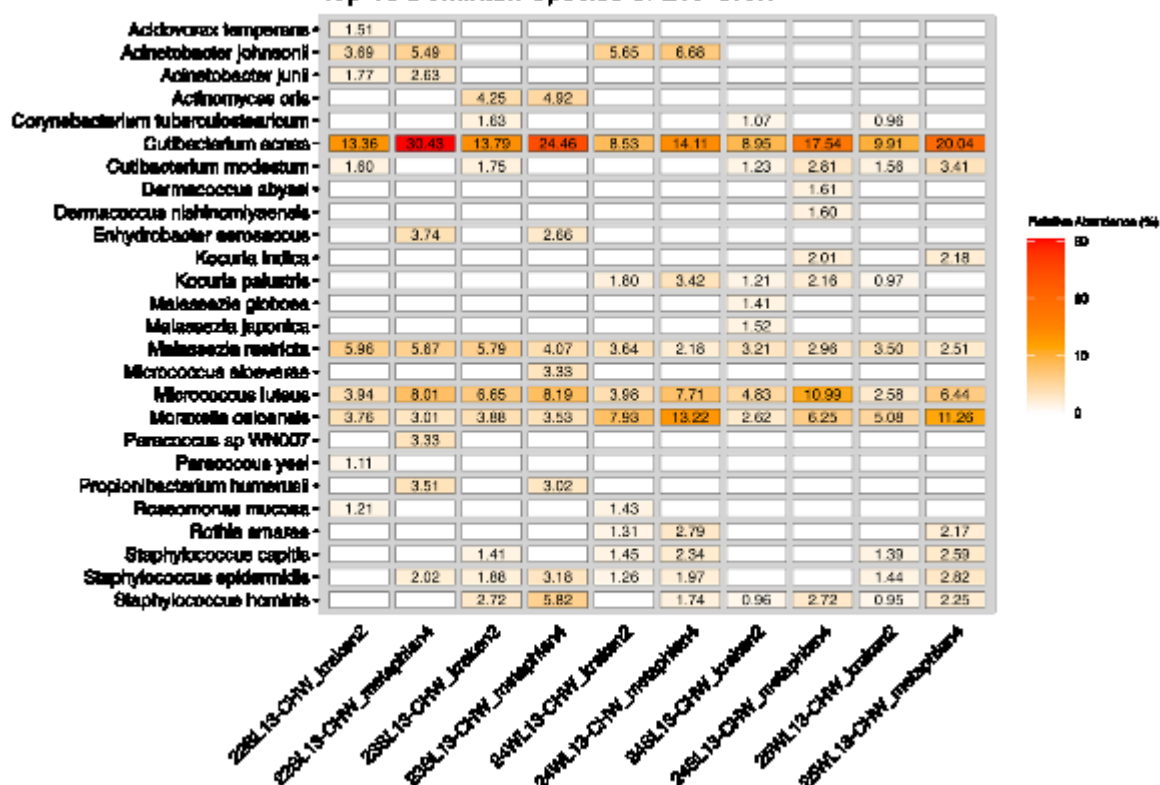
Top 10 Dominant Species of L11-LET



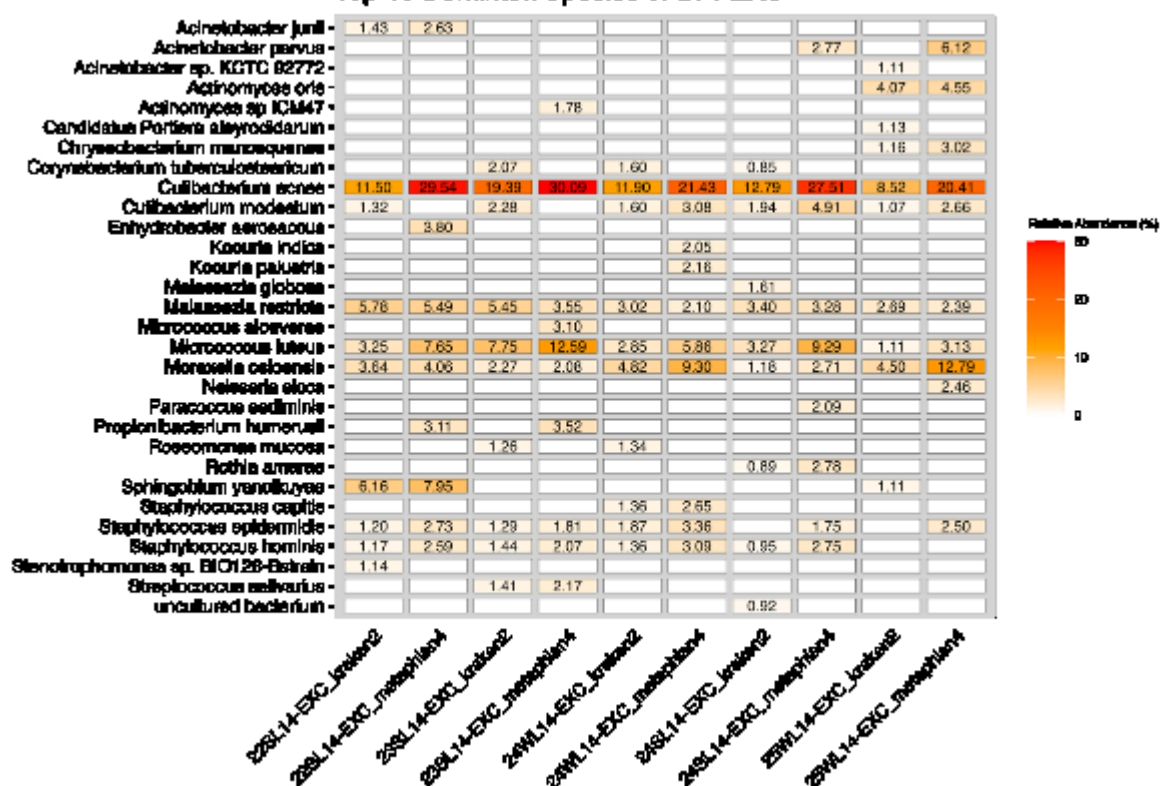
Top 10 Dominant Species of L12-CCP



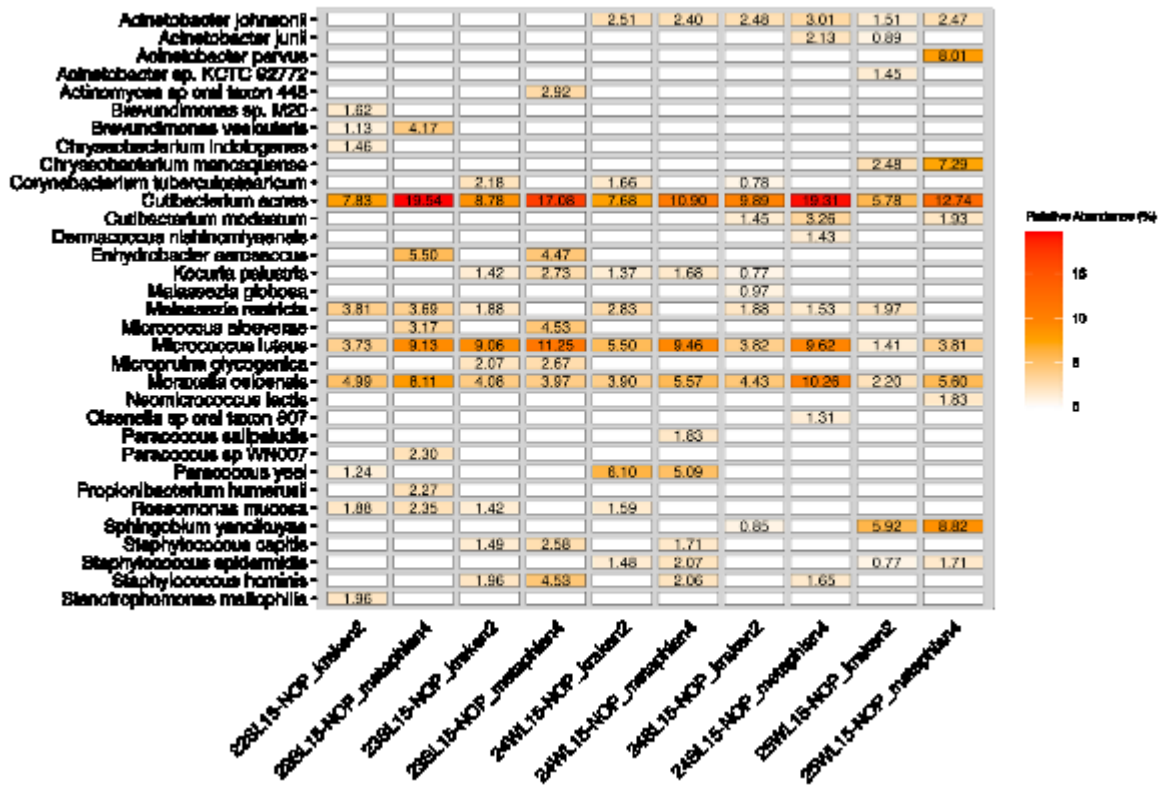
Top 10 Dominant Species of L13-CHW



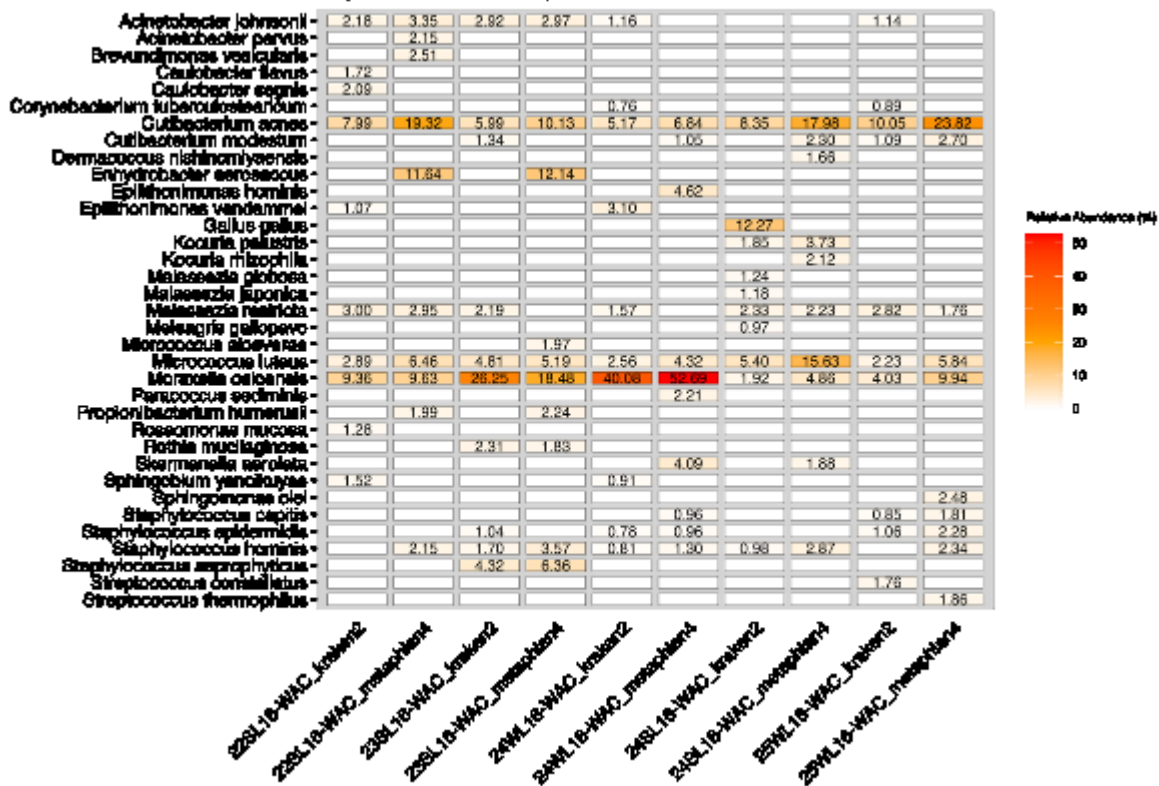
Top 10 Dominant Species of L14-EXC



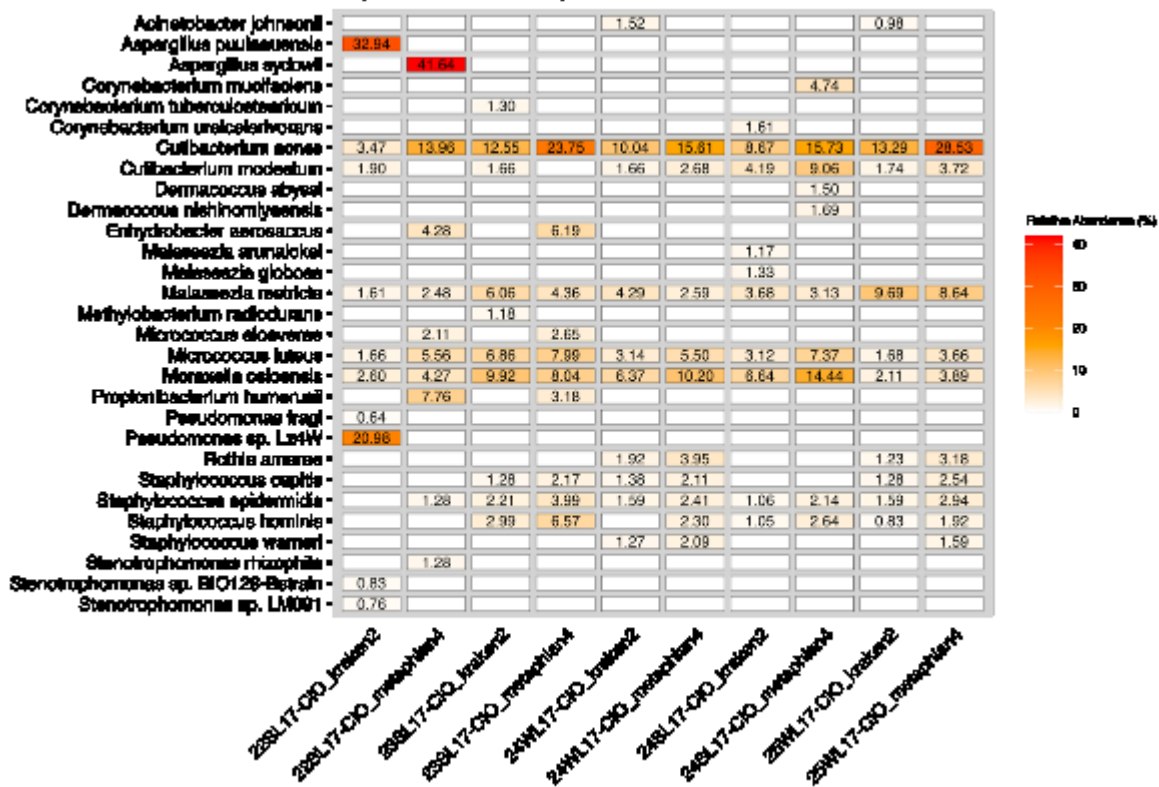
Top 10 Dominant Species of L15-NOP



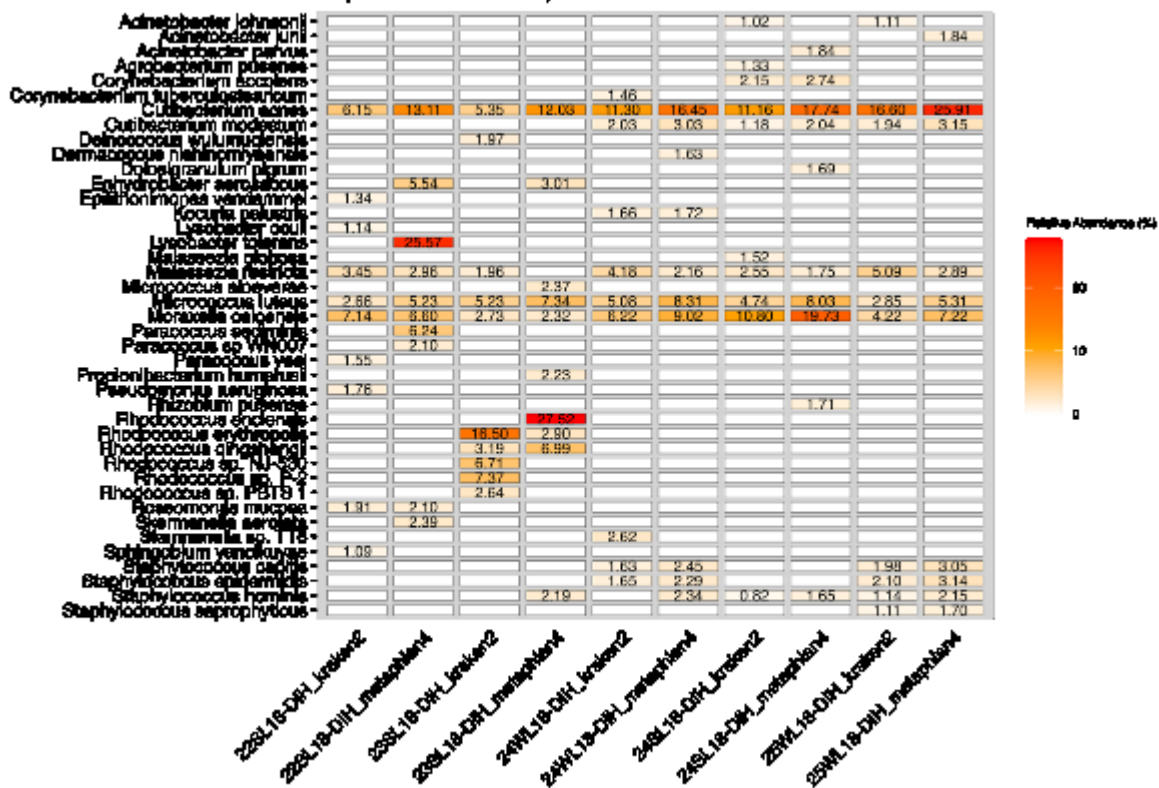
Top 10 Dominant Species of L16-WAC



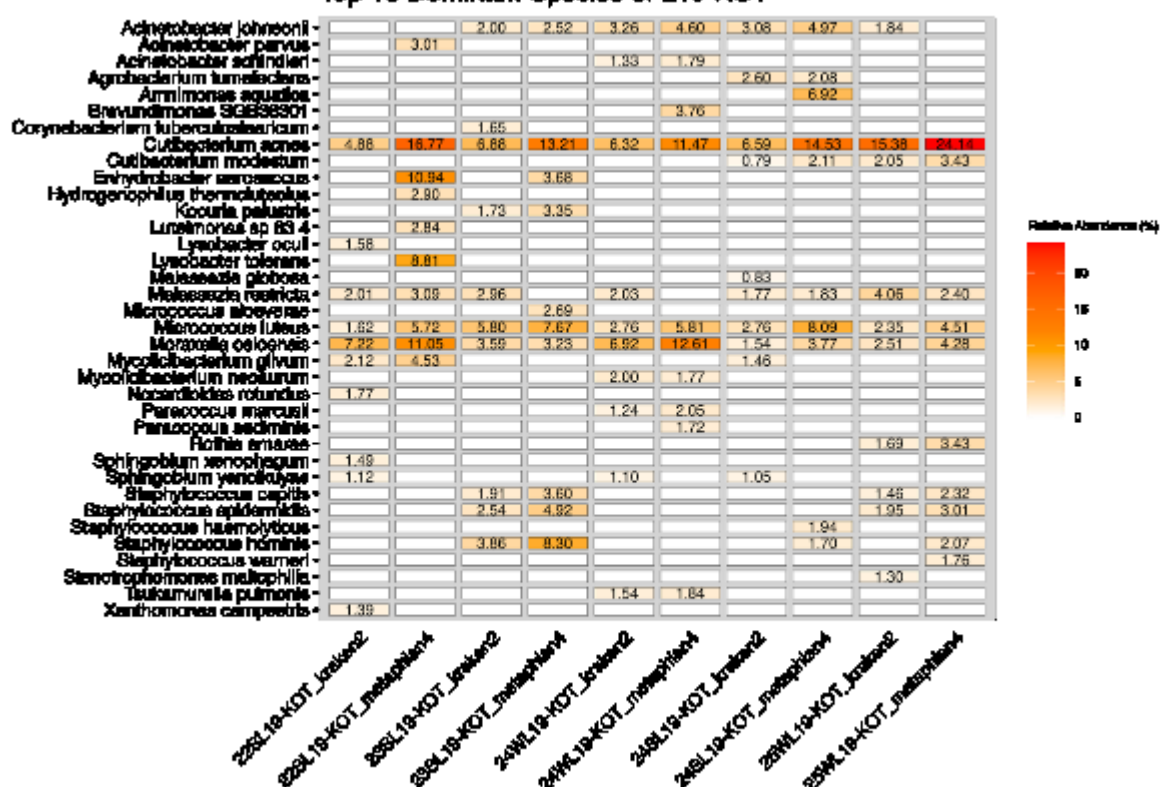
Top 10 Dominant Species of L17-C10



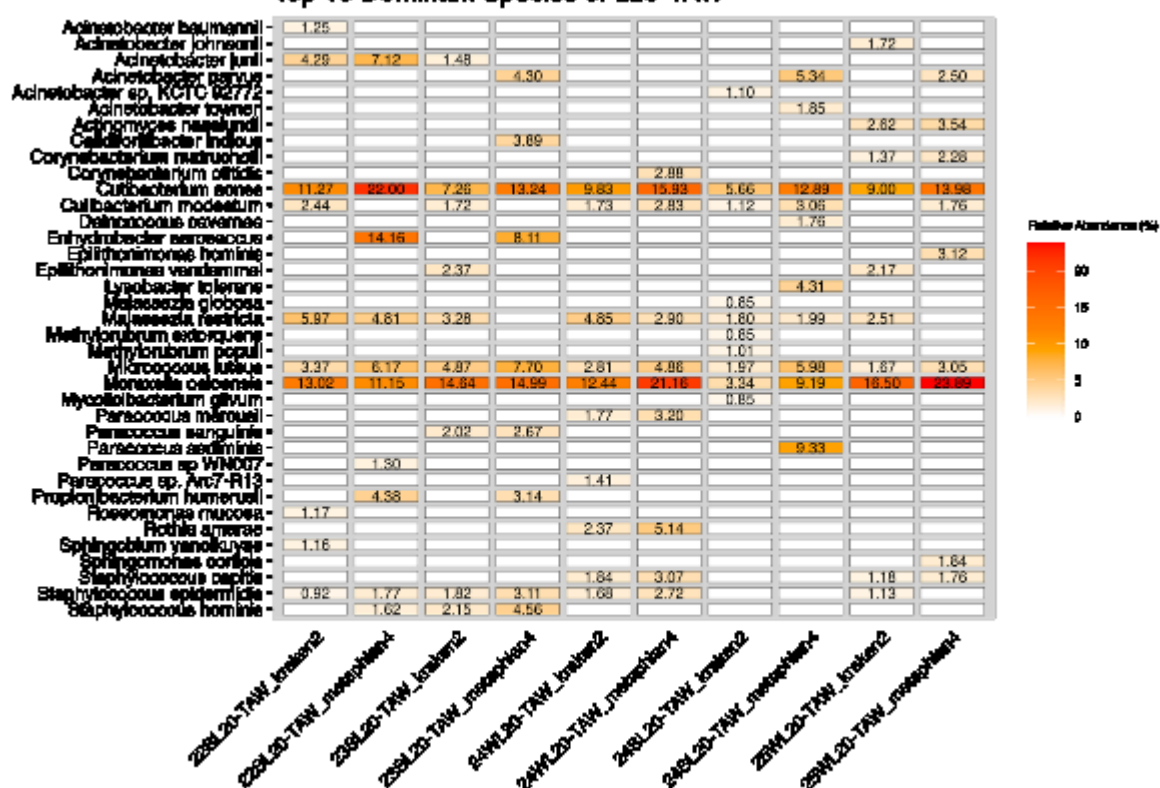
Top 10 Dominant Species of L18-D1H



Top 10 Dominant Species of L19-KOT



Top 10 Dominant Species of L20-TAW



Top 10 Dominant Species of L21-HAH

<i>Acinetobacter junii</i>	1.84	3.55							
<i>Acinetobacter parvus</i>						1.69			
<i>Actinomyces sp. oral taxon 446</i>						2.00			
<i>Corynebacterium tuberculoaerarium</i>	1.16		1.68	1.34			0.82		
<i>Cutibacterium acnes</i>	7.60	20.47	9.05	15.97	5.38	8.40	15.35	26.71	9.15
<i>Cutibacterium modestum</i>			1.14				1.98	4.18	1.19
<i>Demacoccus abyssi</i>								1.37	
<i>Demacoccus nishinomiyaensis</i>					0.88		0.94	1.79	
<i>Enhydrobacter aerococcus</i>		5.82		3.57					
<i>Escherichia coli</i>	1.26								
<i>GAB2722 GAB3553</i>								1.97	
<i>Kocuria indica</i>						2.11			
<i>Kocuria palustris</i>					1.24	1.70	0.77	1.27	1.23
<i>Makaseezia restricta</i>	2.88	3.01	3.53	2.31	1.76		2.44	1.98	3.08
<i>Micrococcus elovexensis</i>				2.61					1.75
<i>Micrococcus luteus</i>	3.44	8.23	7.03	9.46	3.91	6.89	4.13	9.86	2.21
<i>Moraxella osloensis</i>	4.10	5.40	4.22	3.66	5.39	8.88	2.14	4.38	12.72
<i>Moraxella sp. DQX410</i>									1.02
<i>Paracoccus sp. VN007</i>		3.13							
<i>Paracoccus yeai</i>	1.14								
<i>Prevotella histiola</i>						1.65			
<i>Propionibacterium humerale</i>		2.38		1.96					
<i>Roseomonas mucosa</i>	2.43	3.52	1.23		1.60				
<i>Sphingobium yanoikuyae</i>							0.91		
<i>Staphylococcus capitis</i>			1.56	2.66				1.10	1.87
<i>Staphylococcus epidermidis</i>		2.41	2.43	4.19				1.20	2.12
<i>Staphylococcus hominis</i>			4.73	10.10				1.20	0.87
<i>Streptococcus mitis</i>								0.87	1.90
<i>Streptococcus mutans</i>					0.99	2.28			1.19
<i>Streptococcus salivarius</i>					1.15	2.17			
<i>Stutzerimonas stutzeri</i>	1.96								
uncultured bacterium							0.92		

Relative Abundance (%)



23SL21-HAH_Iraken2
23SL21-HAH_metaphlan4
23SL21-HAH_Iraken2
23SL21-HAH_metaphlan4
24WL21-HAH_Iraken2
24WL21-HAH_metaphlan4
24SL21-HAH_Iraken2
24SL21-HAH_metaphlan4
25WL21-HAH_Iraken2
25WL21-HAH_metaphlan4

Top 10 Dominant Species of L22-KWT

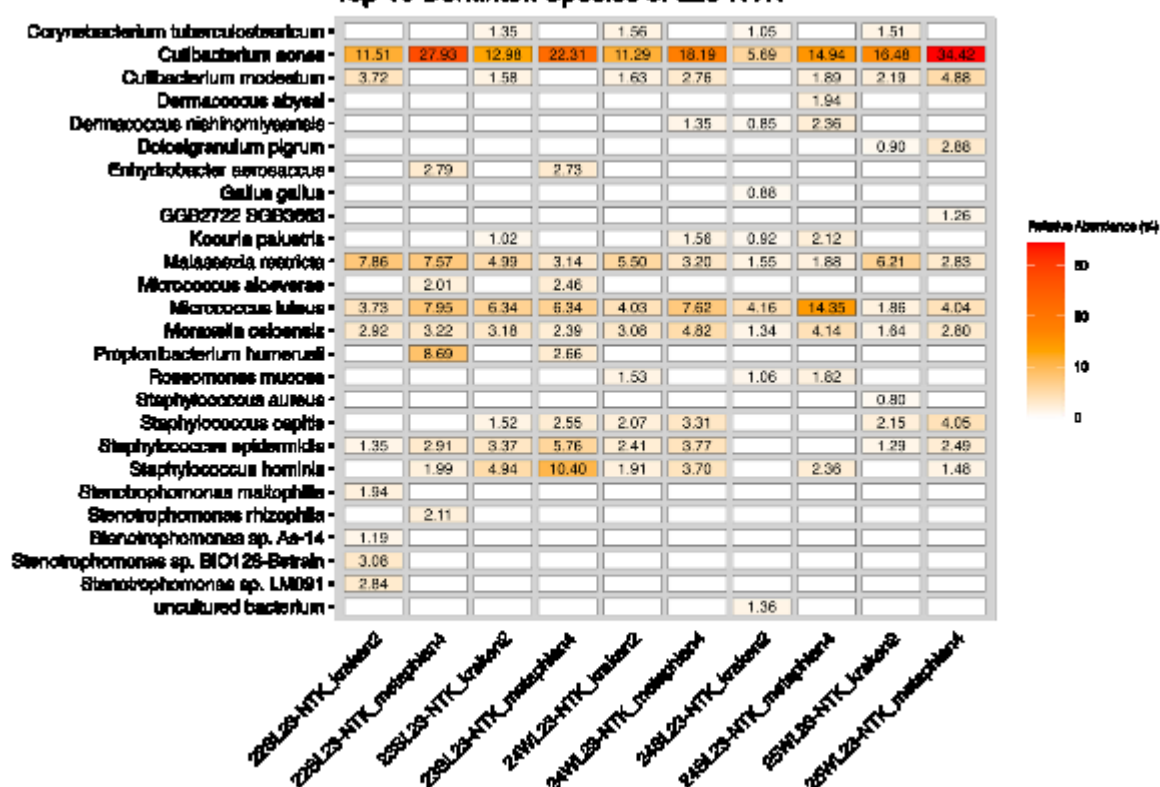
<i>Acinetobacter baumannii</i>		0.49	0.91		1.23	0.95			3.24	4.22
<i>Acinetobacter johnsonii</i>									1.28	1.28
<i>Acinetobacter junii</i>		1.12		1.11		1.03				5.99
<i>Acinetobacter sp. 1335</i>									1.44	
<i>Corynebacterium tuberculoaerarium</i>									2.94	
<i>Cutibacterium acnes</i>	0.76	3.75	0.62	7.29	4.97	6.71	7.73	17.31	5.25	9.23
<i>Cutibacterium modestum</i>			4.74			1.01	1.06	2.81		
<i>Enhydrobacter aerococcus</i>								2.13		
<i>Epithomonas variabile</i>		0.80		17.70						2.11
<i>Kocuria indica</i>						1.30			1.15	
<i>Kocuria palustris</i>								2.13		
<i>Makaseezia restricta</i>								1.22		
<i>Methylobacterium elovexensis</i>		0.82	2.25	1.30	1.90			1.03		
<i>Methylobacterium sp. 1335</i>					0.75			2.57		
<i>Moraxella osloensis</i>				1.17	1.53					
<i>Moraxella sp. DQX410</i>										
<i>Moraxella sp. 1335</i>		1.51	3.01	1.10	2.22	3.49	4.81	13.44		2.10
<i>Moraxella sp. 1335</i>				2.80						
<i>Moraxella sp. 1335</i>				1.25						
<i>Moraxella sp. 1335</i>	0.60	1.34	43.45	32.20	35.03	47.00	1.74	4.37	12.41	25.60
<i>Propionibacterium humerale</i>		0.47			0.82	0.96				
<i>Pseudomonas aeruginosa</i>	0.68								2.10	
<i>Pseudomonas fluorescens group</i>		7.29								5.62
<i>Pseudomonas fluorescens group</i>	0.52									
<i>Pseudomonas sp. 1335</i>			29.34							
<i>Pseudomonas sp. 1335</i>	0.54									
<i>Pseudomonas sp. 1335</i>	40.31									
<i>Pseudomonas sp. 1335</i>									4.18	
<i>Pseudomonas sp. 1335</i>										
<i>Pseudomonas sp. 1335</i>	47.76									
<i>Pseudomonas sp. 1335</i>	0.85									
<i>Pseudomonas sp. 1335</i>									1.86	
<i>Pseudomonas sp. 1335</i>	0.82									
<i>Sphingomonas elaeophila</i>					0.86		0.98		1.43	
<i>Sphingomonas elaeophila</i>						2.60				1.79
<i>Staphylococcus capitis</i>			0.82							
<i>Staphylococcus epidermidis</i>			1.41	1.85	0.83	1.10		1.63		
<i>Staphylococcus hominis</i>			10.26	17.64			0.88	2.83		
<i>Stutzerimonas stutzeri</i>	0.50									
uncultured bacterium							1.42			

Relative Abundance (%)

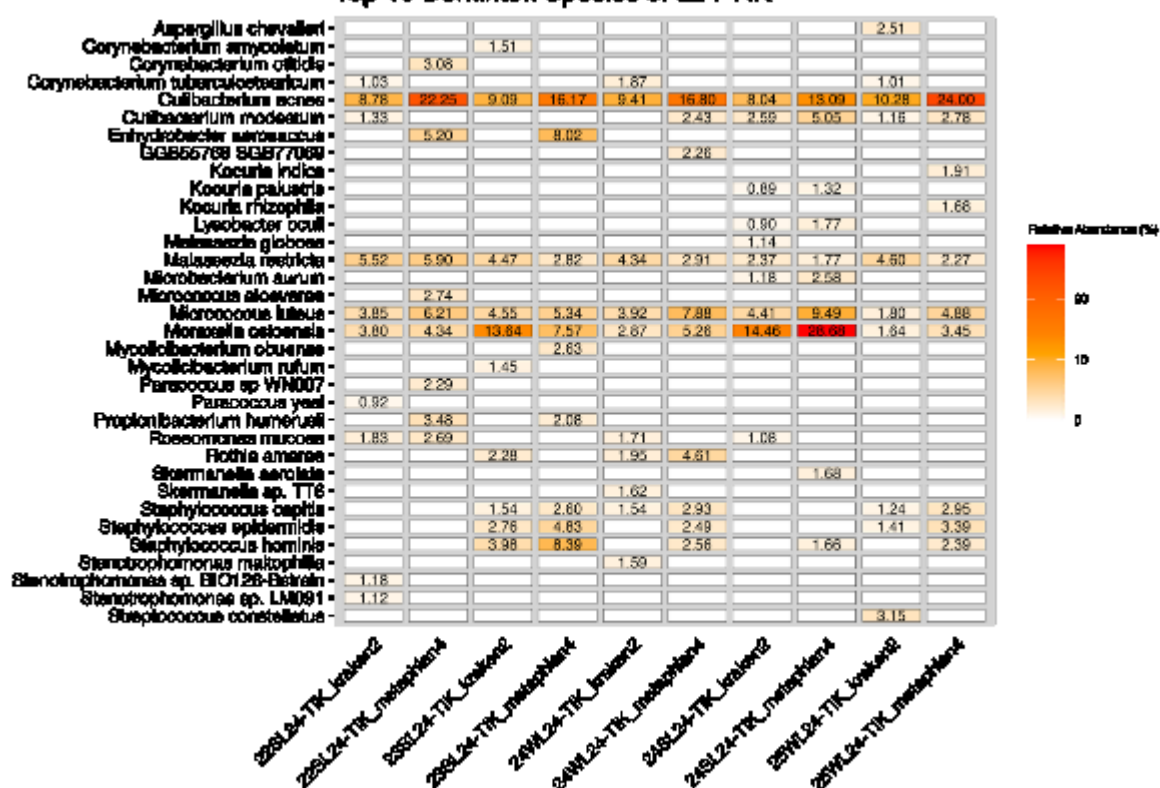


23SL22-KWT_Iraken2
23SL22-KWT_metaphlan4
23SL22-KWT_Iraken2
23SL22-KWT_metaphlan4
24WL22-KWT_Iraken2
24WL22-KWT_metaphlan4
24SL22-KWT_Iraken2
24SL22-KWT_metaphlan4
25WL22-KWT_Iraken2
25WL22-KWT_metaphlan4

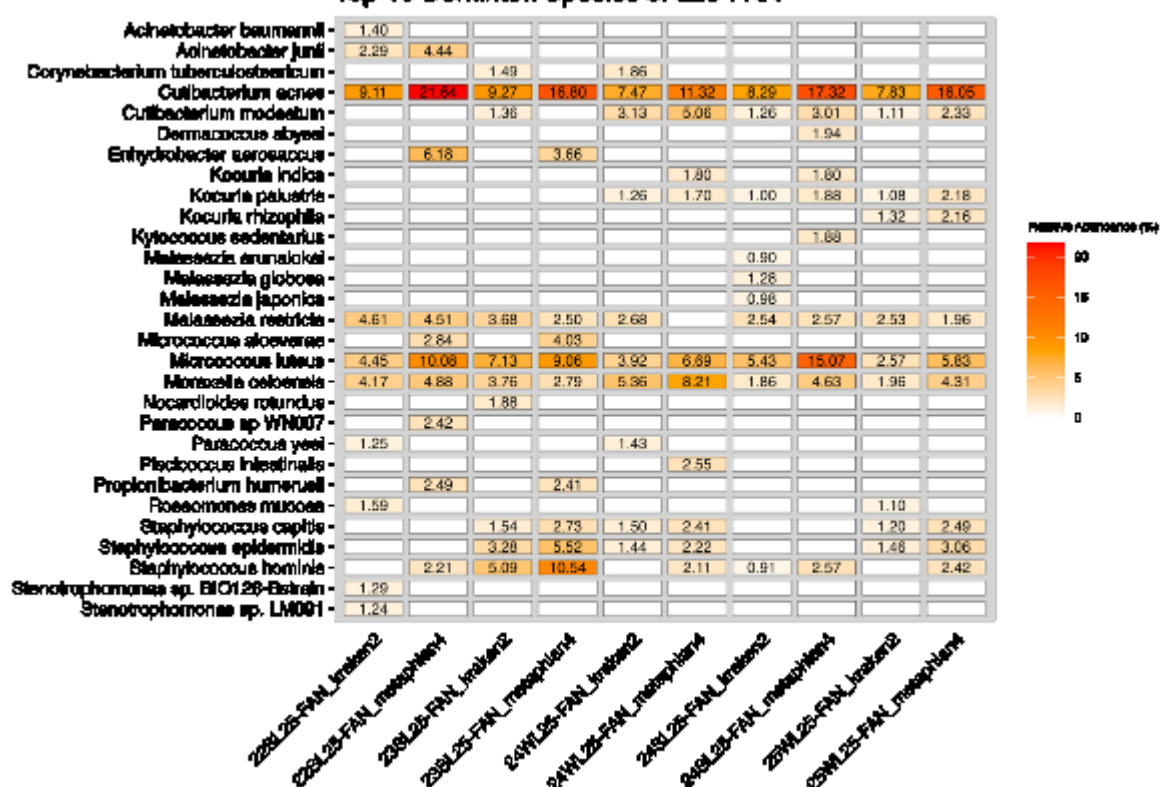
Top 10 Dominant Species of L23-NTK



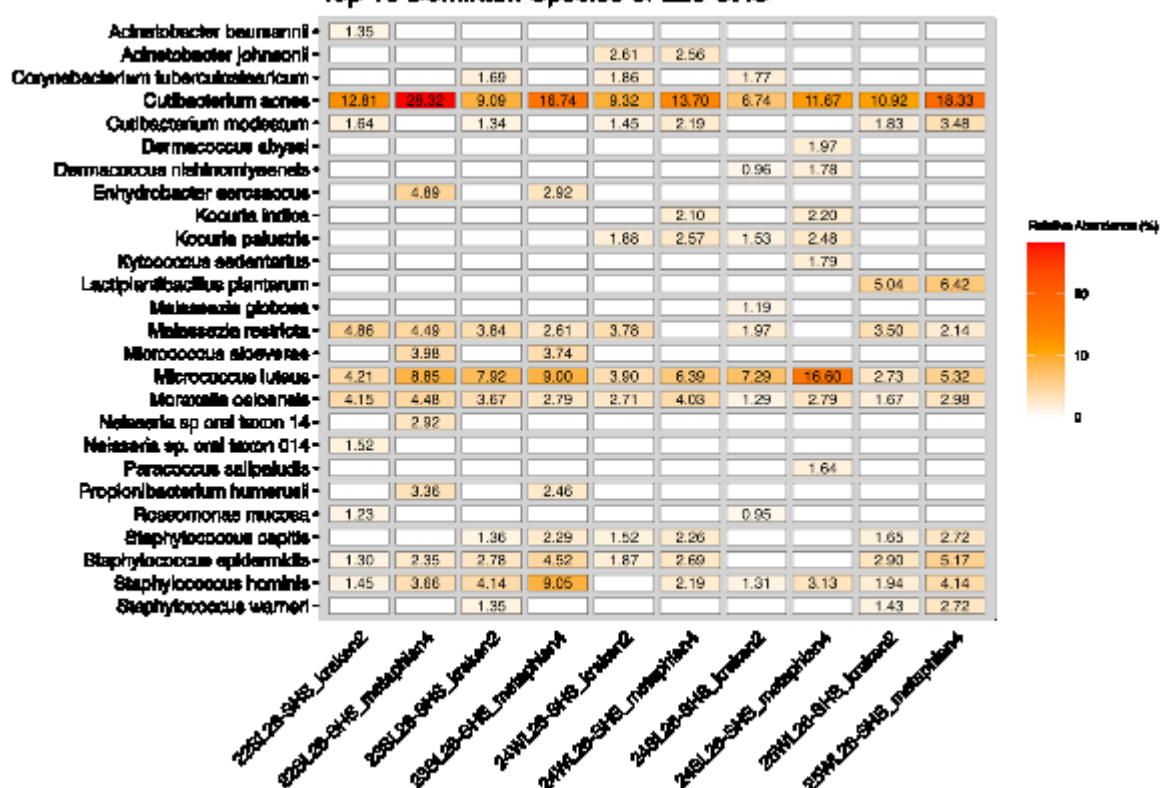
Top 10 Dominant Species of L24-TIK



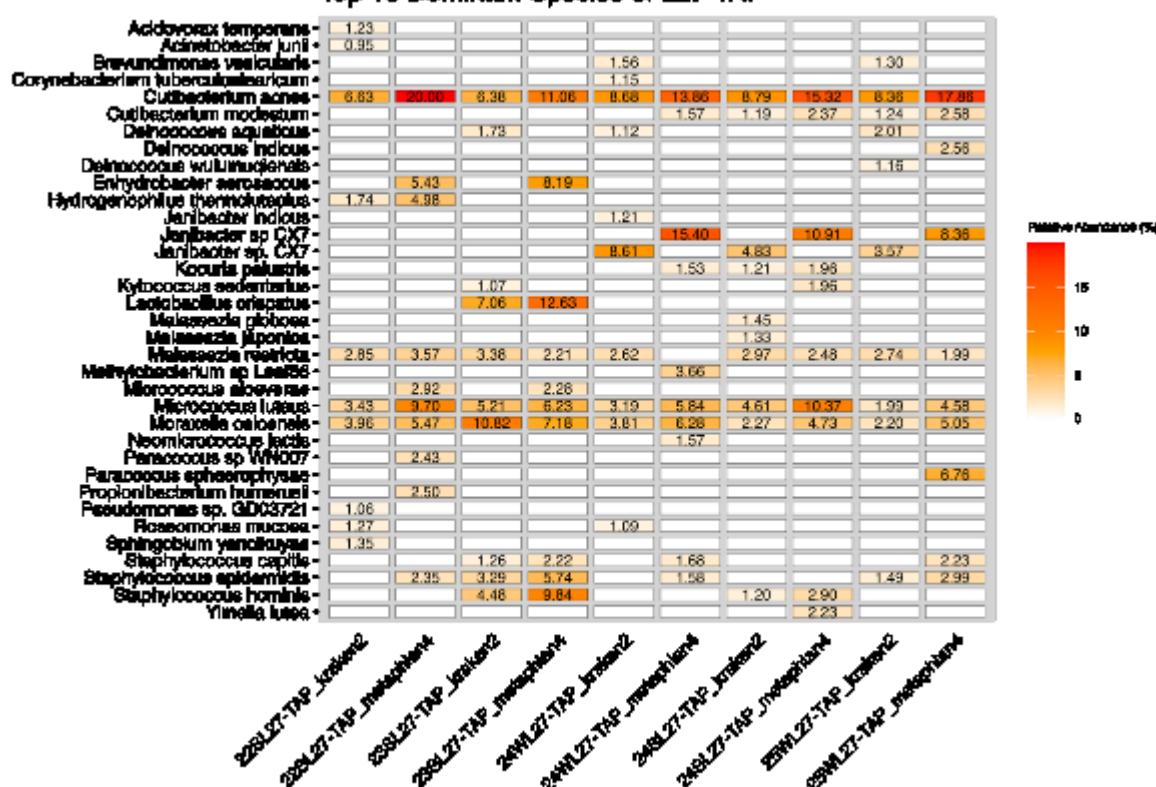
Top 10 Dominant Species of L25-FAN



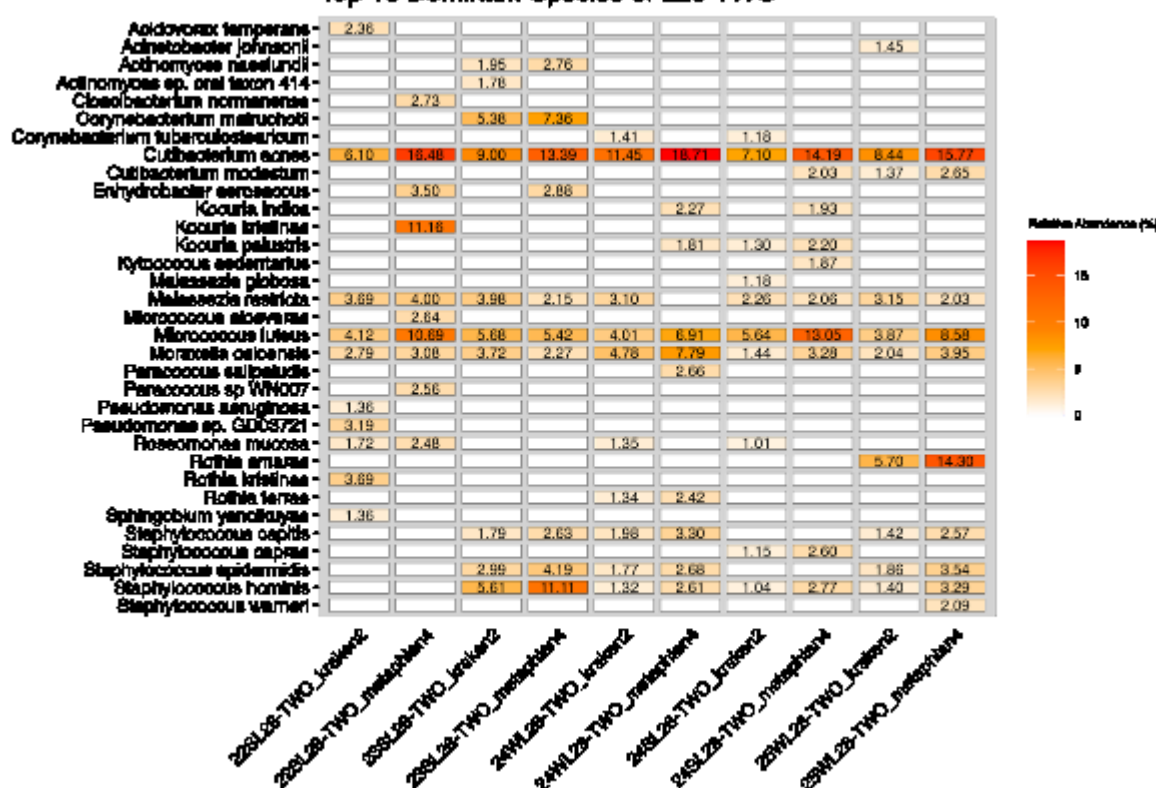
Top 10 Dominant Species of L26-SHS



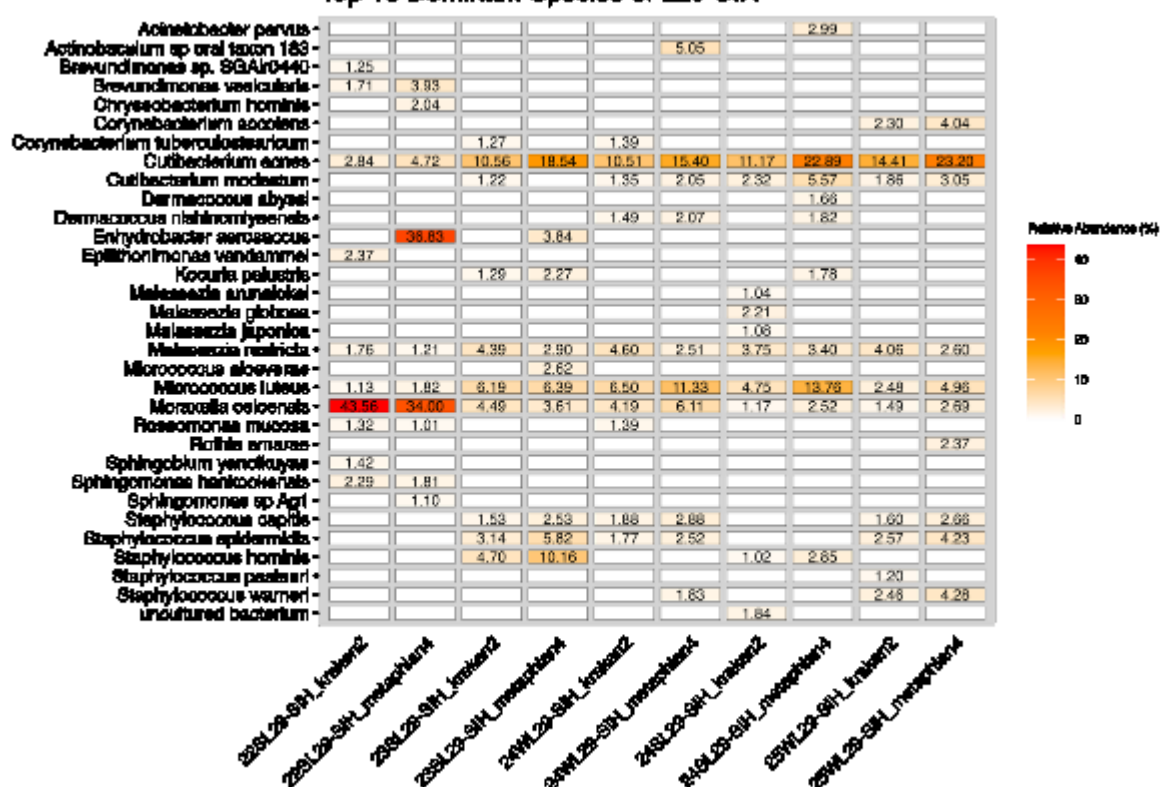
Top 10 Dominant Species of L27-TAP



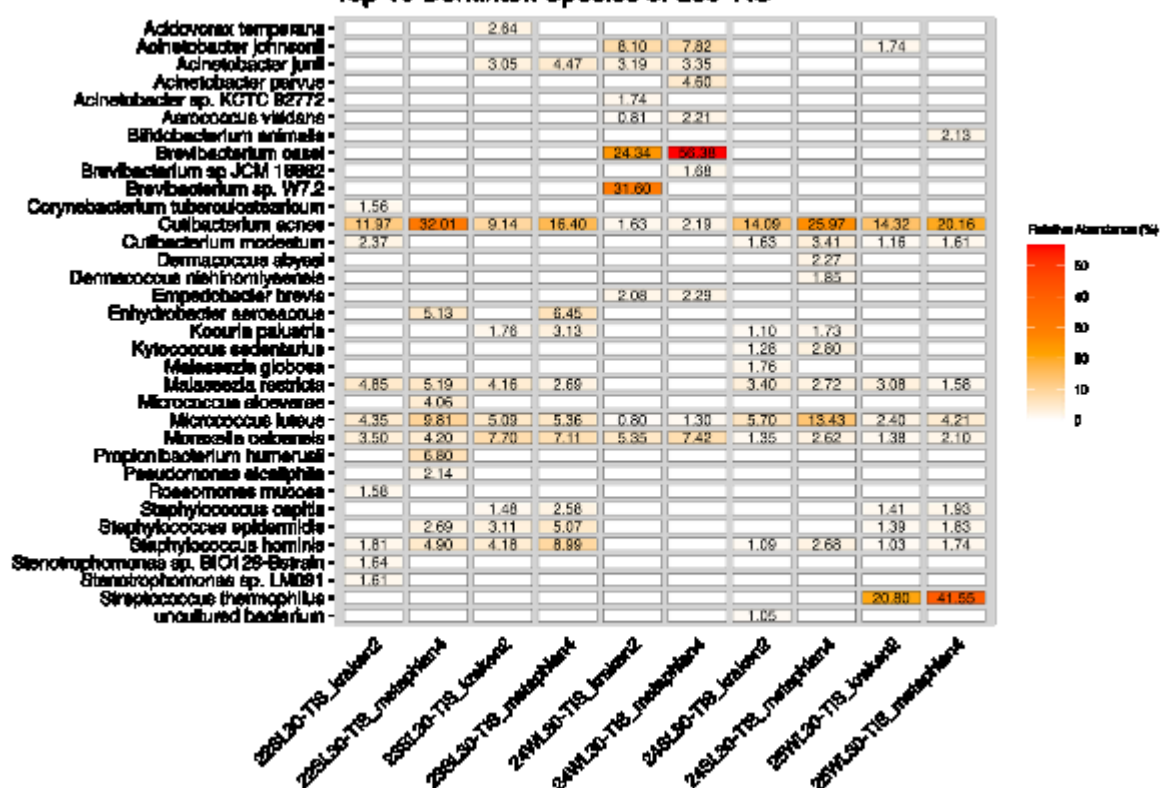
Top 10 Dominant Species of L28-TWO



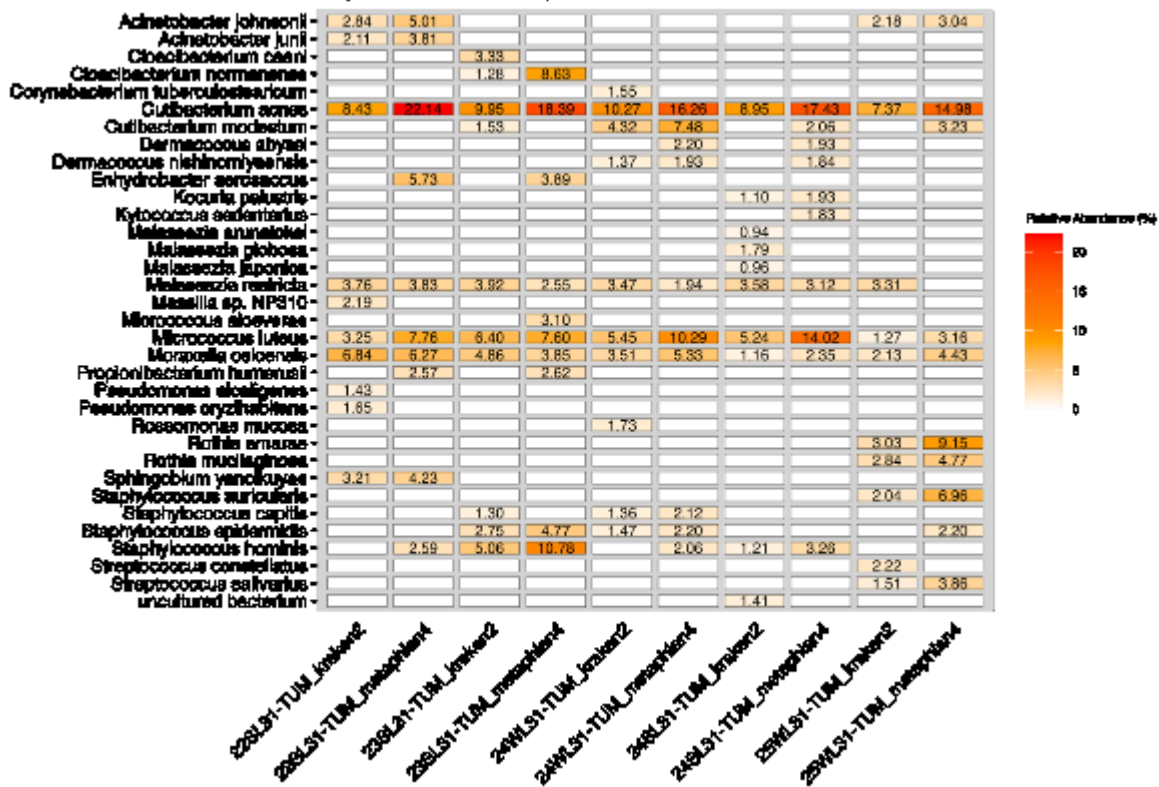
Top 10 Dominant Species of L29-SIH



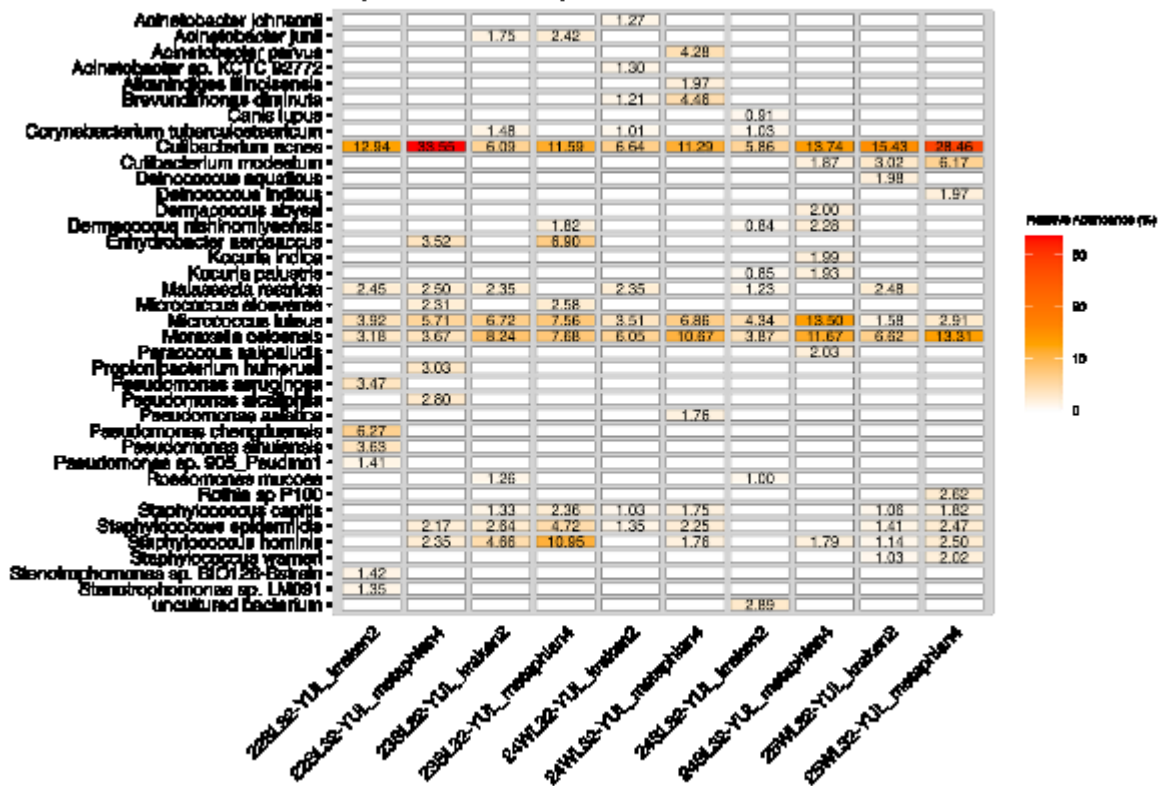
Top 10 Dominant Species of L30-TIS



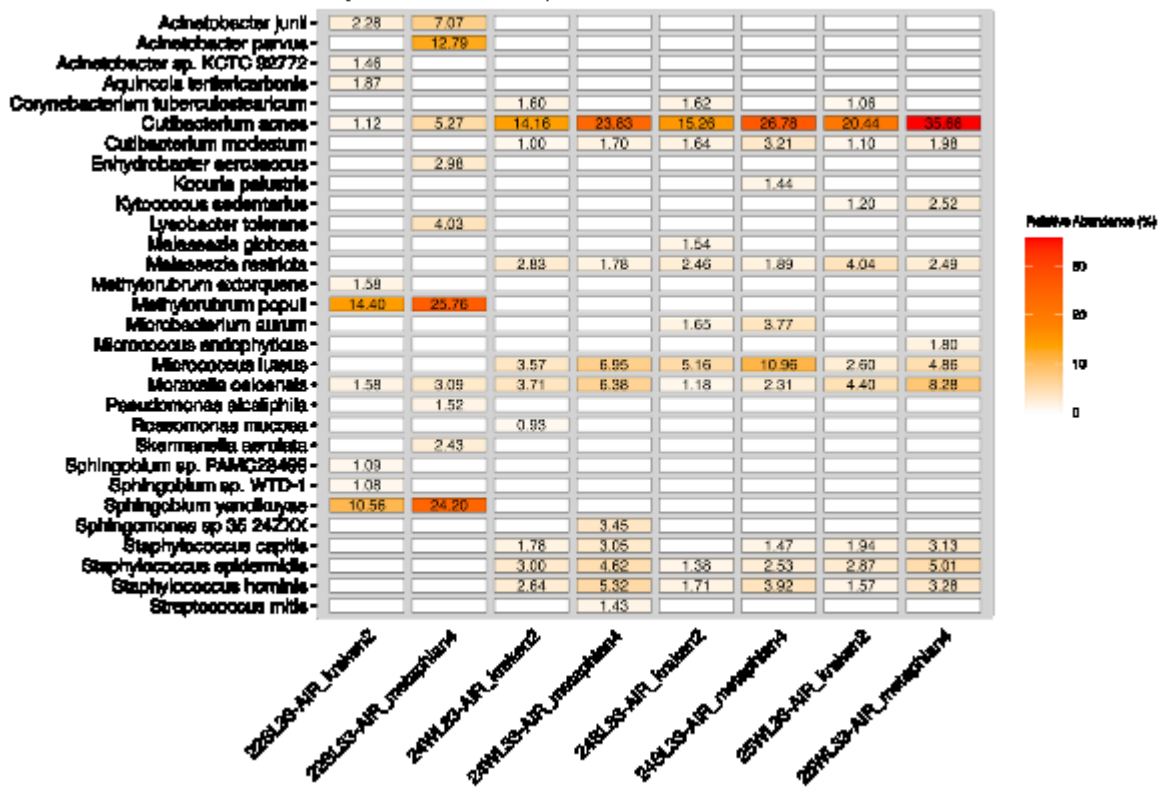
Top 10 Dominant Species of L31-TUM



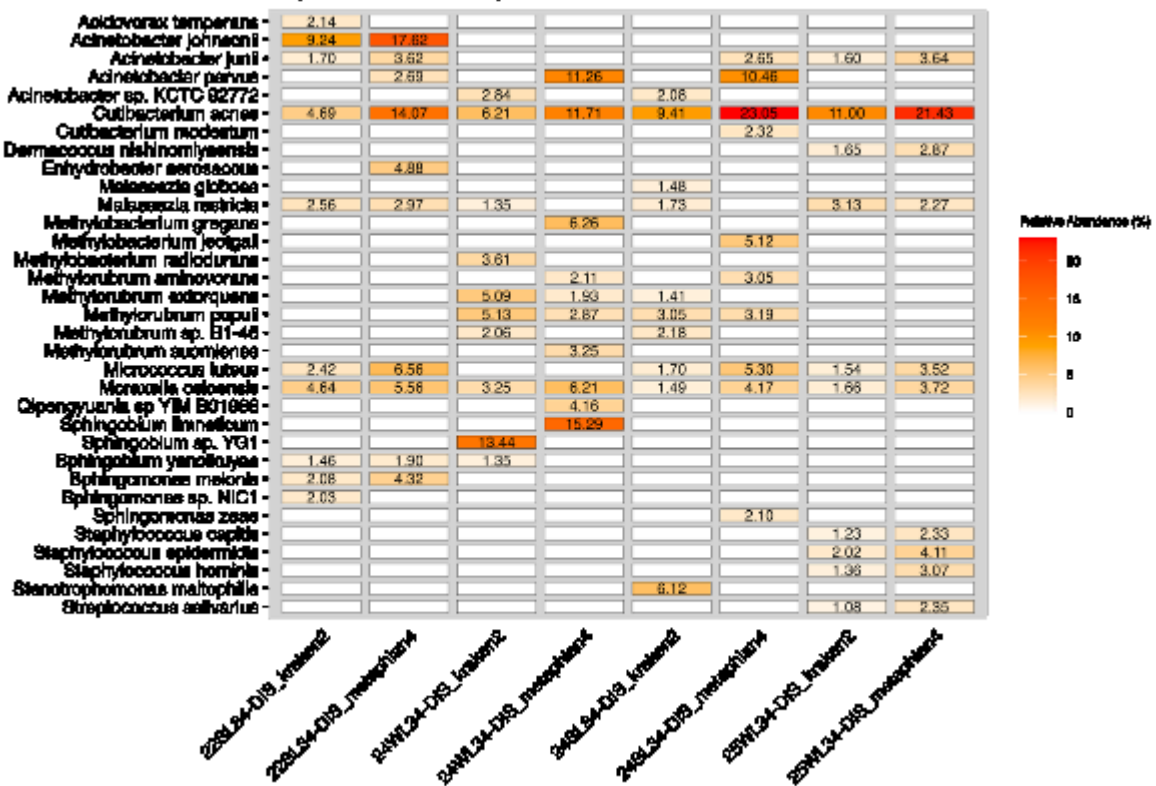
Top 10 Dominant Species of L32-YUL



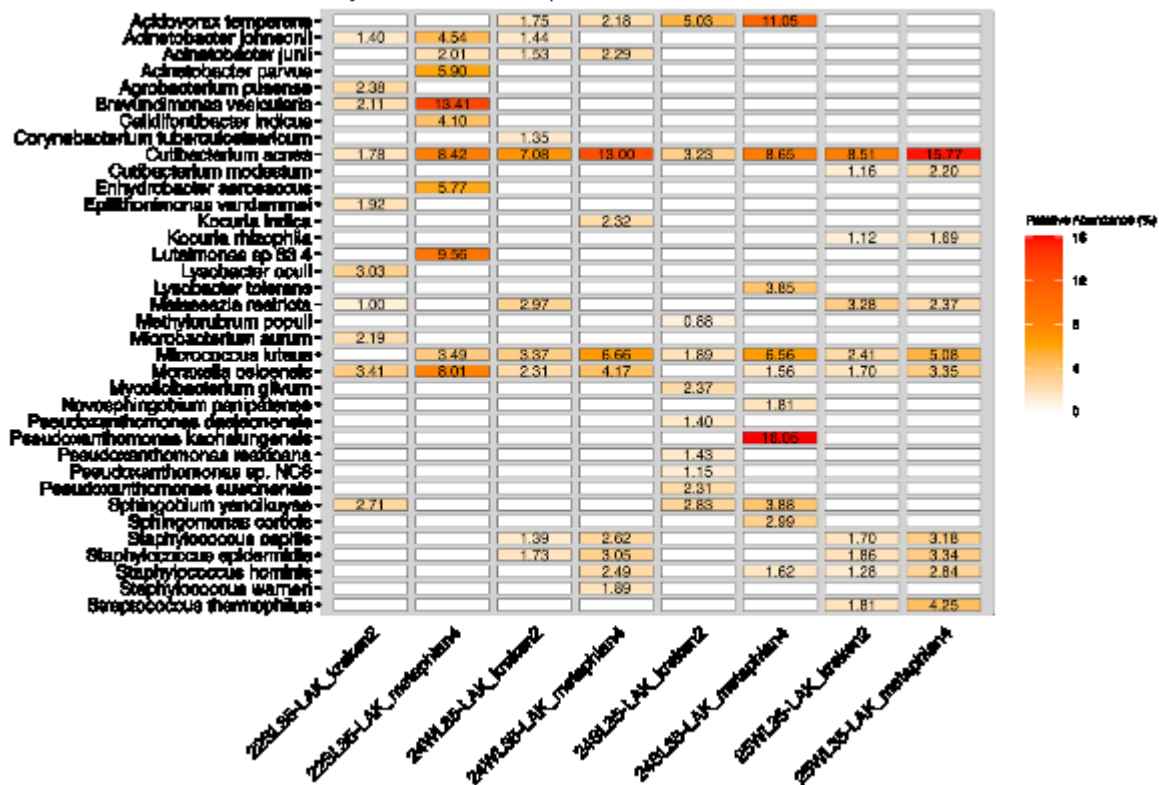
Top 10 Dominant Species of L33-AIR



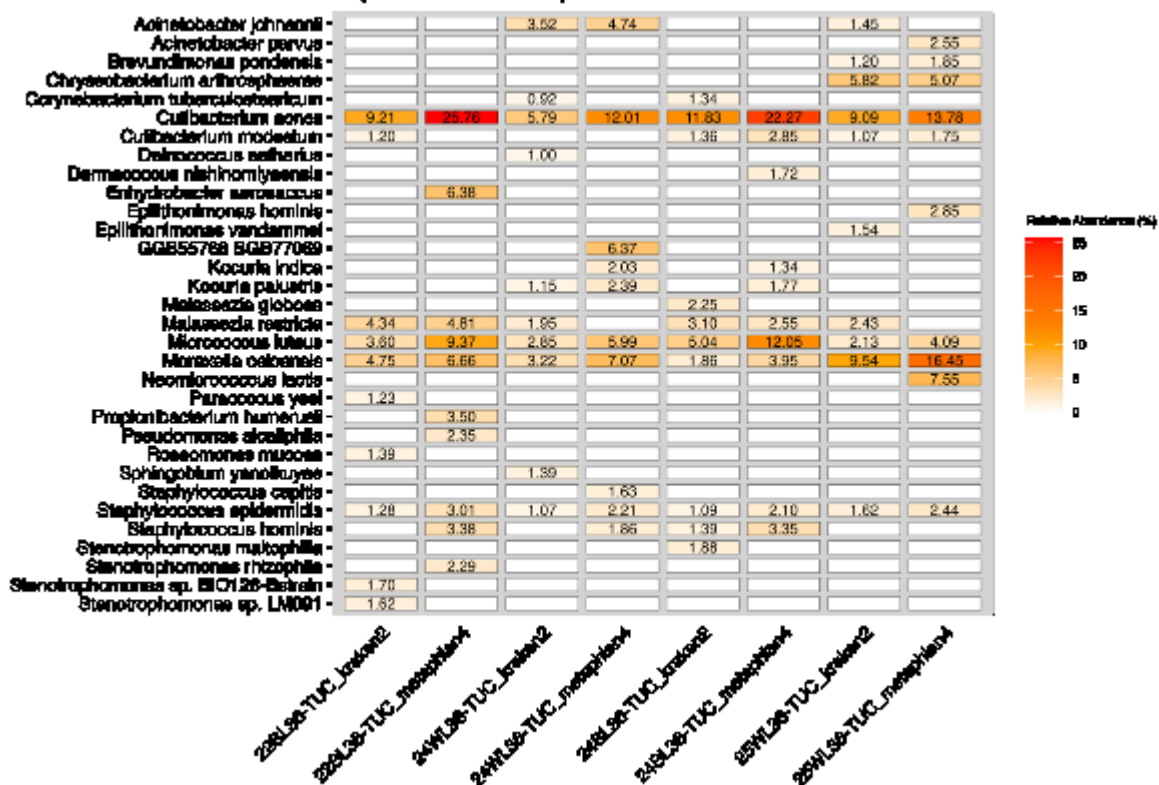
Top 10 Dominant Species of L34-DIS



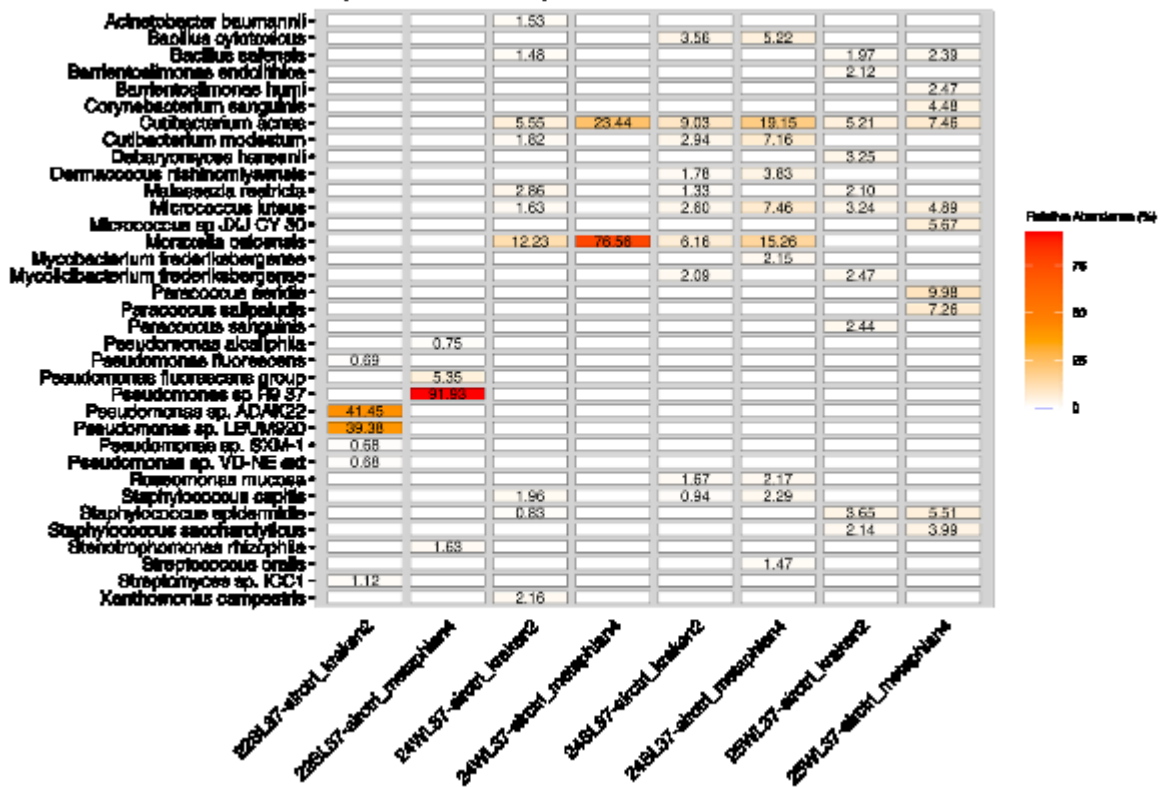
Top 10 Dominant Species of L35-LAK



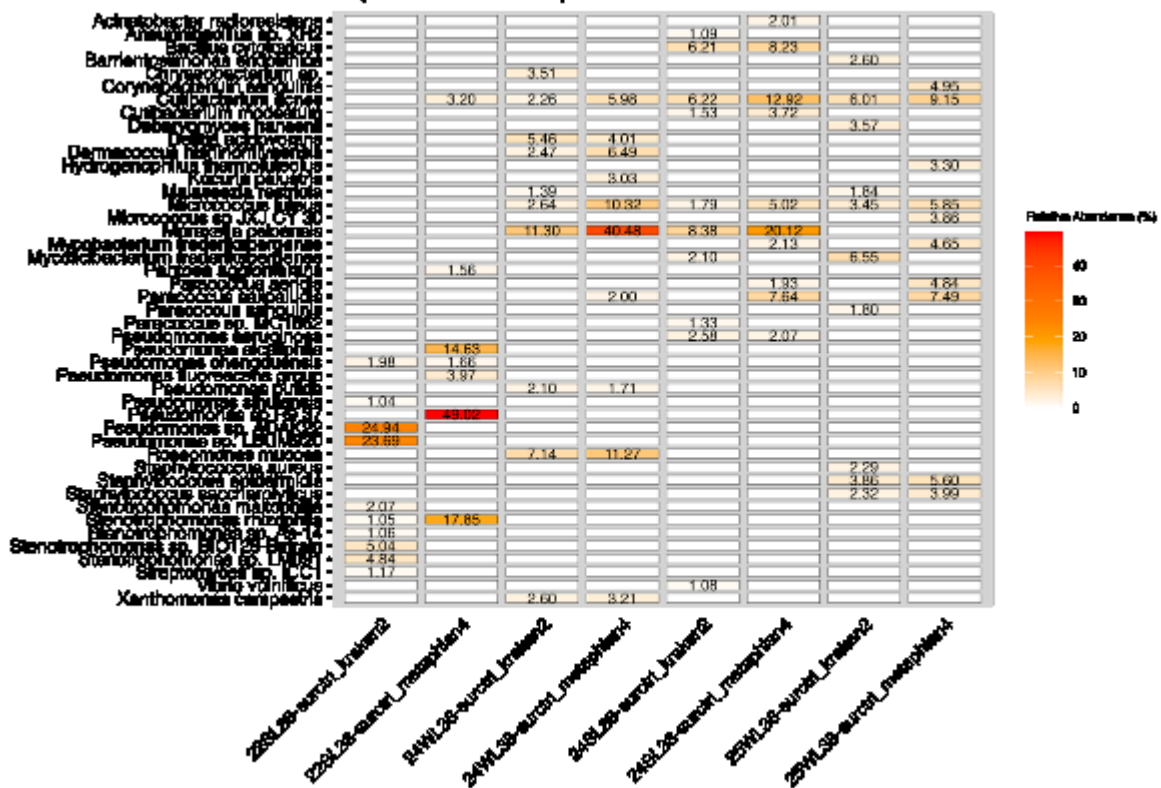
Top 10 Dominant Species of L36-TUC



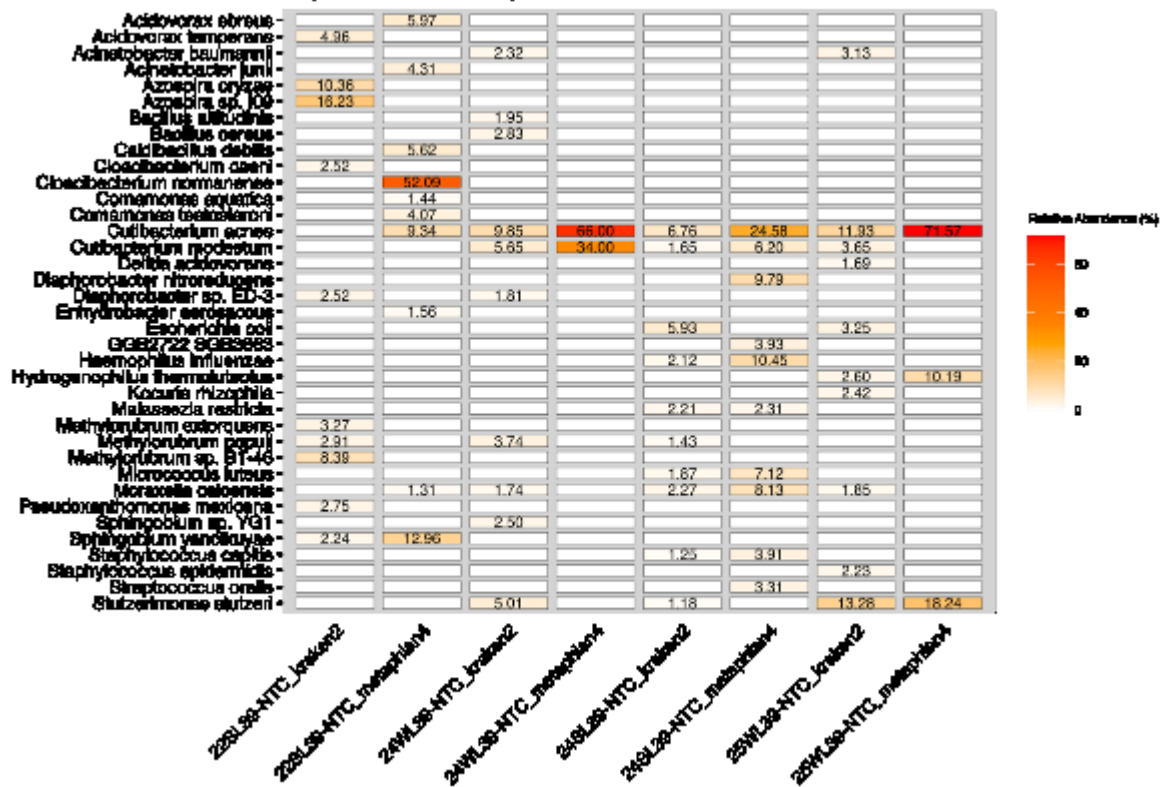
Top 10 Dominant Species of L37-airctrl



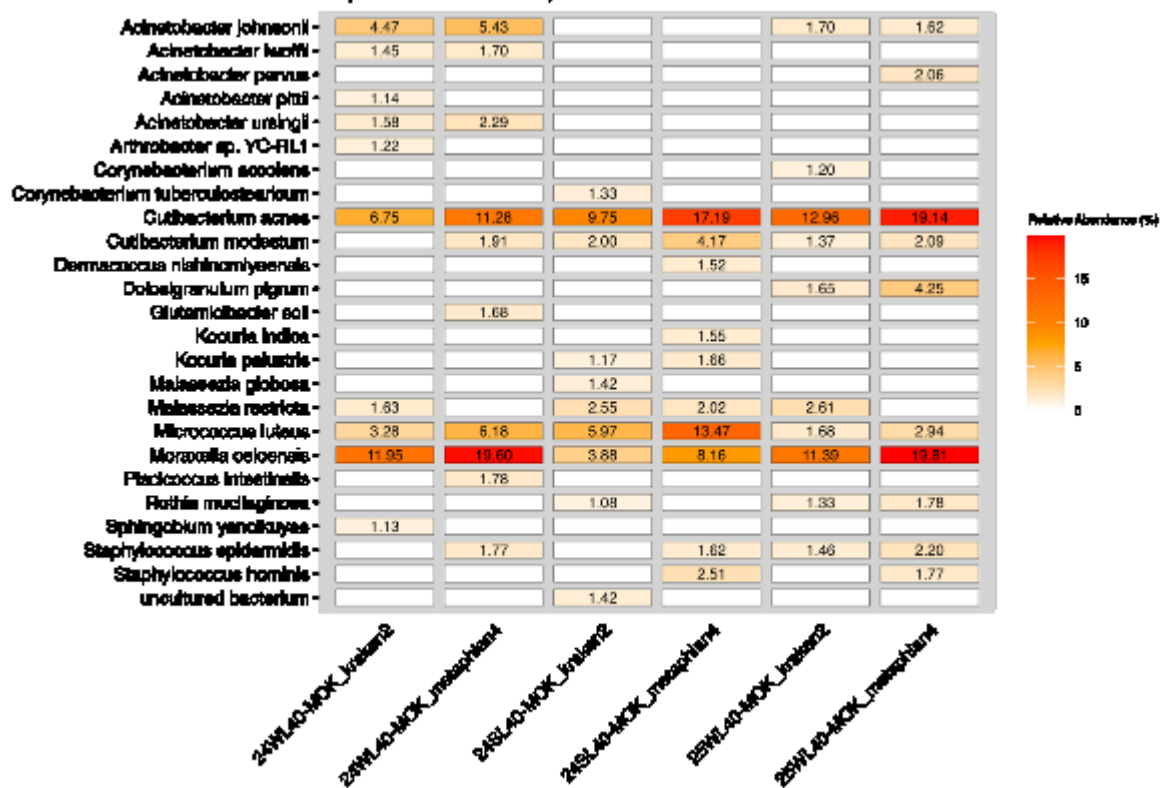
Top 10 Dominant Species of L38-surctrl



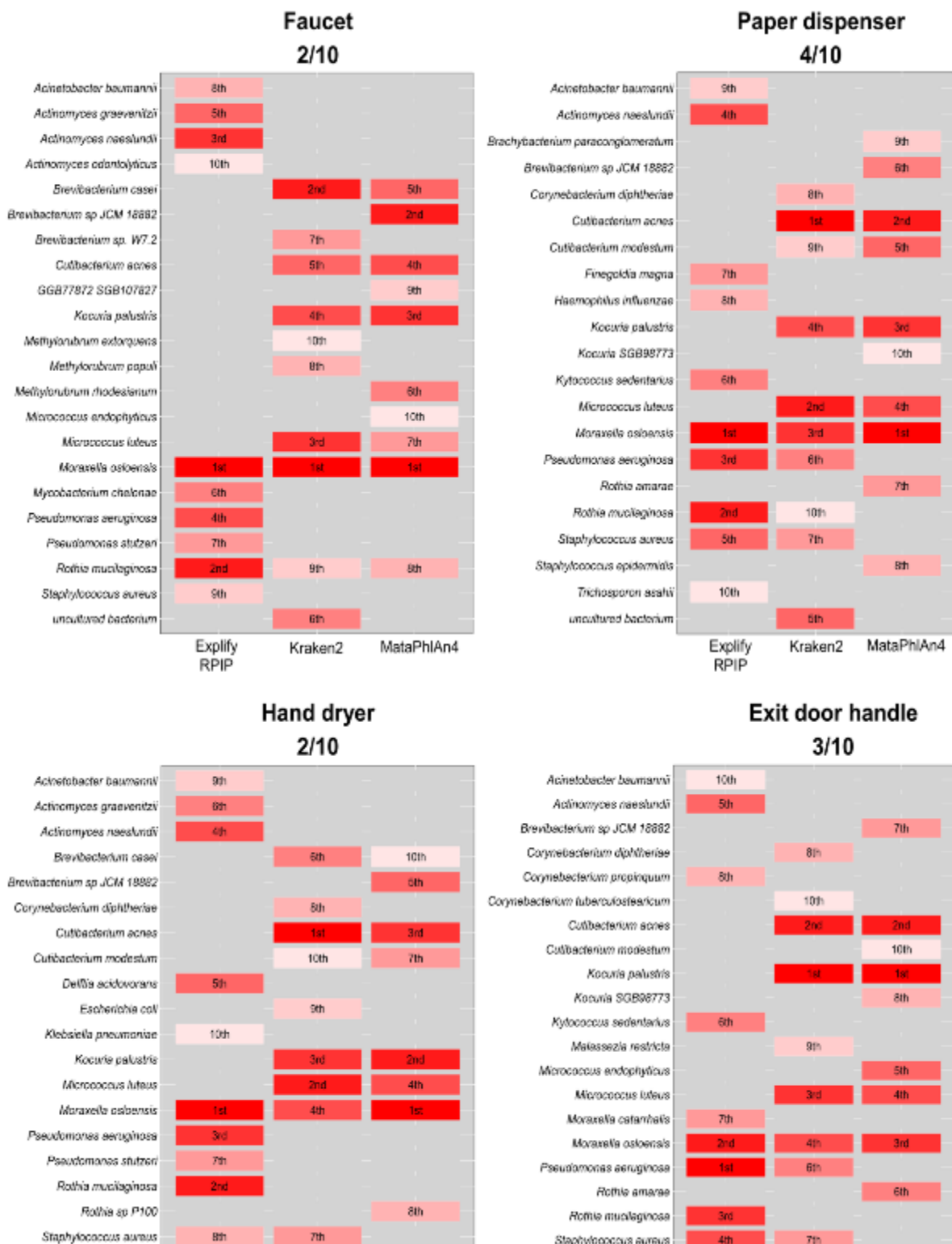
Top 10 Dominant Species of L39-NTC



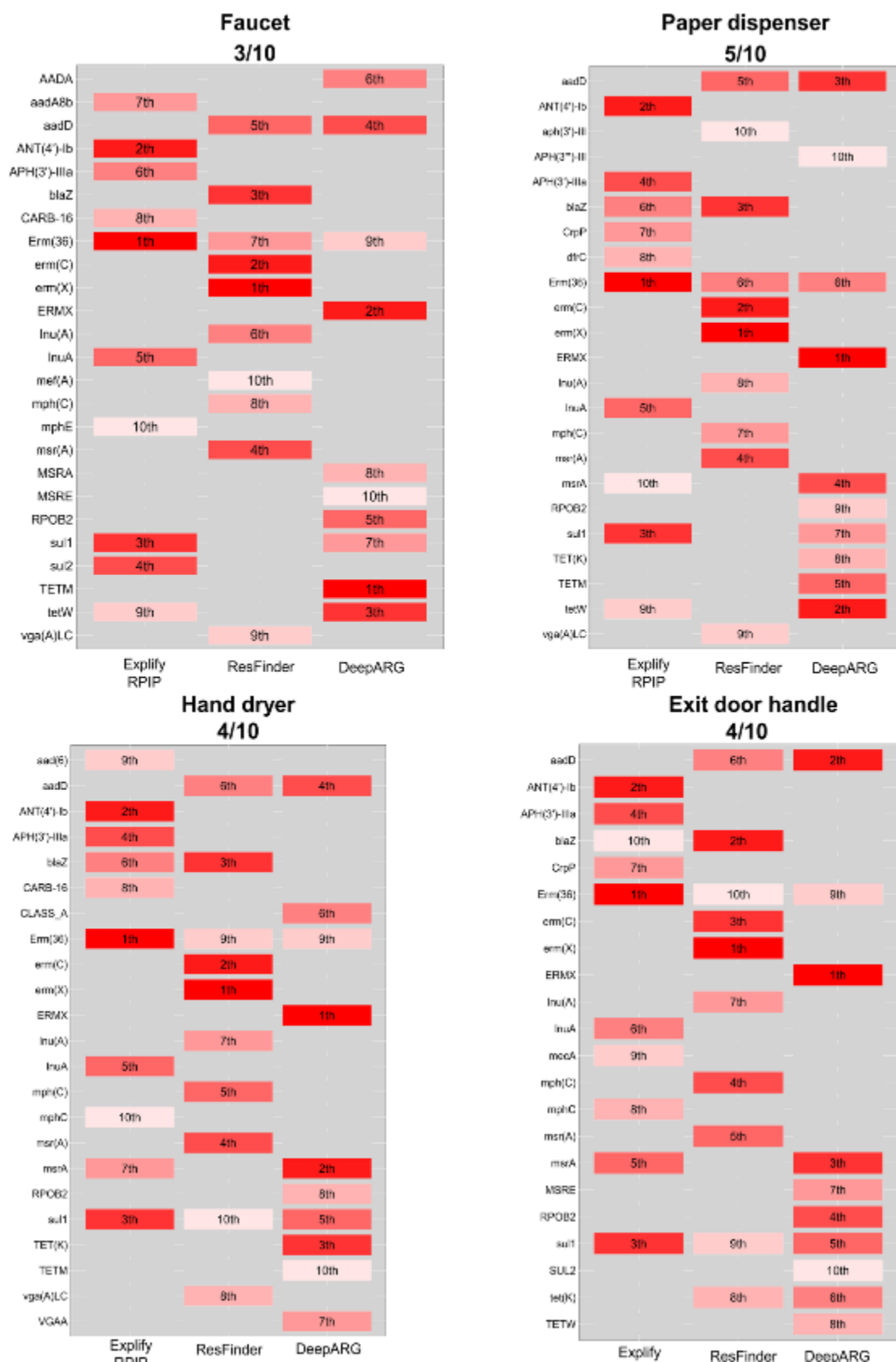
Top 10 Dominant Species of L40-MOK



Supplementary Data 3. Comparison of taxonomic classification and ARG profiling results between Explify RPIP and third-party bioinformatic tools.



Supplementary Figure S5. Comparison of top 10 dominating species classified by Explify RPIP, Kraken2 and MetaPhlAn4.



Supplementary Figure S6. Comparison of top 10 dominating ARGs classified by Explify RPIP, ResFinder and DeepARG.

Supplementary Tables

Supplementary Table ST1. Information of stations in the Hong Kong subway system included in the subway microbiome study.

S/N	Station name	Station code	MTR Line	District	Line Interchange Station	Hospital within 1km
1	Hung Hom	HUH	East Rail line and Tuen Ma line	Kowloon City	Yes	NA
2	Kwai Hing	KWH	Tsuen Wan line	Kwai Tsing	No	NA
3	Mei Foo	MEF	Tsuen Wan line and Tuen Ma line	Sham Shui Po	Yes	Princess Margaret Hospital
4	Tsuen Wan	TSW	Tsuen Wan line	Tsuen Wan	No	NA
5	Jordan	JOR	Tsuen Wan line	Yau Tsim Mong	No	Queen Elizabeth Hospital
6	Sham Shui Po	SSP	Tsuen Wan line	Sham Shui Po	No	NA
7	Wong Tai Sin	WTS	Kwun Tong line	Wong Tai Sin	No	NA
8	Yau Ma Tei	YMT	Tsuen Wan line and Kwun Tong line	Yau Tsim Mong	Yes	Kwong Wah Hospital
9	Admiralty	ADM	Island line	Central and Western	Yes	NA
10	Kennedy Town	KET	Island line	Central and Western	No	NA
11	Lei Tung	LET	South Island line	Southern	No	NA
12	Ocean Park	OCP	South Island line	Southern	No	Wong Chuk Hang Hospital; Grantham Hospital
13	Chai Wan	CHW	Island line	Eastern	No	Pamela Youde Nethersole Eastern Hospital
14	Exhibition Centre	EXC	East Rail line	Wan Chai	No	NA
15	North Point	NOP	Island line	Eastern	Yes	NA
16	Wan Chai	WAC	Island line	Wan Chai	No	Ruttonjee Hospital
17	City One	CIO	Tuen Ma line	Sha Tin	No	Prince of Wales Hospitals
18	Diamond Hill	DIH	Kwun Tong line	Wong Tai Sin	Yes	NA
19	Kowloon Tong	KOT	Kwun Tong line and East Rail line	Kowloon City	Yes	NA
20	Tai Wai	TAW	East Rail line and Tuen Ma line	Sha Tin	Yes	NA
21	Hang Hau	HAH	Tsuen Kwan O line	Sai Kung	No	NA
22	Kwun Tong	KWT	Kwun Tong line	Kwun Tong	No	United Christian Hospital
23	Ngau Tau Kok	NTK	Kwun Tong line	Kwun Tong	No	NA
24	Tiu Keng Leng	TIK	Tsuen Kwan O line	Sai Kung	Yes	NA
25	Fanling	FAN	East Rail line	North	No	NA
26	Sheung Shui	SHS	East Rail line	North	No	North District Hospital
27	Tai Po Market	TAP	East Rail line	Tai Po	No	NA
28	Tai Wo	TWO	East Rail line	Tai Po	No	NA
29	Siu Hong	SIH	Tuen Ma line	Tuen Mun	No	Tuen Mun Hospital
30	Tin Shui Wai	TIS	Tuen Ma line	Yuen Long	No	NA
31	Tuen Mun	TUM	Tuen Ma line	Tuen Mun	No	NA
32	Yuen Long	YUL	Tuen Ma line	Yuen Long	No	NA
33	Airport	AIR	Airport express	Islands	No	NA
34	Disneyland Resort	DIS	Disneyland Resort line	Islands	No	NA
35	Lai King	LAK	Tsuen Wan line	Kwai Tsing	Yes	NA
36	Tung Chung	TUC	Tung Chung line	Islands	No	NA
37	Mong Kok	MOK	Tsuen Wan line and Kwun Tong line	Yau Tsim Mong	Yes	NA

Supplementary Table ST2. Sampling information of the subway microbiome study.

Sampling period	Sample Collection Date (DD/MM/YY)	Mean Temperature (°C)	Mean Relative Humidity (%)	Total rainfall (mm)
Summer 2022	21/7/2022	30.9	74	0.3
	8/8/2022	28.3	87	33.3
Summer 2023	31/7/2023	29.1	84	46.5
	2/8/2023	30.4	70	0
	14/8/2023	29.4	82	3.6
	16/8/2023	30.6	78	0
	19/2/2024	22.7	88	0
Winter 2024	26/2/2024	18.2	76	Trace
	4/3/2024	19.7	91	1.4
Summer 2024	8/7/2024	31.1	72	0.2
	15/7/2024	29.7	85	13.6
	22/7/2024	30.6	80	0.2
Winter 2025	3/2/2025	15.8	74	0.1
	10/2/2025	15.9	63	0.0
	17/2/2025	18.9	70	Trace

Hong Kong Observatory, 2025. Daily Extract of Meteorological Observations.
<https://www.hko.gov.hk/en/cis/dailyExtract.htm?y=2025&m=2>

Supplementary Table ST3. Antibigram and molecular typing results of MRSA isolates from the Hong Kong subway system

MDRO information				Antibiogram																	Molecular typing			ARG profile															
Isolate ID	Sampling log	Subway station	Site	CN	RD	FOX	CPT	LEV	SXT	FA	VA	DA	E	NF	LZD	P	C	QD	TE	Sequence type	ACR group	SCCmec type	spa type	Aminoglycoside	Beta-lactam	Fosfomycin	Streptogramin	Trimethoprim-sulfamethoxazole	Macrolide-lincosamide	Phenicol	Quaternary ammonium	Tetracycline	Rifampicin	Fusidic acid	Quinolone	Fluoroquinolone	Trimethoprim		
S1_TIK4_MRSA_1/UP01294	22S	TIK	Escalator handrails	S	S	R	S	S	S	S	S	R	R	S	R	R	S	S	S	ST508	gp1	IV, IVi	t050	-	blaI, blaR1, blaZ, mecA, mecR1	-	erm(C)	-	catA	-	tet(38)	-	-	-	mepA	-			
HH_ATM_MRSA/UP01295	22S	HUH	ATM	R	R	R	S	S	S	S	S	R	R	S	R	R	S	S	S	ST121	gp4	V	t5110	aac(6)-Ic/aph(2'')-Ia	blaI, blaR1, blaZ, mecA	fosB, glpT_A100V, glpT_L27F, murA_E291D, murA_T396N	-	lmrS	-	-	mcpA, tet(38)	-	-	parC_S80F	mcpA	-			
HH_ESC_MRSA/UP01296	22S	HUH	Escalator handrails	R	R	R	R	R	S	S	S	R	R	S	R	R	S	S	S	ST630	gp1	-	04-02-12-02-12-21-17-34-22-25	aac(6)-Ic/aph(2'')-Ia	blaI, blaR1, blaZ	fosB	-	lmrS	-	qacC	mepA, tet(38)	-	-	gyrA_S84L, fusB, parC_S80Y	mcpA	-			
S1_MEF4re_MRSA_3/UP01297	22S	MEF	Escalator handrails	S	S	R	S	S	S	S	S	R	R	S	R	R	S	S	S	ST159	gp1	IV, IVa	t441	ant(6)-Ia, aph(3'')-IIIa	blaI, blaR1, blaZ, mecA, mecR1	glpT_A100V, murA_E291D, murA_T396N	erm(B)	lmrS	-	-	mcpA, tet(38)	-	-	-	mcpA	-			
UP00192/UP00099_MRSA_1	23S	FAN	Elevator	R	R	R	S	S	S	S	S	S	R	R	S	R	R	S	S	ST1047	gp1	IV, IVa, IVn	08-16-208-43-34-17-34	aac(6)-Ic/aph(2'')-Ia, aadD1	blaI, blaR1, blaZ, mecA, mecR1	-	msr(A)	-	-	-	tet(38)	-	-	-	mcpA	-			
UP00245/UP00119_MRSA_1	23S	TIS	Elevator	S	S	R	S	S	S	S	S	S	R	R	S	R	R	S	S	ST6	gp1	IV, IVa, IVn	t304	-	blaI, blaR1, blaZ, mecA, mecR1	fosB	-	-	-	-	tet(38)	-	-	-	mcpA	-			
UP00247/UP00125_MRSA_1	23S	YUL	ATM	S	S	R	S	S	S	S	S	R	R	S	R	R	S	S	S	ST1232	gp1	V	t011	ant(9)-Ia	blaI, blaR1, blaZ, mecA	-	-	-	-	-	tet(38), tet(K)	-	-	-	mcpA	-			
UP00562/UP00445_MRSA1	24W	JOR	Elevator	S	S	R	S	R	R	S	R	S	S	S	S	R	R	S	S	ST5	gp2	V	t311	-	blaI, blaR1, blaZ, mecA	fosB	lmrS	-	-	mepA, tet(38)	-	-	gyrA_S84L, fusC, parC_S80F	mcpA	dfbG				
UP00563/UP00453_MRSA1	24W	SSP	Elevator	S	R	R	S	S	S	S	S	R	R	S	R	R	S	S	I	ST59	gp1	IV, IVa	t437	ant(6)-Ia, aph(3'')-IIIa	blaI, blaR1, blaZ, mecA, mecR1	glpT_A100V, murA_E291D, murA_T396N	erm(B)	lmrS	-	-	mepA, tet(38), tet(K)	rpoB_H481Y	-	-	mcpA	-			
UP00565/UP00454_MRSA1	24W	SSP	Escalator handrails	S	R	R	S	S	S	S	S	R	R	S	R	R	S	S	R	ST59	gp1	IV, IVa	t437	ant(6)-Ia, aph(3'')-IIIa	blaI, blaR1, blaZ, mecA, mecR1	glpT_A100V, murA_E291D, murA_T396N	erm(B)	lmrS	-	-	mepA, tet(38), tet(K)	rpoB_H481Y	-	-	mcpA	-			
UP00800/UP00622_MRSA_1	24S	MEF	Elevator	S	S	R	S	S	S	S	S	S	S	S	S	R	R	S	R	ST1456	gp3	IV, IVc	t1133	-	blaI, blaPC1, blaR1, blaZ, mecA, mecR1	fosB, glpT_A100V, glpT_V213I, murA_D278E, murA_E291D	-	lmrS	-	-	mcpA, tet(38)	-	-	-	-	-	-		
UP00808/UP00687_MRSA_1	24S	TIK	Escalator handrails	R	S	R	S	S	S	S	S	S	S	R	R	S	R	S	I	ST8	gp1	IV, IVi	-	aac(6)-Ic/aph(2'')-Ia, aadD1	blaI, blaR1, blaZ, mecA, mecR1	fosB	-	lmrS	-	qacB, qacR	mepA, tet(38)	-	-	-	-	-	-		
UP00810/UP00694_MRSA_1	24S	NTK	Elevator	S	S	R	S	R	R	S	S	S	S	S	S	R	R	S	S	ST1809	gp1	IV, IVa, IVn	t304	-	blaI, blaR1, blaZ, mecA, mecR1	fosB	-	lmrS	-	-	mcpA, tet(38)	-	-	gyrA_S84L, parC_S80F	-	-	-		
UP01288/UP01143_MRSA_1	25W	HUH	Escalator handrails	S	S	R	S	S	S	S	S	R	R	S	R	R	S	S	S	ST22	gp1	V, Vb	t309	-	blaI, blaR1, blaZ, mecA	-	-	lmrS	-	-	mcpA, tet(38)	-	-	-	-	dfbA_135T	-		
UP01290/UP01258_MRSA_1	25W	FAN	Elevator	S	S	R	S	S	S	S	S	R	R	S	R	R	S	S	S	ST59	gp1	IV, IVa	t437	ant(6)-Ia, aph(3'')-IIIa	blaI, blaR1, blaZ, mecA, mecR1	glpT_A100V, murA_E291D, murA_T396N	erm(B)	lmrS	-	-	mepA, tet(38)	-	-	-	-	-	-		
UP01292/UP01264_MRSA_1	25W	TAP	ATM	S	S	R	S	R	R	S	S	S	S	S	S	R	R	S	R	ST1047	gp1	V	t1081	-	blaI, blaR1, blaZ, mecA	-	-	lmrS	-	-	mepA, tet(38), tet(K)	-	-	gyrA_S84L, parC_S80F	-	-	-		

CN, Gentamicin; RD, Rifampin; FOX, Cefoxitin; CPT, Cefaroline; CIP, Ciprofloxacin; LEV, Levofloxacin; SXT, Trimethoprim-sulfamethoxazole; FA, Fusidic acid; VA, Vancomycin; DA, Clindamycin; E, Erythromycin; NF, Nitrofurantoin; LZD, Linezolid; P, Penicillin G; C, Chloramphenicol; QD, Quinupristin-dalfopristin; TE, Tetracycline; R, resistant; S, sensitive; I, intermediate.

Supplementary Table ST4. Antibigram and molecular typing results of ESBL-producing *Enterobacterales* isolates from the Hong Kong subway system

MDRO information				Antibiogram																								Molecular typing	ARG profile					
Isolate ID	Sampling log	MDRO species	Subway station	Site	CN	AMP	AMC	ERT	IPM	MEM	DOR	CXM	FOX	CAZ	CTX	CEF	CPT	CIP	LEV	ATM	TZP	TE	C	SXT	SAM	ESBL	Sequence type	Beta-lactam	Colistin	Fosfomycin	Phenicol	Phenicol/quinolone		
S1_DIS3re_ESBL_1/UP01308	22S	Enterobacter roggenkampii	DIS	Elevator	S	R	R		S	S	S	R	R	R	R	R	R	S	S	S	R	S	S	S	R	R	ESBL+	-	blaMIR-9	mer-10	fosA	catA	oqxB	
S1_YMT3_ESBL_1/UP01303	22S	Enterobacter cloacae	YMT	Elevator	S	R	R		S	S	S	R	R	R	R	S	D	R	S	S	R	S	S	S	R	R	ESBL+	ST346	blaACT-15	-	fosA	catA	oqxB	
S1_TWO1_ESBL_2/UP01304	22S	Enterobacter cloacae	TWO	ATM	S	R	R		S	S	S	R	R	R	R	R	R	S	S	R	S	S	S	S	R	R	ESBL+	-	blaACT-17	-	fosA	catA	oqxB	
S1_TAW1_ESBL_1/UP01305	22S	Enterobacter cloacae	TAW	ATM	S	R	R		S	S	S	R	R	R	R	S	D	R	S	S	R	S	S	S	R	R	ESBL+	-	blaACT	-	fosA	catA	oqxB	
S1_JOR3_ESBL_1/UP01306	22S	Enterobacter cloacae	JOR	Elevator	S	R	R		S	S	S	R	R	R	R	S	D	R	S	S	R	S	S	S	R	R	ESBL+	ST809	blaACT-17	-	fosA, fosA7	catA	-	
S1_HAH3_ESBL_2/UP01307	22S	Enterobacter cloacae	HAH	Elevator	S	R	R		S	S	S	R	R	R	R	S	D	R	S	S	R	S	S	S	R	R	ESBL+	-	blaACT	-	fosA	catA	oqxB	
UP00222/UP00136_ESBL_1	23S	Enterobacter hormaechei	DIS	Escalator handrails	S	R	R	S	S	S	S	R	R	R	R	S	D	R	S	S	R	R	S	S	S	R	ESBL+	ST151	blaACT-17	-	fosA		oqxA, oqxB	
UP00255/UP00136_CRE_2	23S	Enterobacter hormaechei	DIS	Escalator handrails	S	R	R	S	S	S	S	R	R	R	S	S	S	S	S	S	S	S	D	D	I	I	S	ESBL+	ST151	blaACT-17	-	fosA		oqxA, oqxB
UP00594/UP00463ESBL1	24W	Enterobacter hormaechei	NOP	ATM	S	R	R	S	S	S	S	R	R	R	I	R	S	D	R	S	S	S	S	S	R	R	ESBL+	ST527	blaACT-108	-	fosA		oqxA, oqxB	
UP00814/UP00711_ESBL_1	24S	Enterobacter hormaechei	WAC	Escalator handrails	S	R	R	S	S	S	S	R	R	R	R	S	D	R	S	S	R	R	S	S	S	R	ESBL+	ST346	blaACT-15	-	fosA	catA	oqxB	

CN, Gentamicin; AMP, Ampicillin; AMC, Amoxicillin-clavulanic acid; ERT, Ertapenem; IPM, Imipenem; MEM, Meropenem; DOR, Doripenem; CXM, Cefuroxime; FOX, Cefoxitin; CAZ, Ceftazidime; CTX, Cefotaxime; CEF, Cefepime; CPT, Ceftazoline; CIP, Ciprofloxacin; LEV, Levofloxacin; ATM, Aztreonam; TZP, Piperacillin-tazobactam; TE, Tetracycline; C, Chloramphenicol; SXT, Trimethoprim-sulphamethoxazole; SAM, Ampicillin-sulbactam; ESBL, Extended-Spectrum Beta-Lactamase; R, resistant; S, sensitive; I, intermediate; SDD, susceptible dose-dependent.

Supplementary Table ST5. Antibigram and molecular typing results of CRAB isolates from the Hong Kong subway system

MDRO information					Antibiogram														Molecular typing	ARG profile			
Isolate ID	Sampling log	MDRO species	Subway station	Post-handwashing facilities	CN	IPM	MEM	DOR	CIP	LEV	TZP	CTX	CRO	CAZ	CEF	SXT	SAM	TE	Sequence type	Beta-lactam	Phenicol	Fluoro	MecAlike
S1_HAH4_MDR_A_1/UP01298	22S	Acinetobacter pittii	HAH	Escalator handrails	S	R	S	S	S	S	R	I	R	S	S	S	S	S	ST93	blaADC-18, blaOXA-500, blaOXA-58	-	adeD, adeE, amvA	mph(E), msa(E)
S1_WTS1_MDR_A_1/UP01299	22S	Acinetobacter pittii	WTS	ATM	S	R	S	S	S	S	I	R	R	S	S	S	S	S	ST457	blaADC-132, blaOXA	-	adeD, adeE, amvA	-
UP00186/UP01300/UP00097_ESB_L_1	23S	Acinetobacter baumannii	FAN	ATM	I	R	S	S	S	S	S	I	I	I	S	S	S	S	ST768	bla, blaADC	expE	adeC, adeD, adeE, amvA	-

CN, Gentamicin; IPM, Imipenem; MEM, Meropenem; DOR, Doripenem; CIP, Ciprofloxacin; LEV, Levofloxacin; TZP, Piperacillin-tazobactam; CTX, Cefotaxime; CRO, Ceftriaxone; CAZ, Ceftazidime; CEF, Cefepime; SXT, Trimethoprim-sulfamethoxazole; SAM, Ampicillin-sulbactam; TE, Tetracycline; R, resistant; S, sensitive; I, intermediate.

Supplementary Table ST6. Antibigram and molecular typing results of CRPA isolates from the Hong Kong subway system

MDRO information				Antibiogram											Molecular typing	ARG profile					
Isolate ID	Sampling log	Subway station	Post-handwashing facilities	CN	IPM	MEM	DOR	CAZ	CEF	CIP	LEV	TZP	SXT	ATM	RD	Sequence type	Aminoglycoside	Beta-lactam	Phenicol	Fosfomycin	Efflux
S1_SSP2_MDRA_4/U_P01301	22S	SSP	Ticket vending machine	S	R	R	S	S	S	S	S	I	S	S	S	ST357	aph(3')-IIb	blaOXA-846, blaPDC-11	catB7	fosA	mexA, mexE, mexX

CN, Gentamicin; IPM, Imipenem; MEM, Meropenem; DOR, Doripenem; CAZ, Ceftazidime; CEF, Cefepime; CIP, Ciprofloxacin; LEV, Levofloxacin; TZP, Piperacillin-tazobactam; SXT, Trimethoprim-sulfamethoxazole; ATM, Aztreonam; RD, Rifampin; R, resistant; S, sensitive; I, intermediate.

Supplementary Table ST7. Information of public washrooms included in the washroom microbiome study.

Washroom ID	Location	District	Sample Collection Date (DD/MM/YY)	Female Toilet Collected	Male Toilet Collected	Accessible Toilet Collected
W1	VA Student Canteen, G/F, Shaw Amenities Building, The Hong Kong Polytechnic University	Yau Tsim Mong	03/07/24	Yes	Yes	Yes
W2	Man Fai Street Public Toilet	Western	02/09/24	Yes	Yes	Yes
W3	L1, IFC mall, 8 Finance Street, Central	Western	02/09/24	Yes	Yes	No
W4	Food Playground, B2, K11 MUSEA, Victoria Dockside, 18 Salisbury Road	Yau Tsim Mong	02/09/24	Yes	Yes	No
W5	LG4, The One, 100 Nathan Road	Yau Tsim Mong	02/09/24	Yes	Yes	Yes
W6	L1, Hong Kong Science Museum, 2 Science Museum Road	Yau Tsim Mong	02/09/24	Yes	Yes	No
W7	1/F, Queen Elizabeth Hospital, 30 Gascoigne Road	Yau Tsim Mong	09/09/24	Yes	Yes	Yes
W8	Eaton Food Hall, B1/F, Eaton HK, 380 Nathan Road	Yau Tsim Mong	09/09/24	Yes	Yes	Yes
W9	Mong Kok Station	Yau Tsim Mong	09/09/24	Yes	Yes	No
W10	L4 F2 Food Court, Langham Place, 8 Argyle St, Mong Kok	Yau Tsim Mong	09/09/24	Yes	Yes	Yes
W11	Thistle street Rest Garden Public Toilet, Thistle Street	Yau Tsim Mong	23/09/24	Yes	Yes	No
W12	Festival walk, 80 Tat Chee Avenue	Kowloon Tong	09/09/24	Yes	Yes	Yes
W13	Chai Wan Sports Centre, 6 Yee Shun Street	Eastern	09/10/24	Yes	Yes	Yes
W14	G/F, Wan Chai Market, 258 Queen's Road East	Wan chai	11/09/24	Yes	Yes	Yes
W15	Lobby Level, Grand Hyatt Hong Kong, 1 Harbour Road	Wan chai	11/09/24	Yes	Yes	No
W16	G/F, Hong Kong Convention and Exhibition Centre, 1 Expo Drive	Wan chai	11/09/24	Yes	Yes	No
W17	Victoria Park, 1 Hing Fat Street	Wan chai	11/09/24	Yes	Yes	Yes
W18	Hong Kong Central Library, 66 Causeway Road	Wan chai	09/10/24	Yes	Yes	No
W19	3/F, Regal Hong Kong Hotel, 88 Yee Wo Street	Wan chai	11/09/24	Yes	Yes	No
W20	27/F, The Park Lane Hong Kong, 310 Gloucester Road	Wan chai	11/09/24	Yes	Yes	No
W21	Hyatt Regency, 18 Chak Cheung Street	Sha tin	25/09/24	Yes	Yes	Yes
W22	Sha Tin Town Hall, 1 Yuen Wo Road	Sha tin	25/09/24	Yes	Yes	Yes
W23	Yuen Long Station	Yuen Long	23/09/24	Yes	Yes	Yes
W24	G/F and 1/F, Tuen Mun San Hui Market, 180 Ho Pong St	Tuen Mun	23/09/24	Yes	Yes	Yes
W25	Tuen Mun Recreation and Sports Centre, 54 Lung Mun Road	Tuen Mun	23/09/24	Yes	Yes	Yes
W26	G/F, Princess Margaret Hospital, 2-10 Princess Margaret Hospital Road	Kwai Tsing	23/09/24	Yes	Yes	Yes
W27	Entrance Toilet at Hong Kong Disneyland	Tung Chung	25/09/24	Yes	Yes	Yes
W28	Arrival Hall A Toilet, Hong Kong International Airport Terminal 1	Tung Chung	25/09/24	Yes	Yes	No

Supplementary Table ST8. Sampling information of the washroom microbiome study.

Sample Collection Date (DD/MM/YY)	Mean Temperature (°C)	Mean Relative Humidity (%)	Total rainfall (mm)
7/3/2024	30.5	78	0.0
9/2/2024	30.6	78	Trace
9/9/2024	27.8	85	13.0
9/11/2024	30.4	76	0.0
9/23/2024	25.7	90	24.9
9/25/2024	28.5	83	5.4
10/9/2024	26.4	68	Trace

Hong Kong Observatory, 2024. Daily Extract of Meteorological Observations.

<https://www.hko.gov.hk/en/cis/dailyExtract.htm?y=2024&m=09> (accessed 30 12 2024)

Supplementary Table ST9. Antibigram and molecular typing results of MRSA isolates from the post-handwashing areas of public washrooms.

MDRO information		Antibiogram														Molecular typing			ARG profile										
Isolate ID	Post-handwashing facilities	CN	RD	FOX	CPT	CIP	LEV	SXT	FA	VA	DA	E	NF	LZD	P	QD	TE	Sequence type	ACR group	SCCmec type	spa type	Aminoglycoside	Beta-lactam	Fosfomycin	Macrolide/Lincosamide/Streptogramin	Macrolide/phenicol	Phenicol	Quaternary ammonium	Tetracycline
UP00915_MRSA_1	Paper dispenser	R	S	R	S	S	S	S	S	S	R	R	S	S	R	R	S	ST338	gp1	V/Vb	t437	aac(6)-Ic/aph(2)-Ia, ant(6)-Ia, aph(3)-IIIa	blaI, blaR1, blaZ, mecA	glpT_A100V, murA_E291D, murA_T396N	erm(B)	lmrS	catA	qacA, qacR	mepA, tet(38)
UP01030_MRSA_1	Hand dryer	S	S	R	S	S	S	S	S	S	S	S	S	S	R	S	S	ST1457	gp2	IV	t067	-	blaI, blaR1, blaZ, mceA, mecR1	fosB	-	lmrS	-	-	mepA, tet(38)

CN, Gentamicin; RD, Rifampin; FOX, Cefoxitin; CPT, Ceftaroline; CIP, Ciprofloxacin; LEV, Levofloxacin; SXT, Trimethoprim-sulfamethoxazole; FA, Fusidic acid; VA, Vancomycin; DA, Clindamycin; E, Erythromycin; NF, Nitrofurantoin; LZD, Linezolid; P, Penicillin G; C, Chloramphenicol; QD, Quinupristin-dalfopristin; TE, Tetracycline; R, resistant; S, sensitive.

Supplementary Table ST10. Antibigram and molecular typing results of CRPA isolates from the post-handwashing areas of public washrooms.

MDRO information			Antibiogram																				Molecular typing			ARG profile														
Isolate ID	MDRO species	Post-handwashing facilities	CN	AMP	AMC	ERT	IPM	MEM	DOR	CXM	FOX	CAZ	CTX	CEF	CPT	CIP	LEV	ATM	TZP	TE	C	SXT	SAM	ESBL	Sequence type	Serotype (e. coli only)	Phylogroup (E. coli only)	Aminoglycoside	Beta-lactam	Fosfomycin	Macrolide	Phenicol	Phenicol/quinolone	Quaternary ammonium	Quinolone	Sulfonamide	Tetracycline	Trimethoprim	Efflux	
UP00763_EsBL_3	Enterobacter hormaechei	Hand dryer	S	R	R	S	S	S	S	R	R	R	R	S	R	D	D	S	R	R	S	I	R	+	ST68	-	-	blaACT-147	-	-	cat A	oqx B	-	-	-	-	-	-	-	
UP00766_EsBL_1	Enterobacter koebeli	Exit door handle	S	R	R	S	S	S	S	R	R	R	R	S	R	S	S	R	R	R	S	S	S	R	+	-	-	blaACT-143	fosA	-	cat A	oqx B	-	-	-	-	-	-	-	
UP00820_EsBL_1	Klebsiella aerogenes	Exit door handle	S	R	R	S	S	S	S	R	R	R	R	S	R	D	D	S	R	R	R	S	S	R	+	-	-	-	ampC	-	-	-	oqx B	-	gyrA_S83T	-	-	-	-	emrD
UP00848_EsBL_2	Enterobacter hormaechei	Faucet	S	R	R	S	S	S	S	R	R	R	R	S	R	D	D	S	R	R	R	S	S	R	+	ST597	-	-	blaACT-46	fosA	-	cat A	oqx B	-	-	-	-	-	-	
UP00892_EsBL_1	Enterobacter hormaechei	Exit door handle	S	R	R	S	S	S	S	R	R	R	R	S	R	D	D	S	R	R	R	S	S	R	+	ST331	-	-	blaACT-25	fosA	-	cat A	oqx B	-	-	-	-	-	-	
UP00949_EsBL_1	Enterobacter bugandensis	Faucet	S	R	I	S	S	S	S	R	R	S	R	R	R	I	S	R	S	D	R	S	R	R	+	-	-	-	ampC, blaCTX-M-3, blaLAP-2, blaTEM-1	fosA	-	cat A	oqx B	-	qnrS1, qnrS1	sul2	tet(A)	dfrA14	-	
UP01038_EsBL_1	Enterobacter hormaechei	Hand dryer	S	R	R	S	S	S	S	R	R	R	R	S	R	D	D	S	R	R	R	S	S	R	+	ST133	-	-	blaACT-17	fosA	-	cat A	oqx B	-	-	-	-	-	-	
UP01039_EsBL_1	Escherichia coli	Faucet	S	R	S	S	S	S	S	R	S	R	R	R	R	R	R	R	S	R	S	S	R	+	ST1193	O75:H5	B2	aadA5, aph(3'')-Ib, aph(6)-Id	blaCTX-M-55, blaEC-5, blaTEM	uhpT_E350Q	mph(A)	-	-	qacEdelta1	gyrA_D87N, gyrA_S83L, parC_S80I, parE_L416F	sul1, sul2	tet(A)	dfrA17	acrF, emrE	
UP01046_EsBL_1	Enterobacter bugandensis	Hand dryer	S	R	R	S	S	S	S	R	R	R	R	S	R	S	S	R	R	R	S	S	S	R	+	ST599	-	-	blaACT-49	fosA	-	cat A	oqx B	-	-	-	-	-	-	
UP01051_CRE1	Enterobacter roggenkampii	Faucet	S	R	R	S	S	S	S	I	R	I	I	S	R	D	D	S	S	S	R	S	S	R	+	ST1709	-	-	blaMIR	fosA	-	cat A	oqx B	-	-	-	-	-	-	

CN, Gentamicin; AMP, Ampicillin; AMC, Amoxicillin-clavulanic acid; ERT, Ertapenem; IPM, Imipenem; MEM, Meropenem; DOR, Doripenem; CXM, Cefuroxime; FOX, Cefoxitin; CAZ, Ceftazidime; CTX, Cefotaxime; CEF, Cefepime; CPT, Ceftaroline ; CIP, Ciprofloxacin; LEV, Levofloxacin; ATM, Aztreonam; TZP, Piperacillin-tazobactam; TE, Tetracycline; C, Chloramphenicol; SXT, Trimethoprim-sulphamethoxazole; SAM, Ampicillin-sulbactam; ESBL, Extended-Spectrum Beta-Lactamase; R, resistant; S, sensitive; I, intermediate; SDD, susceptible dose-dependent.

Supplementary Table ST11. Antibigram and molecular typing results of ESBL-producing *Enterobacterales* isolates from the post-handwashing areas of public washrooms.

MDRO information		Antibiogram												Molecular typing		ARG profile					
Isolate ID	Post-handwashing facilities	CN	IPM	MEM	DOR	CAZ	CEF	CIP	LEV	TZP	SXT	ATM	RD	Sequence type	Type	Aminoglycoside	Beta-lactam	Phenicol	Fosfomycin	Fluoroquinolone	Efflux
UP00760_PA_2	Faucet	S	R	S	S	S	S	S	S	S	R	S	R	ST1341	O6	aph(3')-IIb	blaOXA-396, blaPDC-5, oprD_V35 9L	catB7	fosA	crpP	mexA, mexE, mexX
UP01002_PA_1	Faucet	S	R	S	S	S	S	S	S	S	R	R	R	ST1182	O6	aph(3')-IIb	blaOXA-851, blaPDC-46	catB7	fosA	crpP	mexA, mexE, mexX, tbtB, tbtM, tbtR
UP01018_PA_1	Faucet	S	R	S	S	S	S	S	S	S	R	I	R	ST313	O1	aph(3')-IIb	blaOXA-488, blaPDC-37	catB7	fosA	crpP	mexA, mexE, mexX
UP01037_PA_1	Faucet	S	R	S	S	S	S	S	S	S	R	S	R	ST27	O1	aph(3'')-Ib, aph(3')-IIb, aph(6)-Id	blaOXA-494, blaPDC-15, oprD_V35 9L	catB7	fosA	crpP	mexA, mexE, mexX
UP01039_PA_1	Faucet	S	R	S	S	S	S	S	S	S	R	I	R	ST27	O1	aph(3'')-Ib, aph(3')-IIb, aph(6)-Id	blaOXA-494, blaPDC-15, oprD_V35 9L	catB7	fosA	crpP	mexA, mexE, mexX

CN, Gentamicin; IPM, Imipenem; MEM, Meropenem; DOR, Doripenem; CAZ, Cefazidime; CEF, Cefepime; CIP, Ciprofloxacin; LEV, Levofloxacin; TZP, Piperacillin-tazobactam; SXT, Trimethoprim-sulfamethoxazole; ATM, Aztreonam; RD, Rifampin; R, resistant; S, sensitive; I, intermediate.

Appendix

Appendix 1 – Procedures for isolation of *Candida* spp. and *Candidozyma auris* from environmental samples.

1. Prepare Salt Sabouraud Dulcitol broth (SSD) as follows: Dissolve 5g casein peptone (Fisher Scientific, cat.# R451102), 5g peptic digest of animal tissue (Fisher Scientific, cat.# 7181A), 20g dulcitol powder (Fisher, cat.# AC117701000), and 100g sodium chloride (NaCl) in 1000 mL of culture-grade water.
2. Autoclave mixture for SSD at 121°C for 15 mins. Bring down to room temperature.
3. Add gentamicin (Sigma-Aldrich, cat.# R4408-10ML) and chloramphenicol (Sigma-Aldrich, cat.# RG1397-10ML) at a final concentration of 50 mg/L.
4. Aliquot 2 mL of SSD broth into sterile 15 mL tubes.
5. Add 100 surface sample suspension and incubate at 40 °C for 5 days.
6. Subculture incubated SSD broth onto CHROMagarTM Candida Plus (CHROMagar, Paris, France, cat.# CA242) and grow for 4 days.
7. Perform MALDI-ToF and ITS PCR for all yeast-like colonies.

Appendix 2 – Working concentrations of antibiotics in BHI broth.

Antibiotics-BHI broth	Antibiotics	Concentration in BHI broth (mg/L)
CR	Vancomycin	10
	Meropenem	0.5
ESBL	Vancomycin	10
	Cefotaxime	1
VRE	Vancomycin	3
MRSA	Cefoxitin	3.5
	Aztreonam	20

Appendix 3 – List of chromogenic agars and target drug-resistant organisms.

Chromogenic agar	Brand	Target Organisms	Positive QC (ATCC catalog number)	Negative QC (ATCC catalog number)
CHROMagar Acinetobacter	CHROMagar	Multidrug-resistant <i>Acinetobacter</i> (MDRA)	<i>Acinetobacter baumannii</i> Bouvet and Grimont (BAA-1605)	<i>Enterococcus faecalis</i> (Andrewes and Horder) Schleifer and Kilpper-Balz (29212)
CHROMagar Candida Plus	CHROMagar	<i>Candida albicans</i> , <i>Candida auris</i> , <i>Candida glabrata</i> , <i>Candida krusei</i> , <i>Candida orthopsilosis</i> , <i>Candida parapsilosis</i> , <i>Candida tropicalis</i>	<i>Candida auris</i> Satoh et Makimura (MYA-5001)	<i>Escherichia coli</i> (Migula) Castellani and Chalmers (25922)
CHROMagar ESBL	CHROMagar	ESBL-producing <i>Enterobacteriaceae</i> (ESBL-E)	<i>Klebsiella quasipneumoniae</i> Brisse et al (700603)	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> Rosenbach (25923)
CHROMagar MRSA	CHROMagar	Methicillin-resistant <i>Staphylococcus aureus</i> (MRSA)	In-house MRSA positive control	<i>Enterococcus faecalis</i> (Andrewes and Horder) Schleifer and Kilpper-Balz (29212)
CHROMagar m SuperCARBA	CHROMagar	Carbapenem-resistant <i>Enterobacteriaceae</i> (CR-E)	<i>Klebsiella pneumoniae</i> (Schroeter) Trevisan (BAA-1705)	<i>Enterococcus faecalis</i> (Andrewes and Horder) Schleifer and Kilpper-Balz (29212)
CHROMagar VRE	CHROMagar	Vancomycin-resistance <i>Enterococcus</i> (VRE)	In-house VRE positive control	<i>Enterococcus faecalis</i> (Andrewes and Horder) Schleifer and Kilpper-Balz (29212)
RapID PA agar	Bio-Rad	Multidrug-resistant <i>Pseudomonas aeruginosa</i> (MDR-PA)	<i>Pseudomonas paraaeruginosa</i> Rudra et al. (9027)	<i>Enterococcus faecalis</i> (Andrewes and Horder) Schleifer and Kilpper-Balz (29212)

Appendix 4 – List of drugs tested for *Staphylococcus aureus*.

S/N	Drug class	Antibiotics name	Abbr.	Disc content
1	Aminoglycosides	Gentamicin	CN	10µg
2	Ansamycins	Rifampin	RD	5µg
3	Anti-MRSA cephalosporin	Ceftaroline	CPT	30µg
4	Anti-staphylococcal beta-lactams (or cephamycins)	Cefoxitin	FOX	30µg
5	Fluoroquinolones	Ciprofloxacin	CIP	5µg
		Levofloxacin	LEV	5µg
6	Folate pathway inhibitors	Trimethoprim-sulfamethoxazole	SXT	25µg
7	Fucidanes	Fusidic acid	FA	10µg
8	Glycopeptides	Vancomycin	VA	30µg
9	Lincosamides	Clindamycin	DA	2µg
10	Macrolides	Erythromycin	E	15µg
11	Nitrofurans	Nitrofurantoin	F	300µg
12	Oxazolidinones	Linezolid	LZD	30µg
13	Penicillins	Penicillin G	P	10µg
14	Phenicol	Chloramphenicol	C	30µg
15	Streptogramins	Quinupristin-dalfopristin	QD	15µg
16	Tetracyclines	Tetracycline	TE	30µg

Criteria for defining MDR, XDR and PDR in *Staphylococcus aureus*

MDR: non-susceptible to ≥ 1 agent in ≥ 3 antimicrobial categories.

XDR: non-susceptible to ≥ 1 agent in all but ≤ 2 categories.

PDR: non-susceptible to all antimicrobial agents listed.

Appendix 5 – List of drugs tested for *Enterobacterales*.

S/N	Drug class	Antibiotics name	Abbr.	Disc content
1	Aminoglycosides	Gentamicin	CN	10µg
2	Anti-MRSA cephalosporins	Ceftaroline	CPT	30µg
3	Antipseudomonal penicillins + β -lactamase inhibitors	Piperacillin-tazobactam	TZP	110µg
4	Carbapenems	Ertapenem	EPT	10µg
		Imipenem	IPM	10µg
		Meropenem	MEM	10µg
		Doripenem	DOR	10µg
5	Cephameycins	Cefoxitin	FOX	30µg
6	Combined disc	Ceftazidime-clavulanate	CAZ-CLA	30/10µg
		Cefotaxime-clavulanate	CTX-CLA	30/10µg
7	Extended-spectrum cephalosporins; 3rd and 4th generation cephalosporins	Cefotaxime	CTX	30µg
		Ceftazidime	CAZ	30µg
		Cefepime	CEF	30µg
8	Fluoroquinolones	Ciprofloxacin	CIP	5µg
		Levofloxacin	LEV	5µg
9	Folate pathway inhibitors	Trimethoprim-sulphamethoxazole	SXT	25µg
10	Monobactams	Aztreonam	ATM	30µg
11	Non-extended spectrum cephalosporins; 1st and 2nd generation cephalosporins	Cefuroxime	CXM	30µg
12	Penicillins	Ampicillin	AMP	10µg
13	Penicillins + β -lactamase inhibitors	Amoxicillin-clavulanic acid	AMC	30µg
		Ampicillin-sulbactam	SAM	20µg
14	Phenicol	Chloramphenicol	C	30µg
15	Tetracyclines	Tetracycline	TE	30µg

Criteria for defining MDR, XDR and PDR in *Enterobacterales*

MDR: non-susceptible to ≥ 1 agent in ≥ 3 antimicrobial categories.

XDR: non-susceptible to ≥ 1 agent in all but ≤ 2 categories.

PDR: non-susceptible to all antimicrobial agents listed.

Appendix 6 – List of drugs tested for *Acinetobacter baumannii*.

S/N	Antimicrobial category	Antibiotics name	Abbr.	Disc content
1	Aminoglycosides	Gentamicin	CN	10µg
2	Antipseudomonal carbapenems	Imipenem	IPM	10µg
		Meropenem	MEM	10µg
		Doripenem	DOR	10µg
3	Antipseudomonal fluoroquinones	Ciprofloxacin	CIP	5µg
		Levofloxacin	LEV	5µg
4	Antipseudomonal penicillins + β-lactamase inhibitors	Piperacillin-tazobactam	TZP	110µg
5	Extended-spectrum cephalosporins	Cefotaxime	CTX	30µg
		Ceftriaxone	CRO	30µg
		Ceftazidime	CAZ	30µg
		Cefepime	CEF	30µg
6	Folate pathway inhibitors	Trimethoprim-sulphamethoxazole	SXT	25µg
7	Monobactams	Aztreonam	ATM	30µg
8	Penicillins + β-lactamase inhibitors	Ampicillin-sulbactam	SAM	20µg
9	Rifamycin	Rifampin	RD	5µg
10	Tetracyclines	Tetracycline	TE	30µg

Criteria for defining MDR, XDR and PDR in *Acinetobacter baumannii*

MDR: non-susceptible to ≥1 agent in ≥3 antimicrobial categories.

XDR: non-susceptible to ≥1 agent in all but ≤2 categories.

PDR: non-susceptible to all antimicrobial agents listed.

Appendix 7 – List of drugs tested for *Pseudomonas aeruginosa*.

S/N	Drug class	Antibiotics name	Abbr.	Disc content
1	Aminoglycosides	Gentamicin	CN	10µg
2	Antipseudomonal carbapenems	Imipenem	IPM	10µg
		Meropenem	MEM	10µg
		Doripenem	DOR	10µg
3	Antipseudomonal cephalosporins	Ceftazidime	CAZ	30µg
		Cefepime	CEF	30µg
4	Antipseudomonal fluoroquinones	Ciprofloxacin	CIP	5µg
		Levofloxacin	LEV	5µg
5	Antipseudomonal penicillins + β -lactamase inhibitors	Piperacillin-tazobactam	TZP	110µg
6	Folate pathway inhibitors	Trimethoprim- sulfamethoxazole	SXT	25µg
7	Monobactams	Aztreonam	ATM	30µg
8	Rifamycin	Rifampin	RD	5µg

Criteria for defining MDR, XDR and PDR in *Pseudomonas aeruginosa*

MDR: non-susceptible to ≥ 1 agent in ≥ 3 antimicrobial categories.

XDR: non-susceptible to ≥ 1 agent in all but ≤ 2 categories.

PDR: non-susceptible to all antimicrobial agents listed.