



Faculty of Medicine and Health Sciences

**Investigating the Diversity of Bacteria and Fungi in Rock Pigeon
(*Columba livia*) Stool using Metagenomics in Sarawak, Malaysian
Borneo.**

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Investigating the Diversity of Bacteria and Fungi in Rock Pigeon (*Columba livia*) Stool using Metagenomics in Sarawak, Malaysian Borneo.

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.



.....

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Proverbs 16:3

ABSTRACT

Rock pigeon (*Columba livia*), are domesticated birds, commonly found in urban areas worldwide. Apart from being a nuisance, pigeons are associated with 110 pathogens, with most studies focused on the bacterial and fungal diversity due to its zoonotic potential. Previously, culture-dependent techniques in diversity studies were preferred, including bacterial and fungal studies of pigeon stool. However, the technique underestimates presence of unculturable bacteria and fungi, ultimately inaccurately reporting the actual diversity. To this day, only one study respectively has described on the bacterial and fungal diversity using culture-independent techniques worldwide. Additionally, any microbiome data on pigeon stool has not been reported in Malaysia previously. Thus, to fill this knowledge gap, this study aims to characterise the bacterial and fungal diversity and to identify potentially pathogenic bacteria and fungi in pigeon stool found in towns of Sarawak using high-throughput amplicon sequencing and to identify potentially pathogenic bacterial and fungal genera. Six samples consisting of fresh and dry stool samples were collected from four towns in Sarawak, and its microbial composition was identified through sequencing of the V3 – V4 16S rRNA and ITS2 rRNA regions. A total of 1381 and 481 bacterial and fungal amplicon sequence variants (ASVs), respectively, were identified in pigeon stool, with dry pigeon stool having higher bacterial and fungal ASVs than fresh pigeon stool. Firmicutes was the dominating bacterial pigeon stool contributed by the presence of probiotic bacteria such as *Sporosarcina*, *Clostridium sensu stricto* 1, *Romboutsia*, *Lactobacillus* and *Anerosporobacter*, reflecting the pigeon gut microbiome. Whereas, the dominance of Proteobacteria in dry pigeon stool was attributed to the presence of enteric bacteria such as *Escherichia-Shigella* and *Enterococcus*, which have been found to proliferate upon the excrement from the gastrointestinal tract. On the other hand, dominance of Ascomycota is

contributed by the presence of *Kazachstania* and *Neocucurbutaria*. However, environmental fungal genera including *Aspergillus*, *Trichosporon*, *Penicillium*, *Iodophanus*, and *Cephalophora* contributed to the dominance of Ascomycota in dry pigeon stool. Additionally, 18 bacterial and fungal genera, respectively, were flagged as potentially pathogenic. Of these, four and six bacterial and fungal genera, respectively, were not previously associated with pigeon stool. This study gives insight into the importance of utilising high-throughput amplicon sequencing for bacterial and fungal diversity characterisation, depicting the important role of fresh and dry pigeon stool in the environment. Additionally, the detection of additional pigeon-related potentially pathogenic pathogens in study shows that geographical location and immune status of the bird plays a role in the prevalence of microbes.

Keywords: Pigeon, bacteria, fungi, metagenomics, potentially pathogenic

Menyiasat Kepelbagaian Bakteria dan Kulat dalam Najis Burung Merpati (Columba livia) Menggunakan Metagenomik di Sarawak, Borneo Malaysia.

ABSTRAK

Burung merpati batu (Columba livia), ialah burung domestik, yang biasanya ditemui di kawasan bandar di seluruh dunia. Selain menjadi gangguan, burung merpati dikaitkan dengan 110 patogen, di mana kebanyakan kajian tertumpu pada kepelbagaian bakteria dan kulat kerana potensi zoonotiknya. Sebelum ini, teknik 'culture-dependent' menjadi pilihan dalam kajian, termasuk kajian berkaitan dengan komuniti bakteria dan kulat dalam najis burung merpati. Walau bagaimanapun, teknik tersebut memandang rendah kehadiran bakteria dan kulat yang tidak boleh dibiakkan, akhirnya secara tidak langsung melaporkan kepelbagaian yang tidak tepat. Sehingga kini, hanya satu kajian masing-masing menerangkan kepelbagaian bakteria dan kulat dalam najis burung merpati. Tambahan pula, data tentang mikrobiom najis burung merpati dari Malaysia tidak pernah dilaporkan. Justeru, bagi mengisi jurang pengetahuan ini, kajian ini bertujuan untuk mencirikan kepelbagaian bakteria dan kulat serta mengenal pasti bakteria dan kulat yang berpotensi patogenik dalam najis burung merpati yang terdapat di bandar-bandar Sarawak menggunakan penjujukan amplicon berkapasiti tinggi. Enam sampel yang terdiri daripada sampel najis segar dan kering dikumpul dari empat bandar di Sarawak, dan komposisi mikrob dikenalpasti melalui penjujukan kawasan V3 – V4 16S rRNA dan ITS2 rRNA. Sejumlah 1381 dan 481 variasi rangkaian amplicon (ASVs) bakteria dan kulat masing-masing telah dikenal pasti dalam najis merpati, dengan najis merpati kering mempunyai ASVs bakteria dan kulat yang lebih tinggi berbanding dalam najis merpati segar. Firmicutes mendominasi najis burung merpati disebabkan oleh kehadiran bakteria probiotik seperti Sporosarcina, Clostridium sensu stricto 1, Romboutsia, Lactobacillus dan Anerosporobacter, yang mencerminkan mikrobiom usus burung merpati. Manakala,

dominasi *Proteobacteria* dalam najis kering burung merpati dikaitkan dengan kehadiran bakteria enteric seperti *Escherichia-Shigella* dan *Enterococcus*, yang didapati membiak selepas melalui saluran gastrousus. Sebaliknya, dominasi *Ascomycota* disumbangkan oleh kehadiran *Kazachstania* dan *Neocucurbutaria*. Namun begitu, genera kulat persekitaran termasuk *Aspergillus*, *Trichosporon*, *Penicillium*, *Iodophanus*, dan *Cephalophora* menyumbang kepada dominasi *Ascomycota* dalam najis kering burung merpati. Selain itu, 18 genera bakteria dan kulat masing-masing telah dikenal pasti sebagai berpotensi patogenik. Daripada jumlah ini, empat dan enam genera bakteria dan kulat masing-masing tidak pernah dikaitkan dengan najis burung merpati sebelum ini. Kajian ini memberikan gambaran tentang kepentingan menggunakan penjujukan amplicon berkapasiti tinggi untuk pencirian kepelbagaian bakteria dan kulat, menggambarkan peranan penting najis segar dan kering burung merpati dalam persekitaran. Tambahan pula, pengesanan patogen tambahan yang berpotensi patogenik yang berkaitan dengan burung merpati dalam kajian ini menunjukkan bahawa lokasi geografi dan status imun burung memainkan peranan dalam kekerapan mikrob.

Kata kunci: Burung merpati, bakteria, kulat, metagenomik, berpotensi patogenik

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LIST OF ABBREVIATIONS

°C	Degree Celsius
μL	Microliter
16S rRNA	16S ribosomal RNA
ANCOM	Analysis of Composition of Microbiomes
ANOSIM	Analysis of Similarities
ASV	Amplicon Sequence Variant
bp	Base pair
cm	Centimeter
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
DPS	Dry pigeon stool
e.g.	Exempli gratia
EDTA	Ethylenediaminetetraacetic acid
FPS	Fresh pigeon stool
g	Gram
gDNA	Genomic deoxyribonucleic acid
HTAS	High-throughput amplicon sequencing
ITS rRNA	Internal Transcribed Sequence ribosomal rRNA
kbp	Kilobase pair
kg	Kilogram
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
mg	Milligram

Mins	Minutes
mL	Microliter
MRSA	Methicillin-resistant <i>Staphylococcus. aureus</i>
NaCl	Sodium chloride
NanoSIMS	Nano-scale secondary ion mass spectrometry
NGS	Next-generation sequencing
OTU	Operational Taxonomic Unit
PCoA	Principal Coordinate Analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
QIIME2	Quantitative Insights into Microbial Ecology 2
qPCR	Quantitative polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
x g	G-force (relative centrifugal force)
rRNA	Ribosomal nucleic acid
SD	Standard deviation
ST	Strain type
TBE	Tris/Borate/ Ethylenediaminetetraacetic
V	Volts
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

1.1 Study background

Rock pigeon (*Columba livia*; Family: Columbidae), also referred to as feral pigeon, are urban birds with worldwide distribution. With an average population of one pigeon per 10 – 20 city inhabitants, their world population is estimated to be around 166 - 330 million (Giunchi et al., 2012). Pigeons were first domesticated during the Mesopotamian era for livestock, spiritual offering and its homing abilities (Mench, 2009; Sossinka, 2013). But have then since become feral due to abandonment by owners (Mench, 2009). Regardless, their importance to humans still remains as pigeons serve as food source, pets and for pigeon racing (Bupasha et al., 2020; Dolka et al., 2020; Sayed et al., 2023).

However, it has become clear that pigeons are a significant pest in urban settlements. An individual rock pigeon produces about 12 kg of excrement annually containing high nitrogenous waste, such as uric acid and urea (Sossinka, 2013). Not only does it produce a foul smell and unpleasing sight in public areas, but the acidity of the excrement deteriorates calcareous stones, destroying historic monuments and architecture. Many of which increases the local government annual expenditure for restoration (Spennemann et al., 2017).

Additionally, literatures have reported pigeons as reservoirs of up to 110 zoonotic and allergen-causing pathogens and spreading them either through consumption of undercooked meat, inhalation of feather dust and dust from withered excrete and pigeon coop (Haag-Wackernagel & Moch, 2004; Madsen et al., 2022; Mia et al., 2022; Perez-Sancho et al., 2020). Additionally, the high nitrogenous content of pigeon stool serves as an excellent nutrient source to support the growth of stool-related and environmental pathogens,

contributing to the abundance of opportunistic microbes (Lee et al., 2017; Nualmalang et al., 2023).

According to Haag-Wackernagel & Moch (2004), 110 pigeon pathogens are associated pigeon, and transmission to human occurs either through contact with their excrete in public places or inhalation of withered pigeon stool, infecting mainly the immunocompromised community. Most research has dedicated its objective to understanding the diversity of pathogenic bacteria and fungi in fresh and dry pigeon stool, primarily because infections caused by pigeon-related viruses and helminths are generally underreported. Apart from that, pigeon-related bacteria and fungi have been flagged as priority pathogens in two lists released by the World Health Organisation (WHO) to focus research on these high-virulence and antimicrobial-resistant pathogens (World Health Organisation, 2017; World Health Organization, 2022).

However, many of the studies that have deduced their importance utilised culture-dependent methods such as isolation method (e.g. agar plating, cell culture) or culture-independent method (e.g. species-specific identification, whole or partial 16S rRNA gene sequencing, whole or partial ITS rRNA gene sequencing). This leads to literature underestimating the diversity, especially of unculturable microbes (Bodor et al., 2020). With the increasing number of sporadic infections caused by emerging pathogenic bacteria and fungi, many researchers have moved forward with the inclusion of next-generation sequencing technologies such as high-throughput amplicon sequencing for diversity and surveillance studies, which is more reliable, cost-effective and does not underestimate the diversity of microbes in a sample.

To date, only Oh and colleagues (2023) have utilised next-generation sequencing to understand the microbiome of fresh pigeon stool and reported an additional four genera that have yet to be associated with pigeon previously. Apart from that, no studies have reported on the microbiome of dry pigeon stool itself. However, a study conducted by Madsen and colleagues (2022) found high abundance of inhalable particle size of *Escherichia-Shigella* and *Enterococcus* in dust of pigeon coops, in which air-dispersed dust is one of the contributors to its bacterial diversity. On the other hand, only one study on both fresh and dry pigeon stool have been reported by Lee and colleagues (2017), detecting 28 fungal species not previously reported in pigeon stool.

1.2 Problem statement

Pigeon have been labelled as reservoirs of pathogenic bacteria and fungi. Zoonotic transmission may potentially occur due to close demographics with humans, including the immunocompromised community. Although numerous studies have been conducted and have reported the presence of pathogenic bacteria and fungi, these studies are dependent on microbial isolation and molecular identification using either species-specific primers or universal bacteria or fungi primers. The techniques are considered the gold standard, however, they often fail to identify unculturable microbes, thus underestimating the actual diversity. Apart from that, there is a gap in classifying bacterial and fungal microbiome from pigeon stool using culture-independent techniques such as next-generation sequencing, especially in Malaysia.

1.3 Objectives

The objectives of this study are:

1. To characterise the bacterial diversity of pigeon stool using high-throughput amplicon sequencing.
2. To characterise the fungal diversity of pigeon stool using high-throughput amplicon sequencing.
3. To identify the potentially pathogenic bacteria from pigeon stool.
4. To identify the potentially pathogenic fungi from pigeon stool.

1.4 Research hypothesis

H01: There is no difference in bacterial diversity between fresh and dry pigeon stool through high-throughput amplicon sequencing.

HA1: There is a difference in bacterial diversity between fresh and dry pigeon stool through high-throughput amplicon sequencing.

H02: There is no difference in fungal diversity between fresh and dry pigeon through high-throughput sequencing.

HA2: There are differences in fungal diversity between fresh and dry pigeon through high-throughput sequencing.

H03: There are no potentially pathogenic bacteria in pigeon stool.

HA3: There are potentially pathogenic bacteria in pigeon stool.

H04: There are no potentially pathogenic fungi in pigeon stool.

HA4: There are potentially pathogenic fungi in pigeon stool.

CHAPTER 2

LITERATURE REVIEW

2.1 *Columba livia*



Figure 2.1: A litter of rock pigeons at an opened-space public area in Kuching Waterfront, Sarawak (Image of flocking pigeons courtesy of Vaenessa, April 2023).

Rock pigeon (*Columba livia*), also known as rock doves or feral pigeon (Family: Columbidae) (Figure 2.1), are descendants of the wild rock dove cliff-dwellers. Domestication of rock pigeon was believed to have started at least 5,000 – 10,000 years ago during the Mesopotamia and Egypt era mainly as food source, spiritual offerings and homing abilities (Sossinka, 2013). Rock pigeons are the oldest and most common cosmopolitan human commensal species as they became feral due to abandonment by their owner and their escape during their long flights (Mench, 2009). Nowadays, people have taken an increasing interest in pigeon rearing as pets, poultry and for racing purposes (Bupasha et al., 2020; Dolka et al., 2020; Sayed et al., 2023).

2.1.1 Urbanisation of pigeon

The ability of rock pigeon to establish their colonies in urban settlements is to be applauded, as wild animals, especially birds, have found it difficult to exploit and adapt to urban resources (Sol et al., 2014). The presence of human-related structures such as windows and moving vehicles are the main cause of the high mortality rate of birds in urban settings (Benítez-López et al., 2010; Klem, 2014). Apart from that, scarcity of food resources catering to specific dietary requirements and the inability of birds to nest due to the lack of available nesting sites and nesting materials also threatens the colonisation of bird species in urban settings.

The main key driver of pigeon colonisation success in urban settlements is the adaptation of its diet. Pigeons were primarily granivorous; however, they have adapted their diet to omnivorous to be able to survive off non-grain food resources such as soil, insects and grass and feed off human scraps (Spennemann & Watson, 2017). But most importantly, feeding by pigeon enthusiasts has endorsed unlimited food availability, which, in return, allows the pigeon to spend more energy in breeding all year round (Haag-Wackernagel & Geigenfeind, 2008).

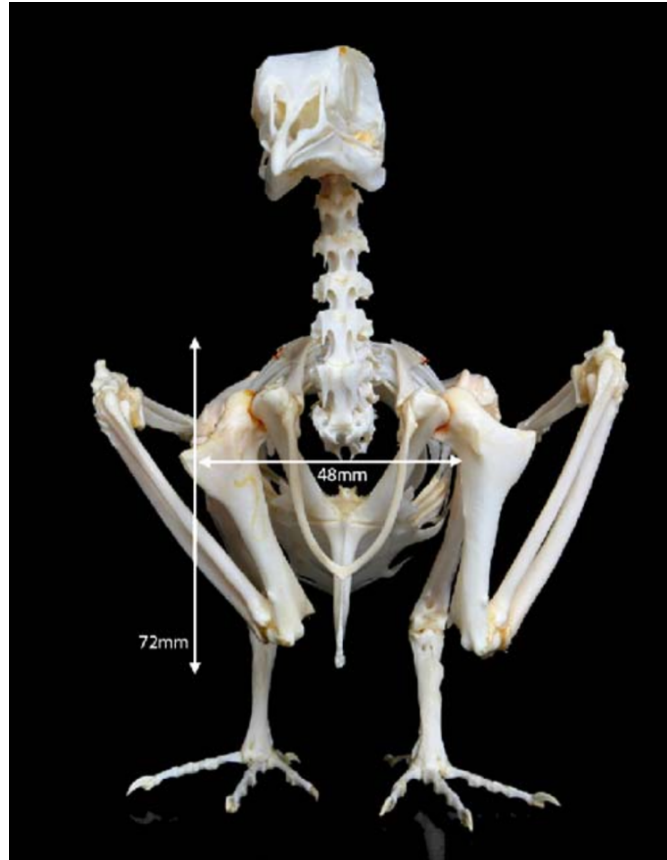


Figure 2.2: The skeletal structure of a feral pigeon. The width distance between the keel of sternum and thoracic vertebrae is labelled in the figure (Haag-Wackernagel & Geigenfeind, 2008)

Another factor that has allowed pigeons to colonise urban settlements is their specific behaviours, which aid in avoiding predators. Pigeons often opt to utilise small and narrow passages of buildings as their roosting and breeding sites, which are often inaccessible to their predators (Haag-Wackernagel & Geigenfeind, 2008). This is attributed to their short chest width, as depicted in the skeletal structure in Figure 2.2, however buildings hollows smaller than 6cm becomes an obstacle for them. Apart from that, pigeons are able to utilise steep building roofs for daytime resting and high building ledges for nesting, allowing them to avoid urban predators, a trait passed down from their ancestors (Lim et al., 2023; Spennemann & Watson, 2017).

2.1.2 Pigeon: friend or foe?

Pigeons are well taken care of in urban areas, especially by pigeon enthusiasts, showing their affection by providing endless sources of food, aiding their population bloom in urban and peri-urban areas. However, their burgeoning population incurs damage to its surrounding and those around in them. To start, pigeons produce around 12 kg of excretion annually, with most accumulating in their feeding and perching sites, setting off a foul smell to its surrounding environment as well as contaminating soil, surface water and food supplies consumed by other urban or companion animals (Stock & Haag-Wackernagel, 2013). Due to the physiology of birds, the excrete of pigeons also includes high concentrations of uric acid and other soluble salt, together with the solid waste, which have been reported to aid in corrosion, decaying and white staining of historical architectures, incurring high cost to city councils for building cleaning and maintenance historical buildings, incurring high cost to city councils for building cleaning and maintenance (Haag-Wackernagel & Geigenfeind, 2008; Spennemann et al., 2017). Additionally, the excreted nitrogenous compounds serve as an important nutrient medium, promoting the growth of stool-related and environmental pathogens (Lee et al., 2017; Nualmalang et al., 2023).

Apart from that, pigeons are natural reservoirs for pathogens that have been observed to cause emerging and re-emerging diseases in humans, carrying about 110 allergen-causing opportunistic and potentially zoonotic pathogens comprising of bacteria, fungi, viruses and parasites (Haag-Wackernagel & Moch, 2004; Mia et al., 2022), with varying effects to humans from light infection to mortality. Among the 110 identified pigeon-related pathogens, with bacterial, viral and helminths studies reported from fresh pigeon stool, whereas fungi was reported from dry pigeon stool (Lee et al., 2017). Notable roles of pigeon stool in spreading zoonotic diseases are 1) respiratory viruses, especially of those from the

family of Orthomyxoviridae (e.g. highly pathogenic avian influenza) and Paramyxoviridae (e.g. avian paramyxovirus), 2) respiratory-related fungi such as cryptococcal meningitis-causing *Cryptococcus neoformans* yeast and histoplasmosis-causing *Histoplasma capsulatum*, 3) pathogenic bacteria especially from the *Escherichia*, *Staphylococcus*, and *Enterococcus* genera and 4) mammal-infecting parasites such as *Toxoplasma gondii* and *Enterocytozoon bieneus* (Haag-Wackernagel & Moch, 2004; Kuiken et al., 2018; Mia et al., 2022; Soltani et al., 2013). Common transmission routes to their host are through consumption of uncooked or poorly cooked pigeon meat, vector-borne and inhalation of air-borne pathogens such as from dust of pigeon feathers or withered excrete (Mia et al., 2022).

Among the pathogens mentioned, research on the diversity of bacteria and fungi from pigeon stool is of high interest as it has been deduced that pigeons are reservoirs of pathogenic bacteria and fungi infecting the immunocompromised (Haag-Wackernagel & Moch, 2004; Soltani et al., 2013), and that infection caused by pigeon stool-related virus or helminths are generally underreported.

2.2 Pathogenic bacteria in pigeon stool

In general, pigeon-related bacteria were first focused on *Chlamydia psittaci*, which will be further discussed in the following subsections. However, in recent years, the interest has shifted to understanding the diversity of bacterial genera with reported antimicrobial resistant properties such as resistance and the presence of resistant gene(s). This is due to reports stating that wild birds, including pigeons, are good sentinels due to their exposure to migratory birds, the main reservoirs of antimicrobial resistant strains, and human waste and sewage, eventually facilitating horizontal transmission to humans and livestock and

companion animals in its vicinity (Martín-Maldonado et al., 2022; Wang & Hu, 2022). These include bacterial species under the Enterobacteriaceae family and *Staphylococcus aureus*.

Apart from that, the increase in bacterial studies from pigeons are contributed by the release of the first pathogen prioritisation list by WHO, which intended to highlight 12 bacterial genera with high virulence and antimicrobial resistance (World Health Organisation, 2017). Among which, ten have been reported in pigeons, including *Salmonella*, *Campylobacter*, *Streptococcus pneumoniae*, *Shigella*, *Enterobacter*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterococcus* species (World Health Organisation, 2017).

Most studies on pigeon-bacterial studies used fresh pigeon stool as their sample and utilised culture-dependent methods for bacterial isolation and culture-independent method such as molecular identification using either species-specific primers or the whole or partial 16S rRNA gene for species identification.

Only two studies utilised high-throughput sequencing for bacterial diversity from fresh pigeon stool and pigeon dust focused in South Korea and Denmark. To the author's knowledge, there are no reports on the bacterial diversity from pigeon stool in Malaysia, or Sarawak for that matter.

2.2.1 Enterobacteriaceae bacteria

Among the bacteria reported in pigeon stool, enteric bacteria especially those from the Enterobacteriaceae, are the most commonly studied. Enterobacteriaceae are Gram-negative bacilli with distribution broadly throughout the environment and gastrointestinal tract of many animals, including humans (Hata et al., 2018). Many of these bacteria play an important role in the gut and are regarded as either non-harmful or opportunistic bacteria, however have been identified as the causal agent food poisoning from consumption of under-prepared pigeon meat or food contaminated with fresh pigeon stool.

Ten genera from the Enterobacteriaceae family have been reported in pigeon stool including *Campylobacter*, *Salmonella*, *Escherichia*, *Enterococcus*, *Klebsiella*, *Bacillus*, *Cronobacter*, *Enterobacter*, *Pantoea* and *Lecleria* (Table 2.1). Among the genera mentioned above, *Campylobacter*, *Salmonella*, and *Enterococcus* are reported with worldwide prevalence. As most of the Enterobacteriaceae bacteria reported in pigeon stool can grow in aerobic conditions, almost all except for one opted to utilise molecular method for identification up to species level instead of the isolation.

Table 2.1: Compilation of Enterobacteriaceae genus reported from pigeon stool or cloacal swab between 2010 – 2024.

Author (Year)	Year of sampling	Country	Type of sample	Identification method	Genus detected
Radimersky et al. (2010)	Jan – March 2006	Czech Republic	Cloacal swab	Isolation, molecular	<i>Escherichia</i> <i>Enterococcus</i>
Abulreesh (2010)	June 2009 – Mac 2010	Makkah	Stool	Isolation	<i>Escherichia</i> <i>Salmonella</i>
Vázquez et al. (2010)	2006 – 2007	Spain	Cloacal swab	Molecular	<i>Campylobacter</i>
Ghanbarpour & Daneshdoost (2012)	Sept 2009 – Oct 2010	Iran	Fresh stool	Isolation	<i>Escherichia</i>
Gargiulo et al. (2014)	Dec 2007 – May 2010	Italy	Cloacal swab	Isolation	<i>Escherichia</i> <i>Campylobacter</i> <i>Salmonella</i>
Gabriele-Rivet et al. (2016)	June – July 2011	Canada	Stool	Isolation, molecular	<i>Campylobacter</i>
Marenzoni et al. (2016)	-	Poland	Cloacal swab	Isolation, molecular	<i>Salmonella</i> <i>Campylobacter</i>
Haesendonck et al. (2016)	-	Belgium	Cloacal swab	Isolation, pulse-field	<i>Salmonella</i>
Dudzic et al. (2016)	-	Poland	Cloacal swab, stool	Isolation, molecular	<i>Campylobacter</i>
Zigo et al. (2017)	-	Slovakia	Cloacal swab	Isolation	<i>Escherichia</i> <i>Enterococcus</i> <i>Bacillus</i>

Table 2.1 continued

Hata et al (2018)	May – June 2014	Japan	Fresh stool	Isolation, molecular	<i>Escherichia</i> <i>Enterobacter</i> <i>Cronobacter</i> <i>Pantoea</i> <i>Leclercia</i> <i>Klebsiella</i> <i>Salmonella</i>
Dolka et al. (2020)	-	Poland	Cloacal swab	Isolation, molecular	<i>Enterococcus</i>
Bupasha et al. (2020)	Feb – April 2018	Bangladesh	Cloacal swab	Isolation	<i>Salmonella</i>
Ghanbarpour et al. (2020)	-	Iran	Cloacal swab	Isolation	<i>Escherichia</i>
Carvalho et al. (2020)	2013	Brazil	Fresh stool	Isolation, molecular	<i>Escherichia</i> <i>Salmonella</i>
Kaczorek-Łukowska et al. (2021)	2015 - 2017	Poland	Cloacal swab	Isolation, molecular	<i>Salmonella</i>
Naguib et al. (2021)	-	Egypt	Cloacal swab	Isolation, molecular	<i>Escherichia</i> <i>Salmonella</i>
Wang & Hu (2022)	July – Aug 2021	China	Fresh stool	Isolation, molecular	<i>Escherichia</i>
Loucif et al. (2022)	May – June 2018	France and Algeria	Fresh stool	Isolation, molecular	<i>Klebsiella</i>
Vasconcellos et al (2022)	July – Aug 2018	Brazil	Fresh and dry stool	Isolation, molecular	<i>Enterococcus</i>
Sayed et al. (2023)	Aug 2014 – Dec 2019	Egypt	Fresh stool	Isolation, molecular	<i>Campylobacter</i>

2.2.2 *Chlamydia* species

As mentioned, genus *Chlamydia* is of particular interest due to its ability to remain infectious in the environment after months and its pathogenicity in humans (Kowalczyk & Wójcik-Fatla, 2022). Pigeons are the known reservoirs of the bacteria and typically asymptomatic. Although very rarely associated with mortality, common subclinical symptoms in humans includes fever, chills, headache, myalgia and malaise, and psittacosis including in the immunocompetent community (Beeckman & Vanrompay, 2009).

Chlamydia psittaci was first thought to be the only *Chlamydia* species in pigeons. However, Sachse and colleagues (2014) proposed a novel species member of the Chlamydiaceae family, *C. avium*, which is also commonly found in pigeons and psittacine birds and found to cause infection in host with subclinical symptoms which may eventually lead to mortality. Nevertheless, its zoonotic potential unlike its counterpart is still unclear, with only assumptions of contribution to respiratory diseases and/or diarrhoea to its breeders (Borel & Sachse, 2023).

Nine studies have reported on the prevalence of *Chlamydia* from either pigeon stool or cloacal swab (Table 2.2), with *C. psittaci* being the most commonly identified among other species. In which, studies are more concentrated in the European and Western Pacific WHO region.

Table 2.2: Compilation of worldwide prevalence of *Chlamydia* species identified from pigeon stool or cloaca between 2010 – 2024.

Author (year)	Year of sampling	Country	Type of sample	Identification method	Species detected
Geigenfeind et al. (2012)	Feb 2007 – Feb 2008	-	Cloacal swab	Molecular	<i>C. psittaci</i>
Sachse et al. (2012)	April – May 2009	Germany	Cloacal swab, stool	Isolation, molecular	<i>C. psittaci</i> <i>C. abortus</i> <i>C. trachomatis</i> <i>C. pecorum</i>
Teske et al. (2013)	July – Sept 2008; Feb – Mac 2008	Germany	Cloacal swab	Molecular	<i>C. psittaci</i>
Sariya et al. (2015)	May – Sept 2013	Thailand	Cloacal swab	Molecular	<i>C. psittaci</i>
Marenzoni et al. (2016)	-	Poland	Cloacal swab	Molecular	<i>Chlamydia</i> sp.
Wannaratana et al. (2017)	-	Thailand	Cloacal swab	Molecular	<i>C. psittaci</i>
Burt et al. (2018)	May 2017	Netherlands	Fresh and dry stool	Molecular	<i>C. psittaci</i> <i>C. avium</i>
Mattmann et al. (2019)	May 2013 – Oct 2018	Switzerland	Cloacal swab	Molecular	<i>C. psittaci</i>
Kowalczyk & Wójcik-Fatla (2022)	Apr – Sept 2021	Poland	Fresh and dry stool	Molecular	<i>C. psittaci</i>

2.2.3 *Staphylococcus aureus*

Within the *Staphylococcus* genus, *Staphylococcus aureus* is an opportunistic bacteria commonly isolated human and animal. The species is of particular interest as it is typically viewed as a harmless colonizer of the skin microbiome. However, due to the excessive use of methicillin antibiotics, the increase prevalence of methicillin-resistant *S. aureus* (MRSA) strains have exponentially increased the severe and life-threatening infections in humans (Foster, 2017). The species have been reported in fresh pigeon stool (de Vasconcellos et al., 2022; Wilson et al., 2024; Zigo et al., 2017). Among which, the highest prevalence was found in samples collected from pigeon stool in the vicinity of hospitals in Brazil and belonged to strain-type 188 (ST188), which has been found to cause severe infection in immunocompromised patients in China due to its virulent trait such as biofilm production (de Vasconcellos et al., 2022; Wang et al., 2018).

2.2.4 Diversity analysis of pathogenic bacteria using next-generation sequencing

Taking advantage of the advancement of technology, recent studies have opted to use high-throughput sequencing technology such as Illumina to combat the underestimation of bacterial diversity in a sample.

This is shown in a study conducted by Oh and colleagues (2023), which identified 983 bacterial taxa from 144 domestic pigeon of South Korea. Of which, 12 genera and family were classified as potentially pathogenic with four genera and one family not previously reported in fresh pigeon stool (*Peptostreptococcus*, *Corynebacterium*, *Curtobacterium* and *Brevibacterium*, Listeriaceae, and *Pseudomonas*).

Another study by Madsen and colleagues (2022) identified 180 genera dominated by *Acinetobacter* from coops in Denmark. Although metagenomics was used to understand the difference in alpha diversity between indoor and outdoor bacterial diversity of pigeon coop, numerous genera have also been identified using the MALDI-TOF protocol, which found eight genera that were not previously associated with pigeon (*Brevibacterium*, *Brevundimonas*, *Corynebacterium*, *Erwinia*, *Kocuria*, *Micrococcus*, *Pseudomonas* and *Springobacterium*) (Madsen et al., 2022).

2.3 Pathogenic fungi in pigeon stool

Recent assessments suggest that more than 150 million people are affected by serious fungal infections, with more than 1.7 million each year dying from this disease (Kainz et al., 2020). Pigeon have been long recognised as spreaders of fungal diseases. In fact, pigeon stool is more commonly associated with reservoirs of pathogenic fungi than bacteria, with studies suggesting that the high nitrogenous content in pigeon stool facilitates fungal spore propagation and supplies nutrients for fungal metabolism (Lee et al., 2017). The primary mode of transmission of pigeon-related fungi is through inhalation of its spores. Pigeon-related fungal infections are more commonly associated with immunocompromised patients (e.g. HIV/AIDS, chemotherapy, hematopoietic stem cell transplant, solid organ transplant, elderly patients) (Soltani et al., 2013).

In 2022, World Health Organisation published its first fungal priority pathogen list, which mirrors the global priority list of antibiotic resistant list (World Health Organization, 2022). The list highlights neglected invasive fungal diseases associated with severe risks of mortality and morbidity and aims to guide researchers in the surveillance and development of therapeutic and diagnostic tools and public health against invasive fungal diseases (Fisher

& Denning, 2023). The list categorised pathogens into three groups, critical, high and medium priority, consisting of 19 pathogenic fungi. Of which, *Cryptococcus neoformans*, *Histoplasma capsulatum*, and *Candida species* (*C. albicans*, *C. glabrata* @ *Nakaseomyces glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*) were categorised in all priority group are commonly isolated from pigeon excrete. Indeed, other fungal genera are widely associated with pigeon, such as *Trichosporon* and *Rhodoitorula*, but are not included in the list due to less infection severity caused by the fungi.

To the author's knowledge, only one study on fungal diversity from pigeon stool has been conducted in Malaysia by Tay and colleagues (2005) on the prevalence of *C. neoformans*, which will be further explained in the following subsections. However, no fungal diversity studies from pigeon stool were reported in Sarawak. Apart from that, only one study in South Korea has opted for next-generation sequencing to identify unculturable fungi from fresh and dry pigeon stool.

2.3.1 *Cryptococcus species*

Cryptococcus (Order: Tremellales), coined from the Greek term “hidden sphere”, is a spherical yeast covered in a thin layer of glycoprotein capsular material with a gelatine-like consistency. Among the *Cryptococcus* species, *Cryptococcus neoformans* has always been a topic of interest associated with pigeons, as the in-depth investigation found cryptococcosis patients have had prolonged exposure to pigeon excretion. This has been confirmed through extensive research on its molecular type and strain type (ST), which found molecular type VNI and VNII, and ST4, ST5 and ST6 in both infected patients and pigeon stool or pigeon-fertilised soil mainly found in Asian countries, including Thailand and China (Chen et al., 2021; Kaocharoen et al., 2013).

Apart from *C. neoformans*, other non-*neoformans* *Cryptococcus* have been reported in pigeon including, *C. laurentii*, *C. liquefaciens* and *C. uniguttulatus*, *C. magnus*, *C. adeliensis*, *C. randhawii* and *C. albidosimilis* as an opportunistic yeast causing superficial infection with no cryptococcal antigen detected in patients (Cano et al., 2020; Morales-López & Garcia-Effron, 2021).

A summary of studies on *Cryptococcus* identification from pigeon stool and cloacal swab between 2010 - 2024 can be found in Table 2.3. Overall, *Cryptococcus* was reported in all WHO regions, including Malaysia. Tay and colleagues (2005) reported the low prevalence of *C. neoformans* from pigeon stool in Klang Valley, Malaysia. The study was not included in the Table 2.3 as the paper was published before 2010.

2.3.2 *Candida* species

Candida (Order: Saccharomycetales) is the cause of almost all fungal infections worldwide, causing 700,000 cases of invasive candidiasis (Bongomin et al., 2017). As *Candida* species are common members of both human and animal mycobiomes, infections are typically of endogenous sources. However, the increasing exogenous-source infections suggest that wild birds, including pigeon, are reservoirs of pathogenic *Candida* in urban settlements (Lord et al., 2010; Magalhães Pinto et al., 2019).

Apart from that, virulence factors such as biofilm formation, moderate adhesion to epithelial cells, and secretion of lytic enzymes such as haemolysins, proteinases and phospholipases, which were previously observed in clinical strains, have been reported in *Candida* species isolated from pigeon stool (Magalhães Pinto et al., 2019). In the WHO fungal priority list, all *Candida* species have been isolated from pigeon except for *C. auris* (World Health Organization, 2022). Other *Candida* species reported as opportunistic fungi

in humans but are not considered as priorities are *C. guilliermondi*, *C. famata*, *C. zeylanoides*, *C. pelliculosa* and *C. lusitaniae*.

A compilation of literature reporting the prevalence of *Candida* from pigeon stool and cloacal swab can be found in Table 2.4. In summary, the presence of *Candida* in pigeon stool has been reported in all WHO regions except for the African and South-East Asian regions.

2.3.3 *Histoplasma capsulatum*

Histoplasma capsulatum (Order: Onygenales) is a dimorphic fungus, which exists as 1) mould in the environment (temperature less than 35°C) and 2) small budding yeast-like form at 37°C. Transmission of the fungus is through inhalation of spores of the fungus and often infects the immunocompromised community (eg. HIV/AIDS, steroid medication). Generally, *H. capsulatum* var. *capsulatum* occurs most commonly in North and South America but does exist in other areas throughout the world, whereas *H. capsulatum* var. *dubloisii* is thought to exist predominantly in South Africa (Hepler et al., 2022). It is commonly believed that they are reservoirs of the fungus. However, recent literature mentioned that the presence of *H. capsulatum* is instead strongly associated with soil with high nitrogenous content, such as those soiled with birds (e.g. pigeon and chickens) and bat guano (Deepe, 2018; Hepler et al., 2022). Additionally, due to its hardy exterior, the fungus is able to survive years in soiled soil even without the presence of the additional nutrients from guano (Norkaew et al., 2013).

Unlike *Cryptococcus* and *Candida*, research on *H. capsulatum* prevalence in environmental samples focuses only on cave guano samples due to the increase in histoplasmosis among travellers (Bahr et al., 2015). Interestingly, in contrast to the common

belief that pigeon are the progenitor of the fungus, prevalence data of *H. capsulatum* from pigeon is very much lacking worldwide. Nevertheless, a notable study by Norkaew and colleagues (2013) reported its low prevalence of about from pigeon-soiled soil in Chiang Mai, Thailand.

2.3.4 Other fungal species

Due to the priority of reporting the prevalence of *Cryptococcus* and *Candida* from pigeon stool, other genera of commonly found fungi from pigeon excrete are often overlooked, as the other fungi are considered as ubiquitous and does not necessarily implicate disease in humans.

Among the fungi is *Rhodorotula* (Order: Sporidiobolales), a mucoid yeast with a distinct pink colour on an agar plate. Previously considered non-pathogenic, increasing cases of colonisation in both animals and humans suggest otherwise. Among the emerging *Rhodorotula* species often isolated from pigeon stool are *R. mucilaginosa*, *R. rubra*, *R. minuta* and *R. glutinis* (Table 2.5). On the other hand, *Trichosporon* (Order: Trichosporonales), an opportunistic yeast that is often isolated from soil, animal droppings and most often part of the human skin and infection does not necessarily manifest only in the immunocompromised community. Among the 12 species under the genus, *T. asahii*, *T. beigelii*, *T. harzianu* and *T. mucoides* have been reported in pigeon stool (Table 2.5). Both *Rhodorotula* and *Trichosporon* has also been reported in all WHO regions except the African and South-East Asian regions.

Table 2.3: Compilation of worldwide prevalence of *Cryptococcus* species identified from pigeon stool or cloaca between 2010 – 2024.

Author (year)	Year of sampling	Country	Type of sample	Identification method	Species detected
Zarrin et al. (2010)	2007	Iran	Stool	Isolation	<i>Cryptococcus</i> sp.
Jang et al. (2011)	April – May 2009	South Korea	Fresh and dry stool	Isolation, API 20C	<i>C. albidus</i> <i>C. laurentii</i> <i>C. neoformans</i>
Chae et al. (2012)	Feb – March 2011	South Korea	Dry stool	Isolation, molecular	<i>C. neoformans</i> <i>C. laurentii</i>
Wu et al. (2012)	May – August 2013	China	Fresh stool	Isolation, molecular	<i>C. neoformans</i> <i>C. albidus</i> <i>C. kuetzingii</i> <i>C. magus</i> <i>C. albidosimilis</i> <i>C. adeliensis</i> <i>C. randwii</i>
Soltani et al. (2013)	Sept 2010 – May 2011	-	Pigeon stool	Isolation	<i>C. neoformans</i>
Canónico-González et al. (2013)	August – Sept 2010	Brazil	Stool	Isolation, molecular	<i>C. neoformans</i> <i>C. albidus</i>
Kaocharoen et al. (2013)	2002 – 2005	Thailand	Stool	Isolation	<i>C. neoformans</i> <i>C. gattii</i>
Abulreesh et al. (2015)	July – Dec 2014	Saudi Arabia	Dry stool	Isolation	<i>C. neoformans</i>
Rosa et al. (2016)	March – Sept 2014	Brazil	Dry stool	Isolation	<i>Cryptococcus</i> sp.

Table 2.3 continued

Krangvichain et al. (2016)	2011 – 2012	Thailand	Fresh and dry stool	Isolation, molecular	<i>Cryptococcus</i> sp.
Marenzoni et al. (2016)	-	Poland	Cloacal swab	Isolation, MALDI-TOF	<i>Cryptococcus</i> sp.
Ellabib et al. (2016)	2013 – 2015	Libya	Stool	Isolation, molecular typing	<i>C. neoformans</i>
Abulreesh et al. (2019)	May – Nov 2018	Saudi Arabia	Dry stool	Isolation, molecular	<i>C. neoformans</i> <i>C. albidus</i> <i>C. gattii</i> <i>C. liquefaciens</i>
Magalhães Pinto et al. (2019)	April – March 2014	Brazil	Fresh and dry stool	Isolation	<i>Cryptococcus</i> sp.
Chen et al. (2021)	-	China	Stool	Isolation, MLST	<i>C. neoformans</i>
Kankam et al. (2021)	June – Aug 2017	South Africa	Stool	Isolation	<i>Cryptococcus</i> sp.
Nualmalang et al. (2023)	March – June 2021	Thailand	Fresh and dry stool	Isolation, MALDI-TOF	<i>C. neoformans</i>

Table 2.4: Summary of worldwide prevalence of *Candida* species identified from pigeon stool or cloaca between 2010 – 2024.

Author (year)	Year of sampling	Country	Type of sample	Identification method	Species detected
Jang et al. (2011)	April – May 2009	South Korea	Fresh and dry stool	Isolation	<i>C. glabrata</i> <i>C. famata</i> <i>C. krusei</i> <i>C. guilliermondii</i> <i>C. albicans</i>
Chae et al. (2012)	Feb – March 2011	South Korea	Dry stool	Isolation	<i>C. guilliermondii</i> <i>C. zeylanoides</i> <i>C. sake</i>
Soltani et al. (2013)	Sept 2010 – May 2011	-	Stool	Isolation	<i>C. albicans</i> <i>C. tropicalis</i> <i>C. parapsilosis</i> <i>C. guilliermondii</i>
Wu et al. (2012)	May -Aug 2013	China	Fresh stool	Isolation	<i>C. catenulate</i> <i>C. glabrata</i> <i>C. albicans</i>
Abulreesh et al. (2015)	July – Dec 2014	Saudi Arabia	Dry stool	Isolation	<i>C. albicans</i>
Marenzoni et al. (2016)	-	Poland	Cloacal swab	Isolation	<i>Candida</i> sp.

Table 2.4 continued

Medina et al. (2017)	-	Spain	Fresh stool	Isolation	<i>C. guilliermondii</i> <i>C. albicans</i> <i>C. kefyr</i> <i>C. pelliculosa</i> <i>C. rugosa</i> <i>C. zeylanoides</i> <i>C. lusitaniae</i> <i>C. parapsilosis</i>
Abulreesh et al. (2019)	May – Nov 2018	Saudi Arabia	Dry stool	Isolation, molecular	<i>C. glabrata</i>
Pakshir et al. (2019)	2014	Iran	Stool	Isolation	<i>C. famata</i> <i>C. albicans</i> <i>C. glabrata</i> <i>C. krusei</i>
Magalhães Pinto et al. (2019)	April – March 2014	Brazil	Fresh and dry stool	Isolation, molecular	<i>C. parapsilosis</i> <i>C. tropicalis</i> <i>C. krusei</i> <i>C. glabrata</i> <i>C. rugosa</i>
Nualmalang et al. (2023)	March – June 2021	Thailand	Fresh and dry stool	Isolation, MALDI-TOF	<i>C. krusei</i> <i>C. metapsilosis</i> <i>C. glabrata</i> <i>C. tropicalis</i> <i>C. albicans</i>
Glushakova et al. (2021)	Sept – Oct 2017	Russia	Fresh stool	Isolation	<i>Candida</i> sp.

Table 2.5: Compilation of worldwide prevalence of *Rhodotorula* and *Trichosporon* species identified from pigeon stool or cloaca between 2010 – 2024.

Author (year)	Year of sampling	Country	Type of sample	Identification method	Species detected
Jang et al. (2011)	April – May 2009	South Korea	Fresh and dry stool	Isolation	<i>Rhodotorula</i> sp. <i>Trichosporon</i> sp.
Chae et al. (2012)	Feb – march 2011	South Korea	Dry stool	Isolation	<i>R. glutinis</i> <i>R.mucilaginosa</i>
Wu et al. (2012)	May – Aug 2013	China	Fresh stool	Isolation, molecular	<i>R. glutinis</i> <i>R. mucilaginosa</i> <i>R.dairenensis</i> <i>Trichosporon</i> sp.
Soltani et al. (2013)	Sept 2010 – May 2011	-	Stool	Isolation	<i>R. rubra</i> <i>T. beigelii</i>
Abulreesh et al. (2015)	July – Dec 2014	Saudi Arabia	Dry stool	Isolation	<i>R. mucilaginosa</i> <i>T. mucoides</i> <i>T. harzianu</i> <i>T. asahii</i>
Marenzoni et al. (2016)	-	Poland	Cloacal swab	Isolation	<i>Rhodotorula</i> sp.
Rosario Medina et al. (2017)	-	Spain	Fresh stool	Isolation	<i>Trichosporon</i> sp. <i>Rhodotorula</i> sp.
Pakshir et al. (2019)	2014	Iran	Stool	Isolation	<i>R. rubra</i>
Abulreesh et al. (2019)	May – Nov 2018	Saudi Arabia	Dry stool	Isolation, molecular	<i>Rhodotorula</i> sp.

Table 2.5 continued

Magalhães Pinto et al. (2019)	April – March 2014	Brazil	Fresh and dry stool	Isolation	<i>Trichosporon</i> sp. <i>Rhodotorula</i> sp.
Glushakova et al. (2021)	Sept – Oct 2017	Russia	Fresh stool	Isolation	<i>Rhodotorula</i> sp. <i>Trichosporon</i> sp.
Nualmalang et al. (2023)	March – June 2021	Thailand	Fresh and dry stool	Isolation, MALDI-TOF	<i>Trichosporon</i> sp.

2.3.5 Diversity analysis of pathogenic fungi using next-generation sequencing

To date, only one study adopted next-generation sequencing method (pyrosequencing) utilising the ITS2 rRNA region to understand the mycobiome of fresh and dry pigeon stool, especially the unculturable fungi. Lee et al. (2017) detected 35 potentially pathogenic fungal species, with 28 species unique to the study. Interestingly, the author was not able to place *C. neoformans* into the diversity list as the bioinformatic pipeline used in the study was not able to assign a species name for the sequence obtained.

2.4 Culture-dependent versus culture-independent techniques for microbial studies

As seen in the previous subsection, bacterial or fungal diversity studies, also referred to as microbiome studies, have been of particular interest by researchers driven by the advancement of technologies. Generally, the methods of identification of microbes, especially bacteria and fungi can be split into two, 1) culture-dependent and 2) culture-independent, both having their advantages and disadvantages. And, depending on the direction of one's research, researchers may adopt either or both methods to achieve their objective(s).

An explanation of the techniques and the pros and cons of each technique for bacterial and fungal diversity can be found in the following subsection.

2.4.1 Culture-dependent techniques

Culture-dependent techniques involve growing and isolating microbes of interest using a suitable medium (e.g. agar plating, cell culture) and conditions (e.g. aerobic or anaerobic, temperature, pH) to sustain growth. Historically, this method has been longed used and more preferred. Typically, the method is then followed by identification using biochemical characterisation or molecular identification through amplification of conserved genes or protein-coding genes.

The major pros of culture-dependent technique are the ability to evaluate cell viability, quantification of cells, and defining phenotypic characteristics of cells (Figdor & Gulabivala, 2008). Next, possession of viable microbes allows for downstream protocol such as antimicrobial inhibitory concentration testing and multi-locus strain typing to be done. Apart from that, the cost is relatively cheaper in comparison to culture-independent methods.

However, the cons outweighs the pros, due to the inability of culture-independent techniques to isolate metabolically active and viable, but unculturable microbes which has been estimated as 99% of microbes in the environment (Bodor et al., 2020). If not considering the presence of unculturable microbes, this creates a bias in the diversity of the sample set. Apart from that, results produced through isolation may vary based on the operator's experience and minor differences in culture protocol, which can influence the overall result. Most importantly, isolation can be highly time-consuming, with many microbes such as fastidious bacteria and fungi culturing can take up to a month depending on the targeted species (Figdor & Gulabivala, 2008).

2.4.2 Culture-independent techniques

Culture-independent techniques are defined as methods that do not require isolation to detect microbes in a sample. The techniques are highly preferred due to its 1) reproducible and non-ambiguous result, 2) quick result and 3) ability to identify unculturable microbes which is highly prevalent in environmental samples (Su et al., 2012).

2.4.2.1 Molecular method by Sanger sequencing

Sanger sequencing was first developed by Sanger and colleagues (Sanger et al., 1965). Nowadays, the technology is currently frequently utilised for molecular methods, including cloning of environmental DNA, PCR of conserved or protein-coding genes to study microbial communities, restriction fragment length polymorphism (RFLP) and quantitative PCR (qPCR) and also frequently used in microbial diversity studies (Lane et al., 1985; Su et al., 2012). However, molecular detection method is limited by its inability to sequence multiple amplicon fragments with different bases due to the limitation of Sanger sequencing, which may inquire additional cost to the project when dealing with large-scale projects such as microbiome studies (Maki et al., 2019).

2.4.2.2 Molecular method by high-throughput amplicon sequencing

Advancements in technology such as Raman microscopy, nano-scale secondary ion mass spectrometry (NanoSIMS) and sequencing technologies (e.g. next-generation sequencing and third-generation sequencing) have catalysed over the years and established variant fields, including metagenomics, metatranscriptomics, metaproteomics and whole-genome sequencing (Su et al., 2012). However, regarding microbial diversity, metagenomics is the preferred approach.

Metagenomics is defined as the direct analysis of genomes contained within an environment (Thomas et al., 2012), and is inclusive of sequence- or function-based. In general, amplicon sequencing would require amplification of conserved genes of the targeted microbe (ribosomal gene of bacteria or fungi) or randomly shearing of genome into small DNA fragments that are then individually sequenced (Maki et al., 2019). This is followed by high-throughput amplicon sequencing technologies using NGS or third-generation sequencing technologies.

Metagenomics using either high-throughput amplicon sequencing (HTAS) or shotgun sequencing both has its pros and cons. To start, HTAS is highly favoured as 1) robust PCR amplification allows low concentration of starting material to generate large dataset, 2) the method provides comprehensive reads on targeted gene, and 3) it is more cost efficient than microbiome studies utilising Sanger sequencing (Su et al., 2012). Nevertheless, significant cons of HTAS includes 1) the introduction of bias due to PCR, 2) poor taxonomic resolution, especially at the species level and 3) the lack of functional gene content of identified microbes (Maki et al., 2019).

On the other hand, a major advantage of shotgun sequencing is the ability of the method to characterise gene content of microbial communities as sequences obtained are assembled to produce a more extensive DNA sequence assembling almost whole genome sequence (Maki et al., 2019). Regardless, due to the lack of PCR amplification prior to sequencing for shotgun sequencing and the presence of host DNA, data obtained from shotgun sequencing low-abundance features might be difficult to detect (Maki et al., 2019). Apart from that, shotgun sequencing is generally more expensive than HTAS.

2.4.3 Which method is best for this study?

After the extensive review of available literature of the detection of specific pigeon-related bacterial species in section 2.2, pigeon-related fungal species in section 2.3, and pigeon micro and -mycobiome studies, it has revealed that pigeon-related culture-dependent studies tend to hyperfixate on specific genera, which in return neglects the presence of other opportunistic microbes and underestimating its presence which was detected in microbiome studies. Additionally, the lack of any microbiome data of pigeons in Malaysia justifies the need to move forward with high-throughput amplicon sequencing, as for this study is concerned.

2.5 Considerations before opting for amplicon high-throughput sequencing

Before venturing into amplicon high-throughput sequencing for any research, it is essential to consider protocols for genomic DNA (gDNA) extraction, library preparation, and suitable high-throughput sequencing technologies.

2.5.1 Genomic DNA extraction

To obtain high quality HTAS data, extracting high quantity and quality gDNA is of utmost importance. Throughout the years, many extraction kits have been designed specific to sample types. From the traditional chemical lysis extraction protocol (eg. CTAB, phenol-chloroform) to specialised kits for troublesome samples (eg. soil, stool) such as those containing organic or inorganic matter (eg. humic acid, polysaccharides) which can interfere with downstream processes. Additionally, recent protocols developed have emphasised the need to include pre-treatment steps which includes mechanical lysis (eg. bead beating, sonification, grinding with liquid nitrogen), chemical lysis (eg. Proteinase K) or enzymatic treatment to increase cell disruption which in return, improved gDNA yield.

In general, usage of specialised kits or protocols aims to produce reproducible results and provide gDNA concentration and quality sufficient for metagenomics analysis. These characteristics are often defined by 1) minimal degradation on gel electrophoresis and 2) spectrometer reading of 1.8 – 2.0 at A260/A280 and 2.0 – 2.2 at A230/260 assessed using quality assessment devices such as Bioanalyzer 2100, Qubit and spectrophotometer machine. Whichever extraction protocol or kit is typically chosen based on preference and one's budget.

2.5.2 Library preparation

Library preparation refers to the process of converting the representative pool of DNA into DNA fragments that can be sequenced to provide insights into the microbial community composition and function. To prepare the sample for HTAS, library preparation is done through amplification of conserved gene of the targeted microbe using primers ligated with sequencing adapters (Maki et al., 2019).

2.5.2.1 Bacterial library preparation

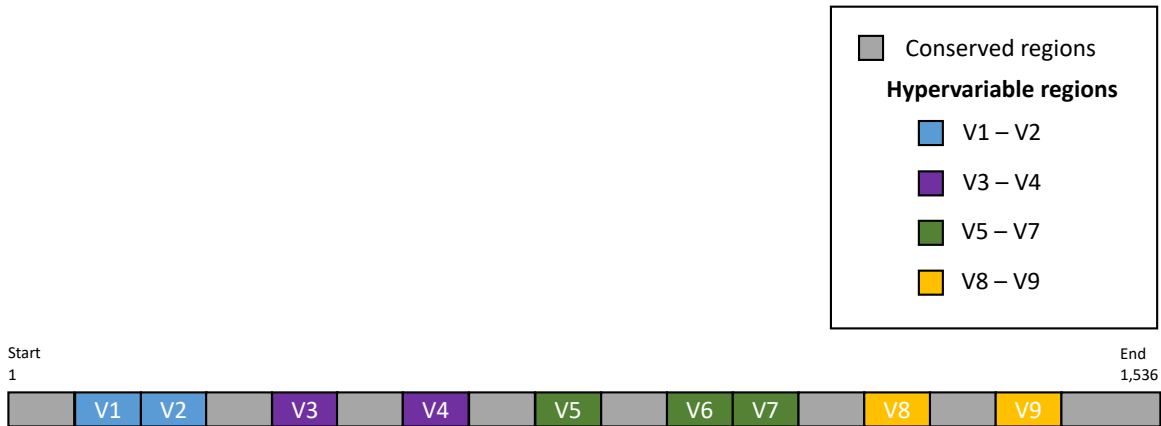


Figure 2.3: Hypervariable regions of the 16S rRNA gene. Adpated from López-Aladid et al. (2023).

In terms of bacteria, library preparation requires the amplification of the hypervariable region of the 16S ribosomal RNA gene (16S rRNA), also known as the V1 to V9 variable regions (Figure 2.3). First discovered by Dubnau et al. (1965), the 16S rRNA gene is specific to bacteria and archaea, and highly conserved between species. It is considered as a useful target to capture most of the bacteria and provide sufficient information to reconstruct the taxonomy of bacterial genera within sample (Abellan-Schneyder et al., 2021).

In general, the V3 - V4 hypervariable region is the most extensively used region, which was developed by Klindworth and colleagues (2013), as it suffers the least from intragenomic heterogeneity in prokaryotes. However, other authors have reported instances of inaccuracy in estimating bacterial diversity when using the V3 – V4 region. Fadeev and colleagues (2021) reported higher alpha diversity when the V4 – V5 hypervariable region was used, being able to detect more Archaea operational taxonomic units (OTUs) and providing insight into the need to consider another hypervariable region when assessing environmental microbial communities. Whereas other researchers have mentioned that using either individual (V4) or paired hypervariable regions (V1 – V2, V5 – V6 regions) have shown to provide better diversity and discrimination (Barb et al., 2016; Prayogo et al., 2020; Walters et al., 2016).

On the other hand, opting to sequence the whole 16S rRNA region consisting of about 1,550 bp long may be an option for better taxonomic resolution with the bonus of gene function and antimicrobial sensitivity prediction (Akaçin et al., 2022).

2.5.2.2 Fungal library preparation

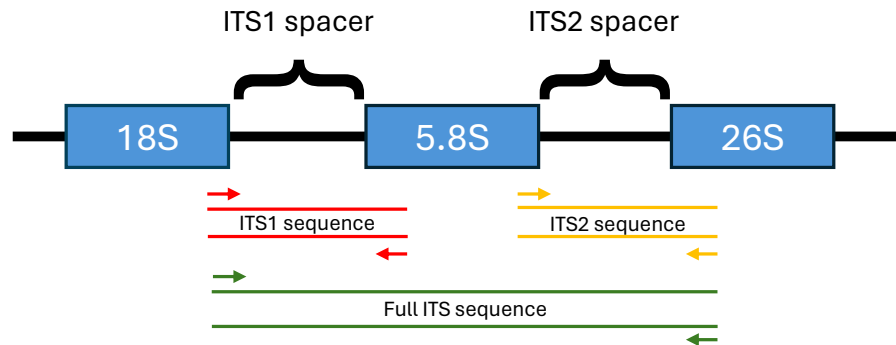


Figure 2.4: Hypervariable regions of the nuclear ribosomal Internal Transcribed Spacer (ITS) region. Adapted from Orhan (2021).

On the other hand, fungal library preparation requires the amplification of the hypervariable region of the nuclear ribosomal Internal Transcribed Spacer ribosomal RNA (ITS rRNA) region, in which ITS1 and ITS2 spacers are found between the 18S and 5.8S gene, and 5.8S and 26S gene respectively (Figure 2.4). Unlike the 16S rRNA gene, which detects only Archaea and bacteria, the ITS region is available in all eukaryotes, including fungi, plants and animals (Nilsson et al., 2008).

Overall, ITS2 appears to be more suited for uncovering the diversity of OTUs within the fungal communities due to its lower GC content, allowing higher specificity of the primer set and sequencing efficiency (Yang et al., 2018). A study conducted by Yang and colleagues (2018) revealed that the ITS2 region has less variables than ITS1 due to the lower evolution rate in the region which results in more consistent sequencing lengths. Additionally, the study also found that the ITS2 sequences observed similar clustering and taxonomy to the whole ITS region (Yang et al., 2018).

To conclude, ITS2 is generally the better option when considering region to use if amplifying the whole ITS region is not an available option.

2.5.3 High-throughput amplicon sequencing technologies

Among the sequencing technologies invented, amplicons with the barcode prepared for metagenomic studies are either sequenced using next-generation sequencing (NGS) (e.g. pyrosequencing, Illumina platform) and third-generation sequencing technologies (TGS) (e.g. Pacific Biosciences, Oxford Nanopore Technology).

The first NGS technology, also known as pyrosequencing technology or second generation technologies, which was first introduced in the 2000s, was the standard for metagenomic as an alternative to conventional Sanger sequencing technology. Pyrosequencing depends on the luminometric detection of pyrophosphate that is released during primer-directed DNA polymerase-catalysed nucleotide incorporation (Fakruddin & Chowdhury, 2012). However, due to the high error rates in accurately detecting variants with long homopolymer stretches (> 5 to 6 bp) and the need for expensive biotinylated primer, pyrosequencing was discontinued (Jeong et al., 2021).

Further development in the sequencing industry produced the Illumina technology, whereby sequencing depends on the characteristics of the wavelengths and intensity of fluorescently labelled deoxynucleotides (dNTPs), which then binds to their complementary bases (Fakruddin & Chowdhury, 2012). Currently, it is extensively employed in diverse metagenomic studies, aiding to reducing high-cost burden associated with NGS (Fadrosh et al., 2014). Nevertheless, Illumina sequencing is limited by the size of fragments (300 – 500 bp) that can be sequenced, which may introduce biasness in identifying chimeric sequences produced during PCR amplification for library preparation and also limits microbial classification up to genus level only according to commonly used 16S rRNA gene-based microbial taxonomy database (Jeong et al., 2021).

To overcome this issue, third-generation sequencing (TGS), or also referred to as long-read sequencing technology was created to overcome the need for massive computational calculations of genome assembly. The fundamentals of the technology works by employing single-molecule sequencing that directly sequences individual DNA or RNA strands present in a sample of interest without prior clonal amplification of the DNA (Roberts, 2013). This is then followed by continuous DNA polymerase which incorporates fluorescently labelled dNTPs, generating continuous DNA synthesis (Eid et al., 2009). The differences between the two leading TGS technologies in the market are the base calling technique, which assigns nucleobases to chromatogram peaks, light intensity signals or electrical currents resulting from nucleotides passing.

While NGS and TGS are both powerful sequencing technologies, deciding the most suitable technology to proceed with metagenomics is crucial. The main limiting factor of choosing either technology is the sequencing cost, with NGS relatively cheaper than TGS when dealing with a smaller sample size. Apart from that, TGS technology is typically used for long sequences and rarely used for short amplicon sequences for metagenomics, in which sequencing accuracy of short sequencing is still unknown. Thus, it is highly appropriate to consider using TGS technology if expected size are above 1000 bp, which is typically obtained when sequencing the whole conserved gene of bacteria or fungi (Akaçin et al., 2022).

2.5.4 Diversity analyses

In most microbiome studies, diversity analyses are used to understand the differences between and across two or more communities. Choosing the right indices and distance matrices is important for deciphering the role of each taxa member between and across communities of any microbiome study.

2.5.4.1 Alpha diversity

Alpha diversity indices quantify within sample diversity and can be compared across groups, with respect to the number of taxa groups (richness) or distribution of abundance of taxa groups (evenness), or both. The indices mentioned below are those commonly used for alpha diversity analysis in microbiome studies. Alpha diversity is commonly visualised using boxplot method.

i. Observed taxa

Observed taxa is defined as the total number of taxa, most often regarded as the number of observed taxonomic units (OTUs) or observed amplicon sequence variants (ASVs) in any given sample (Callahan et al., 2017), as shown in Equation 2.1. The only difference is the algorithm for OTU classification, which takes sequence similarity into consideration and blurs out similar sequences. ASVs take into consideration all sequence variables.

$$S_{richness} = \sum_{s>0} 1_s \quad \text{Equation 2.1}$$

ii. Simpson's index

Simpson's index is an estimator of taxa diversity and takes into consideration both richness and evenness, but is more sensitive to species evenness (Lemos et al., 2011; Simpson, 1949). It indicates the taxa dominance and gives the probability of two individuals that belong to the same taxa being randomly chosen. Based on the equation above, D represents Simpson index; S represents the total number of species in the sample; n_i denotes the number of individuals of the i th species; N denotes the total number of individuals in the species.

$$D = 1 - \sum_{i=1}^S \left(\frac{n_i(n_i - 1)}{N(N - 1)} \right) \quad \text{Equation 2.2}$$

iii. Shannon's index

Shannon's H' index, sometimes referred to as the Shannon-Wiener index, is another popular estimator for taxa diversity (Shannon, 1948). The diversity emphasises the interaction between evenness and richness but is more sensitive to species richness than evenness (Shannon, 1948; Strong, 2016). Shannon's index is more favoured than other indices particularly due to its sensitivity in observing differences between groups (Kers & Saccenti, 2022). Based on Equation 2.3, p_i is the proportion of observations (reads, or abundances) belonging to the i th species

$$H' = -\sum_{i=1}^R p_i \ln p_i \quad \text{Equation 2.3}$$

iv. Chao1 index

Introduced in 1984, the Chao1 index, also known as the Chao1 estimator, is a widely used estimator for species estimation in a sample. The estimator is typically used to estimate the number of OTUs/ASVs present in a sample, taking into consideration the abundance of OTUs/ASVs in a given sample (Chao, 1984). This index is particularly useful for datasets skewed towards low-abundance taxa (Kers & Saccenti, 2022). A higher Chao1 index denotes a larger quantity of OTUs/ASVs, suggesting a relatively higher species diversity within the sample. Based on Equation 2.4, S_{obs} are the number of species observed; n_1 is the number of species occurring only once, n_2 is the number of species that occur twice. However, for microbiome studies, Chao1 distance analysis often removes singletons and doubletons, as it is difficult to effectively differentiate between amplified singletons and real singletons (Kers & Saccenti, 2022).

$$S_{chao1} = S_{obs} + \frac{n_1(n_1 - 1)}{2(n_2 + 1)} \quad \text{Equation 2.4}$$

v. Faith's Phylogenetic Distance

Faith's phylogenetic distance of Faith's PD, is a phylogenetically weighted measure of richness. Unlike the other indices mentioned above, Faith's PD does not take in consideration of the abundance of OTUs/ASVs. Instead, it is defined as the sum of the lengths of all those branches on the tree that span the members of the set, given the phylogenetic tree spanning s taxa (Faith, 2006). Based on Equation 2.5, in which s is the number of observed taxa and b_i is the length of the i th branch in the tree. Index I runs on all branches.

$$PD = \sum_i b_i \quad \text{Equation 2.5}$$

2.5.4.2 Beta diversity

On a bigger scale, beta diversity compares diversity between samples and is often calculated by comparing feature dissimilarity, resulting in a distance matrix between all pairs of samples. Among the many available distance matrices, the four commonly used are those listed below. The visualisation method for beta diversity analysis includes using the dimension-reduction-based ordination method, such as principal coordinate analysis (PCoA) or principal component analysis (PCA). Consideration of appropriate matrices should include whether the presence/absence or abundance is more relevant, whether taxa phylogenetic information is distinct from each other, or both.

i. Bray-Curtis dissimilarities

Bray-Curtis dissimilarities is a quantitative measure that accounts for the taxa abundance between tested communities,. Based on Equation 2.6, i and j represents the two samples; C_{ij} is the sum of common taxa between sample, and S_i and S_j are the total number of taxa counted in both samples (Bray & Curtis, 1957; Kers & Saccenti, 2022).

$$BC = 1 - \frac{2C_{ij}}{S_i + S_j} \quad \text{Equation 2.6}$$

ii. Jaccard distance

Jaccard distance, also known as Jaccard similarity, is a qualitative analysis which only considers the presence/absence of taxa between tested communities. Based on Equation 2.7, the metric calculates the ratio between the number of taxa that are common between the two samples ($i \cap j$), and the total number of members that are distinct to each samples ($i \cup j$), as depicted in the equation below (Kers & Saccenti, 2022).

$$J(i, j) = \frac{|i \cap j|}{|i \cup j|} \quad \text{Equation 2.7}$$

iii. UNIFRAC distance

UNIFRAC distance, both unweighted and weighted, was introduced by Lozupone as an addition to pre-existing tests, such as P and F_{ST} tests which are not able to compare numerous samples simultaneously with clustering or ordination and account for branch length information when comparing samples (Lozupone et al., 2007; Lozupone & Knight, 2005).

The difference between the unweighted and weighted UNIFRAC is the inclusion of abundance information. Unweighted UNIFRAC considers only the presence and absence of species information and counts the fraction of branch length unique to either community (Lozupone & Knight, 2005). Whereas, unlike its counterpart, weighted-UNIFRAC considers the species abundance information and weights the branch length with abundance differences (Lozupone et al., 2007).

CHAPTER 3

MATERIALS AND METHODS

3.1 Sampling site and sample collection

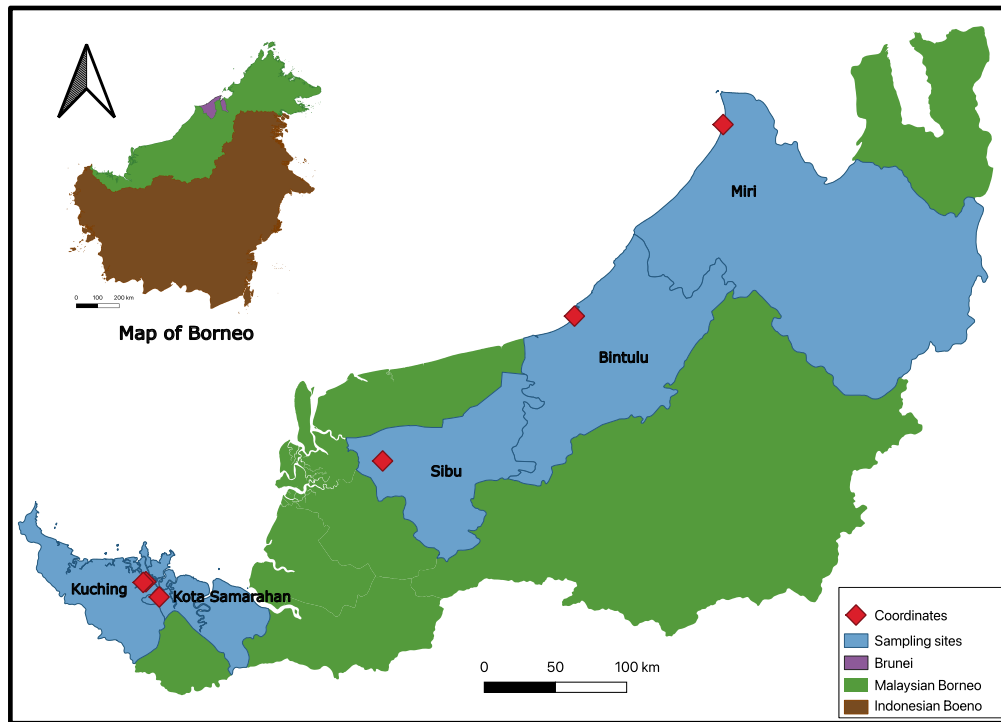


Figure 3.1: Map of Sarawak, Malaysia. Towns included in this study are coloured in blue; Sampling sites are denoted with a red diamond (Map was generated using QGIS v3.32.3).



Figure 3.2: Reference of two types of pigeon stool. Left: Fresh pigeon stool; Right: Dry pigeon stool.

Five towns in Sarawak, 1) Kuching, 2) Kota Samarahan, 3) Bintulu, 4) Sibul and 5) Miri were chosen for sampling for this study as shown in Figure 3.1. In each towns, sampling sites were chosen based on the presence of 1) more than 10 pigeons in the area, and 2) fresh and dry pigeon stool in pigeon roosting sites only. Stool collection was done in the morning, especially for fresh stool, to ensure no extended exposure of the sample type to environmental microbes.

Fresh stools with only the following characteristics were collected: 1) green or brown colour, which is attributed to their grass and seed diet, 2) intact white urates and 3) intact shape of faeces as presented in the left photo in Figure 3.2. On the other hand, dry pigeon stool was collected only in pigeon roosting sites that are shaded (eg. under roofings, beneath aircond outdoor unit), with no signs of roosting of other urban bird species.

Areas with pigeon excrete were then divided into estimated quadrates of 1m x 1m. Samples in each quadrate were then pooled together (5 stools in a container). This was done especially for dry stools, whereby individual stools were difficult to distinguish. Collected stool were then stored in InhibitEX buffer (Qiagen, Germany) and stored in -80°C once in the laboratory. Sampling was conducted between October 2022 – April 2023.

Permissions to sample biological resources were approved by the Sarawak Forestry Corporations (Permit no.: SFC.810-4/6/1(2021)) and Animal Ethics Committee of Universiti Malaysia Sarawak (Reference number: UNIMAS/AEC/R/F07/073).

3.2 Genomic DNA extraction

3.2.1 Materials

3.2.1.1 2% cetyl-hexadecyltrimethylammonium bromide buffer

The 2% cetyl-hexadecyltrimethylammonium bromide (CTAB) buffer was prepared by dissolving 40.90 g of NaCl, 3.78 g of EDTA, 1 g of CTAB powder, 6.05 g of Tris base, topped up with distilled water until reach 500 mL is achieved. Buffer was then autoclaved for 15 mins at 121°C.

3.2.1.2 5M Sodium Chloride solution

The 5M Sodium Chloride (NaCl) solution was prepared by dissolving 29.20 g of NaCl powder in 100mL of distilled water. Solution was then filtered using 0.22µm syringe filter (Sartorius, Germany) and autoclaved for 15 mins at 121°C.

3.2.1.3 Chloroform: isoamyl alcohol

A ratio of 24:1 of chloroform and isoamyl alcohol was mixed well and kept cold in 4°C until use.

3.2.1.4 1X Tris, Boric acid, EDTA buffer

10X Tris, Boric acid, EDTA (TBE) stock solution was made by dissolving 108 g of Tris base, 55 g of Boric acid and 9.30 g of disodium ethylenediaminetetraacetic acid (EDTA) (First Base, Malaysia) topped up with distilled water until 1000 mL solution is achieved. Stock solution was autoclaved for 15 mins at 121°C. To achieve working solution of 1X TBE buffer, buffer stock was diluted with distilled water in 1:10 dilution factor.

3.2.1.5 1% 1X Tris, Boric acid, EDTA (TBE) agarose gel

1% 1X TBE agarose gel was prepared by adding 1g of agarose powder (First Base, Malaysia) into 100 mL of 1X TBE buffer and microwaved for 2 mins.

3.2.1.6 6X loading dye

6X loading dye was produced by mixing 0.01% of bromophenol blue, 4.7mL of glycerol, 1.2 mL 0.5 M Tris and 2.1 mL distilled water.

3.2.1.7 1 kbp molecular ladder

1 kbp molecular ladder was prepared by diluting 100 mL 1kbp molecular ladder (Thermo Scientific, Lithuania) with 100 mL of 6X loading dye (Thermo Scientific, Lithuania) and distilled water at a ratio of 400 mL of distilled water and stored at 4°C.

3.2.2 Method

Genomic DNA (gDNA) of pigeon excrete were extracted using 2% CTAB extraction protocol (Doyle and Doyle, 1987) with slight modification. All centrifugation was done at 10,000 x g for 10 mins. A mixture of stool in InhibitEX buffer and 2% CTAB buffer (1:10 ratio) was incubated at 65°C for one hour and invert-mixed every 10 mins. After incubation, mixture was centrifuged, and supernatant was transferred into a new centrifugation tube. Protein precipitation was done by adding 1 volume of chloroform: isoamyl alcohol into transferred supernatant and invert-mixed vigorously until homogenous. Sample was then centrifuged. Protein precipitation step was done twice. Supernatant was then carefully aspirated while avoiding the organic layer and transferred to a new microcentrifuge tube ½ volume of 5M of NaCl was added to the supernatant, and three volumes of ice-cold absolute ethanol was added. Mixture was slowly invert-mixed and incubated at -20°C for one hour to precipitate the genomic DNA (gDNA). After incubation, mixture was centrifuged, and ethanol was carefully decanted leaving no residue. This is then followed with 1mL of ice cold 70% ethanol was then added, slowly invert mixed, and centrifuged. DNA pellet was then airdried for 15 mins before hydrating with warm ultra-pure water.

Additional gDNA purification was done using the Gel/PCR purification kit (Favorgen, Taiwan) according to the manufacturer's protocol to obtain 260/280 and 260/230 ratios of ~2.0 and ~1.8, respectively. Quantification and purity reading of gDNA was done using Qubit® dsDNA HS Assay kit (Thermo Scientific, Lithuania) and DS-11 Series Spectrophotometer/Fluorometer (DeNovix, USA) respectively. On the other hand, gDNA integrity was determined through gel electrophoresis of 3µL gDNA and 1µL of 6X loading dye on 1% 1X TBE agarose gel, and stained with 0.05 mg of ethidium bromide (Promega, USA). Gel electrophoresis was conducted in cold 1X TBE buffer at 120 V for 45 mins.

3.3 Library preparation and amplicon sequencing

Library preparation was generated through amplification of the V3 – V4 16S rRNA gene for bacterial organism and ITS2 rRNA gene of the fungal organism using primers and master mix components listed in Table 3.1 and Table 3.2. The PCR conditions for bacterial and fungal library preparation were performed using the protocol listed in Table 3.3, and visualised on 0.8% 1X TBE agarose gel.

Purification of PCR product was done using magnetic beads. NGS was conducted using the Illumina Novaseq 6000 platform (Illumina, USA). Automated cluster generation and 250bp paired-end sequencing with dual reads were performed according to the manufacturer's instructions. Library preparation and HTAS was done in a private laboratory (Codon Genomics, Seri Kembangan).

Table 3.1: PCR master mix components for 16S V3 -V4 and ITS2 region library preparation

Reagent	Stock concentration	Volume (μL)	Final concentration
TransStart buffer	10X	1.5	1X
dNTPs	2.5mM	4.0	0.2mM
Forward primer	20mM	1.0	0.2 μM
Reverse primer	20mM	2.0	0.2 μM
TransStart Taq DNA polymerase	250 U	0.5μL	1.25U
DNA Template		20ng	
Nuclease-free water		To make up 20μL	
Total volume		20μL	

Table 3.2: List of primers used for bacterial and fungal library preparation

Gene	Primer name	Primer sequence 5' – 3'	Basepair size (bp)
V3 - V4 16S rRNA	347F	CCT ACG GRR BGC ASC AGK VRV GAA T	~ 500
	802R	GGA CTA CNV GGG TWT CTA ATC C	
ITS2 rRNA	ITS3	GTG AAT CAT CGA RTC	160 - 390
	ITS4	TCC TCC GCT TAT TGA T	

Table 3.3: PCR condition for bacterial and fungal library preparation.

PCR stage	Temperature (°C)	Duration (s)	Number of cycle (s)
Initial denaturation	94	120	1
Denaturation	95	5	24
Annealing	57	90	
Elongation	72	10	
Final elongation	72	300	1

3.4 Bioinformatic and statistical analysis

All bacterial and fungal raw reads were subjected to bioinformatics and statistical analyses were performed in QIIME2 (Quantitative Insights into Microbial Ecology 2) v2023.9 (Bolyen et al., 2019) using the workflow in Figure 3.3.

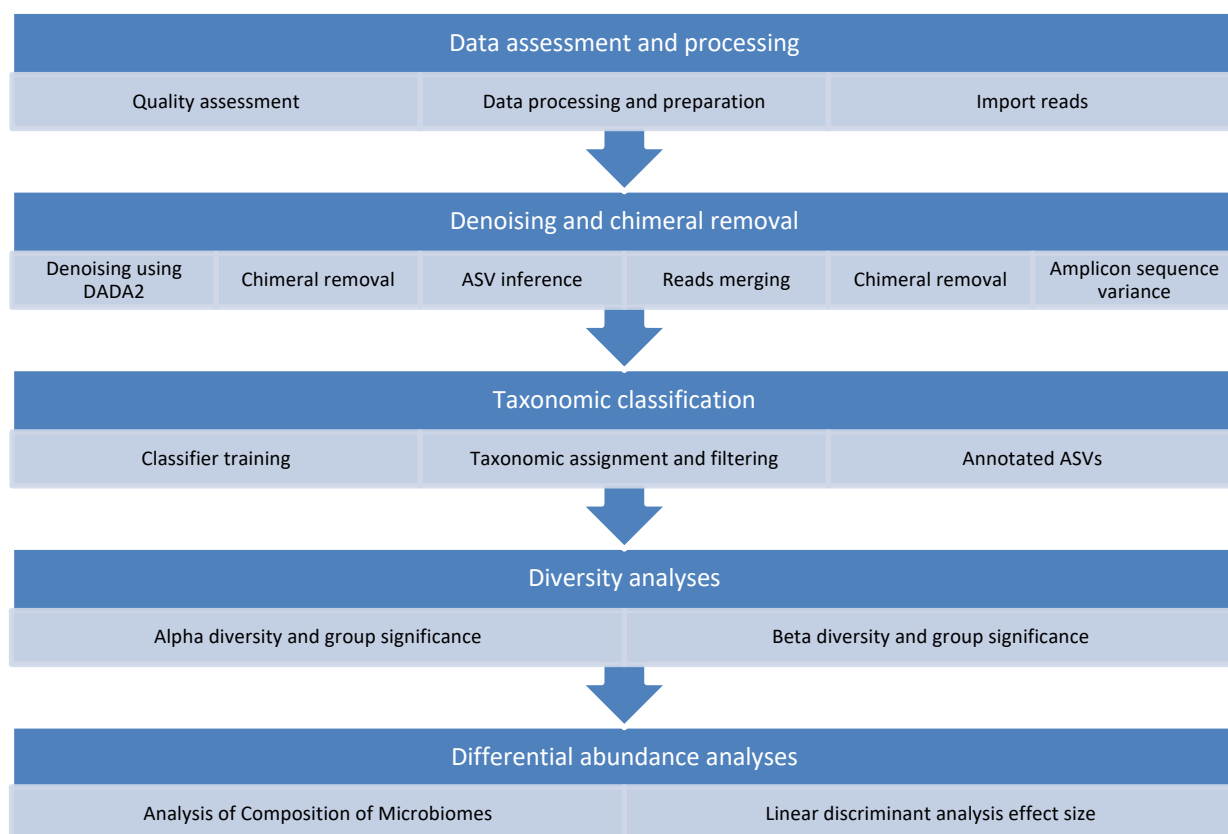


Figure 3.3: Workflow for bacterial and fungal biome analysis

3.4.1 Data assessment and processing

Data quality of the demultiplexed sequencing reads of both 16S V3 – V4 region and ITS2 region reads were assessed using FastQC v0.11.8. and imported into QIIME2 v2023.9 (Bolyen et al., 2019) using the ‘PairedEndFastqManifestPhred33V2’ protocol with manifest file as the input path. Raw forwards and reverse reads were imported into QIIME2 as artefacts files. Raw sequencing reads were filtered according to the following criteria: 1) shorter than 60 bp, 2) quality-read score, Phread score < 30, and 3) contained ambiguous bases or did not exactly match to primer sequences and barcode tags.

3.4.2 Denoising and chimera removal

Denoising for both 16S V3 – V4 and ITS2 rRNA gene raw sequences was done using the ‘denoising-paired’ command in the q2-dada2 (Denoising Amplicon Denoising Algorithm 2) plugin, with parameters used were chosen based on their respective quality plots. Denoised sequences were then inferred for sequence variants, merged and removed for any chimeric and borderline chimeric reads using the ‘uchime-de-novo’ command in the q2-vsearch plugin. However, due to the variable length reads of the fungal ITS2 rRNA gene, an additional step of trimming the paired-reads for equal read length was done using the ‘trim-pair-output-unmerged’ command in the q2-itsxpress plugin.

3.4.3 Taxonomic classification

Two naive-bäyes classifiers were trained for taxonomic classification in this study, 1) a bacterial classifier identifying at 99% similarity utilising the SILVA v138.1 database (<https://www.arb-silva.de/>) (Pruesse et al., 2007) and 2) a fungal classifier identifying at 97% or 99% similarity (dynamic) utilising the UNITE v9.0 database (<https://doi.plutof.ut.ee/doi/10.15156/BIO/2483915>) (Abarenkov et al., 2010, 2024), using

the `classify-naïve-bayes` command. Both trained classifiers were then tested to assign and classify ASVs to their taxonomic groups using the `'classify-sklearn'` command. Mentioned commands are available in the `q2-feature_classifier` plugin. Identified amplicon sequence variance (ASV) were then filtered for features such as mitochondria, chloroplast, archaea and unassigned taxa using the `'filter-table'` command in the `q2-taxa` plugin. Classifiers in this study identified sequences as ASV as the method had demonstrated high sensitivity as well as specificity compared to Operational Taxonomic Unit (OTU), which would provide better differentiated ecological patterns (Callahan et al., 2017).

Overall features detected were grouped based on sample type and visualised as krona plot, bar plots and heatmap at both phylum and genus level. Visualisations were plotted using the `'krona-collapse-and-plot'`, `'barplot'` and `'heatmap'` commands in the `q2-krona`, `q2-feature-table` and `q2-taxa` plugin respectively.

3.4.4 Rarefaction curve and phylogenetic analysis

To determine the minimum reads required to achieve sufficient ASV detection from samples for bacterial and fungal diversity, a rarefaction curve was plotted using the `'alpha-rarefaction'` command in the `q2-diversity` plugin and observed for the number of reads required until curve reached plateau. Samples were then rarefied to an equal number of reads to continue for alpha and beta diversity analysis.

Phylogenetic analysis was performed by aligning sequences using MAFFT, and highly variable regions were masked, phylogenetic construction using FastTree method and rooted at midpoint using the `'align-to-tree-mafft-fasttree'` command in the `q2-phylogeny` plugin. The rooted tree artefact file produced was used in the following alpha and beta phylogenetic analysis.

3.4.5 Alpha diversity analysis

Alpha diversity was calculated using non-phylogenetic Shannon (H'), Simpson (1-dominance), Chao1 species diversity and Faith's PD phylogenetic indices. Shannon's and Simpson's indices reflect the richness and evenness of the sample. On the other hand, Chao1 index estimates the number of ASVs, whereas Faith's PD index calculates the diversity while considering phylogenetic information. Calculations were conducted using the 'alpha' command in the q2-diversity plugin.

Additionally, alpha group correlation and significance between fresh and dry pigeon stool groups were calculated using mentioned indices above and tested statistically using the non-parametric Kruskal-Wallis test using the 'alpha-group-significance' command in the q2-diversity plugin. Boxplots were used to visualise the differences between sample types for both bacterial and fungal diversity.

3.4.6 Beta diversity analysis

Beta diversity was analysed using the non-phylogenetic Bray-Curtis and phylogenetic weighted UNIFRAC distances using the 'beta' and 'beta-phylogenetic' commands, respectively. Both distance matrices consider the total abundance of ASVs present but the latter considers phylogenetic information into the analysis. The results of both distance analyses were visualised as principal coordinate analysis (PCoA) plots. All commands mentioned are available in the q2-diversity plugin.

On the other hand, beta group significance between fresh and dry pigeon stool groups were evaluated using Permutational multivariate analysis of variance (PERMANOVA) and Analysis of Similarities (ANOSIM) with 999 permutations to determine the significant microbiota community changes between groups considering the non-phylogenetic Bray-

Curtis and Jaccard distance matrix and phylogenetic weighted and unweighted UNIFRAC distance matrix. Calculations and boxplot plotting were done using the ‘beta-group-significance’ command available in the q2-diversity plugin.

3.4.7 Differential abundance analysis

Differential abundance analysis was analysed using the Analysis of Composition of Microbiomes (ANCOM) (Mandal et al., 2015) and Linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al., 2011) to detect biomarkers between fresh and dry pigeon stool. Both analyses were tested at each taxonomic level except Kingdom and Species level.

Firstly, ANCOM was tested to identify individual taxa whose relative abundances may differ significantly across sample type groups. Analysis was conducted using the ‘ancom’ command in the q2-composition plugin. Prior to the analysis, features with total abundance of < 50 and handling zeros in feature table by adding one to all counts were done to avoid false discovery rate by using the ‘filter-features’ command in the q2-feature_table and ‘add-pseudocount’ command in the q2-alignment plugin respectively.

Secondly, LEfSe was tested utilising the two-tailed non-parametric Kruskal-Wallis. Biomarkers ranked according to their effect size associated with the class with the highest median are plotted as bar graph and cladogram using the galaxy website (galaxy.biobakery.org) according to suggested parameters by the developer.

CHAPTER 4

RESULTS

4.1 gDNA quality check, library preparation and sequencing

Initially, both fresh and dry pigeon stool were collected at each sampling sites mentioned in Figure 3.1. However, only six genomic DNA of collected stools mentioned in Table 4.1 were proceeded for library preparation of the V3 – V4 16S rRNA and ITS2 rRNA, and HTAS, at the private laboratory.

All six samples mentioned in Table 4.1 were 1) of high MW bands with little to no degradation on gel electrophoresis, 2) in range of 260/280 and 260/230 ratio, and 3) high gDNA concentration on spectrophotometer extracted using the CTAB extraction method were proceed library preparation (gel image of extracted gDNA and PCR amplicons can be found in Appendix B and C respectively). Amplification of targeted gene for library preparation of the six samples also produced sufficient amplicons and proceeded for HTAS (Table 4.1).

A total throughput of 2,230,881 paired raw tags (average reads $\pm$ SD: 371,813 $\pm$ 136,889) or an approximation of 1.04 Gb of data was obtained through 16S V3 – V4 region short-read sequencing. After denoising and chimera removal, a total 1,611,991 reads were retained.

On the other hand, a total throughput of 1,683,005 paired raw tags (average reads $\pm$ SD: 280,500 $\pm$ 66,297) or an approximation of 0.84 Gb of data was obtained through ITS2 region short-read sequencing. After denoising and tag removal, a total of 1,481,846 reads were retained.

Table 4.1: Sample information including locality, sample type and concentration of gDNA and 16S V3 – V4 rRNA region PCR amplification. Concentration readings were taken using Qubit Fluorometer (dsDNA HS Assay kit).

Sample ID	Location	Sample type	Concentration (ng/μL)	Total concentration (ng)	16S V3 – V4 amplification concentration (ng/μL)	ITS2 amplification concentration (ng/μL)
K3DP	Kuching	Dry	210.10	3781.80	22.65	22.67
KS1DP	Kota Samarahan	Dry	84.95	1614.05	26.53	28.85
B1DP	Bintulu	Dry	143.30	2722.70	30.23	28.30
K2FP	Kuching	Fresh	122.25	2322.75	29.37	38.97
S2FP	Sibu	Fresh	206.75	3928.25	34.47	38.97
K3FP	Kuching	Fresh	168.40	3031.20	22.21	26.18

4.2 Bacterial diversity

4.2.1 Bacterial alpha diversity

A total of 1,381 ASVs comprising of 21 phyla, 40 classes, 95 orders, 172 families and 356 genera were identified against the SILVA v138.1 database. The number of ASVs identified in each sample ranged from 63 – 472 ASVs, with an average of 28% of the features detected at > 0.1%. The ASVs were detected from 1,611,464 sequence counts after denoising and chimeric removal. A rarefaction curve plot found that the maximum sampling depth across all samples was 387,560, except for samples K3DP1 and K3FP, as these samples did not reach the sequencing plateau (Appendix D).

Prior to conducting the alpha and beta analysis, sequence counts were normalised by rarefying each sample to the minimum sampling depth of 78,896. Overall, it was found that dry pigeon stool (DPS) contained a higher bacterial composition compared to fresh pigeon stool (FPS), with a total of 934 and 519 ASVs detected, respectively, with 72 ASVs shared between FPS and DPS samples (Figure 4.1). Alpha diversity indices confirmed that samples had higher diversity, richness, evenness and higher phylogenetic information than FPS samples, but is not statistically significant ($p > 0.05$) (Table 4.3).

In general, it was observed that the dominating bacterial phyla in FPS and DPS were in the orders of Firmicutes, Proteobacteria and Bacteroidota (Figure 4.4A; Figure 4.5A). On a genus level, *Sporosarcina* followed by *Escherichia-Shigella* and *Clostridium sensu stricto 1* were the dominating genera in the FPS whereas, *Escherichia-Shigella* followed by *Clostridium sensu stricto 1* and *Paenibacillis* were the three dominant genera in DPS (Figure 4.4B; Figure 4.5B).

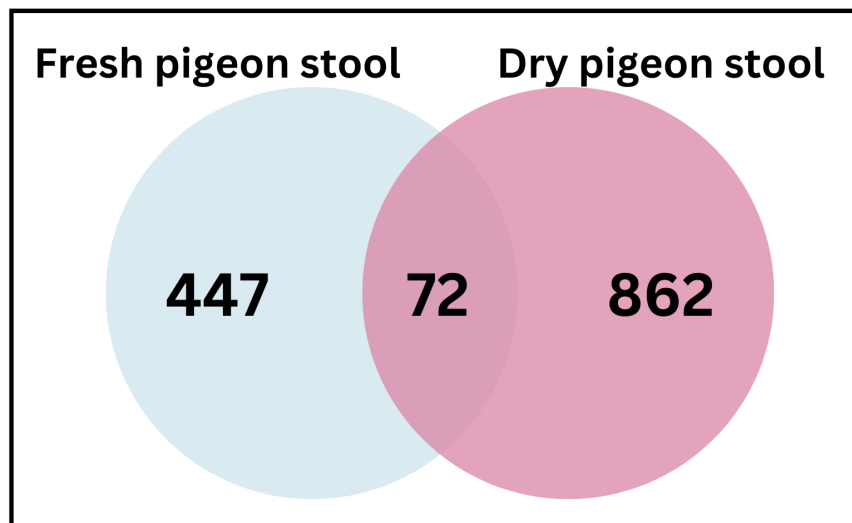


Figure 4.1: Venn diagram demonstrating the sharing bacterial ASVs between fresh and dry pigeon stool collected from four towns in Sarawak.

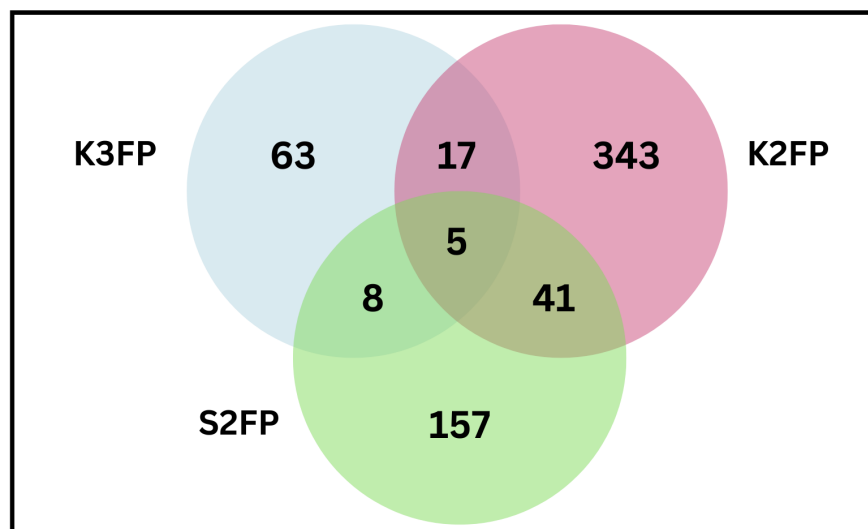


Figure 4.2: Venn diagram demonstrating the sharing bacterial ASVs between fresh pigeon stools collected from three locations around Sarawak.

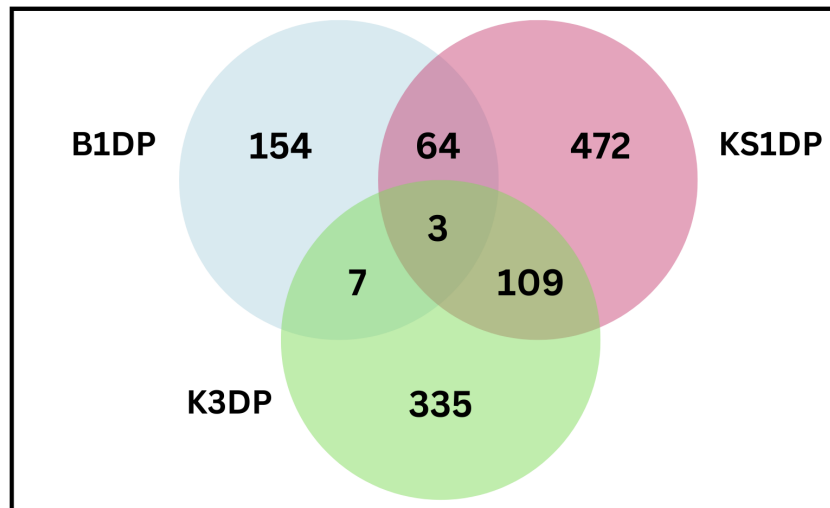


Figure 4.3: Venn diagram demonstrating the sharing bacterial ASV between dry pigeon stools collected from three locations around Sarawak.

Table 4.2: Alpha diversity indices for bacterial diversity of individual fresh and dry pigeon stool samples.

Alpha indices	K3DP	KS1DP	B1DP	K2FP	S2FP	K3FP
#ASVs	335	472	154	343	157	63
Shannon	5.047754	3.843877	2.251917	4.037409	2.215445	3.515776
Simpson	0.85860	0.80754	0.50268	0.84709	0.51603	0.80754
Chao1	337.00000	472.65000	154.00000	343.00000	157.07000	62.50000
Faith's PD	22.61062	27.81707	12.14259	25.20562	12.44489	5.082817

Table 4.3: Alpha diversity indices for bacterial diversity fresh and dry stool. The significance value is presented in the p-value.

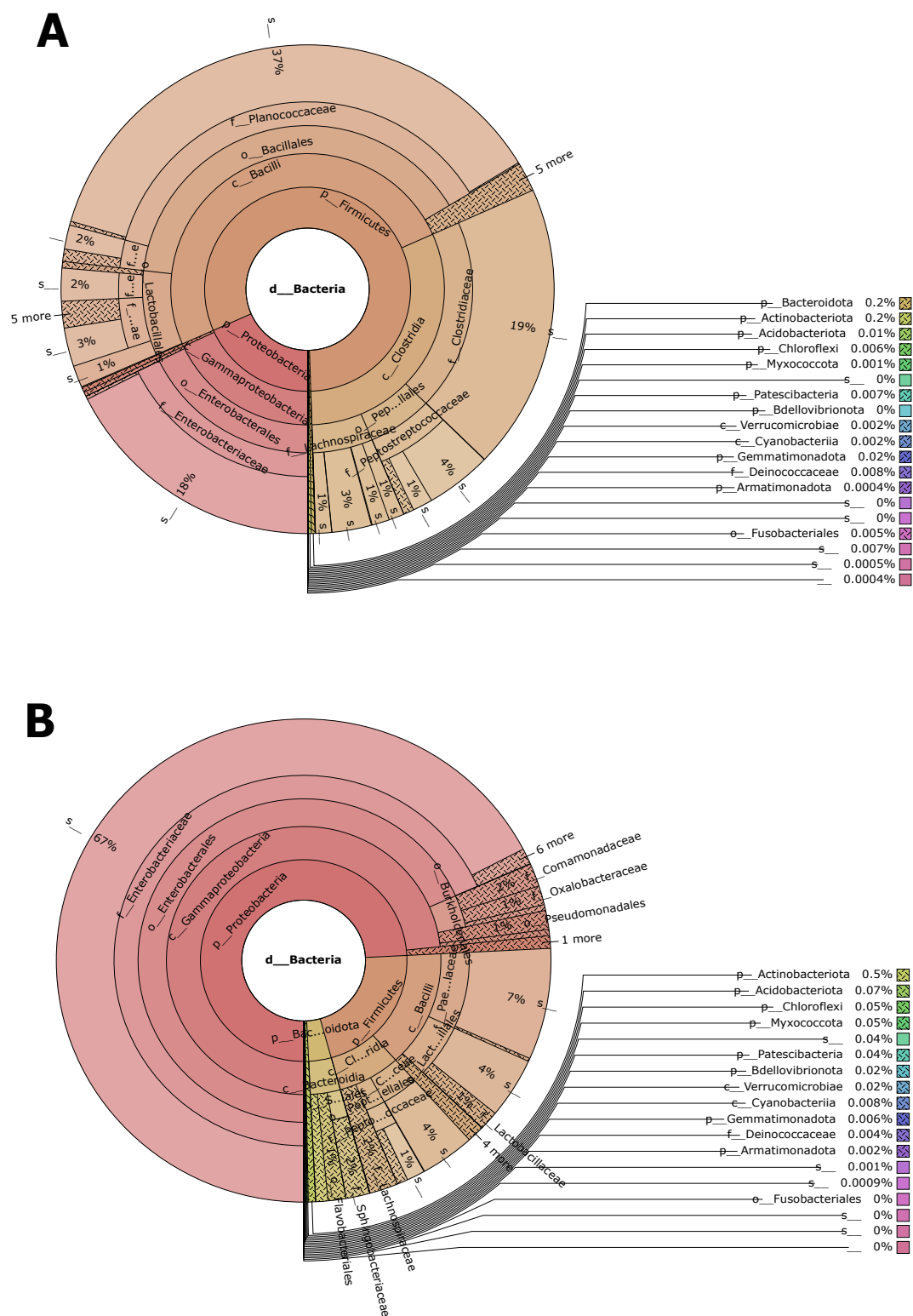
Alpha indices	FPS	DPS	p-value
ASV	187.67 ± 142.50	320.34 ± 345.80	0.1266
Shannon	3.26 ± 0.94	3.72 ± 3.89	0.5127
Simpson	0.72 ± 0.18	0.72 ± 0.74	0.8273
Chao1	187.52 ± 142.71	321.22 ± 346.74	0.1266
Faith's PD	14.24 ± 10.18	20.86 ± 21.86	0.2752

Note: Significance is defined as p-value < 0.05.

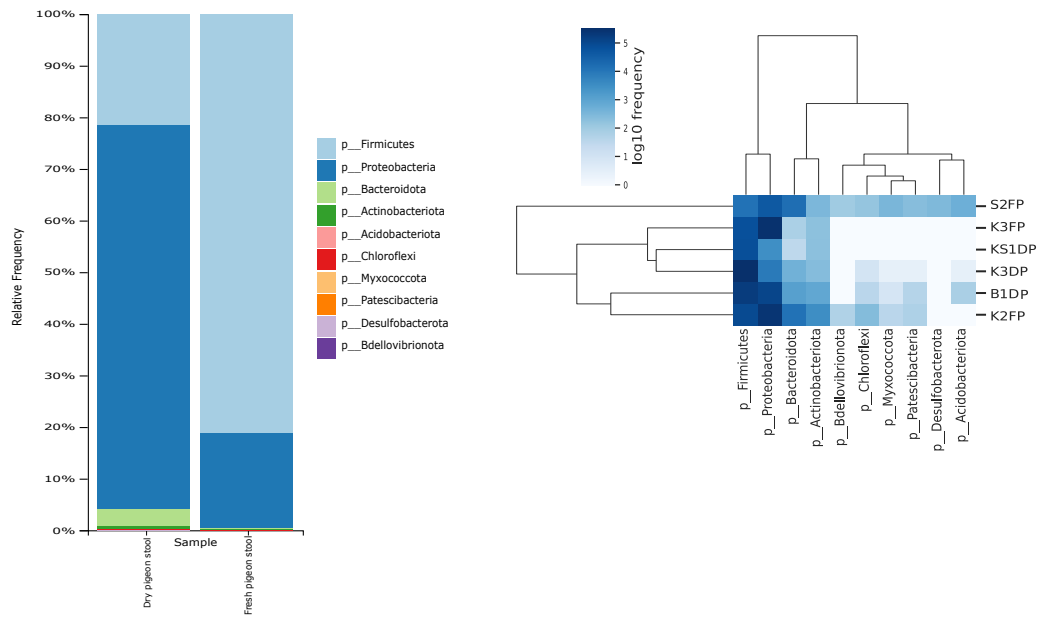
In terms of individual samples, it was found that K3DP had the highest diversity and evenness (ASVs = 335; Shannon's = 5.047754; Simpson's = 0.8586; Chao1 = 337; Faith's PD = 22.61062) among all the samples whereas KS1DP had the highest number of ASVs detected (ASVs = 472) (Figure 4.3). On the other hand, K2FP had the highest diversity and evenness (ASVs = 343; Shannon's = 4.037409; Simpson's = 0.84709; Chao1 = 343; Faith's PD = 25.20562) among the FPS samples (Figure 4.2). The alpha diversity indices values of the individual samples can be found in Table 4.2.

On a phylum level, it was found that BP01DP bacterial microbiome were notably different than the other DPS samples, containing low abundance of phyla Actinobacteriota (Figure 4.5A). Similar was observed in the composition of K3FP which is notably different than those of the FPS samples, not containing Chloroflexi, Myxococcota, Patescibacteria and Acidobacteriota (Figure 4.5A). Apart from that, it was observed that K3DP had higher frequency of Desulfobacterota than the other samples.

On the other hand, at genus level, it was found that FPS sample S2FP and K2FP contained low to high abundance of the top 10 genera (Figure 4.5B). Interestingly, it is observed that K3FP has notably different bacterial composition compared to the other fresh stool sample types, lacking *Paenibacillus*, *Sporosarcina* and *Anaerospobacter*. Then again, it was observed that all DPS samples have low abundance of *Streptococcus*, *Sporosarcina* and *Anaerospobacter* respectively. Similarly, *Sporosarcina* is also observed to be absent from fresh sample K3FP which was taken from the same sampling site of K3DP. This suggests that the sampling site or pigeon in the area lacks *Sporosarcina*.



A



B

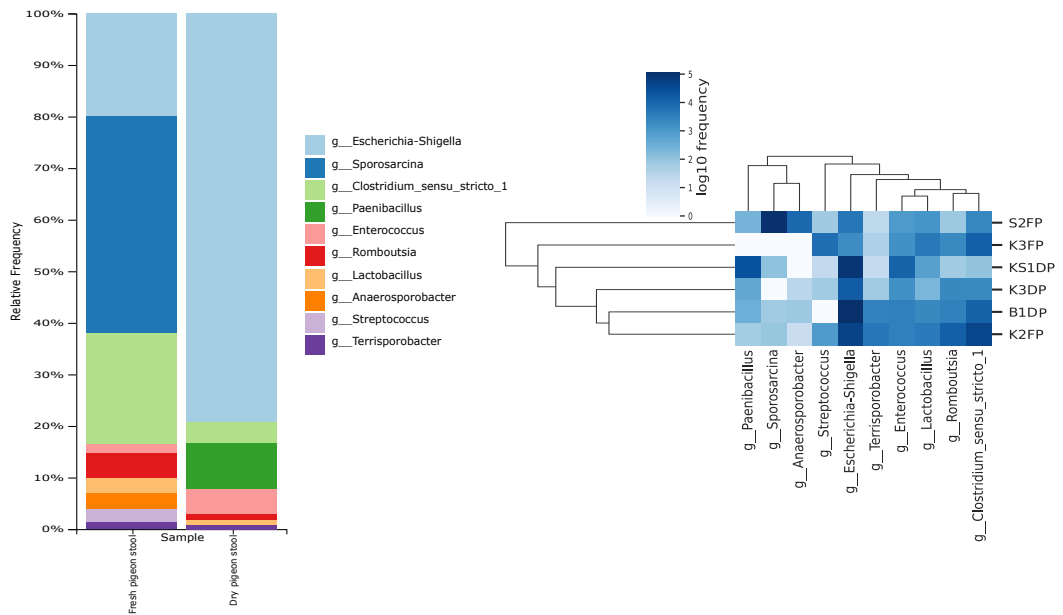


Figure 4.5: Barplot and heatmap of the 10 most abundant bacterial A) phylum and B) genera in fresh and dry pigeon stool.

4.2.2 Bacterial beta diversity

Visualisation of beta diversity through PCoA analysis using Bray-Curtis distance presented no clustering among FPS and DPS samples (Figure 4.6A). Nevertheless, two clusters inclusive of both FPS and DPS groups can be observed. In particular, the cluster indicated with the ‘*’ hailed from the same sampling site, which may indicate that the two samples have highly similar bacterial diversity regardless of sample type.

However, the inclusion of phylogenetic information using weighted-UNIFRAC distance presented obvious separation between FPS and DPS with slight clustering among the samples (Figure 4.6B). Interestingly, no clustering was observed between samples from the same sampling site. The observations mentioned indicates that the bacterial composition when phylogenetic information is taken into consideration between sample types are different, with composition of sample types highly similar to each other.

Regardless, the two sample types are not significantly different with low dissimilarities from each other (PERMANOVA using 999 permutation: Bray-Curtis: pseudo-F = 0.839589, p-value = 0.687; weighted UNIFRAC: pseudo-F = 4.681383, p-value = 0.096) (ANOSIM using 999 permutation: Bray-Curtis: R = 0.037037, p-value = 0.393; weighted-UNIFRAC: R= 0.518519, p-value = 0.105).

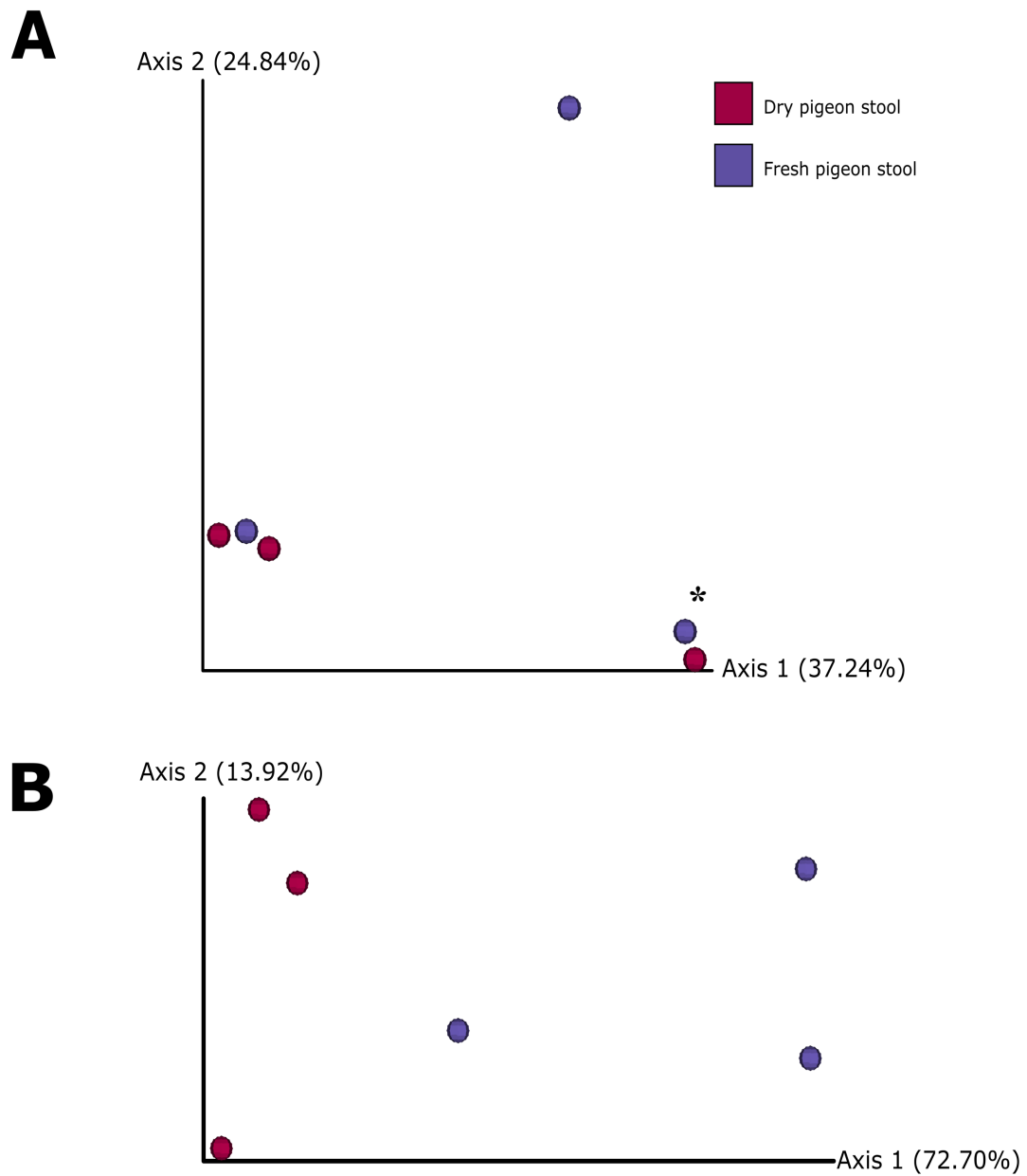


Figure 4.6: Principal coordinate analysis (PCoA) of bacterial beta diversity calculated using the (A) Bray-Curtis and (B) weighted-UNIFRAC distance matrices. Dotted red: dry pigeon stool ($n = 3$); dotted purple: fresh pigeon stool ($n = 3$).

4.2.3 Bacterial differential abundance analysis

Based on the ANCOM analysis, it was revealed that no features were significant to either FPS or DPS. However, further analysis at each level found that four features from the order and family level (order level , $n = 2$; family level, $n = 2$) with W-score of 1, thus rejecting the null hypothesis only once (Figure 4.7). Regardless, the W-score produced were not significant due to the low W-score for each feature.

Additionally, LEfSe analysis at genus level found 17 features as discriminative biomarkers between fresh and dry sample type, with observed LDA scores of > 2.0 and alpha value < 0.05 (Figure 4.8; Figure 4.7). Genus *Ureaplasma* is the only discriminating feature in FPS whereas other 16 features comprising one phylum (Proteobacteria), two classes (Verrucomicrobiae, Gammaproteobacteria), three orders (Verrucomicrobiales, Pseudomonadales, Paenibacillales), three families (Enterobacteriaceae, Pseudomonadaceae, Panenibacillaceae) and six genera (*Stenotrophomonas*, *Lysinibacillus*, *Acinetobacter*, *Pseudomonas*, *Paenibacillus*, *Escherichia-Shigella*) were found to be discriminating features of DPS.

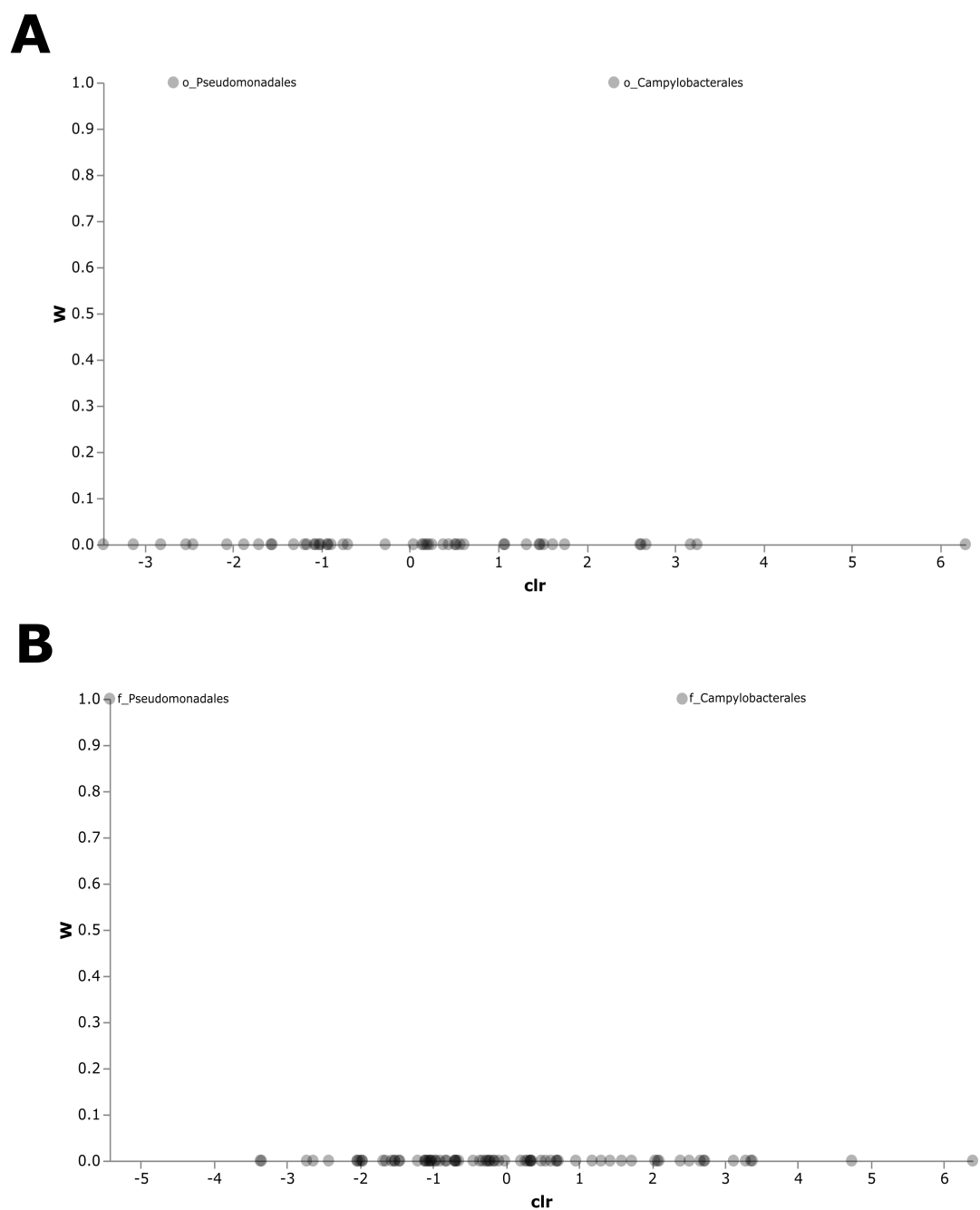


Figure 4.7: ANCOM analysis of bacterial features between fresh and dry pigeon stool at A) order and B) family level.

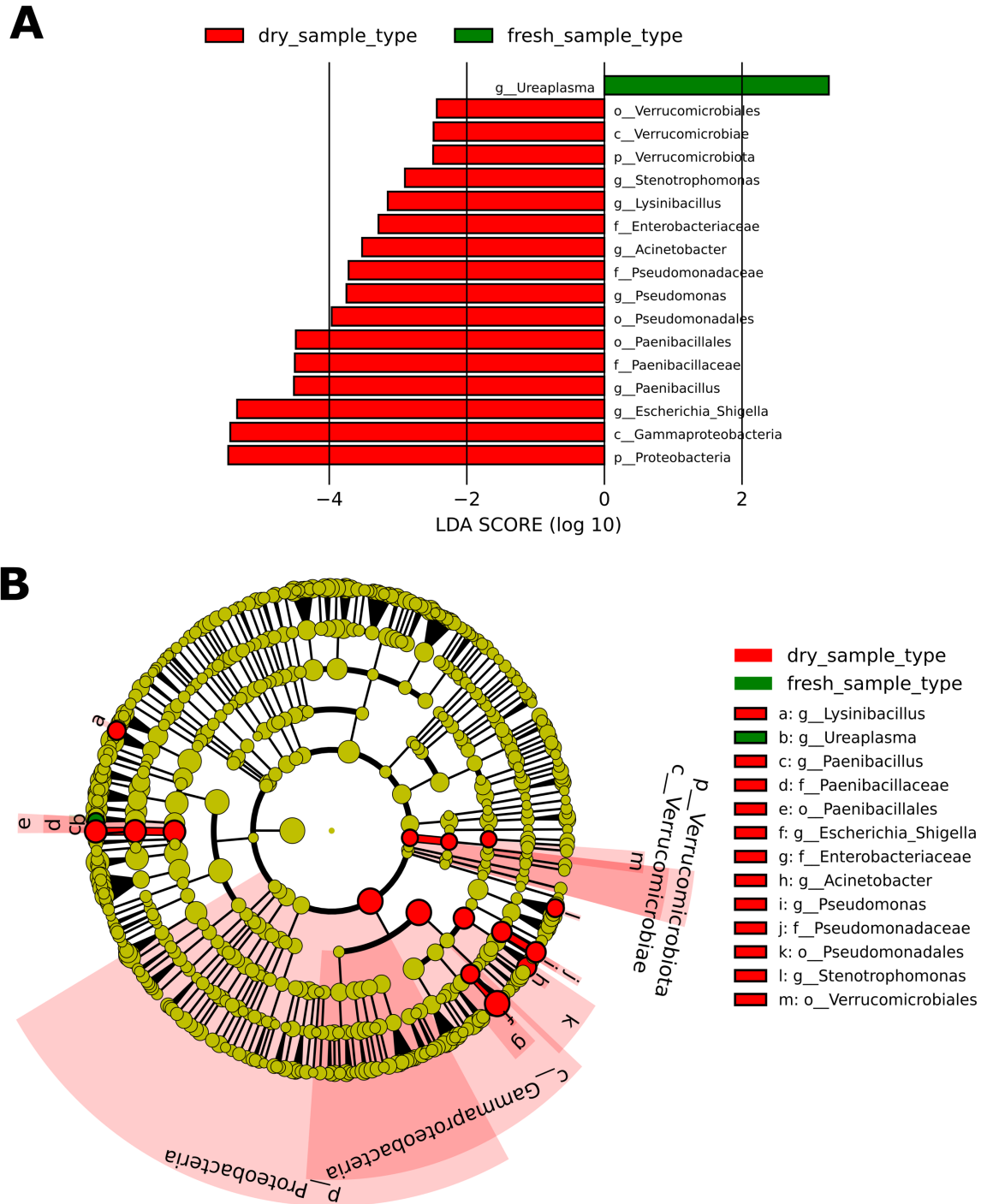


Figure 4.8: LEfSe plot of bacterial biomarkers with LDA log10 score of > 2 in A) vertical bar graph with log10 LDA score and B) cladogram describing the differences fresh and dry pigeon stool at genus level.

4.2.4 Bacteria of public health importance

Among the 356 bacterial genera detected in FPS and DPS, 18 genera potentially pathogenic bacteria were detected in this study (Table 4.4). Among the 18 genera, 14 were identified in both FPS and DPS. Interestingly, *Stenotrophomonas* and *Brevibacterium* was found exclusively in DPS whereas *Campylobacter* and *Ureaplasma* was detected exclusively in FPS. Additionally, *Campylobacter* and *Ureaplasma* were found in the same sample from Kuching, whereas *Brevibacterium* was observed in the DPS sample from Kota Samarahan only. This indicates that the three genera mentioned are specific to the sampling sites.

Table 4.4: Potential pathogenic genera detected in four towns in Sarawak. Reads of more than 10 were considered as positive.

	Kuching (n = 3)	Kota Samarahan (n = 1)	Sibu (n = 1)	Bintulu (n = 1)	Total	
					Fresh (n = 3)	Dry (n = 3)
<i>Acinetobacter</i>	2	1	1	1	2	3
<i>Brevibacterium</i>	-	1	-	-	-	1
<i>Campylobacter</i>	1	-	-	-	1	-
<i>Clostridium</i>	3	1	1	1	3	3
<i>Comamonas</i>	2	1	1	1	3	3
<i>Corynebacterium</i>	2	1	-	1	2	3
<i>Enterococcus</i>	3	1	1	1	3	3
<i>Escherichia-Shigella</i>	3	1	1	1	3	3
<i>Klebsiella</i>	2	1	-	-	1	2
<i>Lactobacillus</i>	1	1	1	1	3	3
<i>Mycoplasma</i>	3	-	-	-	2	1
<i>Pseudomonas</i>	2	1	-	1	1	3
<i>Staphylococcus</i>	2	-	1	-	1	2
<i>Stenotrophomonas</i>	1	1	-	1	-	3
<i>Streptococcus</i>	3	1	1	-	3	2
<i>Sporosarcina</i>	1	1	1	1	2	2
<i>Terrisporobacter</i>	3	1	1	1	3	3
<i>Ureaplasma</i>	1	-	-	-	1	-

4.3 Fungal mycobiome

4.3.1 Fungal alpha diversity

A total of 481 ASVs comprising of 4 phyla, 22 classes, 55 orders, 114 families, and 193 genera were identified against the UNITE v9.0.2023 fungal database. The number of ASVs identified in each sample ranged from 35 – 285 ASVs, with an average of 2.3% of the features detected at > 0.1%. The ASVs were detected from 1,481,846 sequence counts after denoising and chimera removal. Rarefaction curve plot across all samples to the maximum sampling depth 318,766 found confirmed that all samples reached sequencing plateau (Appendix D).

Prior to conducting the alpha and beta analysis, sequence count were normalised by rarefying each sample to the minimum sampling depth of 162,395 following the sample with the lowest sequence count. Overall, it was found that DPS had more fungal composition compared to FPS, with a total of 379 and 126 ASVs detected respectively, with 26 ASVs detected between sample types (Figure 4.9). Alpha diversity indices confirmed that FPS and DPS are significantly different from each other ($p > 0.05$), with higher diversity, richness, and evenness and with higher phylogenetic information in DPS (Table 4.5).

On a phylum level, both FPS and DPS had high abundance of Ascomycota followed by Basidiomycota (Figure 4.12A; Figure 4.13A). On the other hand, it was found *Kazachstania* contributed to Ascomycota dominance in FPS samples. Apart from that, it was found that genera *Neocucurbitaria* and *Fennellomyces* were found specific to FPS whereas *Iodophanus* and *Penicillium* were found specifically DPS (Figure 4.12B; Figure 4.13B).

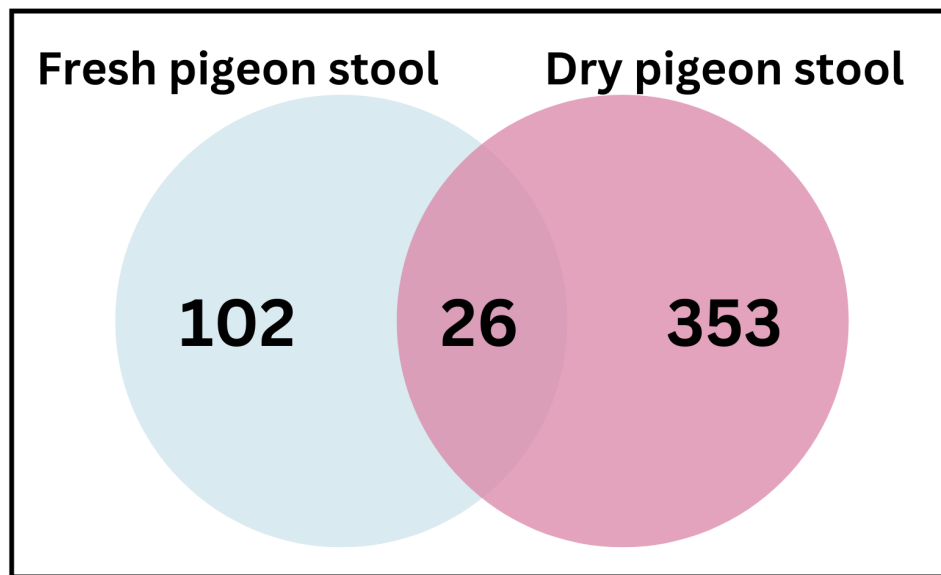


Figure 4.9: Venn diagram demonstrating the sharing fungal ASVs between fresh and dry pigeon stool collected from four towns in Sarawak.

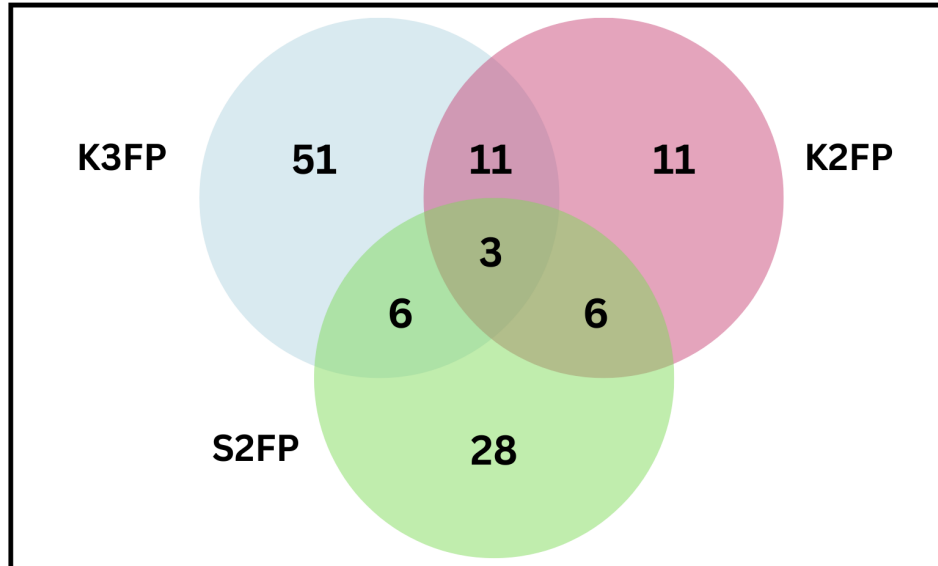


Figure 4.10: Venn diagram demonstrating the sharing fungal ASVs between fresh pigeon stools collected from three locations around Sarawak.

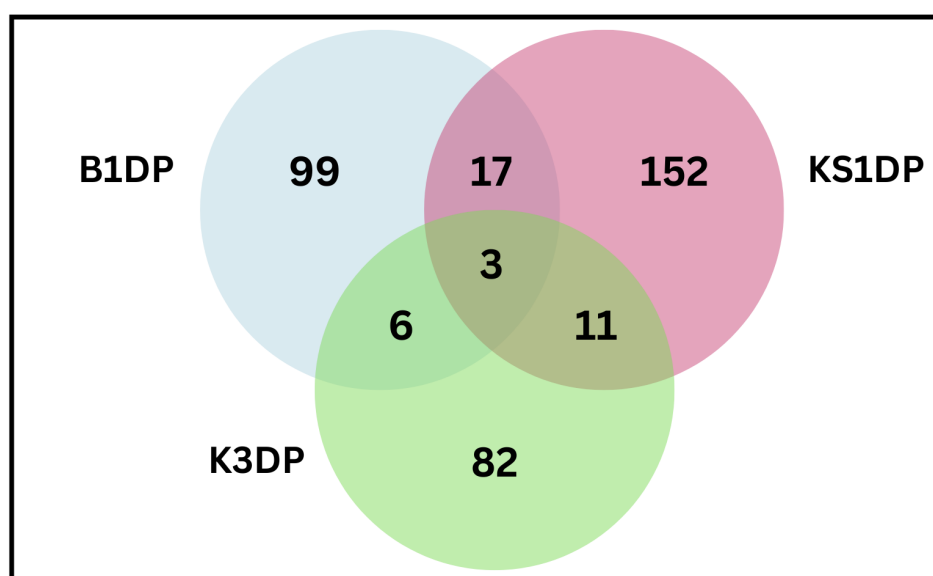


Figure 4.11: Venn diagram demonstrating the sharing fungal ASVs between dry pigeon stools collected from three locations around Sarawak.

Table 4.5: Alpha diversity indices for fungal diversity of individual fresh and dry pigeon stool samples.

Alpha indices	K3DP	KS1DP	B1DP	K2FP	S2FP	K3FP
#ASVs	102	185	125	34	43	72
Shannon	2.003786	3.783630	2.352757	0.105029	0.362646	2.003786
Simpson	0.748451	0.887453	0.625288	0.017823	0.092413	0.617237
Chao1	102.0000	185.0000	125.0000	34.0000	43.0000	71.167000
Faith's PD	30.225360	42.306680	32.186490	11.125870	18.016110	21.202200

Table 4.6: Alpha diversity indices for bacterial diversity of fresh and dry pigeon stool. Significance value is presented in p-value.

Alpha indices	DPS	FPS	p-value
ASV	49.67 ± 19.86	137.33 ± 42.85	0.0495
Shannon	0.82 ± 1.03	2.71 ± 0.94	0.0495
Simpson	0.09 ± 0.62	0.75 ± 0.13	0.0495
Chao1	48.39 ± 20.62	137.33 ± 42.85	0.0495
Faith's PD	16.78 ± 5.15	34.91 ± 6.48	0.0495

Note: Significance is define as p-value < 0.05.

Among the six pooled samples, it was found that KS01DP had the highest diversity, richness and evenness (ASVs = 185; Shannon's = 3.784; Simpson's = 0.887; Chao1 = 185; Faith's PD = 42.306680) among all the samples (Figure 4.11). On the other hand, K3FP had the highest diversity, and evenness (ASVs = 72; Shannon's = 2.004; Simpson's = 0.617; Chao1 = 71.167; Faith's PD = 21.202) among those from the FPS samples (Figure 4.10). However, K2FP had the lowest diversity, with only 34 ASVs detected in the sample. The alpha diversity indices values of the remaining individual samples can be found in Table 4.5.

On a phylum level, it was found that all samples contained medium to high abundance of Chytridiomycota except for sample K2FP whereas Basidiomycota was exclusively in DPS except for sample B1DP (Figure 4.13A). On a genus level, it was observed that all samples had high frequency of *Kazachstania* followed by *Aspergillus* (Figure 4.13B). In particular, *Trichosporon* was observed at medium to high frequency in all samples except K2FP. Similarly, *Dothideomycetes* gen Incertae sedis was observed in low to high frequency in all samples except for sample S2FP. Notably, it was found that the high frequency of *Neocucurbitaria* in FPS and *Fennellomyces* in DPS were contributed by sample K3FP and B1DP respectively (Figure 4.13B).

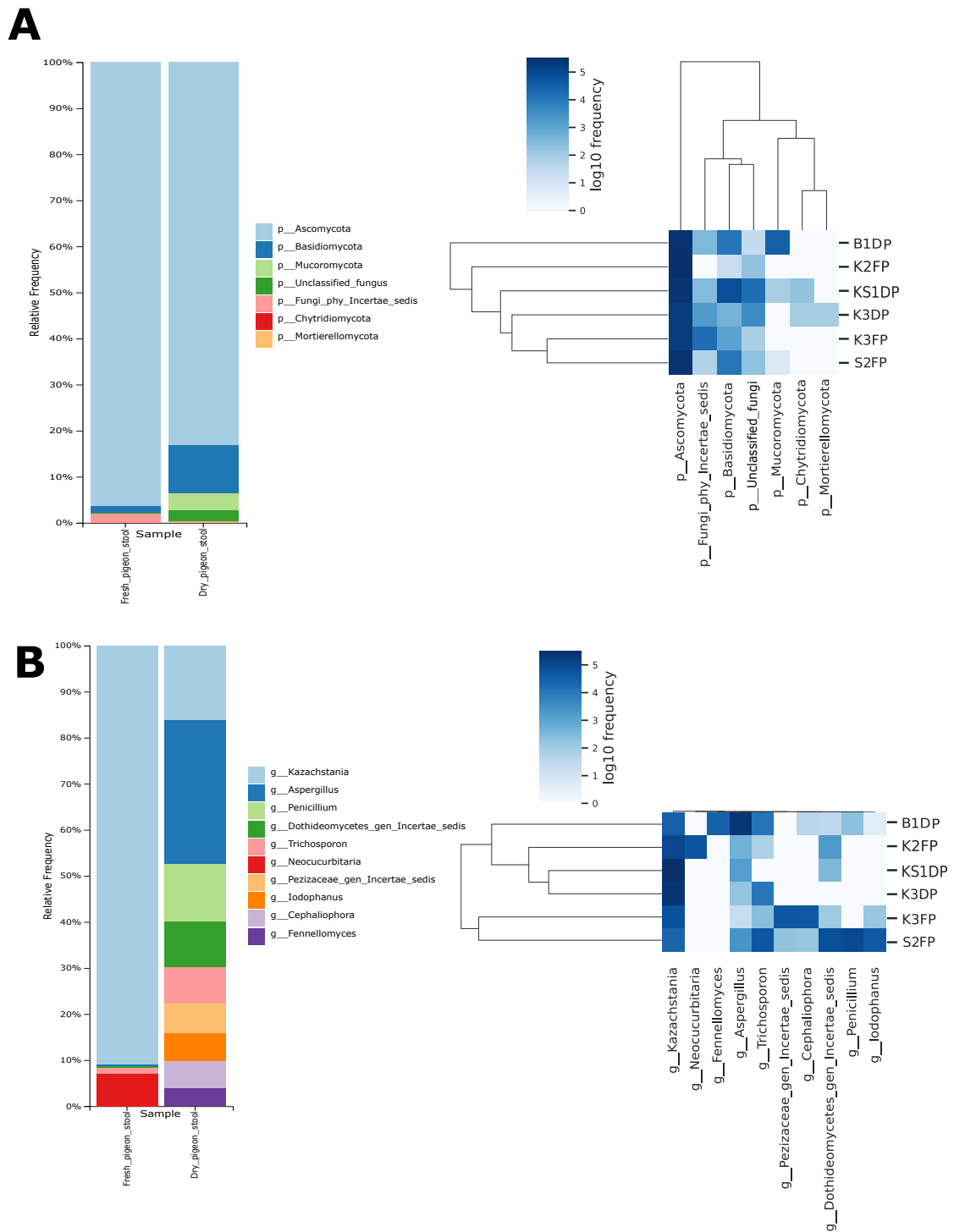


Figure 4.13: Barplot and heatmap of the 10 most abundant fungal A) phylum and B) genera in fresh and dry pigeon stool. Only 7 phyla are shown due to the limited number of phyla detected.

4.3.2 Fungal beta diversity

Visualisation of beta diversity through PCoA analysis using Bray-Curtis and weighted-UNIFRAC presented obvious separation between the FPS and DPS (Figure 4.14). However, it was found that Bray-Curtis distance matrix calculation presented obvious clustering especially between the FPS samples (Figure 4.14A), whereas slight clustering was observed between the sample types when phylogenetic weighted UNIFRAC distance matrix was included (Figure 4.14B).

This indicates that there was an obvious difference in fungal diversity between FPS and DPS, but inclusion of phylogenetic information did not have any effect on clustering between sample types. Instead, weighted UNIFRAC distance matrix provided additional information to indicate phylogenetic difference of individual samples of each sample type.

Nevertheless, the differences were found to be not significant (PERMANOVA using 999 permutation: Bray-Curtis: pseudo-F = 2.42495, p-value = 0.104; weighted UNIFRAC: pseudo-F = 4.681383, p-value = 0.099) (ANOSIM with 999 permutations: Bray-Curtis: R = 0.407407, p-value = 0.207; weighted-UNIFRAC: R = 0.592593, p-value = 0.127).

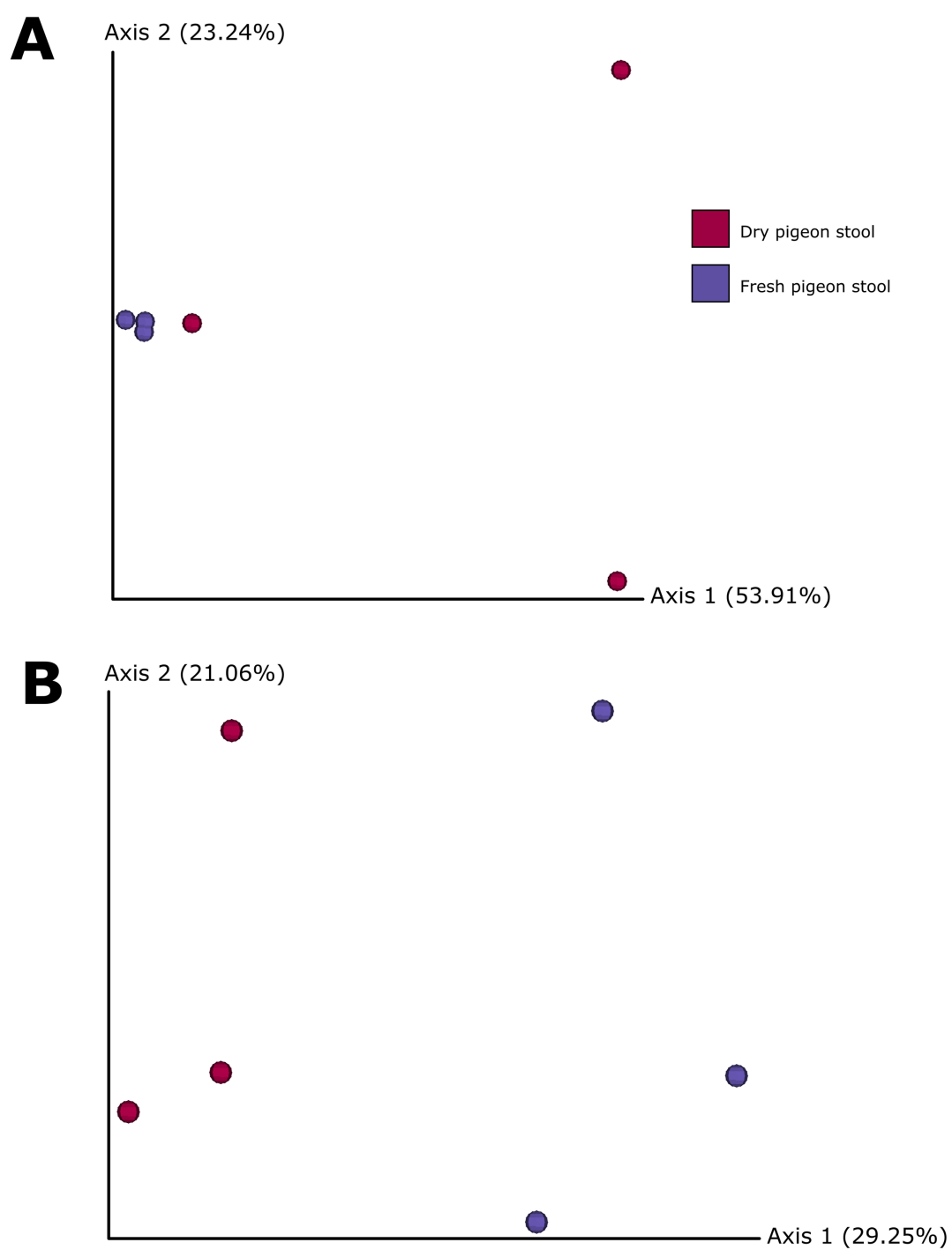


Figure 4.14: Principal coordinate analysis (PCoA) of fungal beta diversity calculated using the (A) Bray-Curtis and (B) weighted-UNIFRAC distance matrices. Dotted red: dry pigeon stool ($n = 3$); dotted purple: fresh pigeon stool ($n = 3$).

4.3.3 Fungal differential abundance analysis

Based on the ANCOM analysis, three ASVs were found to have W-score ranging from 1.0 – 2.0 at genus level, notably *Cephalophora*, *Pezizaceae* gen Incertae sedis and *Kazachstania* (Figure 4.15C). Further analysis on the other taxonomic level found eight features scoring a W-score of 1, from the class and family level (class level, n = 4; family level, n = 4) (Figure 4.15). However, the W-score produced were not significant due to the low W-score for each feature.

On the other hand, LEfSe analysis at genus level found 12 features as discriminative biomarkers between fresh and dry sample type, with observed LDA scores of > 2.0 and alpha value < 0.05 (Figure 4.16). Four features comprising of one class (Saccharomycetes), two families (Saccharomycetaceae, Aspergillaceae) and one genus (*Kazachstania*) were identified as fungal biomarkers from FPS sample. On the other hand, the remaining eight features comprising of one class (Pezizomycetes), one order (Pezizales), three families (Polyporaceae, Bibnectriaceae, Pezizaceae), and two genera (*Iodophanus*, *Pezizales*) are discriminative biomarkers of DPS sample.

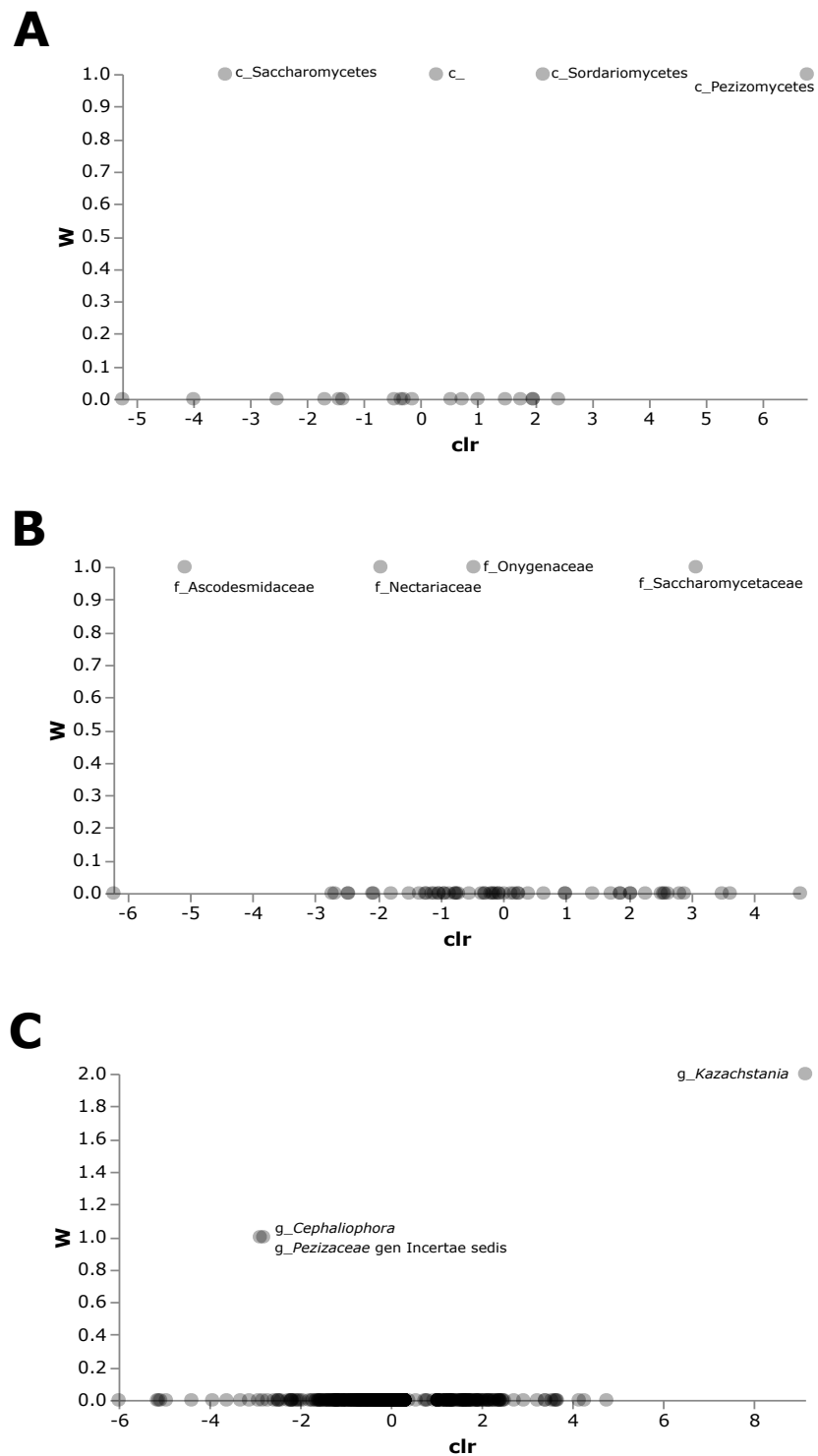


Figure 4.15: ANCOM analysis of bacterial features between fresh and dry pigeon stool at A) class, B) family and C) genus level.

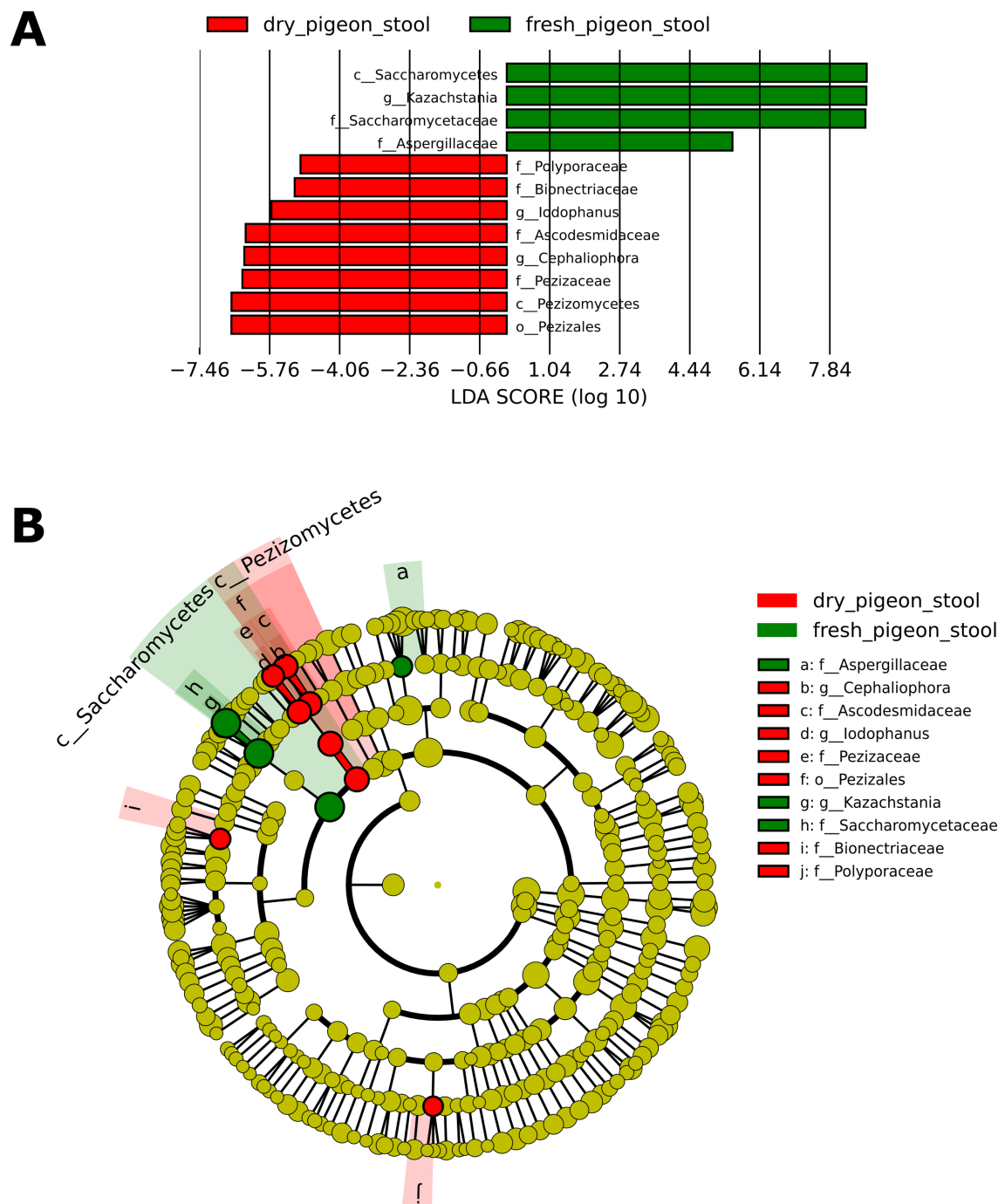


Figure 4.16: LEfSe plot of fungal biomarkers with LDA log10 score of > 2 in A) vertical bar graph with log10 LDA score and B) cladogram describing the differences between fresh and dry pigeon stool.

4.3.4 Fungi of public health importance

Among the 193 genera detected from FPS and DPS, 18 potential pathogenic and allergens fungal genera were detected from this study, with *Aspergillus* present in all samples (Table 4.7). It was found that *Cuteneotrichosporon*, *Cyberlindnera*, *Cystobasidium* were present exclusively in DPS whereas *Nakaseomyces* was present in FPS only. Interestingly, *Cyberlindnera* and *Cystobasidium* was detected in Bintulu only, which may indicate that the fungi is specific to the environment of the sampling site.

Table 4.7: The number of samples and sample type positive for potentially pathogenic fungi from 4 cities in Sarawak on a genus level.

	Kuching (n = 3)	Kota Samarahan (n = 1)	Sibu (n = 1)	Bintulu (n = 1)	Total	
					Fresh (n = 3)	Dry (n = 3)
<i>Aspergillus</i>	3	1	1	1	3	3
<i>Candida</i>	3	-	1	1	3	2
<i>Cladosporium</i>	-	1	1	-	1	1
<i>Curvularia</i>	2	1	1	1	2	3
<i>Cutaneotrichosporon</i>	-	1	-	1	-	2
<i>Cyberlindnera</i>	-	-	-	1	-	1
<i>Cystobasidium</i>	-	-	-	1	-	1
<i>Debaryomyces</i>	-	1	-	-	-	1
<i>Fusarium</i>	2	1	1	1	2	3
<i>Kazachstania</i>	1	1	1	-	2	1
<i>Mucor</i>	-	1	1	1	1	2
<i>Nakaseomyces</i>	-	-	1	-	1	-
<i>Neocucurbitaria</i>	-	1	-	-	-	1
<i>Naganishia</i>	2	-	1	-	2	1
<i>Papiliotrema</i>	1	1	-	-	1	1
<i>Rhodotorula</i>	1	-	1	-	1	1
<i>Talaromyces</i>	-	-	1	-	1	-
<i>Trichosporon</i>	2	1	1	1	2	3

CHAPTER 5

DISCUSSION

The research sought to understand the bacterial and fungal diversity of pigeon in Sarawak by deploying high-throughput amplicon sequencing to avoid biasness in identifying the culturable only. This section intends to further discuss the takeaways from the results.

5.1 Efficacy of 2% CTAB protocol for genomic DNA extraction

A suitable DNA extraction protocol contributes to high sequencing read numbers and should be first considered before proceeding to any NGS protocol. Pigeon stool, like other avian stool have different components than of mammalian stool. This is mainly due to the physiology of avian, in which faecal matter after digestion is often mixed with contents of urogenital and reproductive system. This produces a physio-chemical complex stool component with high uric acid content, which has been proven to cause difficulties in gDNA extraction from avian stool (Eriksson et al., 2017; Vo & Jedlicka, 2014). It has also been observed that commercialised stool extraction kits alone performed poorly, in fact the least successful to obtain gDNA as per the requirements for high-throughput amplicon sequencing analysis (Edwards et al., 2023; Eriksson et al., 2017). Thus, Eriksson et al. (2017) developed a protocol which emphasised on the need to include a pre-lysis treatment, such as bead beating, when using commercialised kits that do not including the said consumable or in its protocol.

However, in real situations, opting for commercialised kits and pre-treatment steps can be expensive or inaccessible, especially when budgeting or when there is a lack of appropriate laboratory equipment. Therefore, this study has proven the utilisation of the 2%

CTAB protocol with slight modification, and the additional step of purification without the need for a pre-treatment step (as described in Section 0) is able to provide high-quality and quantity gDNA for high-throughput amplicon sequencing analysis (Table 4.1). Indeed, previous studies have also proven that the CTAB protocol is able to remove the presence of PCR inhibitors such as humic acid, phenolic compounds, protein, polysaccharides, and cell debris from the samples (Kachiprath et al., 2018).

5.2 Bacterial diversity of pigeon stool

The overall dominating bacteria phyla in pigeon stool samples generally are Firmicutes, Proteobacteria and Bacteroidata (Figure 4.4), with no significant difference in alpha and beta diversity between FPS and DPS (refer to Sections 4.2.1 and 4.2.2). But, inclusion of phylogenetic information plays an important role in discriminating the composition between the two groups. Regardless, it was found that the abundance of specific genera differs between sample types, which ultimately affected the dominating phylum in a sample type.

In FPS, it was observed that Firmicutes is in higher abundance compared to the other phyla, attributed to the high abundance of probiotic bacteria such as *Sporosarcina*, *Clostridium sensu stricto* 1, *Romboutsia*, *Lactobacillus* and *Anaerosporeobacter* (Figure 4.5). The findings reported in this study are similar to those reported from other pigeon microbiota studies (Ji et al., 2020; Oh et al., 2023; Xu et al., 2022). But it's a first for *Anaerosporeobacter*. A study conducted by Ji et al. (2020) found that infections such as those caused by *Trichomonas gallinae* altered the abundance of Firmicutes and Proteobacteria, observing a decrease in the abundance of *Lactobacillus* and an increase of *Enterococcus*. Apart from that, a study steered towards understanding the effects of early weaning on pigeon squab

microbiota by Xu et al. (2022) found that the Firmicutes are predominantly the dominant contributed by the high abundance of *Lactobacillus* and *Romboutsia*, with early weaning affecting the dominance of mainly Proteobacteria and Actinobacteria. On the other hand, Oh et al. (2023) discovered that there was a higher abundance of *Escherichia-Shigella* and those from the Listeriaceae family in samples collected in city areas, whereas *Lactobacillus* was more abundant in samples collected in non-city areas of South Korea. In conclusion, the dominant phylum in FPS is Firmicutes, and the abundance of other phyla such as Proteobacteria, Bacteroidata and Actinobacteriota, reflecting the pigeon gut microbiome and is highly affected by the health status of the bird, early weaning effects, and urbanisation with consideration to the presence of the homeless.

On the other hand, it was observed that Proteobacteria is dominant phyla in DPS, followed by Firmicutes and Bacteroidata (Figure 4.4). The dominance of Proteobacteria is contributed by the high abundance of genera from the Enterobacteriaceae family such as *Escherichia-Shigella* and *Enterococcus*, whereas an increased abundance of *Paenibacillus* from Firmicutes was also observed. Interestingly, previous studies only focused on identifying *Escherichia-Shigella* and *Enterococcus* from FPS (refer to Table 2.1). To this day, there are no reports of bacterial diversity in DPS. Nevertheless, a study conducted on inhalable bacteria from dust of pigeon coop and pigeon exhibition reported similar findings, with high presence of enteric bacteria such as *Escherichia-Shigella* and *Enterococcus* (Madsen et al., 2022, 2023). The factors contributing to the sudden high abundance of enteric bacteria in are unknown, but it is speculated that *Escherichia-Shigella* and *Enterococcus* are able to survive pass the gastrointestinal (GI) tract, remain viable, and found suitable environment (presence of oxygen, suitable temperature, moisture and growth nutrient) to

proliferate upon excrement (Schulz et al., 2016). And that, DPS is the main contributor to the high abundance of *Escherichia-Shigella* and *Enterococcus* in pigeon coop dust.

Interestingly, *Paenibacillus*, a spore-forming bacteria previously reported in FPS was found in high abundance in DPS (Oh et al., 2023). *Paenibacillus* is commonly found in soil, and often associated with plant roots (Grady et al., 2016). It is speculated that *Paenibacillus* is not a common part of the pigeon gut microbiome due to its low abundance in FPS, but rather as a result of the pigeon diet in feeding off grass blades. Subsequently, the bacteria passes through the GI system and finds suitable conditions for growth upon excrement into the environment.

5.2.1 Diversity of potentially pathogenic bacteria in pigeon stool

As mentioned in Section 4.2.4, 18 potentially pathogenic bacteria were identified, with 14 genera found in both FPS and DPS (Table 4.4). Most of the bacteria mentioned in this table have either been isolated in previous studies (refer to Section 2.2) or detected through NGS of pigeon excrete and dust, except for *Commamonas*, *Sporosarcina*, *Terrisporobacter* and *Ureaplasma*.

Enteric bacteria including *Campylobacter*, *Enterococcus*, *Escherichia-Shigella*, *Lactobacillus*, *Klebsiella*, *Actinobacter* and *Clostridium*, are important bacteria naturally found in mammalian and avian gut microbiome which plays an important role in regulating the overall health of the host. However, these bacteria can also act as an opportunistic bacteria, causing infection in the host. It has been reported that enteric bacteria found in pigeon harbour virulence and antimicrobial traits (Morakchi et al., 2017), which is believed to have passed down from migratory birds, livestock and hospital setting (refer to Section 2.2 for its prevalence). Another theory explaining the presence of the potentially pathogenic

enteric bacteria in pigeon is the limiting food source in urban settings, eventually forcing pigeon to feed off human scraps and rubbish, leading to poorer body condition, and an increase in shedding of the bacteria (Grond et al., 2019; Oh et al., 2023). And the presence of these bacteria in the environment should not be taken lightly, as studies have reported on its viability in the dust of pigeon coops and pigeon exhibitions (Madsen et al., 2022, 2023).

Apart from enteric bacteria, 50% and 83.33% of the samples were positive for gram-positive cocci-shaped *Staphylococcus* and *Streptococcus*, from the Proteobacteria phylum. Both genera are regarded as part of the human and pigeon microbiome. However, within the *Staphylococcus* genus, pathogenic *S. aureus* has emerged due to the inappropriate use of antibiotic drugs as treatment, causing a worldwide emergence of methicillin-resistant and multidrug strains from hospitals and farming waste, which is then disseminated in human-populated settlements through pigeon (Madsen et al., 2023; Wilson et al., 2024). Among the *S. aureus* isolated from pigeon, 50.55% of isolates from pigeon dust have been found to contain the *mecA* gene which encodes methicillin-resistance in virulent strains (Madsen et al., 2023; Wilson et al., 2024). On the other hand, within the *Streptococcus* genus, *S. bovis*/*S. gallolyticus* complex is a commensal bacteria of the human rectum and pigeon cloaca (Jans & Boleij, 2018). However, in humans, commensal-turned-pathogenic bacteria was first associated with colorectal cancer in the 1970s, and extensive research found the bacteria described its associated with chronic liver and biliary tract disorder and septicemia (Gonzalez-Quintela et al., 2001; Pasquereau-Kotula et al., 2018; Pompilio et al., 2019). The mechanism used by the species to survive within the human immune system still unclear (Pompilio et al., 2019).

Rarely mentioned bacteria from pigeon stool are *Brevibacterium*, *Corynebacterium* and *Stenotrophomonas*, which were isolated from both FPS and pigeon dust (Madsen et al., 2022, 2023; Oh et al., 2023). However, little is known regarding the pathogenicity of these pigeon opportunistic bacteria apart from the fact that they are commonly found in the environment, but its emergence should not be taken lightly with reports of infection in the immunocompromised communities (Agrawal et al., 2021; Azimi et al., 2021; Munshi et al., 2022).

As mentioned in the first paragraph, four genera, mainly *Commamonas*, *Sporosarcina*, *Terrisporobacter* and *Ureaplasma* were not previously described as members of the pigeon stool microbiota. All genera were present in FPS, indicating that the genera are part of the pigeon microbiome. Relatively little is known about the effects of bacterial infections, as many were regarded as non-pathogenic or of low virulence until case studies were reported.

To start, *Commamonas* sp. are non-fermenting bacilli, previously thought to be low virulence in humans (Ryan et al., 2022). Over the years, there has been increasing reports regarding the infections caused by the bacteria, including septicaemia and endocarditis, in both immunocompetent and immunocompromised patients, and has eventually caused death in 6 patients (Cooper et al., 2005; Farooq et al., 2017; Ryan et al., 2022). *Sporosarcina*, on the other hand, is currently of high interest due to its potential as an antibiotic alternative for poultry treatment in hopes of reducing the emergence of antibiotic-resistant strains (Keshri et al., 2023; Priyodip & Balaji, 2019). However, Wolfgang and colleagues (2012) reported patients with bacteraemia and eye infection caused by *S. newyorkensis* in 11 patients. Next, *Terrisporobacter*, previously classified under the *Clostridium* genus, is an emerging

anaerobic bacteria with little knowledge on its pathogenicity in humans (Cheng et al., 2016). Four reports on *Terrisporobacter* infections have been published, which found that all patients immunocompromised (co-infections with *Terrisporobacter*, Hodgkin's diseases, otitis media, acalculous cholecystitis, cirrhosis) (Cheng et al., 2016; Elsayed & Zhang, 2007; Shah et al., 2022; Van Leer et al., 2009). Lastly, *Ureaplasma*, from the family Mycoplasmataceae, is a unique bacteria known for its ability to hydrolyse urea, and a common bacteria in asymptomatic poultry. Interestingly, the bacteria was previously not associated with pigeon is regularly associated with other bird species such as poultry. Regardless, species from this non-WOAH-listed avian disease-causing genus usually cause urinary and genital tract infections in humans (Cobb & Smith, 2015). But in humans, *Ureaplasma* manifestation is very rare and has only been reported to cause hyperammonaemia (Cobb & Smith, 2015; Kok & Lee, 2022).

Interestingly, no *Chlamydia* was detected in the sample set despite the high prevalence at about 21.42% found in other parts of the world (refer to section 2.2.2). There are a few factors which can induce the shedding of intracellular bacteria, such as 1) breeding season, 2) host infection, 3) compromised immunity of pigeon due to concurring infection with pigeon circovirus and 4) sample collection site, which can affect detection rate (Kowalczyk & Wójcik-Fatla, 2022). Regardless, a low or zero positive prevalence may occur with small sampling size, which may be the case in this study (Oh et al., 2023).

5.3 Fungal diversity of pigeon stool

Overall, the dominant fungal phyla in pigeon stool are Ascomycota, Basidiomycota and Mucormycota (Figure 4.12), with significant differences in alpha diversity across all tested indices and differences in composition between the two groups (refer to Section 4.3.1 and 4.3.2). However, statistical analysis found no significant difference in beta diversity between the two groups, indicating that the fungal composition between the groups are similar.

In FPS, the dominant phyla are Ascomycota and Basidiomycota only, with Ascomycota representing more than 95% of fungi phylum. The findings are similar to those in other avian and mammalian species (Mahtab et al., 2021; Xiang et al., 2021). The dominance of Ascomycota is attributed to the high abundance of *Kazachstania*, representing 90% of the genus detected in FPS, and *Neocucurbitaria*, which has not been reported in pigeon previously. Lee and colleagues (2017) reported on the fungal diversity of pigeon stool, and mentioned that species from the *Kazachstania* genus, such as *K. bovina* uses pigeon as its vector for dissemination into the environment and that it has ability of to either colonise or pass through the GI tract due to its thermophilic characteristics. However, other gut microbiome study have suggested *Kazachstania* are important gut mycobiota and dominated the community, in hand with probiotic bacteria, to aid in decreasing severity of necrotic enteritis in animals (Arfken et al., 2019; West et al., 2023; Yang et al., 2021). *Neocucurbitria* on the other hand, was found in only one sample from Kuching sampling site. Its role in gut is still unknown, and has not been reported in any fungal gut mycobiome studies. Thus, it is speculated that the genus may have utilised pigeon as a mode of dispersion (Lee et al., 2017), but further study is warranted to understand its relation with pigeon.

On the other hand, the dominating phyla in DPS are Ascomycota, Basidiomycota and Mucormycota, with a decrease in relative abundance of Ascomycota than in FPS. The dominance of Ascomycota in dry pigeon stool is attributed to the presence of environmental fungi and human mycobiome such as *Aspergillus*, *Trichosporon*, *Iodophanus*, *Cephalophora* and *Penicillium*. The presence of *Fennellomyces* attributed to the appearance of Mucormycota (Figure 4.12). Among the genera stated above, only *Aspergillus*, *Penicillium* and *Trichosporon* have previously been reported in or from pigeons (Lee et al., 2017; Madsen et al., 2023). The high abundance of environmental fungi can be attributed to their ability to utilise dry pigeon stool as their growth substrate (Lee et al., 2017). The uric acid present in pigeon stool acts as nutrients for urea and uric acid hydrolysing fungus, which has been proven to be abundant in pigeon stool (Borowik et al., 2023; Zuo et al., 2022). However, the presence of novel abundant fungal genera reported in this study may be attributed due to geographical regions, or the fact that studies on pigeon fungal diversity focused on reporting those with clinical importance only.

5.3.1 Diversity of potentially pathogenic fungi in pigeon stool

On the other hand, 18 genera from both FPS and DPS have been identified as potentially pathogenic (Table 4.7). Among which, six genera including *Curvularia*, *Cutaneotrichosporon*, *Cystobasidium*, *Debaryomyces*, *Neocucurbitaria*, and *Talaromyces* were not previously associated with pigeon stool.

Among the fungi found in this study, *Candida*, *Cyberlindnera*, *Kazachstania*, *Rhodotorula* and *Trichosporon* are commonly isolated from pigeon, and have been of interest due to their pathogenicity in humans (refer to 2.3.2 and 2.3.4). Common characteristics of the listed fungi are 1) yeast-like characteristics, 2) the ability to produce

biofilm and 3) antifungal resistant traits toward amphotericin-B and azole drugs due to increase in use for treatments (Farooq et al., 2022; Guinea, 2014; Iturrieta-González et al., 2014; Li et al., 2020; Nunes et al., 2013; Park et al., 2019; Pham et al., 2022). Many of the infections caused by the fungi are observed in immunocompromised community (solid organ transplant, chemotherapy patients, HIV, pulmonary infection), causing either bloodstream, cutaneous, pulmonary infections.

Moreover, moulds found in this study such as *Aspergillus*, *Fusarium* and *Mucor* are also of high public health importance as many of the cases reporting invasive pulmonary infection caused by these moulds often leads to mortality (Shen et al., 2023; Tong et al., 2021). Especially for *Aspergillus*, the mould does not discriminate potential host immune status, with reports finding increase admission of patients from no previous immunocompromised history (Zaragoza et al., 2023).

As mentioned in the first paragraph, six genera found in this study have not been associated with pigeon previously. All six fungi were present only in DPS, indicating that the fungal genera are commonly found in the environment and may have utilised the nitrogenous content of DPS as its growth medium. These environmental fungal genera are said to cause emergent fungal infection with reports focusing on the most suitable treatment due to the lack of reported clinical antifungal breakpoint.

To start, *Curvularia*, from the Dothidiomycetes class, is a mould-like fungus commonly found in dead plants. First reported in humans in the 1980s, inhalation of the fungus have been found to cause rhinosinusitis, onychomycosis and dermatitis in those with co-morbidities and history of drug abuse and perfume inhalation (Gupta et al., 2017; Marinucci et al., 2022; Pham et al., 2022). Next, *Cutaneotrichosporon*, previously classified

under *Trichosporon*, is a yeast-like fungus causing sporadic infection of septicaemia, urinary tract infection, and subcutaneous fungal infection in the immunocompetent, and found to have low MIC towards azole drugs (Nath et al., 2018; Noy et al., 2020; Yoo et al., 2022). *Cystobasidium*, on the other hand, is an ubiquitous fungus commonly found in the environment and have been suggested as a biomarker for studies focusing on understand the changes in human gut microbiome due to *C. difficile* infection (Cao et al., 2022). Although infections caused by the genus is very rare, the genus have been reported to cause angular cheilitis, septicaemia, and joint infection (Cerikcioglu et al., 2005; Inácio et al., 2020; Karajacob et al., 2022).

Subsequently, *Debaryomyces*, formerly classified under *Candida*, is a yeast-like fungus commonly found in animal gut and is of promising potential for probiotic treatment (Angulo et al., 2020). However, although infection caused by the fungus is rare, infections caused by the genus, especially *D. hansenii* (or also referred to as *C. famata*) should not be taken lightly as all cases were reported from immunocompetent patients that eventually required haemodialysis due to chronic renal failure (Karapetsa et al., 2019). Next, *Neocucurbitaria*, especially *N. unguis-hominis* and *N. keratinophila* have been reported to cause toe-nail infection and keratitis respectively in the immunocompetent community (Sáenz-Madrado et al., 2022; Valenzuela-Lopez et al., 2019). Last but not least, *Talaromyces*, is the teleomorph stage of *Penicillium*, especially *T. marneffei* is an opportunistic fungus prevalent in Southeast Asia, causing infection in the immunocompromised caused by HIV or existing co-morbidities, with sporadic cases in the immunocompetent (Kawila & Chaiwarith, 2013; Larsson et al., 2012). Reported symptoms with the infection includes skin lesions, bone or joint pain, oedema, fever and cough (Wei et al., 2021).

Interestingly, *Cryptococcus*, a genus so often associated with pigeon were not detected in this study. In general, the genus is prevalent in pigeon stool worldwide (refer to Section 2.3.1). The absence of the fungus in the samples included in this study is not understood, as *Cryptococcus* has been isolated from Malaysia previously (Tay et al., 2005). However, as Sarawak is on the island of Borneo, geographics may have played a role in its absence. Nevertheless, its counterpart, *Naganishia* was detected in this study. Previously classified as *Cryptococcus*, *Naganishia* sp. (*N. albidus*, *N. liquefaciens*, *N. uzbeiztaniensis*) are opportunistic in pigeon stool, reported to cause central venous catheter-related fungemia, meningitis and atopic dermatitis in the immunocompromised (Morales-López & Garcia-Effron, 2021).

5.4 Limitations of the study

Firstly, due to the lack of isolation method for this study, any test that requires isolates such as biochemical test, antimicrobial resistance testing or multi-locus strain typing was not conducted. This further limits the knowledge on whether the bacteria or fungi identified in the samples were viable or not.

Secondly, although amplification of the short V3 – V4 16S bacterial region and the ITS2 fungal region has been proven to provide sufficient taxonomic information for classification and is able to detect rare genera in stool samples, classification may be inaccurate up to species level (Chen et al., 2019; Yang et al., 2018). Additionally, the use of short-read metagenomics limits the ability to predict functional genes and the presence of antimicrobial resistance in the samples.

Thirdly, the small size of six samples may have affected the detection of common pigeon-related bacteria and fungi not identified in the study and may have skewed the alpha and beta diversity analysis.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

With the utilisation of high-throughput amplicon sequencing and bioinformatic analyses, the study provided insight into the bacterial and fungal microbiome of fresh and dry pigeon stool in Sarawak, Malaysian Borneo. Additionally, the inclusion of the culture-independent method allowed the identification of four and six novel bacterial and fungal genera respectively. Thus, presenting the efficacy of utilising such technologies for future studies.

It was found that the bacterial diversity between fresh and dry pigeon stool is similar to each other, with differences in the abundance of bacterial genera between the sample types playing an important role in the dominance of certain phylum. Additionally, this study is the first to characterise the dry pigeon stool bacterial diversity, and found that genera especially those from the Enterobacteriaceae family in DPS, is able to pass through the GI tract and proliferate in the excreted pigeon stool when exposed to suitable conditions for growth. With this, it can be deduced that dry pigeon stool is the main contributor to highly abundant *Escherichia-Shigella* and *Enterococcus* in pigeon dust, as per reported by other researchers.

On the other hand, the fungal diversity of fresh and dry pigeon stool was significantly different but similar in composition. In general, FPS was dominated by a common gut fungus genus, *Kazachstania*. The diverse fungal genera detected in dry pigeon stool may be contributed by environmental fungi utilising nitrogenous content as their growth medium.

Nevertheless, the absence of certain genera such as *Chlamydia* and *Cryptococcus* may be attributed to the health status of pigeons and geographics of Sarawak.

6.2 Recommendations

To understand the true difference of alpha and beta diversity between the FPS and DPS, increasing the sample size should be considered to achieve greater statistical power and improve the statistical reliability of diversity metrics. Apart from that, opting to amplify the whole 16S and ITS rRNA gene using third-generation sequencing technologies should be considered to enhance the detection of rare taxa, provide precise taxonomic classification up to species level and enable microbial function and antimicrobial resistance prediction.

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APPENDICES

Appendix A

Table 1: Coordinates of sampling sites for collection of fresh and dry pigeon stool samples across Sarawak.

Town	Sampling site	Coordinate	DMS latitude
Kuching	Waterfront	1.558595, 110.342554	1 33' 30.9'' N, 110 20' 33.2'' E
	Satok bridge	1.554226, 110.325906	1 33' 15.2'' N, 110 19' 33.3'' E
Kota Samarahan	PeTARY, UNIMAS	1.464007, 110.426890	1 27' 50.8392'' N, 110 25' 37.1568'' E
Bintulu	Tumbina park	3.204878, 113.047439	3 12' 17.6''N, 113 02' 50.8'' E
Sibu	Rejang Park	2.307107, 111.836919	2 18' 25.6''N, 111 50' 12.9'' E
Miri	Centre Point Sunday Market	4.392388, 113.985611	4 23' 32.6''N, 113 59' 08.2'' E

Appendix B

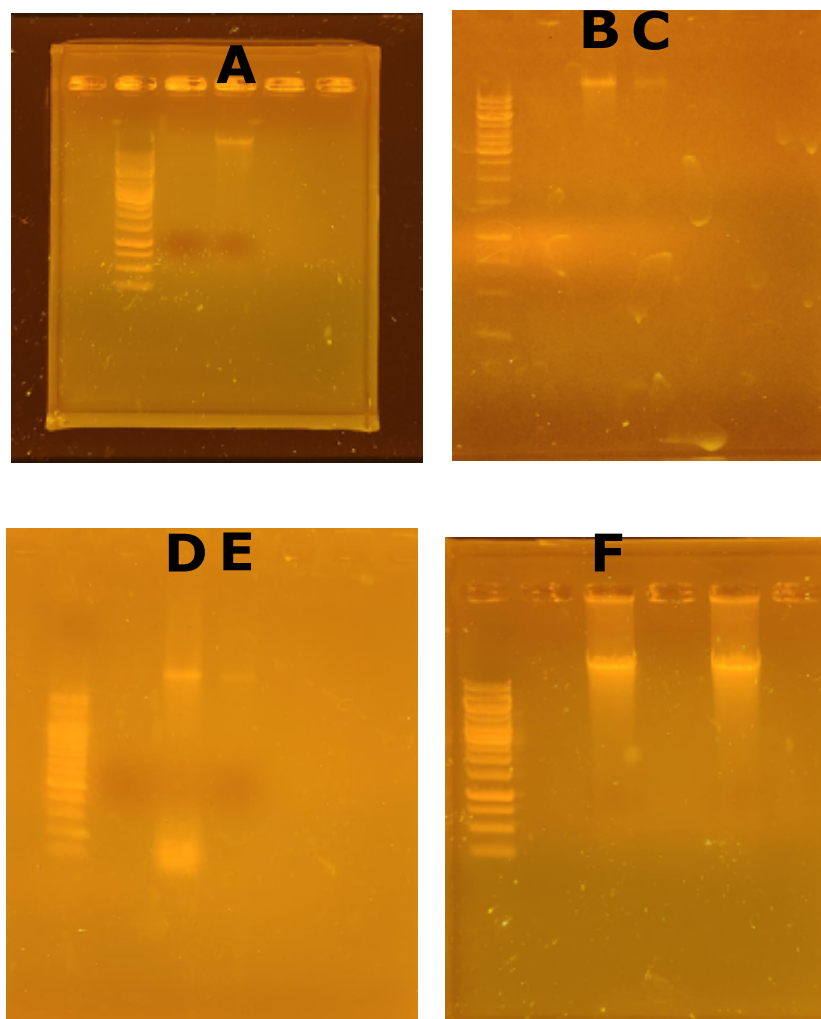


Figure 1: Genomic DNA (gDNA) of pigeon stool samples extracted using 2% CTAB buffer. All gDNA labelled were intact. gDNA visualisation was done on 0.8 1X TBE agarose. A: S2FP; B: K3DP; C: K2FP; D: B1DP; E: K3FP; F: KS1DP.

Appendix C

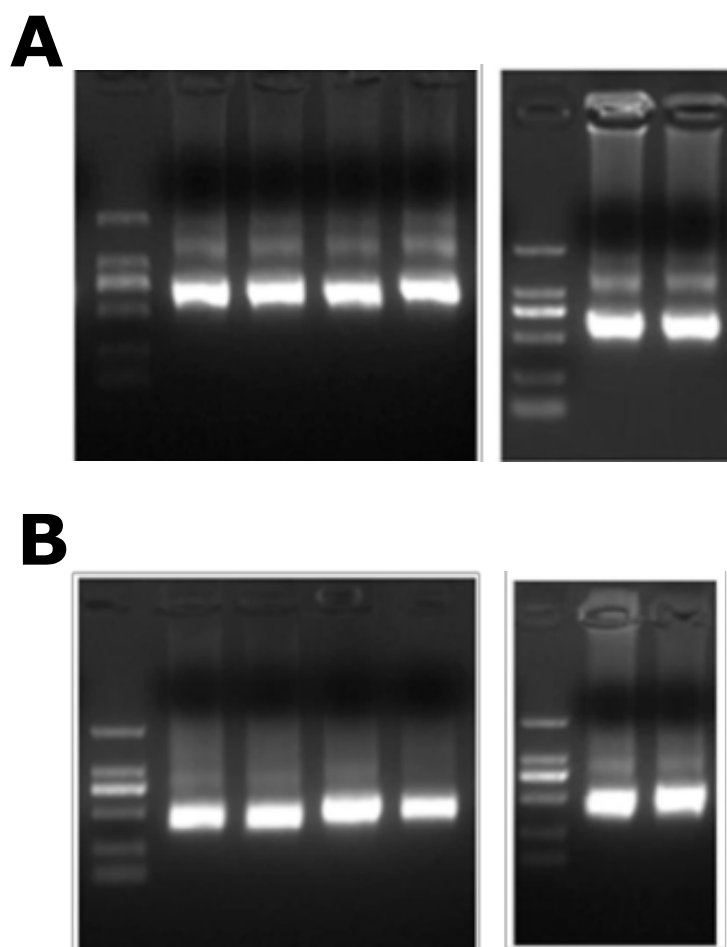


Figure 2: Polymerase chain reaction (PCR) amplicons of the V3 – V4 16S rRNA region and ITS2 rRNA region. Lane 1: S2FP; Lane 2: K3DP; Lane 3: K2FP; Lane 4: B1DP; Lane 5: K3FP; Lane 6: KS1DP.

Appendix D

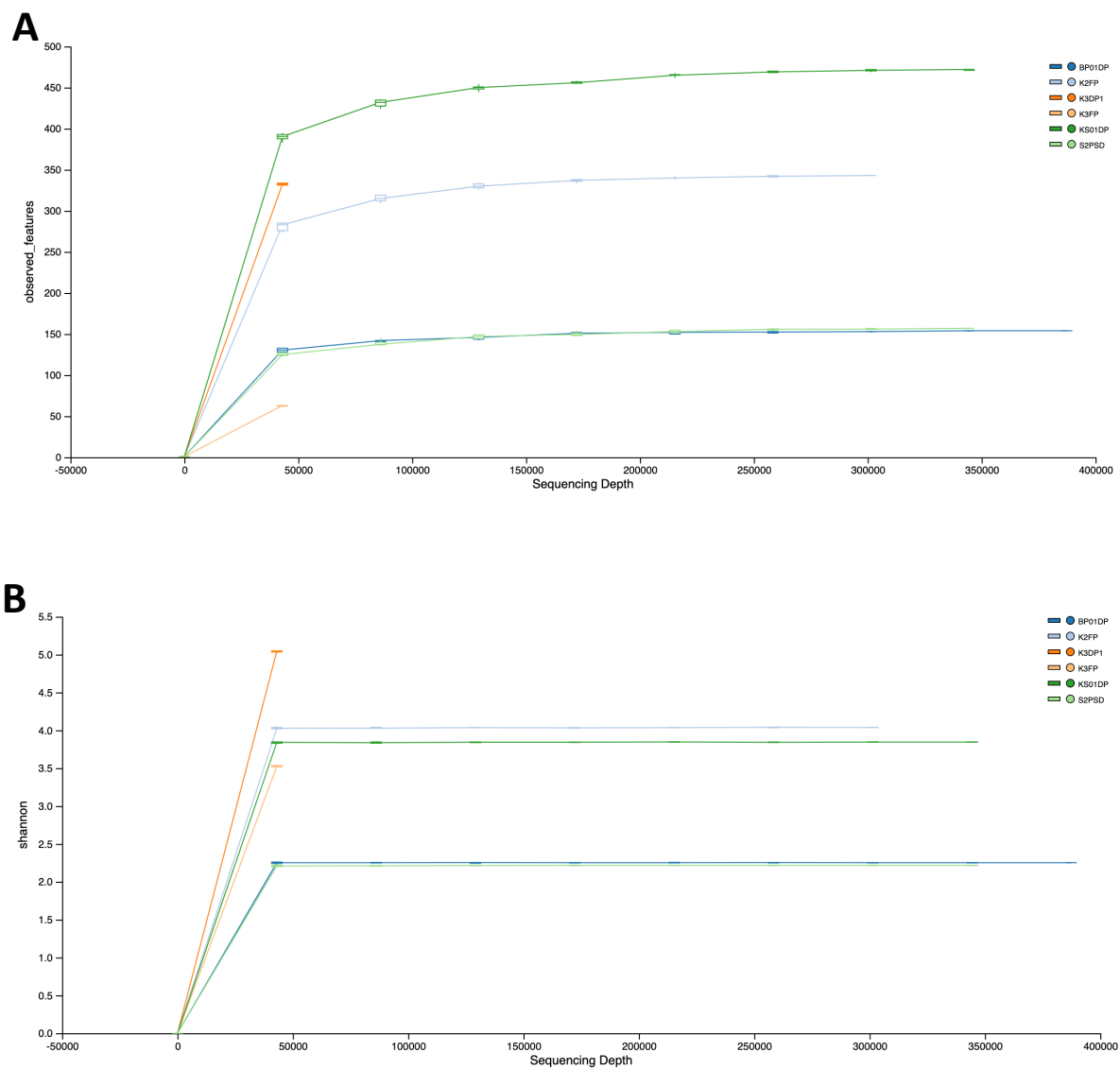


Figure 3: Bacterial A) alpha rarefaction and B) Shannon-index curve of FPS and DPS samples. FPS samples: K2FP, K3FP and S2FP; DPS samples, B1DP, K3DP and KS1DP.

Appendix E

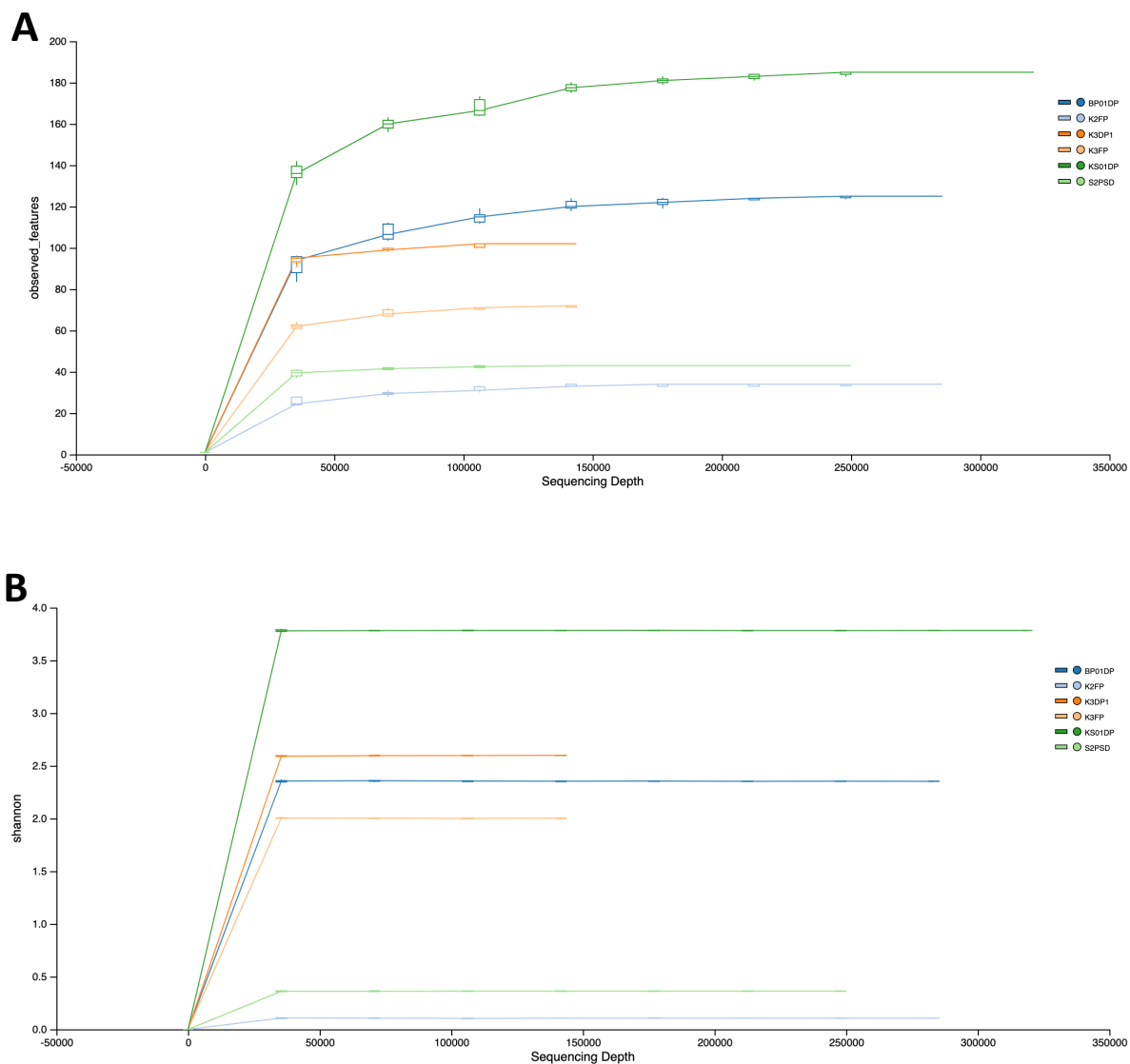


Figure 4: Fungal A) alpha rarefaction and B) Shannon-index curve of FPS and DPS samples. FPS samples: K2FP, K3FP and S2FP; DPS samples, B1DP, K3DP and KS1DP.

Appendix F

Your Ref. : 2021029
Our Ref. : SFC.810-4/6/1 (2021) – 056

Date : 19 November 2021

Dr. Tan Cheng Siang
Faculty of Medicine and Health Sciences
Universiti Malaysia Sarawak
94300 Kota Samarahan
Sarawak, Malaysia

SARAWAK FORESTRY CORPORATION
PARKS & WILD LIFE

Dear Sir/Madam,

PERMISSION TO CONDUCT RESEARCH ON BIOLOGICAL RESOURCES – PERMIT
SFC.810-4/6/1 (2021) – 55

Greetings from SFC!

The application for the above subject matter refers.

2. Enclosed herewith is the permission and approval for you and your team member to conduct research in Sarawak. This approval letter is to be returned to the Controller upon expiry of its validity period.

3. Members of your research group are also required to liaise and report to Sarawak Forestry Corporation (SFC) or Park Warden upon arrival at your research sites (Parks) to ensure that the National Parks and Nature Reserves Ordinance 1998 and Wild Life Protection Ordinance, 1998 are complied with accordingly.

Thank you.

I CARE

Zolkipli Bin Mohamad Aton

ZOLKIPLI BIN MOHAMAD ATON
Controller of Wild Life and
National Parks & Nature Reserves
SARAWAK
nr/

"NEW FRONTIER IN BIODIVERSITY CONSERVATION"

SARAWAK FORESTRY Corporation
Lot 218, KCLD, Jalan Tapang, Kota Sentosa, 93250 Kuching
Tel: (+6) 082 610088 Fax: (+6) 082 629744 www.sarawakforestry.com

Figure 5: Permit approval by the Sarawak Forestry Corporation to conduct research on biological resources.

Appendix G

Deputy Vice Chancellor's Office (Research and Innovation) Animal Ethics Committee (AEC) Tel: 082 581222/23 Fax: 082 665115	UNIVERSITI MALAYSIA SARAWAK 94300 Kota Samarahan
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MEMORANDUM

Reference : UNIMAS/TNC(PI)/09 – 65/03 (5)

To : Associate Professor Dr. Tan Cheng Siang
Faculty of Medicine & Health Sciences

From : Chairman
Animal Ethics Committee (AEC)

Date : 10 June 2022

Subject : **Ethical Approval for Research**

The Animal Ethics Committee (AEC) of Universiti Malaysia Sarawak (UNIMAS) has **approved** this study via email circulation between 14 May 2022 – 31 May 2022. The details of the research are as below:

Project : Molecular Epidemiology and Surveillance of Zoonotic Pathogen in Rock Pigeon (Columba Livia) and Other Species in Sarawak

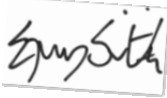
UNIMAS AEC Ref. No : UNIMAS/AEC/R/F07/073

Principal Investigator : Associate Professor Dr. Tan Cheng Siang
(Universiti Malaysia Sarawak)

Co-investigator : Ms. Vaenessa Anak Noni @ Boni
(Universiti Malaysia Sarawak)

Thank you.

Yours sincerely,



Prof Dr Edmund Sim Ui Hang

c.c : Deputy Vice Chancellor (Research & Innovation)
Director, UNIMAS Publisher

Figure 6: Ethics approval for animal-related research given by the Animal Ethics Committee of Universiti Malaysia Sarawak.