METAGENOMIC INSIGHTS INTO FOODBORNE PATHOGENIC BACTERIAL COMMUNITY AND THEIR ANTIBIOTIC RESISTANCE GENES IN FOOD SAMPLES FROM KINTA VALLEY, PERAK

By

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ABSTRACT

METAGENOMIC INSIGHTS INTO FOODBORNE PATHOGENIC BACTERIAL COMMUNITY AND THEIR ANTIBIOTIC RESISTANCE GENES IN FOOD SAMPLES FROM KINTA VALLEY, PERAK

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Foodborne diseases represent an increasing worldwide threat, primarily originating from contamination of bacteria. Despite of a rising prevalence rate in Malaysia, there exists a significant lack of thorough investigations employing metagenomic methodologies to examine foodborne pathogens. This study aimed to determine the feasibility of using shotgun metagenomics to investigate the bacterial community and potential presence of foodborne pathogens, as well as to characterise antibiotic resistance genes (ARGs) present in foodborne pathogens found in food samples, including meats, fruits and vegetables. Shotgun metagenomics revealed the highest DNA sequence reads in vegetables, followed by meats and fruits. Metagenomics analysis of the mock community consisting of *Escherichia coli* ATCC BAA-197, *Campylobacter jejuni* ATCC 33560, *Shigella flexneri* ATCC 29903, *Salmonella enterica* subspecies *enterica* serovar Typhimurium ATCC 700408 and *Shigella flexneri* ATCC 29903 and *Listeria monocytogenes* ATCC 19115 showed that *L. monocytogenes* was undetectable in vegetable and meat samples. ARGs from the mock community such as *aac(3)-lle*, *sul1*, *sco-1*, *floR* and *aph(3')-la* were detected in three spiked samples. Dominant bacterial phyla of Pseudomonadota occurred

across all 27 samples except for deli meat sample collected from Gopeng. Vegetable samples showed high presence of *Proteobacterium*, *Pseudomonas*, *Pectobacterium*, and lactic acid bacteria. Meat samples contained spoilage and pathogenic bacteria such as *Aeromonas*, *Pseudomonas*, and *Lactococcus*. Fruits had *Leuconostoc* and *Enterobacter*. The prevalence of pathogens found in food items varied between locations due to factors like hygiene practices, retail environments, and transport distances. Metagenomics detected pathogens in samples, though some pathogens were not detected when compared to culture methods. Cephalosporin and penam resistance genes were frequent, while deli meat and some fruits showed a lower presence of ARGs compared to vegetables. This suggests that the deli meats underwent processing and that the curing procedure could lower the survival chances of these AMR pathogens while fruits have protective skin or peel. This study demonstrated metagenomics' utility in pathogen detection, microbial profiling, and ARGs prediction, highlighting its potential in enhancing food safety.

Keywords: foodborne pathogens; metagenomics; food safety; pathogen detection; antimicrobial resistance genes

Subject area: QR75-99.5 Bacteria

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LIST OF ABBREVIATIONS

×g	Time gravity
°C	Degree Celsius
AMR	Antimicrobial resistance
ARG	Antimicrobial resistance gene
bacA	Bacitracin resistance protein
BFC	Blood free *Campylobacter* selectivity
bp	Base pair
BLAST	Basic Local Alignment Search Tool
BLEB	Buffered *Listeria* enrichment broth
BPW	Buffered peptone water
CARD	Comprehensive Antibiotic Resistance Database
CFU	Colony forming units
DNA	Deoxyribonucleic acid
EC	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
EMB	Eosin methylene blue
gDNA	Genomic deoxyribonucleic acid
IPB	Illumina purification beads
ITS	Intergenic transcribed spacer
kb	Kilobase

KMA	K-mer alignment
MAC	MacConkey
MALDI	Matrix Assisted Laser Desorption/Ionization
MIC	Minimum inhibitory concentration
NCBI	National Centre for Biotechnology Information
NGS	Next generation sequencing
OD	Optical density
PALCAM	Polymyxin acriflavin lithium-chloride ceftazidime esculin mannitol
PCR	Polymerase chain reaction
QC	Quality control
rRNA	Ribosomal ribonucleic acid
RVS	Rappaport-vassiliadis soy
spp.	Species
STEC	Shiga toxin-producing *Escherichia coli*
TBE	Tris-Borate-Ethylenediaminetetraacetic acid
TSA	Tryptone soya agar
TSB	Tryptone soya broth
VBNC	Viable but non-culturable
WGS	Whole genome sequencing
XLD	Xylose lysine deoxycholate

CHAPTER 1

INTRODUCTION

Foodborne diseases are a major public health issue affecting an estimation of 600 million people worldwide annually (WHO, 2023). Vegetables, fruits and poultry are the major source of outbreaks in the United States. Over the decade, there are numerous foodborne outbreaks associated with *Salmonella* species, *Listeria monocytogenes*, *Campylobacter jejuni*, *Shigella* species and *Escherichia coli*. These pathogens are among the most encountered foodborne pathogens (Azimirad et al., 2021; CDC, 2023b). Most of the vegetables and fruits are consumed raw and minimally processed, which have higher risk of contamination by foodborne pathogens including antibiotic-resistant bacteria (Azimirad et al., 2021; Rahman et al., 2022).

In Malaysia, the presence of Shiga toxin producing *E. coli* (STEC), *L. monocytogenes*, *C. jejuni*, and *Salmonella* spp. were detected in various food items (Kuan et al., 2017b; Saw et al., 2020; Haslinda et al., 2022). Although there is limited information regarding *Shigella* spp. in vegetables from Malaysia, the pathogen was detected in fresh produces in West Java, Indonesia (Hassan and Purwani, 2016). Foodborne pathogens gain access to the human body through the gastrointestinal tract when contaminated or undercooked food, or contaminated

water, is consumed. Therefore, detecting foodborne pathogens in food is essential before it is consumed and to prevent serious foodborne outbreak (Priyanka, Patil and Dwarakanath, 2016). In 2022 alone, there were approximately 14,293 reported cases of food poisoning caused by food and waterborne diseases in Malaysia (Hilmy, 2024). Despite these figures, comprehensive local data on microbial safety, particularly concerning ready-to-eat foods and fresh produce, is limited.

Antibiotic resistance among pathogens is an important food safety issue due to the uncontrolled and excessive use of antibiotics in the clinical and agricultural field (Khalid et al., 2015; Rajaei et al., 2021). Environment such as animal waste, water, sludge, and soil have been identified as reservoirs for antibiotic resistance genes. Recently, studies have demonstrated that antibiotic-resistant bacteria can migrate into plants, thus spreading via the food chain and endangering human health (Cerqueira et al., 2019; Li et al., 2020b; Guo et al., 2021). Studies in Malaysia have shown that several foodborne pathogens isolated from vegetables were resistant to antibiotics. Antibiotic-resistant foodborne pathogens such as *E. coli*, *C. jejuni*, *L. monocytogenes* and *Salmonella* spp. were isolated. This indicates the emerging of antibiotic-resistant foodborne pathogens, which is alarming as infections caused by these pathogens have lower effectiveness of therapeutic treatments (Khalid et al., 2015; Abatcha, Effarizah and Rusul, 2018; Thung et al., 2020; Haslinda et al., 2022; Rahman et al., 2022).

The techniques for foodborne pathogen detection have been expanded over the years (Foddai and Grant, 2020). One of the oldest methods in detecting these pathogens is the culture-based approach, which remains as the gold standard. This method is economical, easy to conduct, sensitive and able to provide quantitative or qualitative information. However, the limitations in culture-based methods include a longer time needed to obtain results; more laborious protocols involving the isolation and culturing of bacteria, followed by complex molecular and biochemical procedures to confirm the pathogen of interest; and additional enrichment protocols to allow the amplification of foodborne pathogens. These reasoning are the main factors of the delay in obtaining results (Priyanka, Patil and Dwarakanath, 2016; Foddai and Grant, 2020). Nevertheless, culture procedures were proved to be more advantageous in obtaining definitive results when isolation is conducted on a positive sample (Ogunremi et al., 2020).

Metagenomic analysis is developed to address the shortcomings of culture-based methods, particularly the time required to obtain definitive results, laborious procedures and overgrowth of contaminant which dominant species overgrows and masks the presence of less abundant pathogenic bacteria causes false negative (Ogunremi et al., 2020). Metagenomics can perform rapid serotyping, multi-locus sequence typing, detection of virulence factors and antimicrobial resistance genes, along with identifying difficult-to-culture bacteria and non-culturable microorganisms (EFSA Panel on Biological Hazards (EFSA BIOHAZ Panel), et al., 2019; Nayfach et al., 2019). In addition, metagenomic data can be used to

reconstruct the genome for functional gene analysis and evolutionary history of foodborne pathogens. These are applicable for identifying antimicrobial resistance genes, tracing sources, and detecting foodborne outbreaks (Allard et al., 2018). Metagenomic approach is pathogen-agnostic, which allows detection and characterisation of foodborne pathogen without prior knowledge on the quantity of unique species present in the sample (Buytaers et al., 2020).

Over the years, advancement of sequencing technologies has reduced costs, promoting the rapid growth of metagenomics research in the detection of foodborne pathogens. However, the optimisation for the procedures of metagenomics remains a challenge to be overcome. Although there have been several studies of metagenomics in Malaysia, most of the research were focused on microbial diversity of the environment (Song et al., 2017; Azli et al., 2022; Hisham et al., 2022). Metagenomics analyses to profile the microbial community and identify foodborne pathogens and associated ARGs found in vegetables, meats and fruits remain scarce in Malaysia, even though it is becoming a key instrument for improving food safety management. Modern food safety is the comprehensive practices, technologies, and systems designed to maintain food as safe for human consumption.

Although foodborne diseases remain a persistent and significant public health issue in Malaysia, local data on microbial safety in fresh food items is limited. Conventional detection methods both time-intensive and laborious while

metagenomic approaches are underutilized in local food safety surveillance. Moreover, the increasing emergence of antibiotic-resistant foodborne pathogens adds to the urgency of effective monitoring. There is a need to explore and evaluate metagenomic sequencing as a feasible tool for pathogen and ARG detection in fresh produce, meats, and fruits commonly consumed by Malaysians.

Thus, this study aims to determine the feasibility of metagenomic shotgun sequencing in detecting foodborne pathogens from meats, vegetables, and fruits collected in Kinta Valley, Perak, Malaysia. To complement this approach, culture-complemented PCR was also performed independently on the same samples analysed via metagenomics, allowing a comparison between conventional and sequencing-based pathogen detection methods. By characterising the bacterial communities and detecting potential pathogens in food items, the study provides insights that can guide specific food safety measures and surveillance efforts in Malaysia. In summary, these are the specific objectives of our study:

i) To determine the feasibility of using shotgun metagenomics to detect foodborne pathogens introduced in a mock community in vegetable, meat and fruit.
ii) To investigate the microbial community and potential foodborne pathogens in randomly obtained vegetables, meats and fruits collected from Kinta Valley comprising of Kampar, Gopeng, and Ipoh areas.

iii) To characterise antibiotic resistance genes (ARGs) in foodborne pathogens found in collected vegetables, meats and fruits using shotgun metagenomics.

Kinta Valley was selected due to its accessibility and diversity in food sources, allowing for practical and representative sampling within the constraints of the study. The Kinta Valley is an ideal location for collecting food items for pathogen detection due to its rich agricultural diversity, high population density, centralised markets, and historical data on food safety (Jusoh et al., 2020). The region's diverse agricultural produce, from vegetables to dairy, combined with the high demand for food in its populous areas, increases the likelihood of encountering various pathogens. Centralised markets in the Kinta Valley aggregate food items from different sources, providing a comprehensive sampling ground (Kaur, 2023). Historical studies on food safety in this region offer a valuable baseline for new research (Asrin and Ismail, 2016). Additionally, the climate and environmental conditions can influence pathogen prevalence, making it a significant area for study. The region's economic significance in agriculture and accessible infrastructure further enhances its suitability for food safety research (Zulkeffeli and Mui'zz Morhalim, 2022).

CHAPTER 2

LITERATURE REVIEW

2.1 Foodborne Illness

Foodborne illness results from consuming food contaminated with bacteria, fungi, viruses, parasites, or biotoxins, with bacteria being the most common cause (Bintsis, 2017; Occupational Safety and Health Administration, 2025). Ingesting food contaminated with pathogens can lead to foodborne infection, while toxins produced by pathogens in food can cause foodborne intoxication. These illnesses are predominantly gastrointestinal and can result in both localised and systemic infections. (Stavropoulou and Bezirtzoglou, 2019). Severe cases of foodborne illness primarily affect the elderly, young children, immunocompromised individuals and healthy individuals exposed to high dose of the pathogen (Bintsis, 2017).

Across the United States, the recorded number of multistate foodborne outbreaks for all food items from 2006 to 2023 were 201. Vegetables had the largest share of outbreaks with 51 cases, followed by poultry at 44 and fruits at 26. The highest number of foodborne outbreaks in the European Union (EU) and USA were caused by *Salmonella* (CDC, 2023b; European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC), 2023). Bacteria were

the cause of most outbreaks in EU, which resulting in the highest hospitalisations and mortality rates. Viruses and parasites were responsible for 353 and 25 outbreaks, respectively. The viral agent that caused the most outbreaks were Norovirus, meanwhile *Cryptosporidium* contributed the most outbreaks in the parasite category (European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC), 2023).

Foodborne illness cases in Malaysia have risen significantly in previous decades. During the 1980s, the incidence remained under 10.2 cases per 100,000 population, but by 1999, it had increased to over 35.0 cases per 100,000. By 2010, the incidence had continued to climb, reaching above 44.0 cases per 100,000 population. This trend is likely influenced by underreporting in the 1980s and 1990s. (Hassan et al., 2023). By 2022, the reported incidence of food poisoning cases in Malaysia was 43.77 per 100,000 population. Typhoid and paratyphoid had an incidence rate of 0.28 per 100,000, with a mortality rate of 0.01. These increases in 2023 with incidence rate of 53.67 food poisoning cases, followed by 0.35 for typhoid and paratyphoid. Meanwhile, dysentery recorded an incidence rate of 0.78, while cholera was reported at 0.02 in 2022 with an increase to 1.54 and 0.14 in 2023 (Ministry of Health Malaysia, 2023; 2025).

The recent trend of consuming raw or minimally cooked fresh vegetables has also heightened the risk of foodborne illness.(Chávez-Ruvalcaba et al., 2021). Most pathogens on food are destroyed during the cooking process; however, spores

of certain pathogenic bacteria may survive. Additionally, pathogens can contaminate ready-to-eat foods through cross-contamination, where they may grow and lead to foodborne illness (Deepa, 2020). The presence of a pathogen in food does not necessarily mean it will cause diseases; a sufficient quantity of the pathogen is required to establish an infection. However, pathogens must survive in the environment and overcome the body's immune defence. There are limited pathogens that can evade host immunity by using mechanisms such as biofilm formation and producing enzymes and toxins that help them survive in harsh environments (Chávez-Ruvalcaba et al., 2021; Musa Moi et al., 2023).

2.2 Foodborne Pathogens

In Malaysia, *Salmonella* contamination has been detected in a range of foods, such as vegetables, beef, chicken and vegetarian burger patties from multiple locations (Nillian et al., 2011; Ain Auzureen et al., 2017; Shafini et al., 2017; Saw et al., 2020; Bahri et al., 2022). *L. monocytogenes* has been found in foods such as frozen burger patties, chicken, vegetables, fruit salads, beef, and offal (Goh et al., 2012; Wong et al., 2012; Jamali, Chai and Thong, 2013; Kuan et al., 2013). Additionally, pathogenic *E. coli* has been found in beef, vegetables, beverages, sausages, fish, pork and chicken, with some samples testing positive for the O157:H7 strain and *Shiga*-toxin producing bacteria (Ain Auzureen et al., 2017; Kuan et al., 2017b; Premarathne et al., 2017b; Nawawee, Bakar and Zulfakar, 2019; Latchumaya et al., 2021; Bahri et al., 2022). Research on *Campylobacter* in Malaysia has revealed

contamination in raw vegetables and chicken (Chai et al., 2007; Sinulingga et al., 2020). Although *Shigella* prevalence has been rarely studied in Malaysia, it has been isolated from vegetables in West Java, Indonesia (Hassan and Purwani, 2016). Studies regarding foodborne pathogens and summary of their findings were tabulated in **Table 2.1**.

Microorganisms that grown in our food is determined by various factors, such as storage condition, type of food, processing methods and microbial contamination. However, the primary sources of microorganism's contamination in our food are zoonotic pathogens from animals which may contaminate soil, water, air, plants, food equipment and food handlers. The soil contains a lot of microorganisms and may contaminate the plants growing on it or animals roaming over it (Alum, Urom and Ben, 2016; Deepa, 2020). Natural water contains waterborne microorganisms coming from natural flora or sewage, soil, animals and even human faeces. Microorganisms found in the air are predominantly bacterial and fungal spores; the numbers of these microorganisms are higher in dry air compared to moist air. Plants contain its own natural flora, but it may be exposed to human pathogens from water, sewage, air, soil and animals (Deepa, 2020). Animal contains many microorganisms residing in the gastrointestinal tract, respiratory tract, and skin surface. Like plants, they can also be contaminated by microorganisms from other sources such as soil, water and air. Animal faeces contain many pathogenic organisms which can contaminate the soil and water. Lastly, microorganisms from handlers may contaminate food products due to poor

improper cleanliness practices, for example not washing hands properly during preparation of food. One of the factors that cause contamination from food equipment are due to cross contamination (Deepa, 2020; Erdoğan and Pamuk, 2020).

Table 2.1: Studies on various foodborne pathogens and their findings.

Findings	Sample Type	Reference
Prevalence of *Salmonella* was 28%, *S.* Enteritidis was 20%, *S.* Typhimurium was 14.3%.	Vegetarian burger patties, tomato, capsicum, cabbage, lettuce, cucumber, carrot	Nillian et al. (2011)
Prevalence of *E. coli* was 23.9% (beef) and 12.9% (chicken). *Salmonella* was 13% (beef) and 9.3% chicken.	Cooked beef products, cooked chicken products	Ain Auzureen et al. (2017)
Prevalence of *Salmonella* was 40.4% (chicken) and 15.4% (beef). Samples from wet market have the highest prevalence of 35.4%, followed by supermarket (26.9%), and butcher shop (21.3%).	Raw chicken meat, raw beef meat	Shafini et al. (2017)

Prevalence of *Salmonella* was 3.3% (cabbage from hypermarket), S. Typhimurium was 10% (cucumber from hypermarket) and 20% (cucumber from wet market).	Cabbages, tomatoes, carrots, lettuce, cucumbers	Saw et al. (2020)
Prevalence of *E. coli* was 3.13% and *Salmonella* was 9.4%.	Indian pennywort, wild parsley, Japanese parsley, bean sprout, winged bean, long bean.	Bahri et al. (2022)
Prevalence of *L. monocytogenes* was 20%.	Chicken meat	Goh et al. (2012)
Prevalence of *L. monocytogenes* was 33.3% in chicken, 22.9% in beef and 10.5% in fish patty.	Raw burger patty (chicken, beef, fish)	Wong et al. (2012)
Prevalence of *Listeria* was 17.9% and *L. monocytogenes* was 11.4%. *L. monocytogenes* in salads and vegetables was 14.7%.	Orange flavoured drink, chicken products, egg products, beef products, seafood products, salads, vegetables, packed lunch	Jamali, Chai and Thong (2013)

Occurence of *L. monocytogenes* were 22.73%, 37.50%, and 41.18% from three different wet markets. *L. monocytogenes* was tested positive in 33.33% of samples.	Beef offal	Kuan et al. (2013)
Prevalence of foodborne pathogen STEC was 0.7%. Prevalence of *Salmonella* and *S. Enteritidis* were 2% and 0.7% respectively. *Listeria* and *L. monocytogenes* had 9.2% and 5.9% of prevalence respectively.	Cabbage, carrot, calamondin, cherry tomato, bird's eye chili, cucumber, eggplant, winged bean, Romaine lettuce, Iceberg lettuce, Looseleaf lettuce, Butterhead lettuce, sweet potato, tomato, white radish	Kuan et al. (2017b)
The highest occurence of *E. coli* O157:H7 was from wet markets (88.5%), followed by hypermarket A (35.4%) and hypermarket B (20%).	Raw beef	Premarathne et al. (2017b)

Prevalence of *E. coli* is 3.2% and was only detected in milk-based drinks. Meanwhile prevalence of *Staphylococcus aureus* was 58.1%.	Cordial-based drinks, milk-based drinks, fruit juices	Nawawee, Bakar and Zulfakar (2019)
Prevalence of *E. coli* was 58% with no detection of O157:H7 and STEC.	Cooked hot dogs, fish, squid gravies, smoked pork products	Latchumaya et al. (2021)
Prevalence of *Campylobacter* was 67.7%, which was the same as *C. jejuni*. *C. coli* had a lower prevalence of 65.7% and *C. fetus* was not detected at all.	Water spinach, yard long bean, winged bean, mung bean sprout, Vietnamese coriander, Japanese parsley, Indian pennywort, Wild cosmos	Chai et al. (2007)
Prevalence of *Campylobacter* was 50.9%. *C. jejuni* was the most frequently identified isolate, followed by *C. coli*, *C. fetus*, and *C. upsaliensis*.	Raw chicken meat	Sinulingga et al. (2020)

The incidence level of *E. coli* was 34%, followed by *Bacillus cereus* at 16%, *Shigella* at 13.3% and *Salmonella* at 10%.	Thai lemon basil, lettuce, tespong leaves, caisim leaves, leaf lettuce, water spinach, spinach, Chinese kale, zucchini/ courgette, Chinese cabbage, yard long bean, handelin leaves, local cucumber, brussels sprouts, white cabbage, tomato, black nightshade, eggplant, cayenne pepper	Hassan and Purwani (2016)

2.2.1 Clinical Impact of Foodborne Pathogens

Pathogenic *E. coli* strains are regularly linked to severe foodborne diseases Although the number of cases is lower compared to other pathogens, there are more recorded hospitalisations and fatalities. Infection by these bacterial strains can cause enterocolitis, gastroenteritis, bloody diarrhoea and even haemolytic-uraemic syndrome (European Centre for Disease Prevention and Control, 2022; Gambushe, Zishiri and El Zowalaty, 2022). According to the European Centre for Disease Prevention and Control (2022), there were a total of 6,534 cases of STEC infection reported by 30 countries in the European region. Germany reported 1,635 cases followed by Ireland with 878 cases, making these the two countries with the highest

numbers in Europe. Among the reported cases, 1,056 required hospitalisations, and the fatality rate was 0.4%. In 2021, there was 36 reported outbreaks across 11 countries in Europe, making STEC the third most frequently detected pathogen in outbreaks.

Infections caused by *L. monocytogenes*, known as listeriosis, present as a mild disease with an incidence rate of 0.3 per 100,000 people. However, 92% of patients with listeriosis require hospitalisation, and fatality rates can reach as high as 16%. The risk of fatality increases in pregnant women, immunocompromised individuals, young children and the elderly (Chen et al., 2017; Jibo et al., 2022; CDC, 2023c). Research indicates that Malaysia reports the highest prevalence of *L. monocytogenes* at 25%, compared to Singapore, Thailand and Indonesia. However, this prevalence has decreased over the past decade (Jibo et al., 2022). Outbreaks related to *L. monocytogenes* are commonly linked to vegetables. In 2020, enoki mushrooms in South Korea were contaminated by the pathogen, causing 31 hospitalisations and four fatalities. In 2021, two outbreaks involving two different brands of packaged salads resulted in 23 hospitalisations and three fatalities in the United States (Gartley et al., 2022).

The Gram-negative bacterium *Shigella* has a low infectious dose and causes an infection known as shigellosis. In 2016, the global incidence rate of shigellosis was estimated at 0.4 per 100,000 people, with a fatality rate of 2.9 per 100,000. In Asia, studies observed that China had the highest prevalence, with 8,135 cases,

followed by Bangladesh with 3,325 cases and Iran with 1,914 cases (Khalil et al., 2018; Salleh et al., 2022). In Japan, an outbreak associated with a kindergarten resulted in 19 confirmed cases, with the most reported symptoms being fever, diarrhoea and abdominal pain. However, the source of infection could not be determined (Kato et al., 2020).

Campylobacteriosis is an illness caused by *Campylobacter* and is usually self-limiting. However, in certain instances, complications including Guillain-Barre syndrome, reactive arthritis, Miller-Fisher syndrome and both respiratory and gastrointestinal infections may occur (Mughal, 2018). In 2016, South Korea reported 18 outbreaks of *Campylobacter*, resulting in 902 cases and contributing to the highest prevalence in Asia. Studies have found that campylobacteriosis incidence was highest in the Czech Republic compared to other countries, while Poland had a low occurrence yet elevated hospitalisation rates of 76.6% (Liu et al., 2022a).

Typhoid is an infection due to the bacterium *Salmonella enterica* serovar Typhi, leading to symptoms such as fever, headache, diarrhoea, malaise, and myalgias. Globally, typhoid fever is estimated to cause 11 to 21 million cases and 200,000 fatalities annually (Muhammad et al., 2020; CDC, 2023a). In Malaysia, there was a rise in typhoid cases in Klang Valley between 2011 and 2015. There was a saw its highest level in 2015 with 98 cases. However, the incidence rate remains low compared to other Asian countries (Muhammad et al., 2020).

2.2.2 Detection of Foodborne Pathogens

Foodborne pathogen detection has its challenges, despite the various methods available for detecting and identifying them. These challenges include interference from other non-target microbes, difficulties in microbial extraction from food matrices, and low concentration of the target organism. Despite the available technology and methods, culture-based methods represent the earliest approach for detection. To this day, it remains the reference method for detecting foodborne bacterial pathogens. This approach is divided into two phases, namely selective enrichment and pre-enrichment. The pre-enrichment stage aids in recovering injured cells and enhancing the concentration of the target organism within the sample. Meanwhile the selective enrichment improves the concentration of a specific pathogen in the food sample. This method of detecting foodborne pathogen is distinctive and selective, as it impedes the growth of unnecessary microbes (Brehm-Stecher et al., 2009; Aladhadh, 2023). Culture-based methods excel once the growth conditions of the target microorganism are established, the culture medium can be tailored to enrich, selectively isolate, or distinguish it from others. Notably, culture-based methods are often coupled with PCR, immunoassays, biosensors and next-generation sequencing (NGS) to detect and identify foodborne pathogens. Fungal growth may require up to seven days of incubation before becoming visible on culture. Like bacteria, detection of foodborne fungi with culture-based methods is usually coupled with other methods. The advantages of the culture method do not stop there, as it is relatively cheap, ease to use, and able to isolate the microorganisms for downstream assays or applications (Kim and Kim,

2021; Patil-Joshi, Rangaswamy and Apte-Deshpande, 2021; Aladhadh, 2023; Kabiraz et al., 2023). This method has limitations, including low sensitivity since not all microorganisms are culturable, and certain bacteria may exist as viable but not culturable (VBNC), which could cause false negative. This method is also laborious and time-consuming, taking up to seven days for bacteria and longer for fungi (Law et al., 2015; Foddai and Grant, 2020; Aladhadh, 2023).

The alternative approach for foodborne pathogen detection is the immunological assays method, which comprises serotyping, lateral flow devices, immunofluorescence, and enzyme-linked sorbent assay (ELISA). It provides rapid and accurate detection of foodborne pathogens, especially bacteria and fungi, together with their biotoxins. The downside of the method is that it is expensive, may produce may yield false positives from cross-reactivity and fail to detect damaged cells (Aladhadh, 2023; Kabiraz et al., 2023). Molecular-based methods for identifying foodborne pathogens are another alternative. As opposed to culture approaches, molecular-based techniques are applicable to foodborne bacteria, fungi, parasites, and viruses. Polymerase chain reaction (PCR) and its variants are commonly used techniques that utilises nucleic acid amplification. The technique amplifies target pathogen genomes in food samples by replicating them with specific primer pairs. This method has a few advantages compared to culture methods due to its higher sensitivity, accuracy, rapidity, specificity, robustness, and minimal risk of contamination. However, there are also limitations to this method: it may not indicate if the target pathogen is viable or not, which requires timely

monitoring, effective surveillance, and sometimes food particles may inhibit the process (Aladhadh, 2023; Kabiraz et al., 2023).

2.3 Metagenomics

Metagenomics refers to the study of genetic material recovered from a particular environmental sample. It investigates the mixed community of microbial organisms directly from the environment by applying modern genomic techniques. These environments include the ocean, soil, animal gut, human gut, and rhizospheres of plants. Initially, studies focused on cloning environmental DNA, functional expression assays, combined with shotgun sequencing of environmental DNA. These studies uncovered vast diversity of functional genes within the microbial realm while proving the principle of the metagenomic approach (Thomas, Gilbert and Meyer, 2012; Mardanov, Kadnikov and Ravin, 2018; Liu et al., 2022).

Metagenomics is categorised into two groups: shotgun sequencing and amplicon sequencing. Amplicon sequencing amplifies marker genes such as 26S rRNA, 18S rRNA, 16S rRNA, and intergenic transcribed spacer (ITS) (De, 2019; Liu et al., 2021). Amplicon approach has a few advantages: it is more sensitive as it requires a lower initial colony-forming unit (CFU) sample concentration, has more established bioinformatics pipeline for data analysis, and is also more cost-effective. However, this approach detects lower species richness and is better at

classifying phylum to genus level (Andersen and Hoorfar, 2018; Ramazzotti and Bacci, 2018; Grützke et al., 2019; Zuo et al., 2022).

On the contrary, shotgun sequencing can overcome limitations of amplicon metagenomics by sequencing all DNA fragments. This approach allows researchers to reconstruct whole genomes to investigate gene structure and function. It demonstrates higher sensitivity, better taxonomic resolution, and lower bias because it is untargeted. However, the data is highly fragmented and consists of a vast range of noise and artifacts, which makes the reconstruction process difficult for less abundant microbial species and requires higher expertise to analyse the data (Berglund et al., 2019; De, 2019).

2.3.1 Limitation and Advantages of Shotgun Metagenomics

Shotgun metagenomics may have the potential for rapid detection, but there are some limitations. One drawback is its sensitivity and specificity in pathogen detection due to the misattribution of detected sequences (Andersen and Hoorfar, 2018). Sensitivity is an important limitation to overcome, especially for the detection of microorganisms. Shotgun metagenomic may have a less sensitive limit of detection, approximately 10^3 CFU/ml. However, it can provide taxonomic information from species to strain level (Leonard et al., 2015; Ottesen et al., 2016; Kim et al., 2018). Researchers have tried increasing the limit of detection by introducing an enrichment process. Research by Leonard, et al. (2015) managed to

increase detection limit to as sensitive as 10 CFU/100g of spinach (Leonard et al., 2015). Another study demonstrated that metagenomics could achieve a detection limit for *Salmonella* at 1 CFU/25g of lettuce by including DNA amplification after an enrichment process (Ogunremi et al., 2020). However, this enrichment process may also introduce bias toward the detection of easy-to-culture or fast-growing species (Leonard et al., 2015; EFSA BIOHAZ et al., 2019).

Moreover, it cannot reliably tell apart active and inactive organisms, as it detects DNA that can originate from both non-viable and viable cells. Although additional procedures can be carried out to overcome this limitation, it has been found that these processes may reduce detection sensitivity (Takahashi et al., 2018). However, the capacity to identify pathogens even when they are no longer viable makes it a useful tool for source-tracking investigations (EFSA BIOHAZ Panel et al., 2019). The sampling step in metagenomics research must be conducted with extra precautions to prevent cross-contamination. In addition, DNA extraction must be performed properly to reduce contamination from host DNA. Earlier research has established specialized protocols to extract DNA from various samples, such as soils, plant tissues, and human faeces (Ahmadi et al., 2018; Echeverría-Beirute et al., 2021; Tourlousse et al., 2021).

2.3.2 Metagenomics in Food Safety

Few years back, metagenomics has been used as a tool to characterise the structure of microbial communities in food samples. The direct detection of microorganisms without the time-consuming protocol of culturing bacterial species for detecting pathogens is a faster alternative (Leonard et al., 2015). Additionally, findings from metagenomics research in the food safety field have proven that this approach is feasible for foodborne pathogen detection. Consequently, researchers have begun using metagenomics to identify foodborne pathogens in several foods, such as ice cream, flour, spinach, and Chinese cabbage (Leonard et al., 2015; Ottesen et al., 2016; Kim et al., 2018; Pfefer et al., 2018). Studies have shown that shotgun sequencing can detect and characterise a variety of spiked pathogenic STEC strains in spinach samples (Leonard et al., 2016). Furthermore, it has been demonstrated that metagenomics is effective in detecting potential foodborne pathogens including *Pantoea*, *Klebsiella, Erwinia*, *Bacillus*, *Staphylococcus*, *Salmonella*, *Yersinia*, *Clostridium*, *Chryseobacterium*, *Sphingomonas*, and *Pseudomonas* in Chinese cabbages from South Korean farms (Kim et al., 2018).

Metagenomics has also provided information regarding the bacterial diversity and composition of vegetables. It was shown that spinach harboured a more varied bacterial community than that of rocket salad, with the most dominant bacterial orders being Enterobacteriales and Pseudomonadales, respectively (Tatsika et al., 2019a). As metagenomics can characterise antibiotic-resistant genes, results from a study show that the bacitracin resistance gene (*bacA)* was the highest

prevalence antibiotic-resistant gene type in ready-to-eat vegetables (Li et al., 2020b). In a study comparing two metagenomic approaches, it was found that shotgun sequencing is a preferred method for the detection of pathogens. This is because genus-level detection using a targeted approach may be inadequate if pathogenicity differs greatly among species of the same genus, such as *Listeria monocytogenes,* which causes listeriosis, and *L. welshimeri,* which is apathogenic (Grützke et al., 2019). Metagenomics was found to provide a similar level of information from an outbreak investigation compared to conventional methods, but the time frame was reduced by over a week (Buytaers et al., 2021).

2.4 Antimicrobial Resistance (AMR) in Foodborne Pathogens

Antimicrobial resistance (AMR) refers to the capacity of bacterial pathogens to withstand the of antibiotics. It occurs due to bacterial adaptation and evolution in response to antibiotic pressure. There are several mechanisms of AMR development, such as spontaneous genetic mutations or through horizontal gene transfer, allowing the exchange of resistance genes among organisms (Azad, 2024; Shahid et al., 2024). Microorganisms employ various strategies to resist antimicrobials, including altering drug target sites, using efflux pumps to expel drugs, modifying porins to prevent drug entry, and enzymatic degradation of drugs. Some bacteria are naturally resistant to certain antimicrobials, while others acquire resistance as a result of mutations or through the uptake of resistance genes from other bacteria (Naaz et al., 2023; Kaur et al., 2024).

2.4.1 The Prevalence Study in Malaysia

In recent years, Malaysia have been receiving increasing reported cases of clinical isolates with antimicrobial resistance (AMR) from food poisoning patients. Patients infected with these antibiotic-resistant pathogens may have higher mortality rates and longer illness duration (Cheesman, Hawala and Cock, 2023). In addition, asymptomatic food handlers from different cities in Malaysia have been found to have different *Salmonella* serovars in their stools, majority of the isolates were resistance to several antibiotic classes (Woh et al., 2017). *Campylobacter* spp. was found to contaminate beef and chicken in Malaysia. Some strains were identified to be resistant to cephalothin, tetracycline, enrofloxacin, gentamicin, ampicillin and some were resistant to three or even four or more antibiotics (Mansouri-najand, Saleha and Soe, 2012; Premarathne et al., 2017a; Sinulingga et al., 2020). *Shigella* spp. strains were isolated from different parts of Malaysia and found to be multi-drug resistant. Among these strains, more than 70% of *Shigella flexneri* showed resistance to a minimum of two antimicrobial agents (Thong et al., 2002).

Throughout Malaysia, pathogenic *Escherichia coli* strains were detected in various foods such as chicken, beef, buffalo, lamb and milk. Some strains have displayed resistance to more than three antibiotics; there were strains that were resistant towards 15 different antibiotics (Sahilah et al., 2010; Chang et al., 2013; Lye et al., 2013). Alternatively, the presence of antimicrobial resistant *Listeria* spp. was found in vegetables and chicken products. The the isolates exhibited resistance to penicillin G, rifampicin, erythromycin, vancomycin, chloramphenicol,

ampicillin, ceftriaxone, gentamicin, tetracycline, ciprofloxacin, and meropenem (Kuan et al., 2017a; Chin et al., 2018).

2.4.2 The Transmission of AMR in Bacterial Communities

The spread of AMR among bacterial communities were primarily through horizontal gene transfer (HGT). This involves the exchange of genetic material, including resistance genes, between bacteria, often facilitated by movable genetic elements, including plasmids and transposons. Moreover, HGT could occur in the gut microbiome, where non-pathogenic bacteria from food are capable of transmitting resistance genes to pathogenic bacteria, thereby enhancing their resistance capabilities (Zinno, Perozzi and Devirgiliis, 2023; Okaiyeto et al., 2024). Moreover, the excessive application and widespread or uncontrolled use of antibiotics in livestock to promote growth and disease prophylaxis substantially contribute to the emergence of AMR. Such practices create reservoirs of antibiotic-resistant bacteria within livestock populations, which possess the potential potentially transferred to humans through the food chain. The presence of residual antibiotics in animal-derived products can additionally encourage the development of antimicrobial-resistant strains, thereby intensifying the severity of AMR (Kim and Ahn, 2022; Okaiyeto et al., 2024).

2.4.3 The Detection of AMR-Bacteria and ARG

The detection methods of AMR bacteria and gene are classified into phenotypic and genotypic resistance types (Galhano et al., 2021). Conventional methods for detection of AMR bacteria are based on biochemical and morphological characteristics of bacteria colonies (Tang et al., 2017). These consist of phenotypic analyses including, antibiotic susceptibility tests, microscopic techniques and enzymatic characterisation (Galhano et al., 2021). The widely used antibiotic susceptibility tests include determining the minimum inhibitory concentration (MIC), designed to determine the minimum level of an antimicrobial necessary to prevent growth of bacteria (Wiegand, Hilpert and Hancock, 2008). The advantages of traditional methods are its simplicity and low cost. However, it has low specificity, time consuming and detection is not possible for viable but non-culturable (VBNC) bacteria (Galhano et al., 2021). Another antibiotic susceptibility test method is the disc diffusion technique, which is conducted by placing an antimicrobial coated disc on a selective agar. This method provides qualitative data, and it is easy to conduct but anaerobic, fastidious microorganisms and some antibiotics that do not diffuse well through agar may affect the results. The elements of both techniques of MIC and disc diffusion can be combined to conduct a method known as E-test® (bioMérieux, France). It is a strip coated with a concentration gradient of antimicrobial that will be placed onto an agar, and it can be used to measure the MIC (Yamin et al., 2023).

In contrast, molecular approaches are applied for AMR detection; these includes microarray, whole genome sequencing (WGS), metagenomics, PCR and its varieties. Unlike traditional method, molecular methods are faster, able to detect VBNC bacteria, simultaneous detection of multiple AMR genes, and some may not even require bacteria culture. However, these techniques are unable to determine MIC, possibility of yielding false negatives and can be costly (Adzitey, Huda and Ali, 2013; Yamin et al., 2023). Modern studies had utilised metagenomics to detect and characterise genotypic resistance of foodborne pathogens (Rodrigues, Ferrari and Conte-Junior, 2018). The technique involves NGS of all genomic DNA in a sample, followed by bioinformatics analyses to obtain valuable metagenomic data. Metagenomics can overcome some limitations of previous traditional and other molecular techniques. This is because it can detect AMR in non-culturable bacteria and does not require prior knowledge on resistance genes for designing primers. However, the cost is high and has the potential limitation of insufficiently deep sequencing of a species' genome in complex samples (Galhano et al., 2021; Yamin et al., 2023).

Research in China have detected different ARG classes including chloramphenicol, bacitracin, tetracycline, aminoglycoside, beta-lactam, and multidrug resistance in RTE foods using metagenomic sequencing. These genes were linked to the antibiotics being administered extensively in human or veterinary medicine (Li et al., 2020b). Moreover, ARGs associated with beta-lactam, tetracycline and aminoglycoside were detected to be more prominent in retail meat

and seafood in Canada. Specifically, aminoglycoside was the most abundant ARG detected in veal, chicken and shrimp samples. Meanwhile, oyster and beef samples were found to have more abundant beta-lactam ARG (Flint et al., 2023). Another study in Hong Kong had detected ARGs in RTE foods such as sashimi, vegetables, pork, beef and chicken. Genes associated to tetracycline and macrolides were found to be prominent in pork samples. On the other hand, chicken samples had higher proportion of ARGs associated with beta-lactam, aminoglycosides and quinolones. Several high risk ARGs were detected from the study, which includes ARGS related to cephalosporin (*bla*^{TEM}, *bla*$^{CTX-M}$, *bla*^{DHA}, *bla*^{SHV}, *bla*^{ACT}, *AmpC*) and carbapenem (*bla*^{IMP}, *bla*^{KPC}, *bla*^{NDM}, *bla*^{VIM}) (Lee et al., 2024).

CHAPTER 3

MATERIAL AND METHODS

3.1 An Overview of the Research Study

An overview of the workflow study is illustrated in **Figure 3.1**. Firstly, the sample types and locations, as well as the processing methods used in the study was determined. The study included the collection and analysis of vegetable, fruit, and meat samples sourced from three locations in Kinta Valley, Perak, Malaysia namely Kampar, Gopeng, and Ipoh. Samples of each type were obtained from three separate vendors at each location. Sample types included lettuce, cabbage and Chinese spinach for vegetables; honeydew, papaya and watermelon for fruits; and raw chicken, ham and roasted chicken for meats.

Next, culture-dependent (culture) and culture independent (metagenomic approach) detection methods were applied parallelly in all the samples for results and analysis. The sliced samples were homogenized and enriched using pathogen-specific broths, followed by serial dilution, spread plating and subculturing on selective agars. DNA was extracted from suspected colonies, amplified via PCR, and analyzed through gel electrophoresis. In parallel, samples were prepared and processed through genomic DNA extraction then quality assessment using gel

electrophoresis and NanoDrop spectrophotometry. This is followed by library preparation, shotgun metagenomic sequencing and bioinformatic analysis.

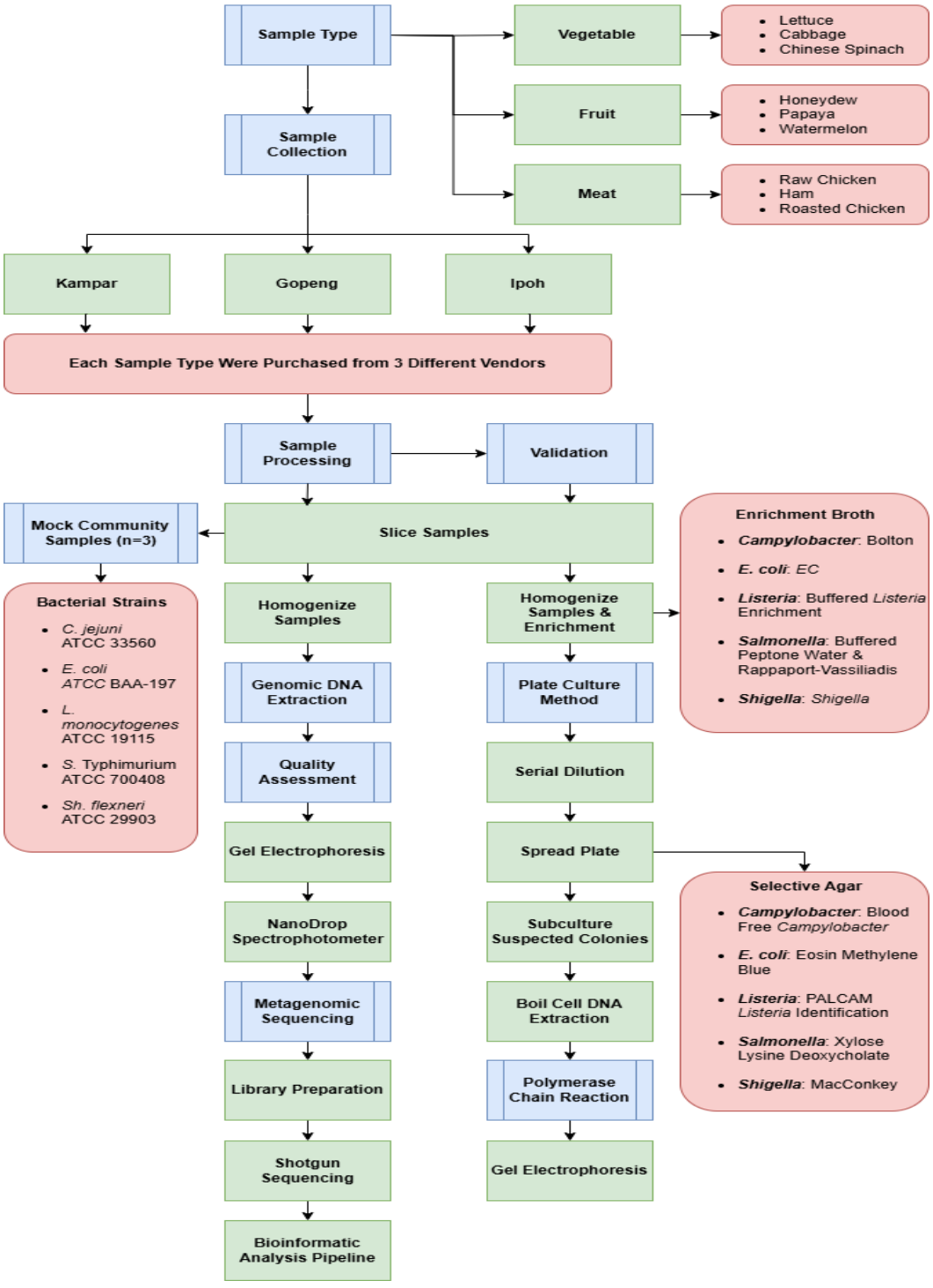


Figure 3.1: An overview of the workflow conducted. The blue boxes represent the main methodology; the green boxes indicate the detailed procedures, and the red boxes show sample types, bacterial strains, enrichment broths and selective agars.

3.2 Bacterial Strains and Inoculum Preparation

This study utilized bacterial strains such as *L. monocytogenes* ATCC 19115, *E. coli* ATCC BAA-197, *Sh. flexneri* ATCC 29903, *S. enterica* subspecies *enterica* serovar Typhimurium ATCC 700408 and *C. jejuni* ATCC 33560. The newly purchased ATCC strains of *S.* Typhimurium and *E. coli* were in lyophilized form. Both was revived by first rehydrating the pellet with 1 mL of TSB containing antimicrobial Ceftazidime pentahydrate (10 mcg/ml) (HiMedia, India) and 1 mL of TSB respectively. The rehydrated pellets were homogenized using a Pasteur pipette and transferred into a 10 mL conical tube containing 5 mL of TSB mixture. The liquid suspension was then streaked on TSA followed by incubation at 37°C for 24 hours. After incubation, two to three colonies were picked up and transferred into a cryovial (Technical Service Consultants Ltd, United Kingdom) containing 25% of glycerol mixture. Glycerol stock cultures were preserved at -80°C freezer until they are ready to use.

Glycerol stock cultures of *Sh. flexneri* ATCC 29903, *E. coli* ATCC BAA-197, *L. monocytogenes* ATCC 19115, *S.* Typhimurium ATCC 700408 and *C. jejuni* ATCC 33560 were revived by aliquoting 0.1 mL of each pure bacteria culture into a Falcon conical tube with 10 mL of TSB. The 15 mL conical tube containing *C. jejuni* ATCC 33560 was subjected to extra incubation conditions in an anaerobic chamber containing microaerobic gas generating sachets (Becton Dickinson, United States). The four other bacterial mixture were incubated at 37°C with shaking at 200 rpm for 24 h, while the *C. jejuni* ATCC 33560 culture was cultured

in a shaking incubator at 200 rpm and 42°C for 24 h. The revived bacteria strains were streaked on TSA and incubated according to plating phase incubation conditions listed in **Table 3.1**. After incubation, two to three colonies were picked up and inoculated into a Falcon conical tube with 10 mL of TSB. This is to minimize risk of contamination of the pure strains. The incubation step was repeated as listed in **Table 3.1**, and the bacteria cultures were ready to be used.

Table 3.1: The bacteria species with enrichment and plating phase conditions, as well as its morphology on the agar.

Bacteria	**Enrichment**		**Plating**		**Morphology of Colony**
	Media	**Incubation Condition**	**Agar**	**Incubation Condition**	
Campylobacter	Bolton broth	AC & microaerobic GGP, 42°C, 48 h	BFC agar	AC & anaerobic GGP, 42°C, 48 h	Grey and flat, with / without green hue
Escherichia coli	EC broth	42°C, 48 h	EMB agar	42°C, 48 h	Green metallic sheen, dark centre
Listeria	BLEB broth	30°C, 48 h	PALCAM agar	30°C, 48 h	Grey green, black halo
Salmonella	BPW, after 24h, aliquot 1 mL to 9 mL RVS broth	BPW at 37°C, 24 h RV at 37°C, 24 h	XLD agar	37°C, 24 h	Pink / red, black centre
Shigella	Shigella broth	AC & anaerobic GGP, 42°C, 20 h	MAC agar	35°C, 20 h	Pale / colourless

*Footnotes:
AC: Anaerobic chamber
GGP: Gas generating pouch
BFC: Blood Free Campylobacter Selectivity
EMB: Eosin Methylene Blue Levine
BLEB: Buffered Listeria Enrichment
BPW: Buffered Peptone Water
RVS: Rappaport-Vassiliadis Soy
XLD: Xylose Lysine Deoxycholate
MAC: MacConkey

3.3 Sample Collection

Nine sample types were used, which classified into three groups: vegetables comprising *Lactuca sativa* (lettuce), *Amaranthus dubius* (Chinese spinach), and *Brassica oleracea* var. *capitata* (cabbage); meats encapsulate raw chicken, roasted chicken, and deli meat and fruits consisting of *Cucumis melo* (honeydew), *Carica papaya* (papaya), and *Citrullus lanatus* (watermelon) (**Table 3.2**). A stratified convenience sampling approach was employed. Each of the samples were bought from three different vendors at Kampar, Gopeng, and Ipoh, respectively. The samples collected from the three vendors at each location were pooled together. Pooling samples provides a cost-efficient strategy that increases the chances of detecting low-abundance pathogens by combining resources and extending findings across the sample group (Anand et al., 2016). Moreover, this strategy enabled broader representation across vendor types. This makes the number of samples in this study totalling at 27 samples, with different labelling as shown in **Table 3.2**. Only items that were visibly fresh and free from spoilage were included. The sampling date and GPS coordinates for each sampling location was also recorded. The samples were transferred into a sterile stomacher bag and were stored in a cooler box containing ice before it was brought back to the laboratory. The processing of all samples occurred within 2 h after purchasing to maintain its integrity.

Table 3.2: Sample grouping and types used in the present study.

Sample Group	Sample	Location	Labelled As	Number of Samples
Vegetables	Lettuce	Kampar	L1K	1
		Gopeng	L2G	1
		Ipoh	L3I	1
	Chinese Spinach	Kampar	S1K	1
		Gopeng	S2G	1
		Ipoh	S3I	1
	Cabbage	Kampar	C1K	1
		Gopeng	C2G	1
		Ipoh	C3I	1
Meats	Roasted chicken	Kampar	RC1K	1
		Gopeng	RC2G	1
		Ipoh	RC3I	1
	Raw chicken	Kampar	CB1K	1
		Gopeng	CB2G	1
		Ipoh	CB3I	1
	Deli meat	Kampar	HM1K	1
		Gopeng	HM2G	1
		Ipoh	HM3I	1
Fruits	Honeydew	Kampar	H1K	1
		Gopeng	H2G	1
		Ipoh	H3I	1
	Papaya	Kampar	P1K	1
		Gopeng	P2G	1
		Ipoh	P3I	1
	Watermelon	Kampar	W1K	1
		Gopeng	W2G	1
		Ipoh	W3I	1

3.4 Sample Processing

The utensils used in the study, including chopping board, gloves, knife and nearby working area were disinfected using 70% of ethanol. The disinfection process was carried out before processing each sample to ensure no cross contamination between samples. The samples were observed and recorded for any visible organisms, including worms or insects, prior to chopping into smaller fragments. Subsequently, 10 g of sample from the three vendors of the same location were pooled to yield 30 g and added into the stomacher bag consisting of 270 mL of saline solution at 0.85% concentration. The stomacher bag was inserted into the BagMixer (Interscience, France) to homogenise for 2 min. The homogenised samples were subjected for genomic DNA (gDNA) extraction.

3.5 Bacteria Spiking for Mock Community Creation

The samples selected for spiking were consisted of one of each: honeydew [H], raw chicken meat [RC] and lettuce [L]. The primary purpose of this spiking step was to validate and optimise the workflow before applying it to the 27 randomly collected food samples. This step ensured that the research workflow was able to successfully detect the target pathogens. The revived bacterial strains grown in TSB (from section 3.2) were placed in an ice box to slow the bacteria growth. Optical density (OD) at 600 nm of the bacterial suspension was determined with a UV–Vis spectrophotometer (GENESYS 10S; Thermo Scientific, Malaysia). The bacterial suspension absorbance was set to 0.4 at OD 600 nm, before it is serially diluted to

ten-folds (OD of 1.0 corresponds to approximately 2.04×10^8 colony-forming units (CFU) per millilitre (Kim et al., 2012)). A 1 mL of the five bacterial strains suspension were aliquoted into the homogenised samples (from section 3.4) and mixed for 1 min. The mixture was then subjected to gDNA extraction. Meanwhile, 100 µL of the diluted bacterial suspensions (10 X dilution) were spread plated onto TSA using a L-shaped spreader (Biologix, United States). The TSA plates were incubated according to the conditions for each bacterium (**Table 3.1**). After incubation, the bacteria were enumerated by counting the colonies presented on the TSA plates.

3.6 Genomic DNA Extraction for Metagenomic Analysis

According to the protocol by Buytaers et al. (2021), 10 mL of the homogenised mixture (from section 3.4) were aliquoted into conical tubes and subjected to centrifugation with Velocity 14R centrifuge (Dynamica Scientific, United Kingdom) at 10,000 × g for 10 min. Following removal of the supernatant, the pellet served as the source for gDNA extraction using the Nucleospin Food Kit (Macherey-Nagel, Germany) according to manufacturer's recommendations. Resuspension of the pellet was carried out with 550 µL of Buffer CF preheated to 65°C. The cells were lysed by transferring the suspension into a 2 mL collection tube and mixed for 15 s before addition of 10 µL of Proteinase K. The suspension was briefly mixed again before incubating for 30 min at 65°C. Once incubation was complete, the mixture was spun using Pico™ 17 centrifuge (Thermo Scientific,

United States) for 10 min at 11,000 × g. A volume of 400 µL of the clear supernatant was transferred to a microcentrifuge tube, while the pellet was discarded. The tube was then added with 400 µL of ethanol and Buffer C4 respectively. The mixture was mixed by vortexing for 30 s before being transferred onto the column of NucleoSpin® Food Column placed in a collection tube. The tube underwent centrifugation at 11,000 × g for 1 min to facilitate DNA binding. The flow-through was removed, and 400 µL of Buffer CQW was applied to the column for washing. Following centrifugation at 11,000 × g for 1 min, the flow-through was discarded. Subsequently, 700 µL of Buffer C5 was pipetted onto the column, centrifuged at 11,000 × g for 1 min, and the flow-through discarded. Another portion of 200 µL Buffer C5 was added to the column and centrifuged at 11,000 × g for 2 min. The column was placed in a new microcentrifuge tube to elute the DNA. Preheated to 70°C, 50 µL of Elution Buffer CE were added to the column membrane. The tube was left at room temperature for 5 minutes, followed by centrifugation at 11,000 × g for 1 min. This procedure was performed again to elute a cumulative DNA volume of 100 µL.

DNA integrity was verified through agarose gel electrophoresis and observing the presence of a single band with minimal smearing (Drouin, Gao and Holmquist, 1996). Next, The purity and concentration of the DNA were measured with NanoDrop™ 1000 spectrophotometer (Thermo Scientific, United States). To perform library preparation, all DNA must have a minimum concentration of 50 µg/mL, an A260/280 ratio within 1.8–2.0, and an A260/230 ratio at least 1.8.

The A260/280 and A260/230 ratios reflect the level of protein contamination in nucleic acid samples and contamination by other organic compounds and salts, respectively (Tantray et al., 2023).

3.7 Culture Dependent Detection of Foodborne Pathogens

Culture plate method was used complementary and comparative approach alongside metagenomic sequencing for evaluating the presence of targeted foodborne pathogens. The same samples used in metagenomic sequencing protocol (from section 3.6) were processed separately for the isolation of five different bacteria: *Campylobacter* species*, Escherichia coli, Listeria* species*, Salmonella* species and *Shigella* species.

The sample processing procedure was similar to the one described above (from section 3.4), with some modifications. 30 g samples were placed into a stomacher bag with 270 mL of the designated enrichment media, and the homogenized mixtures were incubated under the specified environment (**Table 3.1**). Following incubation, 500 µL of the enriched broths were transferred into a 1.5 mL microcentrifuge tube and kept at -20°C for boiled-cell DNA extraction. In parallel, the incubated enrichment broths were diluted tenfold using 0.85 % of saline solution as the diluent. The serially diluted broth was used to perform the spread plate method by aliquoting 100 µL on the selective agar plates. A disposable L-shaped spreader was used to evenly spread the liquid inoculum to ensure that it was

evenly distributed. The agar plates were incubated under controlled settings, after which colony morphology was examined.

3.8 Bacterial Culture Purification

An inoculating loop was used to pick a single presumptive positive bacteria colony on the selective agar and streaked on TSA for subculture purposes. This step was repeated for each presumptive positive bacteria colony and incubated according to the incubation conditions specified for each bacterium as shown in the plating phase (**Table 3.1)**.

3.9 Boiled-Cell DNA Extraction

Initially, 500 µL of the enrichment broths that were incubated (Section 3.7) were subjected to centrifugation for 1 min according to the parameters in **Table 3.3**. Different centrifugation parameters were applied based on each bacterial species. These settings were chosen to optimise bacterial pelleting efficiency and DNA recovery, as recommended in prior protocols and literature. The supernatant was removed, and the pellet was resuspended in 200 µL of distilled water. Meanwhile, in a new microcentrifuge tube, suspected colonies from TSA were isolated with an inoculating loop and resuspended in 200 µL of distilled water. The suspension was mixed thoroughly by vortex. The cell suspension was heated in a heat block (Major Science Co. Ltd., Taiwan) at 100°C for 10 min. Following a 10 min heating step,

the tubes were kept at −20°C for an additional 10 min. The microcentrifuge tubes underwent centrifugation for 3 min according to specific parameters stated in **Table 3.3**. The pellet was discarded, and clear supernatant was pipetted into a new 1.5 mL microcentrifuge tube. The supernatant served as the DNA template for the PCR assay.

Table 3.3: Parameters of centrifugation used for the five bacterial species in boiled-cell DNA extraction.

Bacterial Species	**Centrifugation Parameters**	**References**
Campylobacter	Centrifugation Speed: 12,000 × g For Bolton broth and *Campylobacter spp.* colonies, time was set to 5 min.	Igwaran and Okoh (2020)
E. coli	Centrifugation Speed: 13,400 × g	Kuan et al. (2017b)
Listeria	Centrifugation Speed: 13,400 × g	Kuan et al. (2017b)
Salmonella	Centrifugation Speed: 15,000 × g	Kuan et al. (2017b)
Shigella	Centrifugation Speed: 12,000 × g For *Shigella* broth and *Shigella spp.* colonies, the time was set to 5 min.	Nisa et al. (2021)

3.10 Multiplex Polymerase Chain Reaction (PCR)

Nine forward-reverse primer pairs were employed to perform polymerase chain reaction (PCR) to detect *Listeria* spp*., Campylobacter* spp*., Shigella* spp., *Salmonella* spp., and *E. coli*. The primer pairs, sequence, and targeted gene were listed in **Table 3.4**. The 1X TBE buffer was then used to prepare 1.5% (w/v) agarose gel and as a running buffer for electrophoresis. The 1.5% (w/v) agarose gel contained 2 µL of GelRed for staining purpose. Each well of the gel was loaded with 5 µL of PCR product and 2 µL of a 100 bp DNA ladder as a molecular marker, and underwent electrophoresis at 90 V for 35 min. After gel electrophoresis, the gel was viewed under ultraviolet (UV) light using the gel documentation system (SynGene, United Kingdom).

Table 3.4: The sequences of the primers used, and amplicon sizes expected after PCR.

Targeted Gene	Primer Sequence (5' to 3')	Amplicon Size	Reference
Campylobacter spp. (*cadF*)	cadF-F: TTGAAGGTAATTTAGATATG cadF-R: CTAATACCTAAAGTTGAAAC	400 bp	Shams et al. (2017)
C. jejuni (*hipO*)	hipO-F: GAAGAGGGTTTGGGTGGTG hipO-R: AGCTAGCTTCGCATAATAACTTG	735 bp	Shams et al. (2017)
E. coli (*uidA*)	uidA-F: TATGGAATTTCGCCGATTTT uidA-R: TGTTTGCCTCCCTGCTGCGG	166 bp	Godambe, Bandekar and Shashidhar (2017)
Listeria spp. (16S rRNA)	U1: CTCCATAAAGGTGACCCT LI1: CAGCMGCCGCGGTAATWC	938 bp	Kuan et al. (2017b)
L. monocytogenes (*hlyA*)	LM1: CCTAAGACGCCAATCGAA LM2: AAGCGCTTGCAACTGCTC	702 bp	Kuan et al. (2017b)
Salmonella spp. (Random fragment)	ST11: GCCAACCATTGCTAAATTGGCGCA ST15: GGTAGAAATTCCCAGCGGGTACTGG	429 bp	Kuan et al. (2017b)
S. Typhimurium (*fliC*)	Fli15: CGGTGTTGCCCAGGTTGGTAAT Typ04: ACTGGTAAAGATGGCT	620 bp	Kuan et al. (2017b)
Shigella spp. (*set1A*)	Shig1: TGGAAAAACTCAGTGCCTCT Shig2: CCAGTCCGTAAATTCATTCT	309 bp	Thong et al. (2005)
Sh. flexneri (*ipaH*)	ShET1A-F: TCACGCTACCATCAAAGA ShET1A-R: TATCCCCCTTTGGTGGTA	423 bp	Thong et al. (2005)

*Footnotes:
cadF: *Campylobacter* adhesin to fibronectin
hipO: Hippuricase
uidA: Beta-glucuronidase
hlyA: Haemolysin A
fliC: Flagellin
set1A: *Shigella* enterotoxin 1A
ipaH: Invasion plasmid antigen H

3.11 DNA Library Preparation and Sequencing

The preparation of libraries and subsequent sequencing were conducted by AGTC Genomics Sdn. Bhd. with Illumina DNA Prep kit (Illumina, United States) based on manufacturer's instructions. First, for tagmentation of gDNA, 30 µL of DNA was pipetted to a 96-well PCR plate. The Tagmentation Master Mix was prepared by combining 11 µL of Bead-Linked Transposomes with 11 µL of Tagment Buffer 1. The Tagmentation Master Mix was resuspended by vortexing. Each well with a sample was loaded with 20 µL of Tagmentation Master Mix and the sample was resuspended by pipetting. The plate was placed in the thermal cycler and subjected to cycling conditions at 55°C for 15 min. Then, the plate was added with 10 µL of Tagment Stop Buffer. The well was resuspended by pipetting. The thermal cycler was set to run the plate at 37°C for 15 min. The plate was placed on a magnetic stand to initiate washing, allowing the solution to become transparent. Then, the supernatant was removed and discarded. The plate was taken off the magnetic stand, and 100 µL of Tagment Wash Buffer was added. The liquid was mixed by pipetting, after which the plate was positioned on the magnetic stand. The washing step was repeated for once.

The procedure of amplifying tagmented DNA started with the plate being removed from the magnetic stand. The beads in each well were supplemented with 40 µL of PCR Master Mix containing Enhanced PCR Mix and nuclease-free water. The beads were resuspended by pipetting and the plate was sealed. After shaking the plate at 1600 rpm for 1 min, it was centrifuged at 280 × g for 3 s. The plate was

then added with 10 µL of index adapters and plate was positioned on a shaker and agitated at 1600 rpm for 1 min. The plate was sealed and centrifuged at 280 × g for 30 s. The plate was positioned in a thermal cycler and ran with the conditions as followed; 3 min at 68°C, 3 min at 98°C, followed by 6 cycles of 45 s at 98°C, 30 s at 62°C and 2 min at 68°C, final step was 1 min at 68°C.

Purification of the amplified library procedure was begun with centrifugation for 1 min at 280 × g. The plate was positioned on the magnetic stand and left until the liquid became clear. Next, 45 µL of the supernatant was pipetted onto a MIDI plate. Each well containing the supernatant was added with 40 µL of nuclease-free water followed by 45 µL of Illumina Purification Beads (IPB). The plate was shaken at 1600 rpm for 1 min and subsequently incubated at room temperature for 5 min. After incubation, plate was properly placed on the magnetic stand and left until the solution became clear. A new MIDI plate was added with 15 µL of IPB before transferring 125 µL of supernatant from the first MIDI plate. The sealed plate was shaken at 1600 rpm for 1 min. After a 5 min incubation at room temperature, the MIDI plate was placed on the magnetic stand. The supernatant was discarded once the liquid was clear. The washing step was initiated with 200 µL of 80% ethanol pipetted to the plate, which was then incubated for 30 s. The supernatant was discarded. The washing step was then repeated. The plate was air-dried for 5 minutes prior to removal from the magnetic stand. The well was added with 32 µL of Resuspension Buffer and resuspended by pipetting. The plate was incubated at room temperature for 2 min and placed onto the magnetic stand.

Once the liquid became clear, 30 µL of the supernatant was pipetted into a fresh 96-well plate. The DNA library-containing supernatant was normalised to 1.75 nM and pooled by aliquoting 5 µL of each library in a microcentrifuge tube. The concentration of the library pool was measured with a Qubit Fluorometer (Thermo Fisher, United States). The sample was sequenced on Illumina Novaseq 6000 system (Illumina, United States) at paired end of 150 bp.

3.12 Bioinformatics Analysis

The bioinformatics analysis were carried out by AGTC Genomics Sdn. Bhd. by sorting of paired end reads into individual datasets, a process known as demultiplexing, performed with Illumina DRAGEN v3.9 (Dynamic Read Analysis for GENomics) Bio-IT Platform (**Figure 3.2**). FastQC was used to perform quality control (QC) on the metagenomic reads. The host DNA were removed accordingly to their sample type to minimize contamination. Reads with a Phred quality score (Q-score) below Q30 were trimmed. Adapter sequences were completely removed using Cutadapt to prevent contamination in downstream analyses. Reads shorter than 50 bp after trimming were discarded. These poor-quality reads were trimmed before being mapped against the reference database using KMA (k-mer alignment) (Clausen, Aarestrup and Lund, 2018). The KMA aligner employs the ConClave sorting scheme to ensure highly accurate read mappings by evaluating reference sequences based on every possible mapping of reads. Templates with the highest scores are selected, minimising the occurrence of false-positive alignments.

Subsequently, the data underwent quality filtering and taxonomic classification using CCMetagen (Marcelino et al., 2020).

Taxonomic profiling and species annotation were performed utilising the NCBI taxonomic database, offering comprehensive information on the composition of the bacterial community. Diversity data analysis was also conducted to obtain alpha, beta diversity and rarefaction curve. This database was selected for its comprehensive coverage of prokaryotic organisms, high quality curated sequences, regular updates, and compatibility with the KMA aligner and CCMetagen pipeline. Taxonomic abundance was calculated using CCMetagen's default depth estimation, which applies a correction for template length based on KMA alignment output as seen with the formula below. This method calculates abundance as the total number of nucleotides mapped to a reference sequence divided by the reference genome length, thereby accounting for differences in genome sizes across taxa (Marcelino et al., 2020).

$$Relative\ Abundance = \frac{Total\ nucleotides\ mapped\ to\ reference}{Reference\ genome\ length}$$

The Shannon diversity index was calculated using the formula below (i) where H represents the Shannon diversity index, $\sum$ is the sum of all species, followed by P_i that indicates the proportion of each species. The formula (ii) for calculating beta-analysis specifically where J represents Jaccard index, x_i^A is the species *i*'s abundance within sample A, likewise x_i^B indicates species *i*'s abundance

within sample B. Each analysis was performed using CLC Genomics Workbench pipeline (Qiagen, Germany).

$$\text{i. H} = \sum_{i=1}^{n} P_i log_2 P_i$$

$$\text{ii. J} = 1 - \frac{\sum_{i=1}^{n} \min\left(\boldsymbol{x}_i^A \boldsymbol{x}_i^B\right)}{\sum_{i=1}^{n} \max\left(\boldsymbol{x}_i^A \boldsymbol{x}_i^B\right)}$$

The antimicrobial resistance (AMR) pipeline was executed after completing the data pre-processing procedure. In contrast to the taxonomy assignment protocol, the AMR pipeline's assembly methodology employed a different approach. Mapping of reads was performed using the Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca/) using KMA, while contigs were assembled using SPAdes (Prjibelski et al., 2020). The assembled contigs were subsequently compared to CARD for homology using Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Hits having a sequence identity of at least ≥98% were retained as true predictions. No additional cutoffs for coverage or bit score were applied, so the screening focused solely on high-identity matches. The obtained results were compiled to provide from the platform were aggregated to generate antimicrobial resistance gene (ARG) information and QC matrices (**Figure 3.2**).

Krona plots were generated using the KronaTools package (version 2.8.1) for interactive visualization of the taxonomic composition. Taxonomic output files from CCMetagen were imported into KronaTools to produce a HTML-based interactive chart. Alpha and beta diversity figures were generated using CLC Genomics Workbench pipeline (Qiagen, Germany). Statistical analysis of Cohen's kappa, McNemar's test, and Kruskal-Wallis analysis was conducted with IBM SPSS version 27.0.1.

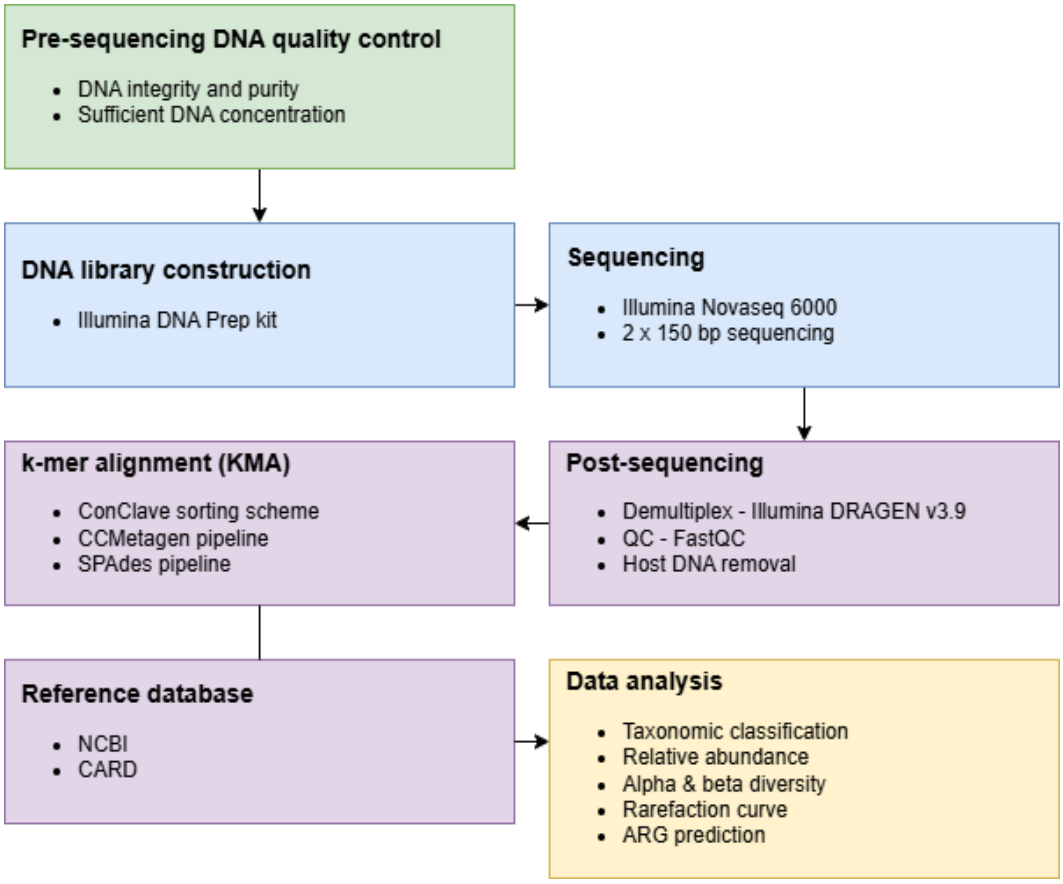


Figure 3.2: Next-generation sequencing workflow. This figure outlines the various steps involved in the process. The green box indicates the pre-sequencing steps; the blue box highlights the sequencing stage; the purple box covers post-sequencing processes, and the yellow box indicates the data analysis phase.

CHAPTER 4

RESULTS

4.1 Quality Control (QC) of DNA, Sequencing Data and Reads

A total of 27 samples comprising vegetables, fruits, and meats purchased from Kampar, Ipoh, and Gopeng, were analysed with shotgun metagenomic sequencing. The extracted DNA were measured before undergoing DNA library preparation and metagenomic sequencing. **Table 4.1** presents the A260/A280 ratios ranging from 1.80 to 1.99 which are within the acceptable range and suggesting minimal contamination. The A260/A230 ratios ranged from 1.82 to 2.05 indicating low levels of polysaccharide, phenolic, or other organic compound contamination. DNA concentrations ranged from 50.5 ng/µL to 147.4 ng/µL, which exceeds the input requirement for shotgun metagenomic library preparation (≥50 ng/µL). Gel electrophoresis results had distinct DNA bands with minimal smearing across all samples.

Table 4.1: DNA purity and concentration measurements of extracted samples.

Sample	A260/A280	A260/A230	Concentration (ng/µL)	Integrity (Gel)
C1K	1.83	1.98	67.9	Intact
S1K	1.92	1.95	123.1	Intact
L1K	1.88	1.82	95.2	Intact
C2G	1.94	1.99	73.2	Intact
S2G	1.81	1.87	70.3	Intact
L2G	1.89	1.82	57.6	Intact
C3I	1.83	1.83	52.0	Intact
S3I	1.94	1.87	120.3	Intact
L3I	1.98	1.82	137.6	Intact
CB1K	1.80	1.83	132.2	Intact
RC1K	1.96	1.97	63.2	Intact
HM1K	1.95	1.98	67.3	Intact
CB2G	1.89	1.90	147.4	Intact
RC2G	1.91	1.95	71.1	Intact
HM2G	1.85	2.05	56.4	Intact
CB3I	1.96	1.84	131.2	Intact
RC3I	1.89	1.86	64.4	Intact
HM3I	1.91	1.94	51.8	Intact
H1K	1.85	1.85	54.9	Intact
P1K	1.90	1.94	64.4	Intact
W1K	1.81	1.98	65.5	Intact
H2G	1.89	2.02	63.8	Intact
P2G	1.84	1.86	51.5	Intact
W2G	1.88	1.97	52.7	Intact
H3I	1.94	2.01	68.9	Intact
P3I	1.88	1.96	50.5	Intact
W3I	1.96	1.80	52.3	Intact
O1L	1.80	1.91	86.4	Intact
O1CB	1.84	1.99	77.8	Intact
O1H	1.99	2.03	63.7	Intact

Total DNA sequence reads were generated for vegetables, fruits, and meats sourced from three separate locations were presented in **Table 4.2**. The mean number of DNA sequence reads obtained for vegetables, fruits, and meats were 117,546,279, 75,082,848, and 102,307,371, respectively (Total of each sample group divided by 3). Read counts differed notably among the sample groups, with vegetables showing the highest number of 352,638,836 reads, followed by the fruit group at 306,922,112 reads, and the least were meat group at 225,248,544 reads. The sequence quality was high, and as all of it achieved Phred score of more than 30 (**Figure 4.1**). A plateau was observed in the rarefaction curves following a specific read count. The curve levels off at different total number ranging nearly from 50 to 300. Overall, the total number was lower in deli meat and fruit samples suggesting that the species diversity and species richness in these samples were lower (**Figure 4.2**).

Table 4.2: Sample groups and the output of sequencing data.

Sample Group	Location	Total Number of Sequenced Reads	Average Q30 Percentage
Vegetable	Kampar	111,398,518	90
	Gopeng	135,821,851	92
	Ipoh	105,418,467	92
Total		**352,638,836**	
Meat	Kampar	88,411,636	92
	Gopeng	70,885,030	92
	Ipoh	65,951,878	90
Total		**225,248,544**	
Fruit	Kampar	115,327,754	91
	Gopeng	104,150,573	90
	Ipoh	87,443,785	92
Total		**306,922,112**	

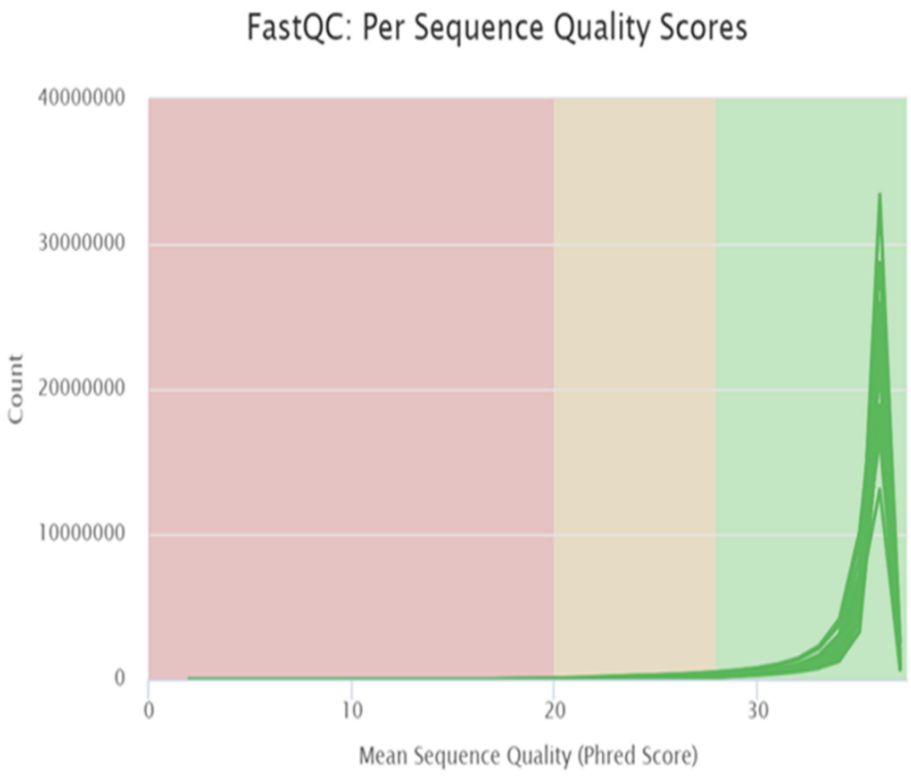
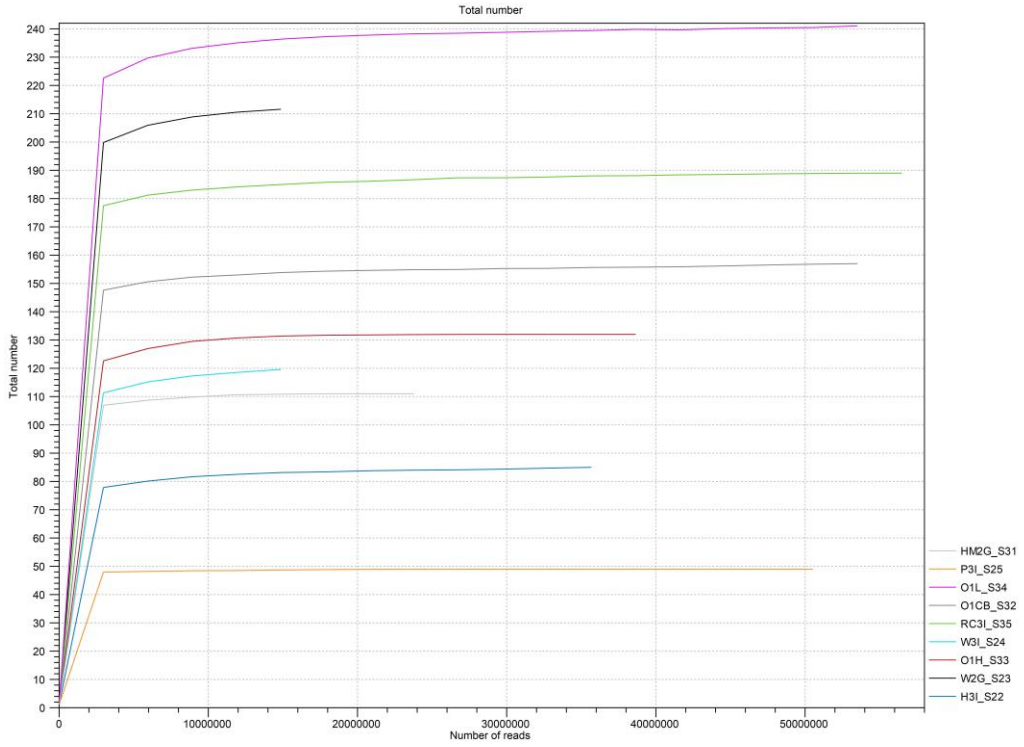
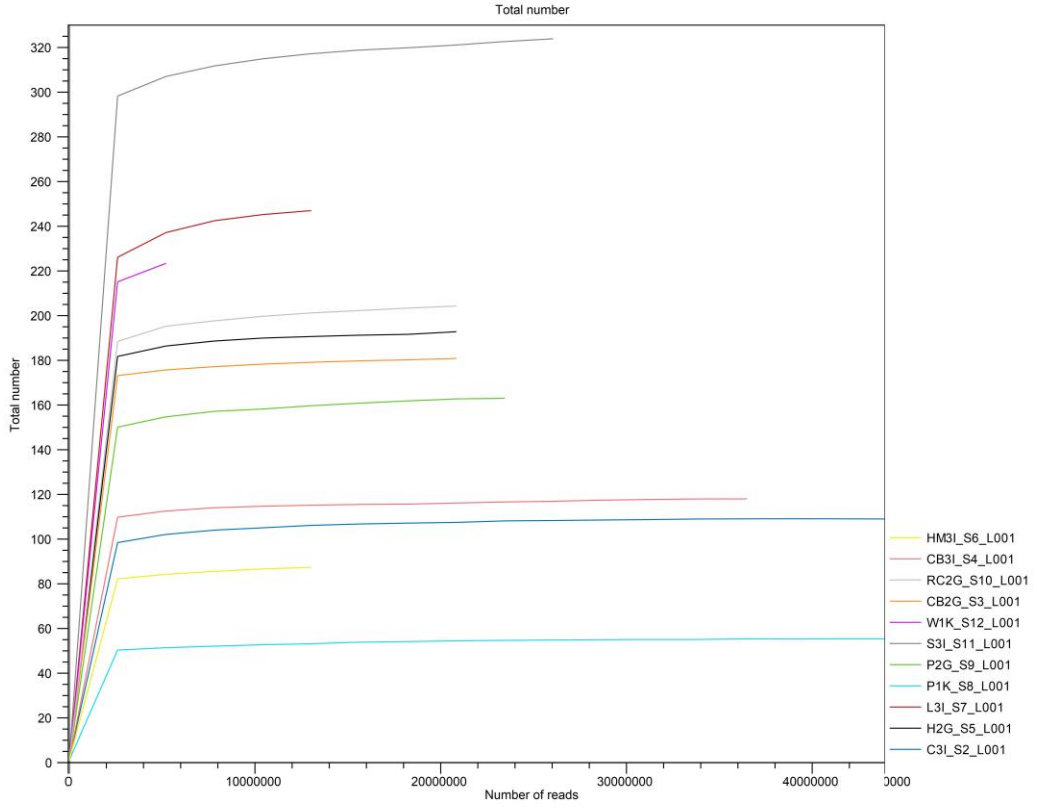


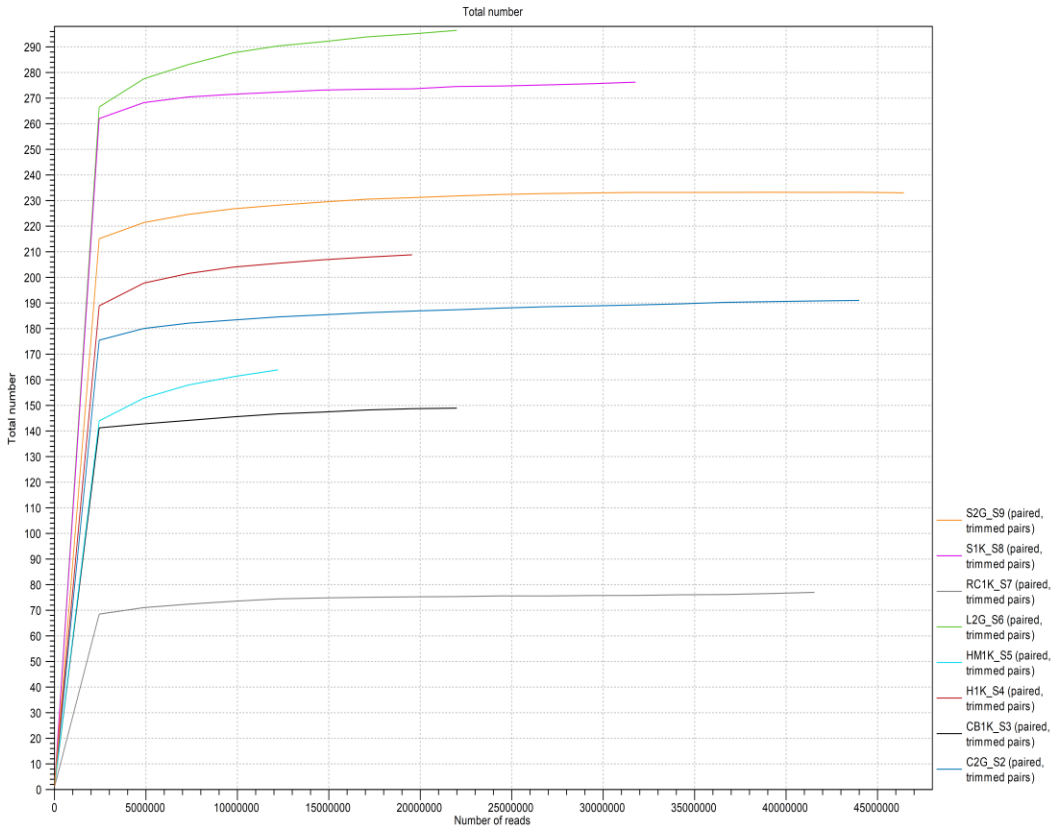
Figure 4.1: The mean sequence quality score of the samples.


Total number
Total number
Number of reads
0
10000000
20000000
30000000
40000000
50000000
HM2G_S31
P3I_S25
O1L_S34
O1CB_S32
RC3I_S35
W3I_S24
O1H_S33
W2G_S23
H3I_S22

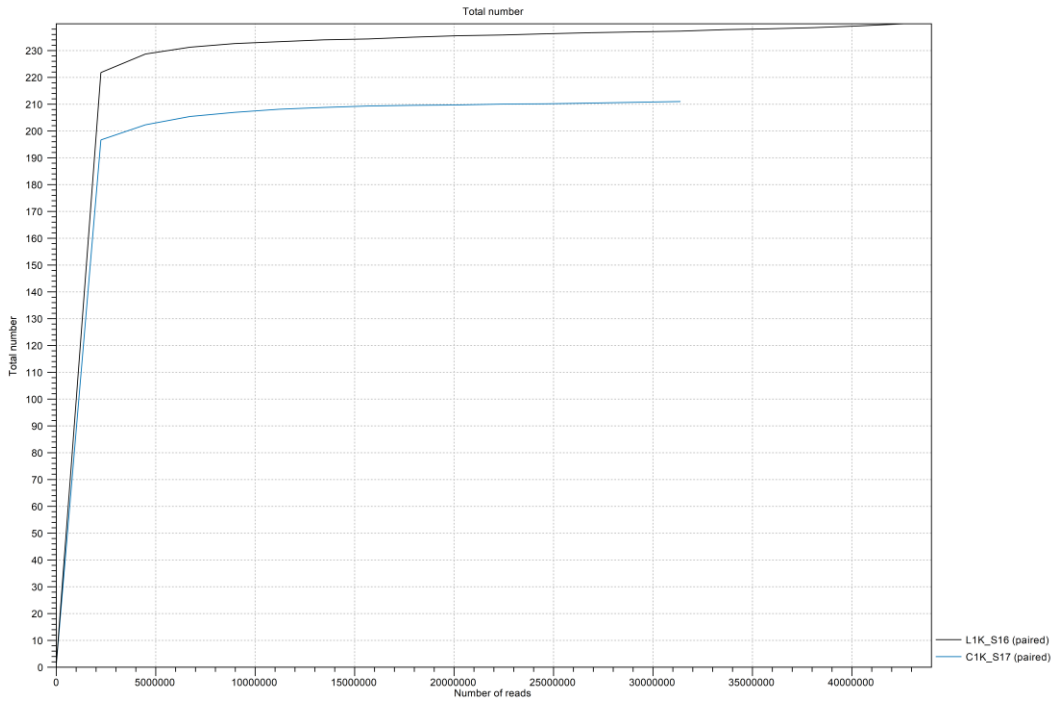
(a)


Total number
Total number
Number of reads
0
10000000
20000000
30000000
40000000
HM3I_S6_L001
CB3I_S4_L001
RC2G_S10_L001
CB2G_S3_L001
W1K_S12_L001
S3I_S11_L001
P2G_S9_L001
P1K_S8_L001
L3I_S7_L001
H2G_S5_L001
C3I_S2_L001

(b)


Total number
Total number
Number of reads
0
5000000
10000000
15000000
20000000
25000000
30000000
35000000
40000000
45000000
S2G_S9 (paired, trimmed pairs)
S1K_S8 (paired, trimmed pairs)
RC1K_S7 (paired, trimmed pairs)
L2G_S6 (paired, trimmed pairs)
HM1K_S5 (paired, trimmed pairs)
H1K_S4 (paired, trimmed pairs)
CB1K_S3 (paired, trimmed pairs)
C2G_S2 (paired, trimmed pairs)

(c)



(d)

Figure 4.2: Rarefaction curves of the samples in different batches **(a)** 9 samples comprising of HM2G, P3I, O1L, O1CB, RC3I, W3I, O1H, W2G, H3I. **(b)** 11 samples comprising of HM3I, CB3I, RC2G, CB2G, W1K, S3I, P2G, P1K, L3I, H2G, C3I. **(c)** 8 samples including of S2G, S1K, RC1K, L2G, HM1K, H1K, CB1K, C2G. **(d)** 2 samples including of L1K and C1K. Plotting activity was highest between 0 to 10 million reads (average) before smoothening and completely plateauing.

4.2 Mock Community

A mock community was generated from five different bacterial species, including *C. jejuni*, *E. coli*, *L. monocytogenes*, *S. enterica* subspecies *enterica* serovar Typhimurium, and *Sh. flexneri*. The corresponding ATCC strains used were 33560, BAA-17, 19115, 700408 and 29903. The mock community were introduced into vegetable, meat and fruit.

4.2.1 Culture-dependent and Metagenomic Detection of Pathogens

The detection of mock community using both culture complemented with PCR and metagenomics analysis were recorded in **Table 4.3**. The two approaches provided complementary perspectives where metagenomics offered a broad and total DNA-based detection, while culture-based methods detected only viable and cultivable cells. Detection of *L. monocytogenes* in vegetable and meat samples was not successful using metagenomics analysis possibly due to filtering of the low-quality reads. The remaining four pathogens were detected consistently across all three sample types using both methods, indicating overlap in detection despite methodological differences. The similarities and differences in detection between the two approaches were examined as part of evaluating the feasibility of metagenomics for pathogen detection.

Table 4.3: Detection of mock community in vegetable, meat and fruit samples using metagenomics analysis and culture methods.

Detection Method / Sample	**Foodborne Pathogen**				
	Campylobacter	*Escherichia*	*Listeria*	*Shigella*	*Salmonella*
Metagenomics					
Vegetable	+	+	/	+	+
Meat	+	+	/	+	+
Fruit	+	+	+	+	+
Culture & PCR					
Vegetable	+	+	+	+	+
Meat	+	+	+	+	+
Fruit	+	+	+	+	+

*Footnotes:
+=Detected
/=Not Detected

4.2.2 AMR Profiles

The three samples containing the mock community bacteria were analysed for the presence of ARGs., and the results are presented in **Table 4.4** and **Table 4.5**. Two of the five ATCC bacterial strains were associated with AMR: *E. coli* and *S.* Typhimurium. The ATCC BAA-197 strain of *E. coli* is an ESBL-producing bacterium, while *S.* Typhimurium ATCC 700408 is resistant to multiple antibiotics, including ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline.

The AMR gene profile of ATCC BAA-197 comprise of seven different ARGs notably *TEM-12*, *sul1*, *APH(3')-Ia*, *AAC(3)-IIe*, *SCO-1*, *catA* and *aadA1*. On the other hand, ATCC 700408 had four different ARGs including *CARB-2*, *aadA16*, *floR* and *sul1*. Metagenomics analysis detected most of the AMR genes of the mock community within the spiked samples. Five ARGs, including *sul1*, *AAC(3)-IIe*, *SCO-1*, *APH(3')-Ia* and *floR* were detected in all three spiked samples. Meanwhile, *catA*, *TEM-12*, and *CARB-2* were not observed in any spiked samples. Additionally, both aminoglycoside nucleotidyltransferase genes (*aadA16*, *aadA1*) were not detected in fruit and vegetable sample respectively. Overall, metagenomics was able to identify the majority of ARGs in the mock community.

Table 4.4: Detection of ARGs of *E. coli* ATCC BAA-197 in vegetable, meat and fruit samples using metagenomics analysis.

Sample	**ARGs of *E. coli* ATCC BAA-197**						
	TEM-12	*sul1*	*AAC(3)-IIe*	*SCO-1*	*APH(3')-Ia*	*aadA1*	*catA*
Vegetable	/	+	+	+	+	/	/
Meat	/	+	+	+	+	+	/
Fruit	/	+	+	+	+	+	/

*Footnotes:
TEM-12=Extended-spectrum class A beta-lactamase
sul1=Sulfonamide-resistant dihydropteroate synthase
AAC(3)- IIe=Aminoglycoside N-acetyltransferase
SCO-1=Class A beta-lactamase
APH(3')-Ia=Aminoglycoside O-phosphotransferase
aadA1=Aminoglycoside nucleotidyltransferase
catA=Type A-1 chloramphenicol O-acetyltransferase
+=Detected
/=Not Detected

Table 4.5: Detection of ARGs of *S.* Typhimurium ATCC 700408 in vegetable, meat and fruit samples using metagenomics analysis.

Sample	**ARGs of *S.* Typhimurium ATCC 700408**			
	sul1	*aadA16*	*floR*	*CARB-2*
Vegetable	+	+	+	/
Meat	+	+	+	/
Fruit	+	/	+	/

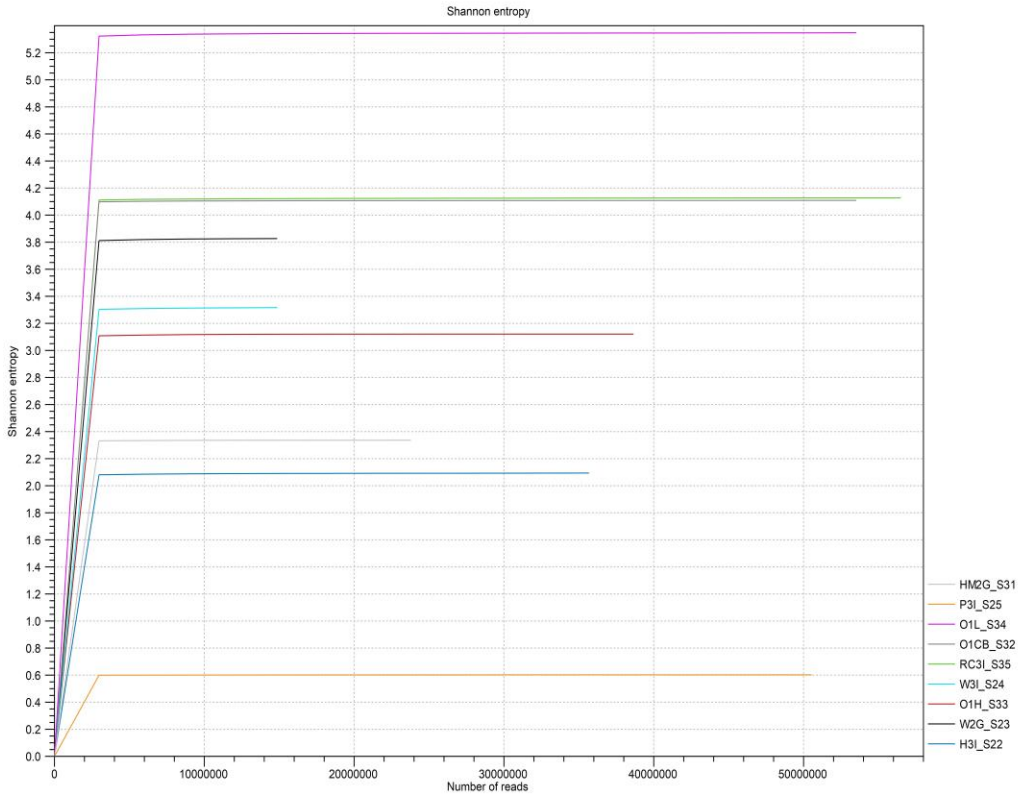
*Footnotes:
sul1=Sulfonamide-resistant dihydropteroate synthase
aadA16=Aminoglycoside nucleotidyltransferase
floR=Chloramphenicol/florfenicol efflux MFS transporter
CARB-2=Carbenicillin-hydrolysing class A beta-lactamase
+=Detected
/=Not Detected

4.3 Microbial Diversity and Pathogen Detection

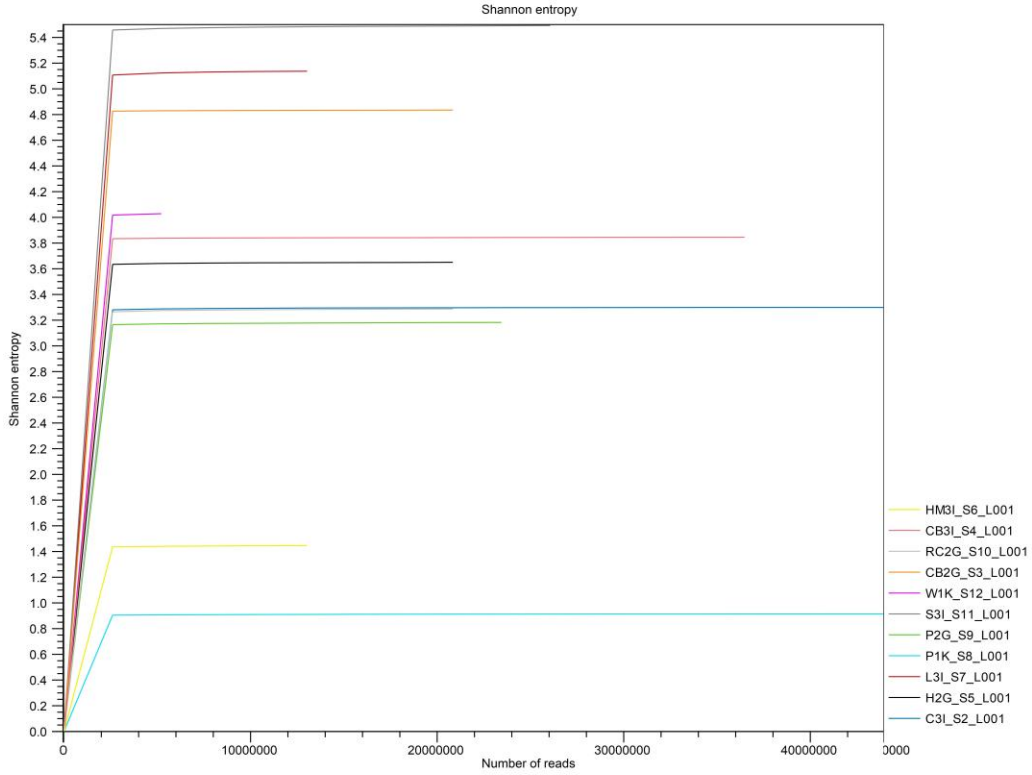
4.3.1 Alpha Diversity

The Shannon index graph reached plateau state across all samples (**Figure 4.3.1**), indicating sufficient sequencing depth. Overall, vegetable samples displayed the highest bacterial diversity with Shannon indices ranging from 3.3 to 5.4. In contrast, fruit samples showed lower diversity, in particular papaya samples (P1K = 0.9, P3I = 0.6). Meat samples particularly raw chicken meat had higher Shannon indices compared to roasted chicken meat and deli meat samples. The highest was CB2G at 4.8 and lowest was HM3I at 1.4. These findings suggest that vegetables had higher richness and more even bacterial communities, while fruits tend to have narrower uneven communities, and meats had greater variability depending on processing and preparation type.

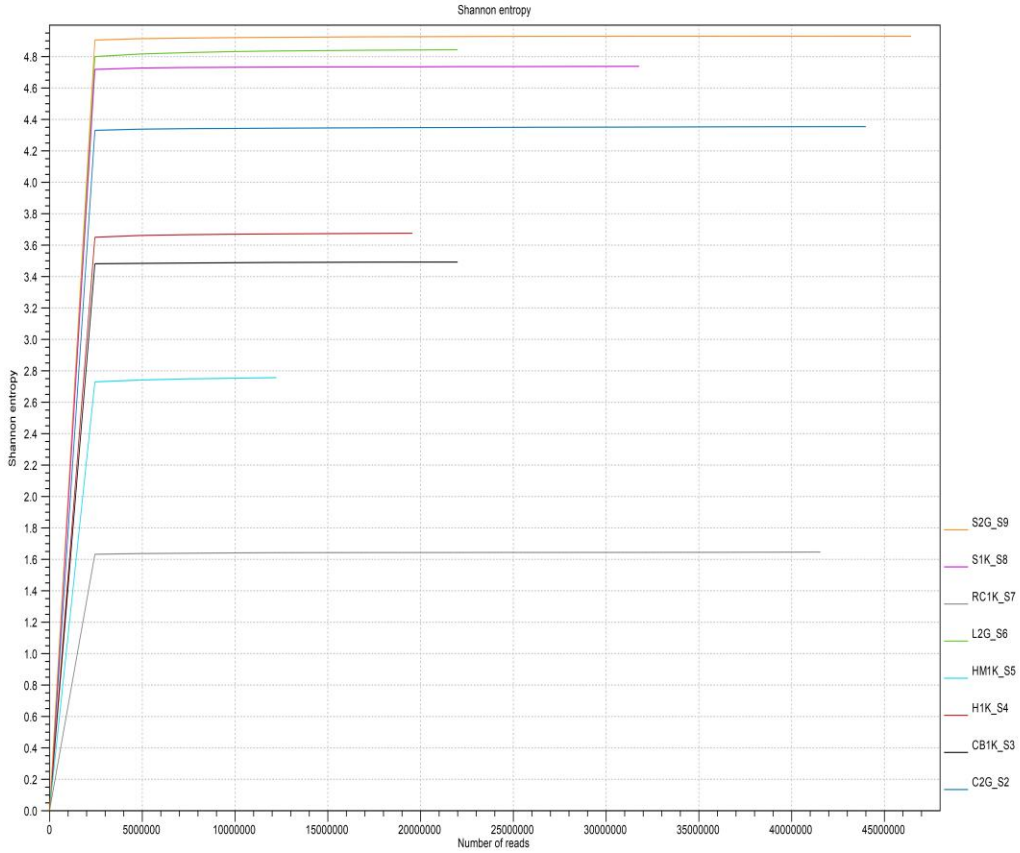
The Kruskal-Wallis test showed significant differences in Shannon diversity index across the three sample types ($p = 0.002$). Post-hoc pairwise comparisons revealed that vegetables differed significantly from both fruits ($p = 0.004$) and meats ($p = 0.012$), while no significant difference was observed between fruits and meats ($p = 1.000$). Additionally, the Shannon diversity index did not differ significantly across locations ($p = 0.709$). This suggests that microbial diversity was relatively similar between Kampar, Gopeng, and Ipoh.


Shannon entropy
Shannon entropy
Number of reads
0
10000000
20000000
30000000
40000000
50000000
HM2G_S31
P3I_S25
O1L_S34
O1CB_S32
RC3I_S35
W3I_S24
O1H_S33
W2G_S23
H3I_S22

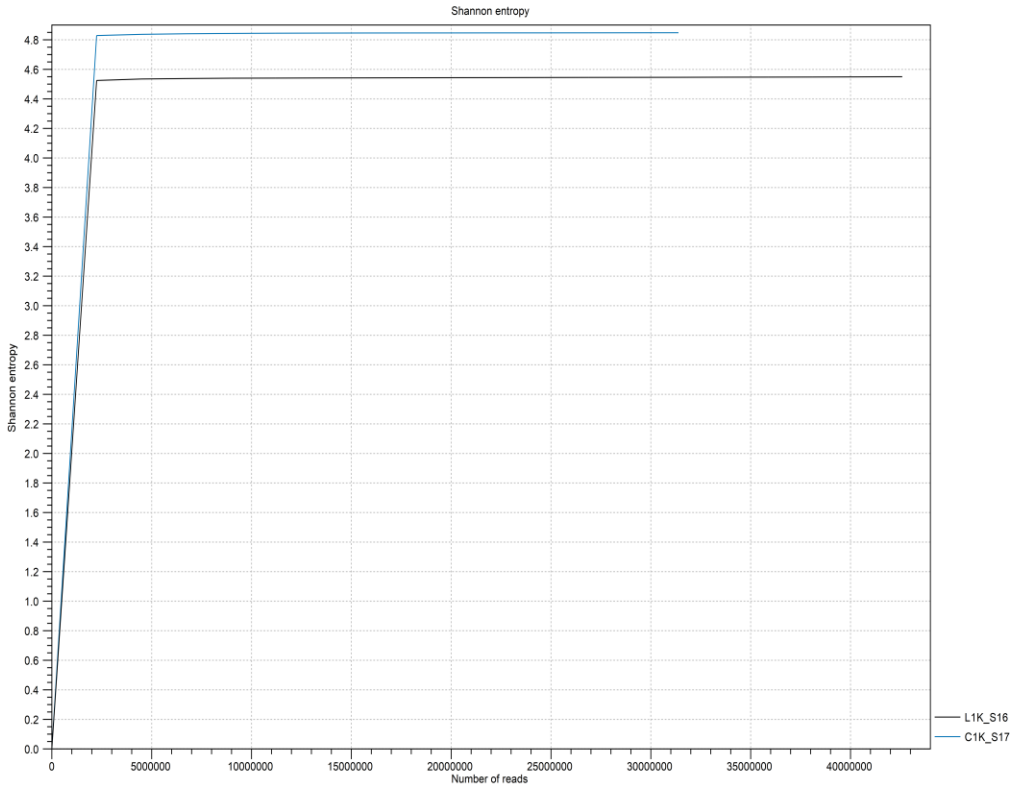
(a)


Shannon entropy
Shannon entropy
Number of reads
0
10000000
20000000
30000000
40000000
HM3I_S6_L001
CB3I_S4_L001
RC2G_S10_L001
CB2G_S3_L001
W1K_S12_L001
S3I_S11_L001
P2G_S9_L001
P1K_S8_L001
L3I_S7_L001
H2G_S5_L001
C3I_S2_L001

(b)


Shannon entropy
Shannon entropy
Number of reads
0
5000000
10000000
15000000
20000000
25000000
30000000
35000000
40000000
45000000
S2G_S9
S1K_S8
RC1K_S7
L2G_S6
HM1K_S5
H1K_S4
CB1K_S3
C2G_S2

(c)



(d)

Figure 4.3.1: Shannon diversity indices of samples in different batches. (a) 9 samples including HM2G, P3I, O1L, O1CB, RC3I, W3I, O1H, W2G, H3I. (b) 11 samples including HM3I, CB3I, RC2G, CB2G, W1K, S3I, P2G, P1K, L3I, H2G, C3I. (c) 8 samples including S2G, S1K, RC1K, L2G, HM1K, H1K, CB1K, C2G. (d) 2 samples including L1K, C1K.

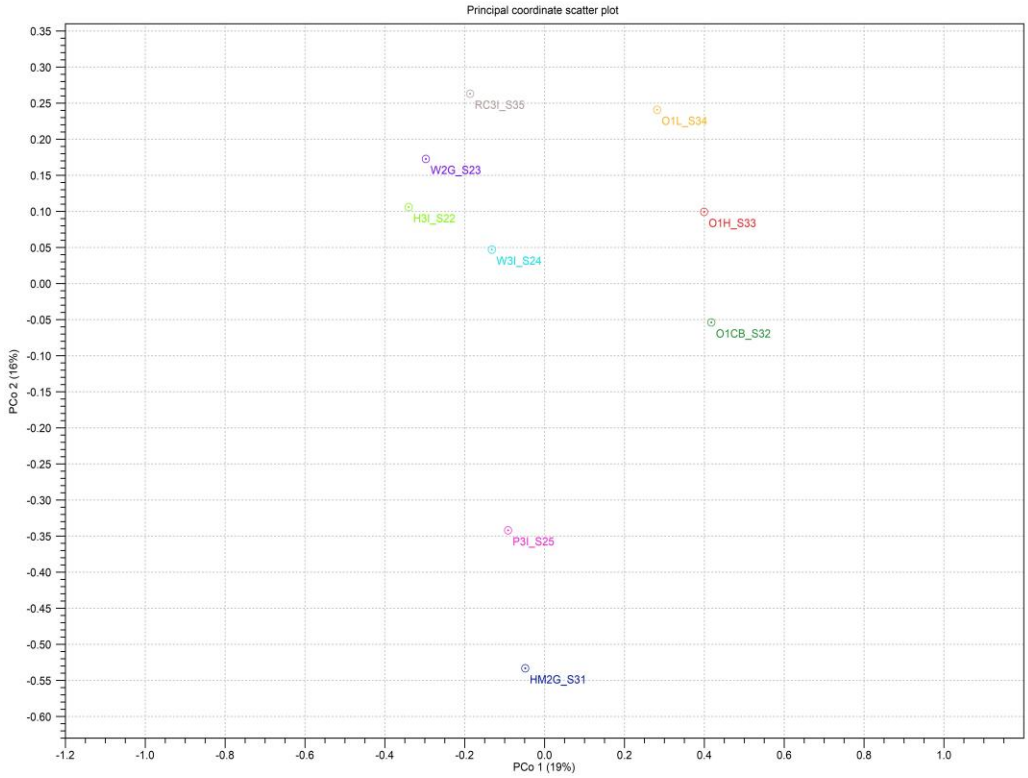
4.3.2 Beta Analysis

The principal coordinates analysis (PCoA) plot based on Jaccard distance (**Figure 4.3.2a**) observed clear separation of bacterial communities among different sample types. Three fruit samples (W3I, W2G, H3I) were clustered more closely, indicating higher similarity in their community composition. In contrast, fruit (P3I) and deli meat (HM2G) sample appeared highly dispersed from other samples.

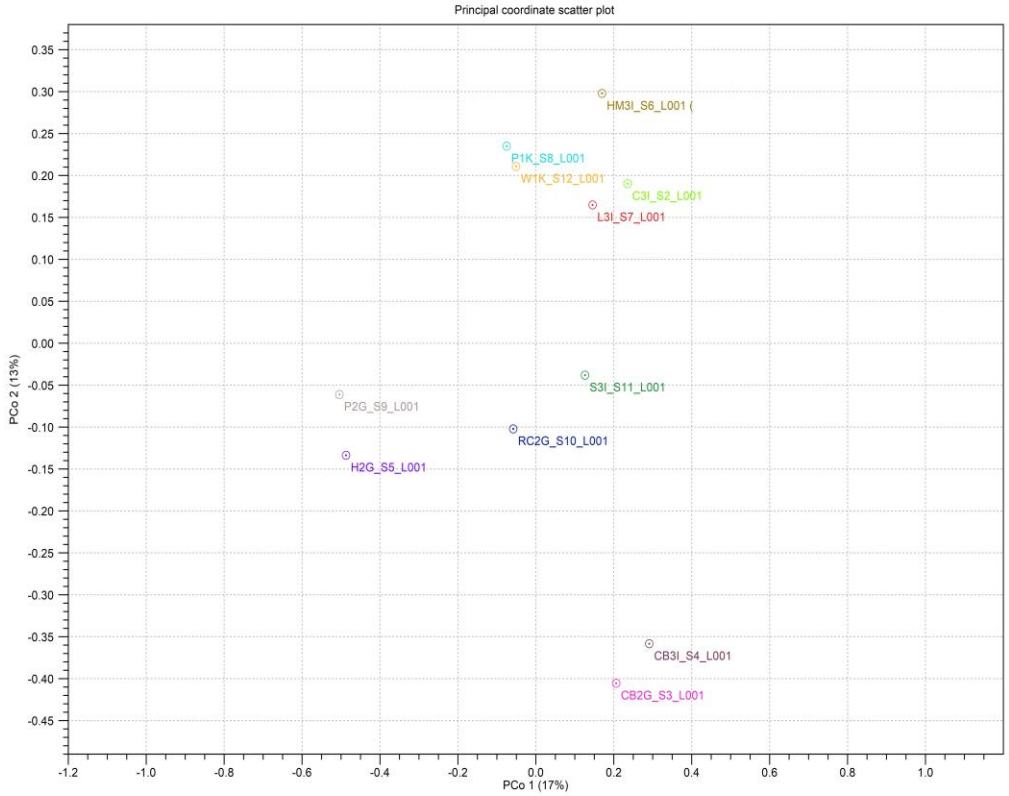
The 11 samples in **Figure 4.3.2b** formed four clusters. Three of the clusters (P2G, H2G; P1K, W1K; L3I, C3I) consisted of samples from the same sampling location, indicating location driven similarity in bacterial communities. However, S3I, which was collected from the same location as L3I and C3I, appeared distinctly separated from their cluster. Additionally, CB3I and CB2G, although collected from different locations, clustered closely together, suggesting that sample type can also influence community composition.

The third batch of samples in **Figure 4.3.2c** showed a similarity in community composition between H1K and C2G, despite differences in both sample type and location. Interestingly, C2G, S2G, and L2G, which were collected from the same location, did not cluster together. Furthermore, meat samples from the same location were distinctly separated, likely reflecting the strong influence of sample type and processing methods on community composition.

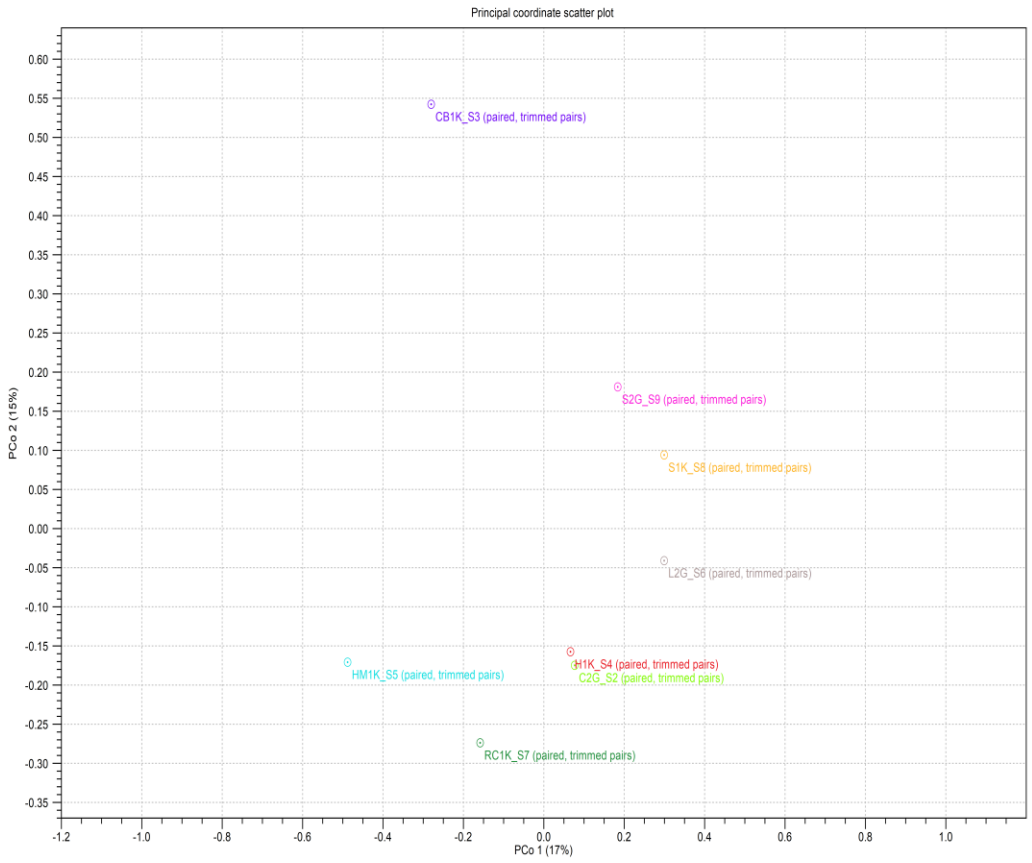
In Figure 4.3.2d, C1K and L1K did not cluster together, likely due to differences in sample type. This separation suggests that community composition was more strongly influenced by the type of produce rather than the sampling location. Since the sequencing was performed in four separate batches, PCoA plots were generated independently for each batch. Therefore, variation percentages on PCoA axes differ across figures and are not directly comparable.


Principal coordinate scatter plot
RC3I_S35
O1L_S34
W2G_S23
H3I_S22
O1H_S33
W3I_S24
O1CB_S32
P3I_S25
HM2G_S31
PCo 2 (16%)
PCo 1 (19%)

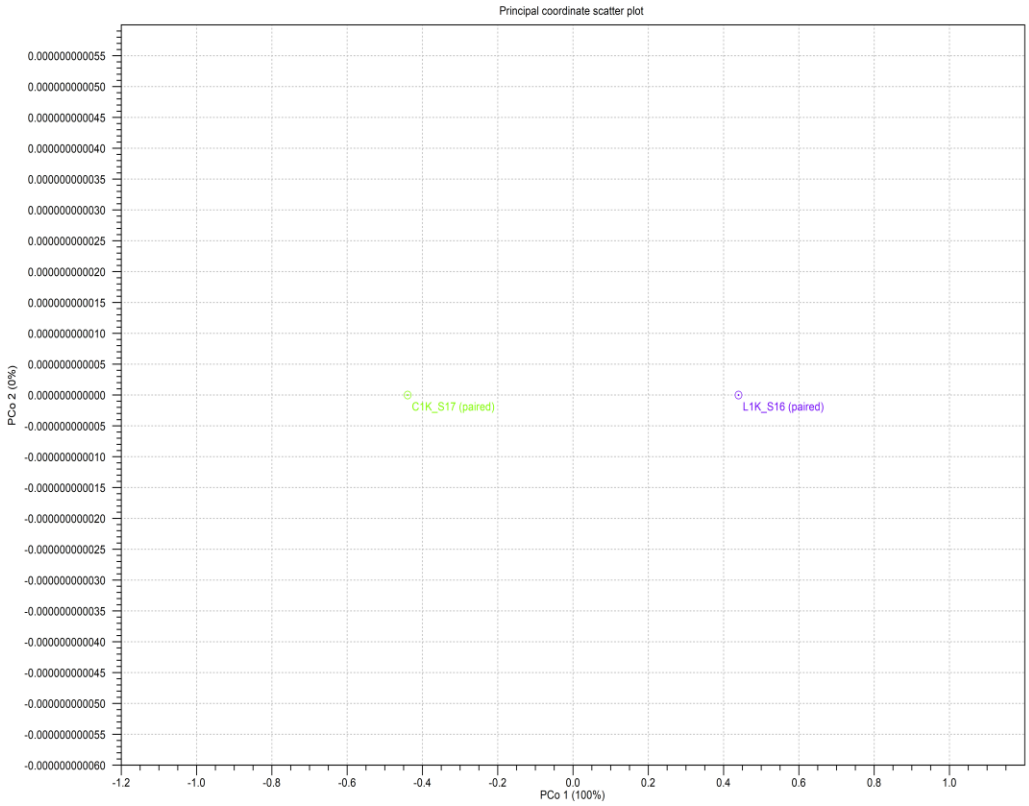
(a)


Principal coordinate scatter plot
HM3I_S6_L001
P1K_S8_L001
W1K_S12_L001
C3I_S2_L001
L3I_S7_L001
S3I_S11_L001
P2G_S9_L001
RC2G_S10_L001
H2G_S5_L001
CB3I_S4_L001
CB2G_S3_L001
PCo 2 (13%)
PCo 1 (17%)

(b)


Principal coordinate scatter plot
CB1K_S3 (paired, trimmed pairs)
S2G_S9 (paired, trimmed pairs)
S1K_S8 (paired, trimmed pairs)
L2G_S6 (paired, trimmed pairs)
H1K_S4 (paired, trimmed pairs)
C2G_S2 (paired, trimmed pairs)
HM1K_S5 (paired, trimmed pairs)
RC1K_S7 (paired, trimmed pairs)
PCo 2 (15%)
PCo 1 (17%)

(c)



(d)

Figure 4.3.2: Principal coordinates analysis based on Jaccard distance in different batches. (a) 9 samples including HM2G, P3I, O1L, O1CB, RC3I, W3I, O1H, W2G, H3I. (b) 11 samples including HM3I, CB3I, RC2G, CB2G, W1K, S3I, P2G, P1K, L3I, H2G, C3I. (c) 8 samples including S2G, S1K, RC1K, L2G, HM1K, H1K, CB1K, C2G. (d) 2 samples including L1K, C1K.

4.3.3 Phylum Level Composition Across Sample

4.3.3.1 Vegetables

In vegetable samples, the bacterial phylum Pseudomonadota dominated, with relative abundances varying from 55% to 93% (**Figure 4.3.3a**). Bacillota were detected in all vegetable samples purchased from Kampar (C1K, S1K, and L1K); however, they were absent in certain samples from Gopeng and Ipoh (S2G and L3I). The abundance of Bacillota was notably higher in the Kampar samples compared to those from Gopeng and Ipoh. The presence of Bacteroidota was relatively low, appearing only in spinach collected from Ipoh (S3I) at 1%. Bacillota were absent in lettuce and spinach samples from Ipoh and Gopeng (L3I and S2G). The category labelled as 'Others' comprised taxa that were either unclassified or present in low abundances. These low abundance phyla include Actinomycetota, Fusobacteriota, Gemmatimonadota and Cyanobacteriota. The highest proportion of 'Others' was observed in lettuce and cabbage samples collected from Ipoh (L3I and C3I), at 32% and 22%, respectively; followed by lettuce sample collected from Gopeng (L2G, 15%).

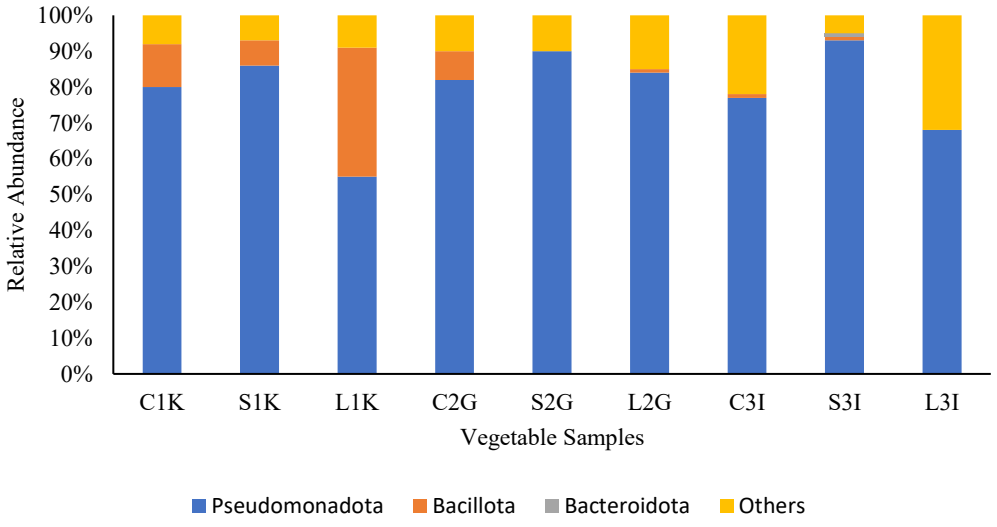
4.3.3.2 Meats

Meat samples were primarily dominated by Pseudomonadota and followed by Bacillota (**Figure 4.3.3b**). The most notable observation was deli meat sample (HM2G), which were dominated by Bacillota. Bacillota had higher abundance in three deli meat samples (HM1K, HM2G, HM3I) compared to other meat samples. Seven meat samples (RC1K, CB1K, RC2G, CB2G, RC3I, CB3I, HM3I) were dominated by Pseudomonadota, with relative abundances ranging from 55% to 96%. The phyla Bacteroidota were present in HM1K sample, with a 7% relative abundance. The 'Others' category, ranging from 2% to 15%, likely includes uncultured bacteria, low abundance taxa and some taxa that have not been classified. This category included of phyla such as Actinomycetota, Gemmatimonadota and Cyanobacteriota.

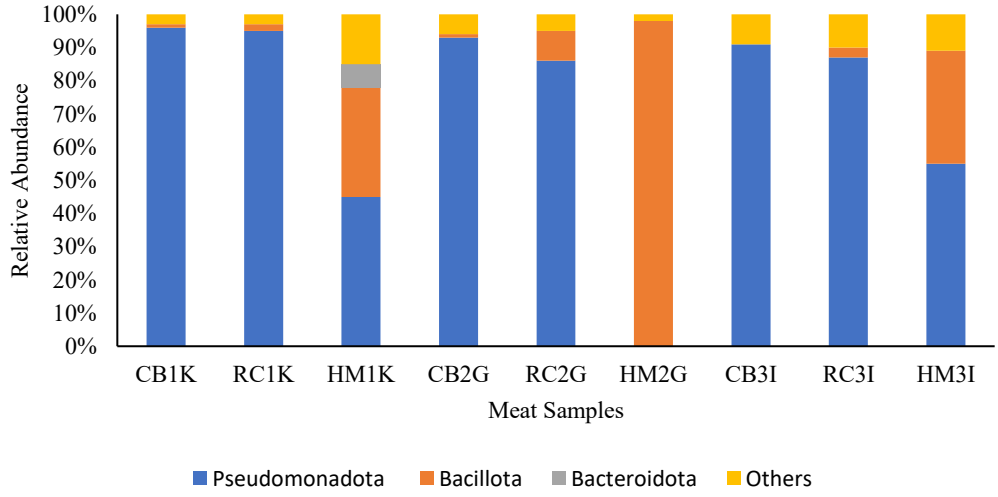
4.3.3.3 Fruits

Among fruit samples, the dominant bacterial phylum was Pseudomonadota, with the highest relative abundance observed in P1K (91%). Bacillota were the second most prevalent phylum but exhibited variability, with relative abundance below 10% in H2G, H3I, W3I, P1K, and P3I (**Figure 4.3.3c**). Samples from Kampar and Gopeng generally showed higher Bacillota abundance compared to those from Ipoh. The 'Others' category accounted for up to 16% of relative abundance in P3I, largely attributed to uncultured bacteria. This group also included low-abundance phyla

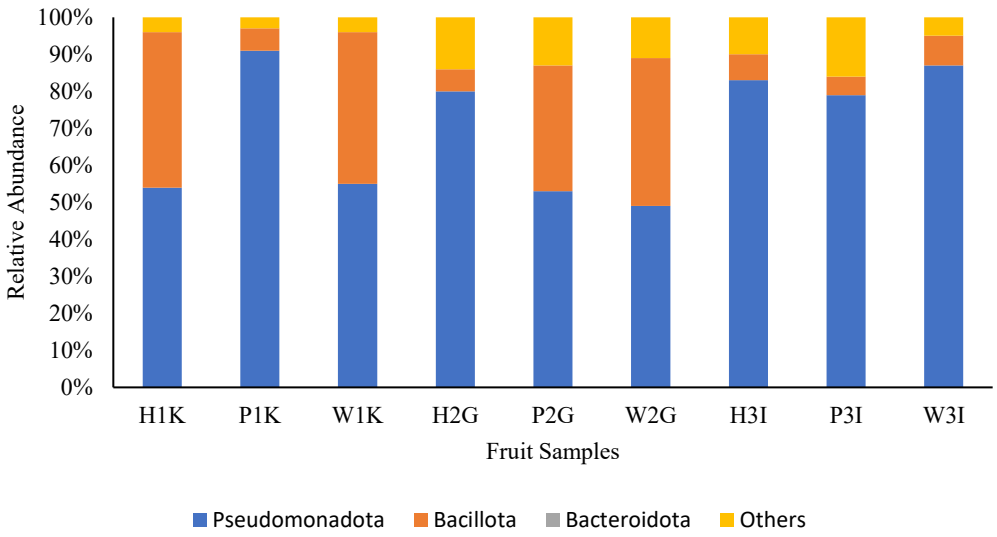
such as Cyanobacteriota and Actinomycetota. In contrast, Bacteroidota were absent across all fruit samples.



(a)



(b)



(c)

Figure 4.3.3: Relative abundance of bacterial phyla in different sample groups from Kampar (1K), Gopeng (2G), and Ipoh (3I). **(a)** Vegetable samples (C, S, L). **(b)** Meat samples (CB, RC, HM). **(c)** Fruit samples (H, P, W).

4.3.4 Genus Level Composition Across Sample

In this section, only the top five most abundant taxa are reported. This approach was chosen to highlight the dominant members of the microbial community, which are most likely to influence the overall composition and food safety. Reporting all detected taxa would result in an overly complex dataset, as many were present in very low abundances.

4.3.4.1 Vegetables

At the genus level, the bacterial abundance of vegetable samples is presented in **Figure 4.3.4**. *Pectobacterium* was observed in all vegetable samples with different relative abundances. The highest abundance was observed in sample S3I (30%), followed by C1K (21%) and S1K (20%). *Pectobacterium* abundance was lower in lettuce samples compared to cabbage and Chinese spinach. *Pseudomonas* represented another major genus found in every vegetable sample, with the highest abundance in cabbage from Gopeng (32%). *Citrobacter* was detected exclusively in cabbage samples (C1K, 6% and C3I, 0.3%). The genus *Enterobacter* had relative abundances ranging from 6% to 11%, appearing in five samples from Gopeng and Ipoh (C2G, S2G, L2G, S3I, and L3I). The abundance of *Pantoea* is highest in lettuce and cabbage samples, showing a low abundance of 0.1% in S3I and being absent in S1K and S2G. *Aeromonas* had the highest relative abundance in sample S2G (41%) but was less abundant in cabbage samples. The 'Others' category, which included genera with low relative abundances and unclassified bacteria, accounted for 15% to 54% of the microbial community across samples.

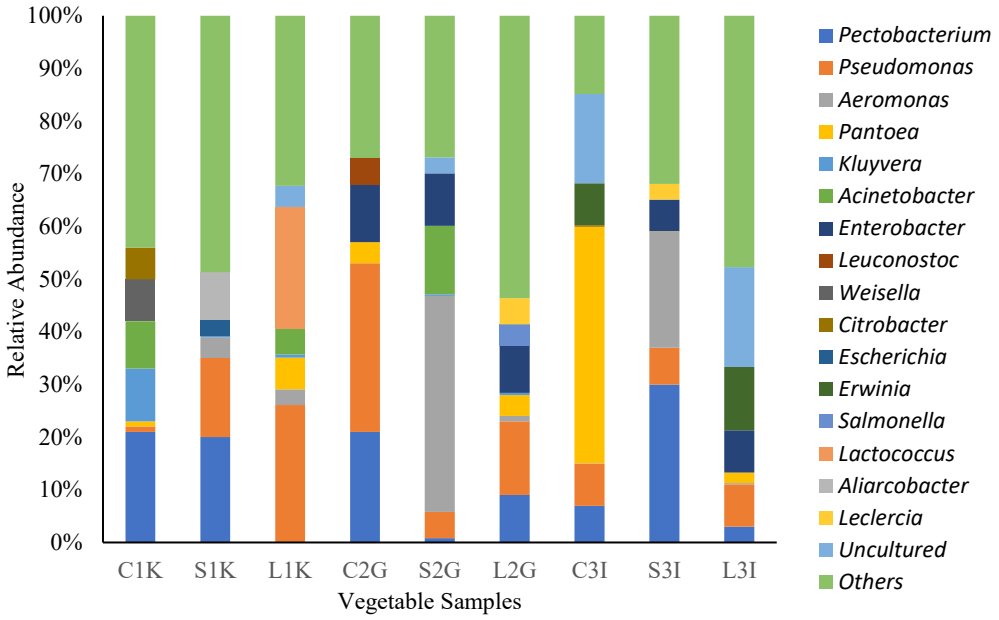


Figure 4.3.4: Major bacterial genera found in vegetable samples ranked by abundance (*Pantoea*, *Aeromonas*, *Pseudomonas*, *Pectobacterium*, *Lactococcus*). The 'Others' category includes low-abundance or unclassified genera.

4.3.4.2 Meats

The bacterial composition at the finer taxonomic level in meat samples is depicted in **Figure 4.3.5**. Notably, the abundance of *Aeromonas* varied substantially across four samples, which ranges 0.3% to 65%, and was predominantly found in raw chicken meat samples. *Pseudomonas* was found in every sample, apart from one deli meat sample from Gopeng (HM2G), with its abundance being lower in deli meats compared to raw and roasted chicken samples. *Moellerella* was identified exclusively in deli meats, with abundances of 4% in sample HM1K and 33% in

sample HM3I. Sample HM2G exhibited a significant presence of *Lactococcus* at 44%, which was absent in other samples. Additionally, *Lactiplantibacillus* and *Leuconostoc* were uniquely identified in deli meat samples HM2G and HM3I. Conversely, *Acinetobacter* was exclusive to raw and roasted chicken samples, with abundances between 1% and 9%. Interestingly, *Vibrio* was dominant in sample RC1K, accounting for 65%, but was undetected in the remaining samples. The category 'Others', representing less abundant genera, ranged from 20% to 52%, with the highest relative abundance found in sample HM1K (deli meat).

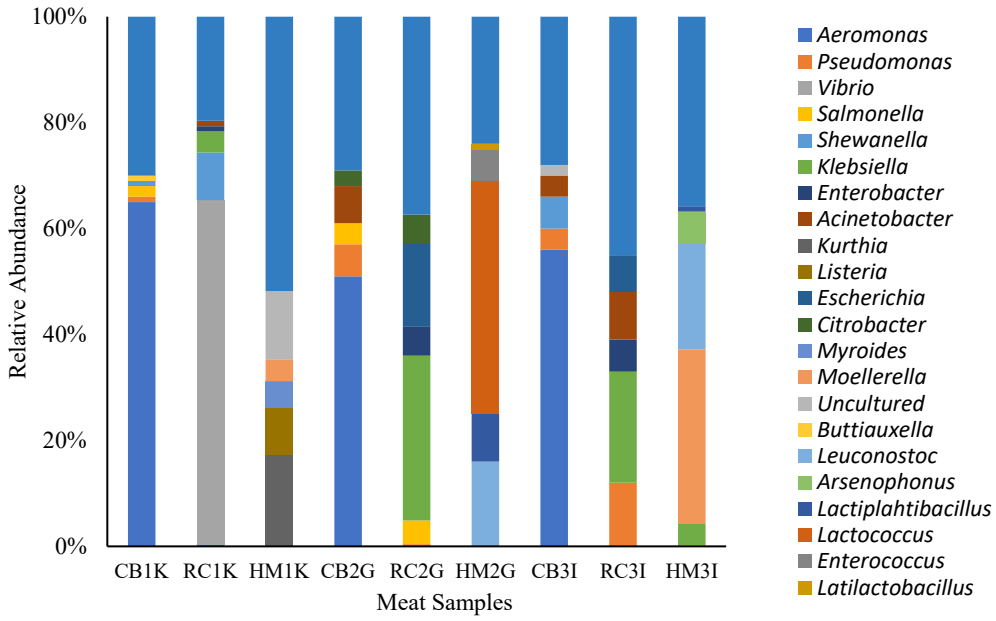


Figure 4.3.5: Predominant bacterial genera detected in meat samples ranked by abundance, including *Aeromonas*, *Vibrio*, *Lactococcus*, *Klebsiella*, *Moellerella*. The 'Others' category encompasses low-abundance or unclassified genera.

4.3.4.3 Fruits

The bacterial abundance at genus level in fruit samples were illustrated in **Figure 4.3.6**. *Leuconostoc* was detected in all of the fruit samples, with abundances ranging from 1% to 23%. Its abundance was lower in fruit samples collected from Ipoh compared to those from Kampar and Gopeng. *Enterobacter* exhibited a wide range of abundance (1% to 74%) across all samples and had highest abundance in the papaya sample (P1K), where it reached 74%. *Klebsiella* were detected exclusively in honeydew samples, with the lowest abundance of 3% in sample H2G and the highest at 26% in sample H3I. Meanwhile, *Acinetobacter* was only detected in samples from Gopeng, with abundances ranging from 6% to 11%. The presence of uncultured bacterium was higher in Gopeng samples, peaking at 11% in sample P2G. Furthermore, sample W1K had a notable abundance of *Tatumella* at 32%, which was not detected in any other samples. The category 'Others' represented a relative abundance of 13% to 57%, with higher values observed in samples from Kampar and Gopeng.

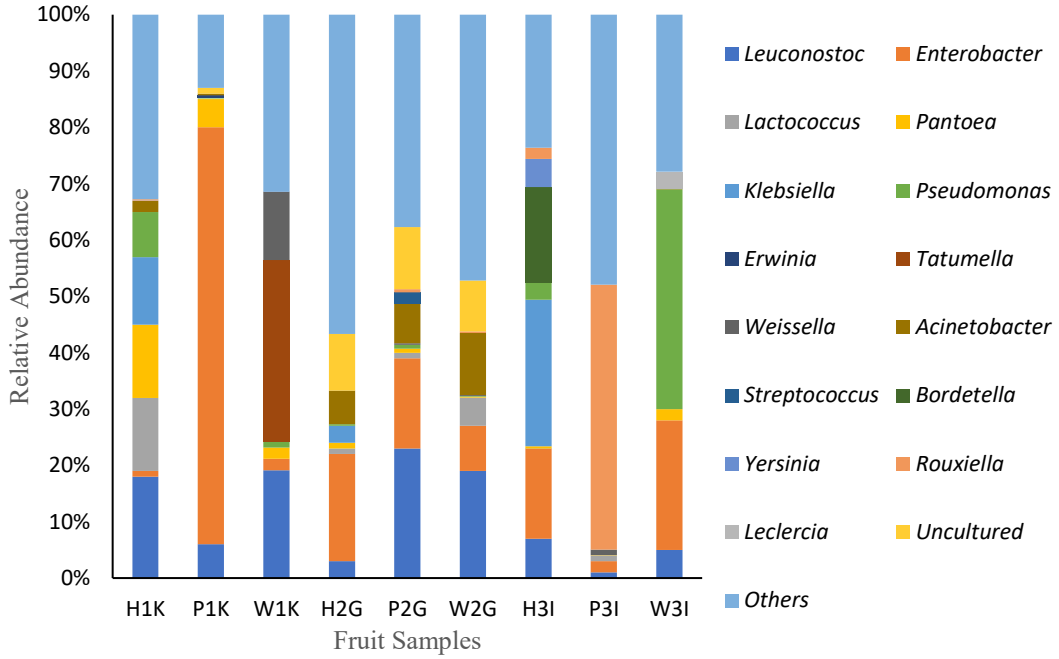


Figure 4.3.6: Top five abundant bacterial genera identified in fruit samples, including *Enterobacter*, *Rouxiella*, *Leuconostoc*, *Tatumella* and *Pseudomonas*. The 'Others' category includes low-abundance or unclassified genera.

4.4 Prevalence of Foodborne Pathogen based on Location

The total counts of the five detected foodborne pathogens are summarised in **Figure 4.4**. Among the vegetable samples, those purchased from Kampar exhibited the highest prevalence, with 13 detections, excluding two negative results for *Sh. flexneri*. This was followed by samples from Gopeng with 10 detections, while vegetables from Ipoh had the lowest prevalence, with a total of 6 detections. For meat samples, the highest prevalence of foodborne pathogens was observed in Gopeng, accounting for 7 detections, followed by Ipoh with 6, and Kampar with 5.

In the fruit sample group, the highest number of foodborne pathogens was detected in samples from Ipoh, totalling 7. This was followed by Gopeng with 6 detections, and Kampar with 5.

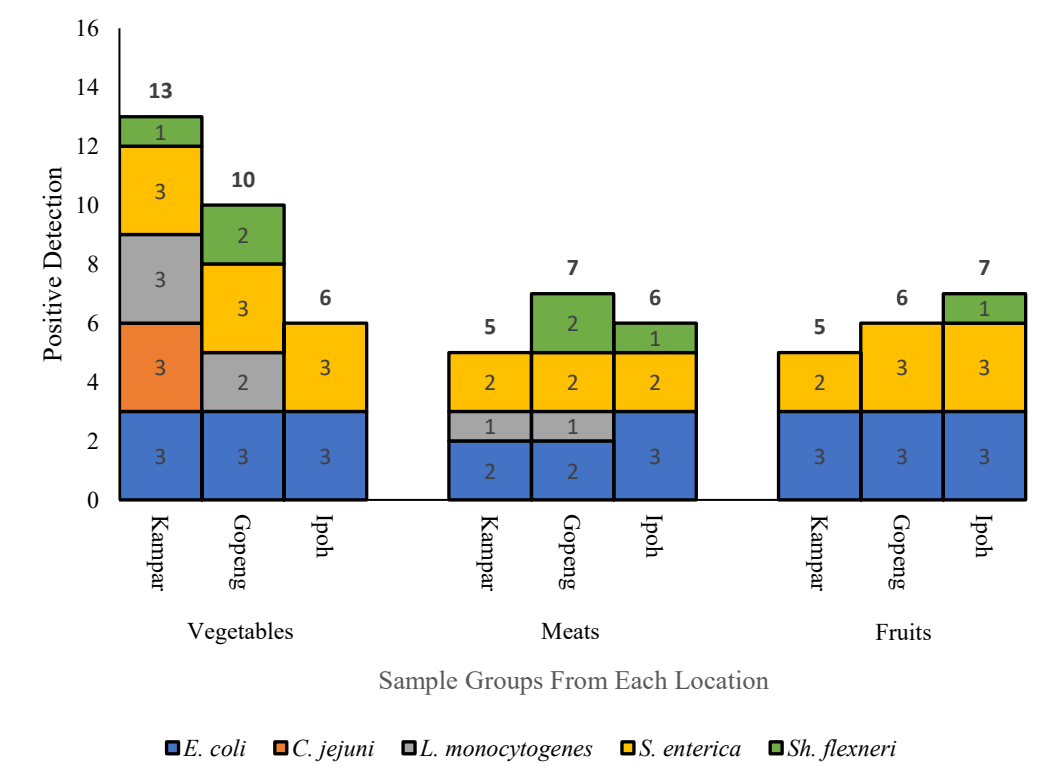


Figure 4.4: Bar chart showing the prevalence of five foodborne pathogens detected in sample groups (vegetables, meats and fruits) collected from Kampar, Gopeng, and Ipoh.

4.5 Foodborne Pathogen Detection

The detection of pathogen in various samples, analysed through metagenomics and culture-based PCR techniques, is summarized in **Figure 4.5** and **Table 4.6**. A '+' symbol indicates detection by both methods, 'M' denotes detection by metagenomics only, 'C' represents detection by culture-based PCR only, and '/' indicates no detection by either method.

Although *E. coli* is not strictly classified as a foodborne pathogen, its presence is significant for food safety, as certain pathogenic strains such as O157:H7 and O104:H4 can cause severe human infections (Bairi, Savalia and Ram, 2024). Both methods detected *E. coli* in all vegetable samples, six meat samples (CB1K, RC1K, CB2G, RC2G, CB3I, RC3I), and seven fruit samples (H1K, W1K, H2G, P2G, W2G, H3I, W3I). Notably, sample HM1K was detected only by metagenomics, whereas a deli meat sample from Ipoh was identified as positive exclusively by the culture-based method.

For *Campylobacter jejuni*, three vegetable samples from Kampar were contaminated, with detection achieved solely by culture-based PCR; metagenomics failed to identify this pathogen in these samples. Similarly, *Listeria monocytogenes* was detected in five vegetable samples and two deli meat samples (HM1K and HM2G), but only through culture-based PCR. Metagenomics analysis could not detect *L. monocytogenes* in any vegetable samples, though both methods successfully identified it in the deli meat samples.

The presence of *Salmonella enterica* was widespread across vegetable samples, with three samples (C2G, C3I, L3I) detected solely by the culture-dependent method. Among meat samples, metagenomics detected *S. enterica* in a deli meat sample from Kampar, while the culture-based method failed to identify it. However, six of the eight meat samples tested positive by the culture-based method. In fruit samples, *S. enterica* was detected in all samples, but three (P1K, P3I, W3I) were exclusively identified by the culture-dependent method. *Shigella flexneri* was detected in three vegetable samples (C1K, C2G, L2G) using culture-based PCR. Among meat samples, *S. flexneri* was detected in CB2G, RC2G, and RC3I by both methods, confirming its presence.

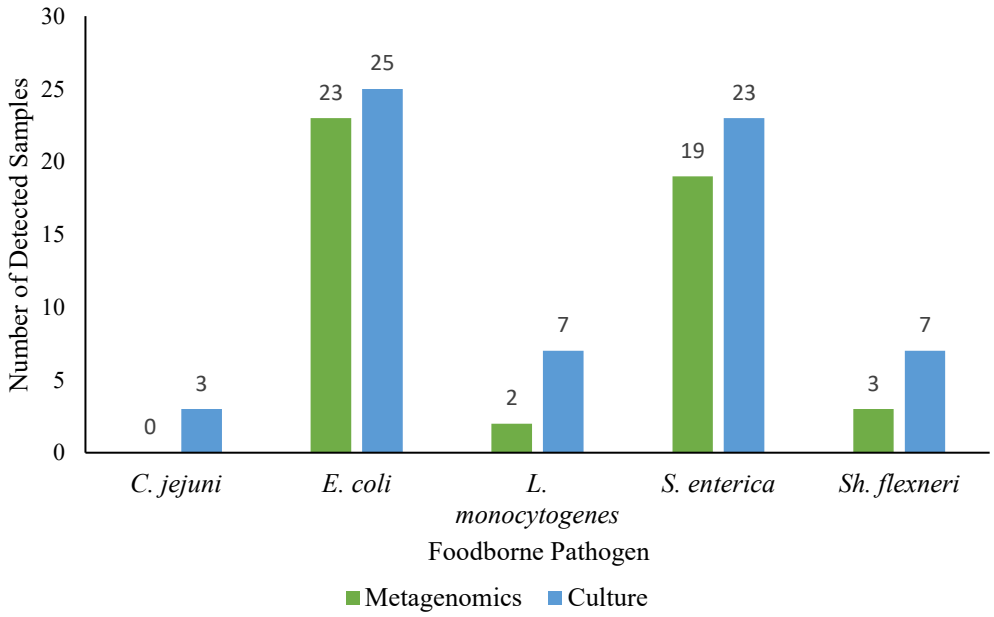


Figure 4.5: The evaluation of detection efficiency for foodborne pathogens between metagenomics and culture.

Cohen's Kappa analysis revealed a substantial level of agreement ($\kappa = 0.655$, $p < 0.001$) between PCR and shotgun metagenomics in detecting foodborne pathogens. However, McNemar's test indicated a statistically significant difference between the two methods ($p < 0.001$), suggesting that the discordant results were not due to chance. The results demonstrate discrepancies between the metagenomics and culture-based PCR methods, highlighting their complementary roles in pathogen detection. The differences in detection could be attributed to limitations in sensitivity, sample preparation, or inherent biases in either method.

Table 4.6: Detection of foodborne pathogens in vegetable (n=9), meat (n=9) and fruit (n=9) samples using metagenomics analysis and culture dependent method.

Samples		**Foodborne Pathogen**				
		C. jejuni	*E. coli*	*L. monocytogenes*	*S. enterica*	*Sh. flexneri*
Vegetable	C1K	C	+	C	+	C
	S1K	C	+	C	+	/
	L1K	C	+	C	+	/
	C2G	/	+	C	C	C
	S2G	/	+	/	+	/
	L2G	/	+	C	+	C
	C3I	/	+	/	C	/
	S3I	/	+	/	+	/
	L3I	/	+	/	C	/
Meat	CB1K	/	+	/	+	/
	RC1K	/	+	/	+	/
	HM1K	/	M	+	M	/
	CB2G	/	+	/	+	+
	RC2G	/	+	/	+	+
	HM2G	/	/	+	/	/
	CB3I	/	+	/	+	/
	RC3I	/	+	/	+	+
	HM3I	/	C	/	/	/
Fruit	H1K	/	+	/	+	/
	P1K	/	C	/	C	/
	W1K	/	+	/	/	/
	H2G	/	+	/	+	/
	P2G	/	+	/	+	/
	W2G	/	+	/	+	/
	H3I	/	+	/	+	/
	P3I	/	C	/	C	C
	W3I	/	+	/	C	/

M=Detected by Metagenomics Analysis.
C=Detected by Culture & PCR.
+=Detected by Both Methods.
/= Not detected by Both Methods.

4.6 Antimicrobial Resistance Gene (ARG)

Metagenomic analysis identified antimicrobial resistance (AMR) genes across 27 food samples, with results presented in tables and visualised in a heatmap to highlight their distribution by sample and antibiotic class (**Figure 4.6**, **Table 4.7**, **Table 4.8**, **Table 4.9**). The table provides a complete list of ARGs detected in each sample, along with total number of ARGs in the sample. The figure uses darker shades to represent greater abundance of specific resistance genes. Resistance genes linked to aminocoumarin, glycylcycline, and phosphonic acid were detected at the lowest levels across all samples.

The full list of AMR genes detected in vegetable samples were provided in **Table 4.7**. The number of ARGs per sample varied widely, ranging from 7 in C3I to as many as 108 in S1K. Commonly detected genes included sul genes (*sul1, sul2*), aminoglycoside resistance determinants such as *APH(3'), APH(6),* and *ANT(3'')* family genes, as well as multiple β-lactamases (*TEM, OXY, OXA, SHV*).

Table 4.7: Antibiotic resistance genes detected in vegetable samples.

Sample	ARGs Detected	Number of ARGs
C1K	*APH(3')-IIIa, aadA3, aadA2, DHA-17, LAP-2, LEN-15, SHV-187, ACT-23, OXY-2-6, ADC-23, APH(6)-Id, APH(3'')-Ib, lnuA, OXY-4-1, APH(3')-Ia, CMY-108, CMY-107, QnrS1, qnrE1, QnrB23, dfrA12, sul1, sul2, dfrA1, dfrA14, KpnE, KpnF, qacEdelta1, tet(A), lmrD, tet(C), tet(39), floR, eptB*	34
S1K	*aadA2, aadA3, APH(3'')-Ib, APH(6)-Id, OXA-850, OXA-486, ANT(3'')-IIa, APH(3')-IIb, tetX, ANT(9)-Ia, FosA6, ACT-16, catB7, APH(3')-IIIa, SAT-4, CMH-1, LAP-2, OXY-1-6, OXY-1-1, OXA-203, LEN-16, ANT(6)-Ia, EreD, OXA-10, AAC(6')-Ib9, TEM-181, PDC-55, ADC-4, CTX-M-8, mphG, HERA-3, catQ, ampH, catI, SHV-213, soxR, ErmB, ErmQ, optrA, QnrD1, tet32, QnrS1, QnrVC4, QnrB58, QnrB23, QnrS2, lsaE, QnrB34, QnrB12, sul2, dfrA14, dfrA1, dfrA16, sul1, cmlA5, qacEdelta1, MexA, bcr-1, mexP, MexG, MexH, AmvA, tet(A), opmE, abeS, emrE, adeI, AcrE, cmx, adeA, mexY, mdtG, mdtH, fexA, tet(39), floR, mdtO, mdtF, mdtE, mexM, OpmD, msbA, emrY, emrK, TriA, TriB, MexC, mefC, tet(C), PmrF, eptA, eptB, LpsB, marA, basS, ArmR, cprR, cprS, adeN, ParS, CRP, adeR, H-NS, adeL, evgA, AcrS, MexL, bacA*	108
L1K	*mphA, AAC(6')-Iz, TEM-181, LEN-13, SHV-187, APH(3')-Ia, LAP-2, aadA3, aadA2, APH(6)-Id, APH(3'')-Ib, lnuG, QnrD1, tetS, QnrS1, dfrA14, sul3, dfrA12, sul2, sul1, dfrA1, tet(39), LptD, CrcB, floR, adeJ, adeI, tet(C), adeH, abeM, qacEdelta1, tet(A), KpnF, KpnE, smeA, smeB, eptB, ArnT, MCR-9.1, OmpA, OmpK37, adeN, adeR, adeL, smeR, smeS*	46
C2G	*ACT-22, FosA6, AAC(6')-Ib-Hangzhou, TEM-181, ANT(9)-Ia, aadA2, qnrE1, QnrS2, sul1, KpnE, tet(C), lmrD, qacEdelta1, eptB*	14
S2G	*TEM-181, OXA-930, OXA-356, lnuG, OXA-91, OXA-413, CTX-M-8, OKP-B-6, OKP-B-4, OKP-B-17, aadA16, arr-3, mphA, LEN-3, LEN-4, APH(3'')-Ib, APH(6)-Id, lnuA, LAP-2, mphE, FosA3, CMY-107, CMY-108, aadA3, aadA2, ANT(3'')-IIa, APH(3')-Ia, MIR-11, OXA-664,*	70

	AAC(6')-Ib-cr4, AAC(6')-Ib-cr6, AAC(6')-Ib-cr1, OXA-10, QnrB17, QnrB6, QnrS1, QnrS2, QnrB29, QnrB13, QnrVC4, qnrE2, QnrD1, sul2, dfrA27, dfrA1, dfrA14, sul1, dfrA10, dfrG, dfrA15, dfrA12, tet(A), lmrD, AbaQ, AbaF, abeS, qacL, tet(C), KpnF, KpnG, acrA, qacEdelta1, floR, tet(39), cmlA5, eptB, ArnT, adeR, ramA, adeL	
L2G	*TEM-181, FONA-6, HERA-6, aadA3, APH(3")-Ib, APH(6)-Id, ACT-7, qnrE1, sul1, lmrD, tet(39), qacEdelta1, tet(C)*	13
C3I	*TEM-181, APH(3")-Ib, APH(6)-Id, CMY-71, QnrB17, QnrB6, sul1*	7
S3I	*APH(3")-Ib, APH(6)-Id, CMH-1, OXY-4-1, aadS, MIR-13, tetX, aadA3, aadA2, MOX-2, SHV-187, FosA6, ACT-35, ACT-7, ACT-32, ACT-27, QnrS2, QnrD1, QnrS1, sul2, sul1, dfrA1, dfrA14, tet(C), floR, tet(39), KpnE, KpnG, qacEdelta1, tet(A), oqxA, eptB, ArnT, OmpK37, ramA*	35
L3I	*APH(3")-Ib, TEM-181, ADC-41, OXA-208, ACT-2, ACT-28, ACT-9, OXA-417, ADC-2, ADC-58, sul1, AbaQ, adeB, abeS, adeH, tet(39), abeM, adeK, adeJ, adeI, adeL, adeN*	22

Detected ARGs in meat samples were presented in **Table 4.8**. The number of ARGs ranged widely, from only 2 in HM2G to as many as 87 in CB2G. Commonly detected genes included *APH(3'), sul, Qnr, dfr* and multiple β-lactamases. In contrast to vegetable samples, meat samples showed a higher prevalence of diaminopyrimidine and quinolone resistance genes.

Table 4.8: Antibiotic resistance genes detected in meat samples.

Sample	ARGs Detected	Number of ARGs
CB1K	*catII, mphE, ampC1, OXA-355, OXA-257, ampC, APH(3')-Ia, APH(3'')-Ib, APH(6)-Id, AAC(6')-Ib9, arr-3, aadA16, catB8, ANT(3'')-IIa, OXA-10, OXA-663, TEM-181, msrE, QnrB5, QnrVC6, QnrD1, QnrB38, tetS, QnrVC1, QnrS2, QnrVC4, sul3, sul2, sul1, dfrA27, qacL, mdtA, emrY, KpnH, emrB, mdtE, acrA, mdtP, tet(E), tet(A), floR, mdtG, qacEdelta1, tet(L), tet(C), tet(39), mdtN, cmlA5, eptA, kdpE, evgA, gadX, CRP, AcrS*	54
RC1K	*FosA6, APH(3'')-Ib, APH(6)-Id, SHV-187, lnuG, CMH-1, OKP-B-1, SAT-2, LAP-2, OXA-55, tetM, QnrA3, QnrD1, sul3, dfrA1, sul2, abeM, oqxB, AmvA, adeH, tet(39), abeS, AbaQ, adeJ, adeK, tet(A), LptD, qacH, floR, qacEdelta1, KpnF, KpnE, tet(K), acrA, KpnG, eptB, ArnT, OmpA, ramA, adeL*	40
HM1K	*aadS, lnuA, SAT-2, APH(3')-Ia, aadA2, aadA3, mphG, tetX, TEM-181, APH(3'')-Ib, APH(6)-Id, tetS, QnrD1, dfrA1, sul1, dfrA12, sul2, tet(L), tet(H), qacEdelta1, mefC, floR, qacH, tet(B)*	24
CB2G	*APH(3')-Ia, OXA-320, OXA-1, SAT-2, APH(4)-Ia, AAC(3)-IV, TEM-181, OXA-230, FosA4, AAC(3)-IId, catB3, FosA3, mphE, catII, ANT(3'')-IIa, LAP-2, OXA-308, APH(3'')-Ib, APH(6)-Id, SHV-187, ampC1, OXA-10, ANT(2'')-Ia, VEB-1, MOX-6, tetX, mphA, MIR-15, AAC(6')-Ib9, QnrS1, QnrVC1, QnrD1, QnrS2, QnrVC4, msrE, tetS, dfrA14, sul3, dfrB4, dfrA12, sul1, sul2, dfrA15, dfrA1, mdtG, mdtE, TolC, AcrF, tet(E), emrB, mdtF, emrA, floR, tet(C), mdtO, msbA, AcrE, acrA, lmrD, oqxB, oqxA, tet(A), tet(D), LptD, cmlA5, KpnE, KpnF, mdtN, tet(39), emrK, emrY, qacEdelta1, eptA, PmrF, eptB, ArnT, OmpA, OmpK37, gadX, baeS, baeR, AcrS, H-NS, kdpE, cpxA, evgA, golS*	87
RC2G	*TEM-181, OXA-383, OXA-78, OXA-338, OXA-386, lnuA, FosA6, APH(3')-Ia, SHV-187, OKP-B-6, aadA27, APH(3'')-Ib, APH(6)-Id, mphA, LAP-2, aadA3, aadA2, APH(3')-VI, aadA5, tetM, QnrS1, tetS, QnrD1, fusc, dfrA15, dfrA1, dfrA14, sul2, sul1, tet(A), KpnE, KpnF,*	48

	LptD, tet(D), tet(L), CrcB, oqxA, oqxB, KpnG, tet(C), acrA, qacH, qacEdelta1, tet(39), floR, ArnT, OmpA, OmpK37	
HM2G	*TEM-181, tetS*	2
CB3I	*APH(3')-Ia, CARB-3, arr-3, ACC-1, APH(3'')-Ib, APH(6)-Id, Yrc-1, catII, AAC(6')-Ib9, ANT(3'')-IIa, aadA3, aadA2, catB8, OXA-10, QnrB19, QnrS2, QnrVC4, QnrA1, tetS, QnrVC1, QnrVC6, QnrD1, dfrA27, sul2, dfrA14, sul1, dfrA1, tet(C), tet(A), tet(39), tet(E), cmlA5, MCR-3.3, MCR-5.1*	34
RC3I	*CTX-M-8, CTX-M-152, CTX-M-78, CMY-150, CFE-1, CMY-124, ACT-25, ACT-16, aadA27, OXA-208, AAC(6')-If, catA4, mphE, lnuA, AAC(3)-IV, MIR-3, APH(6)-Id, APH(3'')-Ib, LAP-2, arr-3, aadA15, aadA3, AAC(6')-Ib7, LEN-10, LEN-20, LEN-55, OXA-355, msrF, msrE, QnrS1, fusC, QnrD1, dfrA14, dfrA1, sul2, sul1, abeM, tet(L), tet(K), tet(J), lmrD, adeI, qacH, KpnE, tet(39), KpnG, LptD, tet(A), tet(C), tet(Y), KpnE, KpnF, qacEdelta1, cmlA6, CrcB, eptB, LpsB, OmpK37, ramA*	59
HM3I	*TEM-117, APH(6)-Id, APH(3'')-Ib, sul2, dfrA1, tet(D)*	6

The ARGs detected in fruit samples are summarised in **Table 4.9**. Overall, the number of ARGs was lower than in vegetables and meat, ranging from 2 in P1K and W1K to 29 in H1K. The most encountered were various β-lactamases family genes, followed by sulfonamide resistance genes. Compared to vegetables and meat, fruit samples exhibited a narrower ARG profile, with resistance largely dominated by β-lactamases.

Table 4.9: Antibiotic resistance genes detected in fruit samples.

Sample	ARGs Detected	Number of ARGs
H1K	*LAP-2, TEM-181, aadA3, aadA2, lnuA, APH(3'')-Ib, ACT-16, MIR-5, MIR-10, MIR-3, LEN-16, LEN-10, LEN-20, LEN-19, LEN-30, ADC-8, QnrS1, tetS, tetM, dfrA14, sul2, sul1, tet(A), floR, KpnE, tet(L), qacEdelta1, tet(39), fexA*	29
P1K	*sul1, ACT-7*	2
W1K	*sul1, ACT-7*	2
H2G	*TEM-181, LEN-24, LEN-13, LEN-11, CTX-M-77, OKP-B-6, OKP-B-4, CMH-1, ACT-2, MIR-10, tetS, QnrS1, sul1, dfrA1, sul2, lmrD, qacEdelta1, tet(A)*	18
P2G	*ACT-35, OKP-B-2, OKP-B-6, LEN-24, LEN-13, LEN-11, TEM-181, APH(6)-Id, APH(3'')-Ib, CTX-M-77, QnrS1, sul2, dfrA14, lmrD, KpnE, tet(39), tet(A), floR, qacEdelta1, OmpA*	20
W2G	*SHV-187, CMH-1, ACT-35, LEN-13, LEN-11, LEN-24, OKP-B-2, OKP-B-6, TEM-181, QnrS1, dfrA1, sul1, sul2, tet(A), tet(39), qacEdelta1*	16
H3I	*APH(3')-Ia, LEN-9, OXY-1-1, MIR-23, TEM-181, ACT-28, acrA*	7
P3I	*TEM-181, LEN-17, LEN-36, ACT-35, tetS*	5
W3I	*OXA-355, aadA3, APH(6)-Id, FosA6, ACT-7, ACT-32, ACT-17, ACT-27, SHV-187, QnrS1, sul1, KpnE, qacEdelta1, eptB*	14

About 50% of the samples showed resistance genes for lincosamide, macrolide, peptide, phenicol, and rifamycin antibiotics. By comparison, resistance genes associated with aminoglycosides, carbapenems, cephamycins, diaminopyrimidines, disinfectants, fluoroquinolones, monobactams, penems, sulfonamides, and tetracyclines were more widespread among the samples.

The highest occurrences of resistance genes were observed for cephalosporin and penam antibiotic classes. Notably, 83% of the cephalosporin resistance genes were predicted to be linked to *Klebsiella aerogenes* and *Klebsiella pneumoniae*. Penam resistance genes were predominantly associated with bacteria such as *Acinetobacter* spp. and *Escherichia coli*.

4.6.1 High ARG Abundance

The raw chicken sample from Gopeng (CB2G) and spinach sample from Kampar (S1K) displayed a higher abundance of resistance genes compared to other samples. Specifically, S1K showed darker regions for fluoroquinolone, macrolide, and phenicol resistance, indicating elevated resistance levels in these antibiotic classes.

4.6.2 Low ARG Abundance

Samples with lower AMR gene abundance included deli meat and certain fruit samples, such as HM2G, HM3I, L2G, P1K, P3I, W1K, W2G, and W3I. Deli meat samples (HM3I and HM2G) exhibited the lowest occurrence of resistance genes across the dataset.

4.6.3 Distribution of ARG Across Food Types

In samples C3I, CB2G, S1K, and S2G, fluoroquinolone resistance genes were highlighted by darker shaded regions, indicating their higher prevalence.

These findings highlight the variability in antimicrobial resistance (AMR) gene distribution across various food types and geographic locations, emphasizing potential risks and the importance of targeted strategies to limit the spread of resistance in food systems.

The Kruskal-Wallis test showed no significant difference in aminocoumarin (p=0.232), aminoglycoside (p=0.375), cephalosporin resistance (p=0.057), cephamycin (p=0.114), diaminopyrimidine (p=0.264), disinfecting agents/antiseptics (p=0.103), fluoroquinolone (p=0.082), glycylcycline (p=0.073), lincosamide (p=0.723), macrolide (p=0.724), monobactam (p=0.128), penem (p=0.480), peptide (p=0.054), phenicol (p=0.394), rifamycin (p=0.262), sulfonamide (p=0.099), and tetracycline (p=0.429). In contrast, there is significant difference in carbapenem (p=0.009), penam (p=0.045) and phosphonic acid (p=0.017).

Post-hoc pairwise comparisons revealed that fruits harboured significantly higher carbapenem resistance gene counts compared to meat ($p = 0.021$) and vegetables ($p = 0.028$), while no significant difference was observed between meat and vegetables ($p = 0.927$). For penam resistance genes, fruits harbored

significantly higher counts compared to meat (p = 0.040). However, no significant differences were observed between fruits and vegetables (p = 0.931) or between meat and vegetables (p = 0.431). Fruits harboured significantly higher phosphonic acid resistance gene counts compared to meat (p = 0.013). No significant differences were observed between meat and vegetables (p = 0.417) or between vegetables and fruits (p = 0.509).

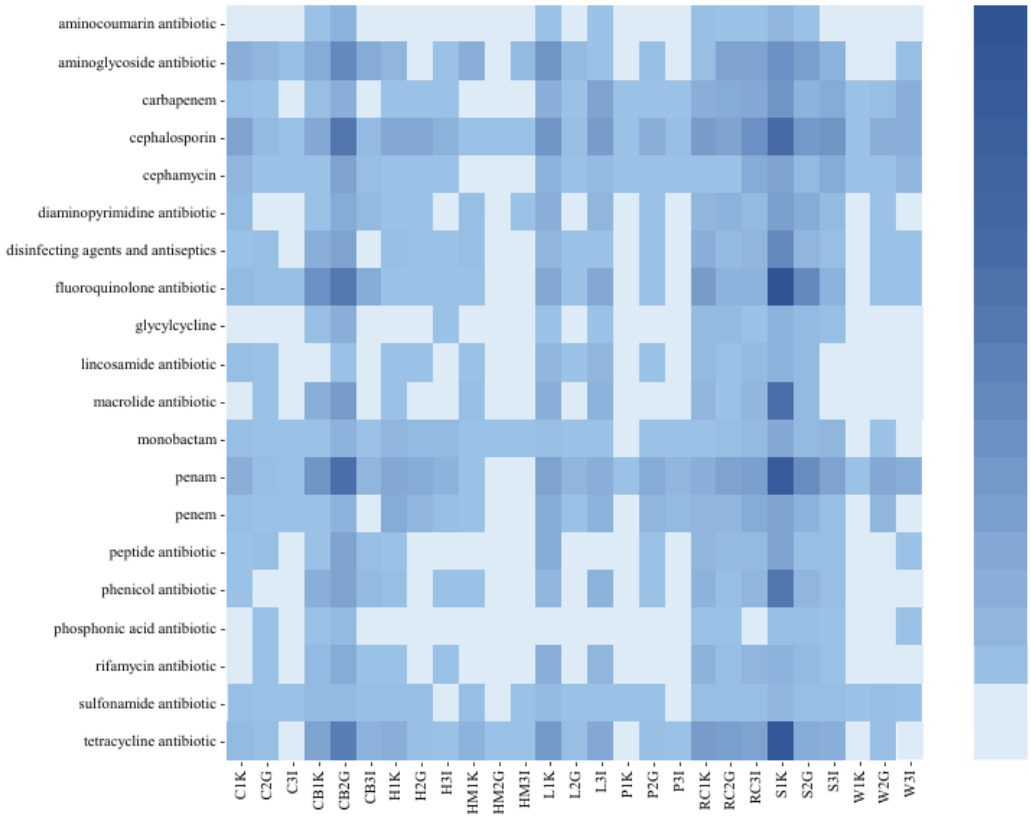


Figure 4.6: Heatmap on the distribution of ARGs among all 27 samples analysed.

CHAPTER 5

DISCUSSION

5.1 QC of Sequencing Data and Reads

The DNA extracted across all samples was of high quality and integrity, with purity ratios and yields meeting the recommended thresholds for shotgun metagenomic sequencing (**Table 4.1**). This suggests that the extraction protocol effectively minimized protein and organic compound contamination from food matrices. Consistently intact bands observed on agarose gels further support the reliability of the extracts, ensuring that downstream sequencing and taxonomic profiling were unlikely to be compromised (Ogunremi et al., 2020; Davis et al., 2023).

All samples achieved a Q30 quality score (Phred score) of 90% and above, indicating that the sequencing data were of high quality and reliable for downstream analyses. This aligns with the recommended threshold for Illumina sequencing (**Figure 4.1**) (Shi, Li and Xu, 2016). The number of reads across the sample group varies differently (**Table 4.2**). For example, vegetable group yielded the highest read count (307 million), while meat group produced fewest read count (225 million). This difference may reflect the complexity of microbial communities associated with each food matrix. Vegetables, which are directly exposed to soil, irrigation water, and post-harvest handling environments, may harbour a broader

and more diverse microbiome compared to animal-derived products (Artimová et al., 2023). In contrast, meats are typically subjected to processing, storage, and preservation practices that can reduce microbial load and diversity, thereby contributing to the lower sequencing read counts observed in this study (Zhao et al., 2022). Importantly, all samples exceeded the commonly recommended threshold of 1 million reads, which ensures reliable taxonomic profiling with <1% dissimilarity from the true community composition (Gweon et al., 2019).

Sufficient sequencing depth significantly enhances the sensitivity and accuracy of results, and in this study, rarefaction curves confirms that the sequencing depth was adequate to proceed with downstream bioinformatics analysis (**Figure 4.2**) Notably, P3I plateaued earlier, suggesting relatively lower diversity, whereas RC2G required higher sequencing depth to capture their broader bacterial community. The combination of high sequence quality and robust QC results further supports the dataset's suitability for advanced analysis. Rarefaction analysis, conducted to address the variability in sequencing depth across samples, normalises the number of sequences, enabling accurate measurement of alpha and beta diversity metrics while ensuring uniform and sufficient sequencing depth (Schloss, 2024).

5.2 Mock Community

5.2.1 Detection of Pathogens in the Mock Community

L. monocytogenes was successfully identified by metagenomic analysis in the fruit sample, but it was not detected in the vegetable or meat samples (**Table 4.3**). The absence of detection in certain samples may be due to factors such as low pathogen abundance, which can fall below the sequencing sensitivity threshold, or exclusion during filtering of low-quality reads (Siegwald et al., 2019; Kibegwa et al., 2020). Despite these variations, multiple pathogens were consistently observed across vegetables, meats, and fruits (**Table 4.3**). These demonstrates that shotgun metagenomics can complement traditional culture-based methods by providing additional insights into bacterial presence, even when some taxa remain below detection thresholds.

5.2.2 AMR Profiles

The analysis of ARGs concentrated on determining the resistance profiles present in the mock community. Beta lactamase associated gene, namely *SCO-1*, were observed in all spiked samples Moreover, ARGs linked to aminoglycoside and sulfonamide were identified. Two genes from ATCC 700408 were detected in all samples (*sul1 & floR*) (**Table 4.4** and **Table 4.5**). Although carbenicillin-hydrolysing class A beta-lactamase (CARB-2) was not detected, associated genes including CARB-3 & CARB-1 were detected. Overall, majority of ARGs

introduced with the ATCC strains were successfully captured, yielding an estimated detection accuracy of 67%. According to a study by Mahfouz et al. (2020), although CARD had a low very major errors rate at 1.17%, the accuracy of CARD can be further improved by the increasing completeness and quality of its curated data (Mahfouz et al., 2020). Overall, majority of the ARGs found in both ATCC strains were detected using metagenomics. The results highlight the ability of metagenomics to recover relevant resistance markers within a controlled community. Importantly, while Malaysian studies have begun to validate the utility of metagenomics for AMR gene surveillance, its deployment in the food safety sector remains limited (Azli et al., 2022; Hanafiah et al., 2024). The outcomes of this study add to the growing evidence base highlighting the potential of metagenomics as a tool for monitoring AMR determinants in foodborne pathogens.

5.3 Microbial Diversity and Pathogen Detection

5.3.1 Alpha Diversity

Vegetables exhibited the highest bacterial diversity compared to fruits and meats **(Figure 4.3.1**). This could possibly be due to agricultural practices and farming environment such as soil and irrigation water associated taxa that shape microbial community in the produce harvested. Moisture content also shapes community composition, which explains a lower diversity in deli meat such as HM3I (Jarvis et al., 2018; Yap et al., 2022). Papaya samples exhibited lower diversity, which may

be attributed to the presence of antimicrobial compounds such as papain that limit microbial colonization (Kumarasinghe et al., 2024). he statistical analysis further reinforced these patterns, showing that vegetables differed significantly in Shannon diversity from both fruits and meats, while no significant difference was observed between fruits and meats. Although previous studies have reported that geographical factors can shape bacterial communities, the present findings suggest that food type exerts a stronger influence on microbial diversity than either preparation method or geography, as no significant differences were detected across Kampar, Gopeng, and Ipoh (Coleman-Derr et al., 2016; Ji, Wei and Lan, 2024).

5.3.2 Beta Diversity

The beta diversity analysis highlighted that community composition was mostly having similarity by sample type (**Figure 4.3.2**). Several fruit and vegetable samples formed distinct clusters in the PCoA plots. Each food provides a different environment that influences the microbial composition. For instance, the dispersion observed in certain fruit and meat samples may be linked to differences in handling, storage, or processing, which can introduce variability in bacterial communities (Wang et al., 2021; Wei et al., 2021). Interestingly, clustering patterns also pointed to a location-driven similarity in P2G-H2G, P1K-W1K and L3I-C3I samples. This indicates that local environmental or handling factors may influence bacterial communities (Leff and Fierer, 2013). Overall, the beta diversity findings complement the alpha diversity results by demonstrating that the composition of

bacterial communities is more strongly determined by sample type than by geographic origin.

5.3.3 Microbial Composition at Phylum Level Across Sample

5.3.3.1 Vegetables

The results align with previous studies that identified Pseudomonadota as the dominant phylum in various vegetables (**Figure 4.3.3a**). Other phyla, such as Bacillota and Bacteroidota, were also detected in the vegetable samples, though at significantly lower abundances (Tatsika et al., 2019b; Giron et al., 2021). Pseudomonadota is a phylum which many human pathogens belong to, are commonly found in agriculture soils, play crucial roles in biogeochemical cycles, particularly in the cycling of carbon, nitrogen, and sulphur. Meanwhile, Bacillota are involved in plant growth promotion, and Bacteroidota contribute to the decomposition of organic matter, highlighting their ecological significance in soil-plant interface (Purbalisa et al., 2022).

The category labelled as 'Others' in the L3I sample, which accounted for 32% of the relative abundance, primarily consisted of uncultured bacteria, contributing 19% of this category. Unculturable bacteria are often present in environments such as agricultural produce and are difficult to isolate using conventional culture-based methods. However, advancements in molecular biology

and bioinformatics have shed light on their diversity and ecological roles. For instances, studies on traditional Chinese fermented foods have identified elusive bacterial species, such as uncultured strains of *Acinetobacter*, as well as *Weissella* and *Lactococcus* (Wang, Hao and Ren, 2023). The uncultured bacteria detected in this study could originate from endophytic bacterial communities residing within plant tissues or from external environmental sources such as soil, water, or air (Kgoale et al., 2023). Their presence underscores the complexity of microbial communities associated with vegetables and the potential need for integrative techniques to better understand their roles and interactions within the ecosystem.

5.3.3.2 Meats

As shown in **Figure 4.3.3b**, Pseudomonadota were the dominant phylum in most meat samples, with relative abundances ranging from 55% to 96% in seven samples (CB1K, RC1K, CB2G, RC2G, CB3I, RC3I, HM3I). This finding is consistent with the know prevalence of Pseudomonadota in retail and processing environments, likely due to contamination during meat handling and storage (Kim et al., 2024). Conversely, Bacillota were more abundant in deli meat samples, particulary HM1K, HM2G, and HM3I, suggesting microbial shifts during processing. Processing steps such as heating, curing, or addition of preservatives can reduce the abundance of heat sensitive bacteria while allowing spore forming or more resilient groups like Bacillota to persist and proliferate. Furthermore, packaging and storage under modified atmospheres can favor anaerobic bacteria, altering the community

structure relative to fresh meat (Xu et al., 2022). Interestingly, the HM1K sample also exhibited a 7% relative abundance of Bacteroidota, diverging from other meat samples. The 'Others' category, notably high in HM1K (13%), likely includes uncultured bacteria introduced during meat processing stages such as cutting, preparation, and packaging (Vinnikova et al., 2019). The observed discrepancies between chicken gut microbiota, where Bacillota typically dominate, and the microbiota in processed meat highlight the impact of environmental and processing conditions (Vinnikova et al., 2019; Xu et al., 2022; Kim et al., 2024).

5.3.3.3 Fruits

Pseudomonadota dominated the microbiome across fruit samples, with the highest relative abundance of 91% in P1K Bacillota (**Figure 4.3.3c**). This finding aligns with previous studies on watermelon and honeydew, where Pseudomonadota were similarly dominant (Glassner et al., 2015; Saminathan et al., 2018). The ability of Pseudomonadota to metabolise diverse carbon sources, such as amino acids, lipids, and carbohydrates, likely supports their prevalence on fruit surfaces under varying environmental conditions (Saminathan et al., 2018). The relatively higher abundance of Firmucutes in Kampar and Gopeng samples may reflect differences in local environmental conditions or agricultural practices. Conversely, the lower Bacillota abundance (<10%) observed in certain samples (e.g., H2G, H3I, W3I, P1K, P3I) may indicate reduced microbial load or specific handling methods. The absence of Bacteroidota across all fruit samples could be attributed to the limited

availability of nutrients required for their growth on fruit surfaces ((Wang, Ranjbaran and Verma, 2024). The 'Others' category, which accounted for 16% in P3I, predominantly included uncultured bacteria. These microorganisms likely originate from environmental sources, such as soil, water, or air, during fruit cultivation and harvesting (Jagessar and Craig, 2024).

5.3.4 Microbial Composition at Genus Level Across Sample Types

5.3.4.1 Vegetables

The bacterial genera observed in vegetable samples were consistent with previous studies that identified genera such as *Pectobacterium* and *Pseudomonas* as dominant in vegetables (**Figure 4.3.4**) (Artimová et al., 2023; Parvin, Taghavi and Osdaghi, 2023). *Pectobacterium* is a plant pathogen that produces cell wall-degrading enzymes, causing tissue maceration and rotting (Golkhandan et al., 2013; Agyemang et al., 2020; Teoh et al., 2023). Its presence in vegetables such as cabbage and Chinese spinach aligns with earlier findings (Zaczek-Moczydłowska et al., 2019; Li et al., 2020a). The lower abundance in lettuce suggests potential host preferences, genetic resistance, or environmental factors in lettuce cultivation that deter its proliferation (Parvin, Taghavi and Osdaghi, 2023; Tang et al., 2024).

Pseudomonas, comprising of pathogenic strains namely *Pseudomonas otitidis*, *Pseudomonas aeruginosa, Pseudomonas oryzihabitans, Pseudomonas*

mendocina, and *Pseudomonas fluorescens* was present in all vegetable samples, with the highest abundance in cabbage from Gopeng (32%) (**Figure 4.4**). This bacterium is ubiquitous in soil, a natural reservoir, and often colonises plant surfaces. While certain strains of *Pseudomonas* provide plant benefits such as nutrient solubilisation, some, like *Pseudomonas aeruginosa*, exhibit pathogenic traits like clinical isolates, necessitating caution (Ruiz-Roldán et al., 2021; Ambreetha et al., 2022).

The detection of *Citrobacter* exclusively in cabbage samples (C1K, C3I) is consistent with reports from other regions, where root and bulb vegetables were more prone to contamination compared to leafy greens (Nigad Nipa et al., 2011; Adegun, Oluduro and Aregbesola, 2019; Nimmanee et al., 2024). This genus may have been introduced during cultivation or post-harvest handling. Two genera from the *Leuconostocaceae* family, namely *Weissella* and *Leuconostoc*, were detected in cabbage samples (C1K, C2G). These genera, belongs to lactic acid bacteria (LAB), which are frequently present in fresh vegetables and fruits (Linares-Morales et al., 2020). LAB are involved in the degradation of proteins and lipids into various compounds, which placed the bacteria in a vital role in the fermentation of vegetables. In addition, LAB were found to produce antimicrobial compounds that may extend the shelf life of foods (Agriopoulou et al., 2020).

Enterobacter, detected in five samples, is commonly associated with soil and irrigation water in agriculture, underscoring its potential entry point into vegetables (Séraphin et al., 2023). Similarly, *Pantoea*, found in lettuce and cabbage

samples, demonstrates dual roles as both a potential pathogen causing plant necrosis and a growth-promoting agent, depending on the strain (Nascimento et al., 2020; Stice et al., 2021; Duchateau et al., 2024). The presence of *Aeromonas*, particularly its high abundance in S2G (41%), may indicate environmental contamination, as this genus thrives in aquatic environments. Lastly, the 'Others' category, which ranged from 15% to 54%, likely includes uncultured or rare taxa. These microorganisms may originate from soil, irrigation water, or post-harvest environments (Agriopoulou et al., 2020).

5.3.4.2 Meats

The detection of *Aeromonas* and *Pseudomonas* in meat samples aligns with earlier studies reporting their presence in various food matrices, including poultry, fish, and meat products (**Figure 4.3.5**) (Eluma et al., 2022; Jawher and Hassan, 2023). *Aeromonas* is particularly prevalent in poultry products, with some studies citing detection rates as high as 74.31% in fresh chicken samples (Enany et al., 2015; Hassan et al., 2020). Its proteolytic and spoilage-related capabilities, especially under refrigerated condition, underscore its potential as a public health concern due to toxin-producing variants (Praveen et al., 2016; Shao et al., 2022). *Pseudomonas* species, known for their psychrotrophic nature, play a key role in meat spoilage under cold storage conditions, affecting the quality of various meat types (Watson et al., 2023). Regional studies, such as those in Iraq, have reported prevalence rates of *Pseudomonas aeruginosa* as high as 39.62% in meat samples, with chicken

meats being particularly susceptible (Jawher and Hassan, 2022; 2023). Similar findings have been documented in other regions, including Saudi Arabia and Iran (Elbehiry et al., 2022; Rezaloo et al., 2022).

The detection of *Lactococcus* species, such as *Lactococcus piscium* and *Lactococcus lactis*, in deli meat samples is noteworthy due to their role in flavour development and metabolic activity in processed meats (Andreevskaya et al., 2015). Their ability to degrade casein, produce lactate, and synthesise aromatic compounds significantly impacts the sensory quality of deli meats (Kuipers, 2001). The dominance of *Vibrio* in sample RC1K highlights a significant concern, as these pathogens, though typically associated with seafood, have also been detected in meat products (**Figure 4.5**) (Azwai et al., 2016; Di et al., 2018; Sen and Mahadeo Garode, 2018). Their presence is attributed to contamination during slaughter, handling, or storage, which facilitates their survival and proliferation(Sen and Mahadeo Garode, 2018).

Lactiplantibacillus, commonly associated with fermented meats such as sausages and salami, plays a critical role in reducing pH levels, enhancing preservation and sensory properties (Parlindungan et al., 2021; Sirini et al., 2023). Its presence in deli meat samples in this study aligns with its known role in processed and fermented meat products. In conclusion, the variation in bacterial genera across the meat samples reflects differences in processing, storage, and

contamination pathways, emphasising the need for stringent quality control measures in the meat industry.

5.3.4.3 Fruits

The detection of *Leuconostoc* species across all fruit samples highlights their dual role in food ecosystems (**Figure 4.3.6**). While they contribute to fermentation processes, enhancing flavour and preservation, their presence in fresh fruits could lead to spoilage. Specific strains, such as *Leuconostoc mesenteroides*, are known to produce bacteriocins with bacteriostatic effects against pathogens like *Listeria monocytogenes*. This trait suggests their potential use as a natural alternative to chemical preservatives in food safety applications (Daba et al., 1991; Kim et al., 2022). However, the unintended impact on product quality necessitates precise identification and management.

Enterobacter, a genus with diverse functional roles, was notably abundant in the papaya sample from Kampar. Members of this genus, such as *Enterobacter cloacae*, are linked to plant diseases, including necrosis and rot in fruits. Additionally, their occurrence in fresh produce raises public health concerns due to their association with antibiotic resistance, including the production of extended-spectrum β-lactamases (ESBL) (Al-Kharousi et al., 2016; Özdemir, 2021; Pintor-Cora et al., 2021). The widespread presence of *Enterobacter* emphasises the potential for fruits to act as reservoirs of pathogenic and resistant bacteria.

Klebsiella, exclusively detected in honeydew samples, is commonly found in fruits and dried vegetables. This bacterium poses a dual threat, causing plant diseases and representing a health hazard to humans, especially through the transmission of opportunistic infections (Ajayasree and Borkar, 2018; Riwu et al., 2022). Its selective presence in certain fruit types, such as honeydew, suggests contamination pathways that warrant further investigation. Although studies on *Acinetobacter* in fruits are limited, its presence in Gopeng samples aligns with its known habitat in soil and water, implying environmental contamination. Certain strains of *Acinetobacter* are opportunistic pathogens and their detection in fresh produce underscores the importance of effective washing and handling practices (Ababneh, Al-Rousan and Jaradat, 2022).

The genus *Tatumella*, found exclusively in sample W1K, has been implicated in plant pathogenesis and food spoilage (Marín-Cevada et al., 2010). Its limited detection in this study highlights its specificity to certain fruits or environmental conditions, meriting further exploration into its ecological and health impacts. In conclusion, the variation in bacterial genera among fruit samples reflects differences in environmental conditions, cultivation practices, and handling methods. The findings underscore the importance of monitoring and managing microbial communities in fruits to ensure both food quality and safety.

5.3.4 Unidentified Taxonomy

Unidentified taxa often represent "microbial dark matter" which is most of the microbial diversity that remains uncultured and uncharacterised. Studies suggest that only 1-5% of environmental microorganisms have been successfully cultured in laboratory conditions. All the samples had 5% or less of unidentified bacteria, however HM3I and L2G each had 10%. Although the relative abundance of unidentified taxa was not dominant in any sample, their consistent presence across datasets reflects a common finding in metagenomic analysis (Solden, Lloyd and Wrighton, 2016; Amorín de Hegedüs, Conesa and Foster, 2023).

Processed meats may carry microbes introduced during processing or from preservation environments. One study found that microbial communities can permanently reside within meat processing facilities, serving as a persistent source of transmission. The same study also revealed a large microbial diversity, including 74 previously undescribed bacterial taxa, which may help explain the higher proportion of unidentified taxa observed in HM3I (Xu et al., 2025). Research on vegetable microbiomes, while expanding, remains less systematically developed compared to other food sources. Studies have shown that soil and water associated bacteria can strongly influence the microbial community structure of fresh produce. Such environmental factors may account for the increased relative abundance of unidentified taxa observed in L2G (Berg et al., 2014; Lee et al., 2024). These unidentified taxa may include potential pathogens or opportunistic microbes that have not yet been recognized. Beyond their possible health implications, they also

represent a reservoir of novel genetic and metabolic diversity. Even if they are not pathogenic, such taxa may encode unique enzymes, metabolites, or bioactive compounds with potential applications in biotechnology, medicine, and food preservation.

5.4 Prevalence of Foodborne Pathogen based on Location

The varying prevalence of foodborne pathogens across the three locations—Kampar, Gopeng, and Ipoh—can be attributed to a combination of agricultural practices, post-harvest handling, retail environments, and supply chain dynamics (**Figure 4.4**).

In vegetables, Kampar exhibited the highest pathogen prevalence, likely influenced by improper cultivation practices and the use of contaminated irrigation water or organic soil amendments. These factors are consistent with findings from India, where improper agricultural practices are significant contributors to microbial contamination in produce (Mohanapriya et al., 2024). Additionally, post-harvest handling plays a critical role; studies have shown that fresh-cut fruits and vegetables are highly susceptible to microbial contamination during processing (Bai et al., 2024).

Retail environments also impact contamination rates. For instance, unpackaged vegetables often exhibit higher contamination levels than packaged

ones due to increased exposure to environmental pathogens (Silva et al., 2017). Poor hygiene conditions in open markets, as evidenced by higher coliform counts in market-sourced vegetables compared to farm-sourced ones, may further exacerbate contamination risks (Mohanapriya et al., 2024). However, contrary evidence suggests that supermarkets may also harbour significant contamination risks, particularly for pathogens like *Salmonella* (Sarré et al., 2023).

In meat samples, Gopeng recorded the highest pathogen prevalence, which may reflect differences in slaughtering practices, hygiene during handling, and storage conditions. Studies in the United States have shown that extended transportation distances for meat (e.g., 194–469 miles) can lead to a higher prevalence of multidrug-resistant (MDR) bacteria, suggesting that logistics and cold chain maintenance significantly influence contamination levels (Innes et al., 2023).

In fruit samples, Ipoh had the highest pathogen prevalence. This could be due to variations in processing, storage, and transportation practices. Fruits are often prone to contamination from waterborne pathogens during washing or irrigation (Alum, Urom and Ben, 2016; Wadamori, Gooneratne and Hussain, 2017). Additionally, the resilience of certain pathogens, such as *Listeria monocytogenes*, in retail environments could contribute to higher contamination levels (Gartley et al., 2022).

The findings underscore the complex interplay of agricultural, logistical, and retail factors that influence the prevalence of foodborne pathogens. Each location demonstrated distinct challenges, reflecting the importance of tailored interventions to reduce contamination risks in vegetables, meats, and fruits.

5.5 Foodborne Pathogen Detection

The results demonstrate the utility and limitations of both metagenomics and culture-based PCR techniques in detecting foodborne pathogens across various food matrices (**Figure 4.6**). While metagenomics analysis offers broad-spectrum pathogen detection capabilities, its sensitivity is sometimes lower than culture-based methods, particularly in samples where the pathogen is present in low abundance. This discrepancy was evident in cases such as *L. monocytogenes* and *C. jejuni*, where detection relied solely on culture-dependent methods. The complexity of the food matrix and the presence of host DNA further compound the challenges in pathogen identification using metagenomics, as previously observed in food production environments (Kocurek et al., 2023). The detection of foodborne pathogens in food samples does not necessarily mean that illness will occur. Disease manifestation depends on multiple factors, including the infectious dose required, the virulence of the strain, host susceptibility, and the influence of the food matrix and processing conditions. Thus, while the presence of pathogens indicates potential risk, actual foodborne illness arises only when these factors align to allow infection (Bhunia, 2008).

5.5.1 Metagenomics and Culture-based PCR

The consistency analysis using Cohen's Kappa revealed substantial agreement in pathogen detection outcomes, indicating that the approaches applied in this study were broadly aligned. The strong Kappa value reflects considerable overlap in detection capacity, demonstrating reliability in capturing foodborne pathogens across food types. However, the McNemar's test indicated that some pathogens were detected more frequently by one approach, suggesting differences in analytical scope and sensitivity rather than conflicting results.

These findings are consistent with previous research showing that relationships between metagenomic sequencing and traditional detection methods can be complex. For example, a systemic review of clinical studies reported only a moderate relationship between metagenomic next-generation sequencing (mNGS) and conventional microbiological tests, with variation depending on sample type and host immune status (Liu et al., 2025). Similarly, in tuberculosis diagnostics, mNGS demonstrated strong sensitivity and specificity across diverse sample types, but the greatest utility was achieved when combined with culture or targeted assays such as GeneXpert, underscoring how different approaches may contribute complementary strengths (Chen, He and Sun, 2020). Taken together, these observations suggest that while shotgun metagenomics is a feasible and reliable strategy for foodborne pathogen detection, methodological differences can influence the detection of individual taxa. Rather than conflicting, these differences

highlight the potential value of integrating culture-based and sequencing-based approaches to achieve more comprehensive pathogen profiling.

5.5.2 Pathogen Prevalence Across Food Matrices

The high prevalence of *E. coli* and *S. enterica* across vegetable, meat, and fruit samples highlights their adaptability and ability to thrive in diverse environments. Contamination typically occurs through exposure to animal faeces, contaminated irrigation water, or improper handling during processing and storage (Kalwaniya et al., 2020; Pal et al., 2020; Mohamed et al., 2022). In Malaysia, *S.* Typhimurium is a dominant serotype detected in 34.1% of pork samples and commonly found in beef and poultry meat (Roseliza et al., 2011). Its occurrence in fresh produce, such as cucumbers, further underscores the widespread nature of this pathogen in food systems (Saw et al., 2020).

The detection of *E. coli* in traditional Malaysian *ulam* samples, albeit at lower levels (3.13%), represents a health risk associated with the consumption of raw vegetables (Bahri et al., 2022). Meanwhile, pathogens such as *C. jejuni*, *L. monocytogenes*, and *Sh. flexneri* were detected less frequently. This aligns with studies suggesting that their presence is often sporadic and influenced by factors such as the specific food matrix and contamination source.

5.5.3 Implications for Food Safety

The results underscore the importance of employing complementary methods for comprehensive pathogen surveillance. While metagenomics provides broad-spectrum insights, culture-based methods remain indispensable for detecting pathogens in low-abundance contexts or in complex microbial communities. The findings also highlight the need for stringent hygiene measures throughout the food production and supply chain to mitigate contamination risks. This includes proper handling practices, sanitation, and the use of clean water for irrigation to minimize exposure to pathogens such as *S. enterica* and *E. coli*.

By combining these detection approaches, food safety monitoring can achieve greater accuracy, facilitating early intervention to prevent outbreaks and ensuring the safety of food products for consumers.

5.6 Antimicrobial Resistance Gene (ARG)

The distribution of ARGs varied across vegetables, meats, and fruits, reflecting both direct and indirect antibiotic exposure in different food production systems. Vegetables showed a particularly broad ARG spectrum, which may be linked to environmental contamination from irrigation water, soil, or fertilizer use (Lewis et al., 2020). Meats, in contrast, exhibited higher prevalence of diaminopyrimidine and quinolone resistance genes. These antimicrobials class, especially quinolones were commonly used in poultry farms in Malaysia (Rachel et al., 2020). Fruits

carried comparatively fewer ARGs, but their consistent detection of β-lactamases indicates that even foods perceived as "low risk" may serve as reservoirs of clinically relevant resistance genes. Together, these findings suggest that fresh produce and animal-derived foods can act as overlapping but distinct reservoirs of ARGs, contributing to the wider dissemination of resistance through the food chain (Kim et al., 2022).

The observed increase in cephalosporin and penam resistance among foodborne pathogens can be attributed to several related factors, including their widespread application in medicine and agricultural practices (**Figure 4.6**). This practice facilitates the emergence and proliferation of resistant strains, as seen with *E. coli* and *Salmonella*, which frequently carry resistance to β-lactam antibiotics such as cephalosporins (Threlfall, 2013). The use of antibiotics in agriculture accelerates the development of resistance, facilitating the spread of ARGs via the food chain. Surveillance data highlight significant resistance trends, such as resistance to quinolones and cephalosporins in *Salmonella enterica* and to quinolones and macrolides in *Campylobacter* spp., underscoring the critical need to regulate antibiotic use in animal farming to mitigate resistance dissemination (Threlfall, 2013; Costa et al., 2022; Yankova et al., 2023). The detection of ARGs in food does not necessarily mean food safety is immediately compromised. The risk depends on whether these genes are carried by viable and pathogenic bacteria capable of transferring resistance to others. Importantly, commensal and non-pathogenic bacteria can act as reservoirs of resistance or virulence genes within the

shared microbial gene pool. These genes may enhance the success of pathogens during infection or be transferred to pathogenic species through horizontal gene transfer, amplifying their clinical impact. Thus, while ARGs in food are not a direct indicator of illness, they represent a potential reservoir that may indirectly threaten food safety and public health (Hall et al., n.d.; Dionisio et al., 2023).

5.6.1 Variability of ARG by Sample Type

The lower detection of ARGs in deli meats and fruits, compared to vegetables and raw chicken, may result from the stringent safety and hygiene measures routinely used in deli meat processing. High-throughput analyses and qPCR studies have demonstrated that tetracycline-resistant (TR) *Enterobacteriaceae* can be detected in meat samples, although their abundance tends to be lower in products processed under stringent safety protocols. Conversely, environmental sources like river sediments and wastewater outputs tend to harbour higher diversity and prevalence of ARGs (Guarddon et al., 2012; Gweon et al., 2019). The presence of ARGs in vegetables and fruits may be attributed to the use of manure-based fertilizers, which are often found to contain antibiotics such as ciprofloxacin and chlorotetracycline (Zhou et al., 2020).

In contrast, vegetables and raw chicken exhibited considerable resistance, especially against cephalosporins and penams. In Malaysia, a notable 25% of *S. enterica* isolates have shown resistance to ceftriaxone, a third-generation

cephalosporin (Puah et al., 2016). Global studies have identified cephalosporin-resistant *Enterobacterales*, including *K. pneumoniae* and *E. coli*, in raw meat. In the United States, between 5.6% and 10.8% of meat samples were found to contain resistant strains, highlighting meat as an important reservoir for ARGs (Rybak et al., 2022) Additionally, *Listeria* isolates from Malaysian vegetable farms and retail markets exhibited total resistance to penicillin G, while *Salmonella* isolates from poultry and vegetable farms displayed widespread resistance to ampicillin and amoxicillin (Kuan et al., 2017a; Subramaniam et al., 2023).

5.6.2 Resistance Trends in Specific Antibiotic Classes

In this study, genes conferring resistance to aminocoumarin, glycylcycline, and phosphonic acid were detected at the lowest frequencies. For aminocoumarins, their limited use and specificity against bacterial gyrase restrict their application, which may explain the low occurrence (Heide, 2009). Tigecycline and other glycylcycline resistance genes were similarly uncommon. A study in China found a low prevalence (0.65%) of tigecycline resistance in *E. coli* from food sources, although higher levels (9.35%) were observed in *Enterobacterales* from porcine origins, highlighting the variability of resistance across bacterial sources (Wang et al., 2022; Fan et al., 2024). Resistance to phosphonic acids, especially genes conferring fosfomycin resistance, was observed at very low levels. However, its growing prevalence in *Campylobacter* and *Salmonella* is concerning, as these pathogens can transmit resistance to humans through food, complicating treatment of infections

(O'Bryan, Crandall and Ricke, 2018; Wei and Kang, 2018; Wang et al., 2024). Fosfomycin's low prevalence in the samples does not diminish its clinical value, underscoring the importance of monitoring and prudent antibiotic administration to limit resistance development.

5.6.3 Antimicrobial Use Policies in the Agriculture Sector

In Malaysia, the Ministry of Agriculture and Food Security has strengthened policies to regulate antimicrobial use (AMU) in the agriculture sector through biosecurity measures, certification schemes such as Malaysian Good Animal Husbandry Practices (MyGAP) and MyOrganic, and the introduction of the National Veterinary Antimicrobial Guideline (2019). To preserve the effectiveness of critically important antimicrobials, several drugs have been progressively banned for use in food-producing animals, beginning with colistin in 2019 and followed by six others in 2021. Farm licensing requirements now mandate AMU reporting, and antimicrobial usage is monitored through WOAH's international reporting format (Ministry of Health Malaysia and Ministry of Agriculture and Food Security, 2022).

A One Health approach underpins national surveillance strategies, linking human, animal, and environmental health. Programmes such as the Code of Practice to Minimise and Contain Foodborne AMR (COP) and the Guidelines on Integrated Monitoring and Surveillance of Foodborne AMR (GLISS) aim to

monitor AMR in livestock, aquaculture, and food products from retail markets. Malaysia's national action plan (My-AP-AMR) sets measurable targets, including phasing out antibiotics as growth promoters, responsible antimicrobial use in husbandry, and achieving a 30% reduction in the use of critically important antimicrobials in food animals, along with a 5% overall reduction in animal-sector antimicrobial use by 2026 (Ministry of Health Malaysia and Ministry of Agriculture and Food Security, 2022).

5.7 Limitations and Future Works

Despite the promising outcomes of this study, several limitations should be acknowledged. First, the sample size was relatively modest and limited to three food categories (vegetables, meats, and fruits) collected from selected vendors in Kinta Valley. While these data provide valuable insights, they may not fully represent the broader diversity of food sources in Malaysia. Second, the metagenomic sequencing depth employed may not have captured all low-abundance microbial taxa or rare antimicrobial resistance genes (ARGs), potentially underestimating their prevalence (Liu et al., 2022b). Third, the reliance on short-read sequencing platforms constrained the resolution of strain-level identification and limited the ability to link ARGs directly to specific pathogens (Seol et al., 2019). Fourth, although culture-based validation was performed, inherent biases of enrichment and selective plating methods may have affected the comparability of results (Hyeon et al., 2018).

Future research should seek to address these limitations. Expanding the study to include larger sample sizes across diverse geographical regions and food supply chains in Malaysia would improve the representativeness of findings. Employing long-read sequencing technologies or hybrid assemblies could enhance taxonomic resolution and enable the direct assignment of ARGs to pathogenic strains. In addition, integrating metatranscriptomics or functional metagenomics could provide deeper insights into the expression and activity of ARGs beyond their genomic presence. Longitudinal surveillance studies are also warranted to monitor seasonal or temporal variations in pathogen distribution and resistance patterns. Finally, the integration of metagenomic approaches into national food safety monitoring frameworks may strengthen early detection systems and support evidence-based policymaking for public health protection.

CHAPTER 6

CONCLUSION

This study addressed three main objectives, yielding significant insights into the application of shotgun metagenomics for food safety.

The first objective was to assess its feasibility for detecting foodborne pathogens in a mock community of vegetables, meats, and fruits. The analysis achieved an overall accuracy of 83% (112/135) in non-mock community samples. Among the five pathogens analysed, *C. jejuni* exhibited the highest detection accuracy (89%, 24/27), while *S. enterica* showed the lowest (74%, 20/27). Importantly, shotgun metagenomics enabled species-level identification, which is crucial for effective foodborne pathogen screening.

The second objective focused on profiling the microbial community in food samples. The dominant phyla observed were Pseudomonadota, Bacillota and Bacteroidota. At a finer taxonomic level, genera such as *Klebsiella, Weisella, Lactococcus, Arcobacter,* and *Pantoea* were identified using metagenomics pipeline. Distinct trends in microbial community composition were noted based on sample type and location, such as the prevalence of *Pseudomonas* in vegetables, *Aeromonas* in meats, and *Enterobacter* in fruits. While metagenomics effectively

characterised the microbial community, accurate species-level identification of foodborne pathogens remains a challenge. These findings demonstrate the capability of shotgun metagenomics to reveal microbial diversity within food products.

The third objective was successfully achieved, as metagenomics predicted ARGs. Resistance genes for cephalosporins and penams were the most prevalent, especially in raw chicken and vegetable samples, while deli meat and fruit samples showed lower levels. These resistance patterns likely reflect agricultural practices and antibiotic usage, underscoring the need for stricter controls in food production. The lower prevalence of resistance to classes including phosphonic acids and glycylcyclines also suggests possibility for strategic preservation of antibiotics.

These outcomes highlight the potential of metagenomics as a complementary and, in certain cases, superior approach to conventional pathogen surveillance. The detection of clinically relevant ARGs underscores the importance of integrating genomic tools into food safety assessments, particularly as antimicrobial resistance continues to pose global health challenges. Looking ahead, the incorporation of metagenomic surveillance into Malaysia's routine food safety monitoring framework could significantly enhance the early detection of foodborne pathogens and ARGs. Such integration would support proactive risk management, safeguard consumer health, and contribute to national and regional food security. Furthermore, the methodologies applied in this study may serve as a foundation for

future large-scale and longitudinal investigations, advancing both scientific knowledge and public health policy in the context of foodborne pathogen surveillance.

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APPENDICES

Appendix A

Detailed Information of Collected Samples Before Pooling

No	Type	Sample	Date	GPS Coordinates	Temp
1	Spinach	S1K	14/8/23	4.33562861922032, 101.1538572269907	Room Temp
2	Spinach	S1K	14/8/23	4.32757855712863, 101.14694516721202	Room Temp
3	Spinach	S1K	14/8/23	4.317725765029743, 101.1483670095423	Room Temp
4	Cabbage	C1K	10/7/23	4.33562861922032, 101.1538572269907	Room Temp
5	Cabbage	C1K	10/7/23	4.32757855712863, 101.14694516721202	Room Temp
6	Cabbage	C1K	10/7/23	4.317725765029743, 101.1483670095423	Room Temp
7	Lettuce	L1K	10/7/23	4.33562861922032, 101.1538572269907	Cold
8	Lettuce	L1K	10/7/23	4.32757855712863, 101.14694516721202	Cold
9	Lettuce	L1K	10/7/23	4.317725765029743, 101.1483670095423	Cold
10	Spinach	S2G	3/9/23	4.493972078685568, 101.15319552517505	Room Temp
11	Spinach	S2G	3/9/23	4.474946945203656, 101.16895905866305	Room Temp

12	Spinach	S2G	3/9/23	4.467389642887472, 101.16367610749442	Room Temp
13	Cabbage	C2G	3/9/23	4.493972078685568, 101.15319552517505	Room Temp
14	Cabbage	C2G	3/9/23	4.474946945203656, 101.16895905866305	Room Temp
15	Cabbage	C2G	3/9/23	4.467389642887472, 101.16367610749442	Room Temp
16	Lettuce	L2G	3/9/23	4.493972078685568, 101.15319552517505	Cold
17	Lettuce	L2G	3/9/23	4.474946945203656, 101.16895905866305	Cold
18	Lettuce	L2G	3/9/23	4.467389642887472, 101.16367610749442	Cold
19	Spinach	S3I	22/10/23	4.544685244708797, 101.07035100074732	Room Temp
20	Spinach	S3I	22/10/23	4.551478834793077, 101.11674350123909	Room Temp
21	Spinach	S3I	22/10/23	4.523955163350567, 101.10586880842031	Room Temp
22	Cabbage	C3I	22/10/23	4.544685244708797, 101.07035100074732	Room Temp
23	Cabbage	C3I	22/10/23	4.551478834793077, 101.11674350123909	Room Temp
24	Cabbage	C3I	22/10/23	4.523955163350567, 101.10586880842031	Room Temp
25	Lettuce	L3I	22/10/23	4.544685244708797, 101.07035100074732	Cold
26	Lettuce	L3I	22/10/23	4.551478834793077, 101.11674350123909	Cold

27	Lettuce	L3I	22/10/23	4.523955163350567, 101.10586880842031	Cold
28	Raw Chicken Meat	CB1K	21/8/23	4.33562861922032, 101.1538572269907	Cold
29	Raw Chicken Meat	CB1K	21/8/23	4.32757855712863, 101.14694516721202	Cold
30	Raw Chicken Meat	CB1K	21/8/23	4.317725765029743, 101.1483670095423	Cold
31	Roasted Chicken	RC1K	21/8/23	4.329412956556465, 101.14359266167965	Room Temp
32	Roasted Chicken	RC1K	21/8/23	4.328611463128176, 101.14382020191708	Room Temp
33	Roasted Chicken	RC1K	21/8/23	4.330632669050978, 101.1455522747263	Room Temp
34	Deli Meat (Ham)	HM1K	14/8/23	4.33562861922032, 101.1538572269907	Freezer
35	Deli Meat (Ham)	HM1K	14/8/23	4.32757855712863, 101.14694516721202	Freezer
36	Deli Meat (Ham)	HM1K	14/8/23	4.317725765029743, 101.1483670095423	Freezer
37	Raw Chicken Meat	CB2G	5/11/23	4.493972078685568, 101.15319552517505	Cold
38	Raw Chicken Meat	CB2G	5/11/23	4.474946945203656, 101.16895905866305	Cold
39	Raw Chicken Meat	CB2G	5/11/23	4.467389642887472, 101.16367610749442	Cold
40	Roasted Chicken	RC2G	5/11/23	4.474624055722571, 101.16801156842995	Room Temp
41	Roasted Chicken	RC2G	5/11/23	4.475394176986795, 101.16808264694761	Room Temp

42	Roasted Chicken	RC2G	5/11/23	4.455328844324272, 101.15926261165122	Room Temp
43	Deli Meat (Ham)	HM2G	8/7/23	4.493972078685568, 101.15319552517505	Freezer
44	Deli Meat (Ham)	HM2G	8/7/23	4.452089115284185, 101.15462226332068	Freezer
45	Deli Meat (Ham)	HM2G	8/7/23	4.467389642887472, 101.16367610749442	Freezer
46	Raw Chicken Meat	CB3I	19/11/23	4.544685244708797, 101.07035100074732	Cold
47	Raw Chicken Meat	CB3I	19/11/23	4.551478834793077, 101.11674350123909	Cold
48	Raw Chicken Meat	CB3I	19/11/23	4.523955163350567, 101.10586880842031	Cold
49	Roasted Chicken	RC3I	7/7/23	4.531459724964557, 101.127049105596	Room Temp
50	Roasted Chicken	RC3I	7/7/23	4.609886325802867, 101.12526902437472	Room Temp
51	Roasted Chicken	RC3I	7/7/23	4.57128896845497, 101.1144372785204	Room Temp
52	Deli Meat (Ham)	HM3I	19/11/23	4.544685244708797, 101.07035100074732	Freezer
53	Deli Meat (Ham)	HM3I	19/11/23	4.551478834793077, 101.11674350123909	Freezer
54	Deli Meat (Ham)	HM3I	19/11/23	4.523955163350567, 101.10586880842031	Freezer
55	Honeydew	H1K	14/8/23	4.329412956556465, 101.14359266167965	Cold
56	Honeydew	H1K	14/8/23	4.309930242368358, 101.15117330050865	Cold

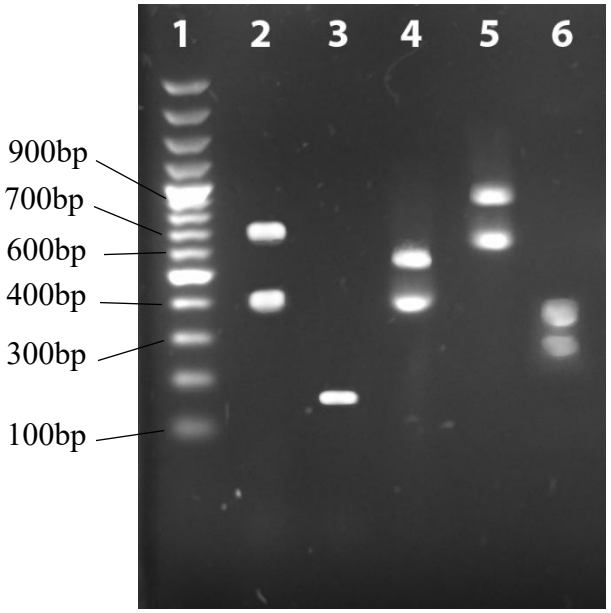
57	Honeydew	H1K	14/8/23	4.309937744165159, 101.15162333148044	Cold
58	Papaya	P1K	26/10/23	4.329412956556465, 101.14359266167965	Cold
59	Papaya	P1K	26/10/23	4.309930242368358, 101.15117330050865	Cold
60	Papaya	P1K	26/10/23	4.309937744165159, 101.15162333148044	Cold
61	Watermelon	W1K	26/10/23	4.329412956556465, 101.14359266167965	Cold
62	Watermelon	W1K	26/10/23	4.309930242368358, 101.15117330050865	Cold
63	Watermelon	W1K	26/10/23	4.309937744165159, 101.15162333148044	Cold
64	Honeydew	H2G	5/11/23	4.47530910771815, 101.16799308315926	Cold
65	Honeydew	H2G	5/11/23	4.455041062498281, 101.15932922514281	Cold
66	Honeydew	H2G	5/11/23	4.456337562941827, 101.15929523865998	Cold
67	Papaya	P2G	5/11/23	4.47530910771815, 101.16799308315926	Cold
68	Papaya	P2G	5/11/23	4.455041062498281, 101.15932922514281	Cold
69	Papaya	P2G	5/11/23	4.456337562941827, 101.15929523865998	Cold
70	Watermelon	W2G	8/7/23	4.47530910771815, 101.16799308315926	Cold
71	Watermelon	W2G	8/7/23	4.455041062498281, 101.15932922514281	Cold

72	Watermelon	W2G	8/7/23	4.456337562941827, 101.15929523865998	Cold
73	Honeydew	H3I	7/7/23	4.596271461386676, 101.08171054328098	Cold
74	Honeydew	H3I	7/7/23	4.595921191140761, 101.08158716920272	Cold
75	Honeydew	H3I	7/7/23	4.595399840737717, 101.08100915226437	Cold
76	Papaya	P3I	7/7/23	4.596271461386676, 101.08171054328098	Cold
77	Papaya	P3I	7/7/23	4.595921191140761, 101.08158716920272	Cold
78	Papaya	P3I	7/7/23	4.595399840737717, 101.08100915226437	Cold
79	Watermelon	W3I	7/7/23	4.596271461386676, 101.08171054328098	Cold
80	Watermelon	W3I	7/7/23	4.595921191140761, 101.08158716920272	Cold
81	Watermelon	W3I	7/7/23	4.595399840737717, 101.08100915226437	Cold
82	Lettuce	O1L	3/7/23	4.33562861922032, 101.1538572269907	Cold
83	Lettuce	O1L	3/7/23	4.32757855712863, 101.14694516721202	Cold
84	Lettuce	O1L	3/7/23	4.317725765029743, 101.1483670095423	Cold
85	Raw Chicken Meat	O1CB	3/7/23	4.33562861922032, 101.1538572269907	Cold
86	Raw Chicken Meat	O1CB	3/7/23	4.32757855712863, 101.14694516721202	Cold

87	Raw Chicken Meat	O1CB	3/7/23	4.33562861922032, 101.1538572269907	Cold
88	Honeydew	O1H	3/7/23	4.329412956556465, 101.14359266167965	Cold
89	Honeydew	O1H	3/7/23	4.309930242368358, 101.15117330050865	Cold
90	Honeydew	O1H	3/7/23	4.309937744165159, 101.15162333148044	Cold

Appendix B

Representative Gel Image



Lane 1: 100 bp DNA ladder (Vivantis, Malaysia)

Lane 2: ATCC 33560 *C. jejuni*: *cadF* (400 bp), *hipO* (735 bp)

Lane 3: ATCC BAA-197 *E. coli*: *uidA* (166 bp)

Lane 4: ATCC 700408 *S.* Typhimurium: Random fragment (429 bp), *fliC* (620 bp)

Lane 5: ATCC 19115 *L. monocytogenes*: *hlyA* (702 bp), 16S rRNA (938 bp)

Lane 6: ATCC 29903 *Sh. flexneri*: *set1A* (309 bp), *ipaH* (423 bp)

Appendix C

Materials Used in the Study

Reagents used in this study and the respective manufacturers.

Materials	Manufacturer, Country
Bolton broth	HiMedia, India
Blood Free Campylobacter selectivity (BFC) agar	HiMedia, India
Microaerophilic gas generating sachets	BD
Escherichia coli (EC) broth	Liofilchem, Italy
Eosin Methylene Blue Levine (EMB) agar	Liofilchem, Italy
Buffered Listeria Enrichment (BLEB) broth	Condalab, Spain
PALCAM Listeria Identification (PALCAM) agar	HiMedia, India
Buffered Peptone Water (BPW)	HiMedia, India
Rappaport-Vassiliadis Soy (RVS) broth	Liofilchem, Italy
Xylose Lysine Deoxycholate (XLD) agar	HiMedia, India
Shigella broth	Biolife, Italy
MacConkey (MAC) agar	HiMedia, India
Bolton Selective Supplement (FD231)	HiMedia, India

CF Supplement I (FD067)	HiMedia, India
Defibrinated lysed horse blood	TCS Biosciences, United Kingdom
Listeria Selective Supplement (PALCAM) (FD061)	HiMedia, India
Novobiocin Antimicrobic Supplement	Biolife, Italy
Sodium Chloride	Stabilab, Malaysia
Glycerol	1Malaysia Bio Lab, Malaysia
Tryptone Soya agar (TSA)	HiMedia, India
Tryptone Soya broth (TSB)	HiMedia, India
Tris-Borate-EDTA (TBE) buffer	Vivantis, Malaysia
Agarose Gel	Condalab, Spain
GoTaq Green Master Mix	Promega, United States
1 kb DNA Ladder (250 bp)	Vivantis, Malaysia
100 bp DNA Ladder (100 bp)	Vivantis, Malaysia
GreenSafe DNA Gel Stain	Canvax, Spain
Nucleospin Food kit	Macherey-Nagel, Germany

Agars and broths used in this study and the preparation methods.

Agar/Broth	Formulation	Function
Bolton broth	One litre was prepared as below: 27.60 g of Bolton broth powder was added to 1 L of distilled water. 2 vials of Bolton Selective supplement, 50 mL of horse blood was added to cooled broth.	For selective enrichment of *Campylobacter* spp.
BFC agar	One litre was prepared as below: 45.50 g of BFC agar powder was added to 1 L of distilled water. 2 vials of CF Supplement I were added to molten agar.	For selective isolation of *Campylobacter* spp.
EC broth	One litre was prepared as below: 37.00 g of EC broth powder was added to 1 L of distilled water.	For selective enrichment of *Escherichia coli*
EMB agar	One litre was prepared as below: 37.50 g of EMB agar powder was added to 1 L of distilled water.	For differential isolation of *E. coli*
BLEB	One litre was prepared as below: 36.00 g of BLEB broth powder was added to 1 L of distilled water.	For selective enrichment of *Listeria* spp.
PALCAM agar	One litre was prepared as below: 68.88 g of PALCAM agar powder was added to 1 L of distilled water.	For selective and differential isolation of *L. monocytogenes*

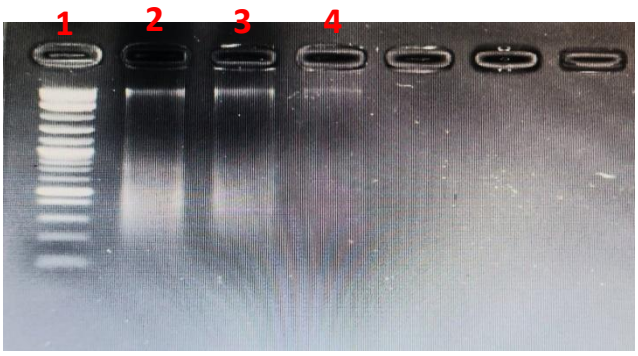
	2 vials of Listeria Selective Supplement were added to molten agar.	
BPW	One litre was prepared as below: 20.00 g of BPW powder was added to 1 L of distilled water.	For pre-enrichment of *Salmonella* spp.
RVS broth	One litre was prepared as below: 26.60 g of RVS broth powder was added to 1 L of distilled water.	For selective enrichment of *Salmonella* spp.
XLD agar	One litre was prepared as below: 56.68 g of XLD agar powder was added to 1 L of distilled water.	For selective and differential isolation of *Salmonella* spp.
Shigella broth	One litre was prepared as below: 31.50 g of Shigella broth powder was added to 1 L of distilled water. 222 µL of Novobiocin Antimicrobic Supplement was added to cooled broth.	For selective enrichment of *Shigella* spp.
MAC agar	One litre was prepared as below: 50.03 g of MAC agar powder was added to 1 L of distilled water.	For selective and differential isolation of *Shigella* spp.
TSA	One litre was prepared as below: 40.00 g of TSA agar powder was added to 1 L of distilled water.	For maintenance and cultivation of bacteria.
TSB	One litre was prepared as below: 30.00 g of TSB broth powder was added to 1 L of distilled water.	For maintenance and cultivation of bacteria.

Reagents used in this study and the preparation methods.

Reagent	**Formulation**
Bolton Selective Supplement	One vial was rehydrated as below: 2.5 mL of 50% ethanol was added.
CF Selective Supplement I	One vial was rehydrated as below: 2 mL of sterile distilled water was added.
Listeria Selective Supplement (PALCAM)	One vial was rehydrated as below: 5 mL of sterile distilled water was added.
Novobiocin Antimicrobic Supplement	One vial was rehydrated as below: 4 mL of sterile distilled water was added.
50% (v/v) glycerol	One vial was rehydrated as below: 500 mL of distilled water was added to 500 mL of 100% glycerol.
0.85% (w/v) saline	One litre was prepared as below: 8.5 g of sodium chloride was added to 1 L of distilled water.
1X TBE buffer	One litre was prepared as below: 100 mL of 10X TBE buffer was added to 900 mL of distilled water.
50% (v/v) ethanol	One litre was prepared as below: 500 mL of 100% ethanol was added to 500 mL of distilled water.

Appendix D

Representative Gel Image



Lane 1: 100 bp DNA ladder (Vivantis, Malaysia)

Lane 2: DNA of Sample O1L

Lane 3: DNA of Sample O1CB

Lane 4: DNA of Sample O1H

Appendix E

McNemar's and Cohen Kappa's

Chi-Square Tests

	Value	Exact Sig. (2-sided)
McNemar Test		.000[a]
N of Valid Cases	135	

a. Binomial distribution used.

Symmetric Measures

		Value	Asymptotic Standard Error[a]	Approximate T[b]	Approximate Significance
Measure of Agreement	Kappa	.655	.063	7.942	.000
N of Valid Cases		135			

a. Not assuming the null hypothesis.
b. Using the asymptotic standard error assuming the null hypothesis.

Appendix F

Kruskal-Wallis Test

Shannon Index across Location Kruskal-Wallis Test	
Total N	27
Test Statistic	.687[a,b]
Degree Of Freedom	2
Asymptotic Sig.(2-sided test)	.709

a. The test statistic is adjusted for ties.
b. Multiple comparisons are not performed because the overall test does not show significant differences across samples.

Shannon Index across Sample Type Kruskal-Wallis Test	
Total N	27
Test Statistic	12.557[a]
Degree Of Freedom	2
Asymptotic Sig.(2-sided test)	.002

a. The test statistic is adjusted for ties.

Appendix F

Kruskal-Wallis Test for Antibiotic Class

	Null Hypothesis	Sig.
1	The distribution of Aminocoumarin is the same across sample type.	.232
2	The distribution of Aminoglycoside is the same across sample type.	.375
3	The distribution of Carbapenem is the same across sample type.	.009
4	The distribution of Cephalosporin is the same across sample type.	.057
5	The distribution of Cephamycin is the same across sample type.	.114
6	The distribution of Diaminopyrimidine is the same across sample type.	.264
7	The distribution of Antiseptics is the same across sample type.	.103
8	The distribution of Fluoroquinolone is the same across sample type.	.082
9	The distribution of Glycylcycline is the same across sample type.	.073
10	The distribution of Lincosamide is the same across sample type.	.723
11	The distribution of Macrolide is the same across sample type.	.724
12	The distribution of Monobactam is the same across sample type.	.128
13	The distribution of Penam is the same across sample type.	.045
14	The distribution of Penem is the same across sample type.	.480
15	The distribution of Peptide is the same across sample type.	.054
16	The distribution of Phenicol is the same across sample type.	.394
17	The distribution of PhosphonicAcid is the same across sample type.	.017
18	The distribution of Rifamycin is the same across sample type.	.262
19	The distribution of Sulfonamide is the same across categories of SampleType.	.099
20	The distribution of Tetracycline is the same across categories of sample type.	.429