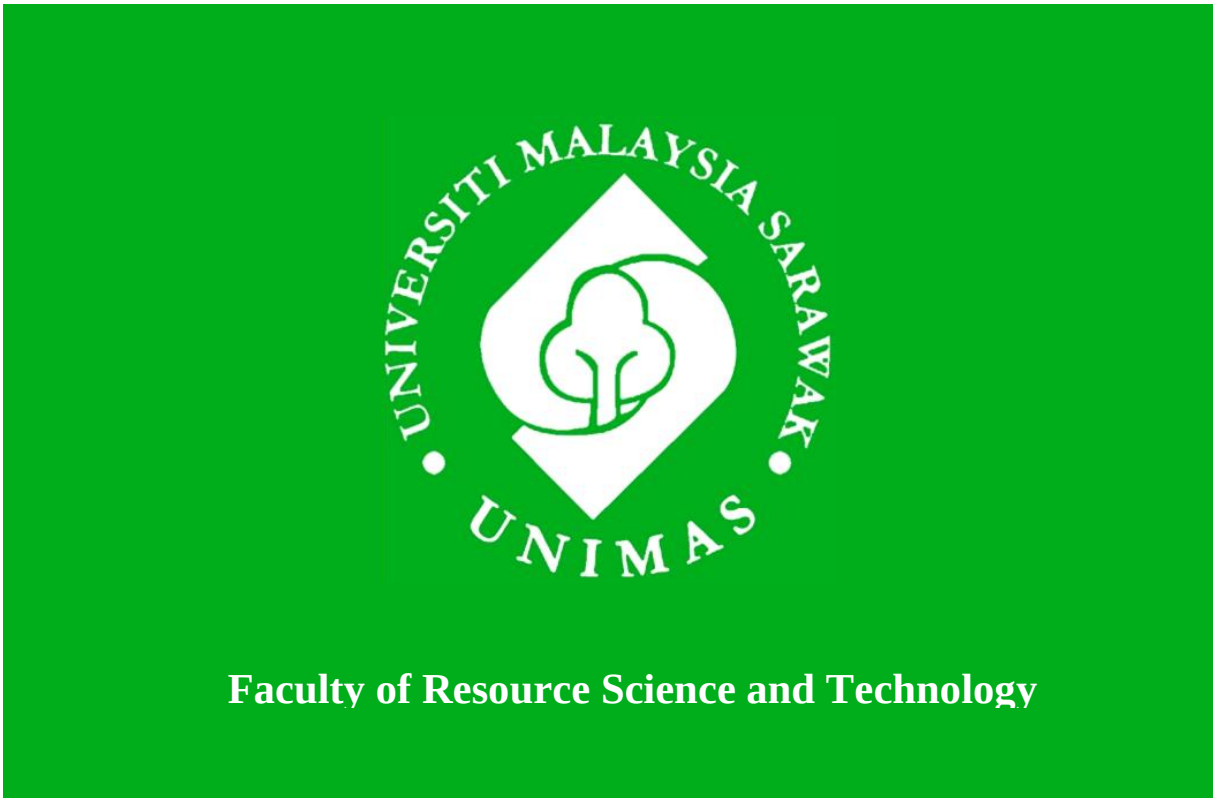

Molecular Characterization of *Listeria* spp. and *Listeria monocytogenes* in Fresh Produce at Farm Level and Determination of their Association with Agricultural Practices

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Master of Science
2016

Molecular Characterization of *Listeria* spp. and *Listeria monocytogenes* in Fresh Produce at Farm Level and Determination of their Association with Agricultural Practices

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A thesis submitted

In fulfillment of the requirements for the degree of

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DECLARATION

I hereby declare that the work entitled, "Molecular Characterization of *Listeria* spp. and *Listeria monocytogenes* in Fresh Produce at Farm Level and Determination of their Association with Agricultural Practices" is my original work. I have not copied from any other students' work or from any other sources with the exception where due reference or acknowledgement is made explicitly in the text, nor has any part of the work been written for me by another person. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

The main aim of this study was to detect, quantify and characterize *Listeria* spp. and *L. monocytogenes* in fresh produces, fertilizers and environment (soil and water) samples from two vegetable farms practicing organic and a conventional farm at Kuching, Sarawak, Malaysia. A total of 386 samples, including vegetable samples, fertilizer samples and environmental samples were collected. PCR analysis revealed that *Listeria* spp. was present in 7.51% (29/386) of all the samples (vegetable, soil, fertilizer and water) collected from all the three farms. This analysis indicated that *Listeria* spp. was present in 6.70% (2/30) and 9.10% (8/88) of vegetables samples from the organic farms, farm A and farm B, respectively. However, *Listeria* spp. was not detected in any vegetables samples collected from the conventional farm, farm C. The vegetable samples collected from organic farm A and farm B are considered unsatisfactory as the plate count exceed 100 CFU/g of *Listeria* spp. In this study, *Listeria* spp. were also present in 16.70% (2/12), 16.70% (4/24) and 8.30% (2/24) of fertilizer samples from organic farm A, organic farm B and conventional farm C, respectively. Other than that, *Listeria* spp. were also detected in 16.70% (2/12) and 8.30% (2/24) of soil samples from organic farm A and organic farm B. *Listeria* spp. were not detected in any soil samples collected from conventional farm C. Furthermore, *Listeria* spp. were only detected in 29.20% (7/24) of water samples from conventional farm C. *Listeria* spp. were found to be absent in the water samples from organic farm A and organic farm B. In the present study, *L. monocytogenes* was found to be absent in vegetable, fertilizer and environment from organic farm A, organic farm B and conventional farm C. The absence of *L. monocytogenes* in this study indicated that the vegetables from the selected farms were safe to consume. Two molecular methods, ERIC-PCR and BOX-PCR were used in this study to analyse the

relatedness among the *Listeria* spp. isolated from the different type of samples and sampling location. The *Listeria* spp. isolated from organic farm A, organic farm B and conventional farm C were found to be genetically diverse and heterogeneous. The Simpson's Index of Diversity, D, of ERIC-PCR and BOX-PCR were calculated to be 0.604 and 0.888, respectively and therefore, the present study showed that BOX-PCR has better discriminatory power compared to ERIC-PCR. Antibiotic susceptibility test revealed that most of the *Listeria* spp. in this study were found to be resistant to ampicillin, rifampin, penicillin G, tetracycline, clindamycin, cephalothin and ceftriaxone. The vegetables, fertilizer and farm environment from selected farms harbor multiple antibiotic resistant *Listeria* spp. The isolates have MAR index ranged between 0.31 and 0.85. This suggests that *Listeria* spp. from vegetables, fertilizer and farm environment from organic farm A, organic farm B and conventional farm C were originated from higher risk source in which they are constantly exposed to antibiotics. The findings in this study provides baseline data on *Listeria* spp. and *L. monocytogenes* contamination at the farm level for future risk assessment work and for establishing food safety standards in both organic and conventional vegetable farms in Sarawak, Malaysia.

Keywords: *Listeria* spp., *L. monocytogenes*, organic vegetable farm, conventional vegetable farm, ERIC-PCR

Pencirian Molekul Listeria spp. dan Listeria monocytogenes dalam Produk Segar pada Tahap Ladang dan Pertalian mereka dengan Amalan Pertanian

ABSTRAK

Tujuan utama penyelidikan ini adalah untuk mengesan, mengira dan mencirikan Listeria spp. dan L. monocytogenes dalam produk segar, baja dan alam sekitar (tanah dan air) dari dua ladang sayur organik dan satu ladang sayur konvensional di Kuching, Sarawak, Malaysia. Sebanyak 386 sampel termasuk sampel sayur-sayuran, sampel baja dan sampel alam sekitar telah dikumpulkan. Analisis PCR *mendedahkan bahawa Listeria spp. dikesan dalam 7.51% (29/386) sampel (sayur-sayuran, tanah, baja dan air) yang dikumpul daripada ketiga-tiga ladang. Analisis ini menunjukkan bahawa Listeria spp. dikesan dalam 6.70% (2/30) dan 9.10% (8/88) sampel sayur-sayuran dari ladang organik A dan ladang organik B, masing-masingnya. Namun demikian, Listeria spp. tidak dikesan dalam mana-mana sampel sayur-sayuran yang dikumpul daripada ladang konvensional C. Sampel sayur yang diambil dari semua ladang-ladang terpilih dianggap tidak memuaskan kerana kiraan plat telah melebihi 100 CFU / g Listeria spp. Dalam kajian ini, Listeria spp. turut dikesan dalam 16.70% (2/12), 16.70% (4/24) dan 8.30% (2/24) sampel baja dari ladang organik A, ladang organik B dan ladang konvensional C, masing-masing. Selain daripada itu, Listeria spp. juga dikesan dalam 16.70% (2/12) dan 8.30% (2/24) sampel tanah dari ladang organik A dan ladang organik B. Listeria spp. tidak dikesan dalam mana-mana sampel tanah yang diambil dari ladang konvensional C. Tambahan pula, Listeria spp. hanya dikesan pada 29.20% (7/24) sampel air dari ladang konvensional C. Listeria spp. tidak dikesan dalam sampel air ladang organik A dan ladang organik B. Dalam kajian ini, L. monocytogenes tidak dikesan dalam sampel sayur-sayuran,*

baja dan alam sekitar dari ladang organik A, ladang organik B dan ladang konvensional C. Ketiadaan L. monocytogenes dalam kajian ini menunjukkan bahawa sayur-sayuran dari ladang-ladang yang terpilih adalah selamat untuk dimakan. Dua kaedah molekul, ERIC-PCR *dan* BOX-PCR *telah digunakan dalam kajian ini untuk mengkaji pertalian di kalangan Listeria spp. yang diasingkan daripada pelbagai jenis sampel dan lokasi persampelan. Genetik Listeria spp. terpencil dari ladang organik A, ladang organik B dan ladang konvensional C didapati adalah pelbagai. Indeks Kepelbagaian Simpson, D,* ERIC-PCR *dan* BOX-PCR *telah dikira dan dipastikan adalah 0.604 dan 0.888 masing-masing dan oleh itu, kajian ini menunjukkan bahawa* BOX-PCR *mempunyai kuasa diskriminasi yang lebih baik berbanding* ERIC-PCR. *Ujian kerentanan antibiotik mendedahkan bahawa kebanyakan Listeria spp. dalam kajian ini didapati tahan terhadap ampisilin, rifampin, penisilin G, tetraksiklina, klindamisin dan seftriakson. Sayur-sayuran, baja dan persekitaran ladang dari ladang-ladang yang dipilih mengandungi Listeria spp. yang tahan terhadap pelbagai antibiotik. Listeria spp. pencilan mempunyai indeks* MAR *antara 0.31 dan 0.85. Ini menunjukkan bahawa Listeria spp. daripada sayur-sayuran, baja dan persekitaran ladang dari ladang organik A, ladang organik B dan ladang konvensional C berasal dari sumber risiko yang lebih tinggi di mana ia sentiasa terdedah kepada antibiotik. Penemuan dalam penyelidikan ini menyediakan data asas pencemaran Listeria spp. dan L. monocytogenes di peringkat ladang untuk kerja-kerja penilaian risiko masa depan dan menubuhkan standard keselamatan makanan di kedua-dua ladang sayur-sayuran organik dan konvensional di Sarawak, Malaysia.*

Kata Kunci: *Listeria spp., L. monocytogenes, ladang sayur organik, ladang sayur konvensional,* ERIC-PCR

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LIST OF ABBREVIATIONS

-	Negative
%	Percentage
+	Positive
act	Actin polymerization factor
AFLP	Amplified Fragment Length Polymorphism
AGE	Agarose Gel Electrophoresis
amp	Ampicillin
AOAC	Association of Official Analytical Chemists
ATCC	American Type Culture Collection
BLEB	Buffered Listeria enrichment broth
bp	Base pairs
℃	Degree Celsius
CAMP	Christie Atkins Munch Petersen
CDC	Center for Disease Control and Prevention
CEN, EN	European Committee for standardization
cf	Cephalothin
CFU	Colony-Forming Units
chl	Chloramphenicol
cli	Clindamycin
CLSI	Clinical and Laboratory Standards Institute
cro	Ceftriaxone
D	Simpson's index of diversity
ddH_2O	Double sterile distilled water
DNA	Deoxyribonucleotide Acid
dNTPs	Deoxynucleotide triphosphates
E. coli	*Escherichia coli*
ERIC	Enterobacterial Repetitive Intergenic Consensus
ery	Erythromycin
EtBr	Ethidium Bromide
FDA	Food and Drug Administration
FSIS	Food Safety and Inspection Service
G	Gram
gen	Gentamicin
hly	Listeriolysin O
hlyA	Listeriolysin O
hr	Hour
inl	Internalin
ISO	International Organization for Standardization
kb	Kilobase
L. grayi	*Listeria grayi*
L. innocua	*Listeria innocua*

L. ivanovii	*Listeria ivanovii*
L. monocytogenes	*Listeria monocytogenes*
L. seeligeri	*Listeria seeligeri*
L. welshimeri	*Listeria welshimeri*
LEB	Listeria Enrichment Broth
Listeria spp.	*Listeria* species
LLO	Listeriolysin O
LPM	Lithium chloride Phenylethanol Moxalactam
M	Molar
MAR	Multiple Antibiotic Resistance
$MgCl_2$	Magnesium Chloride
min	Minute
mL	Millilitre
MLST	Multilocus Sequence Typing
mM	Milimolar
MOX	Modified Oxford agar
mpl	Metalloprotease
MPN	Most Probable Number
MR, VP	Methyl Red, Voges–Proskauer
MSRV	Modified Semisolid Rappaport-Vassiliades
NGFIS	Netherlands Government Food Inspection Services
NMKL	Nordic Committee on Food Analysis
nor	Nitrofurantoin
PALCAM	Polymyxin Acriflavin Lithium-Chloride Ceftazidime
PCR	Polymerase Chain Reaction
penG	Penicillin G
PFGE	Pulsed-field gel electrophoresis
plc	Phospholipase
RAPD	Random Amplification of Polymorphic DNA
REP	Repetitive Extragenic Palindromic
Rep-PCR	Repetitive Sequence Polymerase Chain Reaction
rif	Rifampin
RTE	Ready-To-Eat
sec	Seconds
SLST	Single Locus Sequence Typing
str	Streptomycin
sxt	Trimethoprim/Sulfamethoxazole
Taq	*Thermus aquaticus* DNA polymerase
TBE	Tris-Borate EDTA
tet	Tetracycline
TSA	Tryptone Soy Agar
U	Unit
USDA	United States Department of Agriculture
UV	Ultraviolet

UVM	University of Vermont Medium
VNTR	Variable-Number Tandem Repeat
µg	Microgram
μl	Microlitre
μM	MicroMolar

CHAPTER 1

GENERAL INTRODUCTION

Malaysia's economy grew around 6% yearly since 2000 and it had affected the food consumption pattern in Malaysians. With the growth in income and population, Malaysians tend to choose healthier and nutritious food for consumption. They prefer healthier, safer, hygienic, environmental friendly and higher quality food (Teng et al., 2011). In the United States (US), the heightened of consumers' health awareness had resulted in higher demand for fresh produce (Naanwaab & Yeboah, 2012).

Fruits and vegetables are highly recommended in many countries due to their health-promoting properties. Fruits and vegetables are high in vitamins, minerals, antioxidant and dietary fiber (Slavin & Lloyd, 2012). They also play important roles in reducing the risk of cardiovascular diseases and gastrointestinal cancers (Yen et al., 2011). However, fruits and vegetables especially leafy green, are known to be vehicles for transmission of human pathogens (Berger et al., 2010). There are a few food poisoning bacteria reported to be found in vegetables which include *Salmonella* spp., *Listeria* spp., *Escherichia coli* and *Bacillus cereus* (Ramanathan, 2010).

Listeria is a gram-positive, rod-shaped, and non-spore-forming bacterium (Todar, 2012). Genus of *Listeria* is classified into six species including *Listeria monocytogenes*, *Listeria ivanovii*, *Listeria seeligeri*, *Listeria innocua*, *Listeria welshimeri* and *Listeria grayi* (Adzitey & Huda, 2010). *Listeria* spp. are ubiquitous in nature and are commonly found in soil, water, manure, sewage, vegetation, animal feed and farm environments (Williams, 2010).

In the family of *Listeria*, *L. monocytogenes* is a human pathogen that causes human foodborne infection namely Listeriosis (Jeyaletchumi et al., 2010). This foodborne disease rarely develops clinical symptoms in healthy individual but in some cases, it causes fever, nausea, diarrhea, headache, abdominal pain, gastroenteritis and sometimes myalgia. These symptoms are normally self-limiting and last for only one to three days. However, elderly, pregnant woman, newborns and immunocompromised individuals are more susceptible to listeriosis. Listeriosis results in meningitis, meningoencephalitis, septicemia, abortion, stillbirth and premature birth in this group of people (The Center for Food Security and Public Health, 2005; Center for Disease Control and Prevention (CDC), 2013). Listeriosis has approximately 20-30% mortality rate in susceptible groups of individual (OIE, 2014).

In September 2011 in the US, an outbreak of *L. monocytogenes* associated with consumption of cantaloupe from Jensen farms, Colorado had resulted in 147 illnesses, 33 deaths, and 1 miscarriage among residents of 28 states (CDC, 2012). In August 2014, there was another outbreak of listeriosis linked to bean sprouts from Wholesome Soy Products Inc. of Chicago, US. It was revealed that the outbreak was due to unsanitary conditions of their facilities (CDC, 2014). Early of the year of 2015, US reported an outbreak of listeriosis linked to commercially produced, pre-packaged caramel apples. The outbreak leads to 35 cases of infection and 7 deaths (CDC, 2015). In Malaysia, 33.3% of *Listeria* spp. and 22.5% of *L. monocytogenes* were detected in raw salad vegetables sold at retail level in the wet markets and hypermarkets from the state of Selangor. In the study, *L. monocytogenes* was more frequently detected in Japanese parsley and yardlong bean (Ponniah et al., 2010). Other than that, Jeyaletchumi et al. (2011) have also discovered that the poultry manure, soil samples, cabbages, cucumber, yardlong beans and carrots from vegetable farms in Cameron Highlands,

Malaysia were contaminated with *L. monocytogenes*. The finding of the study suggested that the contamination of vegetables by *Listeria* spp. could be due to the contaminated soil and manure at the farm. Contamination of vegetables by food pathogens at pre-harvest stage may occur through agricultural practices such as irrigation with contaminated water, fertilization with contaminated manure and contamination from soil and animals (Jung et al., 2014). Microbiological contamination of fresh produce is more likely to occur in organic farming due to the use of organic fertilizers, a possible source of pathogenic bacteria (Loncarevic et al., 2005). Maffei et al. (2013) studied that the organic vegetables have higher microbial counts as compared to conventional vegetables. However, this fact does not indicate a trend as it was not observed for all microbial groups tested and in varieties of vegetables analyzed. Therefore, more studies are required to evaluate the microbial profile of vegetables practicing organic and conventional farming.

There are several international standard enrichment protocol commonly used for isolation and identification of *Listeria* spp. which include the US Food and Drug Administration (FDA), the Association of Official Analytical Chemists (AOAC), the European Committee for standardization (CEN, EN), the International Organization for Standardization (ISO), the Netherlands Government Food Inspection Services (NGFIS), the Nordic Committee on Food Analysis (NMKL) and the United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) (Ryser and Marth, 1999; OIE, 2014). In our study, NGFIS enrichment method was used to detect *Listeria* spp. This method involves the use of Listeria Enrichment Broth (LEB) and Polymyxin-Acriflavin-Lithium chloride-Ceftazidime-Aesculin-Mannitol (PALCAM) agar to differentiate *Listeria* spp. from other organisms (Churchhill et al., 2006).

Listeria spp. can also be identified rapidly by biochemical tests such as oxidase, catalase, Methyl Red, Voges–Proskauer (MR, VP), Rhamnose, Mannitol, Xylose and Christie Atkins Munch Petersen (CAMP) tests. Many rapid indentification kits (API Listeria test strip, MICRO-ID Listeria and Microbact 12L Listeria) were used for the biochemical tests as they are more rapid and less tedious (Rocourt & Buchrieser, 2007; USDA-FSIS, 2012). However, culture-based detecting methods often give false-positive/false-negative results. Hence, Polymerase Chain Reaction (PCR) assay can be a good alternative for *Listeria* spp. and *L. monocytogenes* detection (Khan et al., 2013). Normally, primers targeting 16S rRNA region and *hlyA* gene were used in PCR assay to detect *Listeria* spp. and *L. monocytogenes* (Jeyaletchumi et al., 2011).

Repetitive sequence-based PCR (Rep-PCR) is a DNA amplification technique for bacterial genomic fingerprinting by using repetitive DNA elements present within bacterial genome. There are three main types of repetitive sequences used for molecular typing which include repetitive extragenic palindromic sequences (REP elements), enterobacterial repetitive intergenic consensus (ERIC) sequence and BOX elements. ERIC sequence-based PCR (ERIC-PCR) and BOX-PCR were used in this study as they are rapid subtyping methods and have high discrimination power (Olive & Bean, 1999; Jeyaletchumi et al., 2010; Fakruddin et al., 2013).

Listeriosis is usually treated with antibiotic therapy which involves the use of penicillin, ampicillin, rifampin, gentamicin, tetracycline, erythromycin, chloramphenicol or tri-methoprim with sulfamethoxazole alone or in combination (Walsh et al., 2001; Chen et al., 2010). Researchers revealed that *Listeria* spp. are resistant to various types of antibiotics

including clindamycin, daptomycin, oxacillin, tetracycline and nalidixic acid (Adzitey et al., 2013; Moreno et al., 2014; Gomez et al., 2014). Therefore, it is important to monitor antibiotic susceptibility of *Listeria* spp. and *L. monocytogenes* to assure the effectiveness of Listeriosis treatment. In this study, antibiotic susceptibility of *Listeria* spp. in the samples were examined by disk diffusion method (Morobe et al., 2012).

As the presence of microorganisms such as *Salmonella* and *L. monocytogenes* in vegetables raises major concern to consumers, we decided to study on the level of *Listeria* spp. in fresh produce vegetables because vegetables are often consumed raw and minimally processed.. According to Strawn et al. (2013), they suggested that agricultural practices (irrigation with contaminated water, fertilization with contaminated manure and contaminated soil) could increase the risk of bacterial contamination in vegetables. Therefore, we were also investigating on the fertilizer, soil and water from vegetable farms. In addition, no research has been done to detect the presence of *Listeria* spp. and *L. monocytogenes* at conventional and organic vegetable farms in Kuching, Sarawak. Therefore, this study was carried out to assess the contamination levels of *Listeria* spp. and *L. monocytogenes* in vegetables, fertilizer and environmental samples (soil and water) at farm level in Kuching, Sarawak. The findings of this study provided baseline data on *Listeria* spp. and *L. monocytogenes* contamination at the farm level for future risk assessment work and establishment of food safety standards in both organic and conventional vegetable farms at Sarawak, Malaysia.

The main goal of this study was to detect, quantify and characterize *Listeria* spp. and *L. monocytogenes* in fresh produce, fertilizer and environmental samples from two vegetable farms practicing organic farming and one vegetable farm practicing conventional farming in Sarawak, Malaysia. The specific objectives of this study were:

1. To detect *Listeria* spp. and *L. monocytogenes* in fresh produce, fertilizer and environmental samples from vegetable farms practicing organic and conventional farming by using Polymerase Chain Reaction (PCR).

2. To characterize *Listeria* spp. and *L. monocytogenes* in fresh produce, fertilizer and environmental samples by using Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC- PCR) and BOX-PCR.

3. To determine the presence of *Listeria* spp. and *L. monocytogenes* and their association with agricultural practices such as the use of contaminated soil and water and fertilization with contaminated manure.

4. To determine the antibiotic resistance profiles of *Listeria* spp. and *L. monocytogenes* isolated from fresh produce, fertilizer and environmental samples from vegetable farms practicing organic and conventional farming.

CHAPTER 2

LITERATURE REVIEW

2.1 Discovery and Taxonomy of *Listeria*

In 1924, E.G.D. Murray isolated bacterium *monocytogenes* from the blood of laboratory animal, rabbits. At that time, this pathogenic microorganism was not classified to any bacterial genus known (Murray et al., 2005). It was believed that this bacterium was grown by other bacteriologists before Murray without having a clear classification. Later in 1940, Pirie classified the bacterium as genus *Listeria* (Hof, 2003). Figure 2.1 shows the view of *Listeria* spp. cell under microscope.

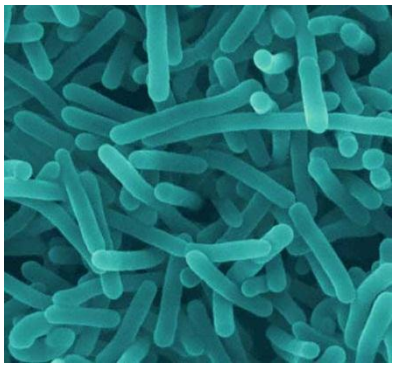


Figure 2.1 Microscopic view of *Listeria* spp. cell (Adapted from AIB, International, 2015)

Listeria spp. is a rod-shaped, Gram-positive and non spore-forming *bacilli*. *Listeria* spp. is generally aerobes or facultative anaerobes, catalase positive and oxidase negative. *Listeria* spp. is motile at the temperature below 30 ℃ as a few peritrichous flagella are grown at that temperature. There are six species found in this genus which are *L. monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri and L. grayi* (FDA, 2012). In this genus, there

are three hemolytic species (*L. monocytogenes, L. ivanovii*, and *L. seeligeri*) and three non-hemolytic species (*L. innocua, L. welshimeri* and *L. grayi*) (Garrido et al., 2010). Among all of the *Listeria* spp., only *L. monocytogenes* and *L. ivanovii* are recognized as pathogens (Muller et al., 2010). *L. monocytogenes* is a causative agent of a severe foodborne disease in human called Listeriosis. A range of mammals can also be infected by *L. monocytogenes* (Ordonez et al., 2015). Other than *L. monocytogenes*, *L. ivanovii* was found to be contributing to animal listeriosis in ruminants. Although rare, it has been reported that *L. ivanovii* is also responsible for human listeriosis cases in susceptible individuals (Wang et al., 2014). In January 2007, a 55-year-old man in Paris, France, was hospitalized and reported to be infected with *L. ivanovii* (Guillet et al., 2010). *L. seeligeri* has both hemolytic and non-hemolytic isolates. Similar to *L. monocytogenes* and *L. ivanovii*, hemolytic *L. seeligeri* also carries a virulence gene cluster (*prfA* cluster) (Sauders et al., 2012). However, *L. seeligeri* is considered a non-pathogenic *Listeria as* the genes of the virulence cluster are not expressed in a correct or functional fashion. Besides that, the non-hemolytic *Listeria* spp. including *L. innocua*, *L. welshimeri* and *L. grayi* are considered as non-pathogenic (Muller et al., 2010).

Listeria spp. are extremophiles. They can survive in acidic, low temperature (refrigeration temperature) and high salt conditions (FDA, 2012). This bacterium is ubiquitous in the environment and can be found in moist environment, soil and decaying vegetation (FDA, 2012). According to Shantha & Gopal (2014), *Listeria* spp. can be found in soil, sewage, silage, water, plants, and in a variety of foods including raw meat, raw vegetables, processed foods and food processing environments. In Ireland, Wilson (1995) found that 5.00% of 8000 ready-to-eat (RTE) foods (meat products, plant products, dairy products, cooked prepared meals, bakery products and miscellaneous products) examined

were found to contain *Listeria* spp. Vitas et al. (2004) also reported on the occurrence of *Listeria* spp. in 8.10% soft cheese and 76.30% raw poultry samples from different industries and markets of Northern Spain. *Listeria* spp. was also detected by Gomez et al. (2015) in RTE meat (sausages and ham) and manufacturing plants from meat industries of 6 Spanish provinces and retails in Zaragoza. Besides that, Dalmasso & Jordan (2013) also found that 33.00% of swab samples from an Irish food processing facility were positive for the presence of *L. monocytogenes*. Other than food products and food processing facility, *Listeria* spp. were also detected in the environment. Linke et al. (2014) revealed that *Listeria* spp. was present in 30.00% and 26.00% of the investigated soil and water samples, respectively, from 12 areas in Austria.

2.2 *Listeria monocytogenes*

2.2.1 Features and Characteristics

L. monocytogenes is a Gram-positive, non-spore forming and rod-shaped bacterium that can grow under both aerobic and anaerobic conditions (Sutherland et al., 2003; Lado & Yousef, 2007; FDA, 2012). This organism is catalase positive, oxidase negative and expresses β-hemolysin. It is also motile between 20 ℃ and 25 ℃ as its flagella produced and assembled at the cell surface in this range of temperatures. However, flagella production of *L. monocytogenes* is markedly reduced at 37 ℃ (O' Bryan et al., 2015).

L. monocytogenes was first discovered more than 100 years ago and was recognized primarily as an animal pathogen. Since 1981, *L. monocytogenes* had broadly been documented to be a foodborne pathogen detected in human body (Donnelly & Gonzalez, 2013). It is also widely present in soil, sewage, silage, water, vegetation, a variety of food processing environments and food products (Thevenot et al., 2006; Jeyaletchumi et al., 2010; Laksanamalai et al., 2012). *L. monocytogenes* had also been isolated from poultry and poultry product (Crespo et al., 2013).

L. monocytogenes consists of 13 serotypes which include 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7. The serotypes most often associated with human illness are 1/2a, 1/2b and 4b (Farber & Peterkin, 1991; FDA, 2012) which account for more than 95% of the isolates from human infection (Pan et al., 2009; Lamon et al., 2015).

2.2.2 Virulence Genes of *Listeria monocytogenes*

L. monocytogenes has a few virulence genes namely, listeriolysin O (*hly*), phospholipases (*plcA* & *plcB*), metalloprotease (*mpl*), actin polymerization factor (*actA*) and internalins (*inlA* & *inlB*) that are involve in the infectious process (Sheehan et al., 1995; Cossart, 2011). *InlA* and *inlB* genes encode two proteins which are internalins A and B. These proteins promote the entry of *L. monocytogenes* into non-phagocytic cells (Vadia et al., 2011). *InlA* binds covalently to the cell wall of epithelial host cells while *inlB* loosely interact with the cell wall. These proteins mediate internalization of *L. monocytogenes* into epithelial host cells (Pizzaro-Cerda et al., 2012). It was also revealed that *inlA* plays an important role in human listeriosis (Werbrouck et al., 2006).

The gene that encodes Listeriolysin O (LLO) is *hlyA* and it was the first virulence gene identified in *Listeria*. LLO is a protein which responsible in the escape of *L. monocytogenes* from bacterial vacuole. It also induces the entry of calcium ions for invasion of epithelial cells. Phospholipases *plcA* and *plcB* encoded by *plcA* & *plcB* gene are involved in the lysis of bacterial secondary vacuole. These two enzymes cooperate with LLO to disrupt primary and secondary vacuole (Camejo et al., 2011). Once LLO enters the cytoplasm, *ActA* gene which encodes *actA* protein, initiates the motility of *L. monocytogenes* (Travier et al., 2013). This protein induces actin filaments polymerization on the bacteria, forming actin comet tails so that *L. monocytogenes* is able to move to neighbouring cells and start a new invasion cycle (Cossart & Lecuit, 1998; Pizzaro-Cerda et al., 2012). This gene is also shown to be engaged in cell attachment and entry (Suarez et al., 2001). Figure 2.2 shows the entry of *L. monocytogenes* into mammalian epithelial cells with the help of virulence factors.

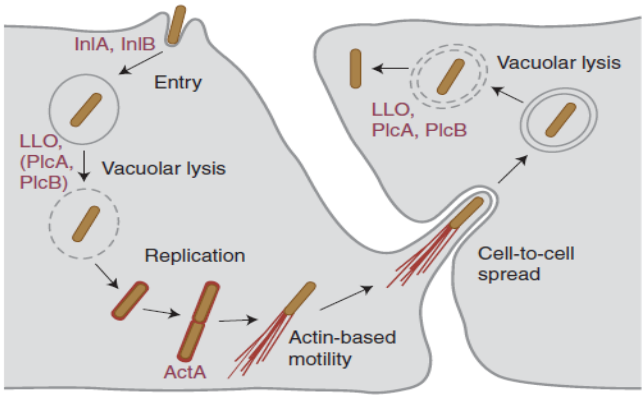


Figure 2.2 Entry of *L. monocytogenes* into mammalian epithelial cells with the help of virulence factors including internalin A (*inlA*) and B (*inlB*), Listeriolysin O (LLO), *plcA* and *plcB* phospholipases and *actA* protein (Adapted from Pizzaro-Cerda et al., 2012)

Lomonaco et al. (2012) selected 7 virulence-associated genes which include *actA*, *inlC*, *inlJ*, *plcA*, *prfA*, *hlyA* and *iap* genes, to detect *L. monocytogenes* from Gorgonzola cheese. However, *hlyA* gene based detection for *L. monocytogenes* has been frequently adopted by many researchers. Swetha et al. (2012), Khan et al. (2013) and Al-Ashmawy et al. (2014) targeted *hlyA* gene for *L. monocytogenes* detection in fish samples, commercial food samples (raw meat and raw milk and milk products) and milk and soft cheeses. Swetha et al. (2012) found that 2 fish samples and 2 fish surface swabs of 60 samples (25 each of fish samples and fish surface swabs and 10 dried salt fish samples) collected from various markets of Hyderabad were positive for *L. monocytogenes* by PCR assay targeting *hlyA* and *iap* genes. Khan et al. (2013) also found the occurrence of *hlyA* gene of *L. monocytogenes* in raw chicken (6.00%), fish meat (4.00%), beef (2.50%), curd (2.00%) and raw milk (1.30%) collected from butcheries, dairy farms and dairy/confectionery shops at various locations in the Bareilly city, Uttar Pradesh, India. Al-Ashmawy et al. (2014) were also revealed that 14.00% (28/200) of market raw milk, bulk tank milk, Damietta cheese and kareish cheese samples were *L. monocytogenes* positive by using colony PCR method targeting *hlyA* gene.

2.2.3 Clinical Manifestation of Listeriosis

L. monocytogenes is an important foodborne pathogen which causes listeriosis. Human listeriosis was first discovered in 1929. Since that time, listeriosis has been recognized as a rare but often fatal illness (Yusuf & Hamid, 2013). Listeriosis is associated with high fatality rate of 30% compared with other foodborne microbial pathogens, such as *Salmonella*, which rarely results in fatalities (Khan et al., 2013). Listeriosis can be present as either non-invasive and invasive form of infection. Non-invasive infection has been documented in several outbreaks. The onset of symptoms for non-invasive infections is short where most patients developed symptoms of gastroenteritis such as diarrhea, fever, headache, and myalgia (Kruszyna et al., 2008). However, the symptoms vary with the infected person. Elderly, immunocompromised individuals, pregnant women, and newborn infants are more susceptible to invasive form of listeriosis. Severe symptoms including meningitis, septicemia, primary bacteremia, endocarditis, non-meningitic central nervous system infection, conjunctivitis, flulike illness (fever, fatigue, malaise, nausea, cramps, vomiting, and diarrhea) and even death, are more likely to occur in this group of individuals (Donnelly & Gonzalez, 2013). Pregnant women may experience fever and other non-specific symptoms, such as fatigue and aches. Listeriosis during pregnancy can also results in more severe outcomes such as miscarriage, stillbirth, premature delivery or life-threatening infection to the newborn (CDC, 2013; Mateus et al., 2013).

2.2.4 Foodborne Listeriosis Outbreak

In 2011, there was an outbreak of *L. monocytogenes* in cantaloupes (orange-flesh melon) at Colorado, United State. In this incident, 147 individuals were infected with Listeriosis and 33 deaths were reported. Miscarriage had also occurred in a pregnant woman (CDC, 2012). In August 2014, there was another outbreak of listeriosis linked to bean sprouts in Chicago. It was revealed that the outbreak was due to unsanitary conditions of the facilities of Wholesome Soy Products Inc (CDC, 2014). In early 2015, Granny Smith and Gala apples produced in California, United States were recalled from the markets in many countries including Thailand, Philippines and Malaysia because environmental testing revealed contamination of *L. monocytogenes* at the Bidart Bros apple-packing facilities, United States. A total of 35 people were infected with the strains of *L. monocytogenes* while 7 deaths and 1 fetal loss were reported (CDC, 2015; Singapore Press Holdings, 2015).

In Malaysia, Ponniah et al. (2010) revealed that 33.30% of *Listeria* spp. and 22.50% of *L. monocytogenes* were detected in raw salad vegetables sold at the wet markets and hypermarkets from the state of Selangor. Their finding shows that *L. monocytogenes* was more frequently detected in Japanese parsley and yardlong bean. Jeyaletchumi et al. (2011) have also studied on the occurrence of *L. monocytogenes* in vegetable farms in Cameron Highlands, Malaysia and they found out that the poultry manure, soil, cabbages, cucumber, yardlong beans and carrots from the selected vegetable farms were contaminated with *L. monocytogenes*. Although *L. monocytogenes* was found to be present in fresh produce, there has been no cases of food borne listeriosis reported in Malaysia as *L. monocytogenes* is rarely tested in food poisoning cases (Jamali et al., 2013).

L. monocytogenes can be isolated from the surface and underground waters, improperly fermented silage, sewage sludge, slaughter wastes, animal and human feces, food, and food industry plants (Marin et al., 2012; Santorum et al., 2012). The presence of *L. monocytogenes* in farm animals is mainly associated with cattle and sheep farming, and less often with pig and poultry farming. *L. monocytogenes* usually transmitted into these animals when they consume contaminated feed. *L. monocytogenes* will then passes through the gastrointestinal tract of the animals and excreted in feces. Therefore, fecal contamination of soil, vegetation, and surface water is the primary source of *L. monocytogenes* contamination in food production (Gelbicova & Karpiskova, 2012).

2.3 Prevalence of *Listeria* spp. in Vegetables and Association with Agricultural Practices at Farm

Conditions and practices taken along the farm to plate continuum, affect the microbial contamination in vegetables (Mritunjay & Kumar, 2015). The main sources contributed to the microbial contamination include contaminated agricultural water, soil amendments, contaminated harvesting equipment, field workers, processing plants and retail handling (Jung et al., 2014). At pre-harvest stage, contamination of *L. monocytogenes* may traverses through agricultural practices such as irrigation with contaminated water, fertilization with contaminated manure and contamination from the soil, air and animals (Jeyaletchumi et al., 2011).

Soil reserves huge diversity of microorganisms which are important for greater plant growth by engaging in the transformations of nitrogen, phosphorus, and sulfur, and forming mutualistic associations with plants (Massenssini et al., 2014). However, foodborne pathogens such as *B. cereus, Clostridium botulinum, Clostridium perfringens* and *L. monocytogenes* were found to be present in soil (European Commission, 2002). Linke et al. (2014) found that *Listeria* was present in 30.00% of the investigated soil collected in 12 distinct geological and ecological sites in Austria. *L. monocytogenes* contamination in vegetables may be contributed by *L. monocytogenes* in the soil at the pre-harvest stage when seeds are sown (Vackachan et al., 2015). Irrigation water (streams, rivers, ponds) have also been reported to be contaminated with *Listeria* spp. (43.30%) including *L. monocytogenes*, *L. innocua* and *L. grayi* at Jos, Plateau State, Nigeria (Mawak et al., 2009). Pathogenic bacteria that were found in the irrigation water for fresh produce, can internalize into a crop through the roots of the plant and survive in it (Galvez et al., 2014). Therefore, water used for irrigation must be of suitable microbiological quality (European Commission, 2002).

Research demonstrates that fertilizer used in farm may also contain pathogenic bacteria that represent a human health concern (Miller, 2011). According to Hofmann et al. (2014), fertilization with animal waste is one of the factors which contribute to bacterial contamination in vegetable as animal waste contains various human pathogenic bacteria. Jeyaletchumi et al. (2011) conducted a study and revealed that 77.80% and 61.10% poultry manure (for fertilization) from vegetable farms in Cameron Highlands, Malaysia were found to contain *Listeria* spp. and *L. monocytogenes*, respectively. These environmental factors at pre-harvest stage affect the prevalence of food-borne pathogens and consequently, impact the

risk of contamination in fresh produce. However, appropriate management of practices could minimize the contamination risks (Strawn et al., 2013).

2.4 Isolation and Enumeration of *Listeria* spp. in Food

There are several conventional methods commonly used to isolate *Listeria* spp. and *L. monocytogenes* which are published in a few international standards including the standard of the United States Food and Drug Administration (FDA), the International Organization for Standardization (ISO), the US Department of Agriculture–Food Safety and Inspection Service (USDA–FSIS) and the Netherlands Government Food Inspection Services (NGFIS) (Churchhill et al., 2006).

According to the FDA standard, sample is enriched in Buffered Listeria enrichment broth (BLEB) containing sodium pyruvate and then streaked on differential selective-agars (eg. PALCAM, Oxford or LPM) in order to isolate *Listeria* spp. (Hitchins & Jinneman, 2011). Wong et al. (2012) had isolated *L. monocytogenes* from 33.30% of chicken burger patties, 22.90% of beef patties, and 10.50% of fish patty samples commercially available at retail level in Malaysia, by using this method. They suggested that common culturing method should be used in combination with PCR-based detection method as conventional culturing method alone can resulted in false negative as in their study.

ISO method was used by Jamali et al. (2013) for isolation and identification of *L. monocytogenes* in RTE foods (beverages, chicken and chicken products, egg and egg products,

beef and beef products, seafood and seafood products and salads and vegetables). In this study, samples were enriched in half Fraser broth for primary enrichment and Fraser broth as second enrichment culture. The enriched Fraser broth-culture is streaked onto different selective agar, Listeria selective agar, PALCAM agar and CHROMagar Listeria to detect *Listeria* spp. In the study, *Listeria* spp. and *L. monocytogenes* were detected in 17.90% and 11.40% respectively, from RTE food. The finding also shows that the detection sensitivity of Listeria selective agar, PALCAM agar and CHROMagar Listeria were improved by almost two fold with extra incubation time of 24 hr.

For USDA-FSIS method, University of Vermont Medium I (UVM I) and UVM II was used as primary enrichment and secondary enrichment, respectively. Enriched culture was then streaked on Modified Oxford agar (MOX) for *Listeria* spp. isolation (USDA-FSIS, 2012). Shamloo et al. (2014) had used USDA method to study the prevalence of *Listeria* spp. in raw milk and traditional non-pasteurized dairy products in Isfahan, Iran. Of 292 samples, 21 (7.14%) and 4 (1.47%) were positive for *Listeria* spp. and *L. monocytogenes*, respectively. For NGFIS standard, *L. monocytogenes* is isolated by using LEB as primary and secondary enrichment and then subculture on PALCAM agar (Churchhill et al., 2006). By using the NGFIS method, Ozbey et al. (2006) isolated *Listeria* spp. and *L. monocytogenes* in 16.00% and 9.00% of camel sausages from retail markets in Turkey, respectively. Hayes et al. (1992) had compared three selective enrichment procedures—FDA method, USDA method, and NGFIS method—for isolating *L. monocytogenes* from contaminated food. The finding shows that USDA and NGFIS methods are better at recovering *L. monocytogenes* as *L. monocytogenes* was detected in 65.00%, 74.00% and 74.00% of the contaminated food by using the FDA, USDA and NGFIS standard, respectively.

Enumeration of microorganisms is the determination of the number of microorganisms in per gram or mL of food or dairy product, in order to assess the level of contamination (Public Health England, 2014). There are several conventional methods, including Most Probable Number (MPN), standard plate count method, Modified Semisolid Rappaport-Vassiliades (MSRV) and direct counting to enumerate microorganisms (Malorny et al., 2008). Among all of the enumeration methods, standard plate count is the most frequently used method for enumerating microorganisms. As this method is time consuming, scientists are working on novel methods for bacteria enumeration. However, standard plate count method still serves as a reference method in the assessment of enteric bacteria, in investigations of different bacterial mutations associated with drug resistance and in detection of urinary track infection (Szermer-Olearnik et al., 2014). By using standard plate count, bacterial samples with appropriate dilution can be spread on selective agar media followed by a particular incubation time. After incubation, bacterial colonies can be counted and colony-forming units (CFU) for the particular bacteria can be calculated (Gracias & Mckillip, 2004). Mugampoza et al. (2011) had enumerated *Listeria* spp. in bulked raw milk and traditionally fermented dairy products in Uganda by plating bacterial enrichment broth (LEB) on selective agar (Oxford agar) for *Listeria* spp. enumeration. In the finding, Bongo, bulked raw milk and locally processed yogurt had total plate count of 9.00 ± 0.13 $\log_{10}$ CFU/mL, 8.40 ± 0.11 $\log_{10}$ CFU/mL and 7.40 ± 0.13 $\log_{10}$ CFU/mL, respectively. The study suggested a high risk of *Listeria* spp. contamination associated with consumption of bulked raw milk and fermented dairy products.

2.5 Identification of *Listeria* spp. and *Listeria monocytogenes*

2.5.1 Cultural and Biochemical confirmation

Selective media can be used to isolate *Listeria* spp. Selective media commonly used are Oxford Medium (OXA), PALCAM agar, Modified Oxford (MOX) agar and Lithium Phenylethanol Moxalactam (LPM) agar, with esculin and Fe^{2+} added in it. *Listeria* spp. colonies are observed to be black in colour and surrounded with black halo on these esculin containing media (Hitchins & Jinneman, 2011). Recently, selective chromogenic differential plating media have been developed to detect pathogenic *Listeria* spp. which include BBL™ CHROMagar™ Listeria, CHROMagar™ Listeria, ALOA™, BCM™ *Listeria monocytogenes* plating medium and Rapid *L. mono* Agar (Odumeru et al., 2005) These media differentiate pathogenic *Listeria* spp. from non-pathogenic *Listeria* spp. by analyzing the colonies and the zone around the colonies (Odumeru et al., 2005; Hitchins & Jinneman, 2011).

Jamali et al. (2013) conducted a study to detect *Listeria* spp. and *L. monocytogenes* in RTE food with various selective culture media and revealed that both Listeria selective agar and PALCAM agar displayed a low sensitivity and specificity in *L. monocytogenes* detection compared to CHROMagar Listeria which demonstrated 96.90% of sensitivity and 99.1% of specificity in *L. monocytogenes* detection. They suggested that selective media does not allow one-step detection of *L. monocytogenes* so further identification with biochemical test or PCR is needed to differentiate between *Listeria* spp.

2.5.2 Nucleic Acid-Based Identification: Polymerase Chain Reaction (PCR)

Culture-based identification methods often give false-positive/false-negative results (Khan et al., 2013). Hence, various nucleic-acid based identifications have been employed for identification of *Listeria* spp. and *L. monocytogenes*. Nucleic-acid based identifications include conventional PCR, multiplex PCR, real-time PCR, nucleic acid sequence-based amplification (NASBA) and DNA microarrays (Churchhill et al., 2006). Among all the nucleic-acid based identifications, PCR was the first and the most common technique used by many researchers and clinical laboratories. PCR is a simple and fast way to amplify specific nucleic acid sequences from the desired organism to an amount that can be viewed by the human eye with detection devices (Jeyaletchumi et al., 2010).

PCR is a chain reaction which produces multiple copies of DNA in continuous doubling of small amount of DNA. This reaction is completed by enzyme known as DNA polymerase that binds individual DNA building blocks together to form long molecular strands. To amplify the targeted DNA, template DNA (nucleotides consisting of the four bases adenine (A), thymine (T), cytosine (C) and guanine (G)), primers (small fragments of DNA anneal to the template DNA) and polymerases are required to complete the reactions. PCR requires a particular segment of DNA, primers and polymerase to selectively amplify the targeted segment of DNA. It can amplify a sufficient amount of DNA visible by gel electrophoresis in approximately 2 hours (Solanki, 2012).

The development of the PCR has changed the field of molecular science with its impact in numerous sub-disciplines in biology. In 1971, Keppe and co-workers outlined the

theoretical process of PCR. However, it took 14 years for the complete PCR procedure to be illustrated and experimentally applied by Kary Mullis in 1985 (Lorenz, 2012). *Taq* DNA polymerase, a thermal stable DNA polymerase isolated from the bacterium *Thermus aquaticus* was introduced in this technique (Roayaei & Galehdari, 2008). PCR utilises *Taq* DNA polymerase and a small amount of starting material (DNA template or target sequence) to generate an ample supply of a specific segment of DNA (Kalle et al., 2014).

There are three major stages involved in the PCR technique which are denaturation, annealing, and extension. In denaturation process, DNA is denatured and separated into two single-stranded DNA at high temperatures from 90-97℃ (Joshi & Deshpande, 2011). At annealing stage, primers anneal to the DNA template strands and then *Taq* polymerase synthesizes two new strands of DNA, using the original strands as templates. This process results in the duplication of the original DNA then the new strands undergo further duplication. The annealing phase usually occurs at a lower temperature, from 50-60℃ to allow the primers to hybridize to their respective complementary template strands. Extension occurs at the end of the annealing phase to create a complementary copy strand of DNA. The extension phase occurs at approximately 72℃ to fill in the protruding ends of newly synthesized PCR products (Joshi & Deshpande, 2011; Virtual Medical Centre, 2014; Bio-Rad Laboratories, 2015).

PCR is a very fast technique to amplify desired DNA compared to other techniques as each PCR cycle doubles the number of copies of the desired DNA strand. The specificity of this technique depends on the primers to recognize and bind to sequences other than the intended target DNA sequences (Joshi & Deshpande, 2011). By using PCR assay, Lakicevic

et al. (2010) detected the presence of *Listeria* spp. and *L. monocytogenes* in food processing environment in Serbia. They used primers targeting the *iap* and *hlyA* genes for *Listeria* spp. and *L. monocytogenes* detection. The result showed 23 out of 147 swab samples from food processing environment in Serbia were positive for *Listeria* spp. Besides that, Aytac et al. (2010) also used PCR to evaluate *L. monocytogenes* contamination on leafy green vegetables. In the study, specific primers targeting the *inlA* gene of *L. monocytogenes* was used in PCR assay and 14 samples (3 basils, 1 dill, 1 garden cress, 2 kales, 1 lettuce, 1 mint, 2 parsleys, 1 purslane and 2 rockets) out of 164 samples were detected with the presence of *L. monocytogenes*. In Malaysia, Wong et al. (2012) also analyzed the prevalence of *Listeria* spp. and *L. monocytogenes* in frozen burger patties by using PCR assay targeting 16S rRNA and *hlyA* regions, respectively. In the study, *L. monocytogenes* was detected in 33.30% of chicken burger patties, 22.90% of beef patties, and 10.50% of fish patties.

Fragments of PCR products are usually separated by standard agarose gel (Burland et al., 1996). Agarose gel electrophoresis (AGE) has proven to be an efficient and effective technique to separate DNA fragments (nucleic acid) of varying sizes ranging from 100 bp to 50 kb (Pal, 2015). Agarose is generally extracted from seaweed genera *Gelidium* and *Gracilaria* and it is made up of repeated agarobiose (L- and D-galactose) subunits (Serwer, 1983). Before AGE was introduced, DNA was separated by using sucrose density gradient centrifugation, which only provides an approximation of size (Das & Dash, 2015). AGE separates DNA by applying an electric field to move the DNA through an agarose matrix (Stellwagen, 2009). When electric field is applied, negative charge of the phosphate backbone of DNA molecules will migrate to the positively charged anode. As DNA has a uniform mass/charge ratio, the distance travelled by DNA molecules is inversely proportional to the

log of its molecular weight. There are a few factors affecting the rate of migration of DNA molecules through agarose gel which include the size of DNA molecule, concentration of agarose gel, DNA conformation, voltage applied to the gel and the buffer for electrophoresis (Lee & Bahaman, 2012). Different forms of DNA migrate through the gel at different rates because of their conformation. Supercoiled plasmid DNA migrates through the gel fastest and open circular DNA travels the slowest. Linear DNA fragment of the same size migrates moderately in the gel (Lee et al., 2012).

Ethidium Bromide (EtBr) is the stain most commonly used to detect DNA in agarose gels. There are other stains which include SYBR Gold, SYBR green, Crystal Violet and Methyl Blue. However, many researchers choose EtBr as the staining agent because of cost, ease of use, and sensitivity (Yilmaz et al., 2012). Electrons in the ethidium molecule are activated when they are exposed to UV light, inducing the release of energy (light). The amount of DNA in a PCR product can be estimated based on the intensity of the band. This is because EtBr intercalates into the major grooves of the DNA in a concentration dependent manner (Caridade et al., 2009; Lee et al., 2012; Yilmaz et al., 2012). Jami et al. (2010), Abay et al. (2012) and Soultos et al. (2014) had separated DNA of *Listeria* spp. and *L. monocytogenes* on AGE and stained with ethidium bromide for identification.

2.6 Molecular Typing of *Listeria* spp. and *Listeria monocytogenes*

Molecular typing are essential epidemiological tools in surveillance of bacterial pathogens in infection prevention and control. They are used to identify different bacterial isolates within the same species (Lin et al., 2014). There are few types of molecular typing methods which include pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), random amplification of polymorphic DNA (RAPD), repetitive-element PCR (rep-PCR), variable-number tandem repeat (VNTR), single locus sequence typing (SLST), multilocus sequence typing (MLST), comparative genomic hybridization, optical mapping and whole genome sequencing. These methods have their specific advantages and disadvantages (Sabat et al., 2013).

2.6.1 Repetitive sequence-based PCR (Rep-PCR)

Repetitive sequence-based PCR (Rep-PCR) is a DNA amplification technique for genomic fingerprinting based on repetitive DNA elements present within bacterial genome (Alves et al., 2004). Repetitive DNA sequences are commonly found in eubacteria throughout the world and the sequences have been utilized to type bacterial genomes molecularly. Rep-PCR has been used to differentiate strains of *L. monocytogenes*, *V. parahaemolyticus*, *Acinetobacter baumanii*, *Burkholderia cepacia*, *Citrobacter diversus*, and *Rhizobium meliloti*. Rep-PCR has good discriminatory power to differentiate between bacterial strains. With this technique, the genetic rearrangement in bacteria strains can be studied (Fakruddin et al., 2013). Dendrogram can also be constructed for Rep-PCR analysis to show the relationships between identical strains and also similar groups of strains (Jablonski et al., 2011). There are three main types of

repetitive sequence used for molecular typing which include REP elements, ERIC sequence and BOX elements. These sequences are genetically stable and differ only in their number and chromosomal locations between species. Therefore, these sequences are used to differentiate bacterial isolates to species, subspecies and strain levels (Fakruddin et al., 2013).

ERIC sequences are usually 124-127 bp long, highly conserved central inverted repeat and located at non-coding transcribed regions of the chromosome of various enterobacteria (Waturangi et al., 2012; Fakruddin et al., 2013). ERIC sequences were first discovered in *Salmonella typhi* and *E. coli* and make up approximately 1% of the bacterial genome. On the other hand, the consensus BOX element is created from 5' to 3', consisting of three subunits, box A (59 bp), box B (45 bp), and box C (50 bp). It is 154 bp long and located in distinct intergenic positions around the genome of the *Streptococcus pneumoniae* chromosome (Fakruddin et al., 2013; Silva & Valicente, 2013).

ERIC sequences are generally used for Gram-negative bacteria, while BOX elements are used for Gram-positive. ERIC-PCR was utilized for typing *Haemophilus somnus*, *Viridans streptococci* and *Vibrio parahaemolyticus* (Fakruddin et al., 2013). Laciar et al. (2006) had used ERIC-PCR as DNA fingerprinting method to compare between *Listeria* spp. strains isolated from from human, animal, and food samples in San Luis, Argentina. They concluded that ERIC-PCR technique can be the alternative way for *Listeria* spp. identification and also discrimination of strains within one species. Besides that, Soni et al. (2014) had also serotyped *L. monocytogenes* isolated from vegetable and soil samples by using ERIC-PCR analysis. It was revealed that *L. monocytogenes* isolates from vegetables and their respective rhizospheric soils had distinct PCR fingerprints. Bolivar et al. (2011) had also studied on

intra-serotype variations among circulating *L. monocytogenes* strains by using ERIC-PCR fingerprinting. Other than that, Bowman et al. (2008) had also employed ERIC-PCR and BOX- PCR to study the relatedness between *L. monocytogenes* strains from salami and rabbit tissue and they found that the fingerprint patterns for these strains were nearly identical, confirming that both strains were genotypically very similar. BOX-PCR was also utilized by Jamali & Thong (2014) to determine the genetic relatedness of *L. monocytogenes* isolated from RTE food in Malaysia. The finding of BOX-PCR analysis indicated that *L. monocytogenes* isolates from RTE food were heterogeneous.

2.7 Antibiotic Resistance Profile of *Listeria* spp. and *Listeria monocytogenes* in Environmental and Fresh Produce

Antibiotics are strong medicines that treat bacterial infections. Antibiotics work by killing the bacteria or restrict them from reproducing. Antibiotics are ineffective against viral infections, such as the common cold, flu, most coughs, bronchitis and sore throats (U. S. National Library of Medicine, 2015). The first real antibiotic namely penicillin, was discovered by Professor Alexander Fleming at 1928. He identified the unordinary strain of Penicillium notatum that inhibited bacterial growth of a wide range of harmful bacteria, such as *staphylococcus*, *streptococcus*, *meningococcus* and the *diphtheria bacillus* (American Chemical Society, 2016). Fleming was among the first who was aware on the potential resistance of penicillin if the antibiotic is improperly used during treatment (Aminov, 2010). Improper or increased applications of antibiotics in human and veterinary medicine often lead to the spreading of drug resistant microorganisms. This poses great risk towards the public

health due to the transmission of resistant strains to humans via the food chain (Fallah et al., 2013). Acquisition of antibiotic resistance strain in most bacteria is usually occurred by mutation in an intrinsic chromosomal gene, acquisition of new genes through conjugative plasmids and transposons, transformation (DNA release and uptake by suitable recipients) and transduction (transfer DNA to another bacterium by subsequent infection) (Chen et al., 2010).

In the past, *Listeria* spp. have been reported as susceptible to antibiotics active against Gram-positive bacteria but, recently the reports on antibiotic resistance among *Listeria* spp. have increased (Walsh et al., 2001). According to Chen et al. (2010), most *Listeria* spp. were susceptible to almost all of the antibiotics, except for cephalosporins and fosfomycin. They also reported that *Listeria* spp. started to show resistance to a wide range of antimicrobial agents. Moreno et al. (2014) revealed that *Listeria* spp. isolated from various sources were resistant to penicillin, ampicillin, tetracycline, streptomycin, clindamycin, and even oxacillin and vancomycin. According to Korsak et al. (2012), *L. monocytogenes* strains isolated from food and food processing environment in Poland were sensitive to ampicillin, amoxicillin, gentamicin, erythromycin, trimethoprim, rifampicin, vancomycin, chloramphenicol and sulfamethoxazol. Only 2 of the *L. monocytogenes* strains were resistant to tetracycline and minocycline due to the presence of tet (M) gene in the strains. Adzitey et al. (2013) also conducted a research to determine antibiotic resistance of *L. monocytogenes* isolated from ducks, their rearing and processing environments in Penang, Malaysia. They revealed that all *L. monocytogenes* isolates were susceptible to ampicillin, cephalothin, chloramphenicol, ciprofloxacin, gentamicin and streptomycin but resistant to nalidixic acid.

Antimicrobial selective pressure and gene transfer mechanism between and amongst *Listeria* spp., *Enterococcus*, *Streptococcus* and *Staphylococcus* species have contributed to multiple drug resistance in *Listeria* spp. (Pandove et al., 2013). Alonso-Hernando et al. (2012) carried out a research to determine the multiple antibiotic resistance (MAR) among *L. monocytogenes* isolates from poultry in Spain and they concluded that multi-resistance (resistance to two or more antibiotics) was less common in 1993 than in 2006 (18.60% and 84.00%, respectively). Therefore, regular monitoring of multi-drug resistance in microorganisms is necessary. Katre et al. (2009) carried out an antibiogram study of *Listeria* spp. from foods of animal origin. They found that the MAR index value of most of the *Listeria* spp. tested were more than 0.20 which indicates that the organism were originated from sources of contamination where antibiotics were often used. Wong et al. (2012) also revealed the presence of multidrug-resistant *L. monocytogenes* isolates in the processed meat products in Malaysia and concluded that the emergence of antibiotic resistance in bacterial strains in the food chain. Al-Ashmawy et al. (2014) also conducted a study on antimicrobial susceptibility of *L. monocytogenes* isolated from milk and soft cheeses and found that all of the *L. monocytogenes* isolates resist to at least 8 of the 13 antimicrobials used. This result indicated that there is a seriously misused of antimicrobials and poses a real threat for public health.

Information on the antibiotic susceptibility of *Listeria* spp. and *L. monocytogenes* isolated from environment and food products is limited (Chen et al., 2010; Granier et al., 2011). In addition, regular monitoring of multi-drug resistance in microorganisms isolated from environment and food products is necessary in order to understand the patterns of resistance to antibiotics and to plan the strategies for prevention of their environmental spread.

Therefore, continuous surveillance of antimicrobial resistance profile is necessary. Antibiotic resistance test can be carried out by disk diffusion method. This method was used by Morobe et al. (2012) to test for the susceptibilities of all isolates of *L. monocytogenes* to different antimicrobial agents. In the analysis, after incubation of bacteria with antibiotic impregnated discs, the diameters of zone of inhibition can be visualized and measured to determine the antibiotic sensitivity of isolates.

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample Collection

Environmental samples (soil, water and fertilizer with manure) and fresh vegetables were collected from two organic farms (organic farm A and organic farm B) and one conventional farm (conventional farm C) in Kuching, Sarawak. Figure 3.1, Figure 3.2 and Figure 3.3 show the farm environment of organic farm A, organic farm B and conventional farm C, respectively.

Figure 3.1 Photographs of organic farm A environment

Figure 3.2 Photographs of organic farm B environment

Figure 3.3 Photographs of conventional farm C environment

The two organic farms practice the techniques of crop rotations, application of composted chicken waste and plant waste as fertilizer, mechanical methods to control weeds and restricted usage of pesticides. The selected conventional farm also practices the usage of composted animal manures and plant waste as fertilizer. Unlike the organic farms, the conventional farm did not practice crop rotation and synthetic chemical pesticides were applied to the produce biweekly. Sampling periods were recorded in Table 3.1.

Table 3.1 Sampling period and agricultural practices for respective sampling sites

Sampling site	Agricultural practices	Sampling trips
N & N organic farm (Organic Farm A)	(i) Crop rotations (ii) Organic fertilizer (iii) Mechanical weed control (iv) No pesticides	19/02/14 13/03/14 24/03/14 21/04/14
Godseffia organic farm (Organic Farm B)	(i) Crop rotations (ii) Organic fertilizer (iii) Mechanical weed control (iv) No pesticides	11/08/14 13/10/14 27/10/14 17/11/14
Fung Fung conventional farm (Conventional Farm C)	(i) No crop rotation (ii) Organic fertilizer (iii) Application of synthetic pesticides	12/05/14 09/06/14 30/06/14 14/07/14

The vegetable samples and soil samples were collected from the fields while the fertilizer samples were collected from the fertilizer storage places. Water samples were collected from the respective water sources (pond or rainwater). The vegetable, soil, fertilizer and water samples collected from the three selected farms were shown in Table 3.2 and Table 3.3. During the transportation of samples to laboratory for analysis, all samples were kept in sterile plastic bags and placed in an ice box (Tunung et al., 2011).

Table 3.2 Vegetable samples collected from organic farm A, organic farm B and conventional farm C

No.	Types of vegetables	Scientific name	No. of samples collected		
			Organic farms		Conventional farm C
			Organic farm A	Organic farm B	
1	Chinese cabbage (Pak Choy)	*Brassica Rapa*	6	14	10
2	Chinese flowering cabbage (Choy Sum)	*Brassica rapa* var. *parachinensis* or *Brassica chinensis* var. *parachinensis*	3	16	10
3	Romaine/cos lettuce	*Lactuca sativa* L. var. *longifolia*	1	16	-
4	Chinese white cabbage (Baby Pak Choy)	*Brassica Rapa subsp. Chinesis*	-	16	-
5	Cucumber	*Cucumis sativus*	6	-	14
6	Yardlong bean	*Vigna unguiculata* subsp. *Sesquipedalis*	4	-	14
7	Chinese Mustard	*Lactuca Sativa L. var longifolia*	1	12	10
8	Lettuce	*Lactuca sativa*	6	-	12
9	Chinese Kale (Kailan)	*Brassica oleracea*	-	8	10
10	Tomato	*Solanum lycopersicum*	3	-	8
11	Local vegetable (Sabi Sative)	-	-	6	-
Total			30	88	88
			206		

- = not available

Table 3.3 Soil, fertilizers and water samples collected from organic farm A, organic farm B and conventional farm C

No.	Types of environment samples	No. of samples collected			
		Organic farms		Conventional farm C	Total
		Organic farm A	Organic farm B		
1	Soil	12	24	24	60
2	Fertilizer	12	24	24	60
3	Water	12	24	24	60
Total (from each farm)		36	72	72	

3.2 Processing and Isolation of Samples

Sample processing was done as described in Ozbey et al. (2006). First, 25 g (or 25 ml) of each samples (soil, fertilizer and water) were weighed or measured and then transferred into conical flasks containing 225 ml of LEB. After that, the flasks were incubated at 30 ℃ for 48 hr. Vegetable samples were weighed (25 g) and cut into pieces before transferred into the conical flasks containing 225 ml of LEB followed by incubation at 30 ℃ for 48 hr.

3.3 Enumeration of *Listeria* spp.

The enriched bacterial cultures (100 μl) from the samples were transferred and spread on PALCAM agar by using micropipette. The PALCAM agar plates were incubated at 37℃ for 48 hr. *Listeria* spp. colonies appeared to be greyish-green or black in colour and surrounded by black halo on PALCAM agar (Zimbro et al., 2009). These colonies were observed, counted and recorded. Colony-forming units (CFU) for the particular bacteria were calculated. Other than that, these colonies were also picked and streaked on Tryptone Soy Agar (TSA) followed by 18-24 hr incubation at 37℃. The TSA were kept for further analysis and identification.

Colony Forming Unit (CFU) per gram (g) or millilitre (ml) of a sample for *Listeria* spp. can be calculated as follow (Yousef & Carlstrom, 2003):

$$\text{CFU/g or CFU/mL} = \frac{\text{number of colonies on plate}}{\text{dilution factor} \times \text{volume plated}}$$

The European legislation consists of microbiological food safety guidelines for assessing the microbiological safety of RTE food. For *L. monocytogenes*, RTE food which exceeds 100 CFU/g of *L. monocytogenes* throughout the shelf life, is considered to be legally unsatisfactory. The producers of RTE food (with higher risk of *L. monocytogenes* contamination) are required to check the food processing areas and equipment for the presence of *L. monocytogenes* (Koutsoumanis & Angelidis, 2007). The presence of *Listeria* spp. in food or food processing environment suggests the possibility that *L. monocytogenes*

may also be present in these foods and the environment. According to Public Health England (2014), samples consisting more than 100 CFU/g of *Listeria* spp. are considered unsatisfactory and investigation is required. Samples with more than 100 CFU/g of *L. monocytogenes* are considered to be harmful to health and immediate investigation is required.

3.4 Morphological Identification-Gram Stain

Presumptive *Listeria* spp. isolates on PALCAM agar were confirmed by gram staining. After that, the isolates were subjected to PCR assay for identification. The procedure for gram staining was carried out based on Smith & Hussey (2013). Firstly, the colony was smeared and heat-fixed on glass slide. After that, the slide was flooded with crystal violet staining reagent for 1 min. Then, the slide was rinsed gently under the tap water. After that, the slide was flooded with Gram's iodine for 1 min. The slide was then rinsed gently under the tap water again. Next, the slide was flooded with decolorizing agent for 15 sec before it was rinsed with tap water. Lastly, the stain was flooded with counterstain, safranin for 30 sec to 1 min then rinsed gently under tap water until no color appears in the effluent. The slide was blotted dry with absorbent paper and was observed under oil immersion using a microscope. Upon completion, Gram-positive and rod-shaped bacterial isolates were subjected to PCR.

3.5 Identification of *Listeria* spp. and *Listeria monocytogenes* in Fresh Produce, Fertilizer and Environment

3.5.1 DNA Extraction

Presumptive *Listeria* spp. colonies were selected from PALCAM agar and subjected to DNA extraction. The DNA was extracted using GF-1 Nucleic Acid Extraction Kits (Vivantis) according to manufacturer's guide. Firstly, 1-3 ml of bacteria culture grown overnight was pelleted by centrifugation at 6,000 x g for 2 min at room temperature. The supernatant was decanted completely. Then, 100 µl Buffer R1 was added to the pellet and the cells was resuspended completely by pipetting up and down. For Gram-positive bacteria strains, 20 µl lysozyme (50 mg/ml) was added into the cell suspension. The mixture was mixed and incubated at 37℃ for 20 min. The digested cells were then pelleted by centrifugation at 10,000 x g for 3 min. The supernatant was decanted completely. After that, the pellet was resuspended in 180 µl of Buffer R2 and 20 µl of Proteinase K was added and mixed thoroughly. The mixture was incubated at 65℃ for 20 min in a shaking water bath. Next, 2 volumes of Buffer BG was added and mixed thoroughly by inverting the tube several times until a homogeneous solution is obtained. The mixture was incubated for 10 min at 65℃. Then, 200 µl of absolute ethanol was added and mixed immediately and thoroughly. The sample was then transferred into a column assembled in a clean collection tube provided and centrifuged at 10,000 x g for 1 min. The flow through was discarded. After that, the column was washed with 750 µl of Wash Buffer and centrifuged at 10,000 x g for 1 min. The flow through was discarded. The column was then centrifuged at 10,000 x g for 1 min to remove residual ethanol. Lastly, the column was placed into a clean microcentrifuge tube and 50-100

µl of preheated Elution Buffer was added directly onto column membrane and stood for 2 min. The column was centrifuged at 10,000 x g for 1 min to elute DNA. DNA template was further subjected to PCR-based analysis.

3.5.2 Polymerase Chain Reaction (PCR) for *Listeria* spp.

The PCR detection of *Listeria* spp. was carried out as described by Jeyaletchumi et al. (2011) and Wong et al. (2012) with slight modification in the concentration of reagents. The primer pairs used for the detection of *Listeria* spp. (genus specific) were 5'-CTC CAT AAA GGT GAT CCT-3' and 5'-CAG CAG CCG CGG TAA TAC-3'. These primers were designed to amplify a 938 bp region in the 16S rRNA gene. To prepare 25 μl of PCR mixture, 0.2 M of forward primer, 0.2 M of reverse primer, 5.5 μl of DNA template, 1.5 µl of 10×*Taq* PCR buffer, 0.2 µl dNTP, 1.5 mM $MgCl_2$, and 1.5 unit of *Taq* DNA polymerase were mixed together.

The PCR reaction was conducted according to the condition in Table 3.4. Lastly, the PCR products were separated on 1% agarose gel with 100 kb DNA ladder for 75 min, stained with ethidium bromide and viewed under a UV transilluminator (Maestrogen).

Table 3.4 PCR conditions for detection of *Listeria* spp.

PCR steps	Temperature (℃)	Duration (min)	Cycle
Initial denaturation	94	5.00	-
Denaturation	94	0.50	
Annealing	53	1.00	30
Elongation	72	2.00	
Final Extension	72	7.00	-

3.5.3 Polymerase Chain Reaction (PCR) for *Listeria monocytogenes*

The PCR detection of *L. monocytogenes* was carried out as described by Awaisheh (2009) and Kargar & Ghasemi (2009) with modification in the concentration of reagents. The primer pairs used for the detection of *L. monocytogenes* are 5'-CAT TAG TGG AAA GAT GGA ATG-3' and 5'-GTA TCC TCC AGA GTG ATC GA-3' which amplify 730 bp region of the listeriolysin (*hlyA*) gene. To prepare 25 μl of PCR mixture, 0.4 M of *hlyA* forward primer, 0.4 M of *hlyA* reverse primer, 5 μl of DNA template, 2.5 µl of 10×*Taq* PCR buffer, 0.2 mM dNTP, 0.8 mM $MgCl_2$, and 2.5 unit of *Taq* DNA polymerase were mixed together.

The PCR reaction was conducted according to the condition in the Table 3.5. Lastly, the PCR products were separated on 1% agarose gel with 100 kb DNA ladder for 75 min. After that, the gel was stained with ethidium bromide and viewed under a UV transilluminator (Maestrogen).

Table 3.5 PCR conditions for detection of *L. monocytogenes*

PCR steps	Temperature (℃)	Duration (min)	Cycle
Initial denaturation	95	5.00	-
Denaturation	95	1.00	
Annealing	62	0.75	34
Elongation	72	1.50	
Final Extension	72	5.00	-

3.6 Genotyping for *Listeria* spp. and *Listeria monocytogenes*

3.6.1 Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR

Genotyping of *Listeria* spp. was carried out by ERIC-PCR as described by Indrawattana et al. (2011) and Laciar et al. (2006). The primer pairs used were 5′-ATGTAAGCTCCTGGGGATTCAC-3′ and 5′-AAGTAAGTGACTGGGGTGAGCG-3′. To prepare 25 μl of PCR mixture, 1.0 M of forward primer, 1.0 M of reverse primer, 3.0 μl of DNA template, 5.0 µl of 5×*Taq* PCR buffer, 0.2 mM dNTP, 2.0 mM $MgCl_2$, and 1.0 unit of *Taq* DNA polymerase were mixed together.

The PCR reaction was carried out according to the condition in the Table 3.6. The PCR products were then separated on 2% agarose gel with 100 kb DNA ladder for 90 min. After that, the gel was stained with ethidium bromide and viewed under a UV transilluminator (Maestrogen). The DNA band patterns were analyzed and a dendrogram was generated for the *Listeria* isolates by using BioNumerics 7.5 software program (Applied Maths, Sint-Martens-

Latem, Belgium) using Dice coefficient and the unweighted pair group method (UPGMA) (Jackson et al., 2012). Simpson's index of diversity, D for ERIC-PCR was also calculated.

Table 3.6 PCR conditions for ERIC-PCR

PCR steps	Temperature (℃)	Duration (min)	Cycle
Initial denaturation	95	5.00	-
Denaturation	90	0.50	
Annealing	50	0.50	30
Elongation	52	1.00	
Final Extension	72	8.00	-

The discriminating power of this typing method was calculated by using Simpson's index of diversity, D (Hunter & Gaston, 1988). The higher the discriminatory index, the greater the effectiveness of a particular fingerprinting method to discriminate different strains (Kqueen et al., 2013). This index was given by the following equation:

$$D = 1 - \left(\frac{1}{N(N-1)}\right)\sum_{j=1}^{s} n_j(n_j - 1)$$

Where,

N = the total number of strains in the sample population

s = the total number of types described

n_j = the number of strains belonging to the jth type

3.6.2 BOX-PCR

BOX-PCR was also used for *Listeria* spp. genotyping. BOX-PCR was carried out as described in Jamali & Thong (2014) and Versalovic et al. (1994) with slight modifications on the reagent concentration and reaction condition. The primer used is BOX A1R (5'-CTA CGG CAA GGC GAC GCT GAC G-3'). To prepare 25 μl of PCR mixture, 400 µM of each dNTPs, 1×PCR buffer, 3 mM $MgCl_2$, 4 µM of primer and 2.5 U *Taq* DNA polymerase (Promega) were mixed together.

The PCR reaction was carried out according to the condition in the Table 3.7. The PCR products were then separated on 2% agarose gel with 100 kb DNA ladder for 90 min. After that, the gel was stained with ethidium bromide and viewed under a UV transilluminator (Maestrogen). The DNA banding patterns were analyzed and a dendrogram was generated for the *Listeria* isolates by using BioNumerics 7.5 software program (Applied Maths, Sint-Martens-Latem, Belgium) using Dice coefficient and the unweighted pair group method (UPGMA) (Jackson et al., 2012). The discriminating power of this typing method was calculated by using Simpson's index of diversity, D (Hunter & Gaston, 1988).

Table 3.7 PCR conditions for BOX-PCR

PCR steps	Temperature (℃)	Duration (min)	Cycle
Initial denaturation	94	5	-
Denaturation	94	1	35
Annealing	40	2	
Elongation	72	2	
Final Extension	72	10	-

3.7 Antibiotic Susceptibility Test

Antibiotic susceptibility of isolated *Listeria* spp. was carried out with disc-diffusion method as described in Chen et al. (2010) with slight modification. The antibiotic discs that were used are as follow: ampicillin (10 µg), cephalothin (30 µg), chloramphenicol (30 µg), clindamycin (2 µg), erythromycin (15 µg), gentamycin (10 µg), penicillin G (10 µg), rifampin (5 µg), streptomycin (10 µg), tetracycline (30 µg), sulfamethoxazole/trimethoprim (23.75 µg/1.25 µg), novobiocin (30 µg), nitrofurantoin (10 µg) and ceftriaxone. All discs were obtained from Oxoid. Firstly, the overnight culture grown in Mueller-Hinton broth was spread uniformly onto the Mueller-Hinton agar plate. Antibiotic discs were then placed onto the surface of each plate (4 antibiotics/petri dish) using antiobiotic-disc dispenser (Oxoid). After incubation at 37℃ for 24 hr, the diameter of growth inhibition zone surrounding each disc was measured and interpreted according to the CLSI (Clinical and Laboratory Standards Institute) 2014 recommendation. CLSI determine the antibiotic resistant, susceptible and intermediate of the isolate by referring to the Zone Diameter Interpretive Criteria (nearest whole mm) of a particular antibiotic. Each antibiotic has its own Zone Diameter Interpretive Criteria. The CLSI criteria for *staphylococci* were applied in this study because CLSI do not provide interpretative criteria for *Listeria* with the exception of susceptibility breakpoints for ampicillin and penicillin.

Multiple Antibiotic Resistance (MAR) index of an isolate was calculated as defined by Krumperman (1983):

$$\text{MAR index} = \frac{a}{b}$$

Where,

a = Number of antibiotics to which the particular isolate was resistant;

b = Number of antibiotics to which the particular isolate was exposed.

MAR index higher than 0.20 indicates that the particular organism is originated from high-risk sources of contamination, where antibiotics are often used. MAR index less than or equal to 0.20, indicates that the isolate is originated from environment where antibiotics are seldom or never used (Krumperman, 1983; Pandove et al., 2013)

CHAPTER 4

RESULTS

4.1 Prevalence of *Listeria* spp. and *Listeria monocytogenes* in Vegetables, Fertilizer and Environment in Organic Farms and Conventional Farm at Kuching, Sarawak

4.1.1 Gram Stain

In this study, a total of 206 vegetables samples, 60 fertilizer samples, 60 soil samples and 60 water samples were collected from 2 organic farms (farm A & farm B) and a conventional farm (farm C). Presumptive *Listeria* spp. was appeared to be greyish-green colonies or black colonies with black halo on PALCAM agar. All presumptive *Listeria* spp. were picked from PALCAM agar and subjected to gram stain for confirmation. Figure 4.1 showed the Gram-positive and rod-shaped *Listeria* spp. under the microscope. These isolates were subjected to PCR analysis.

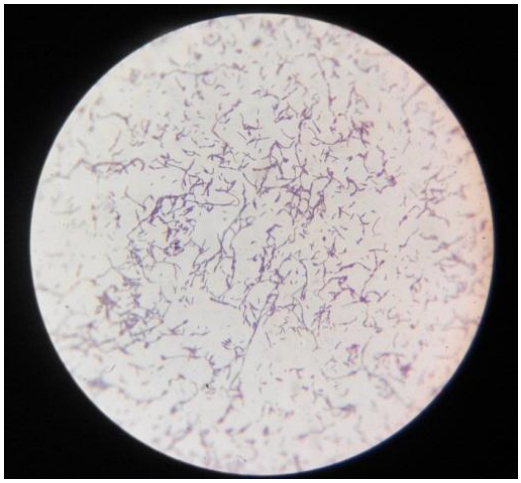


Figure 4.1 Microscopic view of gram stain for V116c isolates obtained in Chinese cabbage (Pak Choy) sample from organic farm B

4.1.2 Identification of *Listeria* spp. by Polymerase Chain Reaction (PCR) assay

Presumptive *Listeria* spp. from vegetable, fertilizer, soil and water samples were subjected to PCR assay for further identification of *Listeria* spp. Detection of *Listeria* spp. was done by using PCR with primers that were designed to amplify a 938 bp region in the 16S rRNA gene. The gel picture for PCR amplification of 16s rRNA gene of *Listeria* spp. was shown in Figure 4.2. The result of this PCR assay revealed that *Listeria* spp. was present in 7.51% (29/386) of all the samples (vegetable, soil, fertilizer and water) collected from all the three farms. It was present in 9.10% (6/66), 8.13% (13/160) and 6.25% (10/160) of all samples collected from organic farm A, organic farm B and conventional farm C, respectively. The prevalence of *Listeria* spp. in vegetable, fertilizer and environmental samples (soil and water) from all the three farms were shown in Table 4.1.

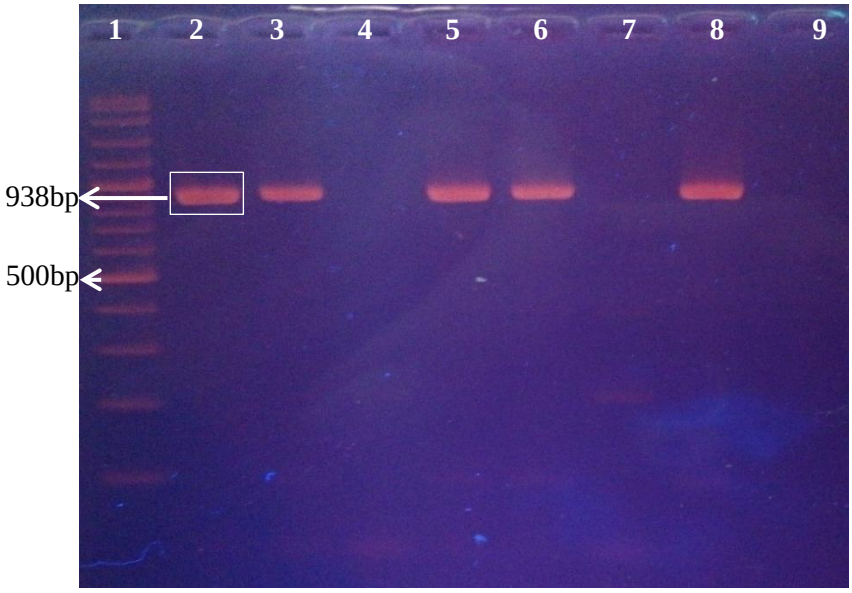


Figure 4.2 PCR amplification of 16s rRNA gene of *Listeria* spp. with expected size of 938 bp, in fertilizer samples obtained from organic farm B. Lane 1: 100 bp ladder; Lane 2: *L. monocytogenes* reference strains ATCC 19155 as positive control; Lane 3-8: presumptive *Listeria* spp. isolates from fertilizer sample; Lane 9: Negative control

Table 4.1 Prevalence of *Listeria* spp. in organic farms and conventional farm in Kuching, Sarawak by PCR assay

No.	Types of sample	Organic farm				Conventional farm	
		Organic farm A	Standard Plate Count (CFU/g)	Organic farm B	Standard Plate Count (CFU/g)	Conventional farm C	Standard Plate Count (CFU/g)
1	Chinese cabbage (Pak Choy)	0% (0/6)	-	14.30% (2/14)	9.50×10^{2}-7.70×10^{3}	0% (0/10)	-
2	Lettuce	0% (0/6)	-	-	-	0% (0/12)	-
3	Cucumber	16.70% (1/6)	2.10×10^{5}	-	-	0% (0/14)	-
4	Yardlong bean	0% (0/4)	-	-	-	0% (0/14)	-
5	Chinese flowering cabbage (Choy Sum)	0% (0/3)	-	0% (0/16)	-	0% (0/10)	-
6	Tomato	0% (0/3)	-	-	-	0% (0/8)	-
7	Chinese Mustard	100.00% (1/1)	2.50×10^{4}	0% (0/12)	-	0% (0/10)	-
8	Romaine/cos Lettuce	0% (0/1)	-	25.00% (4/16)	2.10×10^{3}-9.90×10^{4}	-	-
9	Chinese Kale (Kailan)	-	-	0% (0/8)	-	0% (0/10)	-
10	Chinese white cabbage (Sawi Manis)	-	-	6.30% (1/16)	1.30×10^{4}	-	-
11	Local vegetable (Sabi Sative)	-	-	0% (0/6)	-	-	-
12	Soil	16.70% (2/12)	3.30×10^{3}-6.90×10^{4}	8.30% (2/24)	1.00×10^{5}-1.20×10^{5}	0% (0/24)	-
13	Fertilizer	16.70% (2/12)	1.20×10^{4}-2.20×10^{4}	16.70% (4/24)	5.10×10^{4}-7.00×10^{5}	8.30% (2/24)	4.30×10^{4}-1.50×10^{5}
14	Water	0% (0/12)	-	0% (0/24)	-	33.30% (8/24)	1.00×10^{3}-3.50×10^{6}
	Total	9.10% (6/66)		8.13% (13/160)		6.25% (10/160)	

- = not available

Listeria spp. was present in 6.70% (2/30) and 8.00% (7/88) of vegetables samples from organic farm A and organic farm B, respectively. *Listeria* spp. was detected in a cucumber and a Chinese mustard from organic farm A whereas in organic farm B, *Listeria* spp. was detected in 4 Romaine/cos lettuce, 2 Chinese cabbage (Pak Choy) and a Chinese white cabbage (baby Pak Choy). However, *Listeria* spp. was not detected in any vegetables samples collected from conventional farm C. Vegetable samples from organic farm B have highest prevalence of *Listeria* spp. among the three farms analyzed. For soil samples, 16.70% (2/12) and 8.30% (2/24) of soil samples from organic farm A and organic farm B were detected with *Listeria* spp. Similar to vegetable samples, *Listeria* spp. was also not detected in any soil samples collected from conventional farm C. Among the three farms analyzed, soil samples from organic farm A have highest prevalence of *Listeria* spp. For fertilizer samples, 16.70% (2/12), 16.70% (4/24) and 8.30% (2/24) of fertilizer samples from organic farm A, organic farm B and conventional farm C, respectively, were detected with *Listeria* spp. Fertilizer samples from organic farm A and B have the highest prevalence of *Listeria* spp. among the three farms analyzed. For water samples, *Listeria* spp. was only present in 33.30% (8/24) of water samples from conventional farm C.

Listeria spp. was enumerated by using standard plate count method. *Listeria* spp. which appeared to be greyish-green colonies or black colonies with black halo on PALCAM agar, were counted and calculated. The standard plate count (in CFU/g) of *Listeria* spp. in vegetables, fertilizer and environmental samples (soil and water) in organic farm A, organic farm B and conventional farm C are also shown in Table 4.1. The *Listeria* spp. in cucumber and Chinese mustard from organic farm A have plate count of 2.10×10^5 CFU/g and 2.50×10^4 CFU/g, respectively whereas the *Listeria* spp. in Chinese cabbage (Pak Choy),

Romaine/cos lettuce and Chinese white cabbage (baby Pak Choy) from organic farm B have plate count ranged between 9.50×10^2 CFU/g - 7.70×10^3 CFU/g, 2.10×10^3 CFU/g - 9.90×10^4 CFU/g and 1.30×10^4 CFU/g, respectively. However, *Listeria* spp. was not present in vegetables from conventional farm C. For soil samples, *Listeria* spp. from organic farm A and organic farm B have plate count ranged between 3.30×10^3 CFU/g - 6.90×10^4 CFU/g and 1.00×10^5 CFU/g - 1.20×10^5 CFU/g, respectively. *Listeria* spp. was not detected in conventional farm C. For fertilizer samples, *Listeria* spp. from organic farm A, organic farm B and conventional farm C have plate count ranged between 1.20×10^4 CFU/g - 2.20×10^4 CFU/g, 5.10×10^4 CFU/g - 7.00×10^5 CFU/g and 4.30×10^4 CFU/g - 1.50×10^5 CFU/g, respectively. From all the water samples collected from the three farms, only water samples from conventional farm C were detected with *Listeria* spp. with plate count ranged between 1.00×10^3 CFU/g and 3.50×10^6 CFU/g.

4.1.3 Identification of *Listeria monocytogenes* by Polymerase Chain Reaction (PCR) assay

The PCR detection of *L. monocytogenes* in the samples was carried out by using primer pairs which amplify 730 bp region of the listeriolysin (*hlyA*) gene. Figure 4.3 showed the gel picture for PCR amplification of *hlyA* gene of *L. monocytogenes*. However, the finding shows that *L. monocytogenes* was absent in all the vegetable, fertilizer, soil and water samples from all the three farms analyzed.

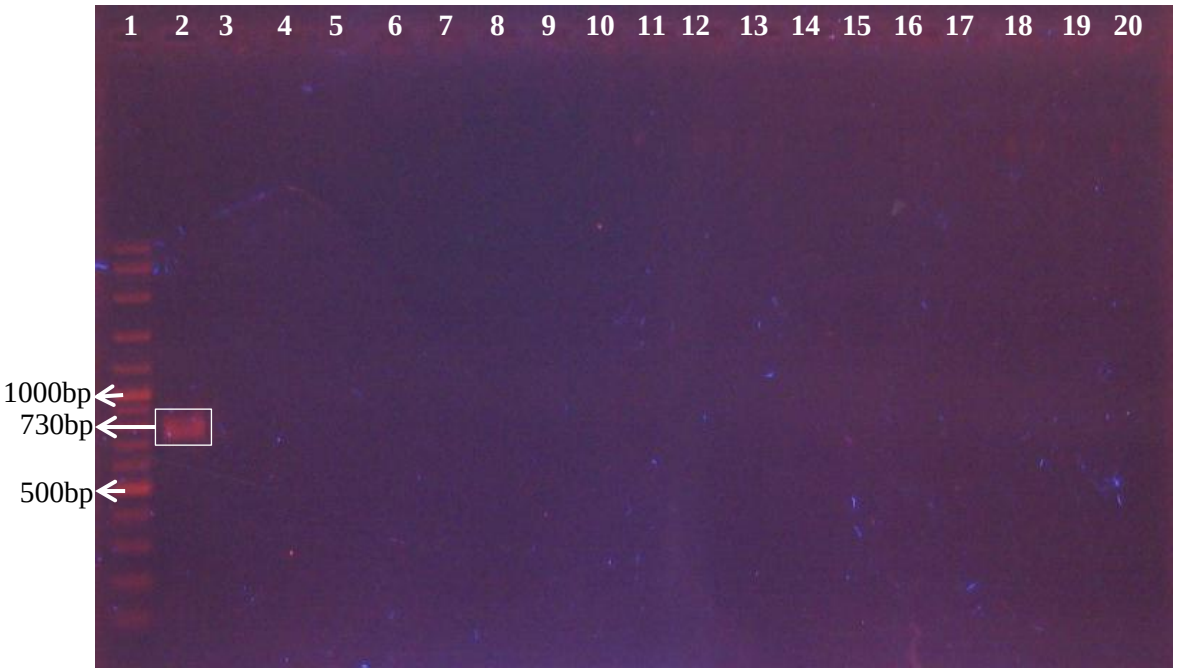


Figure 4.3 PCR amplification of *hlyA* gene of *L. monocytogenes* with expected size of 730 bp, in vegetable, soil and fertilizer samples obtained from conventional farm C. Lane 1: 100 bp ladder; Lane 2: *L. monocytogenes* reference strains ATCC 19155 as positive control; Lane 3: Negative control; Lane 4-13: *Listeria* spp. isolates from vegetable samples; Lane 14-19: *Listeria* spp. isolates from soil sample; Lane 20: M19c *Listeria* spp. isolate from fertilizer sample

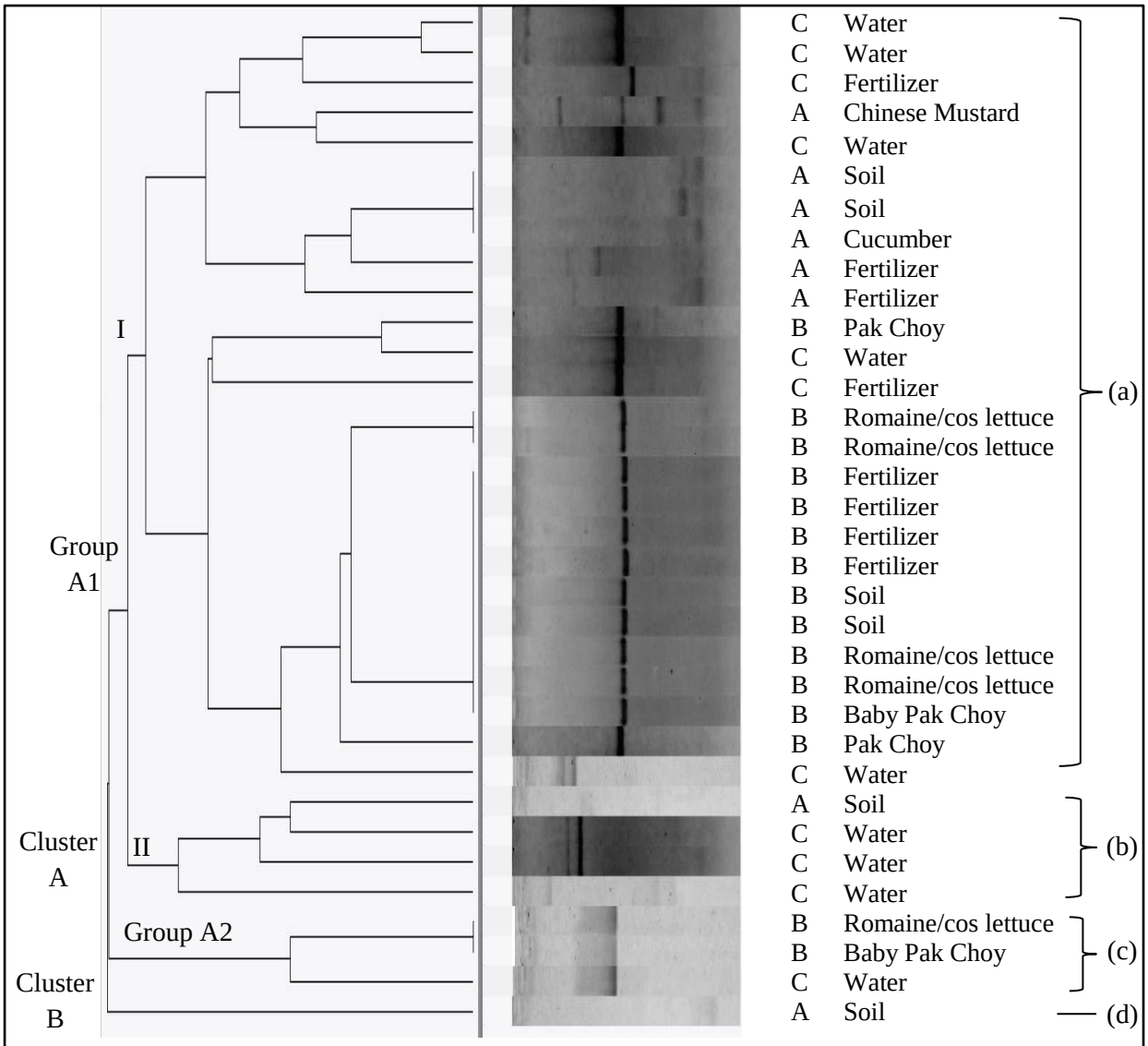
4.2 Genotyping of *Listeria* spp. in Vegetables, Fertilizer and Environment in Organic Farms and Conventional Farm at Kuching, Sarawak

4.2.1 Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR

In this study, 34 *Listeria* spp. isolated from 29 samples (vegetable, soil, fertilizer and water) collected from organic farm A, organic farm B and conventional farm C were subjected to ERIC-PCR. The electrophoretic profile of DNA fragments obtained after ERIC-PCR amplification yielded 1-5 bands with size approximately 120 bp to 1450 bp. A common band with molecular size of approximately 520 bp was observed in the electrophoretic profile from most of the isolates.

The dendrogram shown in Figure 4.4 was constructed for the *Listeria* spp. isolates by using BioNumerics 7.5 software program. The dendrogram showed that all the *Listeria* spp. isolated from the samples collected were distributed into cluster A and cluster B. Cluster A consists of group A1 and A2. Group A1 is further subdivided into subgroup I and II. Group AI subgroup I consists of 26 *Listeria* spp. isolated from vegetable, soil, fertilizer and water samples from organic farm A, organic farm B and conventional farm C. In Group AI subgroup I, there are 6 *Listeria* spp. isolated from vegetable (2), soil (2) and fertilizer (2) samples in organic farm A, 13 *Listeria* spp. isolated from vegetable (7), soil (2) and fertilizer (4) samples in organic farm B, and 7 *Listeria* spp. isolated from fertilizer (2) and water (5) samples in conventional farm C. For group A1 subgroup II, it consists of 4 *Listeria* spp. isolated from soil sample (1) in organic farm A and water samples (3) from conventional farm C.

For group A2, it consists of 3 *Listeria* spp. isolated from organic farm B and conventional farm C. The 2 *Listeria* spp. isolated from organic farm B were collected from leafy green vegetable samples while a *Listeria* spp. isolated from conventional farm C was collected from water sample. Only one isolate fell into cluster B. The isolate was isolated from soil sample from organic farm A. Based on the dendrogram from ERIC-PCR, the *Listeria* spp. isolated from organic farm A, organic farm B and conventional farm C were genetically diverse and heterogeneous as they were not classified into specific cluster neither by sampling area nor the type of samples.



Legend

Label	Description
A	Organic farm A
B	Organic farm B
C	Conventional farm C
(a)	*Listeria* spp. isolated from vegetables, soil, fertilizer and water from organic farm A, organic farm B and conventional farm C
(b)	*Listeria* spp. isolated from soil and water from organic farm A and conventional farm C
(c)	*Listeria* spp. isolated from vegetable and water from organic farm B and conventional farm C
(d)	*Listeria* spp. isolated from soil from organic farm A

Figure 4.4 Dendrogram constructed for ERIC-PCR of *Listeria* spp. in vegetable, fertilizer and environmental samples collected from organic farm A, organic farm B and conventional farm

ERIC-PCR analysis produces 11 different DNA fingerprint profiles. There are 15 *Listeria* spp. isolates from organic farm B have indistinguishable DNA fingerprints. These *Listeria* spp. were isolated from vegetables, fertilizer and soil samples in the farm and 86.70% (13/15) of them were distributed into group A1 subgroup I. The remaining 13.30% (2/15) of these *Listeria* spp. isolates were randomly distributed into different group. *Listeria* spp. from vegetables, fertilizer, soil and water in organic farm A and conventional farm C have distinct DNA fingerprints and they were randomly distributed into different group.

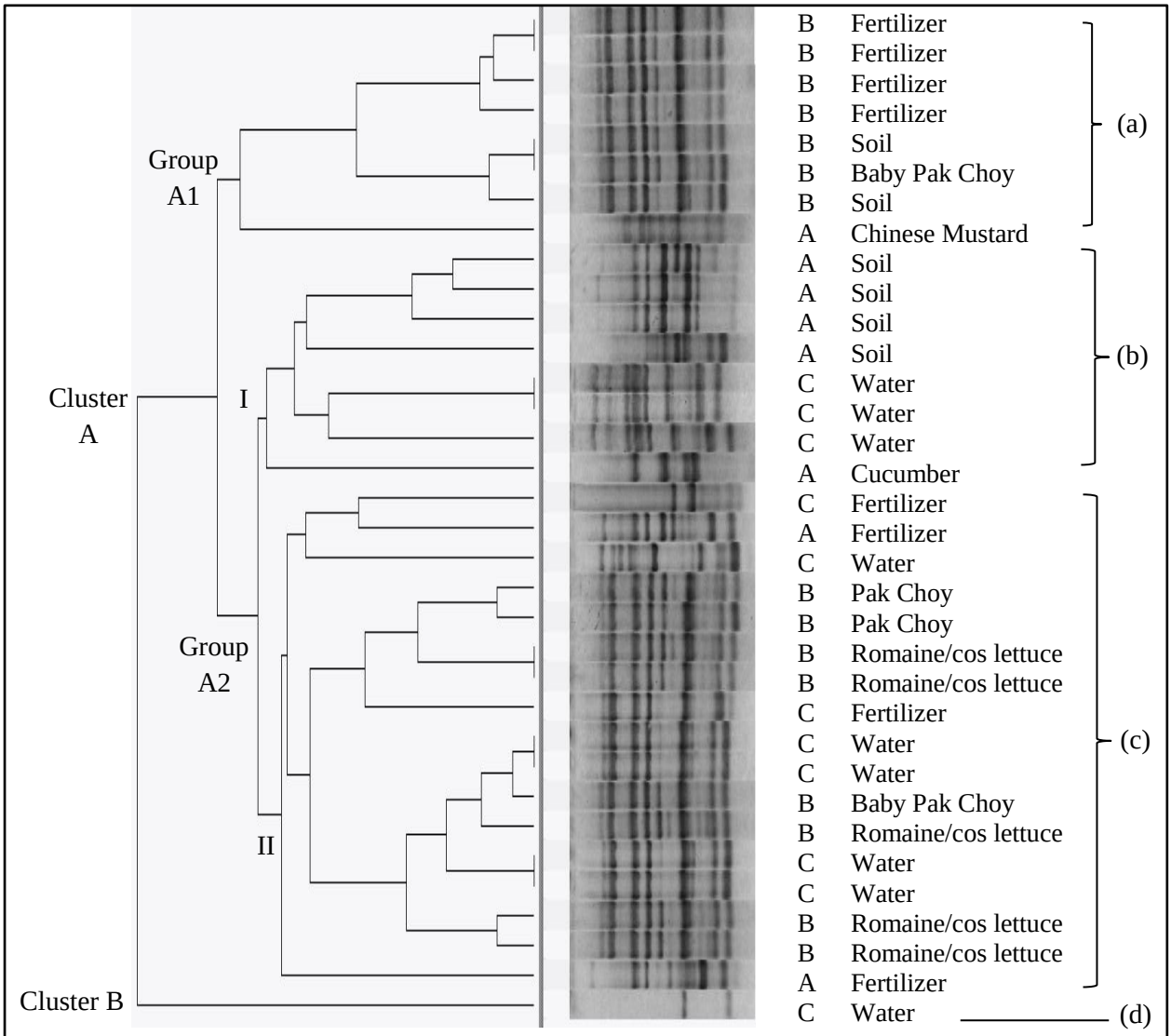
The Simpson's Index of Diversity, D, was calculated for ERIC-PCR based on Hunter & Gaston (1988). The D value of this technique was calculated to be 0.604.

4.2.2 BOX-PCR

In this analysis, 34 *Listeria* spp. isolated from 29 samples (vegetable, soil, fertilizer and water) collected from organic farm A, organic farm B and conventional farm C were subjected to BOX-PCR. The electrophoretic band pattern of BOX-PCR amplification yielded 2-13 bands with size approximately 120 bp to 1550 bp. Common bands with molecular size of approximately 1450 bp, 1280 bp, 1020 bp, 810 bp, 650 bp, 400 bp, 380 bp, 200 bp and 170 bp were observed in 67.65% (23/34), 67.65% (23/34), 85.29% (29/34), 85.29% (29/34), 73.53% (25/34), 79.41% (27/34), 85.29% (29/34), 67.65% (67.65%) and 76.47% (26/34) of the isolates, respectively.

A dendrogram shown in Figure 4.5 was generated for the *Listeria* spp. isolates by using BioNumerics 7.5 software program. All the *Listeria* spp. isolated from the samples collected were distributed into cluster A and cluster B as shown in the dendrogram. Cluster A consists of group A1 and A2. Group A1 consists of 8 *Listeria* spp. isolated from a vegetable (1) sample in organic farm A and vegetable (1), soil (2) and fertilizer (4) samples in organic farm B.

Group A2 is further subdivided into subgroup I and subgroup II. Subgroup I consists of 8 *Listeria* spp. isolated from vegetable (1) and soil (4) samples in organic farm A, and water (3) samples in conventional farm C. For group A2 subgroup II, it consists of 17 *Listeria* spp. isolated from fertilizer (2) samples in organic farm A, leafy green vegetables (8) samples in organic farm B, and fertilizer (2) and water (5) samples in conventional farm C. Lastly, cluster B consists of only one *Listeria* spp. The *Listeria* spp. was isolated from a water sample from conventional farm C. Based on the dendrogram from BOX-PCR, *Listeria* spp. isolated from all the three farms were not classified according to the types of sample nor sampling area. Therefore, these *Listeria* spp. isolates were genetically diverse and heterogeneous.



Legend

Label	Description
A	Organic farm A
B	Organic farm B
C	Conventional farm C
(a)	*Listeria* spp. isolated from vegetables, soil and fertilizer from organic farm A and organic farm B
(b)	*Listeria* spp. isolated from vegetable, soil and water from organic farm A and conventional farm C
(c)	*Listeria* spp. isolated from vegetables, fertilizer and water from organic farm A, organic farm B and conventional farm C
(d)	*Listeria* spp. isolated from water from conventional farm C

Figure 4.5 Dendrogram constructed for BOX-PCR of *Listeria* spp. in vegetable, fertilizer and environmental samples collected from organic farm A, organic farm B and conventional farm C

BOX-PCR analysis produces 14 different fingerprint profiles. There are 73.30% (11/15) *Listeria* spp. isolated from vegetables, fertilizer and soil in organic farm B have indistinguishable DNA fingerprints and 54.55% (6/11) of these *Listeria* spp. were distributed into group A1. The remaining 45.45% (5/11) of these *Listeria* spp. isolates, were randomly distributed into different group. Other than that, 36.40% (4/11) of *Listeria* spp. from conventional farm C also have indistinguishable DNA fingerprints and were distributed into group A2 subgroup II. *Listeria* spp. from organic farm A, organic farm B and conventional farm C with distinct DNA fingerprints, were randomly distributed into different group.

The Simpson's Index of Diversity, D, was calculated for BOX-PCR based on Hunter & Gaston (1988). The D value of this technique was calculated to be 0.888.

4.3 Antibiotic Susceptibility Test

In this analysis, 34 *Listeria* spp. isolated from 29 samples (vegetable, soil, fertilizer and water) collected from organic farm A, organic farm B and conventional farm C were subjected to antibiotic susceptibility test. *Listeria* spp. isolates were most resistant to clindamycin followed by penicillin G, tetracycline, ceftriaxone, cephalothin, ampicillin, rifampin, erythromycin, nitrofurantoin, chloramphenicol, streptomycin, sulfamethoxazole/trimethoprim and gentamicin with the percentages of 97.06% (33/34), 94.11% (32/34), 91.80% (31/34), 91.80% (31/34), 73.53% (25/34), 67.65% (23/34), 55.88% (19/34), 52.94% (18/34), 44.11% (15/34), 38.24% (13/34), 38.24% (13/34), 32.35% (11/34) and 17.65% (6/34), respectively. The antibiotic resistance profile is shown in Table 4.2, Table 4.3 and Table 4.4.

Table 4.2 The antibiotic resistance profile of *Listeria* spp. isolates from organic farm A

Antimicrobial agent	Percentage of isolates resistance (%)				
	Organic farm A				
	Vegetables	Soil	Fertilizer	Water	Total
	n =2	n = 4	n = 2	n = 0	n = 8
Ampicillin	50.00 (1/2)	50.00 (2/4)	100.00 (2/2)	-	62.50 (5/8)
Rifampin	100.00 (2/2)	100.00 (4/4)	50.00 (1/2)	-	87.50 (7/8)
Penicillin G	100.00 (2/2)	75.00 (3/4)	100.00 (2/2)	-	87.50 (7/8)
Gentamicin	50.00 (1/2)	0 (0/4)	50.00 (1/2)	-	25.00 (2/8)
Trimethoprim/ sulfamethoxazole	0 (0/2)	25.00 (1/4)	100.00 (2/2)	-	37.50 (3/8)
Erythromycin	100.00 (2/2)	50.00 (2/4)	100.00 (2/2)	-	75.00 (6/8)
Tetracycline	100.00 (2/2)	75.00 (3/4)	100.00 (2/2)	-	87.50 (7/8)
Chloramphenicol	50.00 (1/2)	75.00 (3/4)	100.00 (2/2)	-	75.00 (6/8)
Clindamycin	100.00 (2/2)	100.00 (4/4)	100.00 (2/2)	-	100.00 (8/8)
Nitrofurantoin	100.00 (2/2)	75.00 (3/4)	50.00 (1/2)	-	75.00 (6/8)
Streptomycin	0 (0/2)	75.00 (3/4)	0 (0/2)	-	37.50 (3/8)
Cephalothin	50.00 (1/2)	100.00 (4/4)	100.00 (2/2)	-	87.50 (7/8)
Ceftriaxone	50.00 (1/2)	100.00 (4/4)	100.00 (2/2)	-	87.50 (7/8)

Table 4.3 The antibiotic resistance profile of *Listeria* spp. isolates from organic farm B

Antimicrobial agent	Percentage of isolates resistance (%)				
	Organic farm B				
	Vegetables	Soil	Fertilizer	Water	Total
	n = 9	n = 2	n = 4	n = 0	n = 15
Ampicillin	100.00 (9/9)	100.00 (2/2)	25.00 (1/4)	-	80.00 (12/15)
Rifampin	66.70 (6/9)	0 (0/2)	0 (0/4)	-	40.00 (6/15)
Penicillin G	100.00 (9/9)	100.00 (2/2)	100.00 (4/4)	-	100.00 (15/15)
Gentamicin	22.20 (2/9)	0 (0/2)	0 (0/4)	-	13.30 (2/15)
Trimethoprim/ sulfamethoxazole	33.30 (3/9)	50.00 (1/2)	0 (0/4)	-	26.70 (4/15)
Erythromycin	33.30 (3/9)	50.00 (1/2)	75.00 (3/4)	-	60.00 (9/15)
Tetracycline	100.00 (9/9)	100.00 (2/2)	100.00 (4/4)	-	100.00 (15/15)
Chloramphenicol	22.20 (2/9)	0 (0/2)	0 (0/4)	-	13.30 (2/15)
Clindamycin	100.00 (9/9)	100.00 (2/2)	100.00 (4/4)	-	100.00 (15/15)
Nitrofurantoin	55.60 (5/9)	0 (0/2)	25.00 (1/4)	-	40.00 (6/15)
Streptomycin	44.40 (4/9)	0 (0/2)	25.00 (1/4)	-	33.30 (5/15)
Cephalothin	100.00 (9/9)	100.00 (2/2)	75.00 (3/4)	-	93.30 (14/15)
Ceftriaxone	100.00 (9/9)	100.00 (2/2)	100.00 (4/4)	-	100.00 (15/15)

Table 4.4 The antibiotic resistance profile of *Listeria* spp. isolates from conventional farm C

Antimicrobial agent	Percentage of isolates resistance (%)				
	Conventional farm C				
	Vegetables	Soil	Fertilizer	Water	Total
	n = 0	n = 0	n = 2	n = 9	n = 11
Ampicillin	-	-	50.00 (1/2)	55.60 (5/9)	54.50 (6/11)
Rifampin	-	-	100.00 (2/2)	44.40 (4/9)	54.50 (6/11)
Penicillin G	-	-	50.00 (1/2)	100.00 (9/9)	90.90 (10/11)
Gentamicin	-	-	0 (0/2)	22.20 (2/9)	18.20 (2/11)
Trimethoprim/ sulfamethoxazole	-	-	0 (0/2)	44.40 (4/9)	36.40 (4/11)
Erythromycin	-	-	50.00 (1/2)	22.20 (2/9)	27.30 (3/11)
Tetracycline	-	-	50.00 (1/2)	88.90 (8/9)	81.80 (9/11)
Chloramphenicol	-	-	50.00 (1/2)	44.40 (4/9)	45.50 (5/11)
Clindamycin	-	-	100.00 (2/2)	88.90 (8/9)	90.90 (10/11)
Nitrofurantoin	-	-	0 (0/2)	33.30 (3/9)	27.30 (3/11)
Streptomycin	-	-	50.00 (1/2)	44.40 (4/9)	45.50 (5/11)
Cephalothin	-	-	50.00 (1/2)	44.40 (4/9)	45.50 (5/11)
Ceftriaxone	-	-	100.00 (2/2)	77.80 (7/9)	81.80 (9/11)

Listeria spp. from organic farm A were 100.00% (8/8) resistant to clindamycin. In this farm, 87.50% (7/8), 87.50% (7/8), 87.50% (7/8), 87.50% (7/8), 87.50% (7/8), 75.00% (6/8), 75.00% (6/8), 75.00% (6/8), 62.50% (5/8), 37.50% (3/8), 37.50% (3/8) and 25.00% (2/8) of *Listeria* spp. were resistant to rifampin, penicillin G, tetracycline, ceftriaxone, cephalothin, erythromycin, chloramphenicol, nitrofurantoin, ampicillin, streptomycin, trimethoprim/sulfamethoxazole and gentamicin, respectively. For organic farm B, *Listeria* spp. isolated were 100.00% (15/15) resistant to penicillin G, tetracycline, clindamycin and ceftriaxone. In this farm, 93.30% (14/15), 80.00% (12/15), 60.00% (9/15), 40.00% (6/15), 40.00% (6/15), 33.30% (5/15), 26.70% (4/15), 13.30% (2/15) and 13.30% (2/15) of *Listeria* spp. were resistant to cephalothin, ampicillin, erythromycin, rifampin, nitrofurantoin, streptomycin, trimethoprim/sulfamethoxazole, gentamicin and chloramphenicol, respectively. For conventional farm C, 90.90% (10/11), 90.90% (10/11), 81.80% (9/11), 81.80% (9/11), 54.50% (6/11), 54.50% (6/11), 45.50% (5/11), 45.50% (5/11), 45.50% (5/11), 36.40% (4/11), 27.30% (3/11), 27.30% (3/11) and 18.20% (2/11) of *Listeria* spp. were resistant to penicillin G, clindamycin, tetracycline, ceftriaxone, ampicillin, rifampin, chloramphenicol, streptomycin, cephalothin, trimethoprim/sulfamethoxazole, erythromycin, nitrofurantoin and gentamicin, respectively. *Listeria* spp. from organic farm A, organic farm B and conventional farm C showed highest resistance towards clindamycin and least resistance towards gentamicin.

Listeria spp. was isolated from 11 vegetable samples, 2 (Chinese mustard and cucumber) from organic farm A and 9 (Chinese cabbage, Romaine/cos lettuce and Chinese white cabbage) from organic farm B. No *Listeria* spp. was isolated from the vegetable samples collected from conventional farm C. Antibiotic resistance graph of vegetable samples from the 3 selected farms is shown in Figure 4.6. For organic farm A, 100.00% (2/2) of

Listeria spp. isolated from both Chinese mustard and cucumber samples were resistant to rifampin, penicillin G, erythromycin, tetracycline, clindamycin and nitrofurantoin. The Chinese mustard was also resistant to ampicillin, gentamicin, chloramphenicol, cephalothin and ceftriaxone. However, 100.00% (2/2) *Listeria* spp. from both Chinese mustard and cucumber samples were susceptible to trimethoprim/sulfamethoxazole and streptomycin. For organic farm B, 100.00% (9/9) of *Listeria* spp. isolated from Chinese cabbage (Pak Choy), Romaine/cos lettuce and Chinese white cabbage (baby Pak Choy) were resistant to ampicillin, tetracycline, penicillin G, clindamycin, cephalothin and ceftriaxone. *Listeria* spp. from Chinese cabbage (Pak Choy) were also resistant to rifampin, erythromycin and nitrofurantoin. Other than that, *Listeria* spp. from Romaine/cos lettuce were also resistant to rifampin, gentamicin, trimethoprim/sulfamethoxazole, erythromycin, chloramphenicol, nitrofurantoin and streptomycin while *Listeria* spp. from Chinese white cabbage (baby Pak Choy) were resistant to rifampin, trimethoprim/sulfamethoxazole and nitrofurantoin. In this study, it was revealed that all the *Listeria* spp. isolated from vegetable samples from both organic farm A and organic farm B, were resistant to penicillin G, tetracycline and clindamycin.

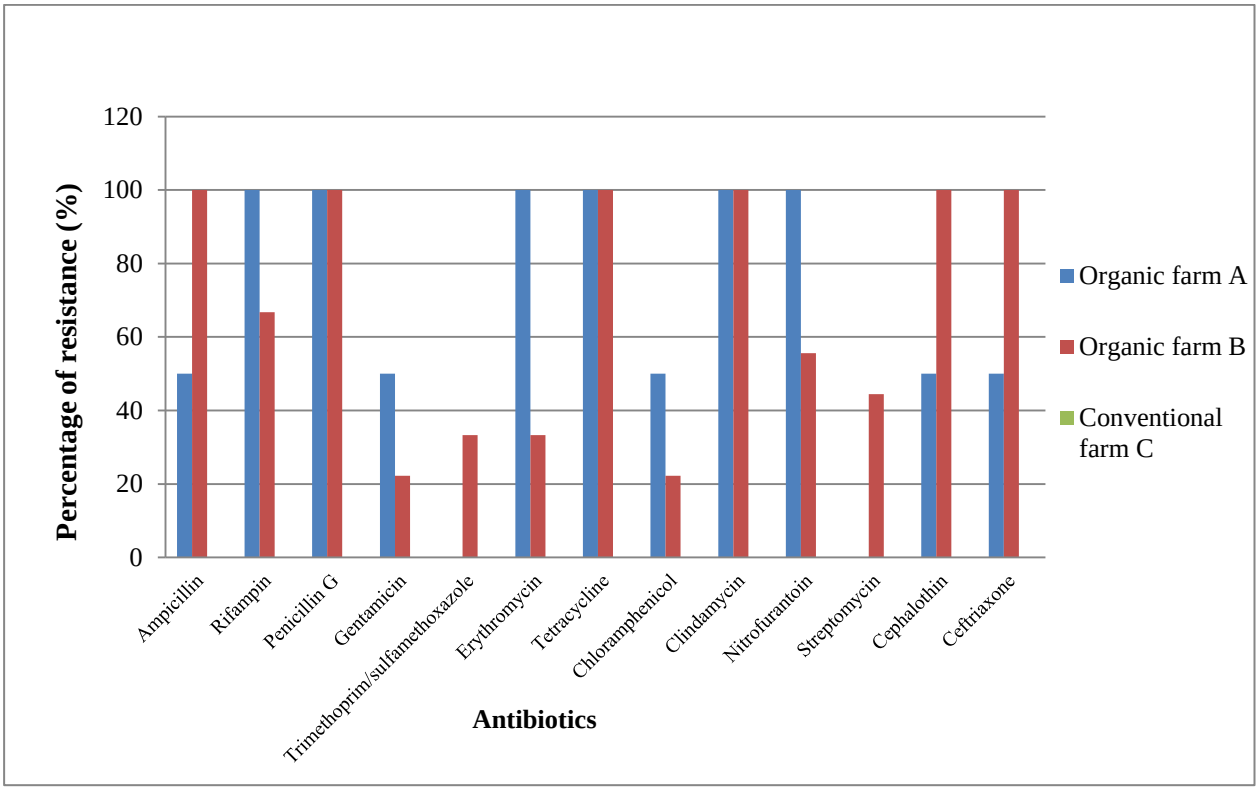


Figure 4.6 Percentage of antibiotic resistance of *Listeria* spp. from vegetable samples from organic farm A, organic farm B and conventional farm C

Listeria spp. was also isolated from 6 soil samples, 4 from organic farm A and 2 from organic farm B. No *Listeria* spp. was isolated from the soil samples in conventional farm C. Antibiotic resistance graph of soil samples from the 3 selected farms is shown in Figure 4.7. The results showed that 100.00% (4/4) of *Listeria* spp. from soil samples collected from organic farm A were resistant to rifampin, clindamycin, cephalothin and ceftriaxone. Besides that, 75.00% (3/4), 75.00% (3/4), 75.00% (3/4), 75.00% (3/4), 75.00% (3/4), 50.00% (2/4), 50.00% (2/4) and 25.00% (1/4) of *Listeria* spp. from soil samples collected from organic farm A were also resistant to penicillin G, tetracycline, chloramphenicol, nitrofurantoin, streptomycin, ampicillin, erythromycin and trimethoprim/sulfamethoxazole, respectively. Soil samples collected from organic farm A were sensitive to gentamicin. For organic farm B,

100.00% (2/2) of *Listeria* spp. from soil samples were resistant to ampicillin, penicillin G, tetracycline, clindamycin, cephalothin and ceftriaxone. Other than that, 50.00% (1/2) of *Listeria* spp. from soil samples collected from organic farm B were also resistant to trimethoprim/sulfamethoxazole and erythromycin. The result also showed that 100.00% (2/2) of the *Listeria* spp. from soil samples collected from organic farm B were susceptible to streptomycin, nitrofurantoin, chloramphenicol, gentamicin and rifampin. In this study, all *Listeria* spp. isolated from soil samples collected from both organic farm A and organic farm B, were resistant to clindamycin, cephalothin and ceftriaxone.

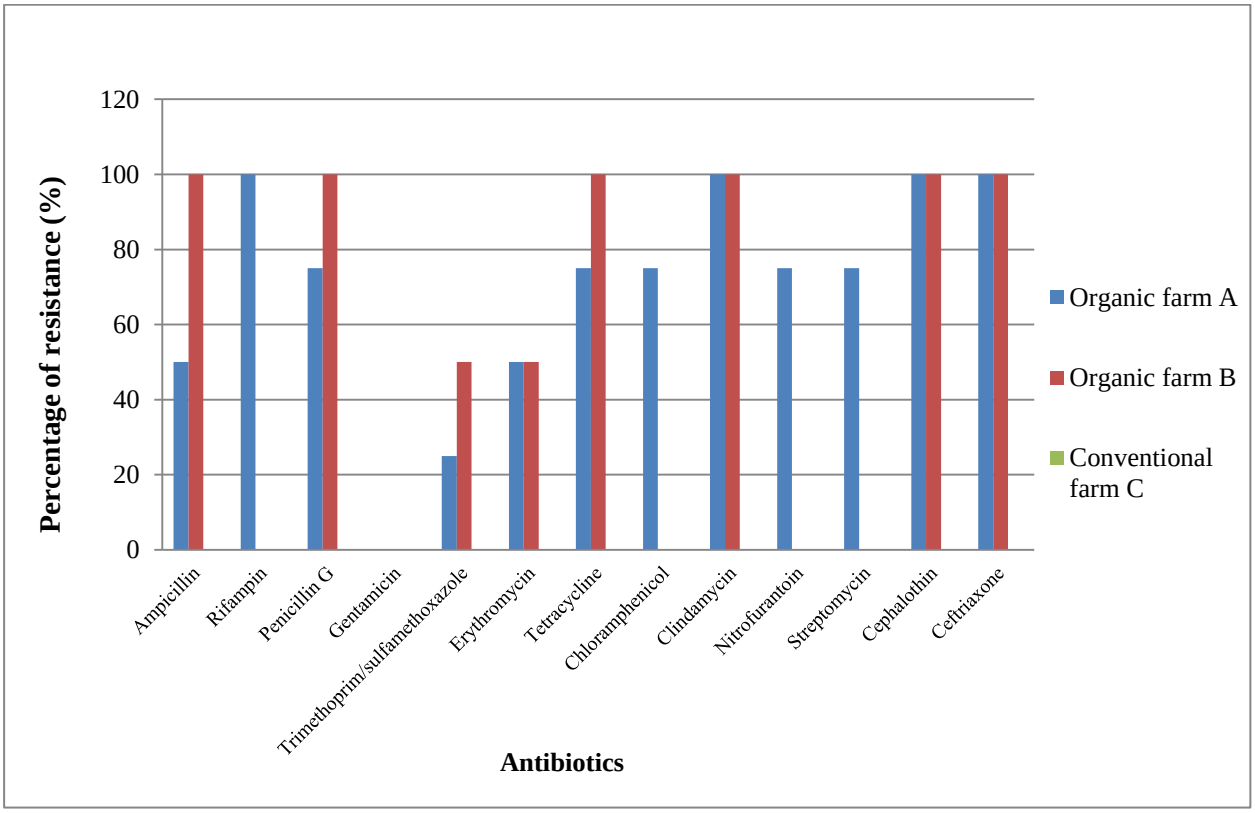


Figure 4.7 Percentage of antibiotic resistance of *Listeria* spp. from soil samples from organic farm A, organic farm B and conventional farm C

Fertilizer samples from organic farm A, organic farm B and conventional farm C were also detected with *Listeria* spp. There are 2 *Listeria* spp., 4 *Listeria* spp. and 2 *Listeria* spp. isolated from the soil samples collected from organic farm A, organic farm B and conventional farm C, respectively. Antibiotic resistance graph of fertilizer samples from the 3 selected farms is shown in Figure 4.8. In organic farm A, 100.00% (2/2) of *Listeria* spp. isolates from fertilizer samples were found to be resistant to ampicillin, penicillin G, trimethoprim/sulfamethoxazole, erythromycin, tetracycline, chloramphenicol, clindamycin, cephalothin and ceftriaxone. Other than that, it was also found out that 50.00% (1/2) of *Listeria* spp. isolates from fertilizer samples collected from organic farm A were also resistant to rifampin, gentamicin and nitrofuratoin. In this study, 100.00% (2/2) of *Listeria* spp. were found to be susceptible to streptomycin. For organic farm B, 100.00% (4/4) of *Listeria* spp. isolates from fertilizer samples were resistant to penicillin G, tetracycline, clindamycin and ceftriaxone. It was also found out that 75.00% (3/4), 75.00% (3/4), 25.00% (1/4), 25.00% (1/4) and 25.00% (1/4) of *Listeria* spp. from fertilizer samples were also resistant to erythromycin, cephalothin, ampicillin, nitrofuratoin and streptomycin. In our study, 100.00% (4/4) of *Listeria* spp. isolates from fertilizer samples from organic farm B were susceptible to rifampin, gentamicin, trimethoprim/sulfamethoxazole and chloramphenicol. For conventional farm C, 100.00% (2/2) of *Listeria* spp. from fertilizer samples were resistant to rifampin, clindamycin and ceftriaxone. It was found out that 50.00% (1/2) of *Listeria* spp. isolates from fertilizer samples collected from conventional farm C were resistant to ampicillin, penicillin G, erythromycin, tetracycline, chloramphenicol, streptomycin and cephalothin. In this study, 100.00% (2/2) of the *Listeria* spp. isolates from fertilizer samples were susceptible to gentamicin, trimethoprim/sulfamethoxazole and nitrofurantoin. The results revealed that all *Listeria* spp. isolated from fertilizer samples were resistant to clindamycin and ceftriaxone.

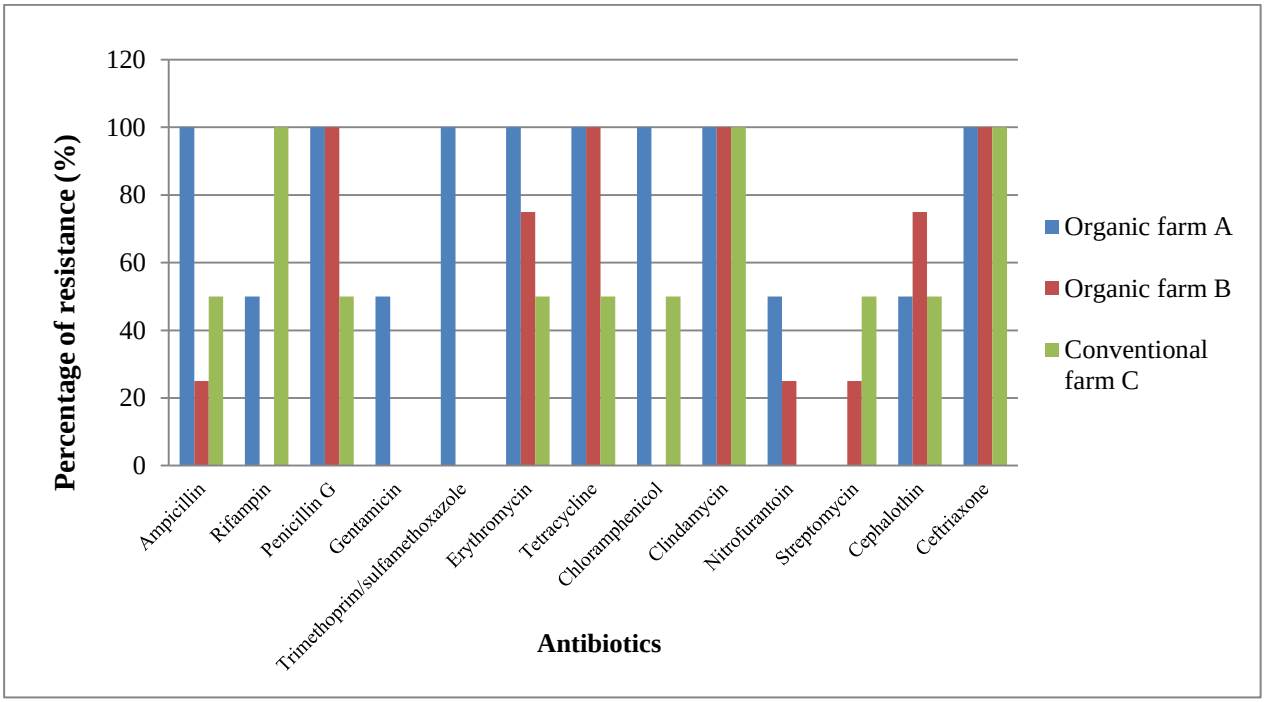


Figure 4.8 Percentage of antibiotic resistance of *Listeria* spp. from fertilizer samples from organic farm A, organic farm B and conventional farm C

In this study, conventional farm C was the only farm that the water samples were detected with *Listeria* spp. *Listeria* spp. was absent in the water samples from organic farm A and organic farm B. In conventional farm C, there were 9 *Listeria* spp. isolated from water samples. Antibiotic resistance graph of water samples from the 3 selected farms is shown in Figure 4.9. It was found out that 100% (9/9) of the *Listeria* spp. were found to be resistant to penicillin G. It was also found out that 88.90% (8/9), 88.90% (8/9), 77.80% (7/9), 55.60% (5/9), 44.40% (4/9), 44.40% (4/9), 44.40% (4/9), 44.40% (4/9), 44.40% (4/9), 33.30% (3/9), 22.20% (2/9) and 22.20% (2/9) of *Listeria* spp. from water samples were resistant to tetracycline, clindamycin, ceftriaxone, ampicillin, rifampin, trimethoprim/sulfamethoxazole, chloramphenicol, streptomycin, cephalothin, nitrofurantoin, gentamicin and erythromycin, respectively.

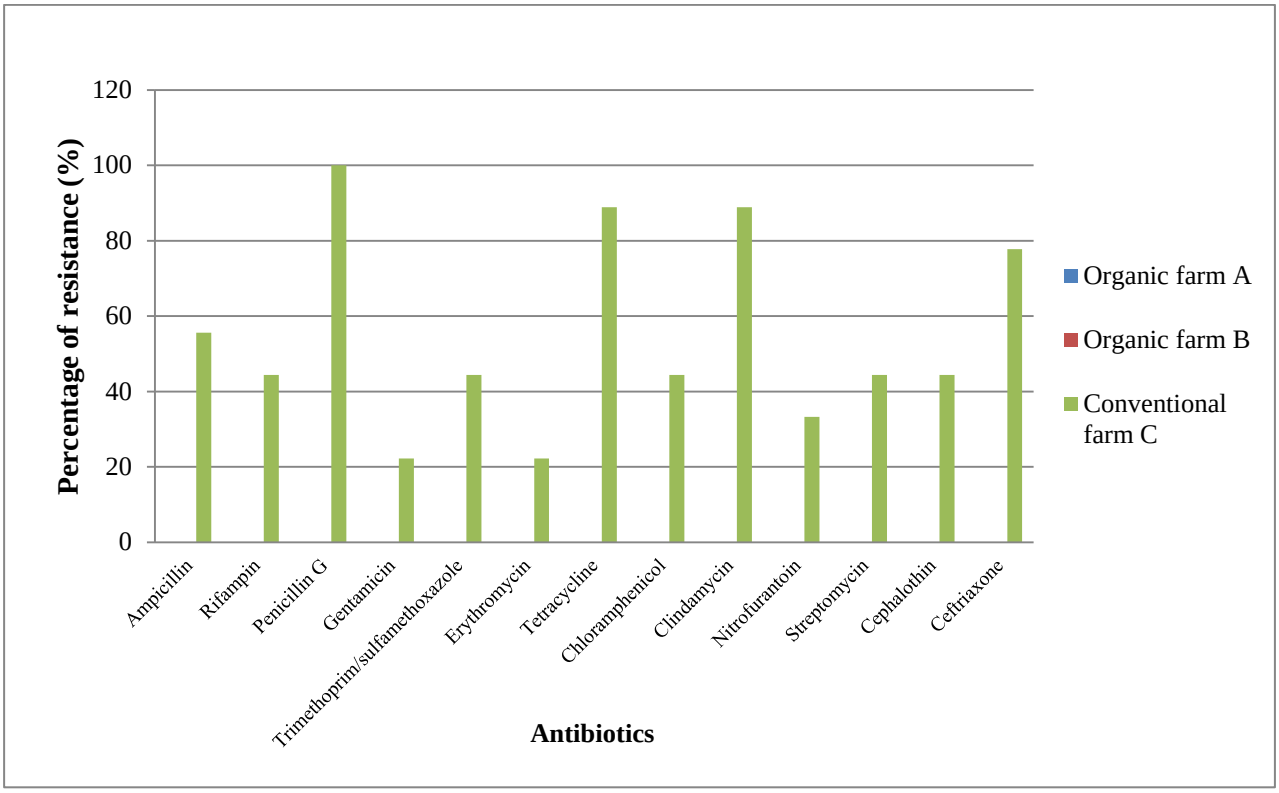


Figure 4.9 Percentage of antibiotic resistance of *Listeria* spp. from water samples from organic farm A, organic farm B and conventional farm C

MAR index defined by Krumperman (1983), was assigned for all the isolates. In this study, *Listeria* spp. isolates demonstrated MAR. These isolates were resistant to at least four of the thirteen antibiotics tested. The MAR indexes for all *Listeria* spp. isolates were recorded in Table 4.5 and Table 4.6.

For organic farm A, *Listeria* spp. isolated from Chinese mustard and cucumber have MAR indexes of 0.85 and 0.46, respectively. For organic farm B, the 2 *Listeria* spp. isolated from Chinese cabbage (Pak Choy) have MAR index of 0.62 while the 5 *Listeria* spp. isolated from Romaine/cos lettuce have MAR indexes of 0.69, 0.69, 0.69, 0.69 and 0.85. The 2 *Listeria* spp. isolated from Chinese white cabbage (baby Pak Choy) have MAR indexes of

0.77 and 0.62. *Listeria* spp. was not isolated from vegetable samples from conventional farm C. *Listeria* spp. in Chinese mustard from organic farm A and Romaine/cos lettuce from organic farm B have the highest MAR index which is 0.85.

For soil samples, the 4 *Listeria* spp. isolated from organic farm A have MAR indexes of 0.77, 0.69, 0.69 and 0.61 while the 2 *Listeria* spp. isolated from organic farm B have MAR index of 0.54. *Listeria* spp. was not isolated from soil samples from conventional farm C. *Listeria* spp. in soil samples from organic farm A has the highest MAR index which is 0.69. For fertilizer samples, the 2 *Listeria* spp. isolated from organic farm A have MAR indexes of 0.85 and 0.77 while the 4 *Listeria* spp. isolated from organic farm B have MAR indexes of 0.54, 0.54, 0.46 and 0.38. The 2 *Listeria* spp. isolated from fertilizer samples from conventional farm C have MAR indexes of 0.54 and 0.46. *Listeria* spp. in fertilizer samples from organic farm A has the highest MAR index which is 0.85. For water samples, *Listeria* spp. was not isolated from organic farm A and organic farm B. The 9 *Listeria* spp. isolated from water samples from conventional farm C have MAR indexes of 0.31, 0.38, 0.46, 0.46, 0.54, 0.62, 0.69, 0.69 and 0.77. *Listeria* spp. in water samples from conventional farm C has the highest MAR index which is 0.77.

Table 4.5 MAR index for all *Listeria* spp. isolates from organic farm A

No	Source	Sample	*Listeria* spp. Isolates	Antibiotic Resistance Pattern	MAR index
1	Organic farm A	Chinese Mustard	V9b	Amp, Rif, PenG, Gen, Ery, Tet, Chl, Cf, Cli, Nor, Cro	0.85
2		Cucumber	V11a	Rif, PenG, Ery, Tet, Cli, Nor	0.46
3		Soil	S4a	Amp, Rif, PenG, Tet, Str, Cf, Cli, Nor, Cro	0.69
4			S4b	Rif, PenG, Sxt, Tet, Chl, Str, Cf, Cli, Cro	0.69
5			S4e	Rif, PenG, Ery, Chl, Cf, Cli, Nor, Cro	0.61
6		Soil	S5b	Amp, Rif, Ery, Tet Chl, Str, Cf, Cli, Nor, Cro	0.77
7		Fertilizer	M4a	Amp, Rif, PenG, Gen, Stx, Ery, Tet, Chl, Cf, Cli, Cro	0.85
8		Fertilizer	M5b	Amp, PenG, Sxt, Ery, Tet, Chl, Cf, Cli, Nor, Cro	0.77

Table 4.6 MAR index for all *Listeria* spp. isolates from organic farm B and conventional farm C

No	Source	Sample	*Listeria* spp. Isolates	Antibiotic Resistance Pattern	MAR index
9	Organic farm B	Pak Choy	V114a	Amp, Rif, PenG, Tet, Cf, Cli, Nor, Cro	0.62
10		Pak Choy	V116c	Amp, PenG, Ery, Tet, Cf, Cli, Nor, Cro	0.62
11		Romaine/cos lettuce	V118a	Amp, Rif, PenG, Gen, Ery, Tet, Cf, Cli, Cro	0.69
12		Romaine/cos lettuce	V118c	Amp, Rif, PenG, Gen, Tet, Cf, Cli, Nor, Cro	0.69
13		Romaine/cos lettuce	V120b	Amp, Rif, PenG, Sxt, Tet, Cf, Cli, Nor, Cro	0.69
14		Romaine/cos lettuce	V120e	Amp, PenG, Sxt, Ery, Tet, Chl, Str, Cf, Cli, Nor, Cro	0.85
15			V120g	Amp, Rif, PenG, Tet, Chl, Str, Cf, Cli, Cro	0.69
16		Sawi Manis	V123e	Amp, Rif, PenG, Sxt, Ery, Tet, Str, Cf, Cli, Cro	0.77
17			V123f	Amp, PenG, Ery, Tet, Str, Cf, Cli, Cro	0.62
18		Soil	S37a	Amp, PenG, Sxt, Tet, Cf, Cli, Cro	0.54
19		Soil	S37c	Amp, PenG, Ery, Tet, Cf, Cli, Cro	0.54
20		Fertilizer	M38a	PenG, Ery, Tet, Cf, Cli, Nor, Cro	0.54
21		Fertilizer	M38f	PenG, Tet, Cf, Cli, Cro	0.38
22		Fertilizer	M39b	PenG, Ery, Tet, Cf, Cli, Cro	0.46
23		Fertilizer	M39f	Amp, PenG, Ery, Tet, Str, Cli, Cro	0.54
24	Conventional farm C	Fertilizer	M16b	Amp, Rif, PenG, Str, Cf, Cli, Cro	0.54
25		Fertilizer	M22b	Rif, Ery, Tet, Chl, Cli, Cro	0.46
26		Water	W21b	Rif, PenG, Gen, Tet	0.31
27		Water	W23a	PenG, Tet, Cf, Cli, Cro	0.38
28		Water	W23b	Amp, Rif, PenG, Tet, Str, Cf, Cli, Nor, Cro	0.69
29		Water	W24a	Amp, Rif, PenG, Sxt, Tet, Chl, Str, Cf, Cli, Cro	0.77
30		Water	W24b	PenG, Tet, Chl, Cli, Nor, Cro	0.46
31			W24c	Amp, PenG, Gen, Sxt, Ery, Tet, Chl, Cf, Cli	0.69
32		Water	W26a	Amp, Rif, PenG, Tet, Str, Cli, Cro	0.54
33		Water	W27a	PenG, Sxt, Ery, Chl, Cli, Cro	0.46
34		Water	W27c	Amp, PenG, Sxt, Tet, Str, Cli, Nor, Cro	0.62

CHAPTER 5

DISCUSSION

5.1 Prevalence of *Listeria* spp. and *L. monocytogenes* in Vegetables, Fertilizer and Environment in Organic Farms and Conventional Farm at Kuching, Sarawak

In this study, a total of 386 vegetable, fertilizer and environmental samples (soil and water) were collected from 2 organic farms (organic farm A and organic farm B) and a conventional farm (conventional farm C). The result of this PCR assay revealed that *Listeria* spp. was present in 7.51% (29/386) of all the samples (vegetable, soil, fertilizer and water) collected from all the three farms. It was present in 9.10% (6/66), 8.13% (13/160) and 6.25% (10/160) of all samples collected from organic farm A, organic farm B and conventional farm C, respectively. The prevalence of *Listeria* spp. in this study is lower as compared to a study conducted by Jeyaletchumi et al. (2011) which they detected *Listeria* spp. in 29.50% (38/129) of samples (poultry manure, soil, water and vegetables) from the selected farms in Cameron Highlands, Malaysia. In the present study, *Listeria* spp. was not detected in any vegetable samples collected from conventional farm C. However, it was present in 6.70% (2/30) and 8.00% (7/88) of the vegetable samples from organic farm A and organic farm B, respectively. The prevalence of *Listeria* spp. in vegetable samples in this study is higher as compared to a study conducted by Schwaiger et al. (2011) which reported that only 1.10% (11/1001) of the vegetables collected from vegetable farms and supermarket in Germany consist of *Listeria* spp. The absence of *Listeria* spp. in vegetable samples from conventional farm C in the present study can relate to a study conducted by Maffei et al. (2013) in which they revealed

that the organic vegetables sold in Brazil, have greater microbial counts than conventional vegetables.

In this study, the results showed higher *Listeria* spp. contamination level in leafy green vegetables as compared to smooth surface vegetables in the three farms analyzed. It was discovered that, 7.64% (12/157) samples of leafy green vegetable (Chinese cabbage, Chinese flowering cabbage, Romaine/cos lettuce, Chinese white cabbage, Chinese mustard, lettuce, Chinese kale and local vegetable) were identified with *Listeria* spp. while only 2.04% (1/49) samples of smooth surface vegetable (cucumber, yardlong bean and tomato) contain *Listeria* spp. This is comparable to a study conducted by Jamali et al. (2013). They were also reported the prevalence of *Listeria* spp. was higher in leafy green vegetables as 11.10% and 15.80% of lettuce and cabbage, respectively, were contaminated with *Listeria* spp. In their study, this organism was only found in 10.50% and 7.10% of cucumber and tomato, respectively. According to Silva et al. (2013), leafy green vegetables provide increased surface area for bacterial colonization. Thus, they are easily contaminated by pathogens.

The *Listeria* spp. from samples of cucumber and Chinese mustard from organic farm A have a plate count of 2.10×10^5 CFU/g and 2.50×10^4 CFU/g, respectively whereas the *Listeria* spp. in samples of Chinese cabbage (Pak Choy), Romaine/cos lettuce and Chinese white cabbage (baby Pak Choy) from organic farm B have plate count of 9.50×10^2 CFU/g - 7.70×10^3 CFU/g, 2.10×10^3 CFU/g - 9.90×10^4 CFU/g and 1.30×10^4 CFU/g, respectively. According to Public Health England (2014), samples consisting more than 100 CFU/g of *Listeria* spp. are considered unsatisfactory and investigation is required. Therefore, the vegetable samples collected from organic farm A and organic farm B in the present study

were considered unsatisfactory. In a study conducted by Zhu & Hussain (2014), they discovered that *Listeria* spp. in all the lettuce, carrot, purple cabbage and green cabbage from market in Canterbury, New zealand, were within the range of safety limits except for one purple cabbage sampled from market in the first week had 150 cfu/g of *Listeria* spp. Their results suggest that food safety risks are associated with fresh produce consumption and further investigations are needed as the ambiguity of *Listeria* poses food safety threat to consumers.

Listeria spp. was not detected in any soil from conventional farm C, similar to the findings in vegetables. However, *Listeria* spp. was present in 16.70% (2/12) and 8.30% (2/24) of soil from organic farm A and organic farm B. This result showed higher prevalence of *Listeria* spp. in soil compared to Vackachan et al. (2015) as only 2% (2/100) of soil collected from fields, near sewages and from farm surroundings in India, were found to be positive for *Listeria* spp. *Listeria* spp. is widely distributed in various environment including soil, vegetation, surface water, sewage, animal feeds, farm environments, and food-processing environments (Sauders et al., 2012). The occurence of *L. monocytogenes* in the soil may contribute to the contamination of vegetables at the pre-harvest stage when seeds are sown. According to Vackachan et al., 2015, the use of contaminated fertilizer and humidity of the soil in the farms may increase the risk of soil contamination. Therefore, measures should be taken for the use of contaminated soil to reduce the presence of *Listeria* spp. in the soil.

In the fertilizer, 16.70% (2/12), 16.70% (4/24) and 8.30% (2/24) of fertilizer sampled from organic farm A, organic farm B and conventional farm C were detected with *Listeria* spp., respectively. In a study conducted by Jeyaletchumi et al. (2011), 77.80% and 61.10%

poultry manure (for fertilization) from selected vegetable farms in Cameron Highlands were found to contain *Listeria* spp. and *L. monocytogenes*, respectively. In comparison, the present study showed lower prevalence of *Listeria* spp. in fertilizers sampled from all the three selected farms. The fertilizer used by the three farms (organic farm A, organic farm B and conventional farm C) in the present study is the compost of animal waste (chicken litter) and plant waste. This fertilizer is low cost organic fertilizer which contain notable amount of nutrients. However, many pathogens such as *Actinobacillus*, *Bordetalla*, *Campylobacter*, *Clostridium*, *Corynebacterium*, *Escherichia coli*, *Globicatella*, *Listeria*, *Mycobacterium*, *Salmonella*, *Staphylococcus*, and *Streptococcus* that can threaten humans, can be found in chicken litter-based fertilizers. Normally, composting of animal waste can inactivate large populations of human pathogens. However, improper composting or cross-contamination results in high survival rate of these pathogens through the composting process. Improper composting may also results in regrow of the pathogens in the finished compost products under favorable conditions (Chen & Jiang, 2014). Consequently, farmers need to practice proper composting process in order to avoid the possible microbiological safety issues for the environment and food crops grown in the farm.

Listeria spp. was found to be absent in water samples from organic farm A and organic farm B. This result is comparable to the study conducted by Jeyaletchumi et al. (2011) which they revealed that the irrigation water from the vegetable farms in Cameron Highlands were found to be free from *Listeria* spp. However, in the present study, *Listeria* spp. was detected in 33.30% (8/24) of water from conventional farm C. The prevalence of *Listeria* spp. in water samples is lower in comparison with a study conducted by Mawak et al. (2009) which reported 43.30% (13/30) of irrigation water (streams, rivers, ponds) at Jos, Plateau

State, Nigeria, were contaminated with *Listeria* spp. including *L. monocytogenes*, *L. innocua* and *L. grayi*. According to Galvez et al. (2014) and Chitarra et al. (2014), pathogenic bacteria such as *Salmonella*, pathogenic *E. coli* and *L. monocytogenes* can be found in irrigation water for fresh produce. These pathogenic bacteria can internalize into a crop through the roots and survive in it. Although this phenomenon can only occur when the crop is exposed to high levels of pathogens but it still poses the risk of contamination.

Vegetable samples from organic farm B have highest prevalence of *Listeria* spp. while the soil collected from organic farm A has highest prevalence of *Listeria* spp. Fertilizer collected from both organic farm A and organic farm B had the highest prevalence of *Listeria* spp. From all the water samples collected from all three selected farms, only water samples from conventional farm C contains *Listeria* spp. In comparison with organic farm A (9.10%) and organic farm B (8.13%), conventional farm C (6.25%) has the lowest occurrence of *Listeria* spp. Low occurrence of *Listeria* spp. in conventional farm C may be due to the application of pesticides in the conventional farm while organic farm A and organic farm B did not practice application of pesticides. According to Telias et al. (2011), a study conducted by Guan et al. (2005) to determine the effect of pesticides on bacterial survival, *Listeria* spp. was revealed to be least able to survive in pesticide solutions, among other bacteria tested.

Detection of *L. monocytogenes* from all the samples was also carried out in this study. PCR targeting 730 bp region of the listeriolysin (*hlyA*) gene of *L. monocytogenes* was used for the detection. However, the findings revealed that *L. monocytogenes* was absent in all the vegetable, fertilizer and environmental samples from organic farm A, organic farm B and conventional farm C. The finding of this present study is comparable to that found by Oliveira

et al. (2010). They reported the absence of *L. monocytogenes* in 144 fresh lettuce samples from organic and conventional production. However, a study conducted by Soni et al. (2014) reported the prevalence of *L. monocytogenes* in 10.00% of vegetables (brinjal, cauliflower, dolichos-bean, tomato, chappan-kaddu and chilli) and 5.00% of soil from the agricultural farm of the Indian Institute of Vegetable Research (IIVR), Varanasi, India. The absence of *L. monocytogenes* in all the samples from the selected farms in the present study may be caused by the inhibitory effect of background microbiota present on the vegetables, fertilizer and farm environment. The absence of pathogenic *L. monocytogenes* in the current study indicates lower chance of listeriosis infections. However, since *Listeria* spp. was detected in vegetable, fertilizer and farm environment samples, there is a need of hygienic measures to be adopted by the farms to avoid the spread of *Listeria* spp. in to the food chain.

5.2 Genotyping of *Listeria* spp. in Vegetables, Fertilizer and Environment in Organic Farms and Conventional Farm at Kuching, Sarawak

Two molecular methods, ERIC-PCR and BOX-PCR were used to study the relatedness among the *Listeria* spp. isolates from different type of samples and sampling areas. Dendrograms were constructed to show the relationships between identical strains and also similar groups of strains. Isolates in the same lineage are usually found to be genetically related and/or indicate possible cross-contamination (Shi et al., 2015). In this study, ERIC-PCR analysis produced 1-5 bands with size approximately 120 bp to 1450 bp. This analysis showed 11 different DNA fingerprint profiles. There are 15 *Listeria* spp. isolated from vegetables, fertilizer and soil samples from organic farm B have indistinguishable DNA

fingerprints. A study conducted by Jamili & Thong (2014) showed 4 *L. monocytogenes* isolates that were isolated from different types of RTE foods (egg products, salad and vegetables, chicken products and packed lunch) in the same location and sampling time, were indistinguishable by both REP-PCR and PFGE. They strongly suggest high prevalence of these isolates in the tested region. In this present study, the 100.00% (15/15) *Listeria* spp. which have indistinguishable DNA fingerprints, were isolated from vegetables, fertilizer and soil samples from organic farm B, suggesting high prevalence of this *Listeria* spp. in organic farm B. Furthermore, by using ERIC-PCR analysis, 86.70% (13/15) of the *Listeria* spp. from organic farm B with indistinguishable DNA fingerprints, were distributed into group A1 subgroup I. This can be explained by a study conducted by Abay et al. (2012) which found out that *L. innocua* isolate of beef minced meat and *L. innocua* isolate of cattle feces were classed in the same group with 99% similarity. They suggested that the beef can be contaminated with *Listeria* spp. of cattle feces. In this present study, vegetables from organic farm B can be contaminated with *Listeria* spp. in soil and fertilizer from the farm.

In this study, BOX-PCR analysis produced 2-13 bands with size approximately 120 bp to 1550 bp. This analysis produces 14 different fingerprint profiles. The 73.30% (11/15) *Listeria* spp. isolated from vegetables, fertilizer and soil samples from organic farm B have indistinguishable DNA fingerprints, suggesting high prevalence of similar *Listeria* spp. in organic farm B. Furthermore, by using BOX-PCR analysis, 54.55% (6/15) of the *Listeria* spp. in vegetables, soil and fertilizers from organic farm B with indistinguishable DNA fingerprints, were distributed into group A1. This result suggests that the vegetables from organic farm B can be contaminated with *Listeria* spp. in soil and fertilizer from the farm. Other than that, 36.40% (4/11) of *Listeria* spp. from conventional farm C also have

indistinguishable DNA fingerprints, suggesting high prevalence of similar *Listeria* spp. in conventional farm C. These *Listeria* spp. with indistinguishable DNA fingerprints, were distributed into group A2 subgroup II. This indicates that the water from conventional farm C may be contaminated by *Listeria* spp. in soil and fertilizer from the farm.

The findings from both ERIC-PCR and BOX-PCR analysis in the present study showed that the *Listeria* spp. isolates were not group together based on the types of samples and the source of isolation. They were not classified into specific cluster neither by sampling area nor the type of samples. This result is comparable to the study conducted by Jamili & Thong (2014) which the *L. monocytogenes* isolates in their study were not classified into specific cluster neither by sampling area nor the type of samples, indicating the *L. monocytogenes* isolates from RTE food were genetically diverse and heterogeneous. In the present study, the *Listeria* spp. isolated from organic farm A, organic farm B and conventional farm C were genetically diverse and heterogeneous. The heterogeneity was expected as the isolates were collected from different types samples (vegetable, soil, fertilizer and water) and sampling locations (organic farm A, organic farm B and conventional farm C). There is a study done by Shi et al. (2015) which also revealed high genetic diversity in *L. monocytogenes* isolated from RTE food in China by using ERIC-and REP-PCR.

The Simpson's Index of Diversity, D, was used to measure the discriminating power of typing methods. The D value was calculated according to Hunter & Gaston (1988). The D value for ERIC-PCR analysis was calculated to be 0.604 while the D value for BOX-PCR analysis was calculated to be 0.888. According to Kqueen et al. (2013), the higher the discriminatory index, the greater the effectiveness of a particular fingerprinting method to

discriminate different strains. Therefore, in the present study, BOX-PCR analysis has greater discriminatory power than ERIC-PCR analysis. The discriminatory power for both BOX-PCR and ERIC-PCR analysis were lower as compared to a study conducted by Jersek et al. (1999) which revealed ERIC-PCR was suitable for the typing of *L. monocytogenes* isolates as the index of discrimination was high (0.98). Another study conducted by Jamali & Thong (2014) reported that the discrimination index for REP-PCR, BOX-PCR, RAPD and PFGE were 0.992, 0.998, 1 and 0.916, respectively. They suggested that different subtyping methods often give different discriminatory powers. Therefore, it is necessary to use more than one subtyping approach to provide a more accurate description of the genetic diversity of microorganisms in the study.

5.3 Antibiotic Susceptibility Test

In this study, antibiotics which were effective in listeriosis treatment including ampicillin, chloramphenicol, erythromycin, gentamycin, penicillin G, rifampin, tetracycline and sulfamethoxazole/trimethoprim, were tested on *Listeria* spp. for antibiotic susceptibility. This present study revealed that 97.06% (33/34) of *Listeria* spp. isolated from vegetables, soil, fertilizer and water from organic farm A, organic farm B and conventional farm C, were resistant to clindamycin. This result is comparable to Chen et al. (2010), which they found out all *Listeria* spp. isolates in catfish fillets and processing environment, were resistant to clindamycin. Gamboa-Marin et al. (2013) also revealed that *L. monocytogenes*, *Listeria* spp. and *L. ivanovii* from swine processing facilities in Colombia have major resistance and intermediate susceptibility to clindamycin.

Other than that, 94.11% (32/34), 91.80% (31/34) and 91.80% (31/34) of *Listeria* spp. isolated from the three selected farms, were resistant to penicillin G, tetracycline and ceftriaxone, respectively. According to Troxler et al. (2000), *Listeria* spp. were naturally resistant to ceftriaxone. This result is comparable to the present study as 91.80% (31/34) of the *Listeria* spp. from the three selected farms were resistant to ceftriaxone. In the present study, 94.11% (32/34) and 91.80% (31/34) of *Listeria* spp. from the three selected farms were resistant to penicillin G and tetracycline, respectively. This is comparable to a study conducted by Walsh et al. (2001) which reported the frequent resistance of *Listeria* spp. isolated from retail food (cooked meat, frozen burger, haddock fish, ice cream and salad), towards tetracycline and penicillin. Rahimi et al. (2012) also revealed the *Listeria* spp. from traditional dairy products (cheese, yogurt, ice-cream, doogh, butter and kashk) in Iran showed resistance against penicillin (47.60%), and tetracycline (33.30%).

Besides that, 73.53% (25/34), 67.65% (23/34), 55.88% (19/34) and 52.94% (18/34) of *Listeria* spp. from the three selected farms were also resistant to cephalothin, ampicillin, rifampin and erythromycin, respectively. However, Moreno et al. (2014) found out that *Listeria* spp. isolated from slaughterhouse environments, pork and human infections in Sao Paulo and Brazilian states, were susceptible to ampicillin and erythromycin. Jamali et al. (2015) also reported 16.5% of *Listeria* spp. from open-air fish market were resistant to cephalothin. The present study showed higher resistance against cephalothin.

There is a study conducted by Yucel et al. (2005) which reported the *Listeria* spp. isolated from meat and meat products exhibited a high degree of susceptibility to chloramphenicol. Soni et al. (2014) also revealed that all the *L. monocytogenes* isolates from

vegetables and soil were susceptible to ampicillin, rifampin, chloramphenicol, streptomycin, sulfamethoxazole/trimethoprim and gentamicin tested. However in the present study, 44.11% (15/34), 38.24% (13/34), 38.24% (13/34), 32.35% (11/34) and 17.65% (6/34) of *Listeria* spp. from the three selected farms were resistant to nitrofurantoin, chloramphenicol, streptomycin, sulfamethoxazole/trimethoprim and gentamicin.

In the present study, *Listeria* spp. from the three selected farms showed lowest resistance against gentamicin. This is comparable to a study conducted by Li et al. (2006) which revealed gentamicin exhibited good activity against *Listeria* spp. from processed bison in USA.

This study showed that *Listeria* spp. isolated from vegetables, fertilizer and environmental samples from organic farm A, organic farm B and conventional farm C were multi-resistant to the antibiotics tested. All of the *Listeria* spp. isolates were resistant to more than one antibiotic and therefore, they demonstrated MAR. The lowest and highest MAR index in these *Listeria* spp. isolates from all farms were 0.31 and 0.85, respectively. According to Krumperman (1983), MAR index value lower than 0.20 indicates that the organism was originated from lower risk source in which the antibiotics are seldom/never used. MAR index value higher than 0.20 indicates that they are originated from higher risk source which is greatly exposed to antibiotics. In a study conducted by Wong et al. (2012), 48.70% of *L. monocytogenes* isolated from frozen burger patties demonstrated MAR. These isolates were found to be resistant to at least two of the eleven antibiotics tested. Another study conducted by Jamali et al. (2013), reported that 8.40% of *Listeria* spp. isolated from raw milk in Iran showed multiple antibiotic resistance. In the present study, all *Listeria* spp.

have MAR index higher than 0.20 suggested that the *Listeria* spp. from vegetables, fertilizer and environmental samples from organic farm A, organic farm B and conventional farm C were originated from higher risk source in which they are constantly exposed to antibiotics. MAR *Listeria* spp. in vegetables, soil and irrigation water could be attributed by the usage of animal waste as fertilizer which may contain antibiotic used to prevent/treat animal diseases and promote animal growth. Hu et al. (2010) conducted a study on migration of antibiotics from manure to soil, from soil to vegetables and groundwater. In the study, they applied manure containing antibiotics to organic vegetable bases and revealed that the soil, vegetables and water were detected with antibiotic residues. This situation poses great risk on the safety of ecosystems and human health. Apart from that, Gullberg et al. (2014) also found that low levels of heavy metals present in polluted external environments could allow selection and enrichment of multidrug resistance bacteria and thus contribute to the emergence, maintenance, and transmission of antibiotic-resistant disease-causing bacteria. This suggests that heavy metals may present in organic farm A, organic farm B and conventional farm C and thereby contributed to MAR in *Listeria* spp.

The findings in the present study showed that *Listeria* spp. isolated from vegetables, fertilizer and environmental samples from organic farm A, organic farm B and conventional farm C were multi-resistant to the antibiotics tested. The presence of *Listeria* spp. that were resistant to antibiotics commonly used to treat human listeriosis (ampicillin, chloramphenicol, erythromycin, gentamicin, penicillin G, rifampin, tetracycline and sulfamethoxazole/trimethoprim), raises the possibility of future acquisition of resistance by *L. monocytogenes*.

CHAPTER 6

GENERAL CONCLUSION

This study provides an overview of occurrence of *Listeria* spp. and *L. monocytogenes* in vegetable, fertilizer and environmental (soil & water) samples from organic (organic farm A & organic farm B) and conventional farm (conventional farm C). The finding showed that organic farm A (9.10%) and organic farm B (8.13%) have higher occurrence of *Listeria* spp. as compared to conventional farm C (6.25%). As discussed, low occurrence of *Listeria* spp. in conventional farm C may be due to the application of pesticides in the conventional farm. However, in the present study, *L. monocytogenes* was found to be absent in vegetable, fertilizer and environmental samples from organic farm A, organic farm B and conventional farm C. This indicates that the vegetables from the selected farms were safe to be consumed. Although *L. monocytogenes* was absent in the selected farms, the finding shows that *Listeria* spp. was present in the vegetable, fertilizer and environmental samples from these farms which indicates that there is a need of hygienic measures to be adopted by farms to avoid the entry of *Listeria* spp. into the food chain.

Two molecular methods, ERIC-PCR and BOX-PCR were used to study the relatedness among the *Listeria* spp. isolates from the vegetable, fertilizer and environmental samples from organic farm A, organic farm B and conventional farm C. In this study, 100% (15/15) and 73.30% (11/15) of the *Listeria* spp. isolated from vegetables, fertilizer and soil from organic farm B have indistinguishable DNA fingerprints by using ERIC-PCR and BOX-PCR, respectively. The finding suggests high prevalence of this particular *Listeria* spp. in organic farm B. *Listeria* spp. from vegetable, fertilizer and environmental samples from

organic farm A and conventional farm C have distinct DNA fingerprints. *Listeria* spp. isolated from all the three selected farms were heterogeneous as they were not classified into specific cluster neither by sampling area nor the type of samples. The Simpson's Index of Diversity, D, was calculated to measure the discriminating power of typing methods. The Simpson's Index of Diversity, D, of ERIC-PCR and BOX-PCR are 0.604 and 0.888, respectively. Therefore, our study showed that BOX-PCR has better discriminatory power compared to ERIC-PCR.

Antimicrobial susceptibility test was carried out in this study on *Listeria* spp. isolated from the vegetable, fertilizer and environmental samples from organic farm A, organic farm B and conventional farm C. In our study, most of the *Listeria* spp. in this study were found to be resistant to ampicillin, rifampin, penicillin G, tetracycline, clindamycin, cephalothin, erythromycin and ceftriaxone. Most of the *Listeria* spp. in this study were least resistant to gentamicin. Besides that, this study revealed that all of the *Listeria* spp. isolates were resistant to at least four of the thirteen antibiotics tested. All of the *Listeria* spp. were multi-resistant to antibiotics tested. These isolates have MAR index ranged between 0.31 and 0.85. MAR index higher than 0.20 suggested that *Listeria* spp. from vegetables, fertilizer and farm environment from organic farm A, organic farm B and conventional farm C were originated from higher risk source in which they are constantly exposed to antibiotics. MAR of *Listeria* spp. in vegetables, soil and irrigation water could be attributed by the usage of animal waste as fertilizer which may contain antibiotic used to prevent/treat animal diseases and promote animal growth. MAR in *Listeria* spp. may also cause by the presence of heavy metals in farms. The prevalence of MAR *Listeria* spp. could be the sign where *L. monocytogenes* gradually became multiple antibiotic resistant by acquiring potential multiple antibiotic resistant genes

from these *Listeria* spp. Therefore, antimicrobial resistance of *Listeria* spp. and *L. monocytogenes* should be monitor frequently to assure the effectiveness of listeriosis treatment.

The findings in this study revealed the absence of *L. monocytogenes* from the vegetable, fertilizer and environmental samples from the organic farm A, the organic farm B and the conventional farm C. However, *Listeria* spp. were isolated from the three selected farms and were also tested for antimicrobial susceptibility. The findings in this study provides baseline data on *Listeria* spp. and *L. monocytogenes* contamination at farm level for future risk assessment work and for establishing food safety standards in both organic and conventional vegetable farms in Sarawak, Malaysia.

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