Occurrence and Quantification of *Escherichia coli* and *Escherichia coli* O157:H7 on Conventional Vegetables at Farm Level

Lillian Sea Shun Yi

Bachelor of Science with Honours (Resource Biotechnology) 2013
ACKNOWLEDGEMENT

The completion of this project must particularly thanks to my project supervisor, Dr. Lesley Maurice Bilung, who been giving many invaluable guidance and insightful comments throughout the project. I am heartily appreciated all her encouragement, advices, supervision and patience, enabling me to accomplish the project with better understanding. I also wish to express my gratitude to my co-supervisor, Dr. Micky Vincent for giving me support, concern and help in the project.

Besides, I am obliged to all master students, Christy Chan Sien Wei and Velnetti Linang for guiding and assisting in handling various equipment and experiment techniques in the laboratory. Their advices and opinions had enabling me to adapt the environment more quickly and enhance my lab skills in the project. Additional thanks also given to lab assistants who provided needs and supplied laboratory apparatus and materials.

Special thanks to my beloved friends, Lim Poh Yiin, Chai Siaw Yew, Chai Sze Fan and Chong Hui Nei. Without their kindness, the progress of the project would unable to accomplish smoothly and even finish on time.

Last but not least, my deepest gratitude goes to my parents Sea Siow Kok and Tan Ah Ling and siblings for their love, support, tolerance and understanding during the ups and downs of the project.
DECLARATION

I hereby declare that this thesis is based on my original work except for question and citation, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions.

[Signature]
Lillian Sea Shun Yi
Resource Biotechnology Programme
Department of Molecular Biology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
# TABLE OF CONTENTS

- **ACKNOWLEDGEMENT**
- **DECLARATION**
- **TABLE OF CONTENTS**
- **LIST OF ABBREVIATIONS**
- **LIST OF FIGURES**
- **LIST OF TABLES**
- **ABSTRACT**
- **CHAPTER 1 INTRODUCTION**
- **CHAPTER 2 LITERATURE REVIEW**
  - 2.1 *Escherichia coli* O157:H7
    - 2.1.1 History
    - 2.1.2 Genus *Escherichia*
    - 2.1.3 General Characteristics
    - 2.1.4 Pathogenesis
    - 2.1.5 Occurrence of human outbreaks
  - 2.2 Occurrence and quantification
    - 2.2.1 Standard plate count
    - 2.2.2 Most Probable Number (MPN) method
    - 2.2.3 Polymerase Chain Reaction (PCR) Detection
    - 2.2.4 Antibiotics Susceptibility
- **CHAPTER 3 MATERIALS AND METHODS**
  - 3.1 Bacterial Strains
  - 3.2 Sample collection
  - 3.3 Detection and enumeration of *E. coli* O157:H7
  - 3.4 DNA extraction using boiled cell method
  - 3.5 Detection using Polymerase Chain Reaction
3.6 Gel electrophoresis 18
3.7 Antibiotic susceptibility 18
3.8 Multiple antibiotic resistances (MAR) calculation 19

CHAPTER 4 RESULTS 20
CHAPTER 5 DISCUSSION 37
CHAPTER 6 CONCLUSION 42
REFERENCES 43
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>°F</td>
<td>Degrees Fahrenheit</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CFU/g</td>
<td>Colony forming unit per gram</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>HCL</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>I</td>
<td>Intermediate</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>mM</td>
<td>Milli molarity</td>
</tr>
<tr>
<td>MPN/G</td>
<td>Most probable number per gram</td>
</tr>
<tr>
<td>pH</td>
<td>Power of hydrogen</td>
</tr>
<tr>
<td>R</td>
<td>Resistance</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolution per minute</td>
</tr>
<tr>
<td>S</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Taq</td>
<td>Thermus aquaticus</td>
</tr>
<tr>
<td>TLTC</td>
<td>Too little to count</td>
</tr>
<tr>
<td>TNTC</td>
<td>Too numerous to count</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>4.1</td>
<td>Green metallic sheen colonies from the positive culture of <em>E. coli</em> O157</td>
</tr>
<tr>
<td>4.2</td>
<td>Result of the PCR assay, amplifying 210 base pair segment of <em>stx</em>1, 292 base pair of <em>rfb</em>E, 484 base pair of <em>stx</em>2 and 625 base pair of <em>flicH</em>7 gene of <em>E. coli</em> O157:H7.</td>
</tr>
<tr>
<td>4.3</td>
<td>Result of the PCR assay, amplifying 210 base pair segment of <em>stx</em>1, 292 base pair of <em>rfb</em>E, 484 base pair of <em>stx</em>2 and 625 base pair of <em>flicH</em>7 gene of <em>E. coli</em> O157:H7.</td>
</tr>
<tr>
<td>4.4</td>
<td>Antibiotic sensitivity against <em>E. coli</em> isolates.</td>
</tr>
<tr>
<td>4.5</td>
<td>Multiple antibiotic resistance index (MAR) of different isolates.</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 3.1.</td>
<td>Conventional vegetables from two farms in Kuching, Sarawak</td>
<td>15</td>
</tr>
<tr>
<td>Table 3.2.</td>
<td>Primer sequences for the detection of <em>E. coli</em> O157:H7 using a multiplex PCR</td>
<td>17</td>
</tr>
<tr>
<td>Table 4.1.</td>
<td>The distribution of <em>E. coli</em> and <em>E. coli</em> O157:H7 in conventional vegetables.</td>
<td>22</td>
</tr>
<tr>
<td>Table 4.2.</td>
<td>Results of occurrence and quantification of <em>E. coli</em> and <em>E. coli</em> O157:H7 in conventional vegetables.</td>
<td>23</td>
</tr>
<tr>
<td>Table 4.3.</td>
<td>Diameter of inhibition zone of <em>Escherichia coli</em> sp. isolates.</td>
<td>34</td>
</tr>
<tr>
<td>Table 4.4.</td>
<td>Antibiotic resistance patterns and multiple antibiotic resistance (MAR) index of <em>E. coli</em> isolates from conventional vegetables in farm level.</td>
<td>35</td>
</tr>
</tbody>
</table>
Occurrence and Quantification of *Escherichia coli* and *Escherichia coli* O157:H7 on Conventional Vegetables at Farm Level

Lillian Sea Shun Yi

Resource Biotechnology
Faculty of Science and Technology
Universiti Malaysia Sarawak

**ABSTRACT**

*Escherichia coli* O157:H7 is the predominant pathogen in the EHEC group which often implicated in human infections worldwide. The recent outbreaks were frequently linked to the consumption of vegetables, instead of solely on meat and poultry products. The aim of this study is to investigate the prevalence and frequency of *E. coli* and *E. coli* O157:H7 on different types of conventional vegetables from two farms by using the combination of MPN and PCR. Another goal is to determine the antibiotic susceptibility profile among the *E. coli* isolates. A total of 11 types of conventional vegetables including Chinese flowering green, Chinese white stem, water spinach, spinach, Chinese broccoli, Chinese chives, okra, French beans, long beans, sweet potato leaves, and betel leaves in Kuching, Malaysia were investigated. The estimated quantity of *E. coli* isolates in all samples is more than 1100 MPN/g and the overall CFU/g in all samples ranged from $8.8 \times 10^7$ to $1.9 \times 10^6$ CFU/g. A full set of target genes for *E. coli* O157:H7 was not detected in the samples, but Chinese flowering green and Chinese white stem samples showed the presence of other type of EHEC. Twelve *E. coli* isolates were subjected to antibiotic susceptibility testing, chloramphenicol and nitrofurantoin were the most effective antibiotic; whereas, cefalothin showed that highest antibiotic resistance. Overall antibiotics resistance pattern of all isolates displayed 1 to 2 resistance patterns with multiple antibiotic resistance (MAR) index ranging from 0 to 0.25 respectively. Hence, the present study indicated that high prevalence of *E. coli* was detected in conventional vegetables, posing a potential risk for raw vegetable consumption in Malaysia.

**Key words:** occurrence, *E. coli*, *E. coli* O157:H7, conventional vegetables, antibiotics susceptibility

**ABSTRAK**

*Escherichia coli* O157:H7 merupakan patogen utama di kumpulan EHEC yang sering menjangkiti manusia di seluruh dunia. Kebelekanan ini, wabak bukan hanya disebabkan pengambilan daging dan produk ternakan sahaja, namun juga sayuran. Tujuan kajian ini adalah untuk mengkaji kejadian dan frekuensi *E. coli* dan *E. coli* O157:H7 di pelbagai jenis sayur konvensional dari dua ladang dengan menggunakan gabungan MPN dan PCR. Matlamat yang seterusnya ialah untuk menentukan profil kerentanan antibiotik antara penala *E. coli*. Sejumlah 11 jenis sayur-sayuran konvensional termasuk kangkong, bayam, kalian, choy sum, pak choy, ku choy, sayur bendi, kangc buncis, kangc panjang, sayur keledek and sayur mani di Kuching, Malaysia telah disiasat. Kuantiti *E. coli* spp. dan *E. coli* O157:H7 dalam semua sampel dianggarkan lebih dari 1100 MPN/g dan jualat CFU/g seluruh sampel dari $8.8 \times 10^7$ ke $1.9 \times 10^6$ CFU/g. Sasaran gen yang lengkap bagi *E. coli* O157:H7 tidak dikesan, tetapi suspek choy sum dan pak choy menunjukkan kehadiran jenis lain EHEC. Dua belas *E. coli* penala melalui ujian kerentanan antibiotik, kloramfenikol dan nitrofurantoin merupakan antibiotik paling berkesan; manakala, cefalotin menunjukkan ketahanan antibiotik yang tertinggi. Keseluruhan corak rintangan antibiotik semua penala mempermaikan 1 ke 2 rintangan corak dengan ketahanan antibiotik (MAR) berbilang indeks melebihi 0 hingga 0.25 masing-masing. Maka, kajian menunjukkan bahawa sebaran *E. coli* yang luas telah dikesan di sayur-sayuran konvensional, menunjukkan potensi risiko memakan sayuran mental di Malaysia.

*Kata kunci:* kejadian, *E. coli*, *E. coli* O157:H7, sayuran konvensional, kerentanan antibiotik
CHAPTER 1

INTRODUCTION

Foodborne disease is a wide spectrum of health illness includes foodborne intoxication and infection which contracted from eating contaminated food or beverages by the contamination of microbial, parasitic or chemical (U.S. Department of Labor, 2005). In the general population, foodborne diseases responsible for high levels of morbidity and mortality, particularly the at-risk groups such as neonates, children, the elderly and the immunocompromised (WHO, 2013). For examples, *Campylobacter jejuni* Clostridium *perfringens*, *Salmonella* spp. and *Escherichia coli* O157:H7 (*E. coli* O157:H7) are the most common bacterial foodborne pathogens implicated in foodborne disease.

Over hundreds of *E. coli* strains are harmless, but the bacterium in enterohemorrhagic group (EHEC), *E. coli* O157:H7 is the prevalent serotype with the production of a potent toxin in human infection called Shiga toxin (Abong'o 2008). According to National Institute of Allergy and Infectious Diseases (2011), Centers for Disease Control and Prevention (CDC) estimates that among 265,000 cases per year of Shiga toxin *E. coli* infections, approximately 36% of the infection are caused by *E. coli* O157:H7. Historically, the outbreak of *E. coli* O157:H7 infection was resulted in the association with undercooked ground beef (Ibekwe *et al.*, 2011). In the recent years, foodborne outbreaks that linked to vegetables had become more common such as lettuce, spinach, and sprouts (Davis and Kendall, 2013).

The first recognition of *E. coli* O157:H7 in year 1982, two hemorrhagic colitis outbreak caused by the consumption of hamburgers in U.S. (Jeshveen *et al.*, 2012). In 1996, the foodborne illness caused the largest outbreak occurred in Sakai City, Japan with the
consumption of contaminated white radish sprouts served at school meals (Pennington, 2009). This outbreak had affected more than 12,000 persons and some with hemolytic uremic syndrome (HUS) which requires kidney dialysis (Doyle et al., 2006; Anderson, 2011). Another major outbreak of \( E. \) \( \text{coli} \) O157:H7 occurred in South Wales in 2005, a majority of school children involved in 44 schools across four local authority areas were infected and 118 out of a total 157 cases were positive with \( E. \) \( \text{coli} \) O157:H7 test (Pennington, 2009). The Outbreak Control Team in South Wales identified that the meats supplied by John Tudor and Son, a catering butchers’ business based in Bridgend was the likely source of the infection (Pennington, 2009).

Due to its long period of survival ability in the environment and low infectious dose, the outbreak of \( E. \) \( \text{coli} \) O157:H7 are sporadic. Furthermore, the rise of the consumption of vegetables in the public had contributed to large scale of produce-associated outbreaks. Hence, microbial safety of vegetables becomes global concern as they might be contaminated through manure, soil and water; several prevention strategies should focus on, especially at farm level. For instance, safe irrigation water, proper manure management and appropriate farm location (Davis and Kendall, 2013).

Therefore, it is important to conduct this study as not much study has been carried out on \( E. \) \( \text{coli} \) O157:H7 on conventional vegetables at farm level especially in East Malaysia (Sarawak). The findings from this study carried out by using Most Probable Method (MPN) to estimate the concentration of viable cell in the sample and quantifying the viable cell number of \( E. \) \( \text{coli} \) spp. which present in green metallic sheen on EMB agar via Colony-Forming Unit (CFU) count. While, the target organism was detected via multiplex Polymerase Chain Reaction (mPCR) which aid in amplifying DNA sequences. The study sites have been focused
on conventional vegetables grown at 3 farms located Serian Road to Matang, Sarawak. Hence, this study was undertaken with the following objectives:

1. To investigate the occurrence of *E. coli* and *E. coli* O157:H7 on different types of conventional vegetables from different farms by using the combination of MPN and PCR.

2. To determine the frequency of *E. coli* and *E. coli* O157:H7 on conventional vegetables

3. To determine the antibiotic susceptibility profile among the *E. coli* isolates.
CHAPTER 2

LITERATURE REVIEW

2.1 *Escherichia coli* O157:H7

2.1.1 History

In 1885, a German pediatrician, Dr. Theodore von Escherich discovered a quick-growing rod-shaped bacterium from infant feces whilst hunting of the cause of fatal intestinal diseases in children (Radhakrishnan, 2012). This bacterium was originally named as *Bacterium coli communum* as this bacterium is universally found in the colon or large intestine. Later, the name was changed into *E. coli* to honor Dr. Escherich after his death.

Later, a pathogenic strain, *E. coli* O157:H7 was first identified due to the cause of two hemorrhagic colitis outbreaks in United States in 1982 (Graf, 2010). The first outbreak occurred in Oregon with 26 cases of which 19 were hospitalized and the second, with 21 cases and 14 hospitalizations (Buchanan and Doyle, 1997). Three months later in Michigan, the consumption of undercooked hamburgers from a fast food chain restaurant was identified as the vehicle due to the isolation of *E. coli* O157:H7 from a frozen ground beef patty and patient who developed bloody diarrhea and severe abdominal cramps after eating hamburgers in a restaurant chain (Buchanan and Doyle, 1997). Due to the high frequency of *E. coli* O157:H7 infection in human, the public awareness had raised in U.S. with a reduction of occurrence (13 outbreaks) in 1993 (United States Department of Agriculture, 1994).

Since then, more outbreaks were reported in continental Europe. In subsequent year, the first outbreak of this new pathogen was emerged in the United Kingdom. Continuously, the first isolation in Belgium, Africa and New Zealand happened in 1987, 1990 and 1993,
respectively (Pennington, 2010). According to United States Department of Agriculture (1997), the number of foodborne outbreaks reported to the Centers for Disease Control and Prevention (CDC) from 1994 to 1996 had decreased from 39 to 29. This may due to increased awareness of disease and improved diagnostics.

In 2005, a research done by Rangel et al. stated that among the 183 foodborne outbreaks reported from 49 states in 1982 to 2002, produce-associated outbreaks occupied 21% of the total. Some of the produce vehicles were lettuce, apple cider/ juice, salad, coleslaw, melons, sprouts, grapes, alfalfa or clover sprout (Rangel et al., 2005). The predominant transmission route of the outbreak mainly via food; therefore, prevention efforts focused on hygiene are needed to reduce transmission.

2.1.2 Genus *Escherichia*

The genus *Escherichia* belongs to the family Enterobacteriaceae in the class of Gammaproteobacteria of the phylum of Proteobacteria (Uniprot Consortium, 2013). Basically, members under Enterobacteriaceae are Gram-negative, aerobic and facultatively anaerobic, catalase-positive and oxidase-negative, non-spore forming, rod shaped bacteria typically 1.1 to 1.5 μm in diameter and 2.0 to 6.0 μm in length (Scheutz and Strockbine, 2007). They are motile by peritrichous flagella or non-motile (Balows et al., 1991). Besides, they have the ability to ferment most of sugar to produce lactic acid (Moder, 2008). Usually most of them reduce nitrates to nitrite and do not produce H2S (Moder, 2008).

There are several species under the Genus *Escherichia*: *E. albertii*, *E. blattae*, *E. coli*, *E. fergusonii*, *E. hermannii*, and *E. vulneris* (UniProt Consortium, 2013). Most of the *E. coli* strains are harmless to human and present naturally in the gut of endothems of warm-blooded animals (Scheutz and Strockbine, 2007). However, some of them are pathogenic with
virulence factors which can cause diarrheal disease even in healthy hosts (Nordqvist, 2007). Pathogenic *E. coli* are categorized into six pathogenic types: enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroaggregative (EAEC), enteroinvasive (EIEC), diffusely adherent (DAEC), and enterohemorrhagic (EHEC) (Feng, 2001).

### 2.1.3 General Characteristics

*Escherichia coli* is a common enteric bacteria which consistently inhabitant as normal flora in the digestive tract of all animals, including humans (Abramochkin, 2004). Most strains of *E. coli* are harmless and serve beneficial function in the body. For instance, *E. coli* compete and suppress the growth of harmful bacteria species that may be present or ingested with food and water and synthesize appreciable amount of vitamins (Feng, 2001). Besides, *E. coli* is also the dominant species found in feces so, it has been used as indicator to track for indirect evidence of fecal pollution and water contamination (Todar, 2012).

Physiologically, *E. coli* is versatile and well-adapted to its characteristic habitats. For instance, it can grow in media with glucose as the sole organic constituent; while, wild-type *E. coli* has no growth factor requirements but it is able to metabolically transform glucose into all of the macromolecular components that make up the cell (Todar, 2012). The optimal temperature of growth is 37.0 °C within a range of 7.2 to 45.5 °C and optimum pH is 6.0 to 8.0 (Snyder, 1999). Furthermore, the serology of *E. coli* is complex as the existed isolates are classified based on 173 somatic (O), 56 flagellar (H), and 80 capsular (K) antigens (Feng, 2001).
2.1.4 Pathogenesis

*Escherichia coli* with serotype O157:H7 is an enterohaemorrhagic strain (EHEC) which often implicated in infections worldwide (Feng, 2001). All EHEC strains produce Shiga toxin 1 (Stx 1) and/or Shiga toxin 2 (Stx 2) which also referred as verotoxin 1 (VT 1) and verotoxin 2 (VT 2) (Buchanan and Doyle, 1997). Therefore, EHEC sometimes also named as Shiga toxin-producing *E. coli* (STEC) or verocytotoxin-producing *E. coli* (VTEC).

The ability to produce both Shiga toxins was acquired from a bacteriophage, presumably directly or indirectly from Shigella through the activity of a lysogenic phage (Buchanan and Doyle, 1997). The toxin is a 70,000 dalton protein which composed of a five B subunits (7.7 kDal) that surrounding by an active A subunit (32 kDal) (Graf, 2010). After recognition and binding of the B subunit with a specific glycolipid receptor, Gb3 on renal endothelial cells, blood vessels, smooth muscle cells and red blood cells, this toxin is then transported into the cell (Buchanan and Doyle, 1997). This action will lead to cell death as it inhibits intracellular protein synthesis and red blood cells are damaged once they are exposed and alter the vasculature (Buchanan and Doyle, 1997). Hence, these toxin-producing strains are associated with two serious extraintestinal diseases known as hemolytic uremic syndrome and thrombotic thrombocytopenic purpura (Balows *et al.*, 1991).

The infective dose of EHEC O157:H7 in human is estimated to be very low and in the range of 10-100 cells (Feng, 2001). Before onset of the illness, the incubation period is usually 3 to 4 days and accompanied with symptoms including abdominal pain, stomach cramps, vomiting and watery diarrhea (Radhakrishnan, 2012). Haemorrhagic colitic (HC) infection is an acute abdominal cramp and bloody diarrhea which cause severe gastrointestinal disease by infecting the intestinal epithelial layer (Feng, 2001). This infection may develop to more severe complications such as hemolytic uremic syndrome (HUS) that is a type of kidney
failure and thrombotic thrombocytopenic purpura (TTP), related to neurological disorder (Tesh and O’Brien, 1991). Usually there is no fever but vomiting may occur (Snyder, 1999).

2.1.5 Occurrence of human outbreaks

Since 1982, E. coli O157:H7 has emerged as the major human pathogenic serotype of EHEC/VTEC in the United Kingdom and North America (Afza et al., 2006). Three of the foodborne illness outbreak is ranked in the top 10 list in the United States, there are Jack in the Box (1993), Sizzler (2000), and natural selection foods (2006).

The year 1993 was a catastrophic for Jack in the Box in Pacific Northwest due to the E. coli outbreak in the Pacific Northwest (Kivett, 2011). It was the biggest record outbreak and highly publicized in the United States with more than 700 people were affected and 4 died (Microbiological Safety of Food, 1995). It was reported that the patties of minced beef were contaminated with fecal matter and not cooked at 155 °F as mandated by Washington state law, but they were not the only food vehicle (Kivett, 2011).

In 1996, an outbreak of E. coli O157:H7 infection occurred among the schoolchildren in Sakai City, Osaka, Japan due to the consumption of white radish sprouts. From the infection, there were 9,451 cases and 12 deaths, representing 16 total outbreaks with the average of more than 10 patients each (7,900 patients) (Michino et al., 1999). In the outbreak, four children died and more than 700 people became sick. In 2000, the steakhouse franchises in Milwaukee, Wisconsin had experienced E. coli O157:H7 outbreak due to contamination of shipped raw meat from the Excel meat packing facility in Colorado, sickened 65 people and a three-year-old girl was killed (Kivett, 2011).

Another outbreak occurred in the late 2006, veggie eaters across America halted the consumption of spinach as a farm in San Benito County, California was suspected irrigation
with possibly cattle feces contamination, causing the contamination of the spinach fields (Kivett, 2011). It was reported a total number of 199 people infected in 26 states and out of the total number, 31 people suffered kidney failure and three died.

Infections by O157: H7 are most commonly caused by the consumption of undercooked, contaminated ground beef or beef product, but illness caused by the organism is transmitted through contaminated drinking water or recreational water, raw milk and person-person contact (Feng, 2001). Other foods like salad vegetables, fruits, alfalfa and radish sprouts, unpasteurized apple cider, mayonnaise, yogurt and salami have also been implicated in recent major outbreaks (Feng, 2001).

2.2 Occurrence and quantification

2.2.1 Standard plate count

To enumerate the population of bacterial in a sample, standard or viable plate count method and spectrophotometric analysis are the most widely used method. The two methods are indirect measurement of cell biomass. Standard plate count method reveals only viable cells in a sample, while spectrophotometric analysis is based on turbidity and indirectly measures all bacteria, both dead and alive (Reynolds and Farinha, 2005).

Initially, the standard plate count method involves series dilution with sterile saline or phosphate buffer diluent. The diluent must not harm the microbes and does not support their growth, so that the microbes do not grow during the analysis (Instructional Microbiology Website, 2013). Then, the diluted sample is plated out on an agar surface via pour plate or spread plate technique, so that the colony becomes visible and enables naked eyes to count (Midlands Technical College, 2012).

To be effective, the final plates in the series should on the average between 30 and 300
colonies (Reynolds and Farinha, 2005). The plate with fewer than 30 colonies is not acceptable for statistical reasons as the presence of a few contaminants will have a drastic effect on the final count. Likewise, if there are more than 300 colonies on the plate, it may result in overlapping colonies and imprecision in the count and designated as too numerous to count (TNTC). Each colony formed is assumed as the growth of bacteria, but not from an individual cell (Midlands Technical College, 2012). Hence, each colony is represented as colony forming unit (CFU) and measured with the formula shown below in CFUs/ml:

\[
\text{Cell density} = \text{The number of colonies} \times \text{Dilution factor of the plate counted}
\]

2.2.2 Most Probable Number (MPN) method

The most probable number (MPN) test is a method to estimate the concentration of viable microorganisms in a sample by means of replicate liquid broth growth in ten-fold dilutions (Sutton, 2010). The methodology of MPN technique required dilution and incubation of replicated cultures across several serial dilution steps (Kirk, 2013). While, the result is based on the observed positive growth response such as turbidity and gas formation in broth tubes (United States Department of Agriculture, 2008).

According to Sutton (2010), there are a few assumptions: microorganism in the sample are randomly distributed, the dilution of the sample through the dilution series is accurate, the microorganism are separated and do not affect each other, that is attract or repel and every tube whose inoculum has a single viable organism will result in visible growth. The advantage of the technique are the result on recovery microbial population is more uniform, mixed populations can be separated into individual colonies, it only measures viable organisms (Kirk, 2013).
2.2.3 Polymerase Chain Reaction (PCR) Detection

PCR was developed in 1987 by Kary Mullis and his associates which is a test tube system to amplify a specific DNA (target) sequence lying between known positions (flanks) on a double-stranded DNA molecule, by producing enormous amplification (identical copies) of a short DNA sequence from a single molecule of the initiated DNA (Redway, 2011).

The amplification process is mediated by a pair of short pieces of single-stranded oligonucleotide primers that, typically 20 to 30 nucleotides long which are designed to match to the segment of target DNA (Genetic Science Learning Center, 2013). Through complementary base pairing, the primers anneal to the flanking region of the target sequence using hydrogen bonding (Redway, 2011). The amplified product is known as an amplicon was undergone three steps, namely, denaturation, annealing and elongation.

Besides amplification, PCR assay is also used to identify bacteria by detecting specific genes of an organism. The size and charge of the amplicon is separated through gel electrophoresis and visualized via UV light after staining with ethidium bromide (Etbr). The use of multiplex PCR method, Apun et al. (2011) reported the isolates were tested for the presence of *Escherichia coli* O157:H7 strain in wildlife from disturbed habitats in Sarawak, Malaysia by targeting the *slt-I, slt-II, rfbE* and *fltCH7* genes. While, Jeshveen et al. (2012) established a protocol for the detection of the pathogen *E. coli* O157:H7 and *E. coli* virulence genes (*eaeA, rfbE, hly, stx1* and *stx2*) in a multiplex PCR protocol using six specific primer pairs. On the other hand, a diagnostic PCR assay was developed and validated in a collaborative trial for the detection of *Escherichia coli* O157 based on amplification of sequence of the *rfbE* O157 gene (Abdulmawjood et al., 2003). In this study, PCR assay was performed to identify enterohemorrhagic *E. coli* O157:H7 and detect the presence of four specific genes, using primers specific for the genes encoding Shiga toxins *stx1* and *stx2* (stx1
and stx2 gene), O157 antigen (rfbE gene) and H7 flagella (flicH7 gene) (Jeshveen et al., 2012).

2.2.4 Antibiotics Susceptibility

Antibiotic susceptibility is the level of sensitivity a bacterium has to a particular type of antibiotic (Smith, 2013). The goal of this test is to predict the success or failure of antibiotic therapy in vivo, which the outcome will be used as guide for antibiotic choice (Sachais, 2007).

One of the oldest approaches is the Kirby-Bauer test, also known as the disk diffusion method, which was first developed and refined in 1950s by W. Kirby and A. Bauer then, standardized by the World Health Organization in 1961 (Reynolds, 2011). Currently, Clinical Laboratory Standards Institute (CLSI) is responsible for through a global consensus process to ensure uniformity of technique and reproducibility of results (Hudzicki, 2009).

Although Kirby-Bauer method has been superseded by automated tests, it stills the method of choice in routine clinical laboratories. This is due to its suitability to test the majority of bacterial pathogens, including the common fastidious bacteria, versatility in the range of antimicrobial agents and require no special equipment (European Committee on Antimicrobial Susceptibility Testing, 2013). This test measures the resistance or sensitivity of aerobes or facultative anaerobes to specific antibiotics, antimicrobial agents, or even herbal extracts by relying on the absence or presence of a clear zone surrounding the impregnated disk (zone of inhibition) (Reynolds, 2011). The diameter of the zone of inhibition corresponds to the degree of susceptibility of the microorganism to the antimicrobial agent, which can then be used by the clinician for treatment of patients with bacterial infection (Biomedical Research and Support Services, 2013).

To ensure standardization and reproducibility, the culture medium used has to be Mueller-Hilton (MH) agar, which is very high in protein and gives satisfactory growth of most
non-fastidious pathogen (Lalitha, 2004). For fastidious organisms, MH agar requires to supplement with additional nutrients or procedural modification (Hudzicki, 2009). Furthermore, the inoculum density must be standardized using McFarland standards. A 0.5 McFarland standard is applied to prepare the suspension at a standard concentration that approximately 1 to $2 \times 10^8 \text{CFU/ml}$ of bacteria inocula (CLSI, 2011).

Lastly, the raw data are interpreted based on the criteria set by CLSI. From the results, the organism will be reported as being susceptible (S), intermediate (I), and resistance (R). According to CLSI (2011), susceptible is defined as a category that implies that isolates are inhibited by the usually achievable concentration of antimicrobial agent when the dosage recommended to treat the site of infection is used, while intermediate category implies clinical efficacy in body sites where the drugs are physiologically concentrated or higher than normal dosage of a drug can be used. Susceptible implies that an infection due to the organism may be treated with the concentration of antimicrobial agent used, unless otherwise contraindicated (CLSI, 2011).
CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial Strains

*Escherichia coli* O157:H7 strain EDL 933 was used in this study as a positive control in PCR assays. The strains were stored at -20 °C in Luria Bertani (LB) broth containing 25% glycerol. Pure cultures of *E. coli* were grown at 37°C for 24 hours in LB broth and the DNA was extracted to obtain the positive control.

3.2 Sample collection

A total of 51 samples from 11 types of vegetables were collected from two farms (Farm A and Farm B) in Kuching, Sarawak. Farm A is located at Serian while Farm B is at Matang area. Each type of vegetables had a triplicate which was randomly picked at farm in Kuching between February and April 2013. Each farm was visited All samples were kept in zip-lock bag and stored in an ice box for transportation, which then were analyzed immediately at the Microbiology Laboratory, UNIMAS. The types of raw conventional vegetable samples are as shown in Table 3.1.

<table>
<thead>
<tr>
<th>Source</th>
<th>Type of vegetable</th>
<th>Local name</th>
<th>Scientific name</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm A</td>
<td>Chinese flowering green</td>
<td><em>Choy Sum</em></td>
<td><em>Brassica chinensis</em></td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Chinese white stem</td>
<td><em>Pak Choy</em></td>
<td><em>Brassica campestris chinensis</em></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Water spinach</td>
<td>Kangkong</td>
<td><em>Ipomea aquatica</em></td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Spinach</td>
<td>Bayam</td>
<td><em>Amaranthus spp.</em></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Chinese broccoli</td>
<td><em>Kailan/ Sawi Bunga</em></td>
<td><em>Brassica rapa var parachinensis</em></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Chinese chives</td>
<td>Ku Choy</td>
<td><em>Allium tuberosum</em></td>
<td>3</td>
</tr>
</tbody>
</table>
3.3 Detection and enumeration of *E. coli* O157:H7

The analytical method performed in this study was as described by Chang *et al.* (2013). Twenty five gram of sample was placed in a stomacher bag and 225 ml of enrichment broth, Luria Broth (LB) was added. The sample was homogenized in a stomacher for 60 s and incubated at 37 °C for 24 hours. Tenfold and hundredfold dilutions of the stomacher fluids were prepared. Hundred microlitres of dilution 10⁻³ to 10⁻⁷ of the stomacher fluid was directly plated onto EMB (Eosin Methylene Blue) agar and incubated for 24 hours at 37 °C.

For MPN enumeration, 1 ml of the aliquot from the first three dilution was transferred into triplicate MPN tubes containing 9 ml of Luria Broth and then, incubated at 37 °C for 24 hours. Turbid MPN tubes are considered positive, which were then subjected to DNA extraction followed by PCR assay for the detection of *stx* 1, *stx* 2, *rfb* E and *flicH₁* genes for *E. coli* O157:H7.

3.4 DNA extraction using boiled cell method

DNA extraction was carried out using boiled cell method as according to Apun *et al.* (2010). One milliliter of sample of each broth was subjected to centrifugation at 13,000 rpm for 5 minutes to pellet the cellular debris. The supernatant was discarded and the cell pellet was