OCCURRENCES OF ESCHERICHIA COLI IN ORGANIC VEGETABLE FARM AND THE SURVIVABILITY IN ACIDIC SOIL.

Law Cheh Tat

Bachelor of Science with Honours (Resource Biotechnology) 2014
OCCURRENCES OF Escherichia Coli IN ORGANIC VEGETABLE FARM AND THE SURVIVABILITY IN ACIDIC SOIL.

This project is submitted in partial fulfillment of the requirement for the degree of Bachelor of Science with Honours

Faculty Resource Science and Technology
(Resource Biotechnology)
2014
ACKNOWLEDGEMENT

First of all, I would like to acknowledgement to my supervisor, Professor Kasing Apun, co-supervisor Dr. Lesley Maurice Bilung, for their keen guidance and advices thought out my write-up proposal and this thesis. Besides that, I would like to deepest gratitude towards post-graduate students in Microbiology Lab, UNIMAS, for their guidance and appropriate practices in order to conduct my experiment. In addition I would like to thanks my course mates Ms Grace June, Ms Cecilia for helping finish up my experiment. I would like to thank Mr. George Lim from N & N farm Sdn. Bhd for their kind hospitality in supporting my experiment and also provide guidance tour in his farm. Last but not least, I would like to thank my family for supporting me in all my studies and financial support.
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<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>HUS</td>
<td>Hemolytic Uremic Syndrome</td>
</tr>
<tr>
<td>STEC</td>
<td>Shiga-toxin producing Escherichia coli</td>
</tr>
<tr>
<td>CDC</td>
<td>Centre for Disease Control and Prevention</td>
</tr>
<tr>
<td>Stx</td>
<td>Shiga toxin</td>
</tr>
<tr>
<td>SOM</td>
<td>Malaysian Organic Scheme</td>
</tr>
<tr>
<td>LB</td>
<td>Luria- Bertani</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
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<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
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<tr>
<td>EMB</td>
<td>Eosin Methylene Blue</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>dNTP</td>
<td>deoxynucleoside triphosphate</td>
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<td>Agarose Gel Electrophoresis</td>
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<td>μL</td>
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<td>ml</td>
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<td>Kg</td>
<td>Kilograms</td>
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<tr>
<td>HCL</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>bp</td>
<td>Base-Pair</td>
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<tr>
<td>OD</td>
<td>Optical Density</td>
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<tr>
<td>TBE</td>
<td>Tris-Borate EDTA</td>
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<tr>
<td>mA</td>
<td>Amperes</td>
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<td>Etbr</td>
<td>Ethidium Bromide</td>
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Occurrences of *Escherichia coli* in Organic Vegetable Farm and the Survivability in Acidic Soil.

Law Cheh Tat (30716)
Resource Biotechnology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

*Escherichia coli* (*E. coli*) is a common microbe that can survive in soil environment. However in acidic environment, *E. coli* adaptation especially pathogenic *E. coli* O157:H7 in the soil was not well studied. This study was carried out to access the occurrence of *E. coli* in organic farm and determine the acidic tolerance of *E. coli* in soil, by using analysis of direct count CFU. Besides that, multiplex PCR was also used to determine the present of *E. coli* O157:H7 strain from the farm. From the result obtained, 6 out of 30 samples showed positive present of *E. coli* in the soil sample. For soil acidic test, *E. coli* growth in different pH showed a total population of $10^7$ CFU/ml for 7 days in pH 4 to 7. Furthermore, result from this study can helps to improve the soil condition in organic farm which indirectly showed free of *E. coli* O157:H7.

**Key words:** *Escherichia coli*, Multiplex PCR, organic farm, soil acidic

ABSTRAK

*Escherichia coli* (*E. coli*) adalah mikrob biasa yang boleh hidup dalam persekitaran tanah. Walau bagaimanapun dalam persekitaran berasid, *E. coli* dalam tanah belum kajian dan sepenuh memahami, terutamanya patogen *E. coli* O157: H7. Kajian dijalankan untuk mengakses berlakunya *E. coli* di dalam ladang organik dan menentukan toleransi berasid *E. coli* dalam tanah, analisis menggunakan kiraan CFU di atas EMB agar. Di samping itu, multiplex PCR juga digunakan untuk mengenalpasti *E. coli* O157: H7 strain dari lading. Berdasarkan keputusan yang diperolehi, 6 daripada 30 sampel menunjukkan positif *E. coli* dalam sampel tanah. Bagi tanah ujian berasid, *E. coli* pertumbuhan dalam pH yang berbeza menunjukkan penduduk berjumlah $10^7$ CFU/ml selama 7 hari dalam pH antara 4 hingga 7. Tambahan pula, hasil dari kajian ini boleh membantu untuk memperbaiki keadaan tanah di ladang organik yang secara tidak langsung menunjukkan bebas daripada *E. coli* O157: H7.

**Kata kunci:** *Escherichia coli*, Multiplex PCR, ladang organik, tanah berasid
1.0 INTRODUCTION

*Escherichia coli (E. coli)* is a common microbe found in stool of warm blooded animals; it colonises in the gastrointestinal tract and gives mutual benefit between bacteria and host (Nataro & Kaper, 1998). Hence, the occurrence of *E. coli* in water, food and soil has commonly been used as indicator microorganism for fecal contamination, and prediction on the presence of Shigella or Salmonella species. Fecal contamination is a major concern to organic agriculture as manure is used as fertilizer for farming. Bacteria or virus from manure can infect the livestock, which subsequently may also affect the human health. Some *E. coli* are harmless towards the host, while some *E. coli* are pathogenic towards the host such as *E. coli* serotype O157:H7, its virulence gene has the ability to produce verotoxin that causes disease such as watery diarrhoea, bloody diarrhoea which known as hemolytic uremic syndrome (HUS), hemolytic anemia and thrombocytopenia (Narato & Kaper, 1998; Gyles, 2006; Sahilah et al., 2010).

*E. coli* is native bacteria found in tropical soil and may not be abundant in soil. The number of *E. coli* can vary from 0% to 100%, because the bacteria need to attach to small particle such as floc to be maintained in soil environment. Besides that, the abundance of *E. coli* in soil and its patchiness in soil distribution was reported by Byappanahalli *et al* (2006). According to Tale (1978), *E. coli* has three times greater survival time in manure soil than sand, and capable to survive inside sterile manure after 10 days of incubation. The organic matter as nutrient source for *E. coli* in manure soil are higher than sand, therefore farming activity in organic farm need practise in precaution. Farming activity in the farm turned soil become acidic because ammonia content in manure decreased, and bacteria to degrade organic
matter producing acid over times. *E. coli* often encounter various environments, such as acidic condition, nutrient depletion condition, different temperature condition, osmotic pressure (Lee et al., 2012). In order to understand the stress tolerance of *E. coli* in acidic soil condition, this research was carried out in organic farm in Siburan Kuching, Sarawak. In addition, the survivability of *E. coli* in acid condition was tested in laboratory. Studies showed by Wang and Suo (2011), *E. coli* O157:H7 strain produces type I and type II Shiga toxin (Stx) that cause food poisoning in food industry. Therefore multiplex PCR was used to detect the Stx gene, O antigen and H antigen in *E. coli* O157:H7. Standard plate count was used to calculate the number of the *E. coli* found in soil.

The objectives of this research are to:-

1. detect the occurrence of *E. coli* present in organic farm soil.

2. evaluate *E. coli* survival in different soil acidity.

3. detect the pathogenic strain *E. coli* O157:H7 in organic farm soil.

The aim of the study is to improve information of *E. coli* survival in organic farm soil, and better understand the survivability and behaviour of *E. coli* in acidic soil.

1.1 Research Hypothesis

It is predicted that the number of *E. coli* found in organic farms are usually high because of the manure used in organic farms. Increase acidic in soil may decrease the survival of *E. coli* in soil.
1.2 Research Question/Research Problem

Will there been a detection of *E. coli* O157:H7 in farms, which is associated with the public safety and health concern? It is always concern that vegetable which eaten raw, are often infected with food poisoning *E. coli* O157:H7 that can cause hemolytic uremic syndrome (HUS), hemolytic anemia and thrombocytopenia (Narato & Kaper, 1998). Therefore, detection of *E. coli* O157:H7 is important in the organic farm, *E. coli* O157:H7 are found in the organic farms, then the risks of exposing pathogenic to human can be reduce and prevent any outbreaks of the disease. Besides that, increase in acidic level which may be caused by organic matter in the soil or other influence are used to study the survival of *E. coli*. There might be a relationship of acidic soil affecting bacteria growth, which helps to better understanding the growth pattern of *E. coli* in soil.
2.0 LITERATURE REVIEW

2.1 Identification and Morphology of *Escherichia coli*

*Escherichia coli* (*E. coli*) is a gram negative bacteria, non endospore-forming, motile and facultative anaerobic bacteria. It is rod shaped with the size of $2.0 \times 0.5 \mu m$ under optical microscope. It belongs to family Enterobacteriaceae and genus Escherichia. When plated on Eosin Methylene Blue (EMB) agar, *E. coli* will show dark colonies with metallic green sheen (Lai & Cheephmam, 2007).

*E. coli* were usually found in intestinal tracts of warm-blooded animals, it was also used as indicator microorganism for fecal contamination in water and soil (Nataro & Kapper, 1998). *E. coli* experience biphasic lifestyle, which can be host-associated in the human intestinal tracts or host independent in soil or water environment (Elsas et al. 2011). In organic agriculture, application of using animal manure as fertilizer was encouraged in Sarawak, as it believe to be more environmental friendly and health to consume. But some pathogenic *E. coli* O157:H7 survive in organic farm soil and maybe hazardous toward customer produce verotoxin to causes diarrhoea, hemolytic uremic syndrome (HUS), thrombocytopenia, haemolytic anemia (Gyles, 2006; Sahilah, 2010; Zhang et al, 2013).

2.2 Survivability of *Escherichia coli* in Soil

Several reports shown that *E. coli* were found more ubiquitous in tropical and subtropical soils (Desmarais et al., 2002; Ishii et al., 2006). In soil, there were several of stress that affecting the survivability of the *E. coli* such as temperature, moisture, variation of soil texture, and
organic matter content, the pH of the soil, and predation (Zhang et al., 2013). Experiment showed by researches that E. coli were found more persistent in silty clay soils, which can survive up to 231 days at 25°C in manure soil (Jiang et al., 2002). Besides that, study by Ling et al. (2009) showed the persistence of E. coli in soil decreased after stimulated rain. This was because the bacteria were able to attach to particles such as floc in the soil that enabled the bacteria to persist at 8% from total population in soil (Muirhead et al., 2005). Furthermore researcher showed that native organisms in soil were also affecting the E. coli survivability (Yao et al., 2013). Therefore the survivability of E. coli in organic agriculture soil maybe favourable as the environment in Kuching, Sarawak is tropical. But the native bacteria inside the soil and other bacteria such as campylobacter species and Salmonella species were also found in manure soil, this different species of bacteria could be affecting E. coli survival inside the soil. In China Jiangsu, there had report of finding E. coli O157:H7 in soil, which was hazardous towards human health (Zhang et al., 2013).

2.3 Food-Borne Related to Escherichia Coli

In agriculture soil, manure was used as fertilizer in farms which posed a serious threat regarding to E. coli O157:H7 towards human. Manure was made up of animals waste or plant waste that used to fertilize the land. There was review showed, E. coli O157:H7 able to survive in manure, soil and water for a period of time (Elsas et al., 2011). E. coli O157:H7 is known as Shiga-toxin producing Escherichia coli (STEC) are an example of the toxin producing bacteria that not only caused infections to human and also caused food-borne outbreaks. According to Elsas et al. (2011), pathogenic E. coli posed a threat in food chain as
it can be found in soil environment, as previously there was an outbreak of fresh spinach that contained *E. coli* O157: H7. Some strain of *E. coli* can produce filamentous structure which helps to attach on the surface of soil and plants, it can colonise the soil, water, plant and the internal plant also can be colonised (Elsas *et al.*, 2011). In organic farm, improper compost of manure could be a good source of pathogen contamination during harvesting and pre-harvest. According to Centre for Disease Control and Prevention (CDC), there had been 73,000 disease related to *E. coli* O157:H7 with 2,000 hospitalize and 60 deaths per year in USA. According to epidemiology study, it was reported that raw vegetable such as lettuce, salad, coleslaw and sprouts were associated with the outbreaks.

2.4 Genotype and Virulence Genes in Shiga-toxin *E. coli*

2.4.1 Stx Gene

Shiga toxin (*Stx*) was found in *E. coli* O157:H7 which caused diarrhoea and hemolytic uremic syndrome (HUS), and *Stx* gene was believed to be the virulence factor of *E. coli* O157:H7. According to Zhang *et al.* (2002), there were two type of toxin, *Stx*1 and *Stx*2, the structural gene of these two toxins showed about 55% similar nucleotide sequences, but *Stx*2 gene showed more heterogeneous compared to *Stx*1. In other bacteria such as bacteriophages H30, H19B, and 933J had identical nucleotide sequences in *Stx*1, even *Shigella dysenteriae* type 1 had one or two amino acid different (Zhang *et al.*, 2002). There were several variants on *Stx* 2 gene, including *Stx* 2c, *Stx* 2d, *Stx* 2e, and *Stx* 2f, these different *Stx*2 alleles were important, as it can help the physician to understand the HUS mechanism in STEC infection (Friedrich *et al.*, 2002).
2.4.2 rfbE Gene

O antigen that was expressed on lipopolysaccharide can be found on *E. coli* O157:H7 caused blood diarrhoea, hemorrhagic colitis and HUS. These *rfb* genes consisted of 12 genes which encoded for O antigen. In total of 12 genes, the fifth gene *rfbE* gene was specific for *E. coli* O157:H7 (Wang & Reeves, 1998; Abdulmawjood *et al*., 2003).

2.5 Acidic Environment Condition for *E. Coli*

Soil environment in farm often turned into acidic because of farming activity that caused the depletion of nitrogen. Excessive raining can cause the leaching of cations. All bacteria including *E. coli* O157:H7 had the ability to respond to pH decrease in nature and expressed their defence mechanism to protect themselves (Bearson *et al*., 1996). Acid was defined as a substance that produced hydrogen ions in liquid and was a proton donor, or in environment acid was cause by biological compound that released hydrogen ions from fermentation or weak acidic waste product in the faces. According to Gorden and Small (1993), Shiga-toxin *E. coli* were good acid tolerant as compared to non pathogenic *E. coli* K12. In different growth phase in *E. coli* showed different acid tolerances. Meanwhile, a study by Lin *et al.* (1996) showed that in stationary phase, *E. coli* was more tolerance to acid compared to lag phase or exponential phase. This was because acid applied on *E. coli* was a stress tolerance but not an adaptation process; it was the gene expression of acid resistance systems. If the acid was from pollution environment, the acid can disrupt the biochemical process inside the bacteria, causing fast proton leakage. If proton loss faster, it eventually caused disruption of cell membrane and death to the bacteria (Bearson *et al*., 1996; Chun *et al*., 2006). Even though three acid resistance glucose catabolise systems, glutamate decarboxylase and arginine
decarboxylase had been tested on *E. coli* O157:H7, none of these encouraged acid resistance, these suggested that were other possible mechanism yet to be discovered (Lin et al., 1996; Chun et al., 2006; Elsas et al., 2011). Research done by Zhang et al. (2013) on survival of *E. coli* O157:H7 in acidic soil showed that *E. coli* O157:H7 were not able to survive in acidic soils of pH 4 and 5 after 3 day of incubation. Meanwhile in soil pH 6, the bacteria were able to survive up to 10 day.
3.0 MATERIALS and METHODS

3.1 Sample Collection

A total of 30 soil samples were collected from an organic farm located at 14th mile Siburan, Kuching Sarawak, located at 1°21'03.0"N 110°25'42.5"E. The organic farm was certified by Malaysian Organic Scheme (SOM). The sample was collected using sterile soil shovel at 3 different locations described in Figure 1, where the first sampling site was at the entrance of the farm, and two sampling sites were in the middle of the farm, samples were taken beside the net farm with the same sampling position described in Figure 1. The samplings were proceeded at 0830 on 6 February 2014, 19 February 2014 and 3 March 2014. A total of 5 of samples collected with duplication at 20 grams each, kept in sterile plastic bag labelled and transported in an ice box to the laboratory. The analysis was conducted within 2 hours upon collection samples.

3.2 Sample Processing

3.2.1 Sample Enrichment

After the collection from the farms, two grams of soil was measured using electronic balance and enriched into 18ml of Luria- Bertani (LB) broth in universal bottle at 135 rpm shaking incubator for 24 hours.
3.2.2 Serial Dilution

After 24 hour of incubation, the incubated sample mixture was subjected to ten-fold serial dilutions. One ml of the sample mixture was diluted with 9 ml of sterile Phosphate Buffer Saline (PBS) at serial dilution at factor $10^{-1}$. Then, one ml of the aliquot from dilution $10^{-1}$ was transferred to second serial dilution factor of $10^{-2}$. The same procedures were carried until dilution factor of $10^{-6}$.

3.2.3 Colony Forming Unit (CFU)

Meanwhile, 100 μl of aliquot from serial dilution $10^{-3}$ until $10^{-6}$ (described in serial dilution) were plated on Eosin Methylene Blue (EMB) agar. The plates were incubated at $37^0\text{C}$ for 16
hours. After incubation, the bacteria detected in EMB plate contained 30 to 300 colonies were selected and calculated using serial dilution protocol described by Reynolds (2005).

\[
\frac{\text{No of Colonies}}{\text{Dilution Factor} \times \text{Volume plated on Media}} = \text{Number of Bacteria (CFU/mL)}
\]

3.2.4 *Escherichia coli* Identification

EMB agar was used as selective media for *E. coli*, and dark colonies with green metallic sheen were assumed to be *E. coli* (Oxoid Microbiology Products). Then a gram staining was performed and viewed under optical microscope to identify the morphology of *E. coli*.

3.3 pH of the Soil

The soil from the farm was tested using glass electrode method (CyberScan pH 510), 10 grams of soil was mix with 20 ml of distilled water until the soil were completely dissolved, then allowed the mixture to stand for 5 minute and swing gently. Then calibrated pH electrode was inserted into soil slurry and waited for the stabilized reading of pH. The pH reading for every soil samples were recorded and measured.

3.4 Growth of *E. coli* in LB broth

Growth of *E. coli* in LB broth Luria-Bertani was tested as described by Sezonov et al. (2007) with modification of volume of LB broth used and incubator method. A single colony of *E.
coli was inoculated in 100 ml of LB broth in scotch bottle, and incubated in 37°C at 150 rpm shaking incubator. 2 mL of the aliquot were withdrawn and tested using spectrophotometer at wavelength 600 nm in 6 hour and 1 mL of aliquot were withdrawn out and put into 9 ml of PBS buffer at 10⁻¹ diluted and serial dilution up to 10⁻⁶. Then withdrawn 0.1 ml of the mixture, plated on EMB and perform CFU count as described. The steps were repeated at 10 hours, and 16 hours.

3.4 Soil Acidic Test

The bacteria soil test was performed using method described by Ishii et al. (2006) with modification of using 5M of hydrochloric acids (HCl) to adjust the pH of the soil and used sterile soil. First, 1.5 kg of soil was collected from organic farms weighed and autoclaved 3 times at 121°C, 20 atm. Then the soil was tested by plating on selective EMB agar to ensure the soil showed absence of E. coli. Next, 100 mL of E. coli growth in LB broth for 16 hours was centrifuged at 8000 rpm at 4°C and washed three times with PBS buffer. Resuspended it in 100 mL of sterile distilled water and poured on sterile soil prepared. The soil was mixed continuously for 5 minute and distributed into 9 sterile plastic bags. Triplicate bags of soil were adjusted to pH range 4.0± 0.2, another triplicates were adjusted to pH range 5.2± 0.3. Meanwhile duplicate of soil were adjusted to pH range 6.3± 2, and 1 soil was used for control of the experiment with original pH 7.8. The pH of the soil and the bacteria survivability count was performed from day 1, 2, 4, 6, 7 by direct plated count using serial dilution up to 10⁻⁶.