DETECTION OF BACTERIA FROM LOCAL VEGETABLES BY PCR-RFLP OF MITOCONDRIAL DNA (mtDNA) 16S rRNA

by

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
mtDNA	Mitochondrial Deoxyribonucleic acid
TBE	Tris borate ethylenediaminetetraacetic acid
Na_2EDTA	Sodium ethylenediaminetetraacetic acid
ddH_2O	Sterile deionized water
NCBI	National Centre for Biotechnology Information
Ver.	Version
HMW	High molecular weight
UV	Ultra violate
PCR	Polymerase chain reaction
G	Guanine
C	Cytosine
A	Adenine
T	Thymine
Tm	Melting temperature
bp	Base pairs
kbp	Kilo base pairs

dNTPs	Deoxynucleotide triphosphates
PCR-RFLP	Polymerase chain reaction-Restriction fragment length polymorphism
WHO	World Health Organization
FAO	Food and Agriculture Organization of the United Nation
USDA	United State Department of Agriculture
EFSA	European Food Safety Authority

LIST OF SYMBOLS

%	Percent
°C	degree Celcius
x g	Gravity force (G-Force)
g	gram
mL	milliliter
µL	microliter
mg/mL	milligram per milliliter
ng/ µL	nanogram per microliter
U/ µL	units per microliter
pmol	picomole
ppm	part per million
V	Volts

ABSTRAK

Penyakit bawaan makanan adalah salah satu masalah kesihatan yang semakin meningkat naik bukan sahaja di Malaysia malah di seluruh dunia. Antara penyebab utama penyakit bawaan makanan adalah mikroorganisma, bahan kimia dan bio-toksin yang terdapat dalam sayur-sayuran mentah atau yang tidak dimasak dengan sempurna. Dalam kajian ini, sepuluh jenis sayur-sayuran tempatan iaitu bayam, kangkung sawi manis, kailan, kubis, tomato, ubi keledek, terung, kacang panjang dan cili telah dipilih untuk mengenalpasti bakteria melalui proses *PCR-RFLP* ke atas gen 16S rRNA mitokondria. Bagi menjalankan proses ini, primer baharu direka berdasarkan gen 16S rRNA mitokondria bakteria yang dipilih, seperti *Pseudomonas syringae*, *Pseudomonas protegens*, *Listeria monocytogenes*, *Salmonella enterica*, *Erwinia amylovora*, *Erwinia carotovora*, *Bacillus cereus*, *Bacillus subtilis*, *Bacillus licheniformis* dan *Escherichia coli*. Urutan separa gen 16S rRNA digandakan melalui PCR dan dicernakan menggunakan enzim pembatasan endonuklase iaitu *Alu* I, *Hha* I, *Hae* III dan *Eco*R I. Pengenalpastian spesies bakteria ditentukan berdasarkan variasi corak khusus yang dihasilkan daripada produk yang dicerna.

ABSTRACT

Foodborne disease is one of the growing public health problems occurred worldwide including Malaysia. The common causes of foodborne disease are due to microorganisms, chemical and bio-toxin which are found in raw or lightly cooked vegetables. In this study, ten local vegetables, namely spinach (bayam), water spinach (kangkong), white flowering cabbage (sawi manis), chinese broccoli (kailan), cabbage (kobis), tomato (tomato), sweet potato (keledek), eggplant (terung), long beans (kacang panjang) and chili (cili) were selected for the identification of bacteria through PCR-RFLP of mitochondrial 16S rRNA gene. A new primer was designed based on mitochondrial 16S rRNA gene sequences of selected bacteria, such as *Pseudomonas syringae*, *Pseudomonas protegens*, *Listeria monocytogenes*, *Salmonella enterica*, *Erwinia amylovora*, *Erwinia carotovora*, *Bacillus cereus*, *Bacillus subtilis*, *Bacillus licheniformis* and *Escherichia coli*. A partial sequence of 16S RrNA gene was PCR amplified and digested by endonuclease enzymes *Alu* I, *Hha* I, *Hae* III and *Eco*R I. Identification of bacteria was assigned based on species-specific variation pattern produced from digested product.

CHAPTER 1: INTRODUCTIONS

1.1 Background of the study

Vegetables serve as a major part of our food supply. Raw vegetables and fruits are known to harbor a number of bacterial populations due to close contact with soil surface during growth process (Rastogi *et al.*, 2012; Tournas, 2005). Common daily vegetables used including salad, cucumber, pepper, tomatoes, onions, red onions, carrots, lettuce, spring onions and radishes and other green leafy or non-leafy vegetables. Vegetables are known to be rich in vitamins, iron, calcium, proteins, fats and minerals, dietary fibers and other nutrients including flavonoids, carotenoids and phenolic compounds that may be useful for health purposes (Osamwonyi *et al.*, 2013; Abdullahi *et al.*, 2010).

Most microorganisms identified on whole fruits and vegetables are soil inhabitants, which are the microbes that responsible for maintaining ecological balance in agricultural systems. These microbes are introduced to the crop on the seed itself, during its growth, during harvesting and postharvest handling, or during storage and distribution (Barth *et al.*, 2009). In addition, the processing of fresh vegetables may alter or increase the number and type of pathogens present on the surface of the product. As reported by Tournas (2005), the properties of vegetables such as high water activity and nearly neutral pH make them susceptible to a wide range of spoilage organisms like bacteria and fungi.

Vegetables have been associated with the outbreaks of food borne disease in many countries including Malaysia. World Health Organization (WHO) defines foodborne diseases as those conditions that are commonly transmitted through ingested food (World Health Organization, 2007). While Centre of Communicable Disease (CDC) defined foodborne disease as any illness resulting from the consumption of foods or beverages contaminated with one or more disease producing agents such as bacteria, parasites, viruses, fungi and their products as well as toxic substances not of microbial origin (National Institute of Diabetes and Digestive and Kidney Diseases, 2016). Cultivation of vegetables may largely account for such pathogenic contamination. The used of manures in many agricultures sectors to promote growth crops and vegetables contain a large number of pathogenic microorganisms including *Salmonella spp.*, *Escherichia coli* O157:H7, *Bacillus anthracis*, *Mycobacterium* spp., *Brucella* spp., *Listeria monocytogenes*, *Yersinia enterocolitica*, *Clostridium perfringens* and *Klebsiella* spp. (Eni *et al.*, 2010).

Malaysia is one of the countries that have high cases of foodborne diseases due to the suitable temperature and condition for the growth of most bacteria (Abdul-Mutalib *et al.*, 2015). Microorganism, chemical and biotoxin are the most common causing agent of the foodborne diseases reported in Malaysia (Sharifa Ezat *et al.*, 2013). The eating practice of raw or lightly cooked food is not good for health since improperly cooked may have a living pathogen. *Salmonella* and *Escherichia coli* O157:H7 are the major pathogens contributing to outbreaks of foodborne illnesses associated with fresh produced (Buck *et al.*, 2003; FDA, 1998). The common symptoms associated with foodborne illness are diarrhoea accompanied by nausea and vomiting (Abdul-Mutalib *et al.*, 2015). As reported by Sharifa Ezat *et al.* (2013) and Ministry of Health (2014),

cholera, typhoid, hepatitis A and dysentery are the common diseases due to food poisoning. According to World Health Organization (2014), the mortality rate due to foodborne illness is 3% and as much 30% of the global population reported to have experienced foodborne diseases annually.

There is no specific method recommended for recovering bacteria from vegetable samples. Study by Kiminos *et al.* (1972), to recover *Pseudomonas aeruginosa* from vegetables, they homogenized all the whole vegetables with sterile distilled water and plated onto cetrimide agar at 42 °C for 24/48 hours. The method used is known as bacteria cultivation. Cultivating is one of the most important techniques in diagnostic microbiology. Culture based method have many benefits such as able to quantify the viable bacteria in a sample, distinguishing the bacteria based on their colonial morphological and provide a pure colony culture for further analysis. However, the major difficulties of culture based method include require more time for bacteria growth which significantly may affect the result produce and also low recovery of target organisms (Kapperud *et al.*, 1990)

Invention of the polymerase chain reaction in 1986 by Kary Mullis had revamped the application of molecular biology in many fields including identification of bacteria. The benefits of molecular methods are less costly, more accessible, high sensitivity and specificity and have ability to identify the organisms down to strain level. There are many methods suitable for bacteria identification include random amplified polymorphic DNA analysis (RAPD), pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP) and DNA sequencing. Recently, next generation sequencing

(NGS) was suggested as a suitable method for identification of organisms from environmental.

1.2 Significance of the study

Vegetables are food that usually consumed by individual either as a raw or cooked product. Although vegetables have beneficial towards human health, but many studies have shown that the intake of raw or improperly cooked vegetables is not good for health since it may introduce new pathogens to a human body. Traditionally, based on culture method, the identification of bacteria was made through colonial morphology. This method has many drawbacks that significantly affect the result produced. Many studies have shown that the application of molecular methods in the identification of the organism from vegetable samples increased sensitivity and specificity as well less labor usage and also cheap. In addition, the selection of DNA markers also plays an important factor in successful of the identification based on molecular methods. The current study uses mitochondrial DNA ribosomal marker 16S rRNA gene as an alternative marker for bacteria identification. Therefore, the aim of the study is to identify the presence of bacteria on vegetables based on PCR-RFLP of 16S rRNA gene.

1.3 Objective of the study

General Objective:

To determine the presence of bacteria from vegetable samples through mitochondrial DNA marker.

Specific objectives:

1. To extract genomic DNA from selected vegetable samples.
2. To design 16S rRNA gene universal primer based on selected bacteria.
3. To perform PCR amplification of extracted DNA samples using 16S rRNA universal primer.
4. To assign the presence of bacteria based on variation profiles produced by PCR RFLP.

CHAPTER 2: LITERATURE REVIEW

2.1 Definition of vegetables

Vegetables can be defined as any plant, part of which is used for food that is not a fruit or seed, but including mature fruits that are eaten as part of a main meal (Sinha *et al.*, 2010; Vainio and Bianchini, 2003). Besides that, mushroom and edible fungi including seaweed although not classified as green plants, also treated as vegetables.

2.2 Benefits of vegetables

Vegetables play an important component of a healthy diet. According to the Dietary Guidelines for American 2010 (USDA, 2010), they suggested one half of plate should consist of fruits and vegetables. Different organizations such as WHO, FAO, USDA and EFSA also recommended and promoted the use of fruits and vegetables as health food (Mohammad *et al.*, 2012). Vegetables as a diverse group of plant foods greatly vary in contents of energy, nutrient, vitamins, minerals and fibers. It has been reported that the fibers intake is linked to lower incidence of cardiovascular disease and obesity (Slavin, and Lloyd, 2012). The vitamins and minerals in vegetables are the sources of phytochemicals that function as antioxidants, phytoestrogens and anti-inflammatory, which are important for human health to improve human well-being (Adjrah *et al.*, 2013).

According to World Health Report (2002), 2.7 million lives could be saved annually if the consumption of vegetables and fruits are sufficiently intake. As minimum of 400 g of fruit and vegetables are required per day for prevention of chronic diseases such as heart disease, cancer, diabetes and obesity especially in third developed country (World Health Report, 2002). Therefore, intake of vegetables is an important way to optimize nutrition to reduce disease risk and maximize good health.

Historically, the consumption of certain plant foods, fruits, vegetables, and legumes was thought to prevent or curve ailments ranging from headaches to heart disease (Steinmetz and Potter, 1996). Early medicine revolved around the prescription of specific foods for certain disorders. In 18th century, vegetarian diets have promoted by individuals who search for physical and spiritual health (Roe, 1968). Based on vegetarian theorists of the ancient philosopher Pythagoras, they believed that diet should be part of an ascetic lifestyle and it was a symbolic of a commitment to health and social reform.

2.3 Contamination sources of vegetables

Fresh vegetables can be a vehicle for transmission of bacterial, parasitic and viral pathogens which is capable of causing human illness. It has been reported that raw vegetables is harboring potential food borne pathogens such as *Escherichia coli*, *Listeria monocytogenes*, *Sallmonella* from raw vegetables (Chaturvedi *et al.*, 2013). Contamination of vegetables with pathogenic organisms can be occurred during pre-harvesting, post harvesting, handling, processing, distribution and marketing or in the home kitchen (Puspanadan *et al.*, 2012; Eni *et al.*, 2010). As stated by Eni *et al.* (2010),

during that process, vegetables can be contaminated with pathogens originating from animals and human sources. Contamination of vegetables can be occurred directly or indirectly from animals or insects, soil, manures, water and also the equipment used along the food chain (Eni *et al.*, 2010).

2.4 Mitochondrial DNA markers

Mitochondria are sub cellular organelles with an extra chromosomal genome which is distinct and separated from nuclear genome (Sykes, 1999). MtDNA sizes are approximately 16,569 base pairs in length, double stranded circular molecules and histone free. Prokaryotic organisms occupied 16S ribosomal gene, while eukaryotic organisms have 18S ribosomal gene (Wang *et al.*, 2014). In contrast, mammalian cells have two mitochondrial genes known as 12S rRNA and 16S rRNA (Sykes, 1999).

2.4.1 Mitochondrial DNA 16S rRNA gene

The breakthrough of the polymerase chain reaction has made the application of molecular markers become more popular for many studies include species identification, microorganism identification, population genetics and phylogenetic study. Identification of bacteria based on molecular markers can be performed either from nuclear DNA or mitochondrial DNA. Nuclear DNA markers usually specific to the organisms and unsuitable as universal markers since involved in enzymes production. In contrast, bacteria ribosomal gene of mitochondrial DNA is universally present in all bacteria and

highly conserved in sequence (Janda and Abbott, 2007). Such features make ribosomal gene suitable as universal marker for identification.

In 1980s, Woese and others has developed a new standard for identifying bacteria based on bacteria small ribosomal subunit for phylogenetic relationship study (Woese *et al.*, 1985). According to Hillis and Dixon (1991), ribosomal RNA sequences have been used to infer phylogenies across a very broad spectrum, from studies among the lineages of life to relationships among closely related species and populations. In fact, mitochondrial ribosomal genes share similar structures and functions in organisms ranging from bacteria to humans, despite their sequences exhibit numerous inter- and intraspecific nucleotide variations (Wang *et al.*, 2014). The highly conserved sequence of mtDNA 16S rRNA made its suitable as a universal marker for identification of organisms (Wang *et al.*, 2014). The degree of conservation is assumed to result from the importance of the 16S rRNA as a critical component of cell function (Clarridge, 2004). The rate of changes in mtDNA 16S rRNA gene sequence is unknown and possibly importance for evolutionary distance and relationship of organism (Harmsen and Karch, 2004).

Due to highly conserve within a species and among species of the same genus mtDNA 16S rRNA gene has been used as the new gold standard for identification of bacteria to the species level (Woo *et al.*, 2003). Traditionally, identification of bacteria based on the basis of phenotypic characterization which is generally not accurate as identification based on genotypic methods. The selection of 16S rRNA gene as a universal marker for bacteria identification is due to the ability of this marker in identification of poorly

described, rarely isolated or phenotypically aberrant strains produced by culture based-method (Clarridge, 2004)

2.5 Polymerase Chain Reaction

Polymerase chain reaction or PCR was first invented in 1985 by a chemist, Kary Mullis. PCR is a powerful technique for amplification of a specific segment of a targeted DNA. Starting with only a very small amount of material, a DNA segment can be multiplied by over a million-fold (Stephenson and Abilock, 2012). Because of this great sensitivity, PCR has found popularity in a wide range of applications. There are several methods for identification based on PCR such as DNA sequencing, amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

2.6 PCR-RFLP

Restriction fragment length polymorphism (RFLP) technique was first applied in forensic analysis in 1986. In this technique, endonuclease enzymes were used to digest total genomic DNA, followed by southern blotting and hybridization with a specific probe. There are two types of RFLP techniques, namely multi locus probes (MLP) and single locus probe, which is based on the number of probe used. Though MLP are highly variable between individuals but the interpretation of the DNA profile was quit challenging due to multibanded pattern produced from multilocus probes (Weedn,

2000). In contrast, the interpretation of SLP was much easier and simple since only one probe was used.

Bellagamba *et al.* (2001) have stated that Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) is an efficient method in identifying raw meats and meats products. This technique also is suitable for taxonomic and phylogenetic analysis (Shekhar *et al.*, 2011; Pereira *et al.*, 2008). PCR-RFLP consists of digestion of the PCR product with restriction endonuclease at specific targeted site by producing fragment based on size variation. Species-specific based on profiles variation created by appropriate restriction endonucleases provide a useful site for diagnostic application (Mackie *et al.*, 1999). Pereira *et al.* (2008), proposed PCR- RFLP as one of the most efficient methods since its offer lower cost, time consuming, robustness and simple technique compared to DNA sequencing.

CHAPTER 3: METHODOLOGY

3.1 Materials

3.1.1 Chemical and Reagents

All reagents, chemicals, commercial kits and consumables used in this study are listed in Appendix 1

3.1.2 Buffer preparations

3.1.2.1 10X Tris Borate EDTA Buffer (10X TBE)

Tris borate EDTA (TBE) buffer stock solution was prepared by dissolving 107.8 g Tris base, 7.4 g Na_2EDTA and 46 g Boric Acid in 800 mL of ddH_20. The pH was adjusted to 8.3 by adding Boric Acid. The solution was made up to 1000 mL by adding ddH_20. The 10X TBE buffer was autoclaved and stored at ambient temperature.

3.1.2.2 0.5X TBE Buffer

TBE buffer 0.5X working solution was prepared by diluting 50 mL of 10X TBE buffer in 950 mL ddH_20.

3.1.2.3 70% Ethanol

The solution was prepared by mixing 30 mL of ddH_20 with 70 mL of absolute ethanol and was stored at ambient temperature.

3.1.2.4 Orange G loading dye

A total of 0.125 g of Orange G powder and 20 g of sucrose were dissolved in 30 mL of distilled water. The solution was made up 50 mL by adding distilled water. The solution then was aliquot into 1.5 mL microcentrifuge tube and stored at – 20°C.

3.1.3 Samples collection

Ten different types of local vegetable are used in this research. All samples were obtained from the local wet market in Kota Bharu, Kelantan. The samples were divided into two groups which were leafy and non-leafy vegetables (Table 3.1).

3.1.4 Sample Preparation

All the vegetables were cut into small pieces and leave at ambient temperature for a dried process. The completely dried process will take 2-5 days. Factors such as humidity, vegetable types and thickness will affect the duration for dried process. The dry samples then crushed into fine powder by using a mortar and pestle prior to DNA extraction. Dried samples were extracted using ZR Plant/Seed DNA Miniprep™ kit following recommended protocols provided by manufacturers. Optimization for DNA extraction was performed by using fresh and dried vegetables samples with different weight starting from 0.15 g, 0.20 g, 0.25 g, 0.30 g, 0.40 g and 0.50 g. This step is very important in order to obtain a good DNA yield that to be used in PCR amplification.

Table 3.1: List of vegetables used in this study

Type of Vegetables	Common name	Scientific name
Leafy	Spinach	*Spinacia oleracea*
	Water spinach	*Ipomoea aquatica*
	White flowering cabbage	*Brassica chinensis var parachinensis*
	Chinese broccoli	*Brassica alboglabra*
	Cabbage	*Brassica oleracea*
Non leafy	Tomato	*Solanum lycopersicum*
	Sweet potato	*Ipomoea batatas*
	Eggplant	*Solanum melongena*
	Long beans	*Vigna unguiculata ssp. sesquipedalis*
	Chili	*Capsicum annuum*

3.2 Method

3.2.1 Primer Design

In this study, new primer was designed based on full sequence of 16S rRNA gene of selected bacteria namely *Pseudomonas fluorescens* (NC_016830.1), *Pseudomonas syringae* (NC_007005.1), *Pseudomonas protegens* (NC_004129.6), *Listeria monocytogenes* (NC_003210.1), *Salmonella enterica* (NC_003198.1), *Erwinia amylovora* (NC_013961.1), *Erwinia carotovora* (NC_004547.2), *Bacillus cereus* (NZ_CM000719.1), *Bacillus subtilis* (NC_000964.3), *Bacillus licheniformis* (NC_006270.3) and *Escherichia coli* (NC_002695.1). The selection of the bacteria was decided based on the previous reported studies (Liao, 1987; Tournas, 2005; Kuczius, 2007; FAO/WHO, 2008; Barth *et al.*, 2009; Warriner *et al.*, 2009). A full sequence of 16S rRNA gene was obtained through the website of the National Centre for Biotechnology Information (NCBI) at http://www.ncbi.nlm.nih.gov/. Related information for selected bacteria was displayed in Table 3.2. Primer designing was done using BioEdit ver. 7.2.5 software (www.mbio.ncsu.edu/BioEdit/page2.html). The primer was designed based on the conserved region of 16S rRNA gene in all bacteria (Appendix 2). The best primer was selected based on the criteria as stated by Innis and Gelfand (2012). The position of 16S rRNA gene universal primers is showed in Figure 3.1.

Table 3.2: A full sequence of 16S rRNA gene for selected bacteria

Type of Bacteria	Accession number	16S rRNA gene sequences (bp)
Pseudomonas fluorescens	NC_016830.1	1546
Pseudomonas syringae	NC_007005.1	1538
Pseudomonas protegens	NC_004129.6	1549
Listeria monocytogenes	NC_003210.1	1546
Salmonella enterica	NC_003198.1	1542
Erwinia amylovora	NC_003198.1	1548
Erwinia carotovora	NC_004547.2	1556
Bacillus cereus	NZ_CM000719.1	1556
Bacillus subtilis	NC_000964.3	1554
Bacillus licheniformis	NC_006270.3	1557
Escherichia coli	NC_002695.1	1542

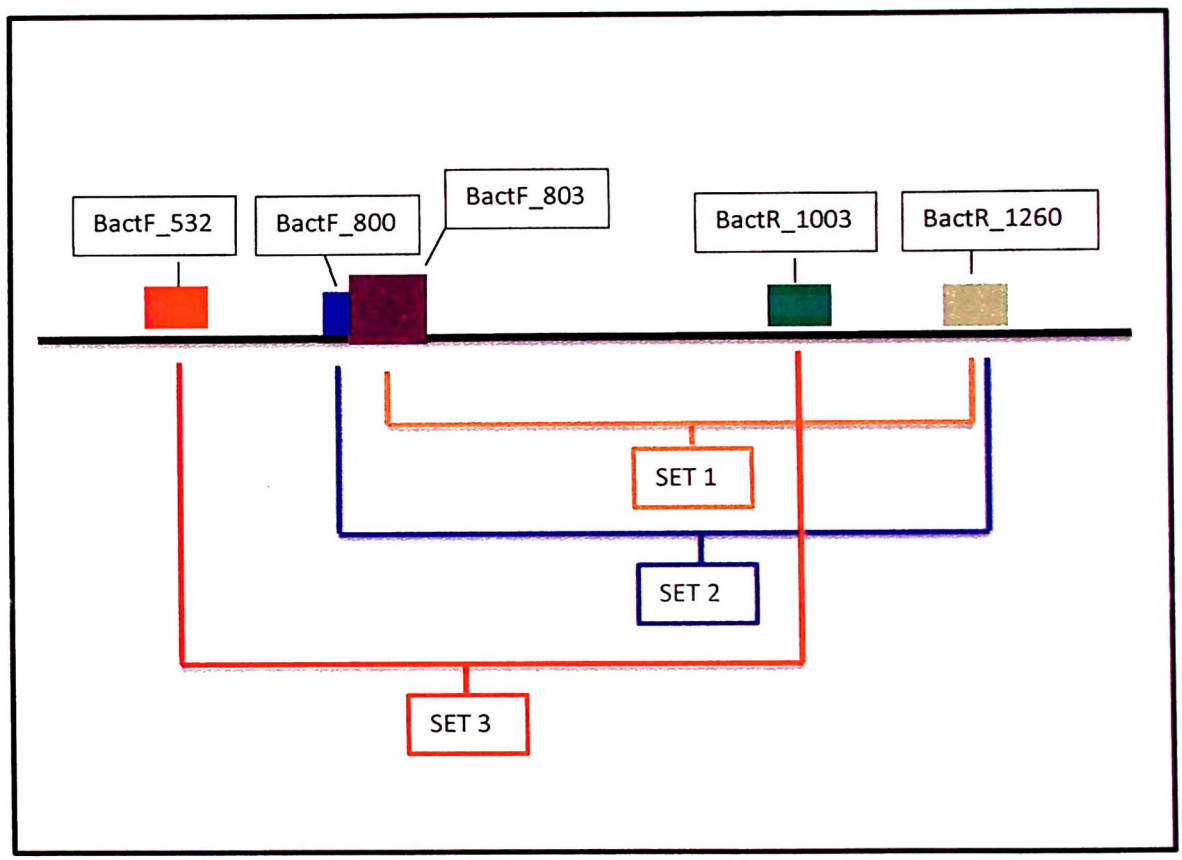

Figure 3.1: Schematic of 16S rRNA universal primers position

3.2.2 DNA Extraction

A commercially ZR Plant/Seed DNA MiniprepTM kit was used for DNA extraction of vegetables sample. According to manufacturer protocol, all the dried samples were hydrated with deionised distilled water (ddH_2O), to improve the DNA extraction efficiency. The sample was added to a ZR BashingBead^{TM} Lysis Tube and followed by adding 750 µL of Lysis Solution. The ZR BashingBead^{TM} Lysis Tube then was centrifuged at 10,000 x g for 1 minute. The collected supernatant approximately 400 µL was transferred into Zymo-Spin^{TM} IV Spin Filter in Collection Tube and centrifuged at 7,000 x g for 1 minute. The filtrate in the collection tube from the previous step was mixed with 1200 µL of Plant/Seed DNA Binding Buffer. The mixture was transferred into Zymo-Spin^{TM} IIC Column in a Collection tube and centrifuged at 10,000 x g for 1 minute. This step was repeated until all the mixture was completely finished. Washing step was performed by adding 200 µL DNA of Pre-Wash Buffer into Zymo-Spin^{TM} IIC Column in a new Collection Tube. The tube was then centrifuged at 10,000 x g for 1 minute. Next, 500 µl of Plant/Seed DNA Wash Buffer was added to the Zymo-Spin^{TM} IIC Column and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin^{TM} IIC Column was transferred into a clean 1.5 mL microcentrifuge tube and 50 µL of DNA Elution Buffer was added directly to the column. The tube was incubated at room temperature for 1 minute to increase the absorption of the DNA elution buffer into Zymo-SpinTM IIC Column before centrifuged at 10,000 x g for 30 seconds. The eluted DNA then was transferred into Zymo-Spin^{TM} IV-HRC Spin Filter in a clean 1.5 mL microcentrifuge tube and centrifuged at 8,000 x g for 1 minute. The eluted DNA was stored at -20°C until further used.

3.2.3 Agarose Gel Electrophoresis of DNA Extraction

The presence of high molecular weight DNA (HMW) was checked using agarose gel electrophoresis. A 1% agarose gel was prepared by adding 1 g agarose gel powder into 100 mL of 0.5X TBE buffer and heated in microwave oven for 3 minutes. The agarose gel solution was allowed to cool down to 60°C before adding 1.5 µL of ethidium bromide (10 mg/mL). The agarose gel solution was poured into horizontal electrophoresis unit and was allowed to solidify at ambient temperature for 30 minutes. A total of 2 µL of extracted genomic DNA was mixed with 1.5 µL of Orange G loading dye and was loaded into the agarose gel. The electrophoresis was performed at 90 V for 45 minutes. The presence of HMW was observed under UV light using Image Analyzer (UVP Bioimagine System).

3.2.4 Primers

Three sets of primer were designed based on 16S rRNA gene full sequence of selected bacteria. The primer sequences were showed in Table 3.3.

Table 3.3: Sequence of 16S rRNA gene universal primer

Primer pair	Primer Sequence (5' – 3')	Primer set
BactF_803	GAT TAG ATA CCC TGG TAG TCC	SET 1
BactR_1260	CAT TGT AGC ACG TGT GTA GC	
BactF_800	CAG GAT TAG ATA CCC TGG TAG	SET 2
BactR_1260	CAT TGT AGC ACG TGT GTA GC	
BactF_532	CCA GCA GCC GCG GTA ATA C	SET 3
BactR_1003	GGT AAG GTT CTT CGC GTT GC	

3.2.5 PCR Optimisation

Polymerase Chain Reaction (PCR) is a great tool for amplification of genetic sequences. Established PCR protocols that have been optimised for a certain gene region may not work or successful for other gene or region (Stephenson and Abilock, 2012). As suggested by Stephenson and Abilock (2012), parameters that need to be optimised include cycle number, annealing temperatures and reagent concentrations. In selecting primers, several guidelines need to be considered such as GC content (40-60%), 3' end of the primer, self-complementarities (between forward and reverse sequence), and the melting temperature (Tm). The annealing temperature for each primer was calculated using the following formulae:

$$\text{Annealing temperature (Tm)} = 2\,(A + T) + 4\,(C + G)$$

Gradient PCR machine was used to find the best annealing temperature for each set of primer. The gradient temperature for each set of primer was showed in Table 3.4. Based on the generated amplified PCR product, the best annealing temperature was selected for PCR amplification.

Table 3.4: PCR optimisation of 16S rRNA gene universal primer

Primer Sets	Gradient Temperature	Annealing temperature	PCR Product (bp)
First set	58°C – 64°C	58°C	457
Second set	60°C – 68°C	62°C	460
Third set	60°C – 68°C	63°C	471

3.2.6 PCR Amplification of 16S rRNA Gene Universal Primer

PCR amplification was carried out in 0.2 mL thin wall PCR tubes containing the following mixtures: 2 µL of 10X PCR buffer $NH_4(SO_4)_2$, 2 µL of 25 mM $MgCl_2$, 0.32 µL of 10 mM dNTPs mix, 1 µl of genomic DNA (10 ng/µL), 1 µL of 10 pmol of each primer, 0.3 µl of Taq polymerase (5 U/µL) and added with 12.38 µL ddH_2O to make up to 20 µL of total reaction mixture. The following PCR thermal cycle conditions were performed: 95°C for 3 minutes, followed by 30 cycles of 95°C for 3 minutes, annealing put in range example 60°C - 62°C for 30 seconds and 72°C for 45 seconds. The PCR ended with last elongation for 5 minute at 72°C and a holding period at 4°C for infinite time.

3.2.7 Agarose Gel Electrophoresis of Amplified PCR Products

A 1% agarose gel was prepared by adding 1 gram of agarose powder into 100 mL of 0.5X TBE buffer and heated in microwave oven for 3 minutes. The agarose gel solution was allowed to cool down to 60°C before adding 1.5 µL of ethidium bromide (10 mg/mL). The solution was poured onto a gel plate and was allowed to solidify at ambient temperature for 30 minutes. A total of 3 µL of PCR products was mixed with 1 µL Orange G dye and was loaded into each well. Two µL of 100 base pair DNA ladder was loaded into the first well. The electrophoresis was performed at 90 V for 45 minutes. The presence of PCR products were observed under UV light using Image Analyzer.

3.2.8 PCR RFLP Analysis

Restriction enzyme is a site specific since its cleave DNA molecules at specific nucleotide sequence (Snustad and Simmons, 2003). Fragment variation produced by restriction digestion can be used for characterization of species (Hartwell *et al.*, 2011). There are two types of cut made by the restriction enzymes known as sticky ends and blunt ends as shown in Figure 3.2 (Snustad and Simmons, 2003).

In the study, PCR-RFLP analysis was carried out using restriction enzymes *Alu* I, *Hha* I, *Hae* III and *Eco*R I to determine the species-specific site for amplified PCR product with universal primer 16S rRNA gene. A total of 15 µl of reaction mixture was prepared (Table 3.5) and incubated at 37°C for 5 hours. The enzyme activity was deactivated at 65°C / 80°C for 20 minutes. The digested product was run in 1.5% agarose gel stained with ethidium bromide. A total of 3 µL of digested product was mixed with 1 µL Orange G dye and 2 µL of 50 base pair DNA ladder was also loaded. The electrophoresis was performed at 100 V for 1 hour and 30 minutes. The presence of PCR products were observed under UV light using Image Analyzer.