Original Article

MOLECULAR IDENTIFICATION OF BACTERIAL COMMUNITIES FROM VEGETABLES SAMPLES AS REVEALED BY DNA SEQUENCING OF UNIVERSAL PRIMER 16S rRNA GENE.

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ABSTRACT

Vegetables are parts of the healthy diet to the individuals. Microbes are introduced to the fresh produces starting from the pre-harvesting, post harvesting and even until end to the consumer. The aim of this study is to identify the bacterial community presence on vegetables sample through DNA sequencing of mitochondrial 16S rRNA gene. In this study, 16 types of vegetables, namely chinese broccoli (S1), water spinach (S2), chinese flowering cabbage (S3), spinach (S4), eggplant (S5), sweet potato (S6), tomato (S7), long bean (S8), chili (S9), scallion (S10), capsicum (S11), cauliflower (S12), pak choy/bok choy (S13), french bean (S14), coriander (S15) and carrot (S16) were selected for PCR amplification using universal primer 16S rRNA gene. The result shows the newly designed universal primer was successfully amplified 460 bp partial sequence of 16S rRNA gene. The bacteria community such as Cyanobacterium sp., Sphingomonadales sp, Bacillales bacterium, Actinobacterium, Deinococcus sp., Trichodesmium and Carnobacterium sp. were successfully identified in this study, suggesting that the identification of bacteria can be performed directly from samples without requirement of bacteria culture.

INTRODUCTION

Vegetables are rich in minerals, vitamins, iron and dietary fibers that are linked to the maintenance of well-being of individuals [1]. In Europe, most of the public health institutions have run awareness campaigns about the importance of the consumption of vegetables and fruits in daily life [2]. Common daily vegetables includes carrots, lettuces, cucumbers, peppers, radishes, salad as well as other green vegetables that easily accessible at markets. Most of the microbes found on fruits and vegetables are soil inhabitants which are responsible for the maintaining of the ecological system in agricultural fields [3]. Exposure to the microbes can occur at all stages starting from pre harvesting, post harvesting, storage and at consumption step [3]. Study has showed that contaminated materials with non-pathogenic microbes show a various consequences on the quality of the produce since it's affecting the rate of the food spoilage [4]. The employment of the manures in agricultural sectors has a numerous beneficial to the plant`s growth and health. However, it promoted a number of pathogenic organisms to the plants such as *Listeria monocytogenes*, *Yersinia enterocolitica*, *Clostridium perfringens*, *Bacillus antracis*, *Salmonella spp*., *Klebsiella spp.* and *Escherichia coli* [5].

Previous studies have shown that the growing number of foodborne illness in recent years is associated with the increased of consumption of fresh produce since there are known as a host of pathogenic and non-pathogenic bacteria [6, 7]. According to Centers for Disease Control and Prevention (CDC), more than 50% of the outbreaks reported in the United State occurred in year the 1973 to 1997 were due to contaminated of fresh produce with *Salmonella spp*. [8]. Other studies also shown that the raw consumption and improperly cooked vegetables may contribute to the increased of outbreaks due to living human pathogens such as *L. monocytogenes, E. coli* and *Salmonella spp.* [9,10] . The common symptoms associated with *Salmonella* infections are fever, abdominal cramps, and diarrhea in individuals and high-risk of fatal to those with weak immune systems [9].

Culture based method is a conventional method for the identification of microorganisms or bacteria. Using this method, the bacteria was identified based

on their phenotypic characteristics, including Gram staining, morphology, culture requirements, and biochemical reaction [11]. However, these techniques have a lot of major limitations such as cannot be used for non-cultivable organisms, the biochemical characteristic of certain bacteria do not fit into patterns of any known genus and species and finally difficulty in identify the slow growing bacteria [11]. The limitation of culture based method was later overcome by molecular techniques with the introduction of the Polymerase Chain Reaction technology (PCR) in 1986 by Kary Mullis [12]. PCR is widely used in clinical biology due to its sensitivity, accuracy and more rapid compared to biochemical methods [12]. The applications of molecular techniques provide a reliable epidemiological data for tracing the source of human infection from foodborne illness cases [13]. Several techniques can be used to identify organism or bacteria such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), pulse-field gel electrophoresis (PFGE) and DNA sequencing.

Recently, many studies were focusing on ribosomal RNA gene of mitochondrial DNA especially 16S rRNA gene. The size of 16S rRNA gene is about 1550 base pairs and the highly conserved sequence of 16S rRNA gene among bacteria and other organisms made this region become popular for identification of unknown organism using universal primer [14]. In addition, database for 16S rRNA gene for known bacteria are available established and consists of large deposited sequence from many sources that can be useful for identification and differentiating of unknown organism or bacteria [15]. The aim of this study was to identify the presence of organisms on vegetable samples through DNA sequencing of ribosomal gene.

MATERIALS AND METHODS

Sample collection

Sixteen (16) different types of vegetables were used in this study. All vegetable samples were purchased from the local wet market and supermarket in Kubang Kerian, Kelantan. The samples were then classified into two groups which were leafy (chinese broccoli, water spinach, chinese flowering cabbage, spinach, scallion, bok choy, coriander) and non leafy (eggplant, sweet potato, tomato, long bean, chili/ cayenne pepper, carrot, cauliflower, french beans, capsicum/ bell pepper).

Primer design

A new set of primers were designed based on 16S rRNA gene full sequence of selected bacteria. The 16S rRNA gene full sequences were available through the website of the National Center of Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). All the primers were manually designed and further confirmed using primer software (BioEdit ver. 4.0 software). The searched sequences were as follows: *Bacillus cereus (*NZ_CM000719.1), *Bacillus subtilis* (NC_000964.3), *Bacillus licheniformis* (NC_006270.3), *Escherichia coli* (NC_002695.1), *Erwinia amylovora* (NC_013961.1), *Erwinia carotovora* (NC_004547.2), *Listeria monocytogenes* (NC_003210.1), *Pseudomonas syringae* (NC_007005.1), *Pseudomonas fluorescens* (NC_016830.1), *Pseudomonas protegens* (NC_004129.6). In this study, two set of universal primer for 16S rRNA gene were used for amplification of unknown bacteria from vegetables samples. The details of the primers sequences are displayed in Table 1.

Sample Preparation and DNA extraction

All the vegetables were cut into small pieces and leave at ambient temperature for a few days until all the samples were completely dried. Prior to DNA extraction the dried samples were crushed into fine powder by using a mortar and pestle. As much 0.3 g of dried samples were used for DNA extraction using modified phenol-chloroform method as recommended by Healey *et al.* [16].

PCR optimisation

The PCR optimisation is necessary to obtain the efficient amplification of specific targets. The parameters need to be considered including cycles number, annealing temperature and the reagent concentrations as recommended by Stephenson and Abilock [17]. The gradient PCR technique was employed for both sets of primers at temperature 62ºC to determine the optimum annealing

Table 1: List of the universal primer for 16S rRNA gene sequence applied in PCR amplification of the unknown organism.

Primer	Primer Sequence (5' – 3')	Primer Set
BactF_355	ACT CCT ACG GGA GGC AGC	SET 1
BactR_722	ATC TAC GCA TTT CAC CGC TA	
BactF_951	GCA CAA GCG GTG GAG CAT GT	SET 2
BactR_1411	AAG GCC CGG GAA CGT ATT CA	

temperature for the primer set during the PCR process. The PCR reaction mix was prepared in 20 µL which consist of 2 µL of 10X PCR buffer $NH_4(SO4)_2$, 2 µL of the 25 mM $MgCl_2$, 0.32 µL of 10 mM dNTPs, 0.3 µL of Taq polymerase (5 U/µL) ,1 µL of 10 pmol of each primers (reverse and forward primers) ,11.38 µL of sterile deionized water (ddH_2O) and 2 µL of genomic DNA. The PCR was performed with initial denaturation at 95ºC for 3 minutes, followed by 30 cycles of denaturation at 95ºC for 3 minutes, primer annealing at 62ºC for 30 seconds, primer extension at 72ºC for 45 seconds and the final elongation at 72ºC for 5 minutes. The amplified products were then analyzed on 1% agarose gel with the loading volume of 3 µL of PCR product and 1 µL of Orange G dye and were allowed to run for 1 hour at 90V. The result obtained was visualized under UV light using gel documentation system (Vilbert Lourmat/Quantum). The annealing temperatures for each primer set were chosen based on the good DNA band intensity on the agarose gel.

PCR Amplification and DNA purification of 16S rRNA Gene

A total of 2 µL genomic DNA was used for PCR amplification using the previously described condition in this study. PCR product was detected by 1 % agarose gel electrophoresis, stained with 1.6 µL ethidium bromide and was allowed to run for 1 hour at 90V. The result obtained was visualized under UV light using gel documentation system (Vilbert Lourmat/Quantum). The PCR amplicon were purified using Gene ALL PCR SV kit (Helix Biotech, Malaysia.) by following manufacturer`s instructions.

Analysis of the DNA Sequence

The PCR products were sequenced using an automated DNA sequencer (Tech Dragon Ltd, Hong Kong, China). The raw DNA sequence data was analyzed using BioEdit ver.4.0 software. The treated DNA sequence then was BLAST using free software at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) in order to identify the bacteria infected vegetable samples.

RESULTS

In this study, a total of 16 samples of dried vegetables were examined for identification of bacteria using newly designed primer of 16S rRNA gene. Based on PCR optimisation results, universal primer SET 2 show a good band intensity with less smear and nonspecific binding were generated at temperature 62ºC (Figure 1). PCR amplification of SET 2 (BactF_951 and BactR_1411,) produced amplicon at 460 bp (Figure 2). Meanwhile, amplification of SET 1 (BactF_355 and BactR_722) which produced amplicon at 367 bp (Figure 2), showed DNA band with more smear and non specific binding compare to PCR amplification of SET 2. Thus, this study has chosen universal primer of SET 2 (BactF_951 and BactR_1411) for PCR amplification of vegetable samples. Out of the 16 samples, 14 samples gave a positive result for the amplification of the 16S rRNA gene (Figure 3). Extracted DNA from sample french beans (S14) and coriander (S15) were failed to amplified using primer BactF_951 and BactR_1411 (SET 2).The failure of amplification also can be caused by the highly degraded genetic material into very small fragments. According to Golenberg *et al*. [18], the initial fragmentation of the template DNA grossly affected the PCR amplification product directly or indirectly by interfere the enzymatic reactions. All the 14 samples then were purified prior to DNA sequencing.

DNA sequencing result show out of the 14 samples, only 11 samples were accepted for further analysis. Three samples that fail for DNA sequencing are sweet potato (S6), tomato (S7) and chili (S9). Based on the electropherogram of the 3 samples, it is suggested that either the low DNA concentration of the purified PCR products or the presence of the impurities in the samples might be the main factors contributing to the failure of DNA sequencing. The unknown sequence profiles generated from the amplification of universal primer 16S rRNA gene further analysed using Basic Local Alignment Search Tool (BLAST) to identify the organism

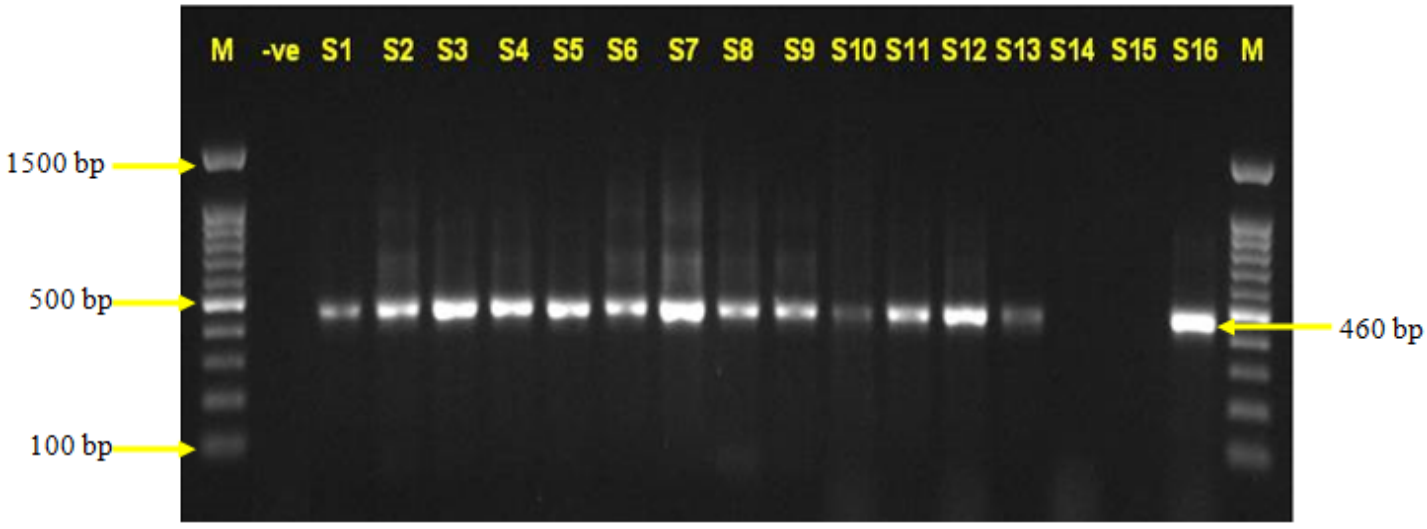


Figure 1: PCR optimisation of universal primer 16S rRNA of SET 2 at 460 bp (BactF_951 and BactR_1411).

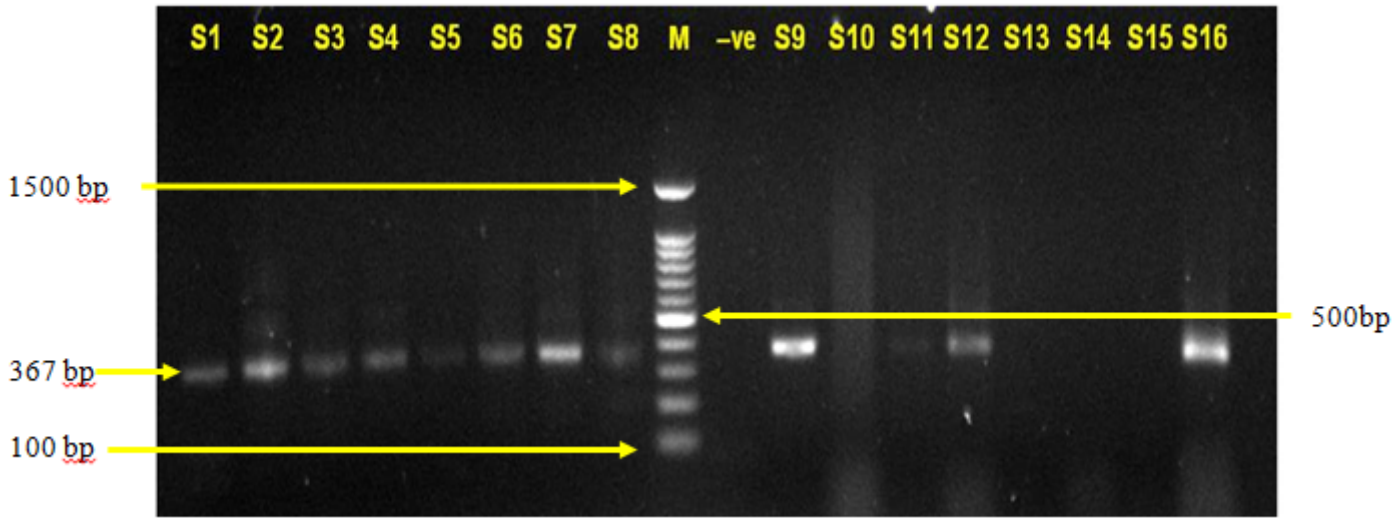
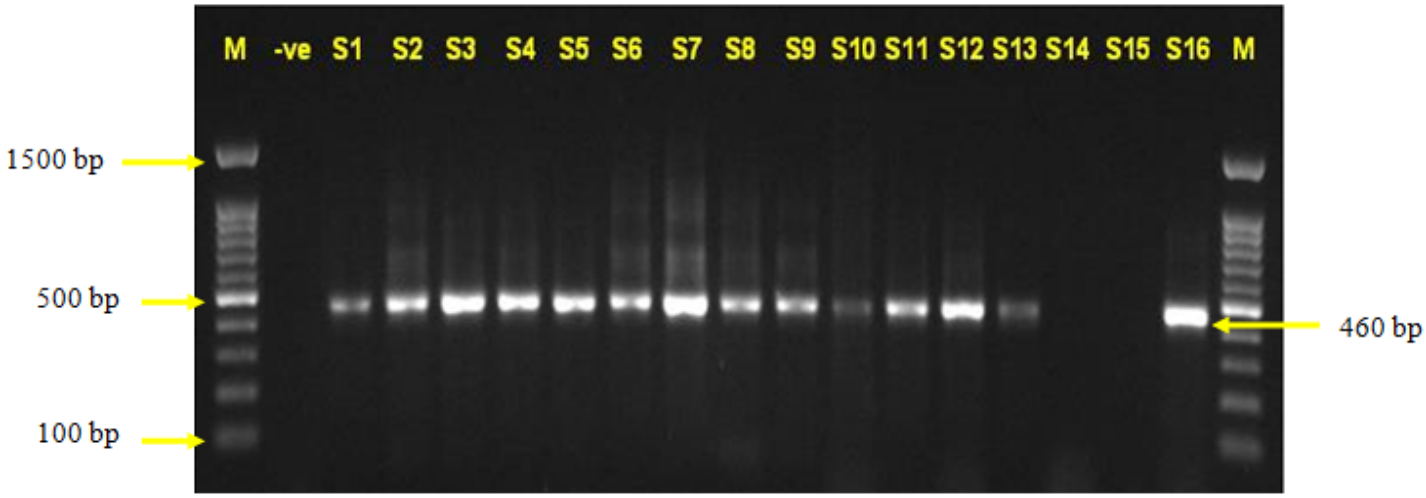


Figure 2: PCR optimisation of universal primer 16S rRNA gene of SET 1 at 367 bp (BactF_355 and BactR_722).



Lane M	: DNA ladder (100bp)	Lane S8	: Long beans
Lane -ve	: Negative control	Lane S9	: Chili
Lane S1	: Chinese broccoli	Lane S10	: Scallion
Lane S2	: Water spinach	Lane S11	: Capsicum
Lane S3	: Chinese flowering cabbage	Lane S12	: Cauliflower
Lane S4	: Spinach	Lane S13	: Pak choy/ Bok choy
Lane S5	: Eggplant	Lane S14	: French beans
Lane S6	: Sweet potato	Lane S15	: Coriander
Lane S7	: Tomato	Lane S16	: Carrot

Figure 3: Amplified PCR product of universal primer 16S rRNA gene SET 2 (BactF_951 and BactR_1411).

presence on the vegetable samples. Based on the Table 2, a diverse bacterial communities were identified from 11 samples, show that the successful of the newly designed universal primer 16S rRNA gene. The bacterial community such as *Bacterium, Cyanobacterium, Actinobacterium, Bacillales, Sphingomonadales, Carnobacterium, Trichodesmium sp.* and *Deinococcus sp.* were identified on the vegetable sample (Table 2). This finding is similar with other previous study reported by [19, 20,6, 21]. All the identified organisms are Gram positive of enteric bacterial. Although, most of the enteric bacteria are harmless and non pathogenic, however, precaution should be taken to avoid health problems. Interestingly, a long bean (S8) is the only vegetable sample was identified with one type of bacteria which is bacterium type. The main reason for that, it could be the long beans may have less contact with soil compared to other vegetable samples. All the vegetable samples were identified with bacterium type (Table 2). The word of bacterium is referring to the single unrecognized bacterium which is submitted by a research in the NCBI website. The unresolved bacteria type indicates that not enough information was generated using partial sequence of 16S rRNA gene to identify the bacteria.

All the identified bacteria from BLAST results were classified into phyla in order to observe the distribution of the bacteria community on the vegetable samples (Figure 4). As shown in Figure 4, five types of phyla were classified namely *Cyanobacteria, Deinococcus, Actinobacteria, Proteobacteria* and *Firmicutes.* The majority of the bacteria were belonging to the *Cyanobacteria* phylum ranging from 60% to 82% (Figure 4). The second common phyla are *Actinobacteria* and *Firmicutes* which ranging from 1-2 % respectively (Figure 4). Phylum *Deinococcus* only seen in a few vegetable samples such as water spinach (S2), spinach (S4), eggplant (S5) and capsicum (S11) with relative abundance ranged between 1-2 % (Figure 4). *Deinococcus sp.* is a bacterium that has a characteristic of highly resistant to environmental hazards [22]. Previous studies have shown that the *Deinococcus sp.* has been isolated from wet sources area such as river [23] and sewage [24]. Classification of phyla cannot be determined for long beans vegetable since there is no specific bacteria was identified (Figure 4).

Table 2: The list of bacteria identified on vegetable samples.

Vegetable names	**List of Bacteria**
Chinese broccoli (S1)	*Bacterium, Cyanobacterium, Actinobacterium, Bacillales Bacterium, Sphingomonadales Bacterium (proteobacteria), Carnobacterium, Trichodesmium sp.*
Water spinach (S2)	*Bacterium, Cyanobacterium, Actinobacterium, Sphingomonadales Bacterium (proteobacteria), Carnobacterium, Trichodesmium sp., Deinococcus sp.*
Chinese flowering cabbage (S3)	*Bacterium, Cyanobacterium, Actinobacterium, Bacillales Bacterium, Sphingomonadales Bacterium (proteobacteria), Carnobacterium, Trichodesmium sp.*
Spinach (S4)	*Bacterium, Cyanobacterium, Actinobacterium, Sphingomonadales Bacterium (proteobacteria), Carnobacterium, Trichodesmium sp., Deinococcus sp.*
Eggplant (S5)	*Bacterium, Cyanobacterium, Actinobacterium, Sphingomonadales Bacterium (proteobacteria), Carnobacterium, Trichodesmium sp., Deinococcus sp., Bacillales Bacterium.*
Long beans (S8)	*Bacterium*
Scallion (S10)	*Bacterium, Cyanobacterium, Actinobacterium, Sphingomonadales Bacterium (proteobacteria), Carnobacterium, Trichodesmium sp.*
Capsicum (S11)	*Bacterium, Cyanobacterium, Actinobacterium, Sphingomonadales Bacterium (proteobacteria), Carnobacterium, Trichodesmium sp., Deinococcus sp.*
Cauliflower (S12)	*Bacterium, Cyanobacterium, Actinobacterium, Sphingomonadales Bacterium (proteobacteria), Carnobacterium, Trichodesmium sp.*
Pak choy/ Bok choy (S13)	*Bacterium, Cyanobacterium, Actinobacterium, Sphingomonadales Bacterium (proteobacteria), Carnobacterium, Trichodesmium sp.*
Carrot (S16)	*Bacterium, Cyanobacterium, Actinobacterium, Sphingomonadales Bacterium (proteobacteria), Carnobacterium, Trichodesmium sp.*

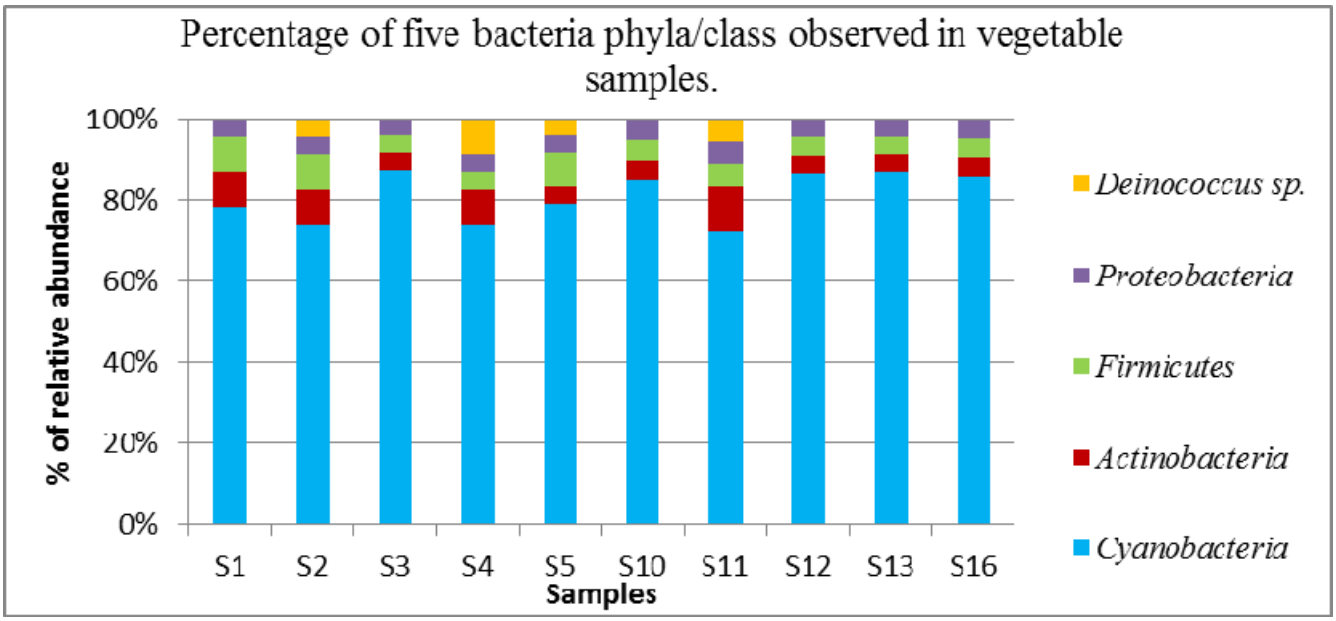


Figure 4: The graph shown the percentage of bacteria phyla/class observed in vegetable samples.

DISCUSSIONS

The abundance of microbial diversity was analyzed deeper into taxonomic level. Phyla *Deinococcus, Firmicutes* and *Cyanobacteria* were attributed into a genus level; meanwhile phyla *Actinobacteria* and *Proteobacteria* were assigned into a family level. *Carnobacterium* and *Bacillales bacterium* are classified under *Firmicutes* phylum. Meanwhile, *Deinococcus sp.* is the genus for *Deinococcus* phylum and *Trichodesmium sp*. is the genus belong to *Cyanobacteria* phylum [25,26]. *Trichodesmium sp.* was usually found in tropical and subtropical oceans. However, as reported by Prufert-Bebout *et al*. [27], *Trichodesmium sp.* was growing successfully in culture laboratory for over 1 year by modifying the medium with seawater and proper handling technique. This suggests that the bacteria can be introduced into different environment as long as the requirement nutrients were fulfilled constantly. *Actinobacterium* family was classified into the most dominant bacteria under the *Actinobacteria* phylum. Similarly, *Sphingomonadales* family was belong to the class *Proteobacteria* classified under *Proteobacteria* phylum [28]. The *Cyanobacteria* phylum was the most prominent found since these bacteria were introduced into vegetables by nitrogen fixing bacteria that originated from soils and water [27]. The vegetables can be exposed to the *Cyanobacterium sp.* during pre-harvest and post-harvest process. It has been reported that the *Cyanobacterium* contaminated the irrigation water during pre-harvest process by producing bioactive compounds like cyanotoxins which causing serious illness if directly consumed [29].

The finding also shows chinese broccoli (S1), chinese flowering cabbage (S3), scallion (S10), capsicum (S12), pak choy/ bok choy (S13) and carrot (S16) were identified with bacteria that classified under the phyla *Proteobacteria, Firmicutes and Actinobacteria* which are responsible for spoilage and deterioration of fresh vegetables [6,21] (Table 2 and Figure 4). Study conducted by Rinland and Gomez [30], described bacteria under these phyla (*Proteobacteria, Firmicutes* and *Actinobacteria*) involved in degradation of different biomass wastes on onion. This study also revealed that the bacteria under *Proteobacteria* and *Firmicutes* phyla were less dominant found on vegetable samples (Table 2 and Figure 4). This finding was contrast to Lopez-Velasco *et al*. [20], since those bacteria phyla are frequently observed in spinach. Pathogenic bacteria or human pathogen such as *Salmonella sp.*, *Listeria sp.* and *E. coli O157:H7* were not detected in this study. This might be due to the presence of the *Actinobacterium* and *Carnobacterium* that suppress the plant pathogens since both of these bacteria have insecticidal and antimicrobial characteristics [31, 32]. The trends and types of bacteria identified from this study were found to be consistent and does not represent all the bacterial communities that associates with plants. Jackson *et al.* [21] pointed out that the different types of fresh produce would have distinct bacterial communities which can be found on any parts of plants like leaves, seeds, or roots. However, certain fresh vegetables could exhibit more similar bacterial communities such as spinach, sprouts and lettuce [21].

CONCLUSION

This study has shown the application of universal primer 16S rRNA gene in identifying the bacterial community from vegetable samples. A newly designed universal primer based on 16S rRNA gene of selected bacteria was successfully amplified 460 bp partial sequence of 16S rRNA gene. The presence of bacteria diversity on vegetable samples indicate that every stage involved starting from pre-harvest process till the end process to the consumer has probability to introduce bacteria into vegetable samples. Though all the identified bacteria were non pathogen and harmless to human being but in certain situation these types of bacteria also can cause illnesses to human such as stomach uncomfortable, vomiting, dizziness and diarrhea. Therefore, the precaution should be taken during preparing vegetable such as wash under the clean water, avoid taking raw vegetables in the menu and properly cooked vegetable. The results also proved that the identification of bacteria using the partial 16S rRNA gene was possibly identified directly from samples without performing culture method. In summary, the molecular approaches that applied in identification of bacterial community from vegetable samples are offering more sensitive, rapid and robust method compared to standard laboratory culture.

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