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# Phenotypic and Molecular Characterisations of Lactic Acid Bacteria Isolated from Malaysian Fruits

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## ABSTRACT

Lactic Acid Bacteria (LAB) are gram-positive, catalase-negative and non-spore forming bacteria known to have many advantages such as starter culture in food fermentation, as antimicrobial agent and plant growth promoter. Limited information on various LAB present in Malaysian fruits hampers further study to explore their potential as autochthonous inoculants in food fermentation, plant disease control and growth promotion. Therefore, the objectives of this study are firstly to isolate and identify LAB from honeydew, ciku, mango and mata kucing by investigating their morphological and biochemical characteristics, secondly to determine the identity of the isolates using 16S rRNA gene sequencing and finally to examine phylogenetic relationship of the LAB present in the fruits. The isolates were subjected to Gram staining, acidity and catalase tests, followed by molecular identification and phylogenetic analysis of the bacteria. Out of 33 isolates, eight isolates were gram- positive, catalase-negative and acid producers, suggesting that they are potentially LAB. 16S rRNA sequencing and NCBI Blast analysis identified the presence of *Lactococcus* sp., *Leuconostoc* sp., *Weissella* sp. and *Aerococcus* sp. in the fruit samples with sequence identity 94-97%. Phylogenetic tree was constructed based on the 16S rRNA sequences using Neighbor-Joining method. This study has assisted in collecting more information about the diversity of LAB in Malaysian fruits, which can be further explored in future for their application as bioinoculant in food fermentation or as biocontrol agent and plant growth promoter in agricultural field.

*Keywords:* Fruits, lactic acid bacteria, phylogenetic analysis, 16S rRNA sequencing

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## INTRODUCTION

Malaysian fruits are highly rich in vitamins and minerals while several of them are known to be helpful in protection against chronic diseases and act as antioxidants (Khoo, Azlan, Kong, & Ismail, 2016). Besides acting as food sources, they also contribute economically to Malaysian agricultural sector. However, excess fruit production and their short shelf- life has led to fruit spoilage, thus prohibiting consumption and increasing waste. Therefore, biopreservation through fermentation process has been one of good ways to preserve fruits and slow down their spoilage (Di Cagno, Coda, De Angelis, & Gobbetti, 2013). Fruits fermentation is mediated by the addition of autochthonous or allochthonous starter culture where Lactic Acid Bacteria (LAB) is usually utilized.

LAB are classified as Gram-positive, non-spore forming, catalase-negative bacteria, which are strictly fermentative and produce lactic acid as a major end product (König, Unden, & Fröhlich, 2017). Some examples of LAB are *Lactobacillus, Lactococcus, Enterococcus and Streptococcus.* They can be grouped into homofermentative or heterofermentative rods and cocci (König et al., 2017). Besides fermentation, LAB also play important roles in food technology where they help to enhance the aroma and texture of food and inhibit the growth of spoilage bacteria (Schleifer et al., 1995). LAB are highly effective against the causal agents of food poisoning and spoilage, thanks to the production of bacteriocin that possesses antimicrobial activites (Zacharof & Lovitt, 2012). In addition, the antibacterial and antifungal substances produced by LAB were reported to be effective in controlling plant pathogens and suppressing pre and post-harvest diseases in crop plants (Belkacem-Hanfi et al., 2014; Hamed, Moustafa, & Abdel-Aziz, 2011; Tsuda et al., 2016).

However, studies on various LAB present in Malaysian fruits are relatively scarce, limiting further strategies to explore their potential as starter culture in food fermentation or as biological control agent for plant diseases. Therefore, this study was carried out to isolate and identify diverse LAB from Malaysian fruits, which can be further studied for their use in industrial and agricultural applications. The isolated LAB can be a great candidate for replacing chemical fertilizers to enhance the uses of biocontrol agents in agriculture besides helping in biopreservation of fruits. Finally, this study is also expected to provide insights into their taxonomic information and distribution in Malaysian fruits.

## MATERIALS AND METHODS

### Samples Collection and Surface Sterilization

Mango (n=10), mata kucing (n=10), honeydew (n=3) and ciku (n=10) were purchased from Serdang, Selangor. 70% ethanol was sprayed onto the surface of fruits skin to remove epiphytic microorganisms. The fruits were rinsed under running tap water and 70% (v/v) ethanol was sprayed on the fruits surface for 3 minutes followed

by rinsing with sterile distilled water. 10% (v/v) sodium hypochlorite was sprayed and left for 3 minutes followed by thorough rinsing with sterilized distilled water. The fruits were cut into two parts by using sterile knife. The seeds and flesh of the fruits were separated and homogenized in a mortar. 10 ml of sterile distilled water was added into the mortar and mixed well. The seeds and flesh extracts (1 ml) were transferred into universal bottle containing 9 ml of de Man, Rogosa and Sharpe (MRS) broth (Oxoid™). The broths were then incubated for 16 to 24 hours at 37 ºC. The overnight cultures were serially diluted in MRS broth (Oxoid™) from $10^{-1}$ until $10^{-6}$. An aliquot of 100 µl of each dilution factor was spread onto MRS agar (Oxoid™) plates for the growth of bacterial colonies. The plates were incubated for 24-48 hours at 37 ºC.

**Isolation of LAB**

Bacterial colonies with different morphologies were picked and streaked on MRS agar (Oxoid™) plates. Sub-culturing of the bacterial colonies was made on MRS agar (Oxoid™) to obtain pure colonies. The morphologies of the isolated colonies on MRS agar (Oxoid™) plates were evaluated based on their size, colour, elevation, shape and consistency using a microscope (Leica ICC50).

**Biochemical Tests**

Gram staining, catalase test and acidity test were performed. Catalase test was conducted by adding one to two drops of 3% hydrogen peroxide ($H_2O_2$) into a cultured broth and the formation of bubbles was observed (Goyal, Dhingra, Bajpai, & Joshi, 2012). Acidity test was performed according to Sobrun, Bhaw-luximon, Jhurry and Puchooa (2012) with a slight modification by streaking bacterial colonies on MRS agar containing Bromocresol purple dye (MRS+ BCP) at final concentration of 0.004% (w/v) (Sobrun et al., 2012).

**Genomic DNA Extraction and Sequencing of the 16S ribosomal RNA Gene**

The extraction of genomic DNA of the isolates was carried out by using Wizard® Genomic DNA Purification Kit (Promega Corporation, USA). Amplification of the genomic 16S rRNA region was then performed in 50 µl reaction containing 5 µl of 10X $NH_4$ reaction buffer, 1.5 µl of $MgCl_2$, 0.25 µl of GoTaq DNA Polymerase (1.25 units), 1 µl of 10 mM dNTPs, 1 µl of 10 µM forward primer 16S 27F (5' –AGA GTT TGA TCC TGG CTC AG -3') and 1492 reverse primer (5' -GGT TAC CTT GTT ACG ACT T -3') (Dai, Li, Wu, & Zhao, 2013), 1 µl of genomic DNA template and 39.25 µl of nuclease-free water. PCR was carried out in Bio-Rad MyCycler Thermal Cycler PCR (Bio Rad Laboratories, Inc) with the following conditions: initial denaturation at 94 ºC for 30 seconds, 30 cycles of denaturation at 94 ºC for 30 seconds, annealing at 56 ºC for 1 min, extension at 72 ºC for 2 minutes and final extension at 72 ºC for 5 minutes. PCR products were analyzed on a 1.0 % (w/v) agarose gel in 1X TAE buffer at 80 V for one hour and purified using the Wizard® SV

Gel and PCR Clean-Up System (Promega Corporation, USA). The purified PCR products were subjected to 1st Base DNA sequencing services and the identity of the isolates was analyzed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

## RESULTS AND DISCUSSIONS

### Morphological and Biochemical Test

Out of 200 colonies, only 33 isolates were picked from all plates and further characterised for LAB properties. These isolates were chosen as representatives of isolates with different morphological properties, whereas those with identical morphologies were not selected. Most of the isolated colonies have small and circular shape, creamy and whitish colour. Gram staining result for the 33 isolates showed that all of them were gram-positive and cocci-shaped. Figure 2 shows the Gram staining result for isolate HDS 9 with purple and cocci-shaped colonies. Acidity test revealed that out of the 33 isolates, 20 isolates were able to produce acid, as shown by a color change from purple to yellow on Bromocresol purple agar (Figure 3). *Lactococcus lactis* was used as positive control while *Bacillus cereus* was used as negative control. Catalase test showed that from the 33 isolates, 17 were catalase-negative as indicated by the absence of bubble formation upon $H_2O_2$ addition, suggesting the absence of catalase enzyme. Most of LAB are catalase-negative, although some of bacteria in this genera may possess this enzyme (Wood & Holzalpfel, 1995). The summary for morphological characteristics and biochemical tests was tabulated in Table 1.

### Molecular Identification of Isolates using PCR and 16S rRNA Gene Sequencing

Since only LAB are targeted in this study, out of 33 isolates, only gram-positive, catalase- negative and acid-producing strains were further characterized for molecular identification using 16S rRNA gene sequencing. Table 2 shows 16S rDNA sequencing results of the isolates. The sequencing of 16S rRNA gene revealed the presence of *Lactococcus* sp., *Leuconostoc* sp., *Weissella* sp., *Aerococcus* sp. and two non-LAB species in the fruits samples with sequence similarity of 94-97%. Isolates HDS 1 and HDS 4 were closely related to *Lactococcus lactis* subsp. *lactis* strain UC06 and *Leuconostoc citreum* KM20 with 96% and 94 % similarity respectively. Isolates HDF 8 and HDF 9 showed 95% and 96% sequence similarity with *Weissella cibaria* strain CMS3 and *Weissella confusa* strain A7Gaf respectively. In addition, the 16S rDNA sequence of isolate MS 3A had 96% similarity with *Leuconostoc citreum* KM20 while isolates MS 6 and KS 5 were 94% and 96% similar with *Lactococcus lactis* strain HadRami9. Whereas, isolate KS 2 displayed 97% sequence similarity with *Aerococcus viridans* strain CCUG4311.

Table 1

*Morphological and biochemical characteristics of isolates*

| No | Isolates | Type and Part of fruit | Size and Form | Colour | Margin | Gram stain | Catalase test | Acidity test |
|---|---|---|---|---|---|---|---|---|
| 1 | MS 1 | [1]MNG , Seed | Small, circular | Creamy | Entire | Gram-positive , cocci | + | + |
| 2 | MS 2 | MNG , Seed | Small, circular | Creamy | Entire | Gram-positive , cocci | + | - |
| 3 | MS 3A | MNG , Seed | Small, circular | White | Entire | Gram-positive , cocci | - | + |
| 4 | MS 3B | MNG , Seed | Small, irregular | White | Undulate | Gram-positive , short rod | - | - |
| 5 | MS 4 | MNG , Seed | Small, circular | Creamy | Entire | Gram-positive , cocci | + | - |
| 6 | MS 6 | MNG , Seed | Small, circular | Creamy | Entire | Gram-positive , cocci | - | + |
| 7 | MS 7 | MNG , Seed | Small, circular | Creamy | Entire | Gram-positive , cocci | - | - |
| 8 | MF 1 | MNG , Flesh | Small, circular | Creamy | Entire | Gram-positive , cocci | - | - |
| 9 | MF 2 | MNG , Flesh | Small, circular | Creamy | Entire | Gram-positive , cocci | + | + |
| 10 | CS 1 | [2]CK , Seed | Small, circular | Creamy | Entire | Gram-positive , cocci | - | + |
| 11 | CS 2 | CK , Seed | Small, circular | White | Entire | Gram-positive , cocci | + | + |
| 12 | CS 3 | CK , Seed | Small, circular | Creamy | Entire | Gram-positive , cocci | + | - |
| 13 | CS 4 | CK , Seed | Small, circular | Creamy | Entire | Gram-positive , cocci | + | + |
| 14 | CF 1 | CK , Flesh | Small, circular | Creamy | Entire | Gram-positive , cocci | + | + |
| 15 | CF 2 | CK , Flesh | [a]Mod, circular | White | Entire | Gram-positive , cocci | + | - |
| 16 | CF 3 | CK , Flesh | Small, circular | White | Entire | Gram-positive , cocci | + | + |
| 17 | CF 4 | CK , Flesh | Small, circular | Creamy | Entire | Gram-positive , cocci | + | + |
| 18 | KS 1 | [3]MK , Seed | Small, circular | Creamy | Entire | Gram-positive , cocci | + | - |
| 19 | KS 2 | MK , Seed | Small, circular | Creamy | Entire | Gram-positive , cocci | - | - |
| 20 | KS 4 | MK , Seed | Small, circular | Creamy | Entire | Gram-positive , cocci | - | + |
| 21 | KS 5 | MK , Seed | Small, circular | Creamy | Entire | Gram-positive , cocci | - | + |

Table 1 *(Continue)*

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 22 | KF 1 | MK , Flesh | Small, circular | White | Entire | Gram-positive, cocci | - | - |
| 23 | KF 2 | MK , Flesh | Small, circular | Creamy | Entire | Gram-positive, cocci | - | + |
| 24 | KF 3 | MK , Flesh | Small, circular | Creamy | Entire | Gram-positive, cocci | + | - |
| 25 | HDS 1 | [4]HD , Seed | Small, circular | Creamy | Entire | Gram-positive, cocci | - | + |
| 26 | HDS 2 | HD , Seed | Small, circular | White | Undulate | Gram-positive, cocci | + | + |
| 27 | HDS 3 | HD , Seed | Small, circular | Creamy | Entire | Gram-positive, cocci | + | + |
| 28 | HDS 4 | HD , Seed | Small, irregular | White | Entire | Gram-positive, cocci | - | + |
| 29 | HDS 5 | HD , Seed | Small, circular | | Entire | Gram-positive, cocci | + | - |
| 30 | HDS 6 | HD , Seed | Small, circular | White | Entire | Gram-positive, short rod | - | - |
| 31 | HDF 2 | HD , Flesh | Small, circular | White | Entire | Gram-positive, cocci | - | + |
| 32 | HDF 8 | HD , Flesh | Small, circular | White | Entire | Gram-positive, cocci | - | + |
| 33 | HDF 9 | HD , Flesh | Small, circular | White | Entire | Gram-positive, cocci | - | + |

Note: [1]MNG indicates mango; [2]CK indicates ciku; [3]MK indicates mata kucing; [4]HD indicates honeydew; [a]Mod indicates moderate size; + indicates positive reaction, - indicates negative reaction

Table 2

*16S RNA gene sequencing analysis of isolates*

| No | Isolates | Closest matches in Genbank | Identity (Base pairs / %) | Accession no. |
|---|---|---|---|---|
| 1 | HDS 1 | *Lactococcus lactis* subsp. *lactis* strain UC06 | 1418/1477 bp (96%) | CP015902.1 |
| 2 | HDS 4 | *Leuconostoc citreum* KM20 | 1204/1286 bp (94%) | DQ489736.1 |
| 3 | HDF 8 | *Weissella cibaria* strain CMS3 | 1200/1267 bp (95%) | CP013934.1 |

Table 2 *(Continue)*

| | | | | |
|---|---|---|---|---|
| 4 | HDF 9 | *Weissella confusa* strain A7Gaf | 1021/1067 bp (96%) | KU324936.1 |
| 5 | MS 3A | *Leuconostoc citreum* KM20 | 1018/1062 bp (96%) | DQ489736.1 |
| 6 | MS 6 | *Lactococcus lactis* strain HadRami9 | 1149/1221 bp (94%) | KU324909.1 |
| 7 | KS 5 | *Lactococcus lactis* strain HadRami9 | 1185/1232 bp (96% | KU324909.1 |
| 8 | KF 2 | *Aerococcus viridans* strain CCUG4311 | 1425/1473 bp (97%) | CP014164.1 |

**Phylogenetic Analysis**

Phylogenetic tree was constructed based on the 16S rRNA gene sequences inferred by the Neighbor-Joining method using Molecular Evolutionary Genetic Analysis 7.0 (MEGA 7.0) in order to identify the relationship of isolates with other different species of LAB according to their ability in producing the antimicrobial substance, bacteriocin. *Klebsiella pneumoniae* strain M5al was used as an out-group. Based on Figure 1, the node placements for all isolates were strongly supported by bootstrap values ranging from 97-100%, indicating reliable placement of the nodes in the phylogenetic tree. Isolates MS3A and HDS 4 were grouped together with *Leuconostoc citreum* KM20 by forming a well-defined cluster at 100% and 98% bootstrap support respectively. *L. citreum* KM20 was previously found predominant in fermented kimchi (Kim et al., 2008). According to Kim et al. (2008), *L. citreum* KM20 can repress the growth of *Bacillus cereus*, *Listeria monocytogenes*, *Micrococcus luteus*, *Pseudomonas aeruginosa* and *Salmonella enterica serovar Typhimurium*. Isolates HDS 4 and MS3A were grouped together with *Leuconostoc mesenteroides* which was reported to be able to produce bacteriocin (Xiraphi et al., 2008). This may suggest that HDS 4 and MS3A also produce bacteriocin, however this needs to be investigated *in vitro* or through whole genome sequencing of the isolates. Other studies have also reported the isolation of other *Leuconostoc* sp. from fruit juices, fresh fruits and vegetables (Emerenini, Afolabi, Okolie, & Akintokun, 2013; Naeem, Haider, Baig, & Saleem, 2012), suggesting that *Leuconostoc* sp. are common in fruits and vegetables.

Isolate HDF 8 and HDF 9 extracted from the flesh of honeydew were both closely related to *Weissella cibaria* and *Weissella confusa* strain A3 respectively. Several studies reported that *Weissella* sp. isolated from fresh fruits and vegetables were able to produce bacteriocin and antimicrobial substances (Goh & Philip, 2015; Papagianni & Papamichael, 2011; Pringsulaka et al., 2012), and therefore are used as biocontrol agent against phytopathogenic bacteria and fungi (Chen, Wu, & Yanagida, 2010; Trias,

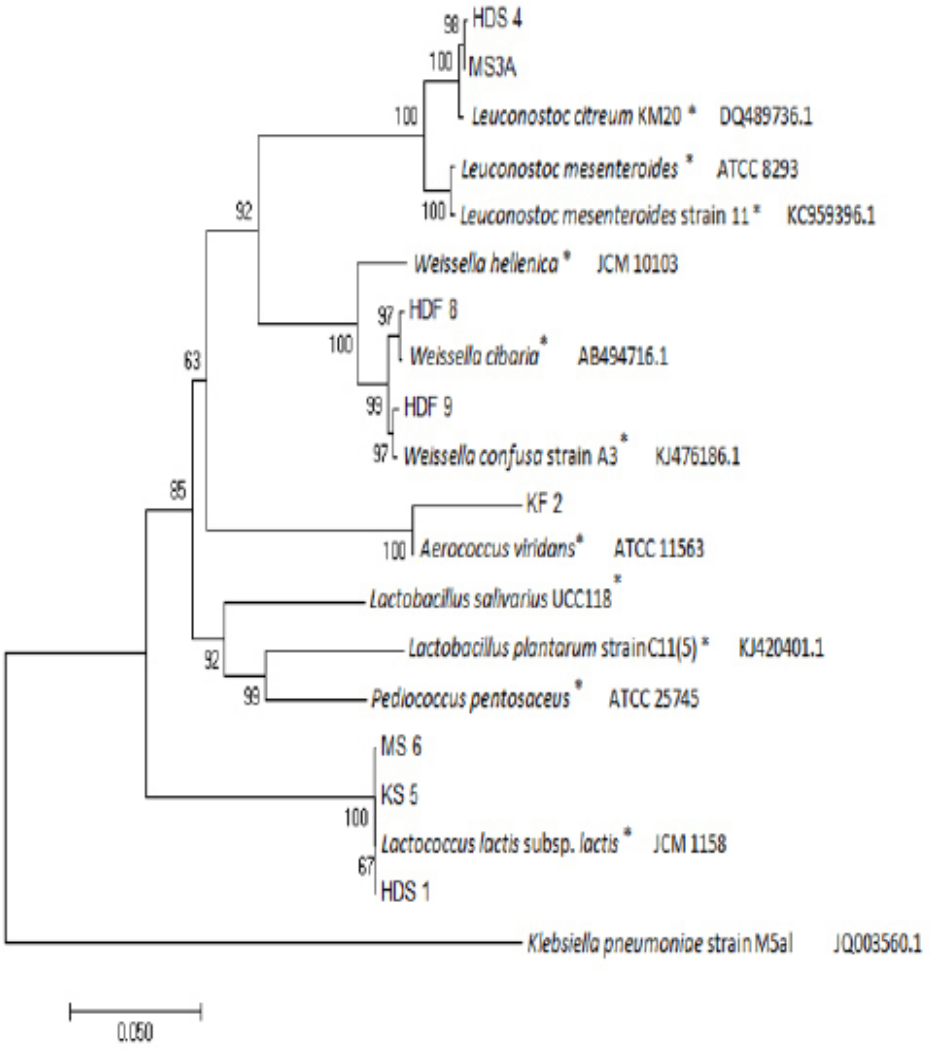


*Figure 1.* Phylogenetic tree of the selected bacterial isolates using 16S rDNA sequence as phylogenetic marker. Phylogenetic tree showing the relative positions of the isolates as inferred by the neighbor-joining method of 16S rRNA sequences. Branch lengths are proportional to the number of nucleotide substitutions. The bootstrap values for a total of 1000 iterations are shown as percentages at the nodes of the tree. *Klebsiella pneumoniae* strain M5aI was chosen as outgroup. The bar indicates 5% sequence divergence. (Note:* indicates bacteriocin producer)

Bañeras, Badosa, & Montesinos, 2008). *Weissella hellenica* was also reported to be able to be bacteriocinogenic that possesses antimicrobial effect towards food-borne pathogens including yeasts and molds indicating possibility in biopreservation (Chen et al., 2010; Leong et al., 2013). Isolate KF 2 from the flesh of mata kucing was closely related to *Aerococcus viridans* with a strong bootstrap support at 100%.

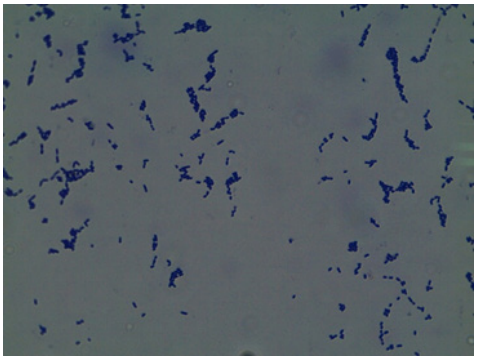

*Figure 2*. Gram staining of representative isolate HDS 9, with purple and cocci-shaped colonies

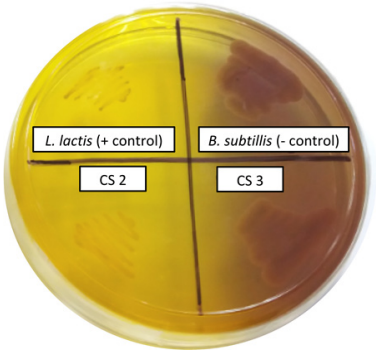


*Figure 3*. Acidity test for representative isolates CS 2 and CS 3. Isolate CS 2 showed positive result for acidity test where yellow colour formed around the colonies on the agar (MRS agar with addition of bromocresol purple) indicates the ability of the isolate to produce acid whereas isolate CS 3 showed negative result as no colour change observed on the agar and remained purple, indicating the absence of acid production. *L. lactis* and *B. subtillis* were used as positive and negative control respectively

*Aerococcus viridans* is human pathogen and often associated with endocarditis, urinary tract infection and urosepsis (Gopalachar, Akins, Davis, & Siddiqui, 2004; Jung et al., 2014; Popescu, Benea, Mitache, Piper, & Horstkotte, 2005). This species is found ubiquitous in housing premises, human skin and raw vegetables. The presence of isolate KF 2 similar to this pathogen on the flesh of mata kucing suggested cross-contaminations might have occurred either during bacterial isolation steps or during transport, storage and handling of the fruits from farm to retailers. Therefore, extra caution has to be taken when consuming raw fruits. The *Aerococcus* genus is also associated with bacteriocin production (Ballester, Ballester, & Belaich, 1980). On the other hand, isolates HDS 1, KS 5 and MS 6 isolated from the seeds of mango and mata kucing showed relatedness to *Lactococcus lactis* subsp. *lactis*. *Lactococcus lactis* strains have been isolated from various fresh fruits and vegetables (Laroute et al., 2017). They are also abundant in various sprouted and unsprouted vegetable seeds, while having antagonistic activities against *Listeria monocytogenes*, owing to the presence of bacteriocin (Kelly, Davey, & Ward, 1998). Meanwhile, a study by Park, Itoh, Kikuchi, Niwa and Fujisawa (2003) had reported the ability of *Lactococcus lactis* subsp. *lactis* isolated from kimchi to produce nisin-Z that had antagonistic properties against foodborne pathogens (Park et al., 2003). This may suggest their potential roles in preserving the fruits from food spoilage and also preventing food poisoning in human.

Settanni and Corsetti (2008) had discussed about the uses of bacteriocins as food additives, such as nisin, which is used in kimchi, mashed potatoes, and fresh-cut products. The authors highlighted about bacteriocin-producing LAB as starter culture in fermented and non-fermented vegetables, which may improve microbial

quality, safety and shelf-life of vegetable-based foods (Settanni & Corsetti, 2008). Trias et al. (2008) described the use of Leconostoc, Lactobacillus, Lactococcus and Weissella genera in inhibiting foodborne human pathogens of Iceberg lettuce and Golden apples The addition of bacteriocin-producing LAB in biopreservation has helped to increase the food safety and shelf life (Fhoula et al., 2013; Kasra-Kermanshahi & Mobarak-Qamsari, 2015).

There were also researches carried out in order to study the antibacterial activity of *Lactococcus lactis* and its uses as biocontrol agents and plant growth promoters in agricultural settings (Chen et al., 2010; Trias et al., 2008). The ability of LAB as biocontrol agents has been shown in the control of post-harvest diseases of fruits and vegetables. For instance, LAB was mentioned to be able to protect cucumber roots against pathogen *Pythium ultimum* and increased the germination rate and seedling emergence of tomato seeds (Lutz, Michel, Martinez, & Camps, 2012). Besides that, a study by El-Mabrok, Hassan, Mokhtar, Hussain and Kahar (2012) had also reported the potential of LAB in suppressing the growth of *Colletotrichum capsici* which was the causal agents of anthracnose in chilli (El-Mabrok et al., 2012). In other studies, their application as biocontrol agents has helped in inhibiting plant pathogens and assisted in plant growth promotion such as increasing the rate of seeds germination (El-Mabrok et al., 2012; Hamed et al., 2011; Murthy, Malini, Savitha, & Srinivas, 2012).

Altogether, these results suggested that all of the isolated strains are potentially bacteriocinogenic as they were clustered together with that of bacteriocin-producers, although some bacterial strains were observed to possess different ability of bacteriocin productions even they belong to the same species (Chen et al., 2010). However, further studies need to be carried out to verify the ability of each isolates to secrete bacteriocins and having antimicrobial properties against various pathogens.

## CONCLUSION

This study has successfully identified eight isolates belonging to *Lactococcus* sp., *Leuconostoc* sp., *Weissella* sp. and *Aerococcus* sp. with sequence identity 94-97%. Morphological and biochemical analysis showed that they were mostly gram-positive cocci, catalase-negative and acidity-positive. All of the isolates were clustered together with bacteriocin producers, indicating their potential in producing the antimicrobial substance. Further work needs to be done to confirm the ability of these strains to secrete bacteriocin. The work has provided preliminary insights into different Lactic Acid Bacteria in Malaysian fruits, paving the way for further analysis and characterisations to explore their potential as starter culture in food fermentation, as biocontrol agent for pre and post-harvest plant disease control or as plant growth promoters to increase plant growth and crop yields in agriculture.

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