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RAPID IDENTIFICATION AND CHARACTERIZATION OF THE *Lactobacillus* STRAINS BY USING 16S-23S rRNA GENE INTERGENIC SPACER REGION AND *recA* GENES

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Abstract

Lactic acid bacteria are known as potential probiotics that contribute beneficial effects for human beings and farmed animals. Identification of potential Lactic acid bacteria isolates was important for potential probiotic characterization. The isolates were under microscopic determination by Gram staining and electron screening microscopy to determine the morphology of the isolate, followed by rapid identification by using PCR amplification with ITS and *recA* genes to identify bacteria species of the isolates. Isolates L8 and L20 showed rod-shaped cells in the cluster while isolate S1 showed coccobacillus-shaped cells after the microscopic observation. L8 and L20 both isolates were identified as *Lactobacillus plantarum* while isolate S1 was identified as *Lactobacillus pentosus* after PCR amplification with ITS and *recA* genes. Our present experiment shows that ITS and *recA* genes can be used with PCR amplification to identify probiotic, *Lactobacillus* spp accurately.

INTRODUCTION

Probiotics are known as microorganisms that could improve balance of commensal host intestinal microflora [1]. Probiotic application is crucial for health of human beings and farmed animal species [2]. Lactic acid bacteria are commonly applied for most of the commercialized and studied probiotics that include *Lactobacillus* spp., *Lactococcus* spp., *Bifidobacterium* spp., *Enterococcus* spp., *Pediococcus* spp. and *Leuconostoc* spp. [3]. Lactic acid bacteria have several important advantages as probiotic that contribute beneficial effects for hosts which include pathogen growth inhibition, adherence to host intestinal epithelial cells and immune system stimulation [4]. However, completely different probiotic properties of lactic acid bacteria are highly dependent on their species and strains [5]. Besides of morphological characteristics of lactic acid bacteria that can be determined by microscopic observation [6], molecular biology technique is recommended for rapid identification of genotypical characteristics of lactic acid bacteria by using Polymerase Chain Reaction (PCR) method which is more accurately compared with the conventional biochemical and physiological tests [7,8]. The 16S rRNA gene is commonly used by many researchers for species identification by PCR amplification but has limitations because low capability of biological discrimination that is used to distinguish microorganisms [9,10]. Therefore, specific genes are required for accurate identification by using PCR amplification. Internal transcribed spacer (ITS) and *recA* genes are suggested because these genes have a discriminative ability for bacteria species identification by PCR amplification [11,12]. Other than that, the advantage of these genes over the morphology-based system is that non-specialists are able to quickly and cheaply identify individual species or strains. In addition, species-specific primers were

tested for the two most common species of related groups. Therefore, ITS and *recA* genes were applied for PCR amplification to identify different species of *Lactobacillus sp.* in this study.

MATERIALS AND METHODS

Materials

MRS (De Man, Ragosa and Sharpe) broth and MRS (De Man, Ragosa and Sharpe) agar were purchased from Difco BD, USA. Crystal violet, Gram's Iodine, Safranin were purchased from Fisher Scientific, USA. Ethanol, phosphate buffer, coverslips (Sarstedt, USA), glutaraldehyde (Sigma Aldrich, USA), light microscope (Leica, United Kingdom), SEM (Live Stereoscopic VPSEM; Hitachi Japan), Mastercycler gradient (Eppendorf, German), One-Tube Bacterial Genomic DNA Extraction Kit (Bio Basic, Canada), EZ-10 Spin Column PCR Products Purification Kit (Bio Basic Inc., Kanada), MyTaq Red Mix Protokol (Bioline, USA).

Preparation of Single Pure Colony Culture

All pure isolates including L8, L20 and S1 were previously isolated from Malaysian fermented fish at Biomedical Science Programme of Faculty Health Science, Unversiti Kebangsaan Malaysia [13]. All pure isolates were kept in MRS broth that contained 20 % (v/v) glycerol at -80 °C as frozen stocks. After the frozen stock were thawed, the isolate was spread on MRS agar. The MRS agar that spread with isolate was incubated in an incubator at 37 °C for 48 h. Then, streak plate on new MRS agar were performed by inoculating a pure colony from the cultured agar followed by incubated in incubator at 37 °C for 48 h. After that, the cultured agar was proceeded for determination of morphological characteristics. A colony was inoculated from cultured agar into 1 mL of MRS broth which then incubated at 37 °C for 48 h. The cultured MRS broth was prepared for phylogenetic identification.

Determination of Morphological Characteristics for Probiotic Candidates by Gram Staining

A colony from pure cultured MRS agar of each isolate was subjected to the examination of their morphological characteristics. The morphological investigation of the isolated strains was carried out by the Gram staining method. The colony of isolate was proceeded for heat-fixed smear on a glass slide. A primary stain, crystal violet was applied on the heat-fixed smear slide. Then, the mordant, Gram's iodine was applied on the heat-fixed smear slide. Afterward, the heat-fixed smear slide was decolorized by using 95% (v/v) ethanol. Lastly, the heat-fixed slide was counterstained by safranin. After the heat-fixed smear slide was dried in room temperature, the heat-fixed smear slide was observed under light microscope (Leica, United Kingdom) with 1000x magnification.

Scanning Electron Microscopy

Overnight cultures of the isolates in MRS broth were centrifuged at 1008 × g for 10 min and the pellets were re-suspended in 0.1 M phosphate buffer (pH7.2) solution to a final concentration of 10^8 CFU/mL. Then, 100 µL of the bacteria suspensions were dropped on the coverslips (13 mm). The bacterial cells were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer for 2 h at room temperature. Then, the bacterial cells were dehydrated in a graded ethanol series (50% v/v, 70% v/v, 80% v/v, 90% v/v and 95% v/v) for 15 min each session, followed by the dehydration step (twice) in 100% ethanol for 30 min. The coverslips containing the cells were air-dried at room temperature for 30 min, mounted on stubs and coated with gold for 15 s. The specimens were then examined through SEM (Live Stereoscopic VPSEM; Hitachi Japan).

Polymerase Chain Reaction (PCR) for *ITS* Gene Amplification

DNA extraction was done by using One-Tube Bacterial Genomic DNA Extraction Kit following the manufacturer's instruction with minor modifications. Two sets of primers that targeted 16S rRNA and ITS genes of probiotic candidates were synthesized by 1ST Base Malaysia company (Malaysia); CGGTGAATACGTTCCCGGGYCTTG for ITS_16F while TTTCRCCTTTCCCTCACGGTA for ITS_23R. The PCR was carried out by using Mastercycler gradient (Eppendorf, German) according to optimized programs based on [14] which are: pre-denaturation at 95 ºC for 5 min, followed by 40 cycles consisting of denaturation at 95 ºC for 1 min, annealing at 55 ºC for 1 min and extension at 72 ºC for 2 min, the reactions were terminated by final extension at 72 ºC for 5 min. In addition, a negative control was set which it was contained same reaction mixture but sterile distilled water instead of DNA template. Amplified DNA product was purified by using EZ-10 Spin Column PCR Products Purification Kit followed the manufacturer's protocol.

DNA Sequencing and Phylogenetic Analysis for *ITS* Gene

Nucleotide sequence of the ITS gene obtained from 1st Base Malaysia (Malaysia) was compared with sequences of GenBank database by using Basic Local Alignment Search Tool (nBLAST) program. The program played role for determining similar degree of identified organisms' sequences in the GenBank database. Multiple sequence alignment for closely related genus of probiotic candidates was done by using ClustalW, then, the phylogenetic tree was constructed for two sets of sequence by using Mega 7 Beta.

For obtaining the information of probiotics' molecular phylogeny, Maximum-Likelihood method [15] and bootstrap analysis [16] were applied to evaluate reliability of phylogenetic tree's nodes.

Identification and Characterization of Probiotic Candidates using *recA* Gene

Since the isolate that not identified accurately for their species level ITS gene, the isolate was identified by using *recA* gene. After the DNA of the isolate was extracted, PCR was carried out by using follwing primers [17]: pentF, 5'-CAGTGGCGCGGTTGATATC-3'; planF, 5'-CCGTTTATGsCGGAACACCTA-3'; pREV, 5'-TCGGGATTACCAAACATCAC-3'. For PCR amplification, MyTaq Red Mix Protokol was used which the mixture that consists of 200 ng DNA template, 1 uL primer (20uM), 25 uL MyTaq Red mix. The total volume was brought up to 50 uL with sterile distilled water. The PCR was carried out by using Mastercycler gradient (Eppendorf, German) according to optimized programs based on [17] which are: pre-denaturation at 94 ^{o}C for 3 min, followed by 30 cycles consisting of denaturation at 94 ^{o}C for 30 s, annealing at 56.8 ^{o}C for 10 s and extension at 72 ^{o}C for 10 s, the reactions were terminated by final extension at 72 ^{o}C for 10 min. In addition, negative control was set which it was contained same reaction mixture but sterile distilled water instead of DNA template. Next, electrophoresis was carried out to observe successful amplified DNA sequence. Fragment sizes of *recA* gene was 218 bp for *L. pentosus* while 318 bp for *L. plantarum*.

RESULTS AND DISCUSSION

Phenotypical and genotypical variations found in many strains of the same species affect the accuracy of bacteria strain identification in research. As an alternative, molecular analysis was suggested to be applied for the identification of probiotics candidates that approached species level [18]. Since the inaccurate biochemical tests on the species identification for the isolates, this experiment used PCR method that targeted on ITS gene for species identification of all isolates accurately.

Determination of Phenotypic and Genotypic Characteristics for Probiotic Candidates

For the experimental result (**Figure 1**), Gram staining proved that all isolates were identified as Gram positive bacteria. Isolates L8 and L20 were showed rod shaped cells that in cluster while isolate S1 was showed coccobacillus shaped cells. For the experimental result (**Figure 2**), isolates L8 and L20 showed rod shaped cells while isolate S1 showed coccobacillus shaped cells. Although the isolates shared closely related genetic relationship, but shapes and sizes of the two species were different based on the Gram staining and screening electron microscopy experiment of Figure 1 and 2. All isolates were Gram positive bacteria which supported by Marhamatizadeh and Sayyadi (2019) [19] who stated *Lactobacillus spp*. is a genus of Gram positive bacteria of high probiotic value. The bacterial shape of isolates L8 and L20 were in rod shape which similar with other strains of *L. plantarum* after microscopic observation [20]. However, bacterial shape of isolate S1 was in coccobacillus shape which different with other typical *Lactobacillus spp* that usually in rod shape after microscopic observation [21]. Hence, the different bacteria strains of same species have different bacterial shape were proved in this experiment and supported by Smith et al. (2016) [22] who showed different bacteria shapes for different *Escherichia coli* strains.

Probiotic candidates were included L8, L20 and S1 that fulfilled the positive criteria as probiotic after the several screening process [13]. Furthermore, isolates of the species were identified by using molecular approach through ITS gene sequence. Many of researchers identified their studied organisms successfully by using partial sequence of the genes. Species identification was crucial in term of safety evaluation for differentiating pathogenic organism or vice versa. Experimental result showed that DNA sequence of the three isolates was amplified successfully at about 750 bp for ITS gene (**Figure 3**). After nBLAST analysis based on the database of genbank, isolate L8 and L20 showed 98% similarity for *L. plantarum*. However, isolate S1 showed 98% similarity for *L. plantarum* and *L. pentosus*. Phylogenetic relationship between the isolates with *L. plantarum* and *L. pentosus* was shown by the dendrogram (**Figure 4**).

Since the inaccurate biochemical tests on the species identification for the isolates, this experiment used PCR method that targeted on ITS gene for species identification of all isolates accurately. Our previous experiment showed that the 16S rRNA gene can identify isolate L8 and L20 which both isolates were *L. plantarum* while isolate S1 was *Lactobacillus pentosus* [13]. However, the result is ambiguous between *L. plantarum* and *L. pentosus* after spatial sequence of isolate S1 was searched by using nBLAST program in GenBank's database. Furthermore, 16S rRNA gene sequence usually used by many researchers for bacteria species identification especially Lactobacillus spp. [22].

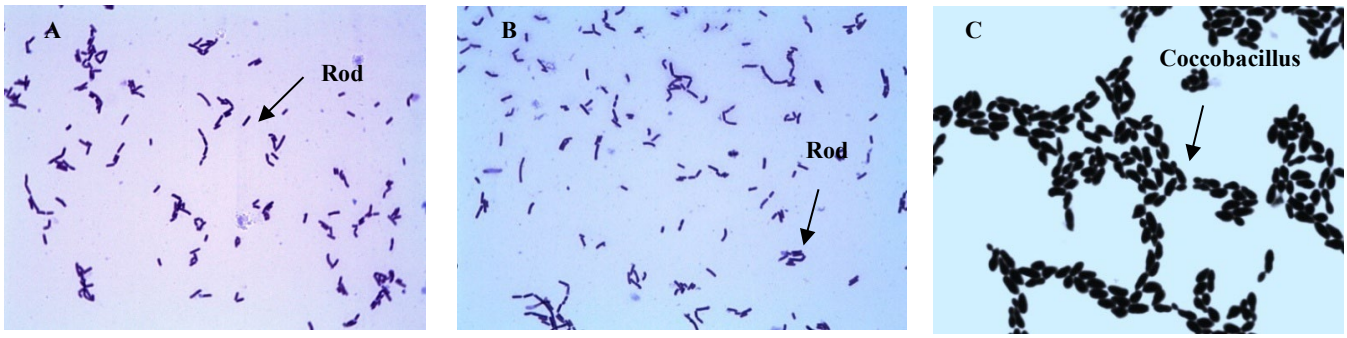

Figure 1. Gram staining results for all isolates under observation of light microscope with 1000X magnification. (A) Isolate L8; (B) Isolate L20; (C) Isolate S1.

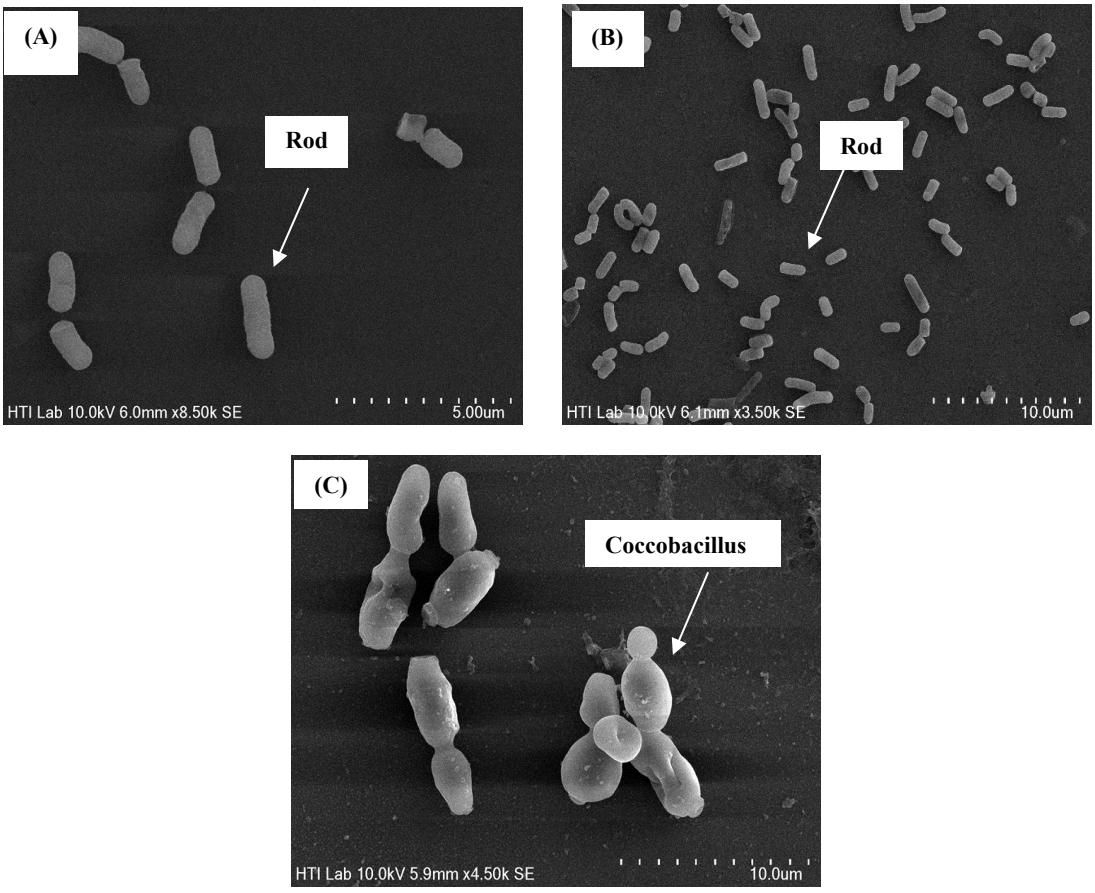

Figure 2. SEM micrographs for all isolates under observation of Scanning Electron Microscopy. (A) Isolate L8; (B) Isolate L20; (C) Isolate S1.

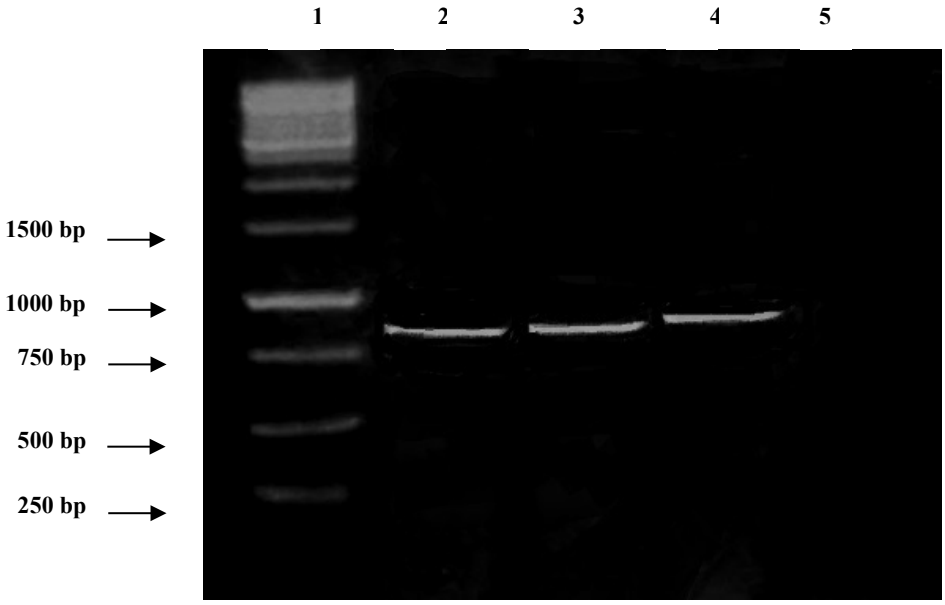

Figure 3. Amplified DNA product of ITS gene showed about 750 bp after using agarose gel electrophoresis. Lane 1 – 1kb DNA ladder; Lane 2 – Isolate L8; Lane 3 – Isolate L20; Lane 4 – Isolate S1; Lane 5 – Distilled water (negative control)

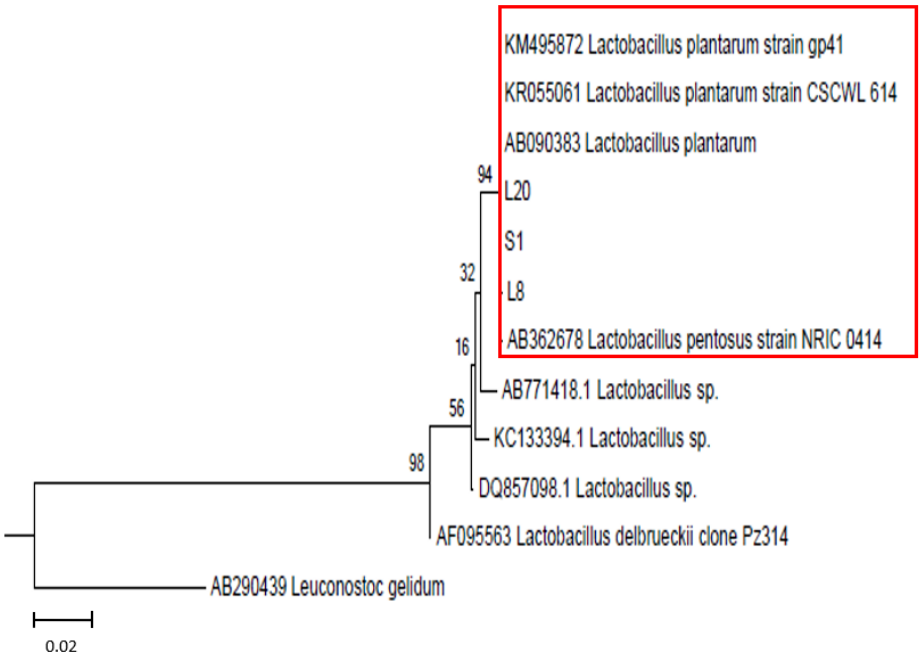

Figure 4. Phylogenetic tree of ITS gene was constructed by using Maximum-likelihood (Chor & Tuller 2005). *L. plantarum* dan *L. pentosus* were in the same monophyletic branch because of highly closely related genotypes.

For further improvement of the identification by PCR amplification, ITS gene can be used as an effective 'tool' for identification and classification of the isolates theoretically because high genetical revolution was conserved by the ITS gene [23]. ITS gene consist of multiple rRNA operon copies that contributed higher variety of sequences for different strain identification among the same species compared with 16S rRNA and 23 rRNA [10]. According to Li et al. (2013) [24], ITS gene have a strong discriminative ability to differentiate closely related species or single species. In addition, ITS gene was considered an important target for fast species differentiation and high interspecific polymorphism of lactococcal isolate identification [10]. Many strains could be discriminated by means of ITS genes,

and the resolution of this method was strikingly higher than that obtained with previously 16s rRNA for *Lactobacillus* strains. However, some researchers not able to identify bacteria species by using amplification of the ITS gene.

Identification and Characterization of Lactobacillus spp. Strain S1 by using *recA* Gene Sequence Analysis

Molecular analysis of ITS gene sequence cannot be identified whether isolate S1 was *L. plantarum* or *L. pentosus*. Therefore, identification and characterization of isolate S1 was identified by using *recA* gene. Experimental result show that DNA sequence of isolate S1 was amplified successfully at about 218 bp for *recA* gene (Figure 5). For identify and characterize isolate S1 by using *recA* gene in this experiment, two different forward primers were used included pentF and planF that targeted on identification of *L. pentosus* and *L. plantarum* respectively. Electrophoresis result (Figure 5) show that isolate S1 was *L. pentosus* based on the successfully amplified DNA product by using the primer pentF compared with isolate S1 that used with primer planF and isolate L8 that used with primer pentF were no DNA product that amplified successfully. In this research, isolate S1 was identified by using *recA* gene and isolate L8 was used as comparison. After the two isolates were tested by using different primers, pentF and planF, result of gel electrophoresis showed that isolate S1 was *L. pentosus* but negative for isolate L8 which proven that isolate L8 was *L. plantarum*. This result was supported by research from Ghotbi et al. (2011) [25] who successfully differentiate *L. plantarum*, *L. pentosus* and *L. paraplantarum* that isolated from Lighvan cheese by using the *recA* gene sequence. Therefore, isolate S1 was identified as *L. pentosus* strain S1. Based on the results, we concluded that probiotic candidates, isolate L8 and L20 were *L. plantarum* while S1 were *L. pentosus.*

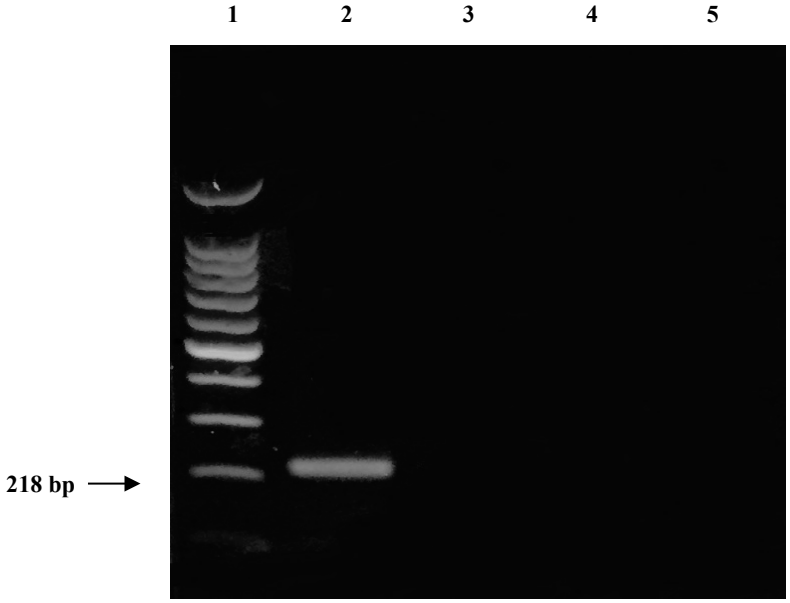

Figure 5. Amplified DNA product of recA gene showed about 218 bp after using agarose gel electrophoresis (1.0%). Lane 1- DNA ladder with 100 bp; Lane 2 – Isolate S1 with primer pentF/pREV; Lane 3 – Isolate S1 with primer planF/pREV; Lane 4 – Isolate L8 with primer pentF/pREV (positive control); Lane 5 – Distilled water (negative control).

Lactobacillus plantarum, *L. pentosus* and *L. paraplantarum* were shared closely related of genotypes and high similarity of phenotypes with each other [17]. Therefore, 16S rRNA sequence are not suitable for identification of the three *Lactobacillus* sp. because high similarity of identity value (>99%) that shared with *L. plantarum* and *L. pentosus* [23]. Consequently, phylogenetic relationship was not feasible because difficulty for identification of the three species [17]. For differentiate the three *Lactobacillus* sp., *recA* gene was suggested as phylogenetic marker [26] which have high discriminative ability for identification of bacteria species that difficultly differentiated, such as *Lactobacillus* sp. included *Bifidobacteria* [18]. Comparison between microscopic observation with sequence analysis by ITS and *recA* gene for the three isolates were shown in Table 1. As a conclusion, the three isolates, L8, L20 and S1 were identified as *Lactobacillus plantarum* strain L8, *L. plantarum* strain L20 and *L. pentosus* strain S1 respectively after microscopic observation and PCR amplification with ITS and *recA* genes. Further analysis will be required for the probiotic application on different fields, especially agriculture and medical.

Table 1. Overall summary of morphological and genotypical analysis for potential probiotic candidate identification

Isolate	Morphology	Taxonomy identification	
		ITS	***recA***
L8	Rod	*Lactobacillus plantarum*	-
L20	Rod	*Lactobacillus plantarum*	-
S1	Coccobacillus	*L. plantarum* / *L. pentosus*	*Lactobacillus pentosus*

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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