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Research

Species Identification of Potential Probiotic Lactic Acid Bacteria Isolated from Malaysian Fermented Food Based on 16S Ribosomal RNA (16S rRNA) and Internal Transcribed Spacer (ITS) Sequences

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ABSTRACT

Taxonomic identification of potential probiotic lactic acid bacteria (LAB) in fermented foods is essential as the microorganisms' benefits are varied and often strain-specific. Using biochemical and physiological methods alone is inadequate to precisely distinguish each strain. In this study, molecular techniques were employed in the identification of 17 LAB isolated from three Malaysian fermented foods: belacan (BE), bosou (BO), and budu (BUM). The respective sizes of polymerase chain reaction (PCR) products from the isolates were approximately 1500 bp and 750 bp when amplified with the 16S ribosomal RNA (16S rRNA) and internal transcribed spacer (ITS) gene primers. The phylogenetic analysis using both gene sequences revealed that all BE and BO isolates were identified as *Lactiplantibacillus plantarum*, while all BUM isolates were identified as *Lacticaseibacillus paracasei.* Both 16S rRNA and ITS genes could disclose the identity of the isolates up to the species level. In summary, the use of the ITS gene in conjunction with the 16S rRNA gene can help with the more effective identification of potential probiotic LAB strains isolated from fermented food.

Key words: 16S rRNA gene, ITS gene, lactic acid bacteria, Malaysian fermented food, PCR

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INTRODUCTION

Despite having an energy-providing diet, consumers have resorted to the functional food category, a diet with adequate nutrients and health benefits such as preventing infections in the digestive system, improving lactose metabolism, reducing cholesterol, and stimulating calcium absorption (Panghal *et al*., 2018). Probiotic foods are an example of a food that falls under this category. Although many efforts have been made to produce food containing probiotics (Perera *et al*., 2017), there are expansions of research in finding probiotics that occur naturally in foods or fermented foods. Examples of probiotic bacteria that have mainly exhibited health advantages include *Lactobacillaceae*, *Bifidobacterium*, *Saccharomyces*, *Enterococcus*, *Streptococcus*, Pediococcus, and *Bacillus* (Fijan 2014). The benefits of probiotics are diverse and strain-specific (Halder *et al*., 2017; Ida Muryany *et al*., 2017; Lim *et al*., 2017). The use of probiotic strains singly or in combinations provides different reactions to the host; some produce a lower impact when used in combinations (Herbel *et al*., 2013). For example, the use of a single strain of *Lactobacillus rhamnosus* GG alone could protect against necrotizing enterocolitis more significantly compared to multi-strain mixtures containing different *Bifidobacterium lactis* strains (McFarland, 2020). Thus, each particular strain must be identified correctly to prevent the lack of probiotic function as desired.

Although numerous microorganisms are found in various fermented foods, they normally fall under the

lactic acid bacteria (LAB) group. Most of the LABs, such as *Lactobacillaceae*, *Pediococcus,* some *Streptococcus*, *Weissella*, and *Enterococcus*, are harmless to humans, making them an ideal agent for food preservation (Martínez-Álvarez *et al*., 2017). LAB consists of Gram-positive bacteria, non-spore-forming, coccus- or rod-shaped, and is most tolerant to low pH values. During the fermentation process, the microorganism will also release various metabolites, including protease, which leads to the major biochemical event, which is the proteolysis of the raw materials (Kilinc *et al*., 2005). The production of these compounds lowers the pH and contributes to the distinct texture and flavor of the fermented food and also their ability to inhibit the growth of pathogens (Nuraida, 2015). As it is a common practice to preserve food, fermentation plays a role in improving the nutritional and functional properties of foods by synthesizing large quantities of peptides and amino acids (Hajeb & Jinap, 2012). Functional foods are foods or food components that offer not only basic nutrition but may also play a role in lowering the risk of certain diseases and health conditions (Wells *et al*., 2017), such as the presence of probiotics, which are commonly found in fermented foods (Bell *et al*., 2017).

The conventional biochemical and physiological methods turn out to have some constraints in distinguishing LAB isolates displaying comparable physiological attributes, which may lead to false identification. The issues and arguments related to biochemical identification include similar strains showing different phenotypic patterns, the non-reactivity of isolates tested, as well as isolates that were not explicitly identified or were misidentified (Herbel *et al*. 2013). Identification using the genotypic approach is more reliable for properly identifying isolates before any biotechnological or industrial applications (Adesulu-Dahunsi *et al*., 2017). For example, in the study by Ida Muryany (2017) using a biochemical identification kit, isolates L8 and S1 were identified as *Bacillus megaterium* and *Pediococcus pentosaceus*, respectively. However, identification using molecular techniques demonstrated that L8 was identified as *Lactobacillus plantarum* while S1 was *Lactobacillus pentosus*. Another study by Diyana-Nadhirah & Ina-Salwany (2016) identified several of their strains as *Aeromonas sobria* using the biochemical test, but molecular identification detected those strains as *Aeromonas hydrophila* and *Aeromonas veronii*, respectively.

In Malaysia, there are several types of fermented aquatic-based products, including belacan, which is made from shrimps, budu, which is made from anchovies, and bosou, which is made by fermenting small fishes. Despite them being favorite dishes by locals, the genotypic identification of potential probiotic LAB isolated from belacan and budu is limited (Liasi *et al*., 2009; Abbasiliasi *et al*., 2011; Sim *et al*., 2012; Haitham, 2017; Khalil *et al*., 2018), while no study has been documented on the molecular identification of LAB isolated from bosou. Therefore, this study aimed to identify the LAB strains found in these samples. All strains were subjected to molecular characterization using two genes, namely the 16S ribosomal RNA (rRNA) gene and the internal transcribed spacer (ITS) gene. The outcomes using both genes were compared to evaluate the most precise species identification.

MATERIALS AND METHODS

LAB strains and growth conditions

Belacan and bosou were purchased from a supermarket in Senawang, Negeri Sembilan, and Tamu Donggongon, Penampang, and Sabah, respectively. The budu was obtained from Perusahaan Warisan Ketereh, Tumpat, Kelantan. The isolation of LAB for all samples was done using MRS agar (Haitham 2017). After 48 h of incubation, the white and creamy colonies with different morphologies and dissolved calcium circles were randomly chosen and transferred onto other MRS agar plates until pure colonies were obtained. The isolates were initially screened for their catalase reactions using the slide method. Each isolate with a negative catalase test was further examined for Gram staining reaction and cell morphology. Only catalase-negative and Gram-positive isolates were identified molecularly. Seventeen LAB strains isolated from belacan (BE3, BE7 & BE16), bosou (BO1, BO8, BO10 & BO16) and budu (BUM5, BUM6, BUM7, BUM12, BUM14, BUM15, BUM18, BUM22, BUM23 & BUM24) were identified using molecular techniques in this study. Each isolate was preserved in 20% glycerol at -20 °C, activated on MRS agar (De Man, Rogosa & Sharpe; Oxoid, UK), and incubated overnight in MRS broth (Oxoid, UK) at 37 °C before use.

DNA extraction

The genomic DNA of each isolate was extracted using the One-Tube Bacterial Genomic DNA Extraction Kit (Bio Basic, Canada) according to the manufacturer's protocol with slight modifications. In brief, the overnight culture grown in MRS broth was adjusted to an optical density of 2.0 at 600 nm. Then, 150 µL of the suspension was transferred into a microcentrifuge tube and centrifuged at 13000 r.p.m for 30 s. The supernatant was discarded before 100 µL of Lysis-Buffer-B was added and vortexed gradually to prevent DNA smearing. The sample was further incubated at 65 °C for 10 min to allow complete digestion. After incubation, 100 µL of Universal Buffer NST was added and vortexed to mix it thoroughly. The mixture was used as a DNA template directly, while the remaining was stored at -20 °C.

Amplification of 16S rRNA and ITS genes

The extracted DNA was used as a template to amplify a segment of the 16S rRNA and ITS genes, respectively, by the polymerase chain reaction (PCR) technique using the universal primers synthesized by Macrogen Laboratory, South Korea (Table 1). The PCR mixture consisted of 0.5 µL of forward and reverse primers, respectively, 1 µL template DNA, and 12.5 µL MyTaq Red Mix (Bioline, UK). The mixture was added to autoclaved distilled water until the volume was 25 µL. The negative controls consisted of all PCR mixture components and autoclaved distilled water to replace the template DNA, while the *Lacticaseibacillus casei* strain Shirota (Yakult, Japan) was used as a reference strain. The PCR reactions were performed using a T100 thermal cycler (Bio-Rad, US) following the standard MyTaq Red Mix protocols. For 16S rRNA gene PCR amplification, the cycle was performed as follows: a cycle of initial denaturation at 95 °C (1 min), followed by 35 cycles of denaturation at 95 °C (15 s), annealing at 50 °C (15 s), and extension at 72 °C (10 s). The final extension was set at 72 °C for 5 min before the reaction ended and was held at 12°C. Meanwhile, amplification conditions for the ITS gene consisted of 1.5 min initial denaturation at 95 °C, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72 °C for 10 s, with a final elongation at 72 °C for 5 min.

Table 1. Universal primers used in DNA amplification

Gene	Universal Primers	Sequences (5'–3')[#]	Fragment size	Reference
16S rRNA	27F	AGAGTTTGATCMTGGCTCAG	1500	Lane (1991)
	1492R	TACGGYTACCTTGTTACGACTT		
ITS	ITS_16SF	CGGTGAATACGTTCCCGGGYCTTG	600-700	Ina-Salwany *et al*.
	ITS_23SR	TTTCRCCTTTCCCTCACGGTA		(2015)

[#]A, adenine; C, cytosine; G, guanine; T, thymine; M, A/C; R, A/G; Y, C/T

PCR product analysis

Electrophoresis's amplicons obtained through PCR reactions were analyzed using HyAgarose™ LE Agarose gel (HydraGene Co., Ltd., China). In the preparation of 1.2% w/v agarose gel, 1.2 g agarose powder was melted in 100 mL of 1× TAE buffer (Promega, US) until dissolved before 3 µL ViSafe Red Gel Stain (Vivantis, Malaysia) was mixed thoroughly. The solidified gel was placed in the gel electrophoresis tank filled with 1× TAE buffer and the gel chamber wells were loaded with 6 µL of amplicons, respectively. To analyze the sizes of DNA samples qualitatively, a 1 kb DNA ladder (Promega, US) mixed with DNA gel loading dye (Thermo Scientific, US) was also loaded as a reference. The agarose gel electrophoresis was run at 85 V for 45 min, and the bands were visualized under the Gel Documentation System (Uvitec Cambridge, UK).

Sequence analysis and phylogenetic tree construction

The PCR products were commercially sequenced by the Macrogen Laboratory (South Korea). The amplified 16S rRNA and ITS gene sequences were further used for the analysis of sequence similarity through the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI). The nucleotide sequences were imported and aligned with MEGA X software (Kumar *et al*., 2018). The isolates were compared to closely related species available in the GenBank database *(http://www.ncbi.nlm.nih.gov/genbank)* and a phylogenetic tree was constructed using the Maximum Likelihood method (Felsenstein, 1981) with 1000 replications. All nucleotide sequences were then deposited into GenBank.

RESULTS

Amplification of probiotic LAB isolates

PCR amplification using primers for 16S rRNA and ITS genes was successfully performed with DNA isolated from all 17 LAB isolates. Both sets of primers used in the study provided good gene amplification for all the isolates. The band patterns of all amplicons and the reference strain for both genes are shown in Figures 1 and 2, respectively. The band size of all amplicons using 27F and 1492R primers corresponded to the expected size of the 16S rRNA gene, which is approximately 1500 bp (Figure 1). In contrast, the band size of amplicons using the ITS_F and ITS_R primers was roughly 750 bp, which matched the ITS gene's size (Figure 2). All gel chamber wells loaded with negative control did not show any bands due to the absence of amplified DNA or contamination of debris, whilst the reference strain showed bands approximately at the same level as the isolated LAB strains.

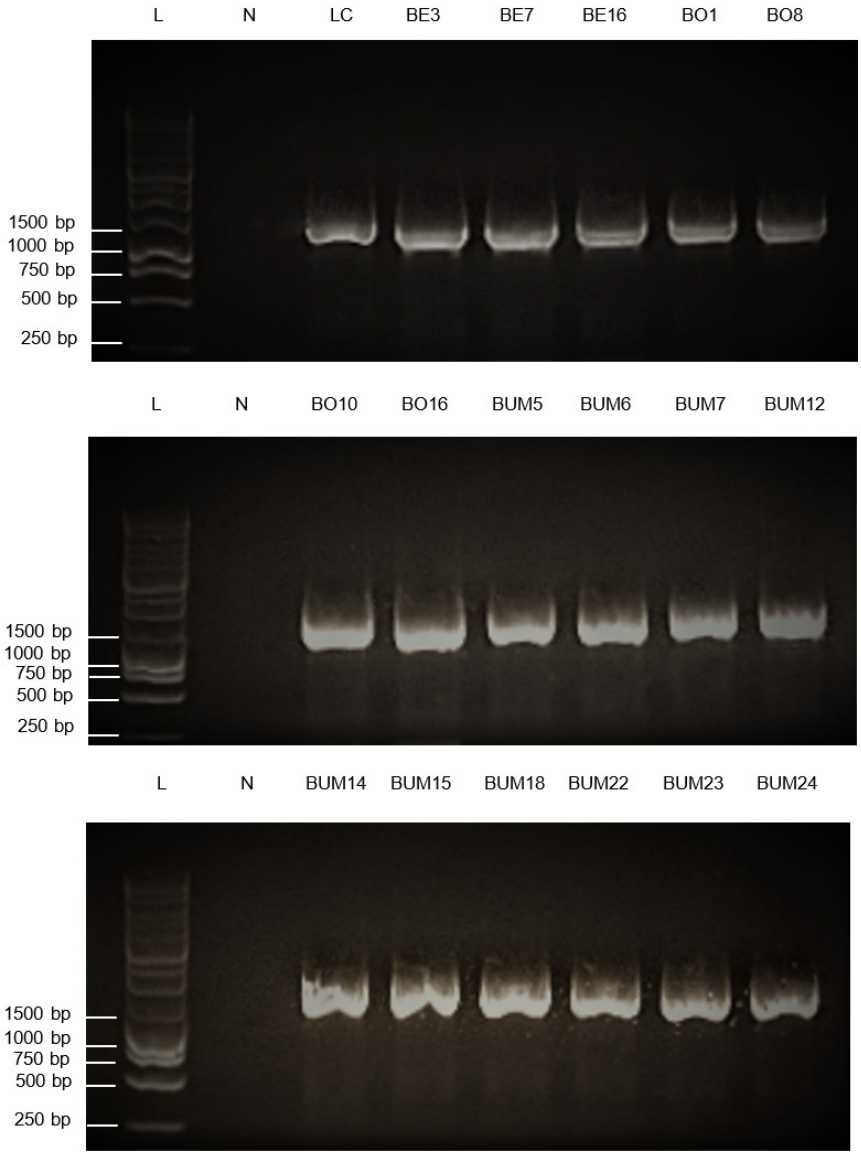

Fig. 1. Agarose gel electrophoresis analysis of PCR amplification of the 16S rRNA gene of BE isolates, BO isolates, BUM isolates, and *L. casei* strain Shirota using 27F and 1492R primers.
L, 1 kb DNA ladder; N, negative control (sterile distilled water); LC, reference strain (*L. casei* strain Shirota).

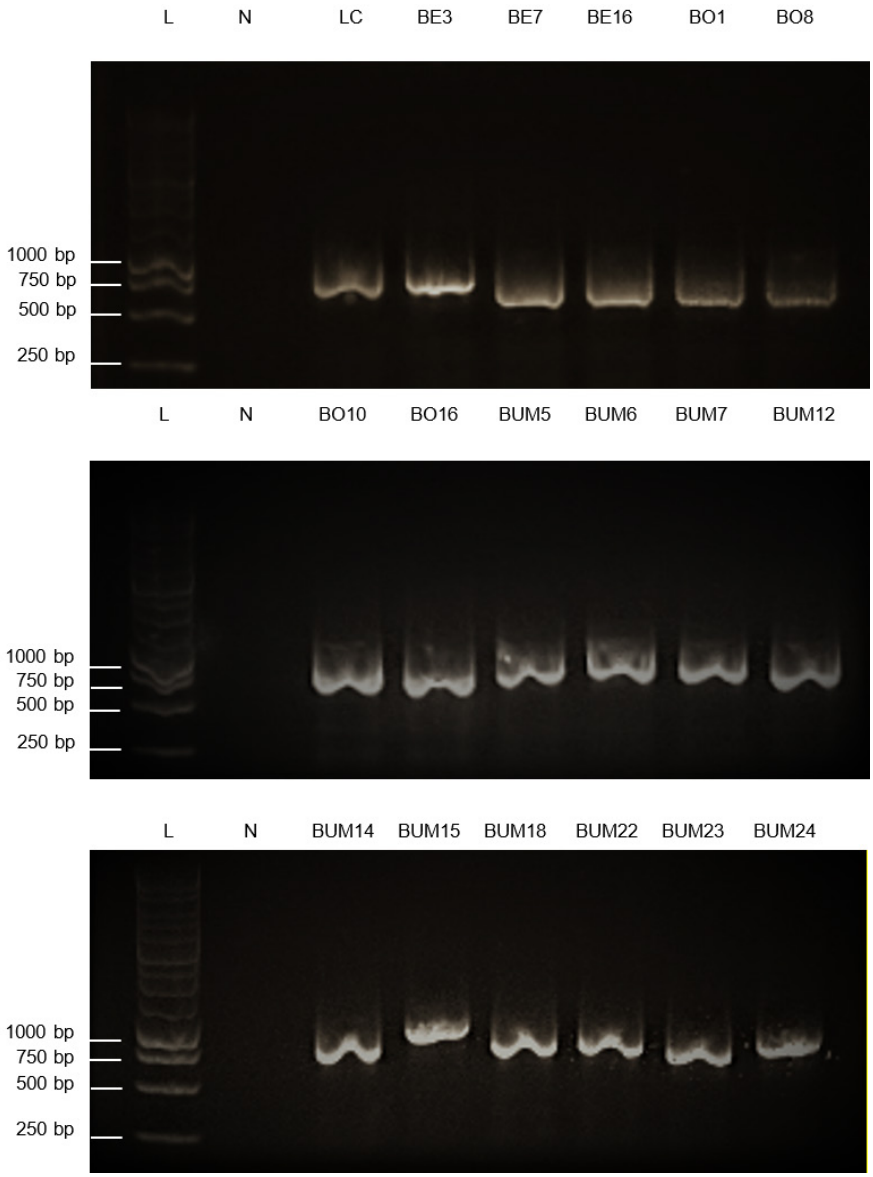

Fig. 2. Agarose gel electrophoresis analysis of PCR amplification of the ITS gene of BE isolates, BO isolates, BUM isolates, and *L. casei* strain Shirota using ITS_F and ITS_R primers.

L, 1kb DNA ladder; N, negative control (sterile distilled water); LC, reference strain (*L. casei* strain Shirota).

Identification and phylogenetic analysis of probiotic lactic acid bacterial isolates using 16S rRNA gene

The results of 17 LAB isolates based on 16S rRNA sequences and their accession numbers are shown in Table 2. The BLASTn results of 16S rRNA gene sequences revealed that all the BE and BO isolates were identified as *Lactiplantibacillus plantarum* with a minimum of 98% similarity to the nucleotide sequences database available in the GenBank. The lineage reports showed that BE and BO isolates recorded a high number of hits (81.2% to 93.5%) on similar species, according to the available taxonomy database. Meanwhile, the BUM strains were identified as *Lacticaseibacillus paracasei* with at least 98% similarity to the GenBank nucleotide sequences database. The lineage reports of BUM strains showed a broader range of the number of hits (58.0% to 87.4%) between the focus strain and the available *L. paracasei* sequences that generated the taxonomy database. A phylogenetic tree of 16S rRNA sequences of the 17 LAB isolates and fourteen *Lactobacillaceae* strains obtained from the Genbank was constructed using MEGA X and displayed in Figure 3. *Staphylococcus aureus* D83357.1 was used as the outgroup. The phylogenetic tree showed that all BUM isolates were in a separate branch from the BE and BO isolates. All BE and BO isolates were grouped with *Lactiplantibacillus plantarum* and *Lactiplantibacillus pentosus* strains from the GenBank (accession no. MZ476213, D79211 & LC638737). Meanwhile, the BUM isolates were clustered together with other *L. paracasei* strains from GenBank (accession no. NR025880 & MW924095).

Table 2. Identified LAB isolates by 16S rRNA gene sequencing with their GenBank accession number

Isolate	Species	Lineage report (%)	Accession number
BE3	*Lactiplantibacillus plantarum*	93.5	MT163337
BE7	*Lactiplantibacillus plantarum*	81.2	MT163338
BE16	*Lactiplantibacillus plantarum*	84.0	MT163339
BO1	*Lactiplantibacillus plantarum*	92.6	MT163340
BO8	*Lactiplantibacillus plantarum*	81.9	MT163358
BO10	*Lactiplantibacillus plantarum*	84.8	MT163341
BO16	*Lactiplantibacillus plantarum*	90.2	MT163342
BUM5	*Lacticaseibacillus paracasei*	65.2	MT163343
BUM6	*Lacticaseibacillus paracasei*	62.6	MT163344
BUM7	*Lacticaseibacillus paracasei*	79.2	MT163345
BUM12	*Lacticaseibacillus paracasei*	58.0	MT163346
BUM14	*Lacticaseibacillus paracasei*	79.4	MT163347
BUM15	*Lacticaseibacillus paracasei*	94.0	MT163359
BUM18	*Lacticaseibacillus paracasei*	69.0	MT163348
BUM22	*Lacticaseibacillus paracasei*	87.4	MT163360
BUM23	*Lacticaseibacillus paracasei*	82.9	MT163349
BUM24	*Lacticaseibacillus paracasei*	81.3	MT163350

Identification and phylogenetic analysis of probiotic lactic acid bacterial isolates using ITS gene

The identified LAB isolates based on ITS gene sequencing and their accession numbers are shown in Table 3. The BLASTn results of ITS gene sequences supported the prior identification of both BE and BO isolates using the 16S rRNA gene, which was identified as *L. plantarum*, with at least 98% similarity to the GenBank nucleotide sequence database. All BUM strains were also identical to *L. paracasei* with a minimum of 98% similarity to the GenBank nucleotide sequence database. The lineage reports revealed that all *L. plantarum* and *L. paracasei* strains showed similar percentages of the number of hits (88.9% & 40.4%) to the taxonomy database available in the GenBank, respectively. A phylogenetic tree of ITS sequences of the 17 LAB isolates and ten *Lactobacillaceae* strains obtained from the Genbank is shown in Figure 4. *S. aureus* DQ256396 was used as the outgroup. The branches formed for BUM isolates were almost identical to those constructed using the 16S rRNA gene, in which the isolates were clustered together with the reference ITS sequence of *L. paracasei* (accession no. AB109028). Meanwhile, BE and BO isolates have formed a separate branch, together with the reference *Lactiplantibacillus plantarum* strain from the GenBank (accession no. AF080101 & AF429622).

DISCUSSION

Research on bacteria with probiotic properties is widely done globally as this microbiota has demonstrated promising benefits (Han *et al*., 2017; Todorov *et al*., 2017; Yasiri *et al*., 2018). In this study, prior identification of the strain isolated from the fermented food was done using molecular techniques. The identification using the 16s rRNA gene confirmed the identity of all BE and BO isolates as different strains of *L. plantarum* species with at least 98% similarity to the GenBank nucleotide sequences database. All BE and BO isolates were further identified using the ITS gene to support the molecular identification using the 16s rRNA gene. Meanwhile, the correct species for BUM isolates was also confirmed by the use of both genes and was identified as a different strain of *L. paracasei*. The identification of these isolates is based on the reclassification of the genus *Lactobacillus* into 25 genera (Zheng *et al*., 2020). It is vital to ensure that identifying each particular strain is done precisely as the specific health advantages and their safety profiles are strain-specific (Fijan, 2014). At the moment, molecular approaches are considered fundamental and crucial methods to identify microorganisms. This method is vital to accurately identifying the genus and species of unknown isolates (Shehata *et al*., 2016; Ida Muryany *et al*., 2017). Identification based on 16S rRNA gene sequencing has been widely utilized to classify strains up to genus level, whilst ITS gene sequencing can discriminate strains up to species level (Diyana-Nadhirah & Ina-Salwany, 2016).

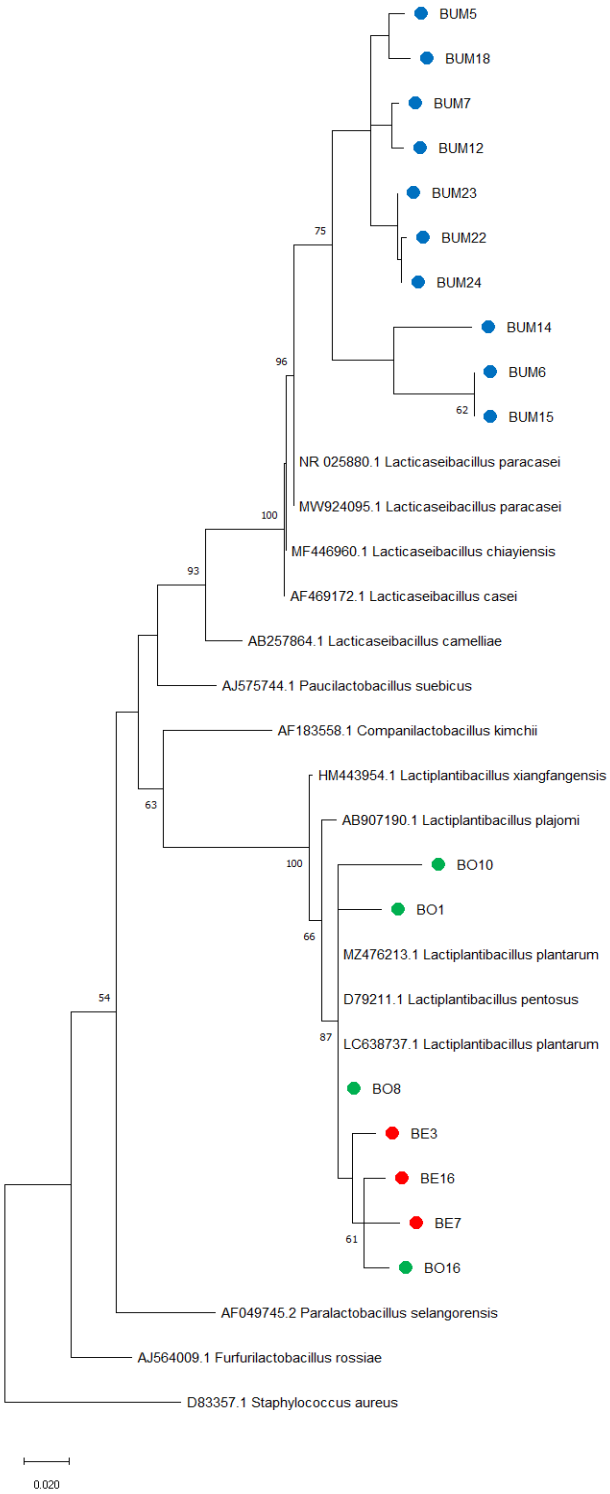

Fig. 3. Maximum-likelihood phylogenetic tree of BE isolates, BO isolates, BUM isolates (dots), and reference strains based on the 16S rRNA gene sequences. Bootstrap values derived from 1000 replications are shown at nodes

Table 3. Identified LAB isolates by ITS gene sequencing with their GenBank accession number

Isolate	*Species*	*Lineage report (%)*	*Accession number*
BE3	*Lactiplantibacillus plantarum*	*88.9*	*MT212063*
BE7	*Lactiplantibacillus plantarum*	*88.9*	*MT212064*
BE16	*Lactiplantibacillus plantarum*	*88.9*	*MT212065*
BO1	*Lactiplantibacillus plantarum*	*88.9*	*MT212080*
BO8	*Lactiplantibacillus plantarum*	*88.9*	*MT212081*
BO10	*Lactiplantibacillus plantarum*	*88.9*	*MT212082*
BO16	*Lactiplantibacillus plantarum*	*88.9*	*MT212083*
BUM5	*Lacticaseibacillus paracasei*	*40.4*	*MT212055*
BUM6	*Lacticaseibacillus paracasei*	*40.4*	*MT995194*
BUM7	*Lacticaseibacillus paracasei*	*40.4*	*MT212056*
BUM12	*Lacticaseibacillus paracasei*	*40.4*	*MT212057*
BUM14	*Lacticaseibacillus paracasei*	*40.4*	*MT212058*
BUM15	*Lacticaseibacillus paracasei*	*40.4*	*MT212059*
BUM18	*Lacticaseibacillus paracasei*	*40.4*	*MT212060*
BUM22	*Lacticaseibacillus paracasei*	*40.4*	*MT212061*
BUM23	*Lacticaseibacillus paracasei*	*40.4*	*MT995195*
BUM24	*Lacticaseibacillus paracasei*	*40.4*	*MT212062*

The absence of other LAB species in belacan, bosou, and budu might be due to their inability to grow in the food samples or MRS broth during prior isolation of LAB. This may be due to the food conditions that are not suitable and may not support the growth of other LAB isolates, for example, its pH values and sodium contents, or the MRS broth that is not modified to enhance their growth. Moreover, the *L. plantarum* found in belacan and bosou, as well as *L. paracasei* isolated from budu, might be dominant compared to the other species and might have entirely inhabited the samples (Haitham, 2017). This view has supported the study by Kopermsub & Yunchalard (2010) who reported that the early stages of the Thai fermented fish making were dominated by *Lactococcus garvieae, Streptococcus bovis,* and *Weissella cibaria*. However, *Lactiplantibacillus plantarum* started to become prevalent after a certain period before dominating the samples until the completion of fermentation. Lactobacilli are non-spore-forming gram-positive and rod-shaped bacteria, with facultatively anaerobic or microaerophilic, acid-tolerant, and catalase-negative characteristics (Huang *et al*., 2018). Organisms that fall under the *Lactobacillaceae* group are widely used as probiotics, such as *L. casei* and *Lactobacillus acidophilus*, found in commercial probiotic drinks Yakult and Vitagen. The isolation of LAB under the *Lactobacillaceae* group from fermented aquatic-based products is not surprising. Previously, Khalil *et al*. (2018) managed to isolate *Lactobacillus fermentum*, a strain that produced exopolysaccharides from budu, different from the outcome of our study which isolated *L. paracasei* from a similar type of food. Haitham (2017) was able to isolate thirteen probiotic LABs from belacan, which were identified as *L. plantarum* and *L. acidophilus,* besides two other species from different genera named *Enterococcus faecium* and *Pediococcus acidilactici*. However, our study was able to isolate multiple strains of *L. plantarum.*

Genotypic characterization of bacteria based on 16S rRNA gene sequence has been used broadly since it exists in nearly all bacteria; it has a sluggish evolutionary rate and constant role, as well as the great size of a gene, which is roughly 1500 bp, which was adequate for bioinformatic purposes (Janda & Abbott 2007). The use of this gene is considered a common and fundamental method for the molecular identification and classification of bacteria. However, the gene is highly conserved, thus a high similarity index of the 16S rRNA gene sequence could affect the identification of bacteria at the genus and/or species level (Tokajian *et al*. 2016). The impact can be seen more obviously if the studied bacteria of interest are phylogenetically very closely related to one another. Due to this difficulty, another phylogenetic marker is needed to correctly identify bacterial species. The ITS region is positioned in the middle of the small-subunit rRNA and large-subunit rRNA genes (Felsberg *et al*., 2015). There is only a single ITS presence in bacteria, located between the 16S rRNA and the 23S rRNA. Thus, it is also called the 16S-23S intergenic spacer region (Liguori *et al*., 2011). When compared to 16S and 23S rRNA genes, the ITS region was the shortest yet the most discriminatory region for identification, differentiation, and systematic analysis of bacterial species and strains (Song *et al*., 2000; Man *et al*., 2010). The ITS region's variability might be due to differences in the base length and sequences, thus offering better information for identifying bacteria (Tokajian *et al*., 2016). Variations found in ITS sequences are sufficient to surmount the 16S rRNA gene sequence's inadequacy in resolving closely related isolates. As most bacteria retain multiple alleles of the ribosomal operon in their complete genome, a significant sequence variation in the ITS region may exist in a different strain of the same species (Felsberg *et al*., 2015). This study's results are consistent with these arguments; the ITS gene's use seemed to be suitable for all BE, BO, and BUM isolate strains' identification and differentiation.

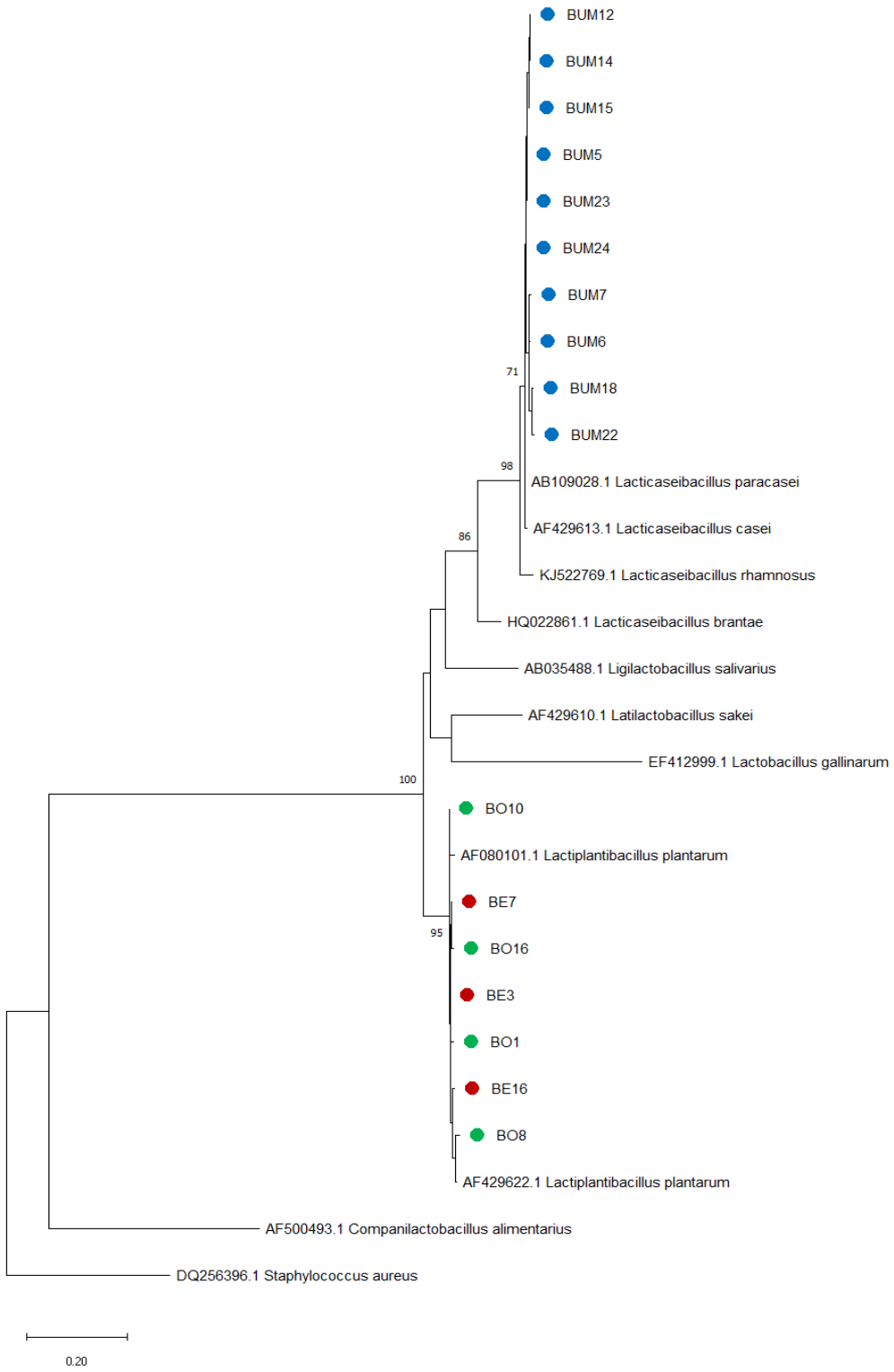

Fig. 4. Maximum-likelihood phylogenetic tree of BE isolates, BO isolates, BUM isolates (dots), and reference strains based on the ITS gene sequences. Bootstrap values derived from 1000 replications are shown at nodes

The ITS gene has been reported as a marker to identify bacterial species other than the 16S rRNA gene. Earlier, Song *et al*. (2000) were able to sequence the ITS region of several *Lactobacillus* species usually found in the human intestine, with amplicons sized between 600 and 700 bp. In another study by Diyana-Nadhirah and Ina-Salwany (2016), the ITS region's amplification on all *Aeromonas* spp. strains isolated from freshwater fishes produced an amplicon of between 1000 and 1200 bp. The different amplicon sizes may be due to the copy number of ribosomal units and tRNA-encoding genes within ITS (Singh *et al*., 2012). Nevertheless, prior molecular identification using the 16S rRNA gene is still needed as the sequences obtained must be matched to the available sequence from the GenBank database for correct identification. The fact that should be emphasized is that the absence of comparable sequences in the GenBank database will cause lower disability to identify the microorganism, as depicted by the lower number of hits in the lineage reports for BUM strains using the ITS gene, compared to the percentages using the 16s rRNA gene.

As variation in lengths and sequences of ITS regions occurs in different bacteria species, this sequence can also be used to design species-specific PCR primers. For example, Chiu *et al*. (2005) have designed PCR primers from the ITS region that are highly sensitive and specific for *Salmonella* spp. but generate negative results for non-*Salmonella* strains. Another study by Dang *et al*. (2012) indicated that their designed primers from the ITS regions could detect and discriminate *L. garvieae* strains from other closely related species isolated from fish and aquaculture environments. However, the primer pairs used in this study are general and non-species specific, which could detect various species from multiple genera such as *Paenibacillus* spp., *Paenibacillus pabuli, Aeromonas A. hydrophila, Aeromonas A. veronii,* and *Bacillus amyloliquefacien* (Ina-Salwany *et al*., 2015; Diyana-Nadhirah & Ina-Salwany, 2016; Azrin *et al*., 2017), as well as the *Lactobacillaceae* strains which have been identified from the BE, BO, and BUM isolat.

CONCLUSION

In conclusion, the study confirmed the presence of LAB strains found in Malaysian fermented foods named belacan, bosou, and budu. The BE and BO isolates were classified as different strains of *Lactiplantibacillus L. plantarum*, and the ten BUM strains were identical to *L. paracasei*, all with at least 98% similarity to the GenBank nucleotide sequence database. There is an association of results obtained by 16S rRNA and ITS genes for the identification of BE, BO, and BUM isolates. The ITS gene, which is not commonly used in the molecular identification of bacterial strains, could effectively identify the *Lactobacillaceae* strains up to the species level. More research is required to unravel the potential probiotic characteristics of each strain and its potential to be applied as a nutraceutical food.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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