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Characterisation of *Lactobacillus plantarum* isolated from pickled cucumber, and its antagonist effect on pathogenic bacteria

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Abstract

Lactic acid bacteria (LAB) have the potentials to produce antimicrobial peptides against pathogens, and grow on traditional fermented foods such as pickled cucumber, which is widely consumed in Indonesia. LAB convert carbohydrates into organic acids, while hydrogen peroxide and antimicrobial peptides inhibit the growth of spoilage bacteria. The present work aimed to identify the antibacterial activity of LAB strains isolated from pickled cucumber. The strains were morphologically and physiologically identified, and selected based on their ability to inhibit the indicator bacteria using the well-diffusion agar method. Furthermore, the identification based on biochemical and molecular tests were carried out on strains with antibacterial activity. The present work isolated four LAB strains, namely S1K2T1, S5K7T1, S5K8T1, and S10.2K6, with antibacterial activities which varied from medium to strong. Strains S1K2T1, S5K7T1, S5K8T1, and S10.2K6 had inhibitory activity against pathogenic bacteria. Strain S1K2T1 had strong inhibition against *Sta. aureus* ATCC 2592.3. Strain S5K7T1 had strong inhibition against *Sal.* Typhi BPE 122.4 CCA. Strain S10.2K6 had strong inhibition against *P. putida* FNCC 0071. Strain S5K8T1 had medium inhibition against both *B. subtilis* ATCC 6633 and *P. putida* FNCC 0071. The four LAB strains were identified as *Lactobacillus plantarum* based on API 50CHL test and phylogenetic analysis of 16S rRNA genes. The plnA gene was detected in the four strains, and identified as a bacteriocin protein from *L. plantarum.*

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Introduction

Lactic acid bacteria (LAB) play an important role in producing agricultural commodities that are safe and delicious for human consumption. Besides acting as a bio-preservative, they also reduce the risk of food-borne diseases, inhibit the growth of pathogenic bacteria, and decrease the formation of biogenic amines (Swetwiwathana and Visessanguan, 2015; Özogul and Hamed, 2017). Lactic acid bacteria are also a major part of the commensal microbial flora of the human digestive tract which play a key role in strengthening the host defence system by inducing an immune response. The bacteria also have a strong resistance to changes in the gastrointestinal environment. They could also increase the absorption rate of fats, proteins, and minerals (calcium, magnesium, iron) in the large intestine. They also produce vitamins such as thiamine, riboflavin, niacin, biotin, pantothenic acid, and folate (Ishiguro *et al.,* 2018). Several studies have shown that LAB and fermented food products are very effective in protecting the body from intestinal pathogen infections, preventing gastric mucosal lesions, and increasing innate immunity (Choi *et al.,* 2015; Aoudia *et al.,* 2016).

Lactic acid bacteria are generally found in traditional fermented foods in most Asian countries, including Indonesia. Some of these bacteria include *Lactobacillus, Pediococcus, Lactococcus, Enterococcus, Weisella,* and *Leuconostoc* (Nuraida, 2015). Lactic acid bacteria could be found as probiotic bacteria in pickled cucumbers, which are among the popular fermented food products consumed in Indonesia. Pickled cucumbers contain nutrients that are beneficial to human health (Naganatha and Hartline, 2015). This vegetable product is generally processed through spontaneous fermentation. During fermentation, LAB convert carbohydrates into organic acids which lead to a pH reduction. The addition of herbs such as salt and sugar during fermentation inhibits the growth of other bacterial contaminants (Paulova *et al.,* 2013; Roy and Ray, 2017). Furthermore, during fermentation, LAB

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produce antimicrobial compounds such as organic acids, hydrogen peroxide, low molecular weight metabolites, and bacteriocin which also aid in inhibiting the growth of pathogenic and spoilage microorganisms (Crispim et al., 2013). Besides inhibiting pathogenic and spoilage microorganisms, LAB also play a role in providing taste, aroma, visual appearance, texture, shelf life, product safety, and nutrition to a food product (Badr *et al.,* 2005; Crispim *et al.,* 2013; Paulova *et al.,* 2013).

Within the LAB group, *Lactobacillus plantarum* has a positive effect on health. *Lactobacillus plantarum* strain 299v has numerous positive benefits against cardiovascular diseases, pancreatic and respiratory infections, gynaecological effects, modulation of immunity in the digestive tract, as well as metabolic and dermatological effects (Darby and Jones, 2017). The test results of *L. plantarum* strain ZLP001 showed its ability to secrete antimicrobial peptides to fight pathogens, modulate the immune system, and prevent intestinal permeability induced by enterotoxigenic *Escherichia coli* (ETEC; Wang *et al.,* 2018). Similarly, *L. plantarum* strain KL-1 isolated from fermented meat has been proven to produce plantaricin which has greater activity than niacin. This bacterium can inhibit pathogenic bacteria such as *Salmonella enterica* serovar Enteritidis DMST 17368, *Pseudomonas aeruginosa* ATCC 15442 and 9027, *E. coli* O157:H7 and *E. coli* ATCC 8739, *Bacillus cereus* JCM 2152T, and *Staphylococcus aureus* TTR 118 (Rumjuankiat *et al.,* 2015).

Indonesia has a diversity of fermented traditional food products comprising of LAB, such as pickled cucumbers. This food is widely used by the majority of Indonesians as a side dish or flavour enhancer in various fast foods. Therefore, it is important to explore the LAB contained in pickled cucumbers to obtain potential strains as probiotic candidates against pathogenic bacteria. This study identifies lactic acid bacteria from the pickled cucumber with antibacterial activity. Lactic acid bacteria isolates proven to inhibit pathogens are used as probiotic strain candidates; therefore, the processed cucumber products have more benefits and economic value.

Materials and methods

Sampling

A total of 10 fresh pickled cucumber samples were obtained from street vendors in Yogyakarta, Indonesia. Each sample was taken three times, and stored at room temperature for 48 - 72 h. This led to the occurrence of a spontaneous fermentation process, thereby allowing the LAB to grow and develop.

Isolation of lactic acid bacteria

The isolation of lactic acid bacteria from pickled cucumber samples was carried out following the procedures described by Roy and Rai (2017). Approximately, 10 g of the sample was inoculated into 90 mL of de Man Rogosa, and Sharpe (MRS, Oxoid, UK) broth enriched with 0.1% NaCl (Merck, Germany). Inoculated broth was incubated at 37°C for 24 h. The inoculum of MRS broth was diluted to 10^{-7} in 0.1% NaCl. Next, approximately 0.1 mL of the last three dilutions (10^{-5}, 10^{-6}, and 10^{-7}) were cultured on MRS agar (Oxoid, UK) enriched with 1% (w/v) $CaCO_3$ (Merck, Germany). Initial screening was conducted based on the phenotypic analysis, including colony morphology, Gram reaction, cell morphology, gas production, growth at temperatures of 10, 30, 37, and 45°C; motility, and catalase reaction (Sneath *et al.,* 2009; Khemariya *et al.,* 2016)

Antibacterial activity test

Cell-free culture supernatant preparation

Lactic acid bacteria strains were cultured in MRS broth (pH 6.5) at 37°C for 18 h. The supernatant was harvested through a 10,000 rpm centrifugation process at 4°C for 10 min. The cell-free culture supernatant (CFCS) was stored in a refrigerator before being used (Baloch *et al.,* 2015).

Pathogenic bacterial culture

Pathogenic bacteria used for antibacterial activity tests consisted of Gram-negative and positive pathogens. Gram-negative bacteria included *Salmonella* Typhi NCTC 786 (PT. Bio Farma, Indonesia), *Sal.* Typhi BPE 122.4 CCA and *Sal.* Typhi BPE 127.1 MC, which were obtained from previous studies (Amarantini *et al.,* 2011; Amarantini and Satwika, 2015), as well as *Sal.* Typhimurium FNCC 0050 and *Pseudomonas putida* FNCC 0071, which were obtained from the Food and Nutrition Culture Collection, Gadjah Mada University, Yogyakarta, Indonesia. Gram-positive pathogenic bacteria used included *Staphylococcus aureus* ATCC 2592.3 and *Bacillus subtilis* ATCC 6633 (non-pathogenic). Each bacterium was cultured in the Brain Heart Infusion (BHI, Merck, Germany) broth at 37°C for 18 h, except for the *P. putida* FNCC 0071 which was cultured at 30°C.

Antimicrobial activity of lactic acid bacteria against pathogens

The antibacterial activity of the LAB strains was examined by the well-diffusion agar method. The indicator bacteria were cultured on Mueller-Hinton Agar (MHA, Oxoid, UK), using sterile cotton

swabs. The CFCS was inserted into the well (8 mm) and incubated according to the pathogen's growth temperature for 24 h. This experiment was carried out three times. Antibacterial activities were observed from the diameter of the clear zone formed around the well (Klayraung and Okonogi, 2009; Fernandez *et al.,* 2013; Baloch *et al.,* 2015). The activity was calculated using Eq. 1:

$$x = D - d \quad \text{(Eq. 1)}$$

where, D = diameter of inhibition zone (mm), and d = diameter of the well (mm). The x value represents the antimicrobial activity (Hu *et al.,* 2019).

Identification of lactic acid bacteria based on biochemical characters using API 50 CHL kit

The LAB strains with antimicrobial activities were selected against several pathogens and identified using the 50CHL Analytical Profile Index (API) kit (BioMérieux, USA). The identification process was carried out based on the fermentation profile of 49 types of carbohydrates according to the procedures used in the API 50CHL test (Martinez *et al.,* 2013).

Molecular characterisation

Molecular characterisation was conducted to detect the plantaricin A (plnA gene) encoding genes (Remiger *et al.,* 1996), and their identification was based on 16S rRNA (Batdorj *et al.,* 2006; Shi *et al.,* 2012).

DNA isolation

DNA was extracted from LAB strains with antibacterial activity against indicator bacteria using the Geneaid kit (Geneaid Biotech Ltd., Taiwan), and following the manufacturer's instruction with slight modification (the addition of ciprofloxacin at the sample preparation stage).

Plantaricin gene detection

The primary pairs of plnA5p (5'-gTACAg-TACTAATgggAG-3') and S7 (5'-CTTACgCCATC-TATACg-3 ') were used to detect plantaricin A (450 bp). The amplification of bacteriocin genes was carried out under PCR conditions with an initial denaturation at 94°C for 3 min, followed by 35 PCR cycles consisting of denaturation at 94°C for 30 s, annealing at 53°C for 1 min, and elongation at 72°C for 1 min. The process ended with a final extension stage at 72°C for 7 min (Remiger *et al.,* 1996).

Molecular identification based on the 16S rRNA gene

DNA was amplified with 1,543 bp using the primary pair fD1 (5'-AgAgTTTgATCCTggCT-CAG-3 ') and rD1 (5'-TAAggAggTgATC-CAgCC-3'). The amplification process comprised of initial denaturation at 94°C for 5 min, followed by 35 PCR cycles at 94°C for 1 min, annealing at 56°C for 1 min, and an extension stage at 72°C for 1 min. The process ended with a final extension stage at 72°C for 7 min (Weisburg *et al.,* 1991; Batdorj *et al.,* 2006).

Statistical analysis

Data were analysed using Microsoft Excel, and expressed as mean ± standard deviation.

Results

Screening of lactic acid bacteria inhibitory potential against pathogenic bacteria

Lactic acid bacteria were cultured on MRS agar enriched using 1% $CaCO_3$. A single colony with the ability to produce strong acids characterised by the formation of clear zones was used for the screening process. A total of 10 of the 49 colonies were selected based on the characteristics of *Lactobacillus*, such as Gram-positive, rod-shaped, homo-fermentative, grow at 10, 30, 37, and 45°C, non-motile, and catalase negative. Four out of the 10 isolates were selected based on the inhibition against pathogens as shown in Table 1. Strain S1K2T1 had inhibitory ability against all indicator bacteria used, and had strong inhibition against *Sta. aureus* ATCC 2592. Strain S5K7T1 had strong inhibition against *Sal.* Typhi BPE 122.4.CCA, and medium inhibition against *B. subtilis* ATCC 6633. Strain S10.2K6 had strong inhibition against *P. putida* FNCC 0071, and medium inhibition against *Sal.* Typhi NCTC 786. Finally, strain S5K8T1 had medium inhibition against *B. subtilis* ATCC 6633 and *P. putida* FNCC 0071.

Identification of Lactobacillus plantarum based on API 50CHL system

The identification of LAB strains isolated from pickled cucumbers using the API 50CHL system showed that strains S1K2T1, S5K7T1, S5K8T1, and S10.2K6 were in fact *L. plantarum* with a similarity index > 99%. These four strains had the ability to ferment 23 kinds of sugars, namely: L-arabinose, ribose, galactose, glucose, fucose, mannose, mannitol, sorbitol, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melezitose, raffinose,

Table 1. Morphology, physiology, and inhibitory zones of antibacterial activity tests of lactic acid bacteria from pickled cucumber.

LAB isolate	Colony diameter (mm)	Diameter of transparent zone (mm)	Gram reaction	Cell morphology	Gas production	Growth at temperature (°C)				Motility	Catalase reaction	Inhibitory zones (mm) of pathogenic bacteria (mean ± SD)						
						10	30	37	45			A	B	C	D	E	F	G
S1K2T1	± 2	± 6	(+)	Rod	Homo	+	+	+	+	-	-	9.00 ± 1.00	11.00 ± 1.41*	3.67 ± 0.58	6.33 ± 0.58	9.50 ± 0.71	4.33 ± 0.58	3.67 ± 0.58
S5K7T1	± 3	± 5	(+)	Rod	Homo	+	+	+	+	-	-	8.17 ± 0.29	0.00	6.67 ± 0.58	12.50 ± 2.12*	0.00	0.00	0.00
S5K8T1	± 4	± 7	(+)	Rod	Homo	+	+	+	+	-	-	8.17 ± 0.29	0.00	6.67 ± 0.58	0.00	0.00	0.00	0.00
S10.2K6	± 2	± 5	(+)	Rod	Homo	+	+	+	+	-	-	0.00	0.00	10.00 ± 1.41*	0.00	0.00	0.00	8.00 ± 1.00

* = strong inhibition (according to Davis and Stout, 1971); A = *Bacillus subtilis* ATCC 6633; B = *Staphylococcus aureus* ATCC 2592; C = *Pseudomonas putida* FNCC 0071; D = *Salmonella* Typhi BPE 122.4.CCA; E = *Salmonella* Typhi BPE 127.1.MC; F = *Salmonella* Typhimurium FNCC 0050; and G = *Salmonella* Typhi NCTC 786.

B-gentibiose, and D-turanose. Rhamnose and gluconate can only be fermented by *L. plantarum* S1K2T1 and *L. plantarum* S10.2K6, while, methyl-D-mannose sugar can only be fermented by *L. plantarum* S5K7T1 and *L. plantarum* S5K8T1. In addition, raffinose can only be fermented by *L. plantarum* S1K2T1, S5K7T1, and S5K8T1.

Molecular characterisation of Lactobacillus plantarum

The antibacterial potential of *L. plantarum* S1K2T1, S5K7T1, S5K8T1, and S10.2K6 were detected molecularly based on plantaricin encoding gene (plnA gene). Figure 1 shows the results of 450 bp DNA fragment amplification, which is a protein amplicon encoded by the plnA gene found in *L. plantarum* ATCC 8014, and *Pediococcus acidilactici* PAF 11 reference isolates. The molecular characterisation based on DNA sequences coding for plnA genes showed that the four isolated strains had similarity with *L. plantarum*. The plnA gene is a bacteriocin protein from *L. plantarum* and similar with the sequences published with accession numbers WP_046947768.1 and WP_057136843.1, as shown in Table 2.

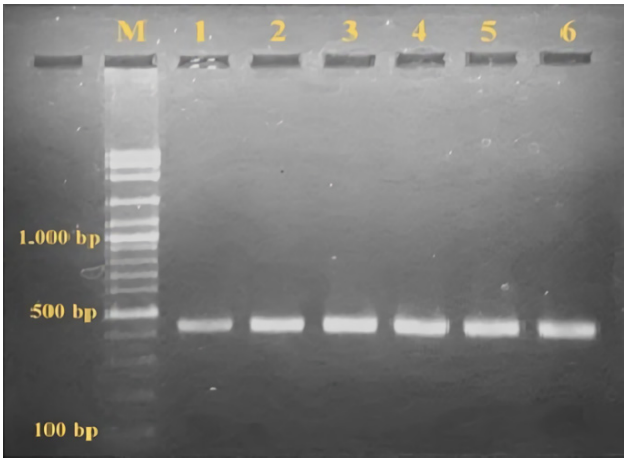

Figure 1. The results of plnA-gene amplification of *Lactobacillus plantarum* isolated from pickled cucumber. M = Marker 1 Kb DNA Ladder (Geneaid); 1 = *Lactobacillus plantarum* ATCC 8014; 2 = *Pediococcus acidilactici* PAF 11; 3 = *L. plantarum* S1K2T1; 4 = *L. plantarum* S5K7T1; 5 = *L. plantarum* S5K8T1; and 6 = *L. plantarum* S10.2K6.

Figure 2 shows that the results of the 16S rRNA sequence analysis were in the cluster of *L. plantarum* with 99% similarity to 16S rRNA sequences published from its member strains.

Table 2. BLAST result of plantaricin A gene of lactic acid bacteria isolated from pickled cucumber.

Isolate	Description of protein/species homolog	Percent similarity	Accession
S1K2T1	bacteriocin [*Lactobacillus plantarum*]	100%	WP_046947768.1
	bacteriocin [*Lactobacillus plantarum*]	100%	WP_057136843.1
	bacteriocin [*Lactobacillus plantarum*]	97.37%	WP_065980438.1
S5K7T1	bacteriocin [*Lactobacillus plantarum*]	100%	WP_046947768.1
	bacteriocin [*Lactobacillus plantarum*]	100%	WP_057136843.1
	bacteriocin [*Lactobacillus plantarum*]	97.37%	WP_065980438.1
S5K8T1	bacteriocin [*Lactobacillus plantarum*]	100%	WP_046947768.1
	bacteriocin [*Lactobacillus plantarum*]	100%	WP_057136843.1
	bacteriocin [*Lactobacillus plantarum*]	97.37%	WP_065980438.1
S10.2K6	bacteriocin [*Lactobacillus plantarum*]	100%	WP_046947768.1
	bacteriocin [*Lactobacillus plantarum*]	100%	WP_057136843.1
	bacteriocin [*Lactobacillus plantarum*]	97.37%	WP_065980438.1

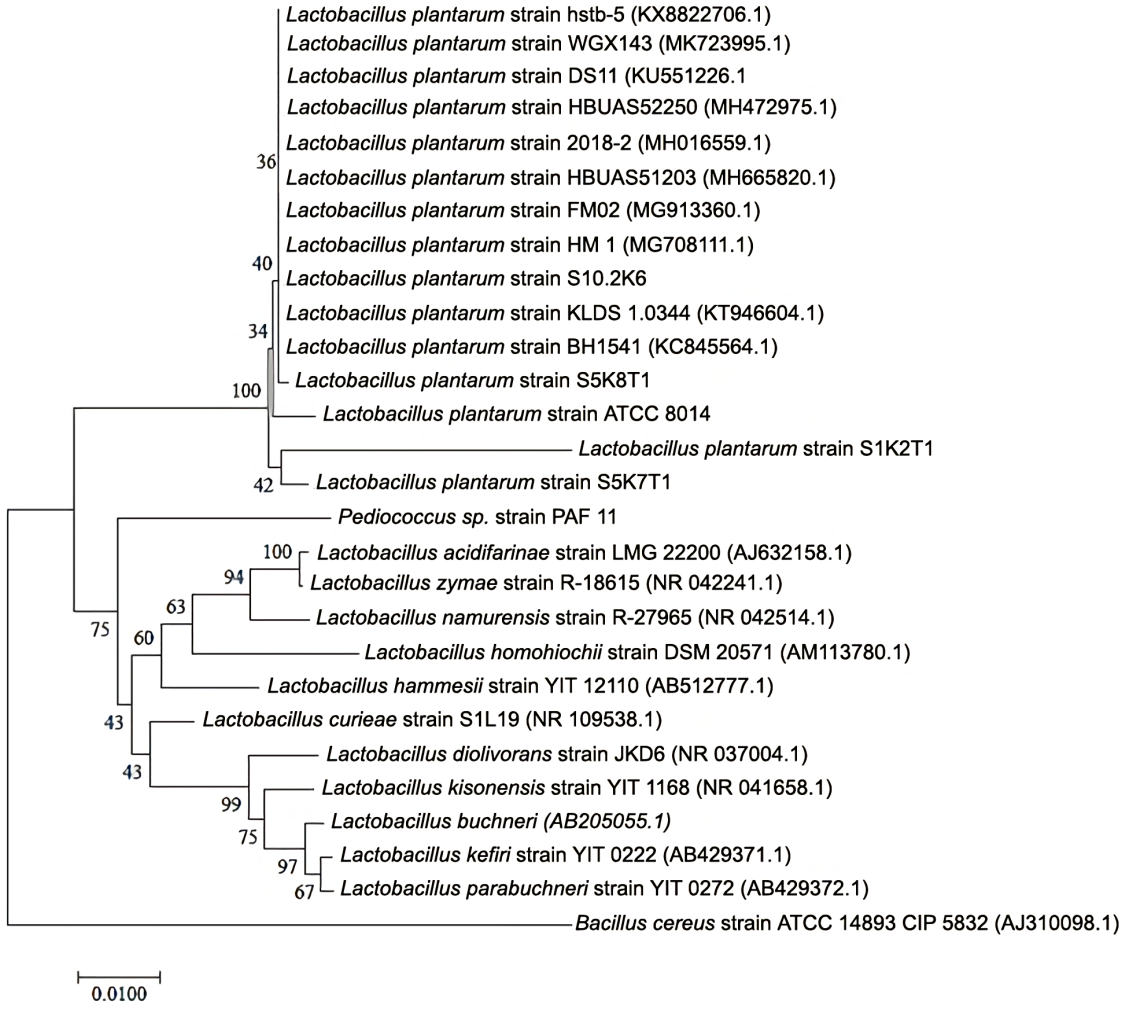

Figure 2. Phylogenetic relationship between lactic acid bacteria isolated from pickled cucumbers and species of lactic acid bacteria belonging to the genus *Lactobacillus* using maximum like-hood (bootstrap = 1000).

Discussion

Screening of lactic acid bacteria inhibitory potential against pathogenic bacteria

Lactic acid bacteria screening was carried out on 10 pickled cucumber samples. They were first incubated at room temperature for approximately 72 h for the fermentation to occur and to obtain maximum amount of LAB. Isolation was carried out using the MRS agar medium enriched with $CaCO_3$ and containing LAB growth material. According to Huang *et al.* (2005), $CaCO_3$ was added to the medium to maintain acidity; therefore, optimal growth and metabolism were achieved. $CaCO_3$ is a basic compound capable of neutralising the acid produced by the LAB. The selected colony was round, and yellowish-white in colour, with a small diameter (0.5 - 2.0 mm). Phenotypic analysis was carried out to determine the cell morphology and physiological characteristics of the isolates. Isolates with Gram-positive reaction, short stem cells (rods), homo-fermentative, growth temperatures of 10, 30, 37, and 45°C, non-motile, and catalase negative were selected for further testing. The cell morphology followed the results of research conducted by Sneath *et al.* (2009) and Khemariya *et al.* (2016).

Screening for LAB isolates was continued based on their ability to inhibit the growth of indicator bacteria. Table 1 shows that a total of four LAB strains had antibacterial activity against the indicator bacteria. The LAB inhibition zone diameter classification of indicator bacteria was divided into four groups by Davis and Stout (1971), namely weak (≤ 5 mm), medium (5 - 10 mm), strong (10 - 20 mm), and very strong (≥ 20 mm). S1K2T1 was the only strain having a broad spectrum, which means that it could inhibit all indicator bacteria. *Staphylococcus aureus* ATCC 2592 is a Gram-positive bacterium that was strongly inhibited by strain S1K2T1. Strain S5K7T1 had strong inhibition against *Sal.* Typhi BPE 122.4.CCA, and medium inhibition against *B. subtilis* ATCC 6633. Strain S10.2K6 had strong inhibition

against *P. putida* FNCC 0071. Strain S5K8T1 had medium inhibition against B. subtilis ATCC 6633 and P. putida FNCC 0071. The ability of LAB to inhibit the growth of indicator bacteria shows that the strains had a bacteriocin-like substance which was determined after the pH of the CFCS solution was neutralised and free of hydrogen peroxide (Anupama and Balasingh, 2018). Research conducted by Sogandi *et al.* (2019) showed that *L. plantarum* had broad-spectrum antibacterial activity against *Sal.* Typhi, *Sta. aureus*, and *B. subtilis*. Variation in antibacterial activity occurs when the isolate is given catalase treatment, where *L. plantarum* is also able to inhibit the growth of *B. subtilis* and *P. aeruginosa*. The results of the present work indicated that each strain had different antibacterial activity. This is consistent with the study conducted by Cleveland *et al.* (2001), which stated that the LAB identified from both fermented and non-fermented foods produced bacteriocin with high diversity. The variations in antibacterial activity occur due to the presence of bacteriocin or other similar metabolites in each strain of bacteria, which differ according to their adaptive response to the environment (Zhou *et al.,* 2014).

Identification of Lactobacillus plantarum based on API 50CHL system

Based on the characterisation test results using the API 50CHL system, strains S1K2T1, S5K7T1, S5K8T1, and S10.2K6 isolated from pickled cucumbers were identified as *L. plantarum* with a similarity index > 99%. The ability of *L. plantarum* to use carbohydrates as a carbon source had diverse variations despite coming from the same habitat. Figure 3 shows that the dendrogram has a similar relationship with the two *L. plantarum* isolates, namely strains S5K7T1 and S5K8T1. Meanwhile, S1K2T1 and S10.2K6 had similar fermentation patterns of 96%. The four *L. plantarum* strains were joined as one cluster with a similarity index of 88.5%.

A similar fermented product from cucumbers commonly consumed in Nepal and Khalpi yielded *L. plantarum, L. brevis,* and *Leuconostoc fallax*. This is consistent with the research carried out by Todorov and De Melo Franco (2010) and Khemariya *et al.* (2016), which stated that *L. plantarum* is naturally found in plant materials and their fermentation products. It is very important to use LAB in the fermentation due to its importance in protecting food against several phytopathogenic microorganisms (Daeschel *et al.* 1987).

Molecular characterisation of Lactobacillus plantarum

The molecular characterisation based on plantaricin A (plnA) gene coding showed that the plnA gene from strains S1K2T1, S5K7T1, S5K8T1, and S10.2K6 had similarity to *L. plantarum* as shown in Figure 1. These results are consistent with the research conducted by Sogandi *et al.* (2019) where the plnA gene isolated from Indonesian traditional fermented foods was found in all strains. Ben Omar *et al.* (2008) also stated that the gene is commonly found in *L. plantarum*.

The four isolates were also identified based on similarity to the 16S rRNA sequence of *L. plantarum* member strains, which was above 99%. Phylogenetic analysis proved that the strains had a close relationship with *L. plantarum* members from many previous studies including *L. plantarum* ATCC 8014 as shown in Figure 2. BLAST analysis using 16S rRNA sequences from all four isolates led to an identification rate above 99%.

The present work showed that the four identified *L. plantarum* strains had strong inhibition

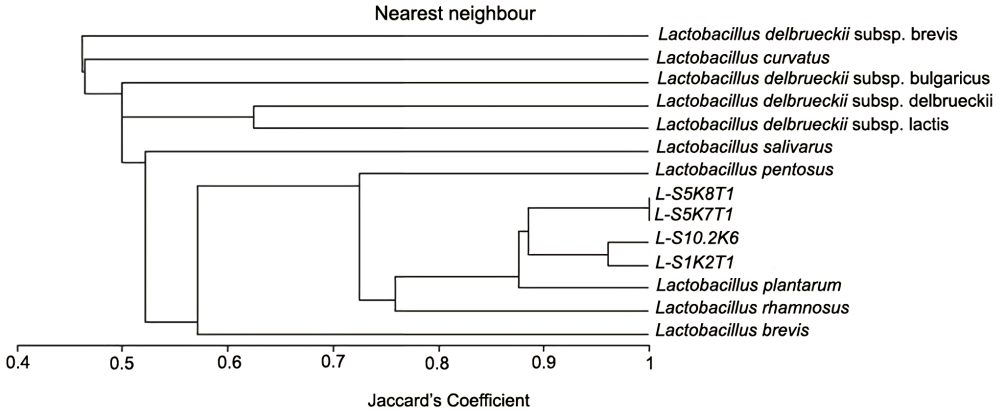

Figure 3. Dendrogram based on the similarity relationship between lactic acid bacteria isolated from pickled cucumbers and species of lactic acid bacteria belonging to the genus *Lactobacillus* using nearest neighbour with Jaccard coefficient.

against Gram-positive and negative pathogens. In addition, plantaricin A gene and *L. plantarum* isolates had the potentials to be used as candidates to produce antibacterial-producing probiotics.

Conclusion

LAB strains S1K2T1, S5K7T1, S5K8T1, and S10.2K6 had inhibitory activity against pathogenic bacteria. Strain S1K2T1 had strong inhibition against *Sta. aureus* ATCC 2592.3. Strain S5K7T1 had strong inhibition against *Sal.* Typhi BPE 122.4 CCA. Strain S10.2K6 had strong inhibition against *P. putida* FNCC 0071. Strain S5K8T1 had medium inhibition against both *B. subtilis* ATCC 6633 and *P. putida* FNCC 0071. The four isolates were identified as *L. plantarum* based on biochemical characters and phylogenetic analysis using 16S rRNA gene sequences. The detected plnA gene is a bacteriocin protein from *L. plantarum*. Therefore, the four *L. plantarum* isolates obtained in the present work were good cultures to be used as candidates for antibacterial-producing probiotics.

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