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Identification of Probiotic Potential *Lactobacillus* from *Mandai* Using Molecular Technique

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Abstract

To date, lactic acid bacteria (LAB) still become the most beneficial microorganisms due to their probiotic potential and antimicrobial activities which able to inhibit the growth of spoilage microorganisms and pathogenic bacteria, thus can maintain the quality and hygienic of the products or host health. In Indonesia, fermented traditional foods such as *tempoyak*, *bekasam*, and *sayur asin* have been found to be the natural habitat of probiotic LAB. In this study, *mandai,* the traditional fermented food made from the fermentation of *cempedak* (*Artocarpus champeden* Spreng.) *dami* was explored. The aim of this research is to identify probiotic potential *Lactobacillus* from *mandai* (a traditional fermented product from *dami* of *Cempedak*) using the molecular technique (DNA fingerprinting). The methods used in this study include: screening probiotic *Lactobacillus* and molecular identification of potential *Lactobacillus*. Based on probiotic screening, 26 *Lactobacillus* isolates were categorized as acid tolerance and 17 isolates were considered as bile salt tolerance bacteria. Fifteen *Lactobacillus* isolates demonstrated excellent inhibitory properties against indicator bacteria. Due to DNA polymorphisms, all species and strains of *Lactobacillus* isolates were difficult to be identified accurately, however, based on phylogenetic tree isolate A14 had the most similarity with *Lactobacillus vaccinostercus* NRIC 0624. Isolate B30 had similarities with *Lactobacillus harbinensis* and *Lactobacillus perolens*. Isolate F71 had similarities with *Lactobacillus casei* and *Lactobacillus paracasei.*

Keywords

lactic acid bacteria, *lactobacillus,* probiotic, identification

1 Introduction

LAB are gram-positive bacteria, fastidious, generally non-sporing, catalase-negative, acid-tolerant, devoid of cytochrome, non-respiring rod or cocci associated by their metabolic and physiological characteristics and produce lactic acid as a major product of fermentative metabolism [1].

LAB has been used for the fermentation of food and feed products in industry as starter cultures [1], contribute to flavor and increased shelf life of fermented food. Moreover, LAB prolongs shelf life and protect foods from pathogenic microorganisms due to the production of metabolite such as lactic and acetic acids, hydrogen peroxide, diacetyl, fatty acids, phenyl lactic and/or bacteriocins [2].

Because of their limited biosynthetic capabilities, LAB has complex nutritional components requirements like carbohydrates, amino acids, peptides, fatty acid esters, and vitamins and must be obtained from their habitats. Indonesia has plenty of local sources that contain these nutritional requirements like sugar units (monomer of carbohydrate) and amino (protein), which, therefore should be fit environments for LAB. In this study, the traditional fermented food which has been explored was *mandai* which made from the fermentation of *cempedak* (*Artocarpus champeden* Spreng) *dami.* In Indonesia, *cempedak* is commonly known in South Kalimantan (Banjarmasin), Sumatera, and West Java. The fermented product of *cempedak dami* is called *mandai*, which is predicted to be a highly potential habitat of LAB, particularly LAB with probiotic and antimicrobial properties. *Mandai* is made by immersing *dami* (inner skin of *cempedak*) into brine water. Brine water often facilitates longer shelf life for the product and leading to microbial selection and succession due to salt contained and also possibly become a good habitat of LAB due to nutrition contained. In this study, the brine water will be used as a substrate for LAB.

Common LAB in fermented food which is used for probiotic preparations includes specific strains of Lactobacilli. *Lactobacillus* are widespread in nature, and many species used in the food industry application. They also distributed in various ecological niches throughout gastrointestinal and genital tracts and

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constitute an important part of the indigenous microflora of man and higher animals. They are rarely associated with a gastrointestinal infection, and the strains are regarded as safe and non-pathogenic microorganisms. Furthermore, they have often called as health promoters [3].

Generally, LAB are classified based on phenotypic and biochemical properties. Due to the fact that in the routine identification of isolates, these properties may not be enough to identify a strain to a particular species [3]. In this research, DNA fingerprinting is used to identify the strain of the most selective probiotic potential LAB isolates. This fingerprinting result then able to determine the position of potential isolates through phylogenetic tree construction thus can visualize the evolutionary relationship between species [4].

2 Methods

2.1 Screening Probiotic Properties of Lactic Acid Bacteria (LAB)

2.1.1 Acid Tolerance

Cultures that have been prepared overnight were inoculated into MRS broth medium with pH of 7 and 3. The pH adjustment of the medium was using HCl 1 M or NaOH 0.5 M. Bacterial growth was observed with the enumerated viable colony at the 0, 1.5, and 3 hours using the pour plate method. Incubations were conducted at 37° C for 48 hours [5, 6] modified.

2.1.2 Bile Salt Tolerance

Cultures that have been prepared overnight were inoculated into MRS broth medium containing bile salt concentration of 0.3% and 0.5%. Bacterial growth was observed with the enumerated viable colony at the 0, 2 and 4 hours using the pour plate method. Incubations were conducted at 37º C for 48 hours [5, 6].

2.2 Detection of Antimicrobial Activity

2.2.1 Preparation of Culture

Overnight cultures which already inoculated into MRS broth medium were centrifuged at 6000 rpm for 10 minutes to separate the supernatant and cells. The culture cells (pellets) were added to 0.85% NaCl. The solution then adjusted with McFarland tube number 5 [7]. One ml solution which contains each culture was added into 100 ml MRS broth media then incubated for 24 hours at 37° C [8] modified.

2.2.2 Preparation of Cell-free Supernatant

The culture extracts were obtained from 24 hours cultures which have been grown in MRS broth. Sterile cell-free supernatant was obtained by centrifugation (6000 rpm for 10 minutes). Detections of antimicrobial activity were done using the agar well diffusion method by measuring the inhibition zone [9].

2.2.3 Detection of Antimicrobial Activity by Agar Well Diffusion Method

Prepared overnight of indicator pathogens (purchased from *Pusat Antar Universitas,* Universitas Gadjah Mada, Yogyakarta) including *Escherichia coli* FNCC 0091 (IFO 3301), *Listeria monocytogenes* FNCC 0156, *Salmonella typhimurium* FNCC 0050, inoculated in NA medium at 37 °C diluted with 0.85% NaCl solution until the turbidity equal with McFarland tube number 3. 10 µl each of indicator bacteria then mixed with 10 ml NA. Three 7 mm of diameter holes at a similar distance were punched and filled with 20 µl of each LAB cell-free supernatant. The Petri dishes were stored at 4 °C for 3 hours to support the diffusion of cell-free supernatant into the medium. After that, the incubation was carried out for 24 hours at 37 °C. The measurement of the clear zone was performed using a caliper. Positive results then recorded when the zone of inhibition of at least 1 mm around the well [8].

2.3 Molecular Identification and Phylogenetic Construction

After selecting the bacterial cultures which have probiotic and antimicrobial properties, the molecular identification of each bacterial isolate was done. This identification was conducted using DNA fingerprinting by polymerase chain reaction (PCR), sequencing and phylogenetic construction using

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bioinformatics software. The whole assays of molecular identification were done in the Microbiology Laboratory of Faculty of Agricultural, Gadjah Mada University, Yogyakarta.

2.3.1 DNA Extraction and Purification

To release DNA from cells, chemical or physical breakdown of cell walls, cell membranes and nuclear membranes (in Eukaryote) is necessary. Molecules such as RNA, proteins, polysaccharides that prevent PCR should be removed to purify DNA. There are many methods to extract DNA from microorganism *i.e.* manual or classical method (Phenol-Chloroform, CTAB method), using commercially available kit (ISOPLANT, DNeasy, etc), and direct method (direct PCR from the colony, DNA extraction by microwave spore-suspension) [9].

2.3.2 Amplification of 16S rRNA Region by Polymerase Chain Reaction (PCR)

DNA is composed of two stranded nucleotide sequences. This conformation is stable under low temperatures but breaks at high temperatures. PCR utilizes the change of chemical conformation of DNA by simple up and down of temperature. Chemicals used in PCR reaction mainly PCR Buffer, $MgCl_2$, dNTPs, Primers (forward and reverse), DNA polymerase, and template DNA. The amplification includes some steps: (1) at room temperature, template DNA is double-strand and all primers and dNTPs are free, (2) denaturation in which double-strand template DNA separated into two single strands at high temperature, (3) annealing in which single stand DNAs are going to combine again with complementary strand during low temperature. As primers are much abundant than template DNA, primers attach to the specific position of the template DNA before template DNA combines with its partner strand, (4) extension in which DNA polymerase synthesizes a new strand by incorporating complementary dNTPs one after another when the temperature is appropriate. The temperature program consisted of an initial heat denaturation step of 95° C for 3 minutes and then 30 cycles of 30 seconds at 95° C, 30 seconds at 55°C, and 1 minute at 72°C, followed by 5 minutes at 72°C [9].

2.3.3 Visualization of PCR Products by Agarose Gel Electrophoresis

Agarose gel electrophoresis is widely used for DNA visualization. Because DNA has a negative electric charge, they are attracted to a positive electrode during electrophoresis. In agarose gel, a small molecule DNA moves faster to a positive electrode than large ones. Using this nature, DNA can be separated depending on their molecular weight. A size standard marker was applied in one or both sides of the gel. The electrophoresis then started in 100V for 20 minutes default. After finished, the gel then put under Ultra Violet (UV) table and documented. The size of bands estimated by referring ladder marker [10]. Purified 16S rRNA gene then amplified using single cycle sequencing PCR, followed by direct sequencing using DNA sequencer.

2.3.4 Phylogenetic Construction

The first step was trimming the 16S rRNA gene sequences data and assembling the sequences to obtain the consensus sequence. After that, the homology search was done to find similar sequences. This homology search was done using BLAST (web-based program to compare nucleotide (or protein) sequences to public databases). Multiple alignments with the closest relatives were done by Clustal_W using Mega software version 7, to construct phylogenetic trees. Eventually, phylogenetic trees were drawn to know the phylogenetic positions of isolates.

3 Results and Discussions

The fermentation of *mandai* was performed using 2 treatments with triplication. For the analysis, the isolate codes used A, B, C for fermentation in 15 % ww^{-1} of salt, and D, E, F for fermentation in 20% ww^{-1} of salt. Lactic acid bacteria (LAB) obtained from the brine water of *mandai* were purified in MRS agar medium supplemented with $CaCO_3$. The screening found 26 isolates identified as *Lactobacillus* genera according to Bergey's Manual of Systematic Bacteriology.

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3.1 Screening Probiotic Properties

3.1.1 Acid Tolerance

The probiotic screening of LAB which obtained from the brine water of *mandai,* was done by analyzing the ability of bacteria to grow (survive) under acid condition (low pH). The selection of acid tolerance bacteria was conducted by culturing each isolate in a medium that has been modified to gastrointestinal pH (pH 3 & 7) and incubated for 2 - 3 hours [6]. From the result, all 26 isolates survived under low pH (pH 3) for 3 hours. Therefore, all 26 isolates were categorized as acid tolerance bacteria (**Table 1**).

According to Corcoran et al [10], acid tolerance properties of *Lactobacilli* are associated with the presence of constant gradient between extracellular and cytoplasmic pH. Cellular functions are generally inhibited when internal pH reaches the threshold value thus causing the cells to die. Gram-positive organisms use the F_0F_1-ATPase mechanism as protection against acidic conditions. F_0F_1-ATPase induced at low pH. The F_0F_1-ATPase is a multiple-subunit enzyme consist of a catalytic portion (F_1) for ATP hydrolysis incorporating the α, β, γ, δ, ε subunits and an integral membrane portion (F_0) for proton translocation including a, b, c subunits, as a membranous channel. The role of F_0F_1-ATPase in organisms without respiratory chain is to generate a proton motive force, *via* proton expulsion. As a result, F_0F_1-ATPase can increase intracellular pH at low extracellular pH.

Lebeer et al [12] and Cotter and Hill [10] reported that amino acid decarboxylase is useful to control the bacteria's environment pH by consuming hydrogen ions as part of decarboxylation reaction. An example of this is glutamate decarboxylase (GAD), which performed by combining an internalized amino acid (glutamate) with a proton and exchanging the resultant product (γ-aminobutyrate) for another amino acid substrate. GAD system has positively associated with pH control of Gram-positive. After the intake of glutamate by a specific transporter into the cell, the cytoplasmic decarboxylation process creates in the consumption of a proton. The reaction product γ-aminobutyrate is exported from the cell via an antiporter. The result is an increase in intracellular pH due to the removal of hydrogen ions.

In acid tolerance assay, the incubation times used were 0, 1.5 and 3 hours. According to Yavuzdurmaz [6], probiotic bacteria will enter the upper intestinal tract which contains bile. In this stage, bacteria strains should be able to resist the digestion process. It is reported that time at the first entrance until release from the stomach takes three hours. Strains should be resistant to stressful conditions while in the stomach (pH 1.5-3) at least during staying time in the stomach.

3.1.2 Bile Salt Tolerance

All isolates which able to survive under low pH (pH 3) for 3 hours were tested subsequently for the ability to survive under bile conditions. The concentrates of bile salt used in this study are 0.3 % ($w \cdot v^{-1}$) and 0.5 % ($w \cdot v^{-1}$), and the incubation time up to 4 hours [13]. In this study, 21 isolates were proceeded to bile salt tolerance test due to the growth problem. From 21 isolates, 17 isolates were able to survive under bile condition (0.3% $w \cdot v^{-1}$ and 0.5% $w \cdot v^{-1}$ of bile salt) for 4 hours. Therefore, 17 isolates were considered as highly bile salt tolerance bacteria (**Table 2**).

Seventeen isolates that have been identified as *Lactobacillus* genera are resistant to bile salt conditions. According to Ruiz et al. [13] and Lebeer et al. [10], bile response is a multifactorial phenomenon involving a variety of detoxification processes of bile and counteracting the elimination effect on bacterial structures. Active efflux of bile acid/salts, bile salt hydrolysis and changes of cell membrane composition and cell wall appear to be the most prevalent bile-specific mechanisms contributing bile resistance in *Lactobacillus* genera. The active extrusion of bile acids and salts accumulated in the cytoplasm through the efflux pump is a common bacterial mechanism to counteract the bile toxicity.

Ruiz et al. [13] and Lebeer et al. [10] also reported that among the different mechanisms released by bacteria to counteract the elimination effect of bile, the activity of bile-salt hydrolases (BSHs) has been proposed to provide protection through bile salt deconjugation. BSHs are generally intracellular enzymes that catalyze the amide bond hydrolysis between the steroid moiety and amino acid side chain of bile acid. BSHs belong to the cholylglycine hydrolase family and have been proposed to have evolved as an adaptation to bile-containing environments. It catalyzes a reaction in which glycine and taurine are deconjugated from bile salts and those unconjugated acids can be further metabolized by other gut bacteria.

In bile tolerance assay, the incubation times used were 0, 2 and 4 hours. According to Yavuzdurmaz [6], intestinal bile concentration is known to be 0.3% ($w \cdot v^{-1}$) and the digestion time of food in the small intestine is approximately 4 hours. Strains should be resistant to the stressful condition of the small intestine at least during staying time.

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3.1.3 Antimicrobial Activity

From previous screening acid and bile salt tolerance, 15 isolates were chosen to perform the antimicrobial activity assay. Based on the result, 15 isolates were able to inhibit the growth of all pathogenic bacteria. From result of group A, B, and C, isolate A2 had the highest inhibition against *L. monocytogenes* (13.37±0.64 mm diameter of inhibition zone) and *E. coli* (12.03±0.11 mm diameter of inhibition zone) among other isolates until 24 hours, while isolate B30 had the highest inhibition against *S. typhimurium* (9.00±1.73 mm diameter of inhibition zone) among other isolates. From result of group D, E, and F, isolate F71 had the highest inhibition against *L. monocytogenes* (10.73±1.48 mm diameter of inhibition zone), *E. coli* (13.73±0.55 mm diameter of inhibition zone), and *S. typhimurium* (6.40±2.33 mm diameter of inhibition zone) among other isolates until 24 hours (**Table 3**).

Based on 15 isolates, three isolates were selected to be analyzed subsequently to species and strain level using DNA fingerprinting. The phylogenetic constructions were used to determine phylogenetic relations (phylogenetic position) of selected isolates among other LAB. The selection of isolates was done based on a comparison of antimicrobial effectiveness against three pathogenic bacteria. From the result, isolates A14, B30, and F71 were selected (Fig 1 – 3).

Table 1 The Ability of Lactic Acid Bacteria to Survive at pH 3 and 7

Isolate	pH 3			pH 7		
	0 hr	1.5 hr	3 hr	0 hr	1.5 hr	3 hr
A2	+	+	+	+	+	+
A3	+	+	+	+	+	+
A6	+	+	+	+	+	+
A13	+	+	+	+	+	+
A14	+	+	+	+	+	+
A15	+	+	+	+	+	+
B20	+	+	+	+	+	+
B21	+	+	+	+	+	+
B26	+	+	+	+	+	+
B27	+	+	+	+	+	+
B28	+	+	+	+	+	+
B30	+	+	+	+	+	+
B82	+	+	+	+	+	+
C31	+	+	+	+	+	+
C32	+	+	+	+	+	+
D41	+	+	+	+	+	+
D42	+	+	+	+	+	+
D44	+	+	+	+	+	+
D45	+	+	+	+	+	+
D49	+	+	+	+	+	+
D50	+	+	+	+	+	+
E51	+	+	+	+	+	+
E52	+	+	+	+	+	+
E55	+	+	+	+	+	+
F65	+	+	+	+	+	+
F71	+	+	+	+	+	+

Key: "+" = bacteria growing
"–" = bacteria not growing

Table 2 The Ability of Lactic Acid Bacteria to Survive at 0.3% and 0.5% of Bile Salt

Isolate	0.3%			0.5%		
	0 hr	2 hr	4 hr	0 hr	2 hr	4 hr
A2	+	+	+	+	+	+
A3	+	+	+	+	+	+
A6	+	+	+	+	+	+
A13	+	+	+	+	+	+
A14	+	+	+	+	+	+
A15	+	+	+	+	+	+
B21	+	+	+	+	+	+
B26	+	+	+	+	+	+
B30	+	+	+	+	+	+
B82	+	+	+	+	+	+
C31	+	+	+	+	+	+
C32	+	+	+	–	–	–
D41	+	+	+	–	–	–
D42	+	+	+	+	+	+
D45	+	+	+	–	–	–
D49	+	+	+	+	+	+
D50	+	+	+	–	–	–
E52	+	+	+	+	+	+
E55	+	+	+	+	+	+
F65	+	+	+	+	+	+
F71	+	+	+	+	+	+

Key: "+" = bacteria growing
"–" = bacteria not growing

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Table 3 Spectrum of Inhibition of Lactic Acid Bacteria

Isolates	Zone of Inhibition (mm)											
	Listeria monocytogenes				*Escherichia coli*				*Salmonella typhimurium*			
	6 hrs	12 hrs	18 hrs	24 hrs	6 hrs	12 hrs	18 hrs	24 hrs	6 hrs	12 hrs	18 hrs	24 hrs
A2	18.87 ±1.10	17.87 ±0.32	14.57 ±0.45	13.37 ±0.64	16.23 ±0.23	13.73 ±1.18	13.43 ±1.15	12.03 ±0.12	14.10 ±0.00	4.77 ±0.58	4.03 ±0.06	2.73 ±0.64
A6	16.87 ±1.25	14.07 ±0.95	12.73 ±1.10	12.07 ±1.00	19.07 ±1.00	14.43 ±1.15	11.73 ±0.64	11.07 ±0.95	13.53 ±2.23	6.73 ±2.12	4.43 ±1.15	4.43 ±1.15
A13	17.07 ±0.95	13.00 ±0.10	9.40 ±1.13	8.07 ±1.67	17.07 ±0.95	14.07 ±1.05	12.77 ±0.58	11.40 ±0.61	12.83 ±0.64	7.73 ±0.55	4.77 ±0.58	5.07 ±0.06
A14	14.77 ±1.15	12.07 ±0.95	9.77 ±1.15	8.87 ±0.68	15.10 ±1.00	12.43 ±1.15	10.77 ±0.58	9.40 ±0.52	14.03 ±0.06	6.07 ±0.06	5.07 ±0.06	5.03 ±0.06
A15	15.40 ±1.13	11.43 ±1.15	9.33 ±1.53	7.40 ±0.40	18.77 ±1.53	13.77 ±1.15	9.73 ±1.97	7.40 ±0.52	12.37 ±0.64	5.40 ±0.61	2.73 ±0.55	4.00 ±0.10
B21	13.73 ±0.64	10.43 ±0.58	8.10 ±1.00	7.03 ±1.00	18.03 ±0.06	14.07 ±1.05	12.10 ±2.00	10.77 ±1.53	14.70 ±0.61	8.43 ±0.58	5.43 ±0.58	4.47 ±0.55
B26	16.07 ±1.79	13.40 ±1.13	11.43 ±0.58	11.07 ±1.00	15.73 ±0.55	8.37 ±0.64	8.40 ±2.52	6.73 ±0.55	14.73 ±1.18	8.67 ±1.15	5.77 ±1.15	5.07 ±1.00
B30	14.77 ±0.58	11.07 ±1.00	10.17 ±0.76	9.70 ±1.13	20.40 ±0.61	15.73 ±0.55	12.60 ±0.87	10.43 ±1.53	13.70 ±1.64	9.73 ±1.58	9.03 ±1.67	9.00 ±1.73
C32	15.43 ±0.58	12.40 ±0.52	11.86 ±0.12	7.66 ±0.76	14.96 ±0.96	12.73 ±0.55	9.73 ±0.55	9.67 ±0.58	14.70 ±1.21	9.73 ±1.10	5.77 ±1.15	5.33 ±0.67
D45	13.07 ±2.00	10.00 ±2.00	9.00 ±1.00	8.10 ±1.00	17.10 ±1.73	14.73 ±1.10	11.40 ±0.61	7.77 ±0.58	14.73 ±1.58	8.40 ±2.14	7.07 ±1.05	4.43 ±0.58
D49	16.37 ±0.55	13.00 ±0.00	9.40 ±0.52	8.07 ±0.06	15.73 ±1.48	14.03 ±1.00	9.37 ±0.55	9.40 ±0.52	13.40 ±0.52	5.63 ±0.57	4.97 ±0.91	4.03 ±0.06
E52	16.37 ±0.64	13.10 ±1.00	9.43 ±2.52	7.37 ±3.18	16.10 ±1.00	16.43 ±1.15	14.10 ±1.00	10.33 ±0.67	15.40 ±0.52	8.10 ±1.00	5.70 ±0.52	6.03 ±1.05
E55	15.07 ±0.95	12.40 ±0.61	8.73 ±0.55	7.77 ±0.58	14.40 ±1.57	10.43 ±1.53	9.07 ±1.05	7.37 ±0.64	14.97 ±0.95	10.07 ±1.05	3.77 ±1.15	3.10 ±0.00
F65	15.70 ±1.04	12.40 ±0.61	9.73 ±0.64	8.67 ±1.15	15.73 ±0.55	12.00 ±0.10	12.00 ±0.10	10.10 ±1.00	16.30 ±0.53	8.33 ±0.69	5.10 ±0.00	5.10 ±0.00
F71	17.77 ±1.15	14.10 ±1.00	11.70 ±0.52	10.73 ±1.48	24.43 ±0.58	19.43 ±0.58	15.60 ±1.32	13.73 ±0.55	15.43 ±1.53	10.07 ±0.95	7.43 ±2.31	6.40 ±2.34

3.2 Molecular Identification

The results of molecular identification on three selected isolates (A14, B30, and F71) in this study were obtained as partial DNA sequences due to limited time and funding. Based on partial sequences, all identified genus of *Lactobacillus* were confirmed. Isolate A14 had the most similarity with *Lactobacillus vaccinostercus* NRIC 0624. Isolate B30 had similarities with *Lactobacillus harbinensis* and *Lactobacillus perolens*. Isolate F71 had similarities with *Lactobacillus casei* and *Lactobacillus paracasei* (Fig. 1–3). However, since all the sequences were partial, the certainty of sister groups (ingroup) in comparison with outgroups were undetermined.

As bacteria in the environment have huge varieties, the range and complexity of the techniques to be used for identification purpose are highly confusing. However, the use of nucleotide sequence data from 16s rRNA gene has been considered to be the most suitable practice to identify and to determine the phylogenetic relationship for all microorganisms. The reasons of using 16S rRNA gene for identification purpose *i.e.* occurrence of genes in all organisms performing same functions, the gene sequence is conserved sufficiently which contain region that is conserved, variable and hyper-variable, and around 1500 bp of sequence size which relatively contains large enough information for identification and phylogeny analysis. This molecular identification technique became popular after adequate deposition of 16s rRNA gene sequences in the database as well as the availability of suitable primers for gene amplification [15]. Since all 16S rRNA sequences of selected isolates (A14, B30, and F71) were partial sequences, similar species and strains were still undetermined. However, species with the most probability to appear in phylogenetic tree were put to discussion.

Based on result, isolate A14 had the most similarity with *Lactobacillus vaccinostercus* NRIC 0624. However, since the complete genome of isolate A14 was not obtained, the similarity to certain species and strain could not fully understood. Therefore, all species which appear at the most number in phylogenetic tree were examined in comparison with screening test results (morphological and physiological characteristic) as well as the source of the isolate. Besides *Lactobacillus vaccinostercus* NRIC 0624, isolate A14 had the probability to be similar to *Lactobacillus suebicus*. *Lactobacillus vaccinostercus* and *Lactobacillus suebicus* are two species which included in *Lactobacillus vaccinostercus* group [16]. According

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to Vos et al. [17], *Lactobacillus suebicus* cells are rounded ends non-motile rods, single or pairs. Obligately heterofermentative. The lowest limits for growth are 10° C and pH 2.8. The habitats for this species are stored apple and pear mashes or fruit mashes. *Lactobacillus vaccinostercus* cells are rounded ends non-motile rods. Occurred usually in pairs. Obligately heterofermentative. They grow in a range of 20–40° C with pH 4.5-7.5 and the habitat for this species are cow dung. Based on the main characteristics of those species, isolate A14 has more probability to close with *Lactobacillus suebicus* instead of *Lactobacillus vaccinostercus*.

Isolate B30 had a similar condition with isolate A14. Due to incomplete genome of this isolate, the similarity to certain species and strain could not be fully understood. Therefore, all species which appear at the most number in phylogenetic tree will also be examined in comparison with screening test results (morphological and physiological characteristic) as well as the source of the isolate. *Lactobacillus harbinensis* and *Lactobacillus perolens* are two species included in *Lactobacillus perolens* group [16]. According to [17] (2009), *Lactobacillus perolens* cells are rounded ends non-motile rods, occurring as single, in pairs, or in short chains. They can grow up to 42° C with an optimum growth temperature of 28–32° C and no growth below 15° C. Optimum growth at pH 5.5-6.5. No growth below pH 3.7. Facultatively heterofermentative, with habitats of spoiled soft drinks and brewery environment. According to Zou et al. [18], *Lactobacillus harbinensis* cells are non-motile rods and are facultatively heterofermentative. They are able to grow at 45° C with 3% NaCl, at pH range at 4.5 –8.0 but no growth at 5 or 50° C or at pH 3.0. These species have been isolated formerly from traditional fermented vegetable "Suan Cai" from Northeastern China.

According to Pisano et al. [19] and Sneath et al. [20], *Lactobacillus plantarum* is heterogeneous and versatile species that can be found in a variety of environmental niches, including dairy, meat, vegetable or plant fermentations as well as GI tract. The cells are rounded ends non-motile rods, straight, occurred as single, in pairs or in short chains and are facultative heterofermentative. They are able to grow at 15° C and some cells are able to grow at 45° C. Some strains are able to survive under low pH (up to 2.5). Some habitats for this species are dairy products and environment, silage, sauerkraut, pickled vegetables, sourdough, cow dung, human mouth, GI tract, stools, and sewage. Based on the main characteristics of those species, isolate B30 has more probability to close with *Lactobacillus harbinensis* or *Lactobacillus plantarum* instead of *Lactobacillus perolens.*

Like the other two isolates, the similarity to certain species and strain of isolate B30 could not be fully understood due to incomplete genome of this isolate. Therefore, all species which appear at the most number in phylogenetic tree will also be examined in comparison with screening test results (morphological and physiological characteristic) as well as the source of the isolate. *Lactobacillus casei* and *Lactobacillus paracasei* are two species which included in *Lactobacillus casei* group [16]. According to Vos et al. [17], and Nezhad et al. [21], *Lactobacillus casei* cells are non-motile rods often with square ends and tend to form chains. They are facultatively heterofermentative. They are able to grow at 15° C but not 45° C with optimum growth pH range 4.0 – 6.5. The habitats for this species are milk, cheese and GI tract. While *Lactobacillus paracasei* cells are rods, often with square ends, occurring singly or in chains and are facultatively heterofermentative. They are able to grow at 10° C and 40° C. Some strains are able to grow at 5° C and 45° C. The habitats of this species are the same with *Lactobacillus casei*. Isolate F71 differs from two previous isolates. Based on the characteristics and habitats of all probable species, F71 has less similarity with either *Lactobacillus casei* or *Lactobacillus paracasei*, thus for further analysis, this isolate needs verification on some screening test.

Based on molecular identification, most of *Lactobacillus* species were included in facultative heterofermentative and thus, different from previous screening results. According to Salminen et al. [3], genus *Lactobacillus* contains species that can be placed in three categories. The first category includes obligately fermentative *Lactobacilli*, which means that sugars only fermented by glycolysis (Embden-Meyerhof-Parnas pathway). The second category includes obligately heterofermentative, means that only 6-phosphogluconate/phosphohexoses (6-PG/PK) pathway available for sugar fermentation. The third category includes *Lactobacilli* that hold the intermediate position. They resemble obligately homofermentative LAB in which they have a constitutive fructose-1,6-diphosphate (FDP) aldolase contributing in hexose fermentation by glycolysis. However, they also use 6-PG/PK pathway to metabolize certain substrates. This third category *Lactobacilli*, therefore, termed as facultative heterofermentative. The result of *Lactobacillus* species might be facultative heterofermentative since the carbon dioxide production test could not determine this category of *Lactobacillus* very well.

Overall, the results of the molecular identification of three isolates were obtained as partial DNA sequences. This condition, therefore, has made the bootstrap value of each isolate in phylogenetic tree low consequently. Bootstrap value is defined as the percentage of appearance of a particular clade and usually displayed at each node in phylogenetic tree [22]. According to Tokumasa [13], if an internal branch has a

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higher bootstrap value, the internal branch can be considered reliable (confident). Thus, the bootstrap value can be used to measure the similarity of two taxa in the internal branch. Based on phylogenetic tree results, isolate A14 and *Lactobacillus vaccinostercus* NRIC 0624 had the bootstrap value of 58%, which means the reliability or similarity of A14 and *Lactobacillus vaccinostercus* NRIC 0624 is only 58%. While isolate B30 had similarity with some *Lactobacillus* strains in bootstrap value of 65%. While isolate F71 had similarity with some *Lactobacillus* strains in bootstrap value of 34% in the nearest node. According to Lemey et al. [23], under normal circumstances, confidence can be given consideration to branches or groups supported by more than 70% or 75%, while branches with support less than 70% should be treated with caution. Therefore, the repetition of molecular identification is necessary to be done to ensure the species and strain based on the full genome.

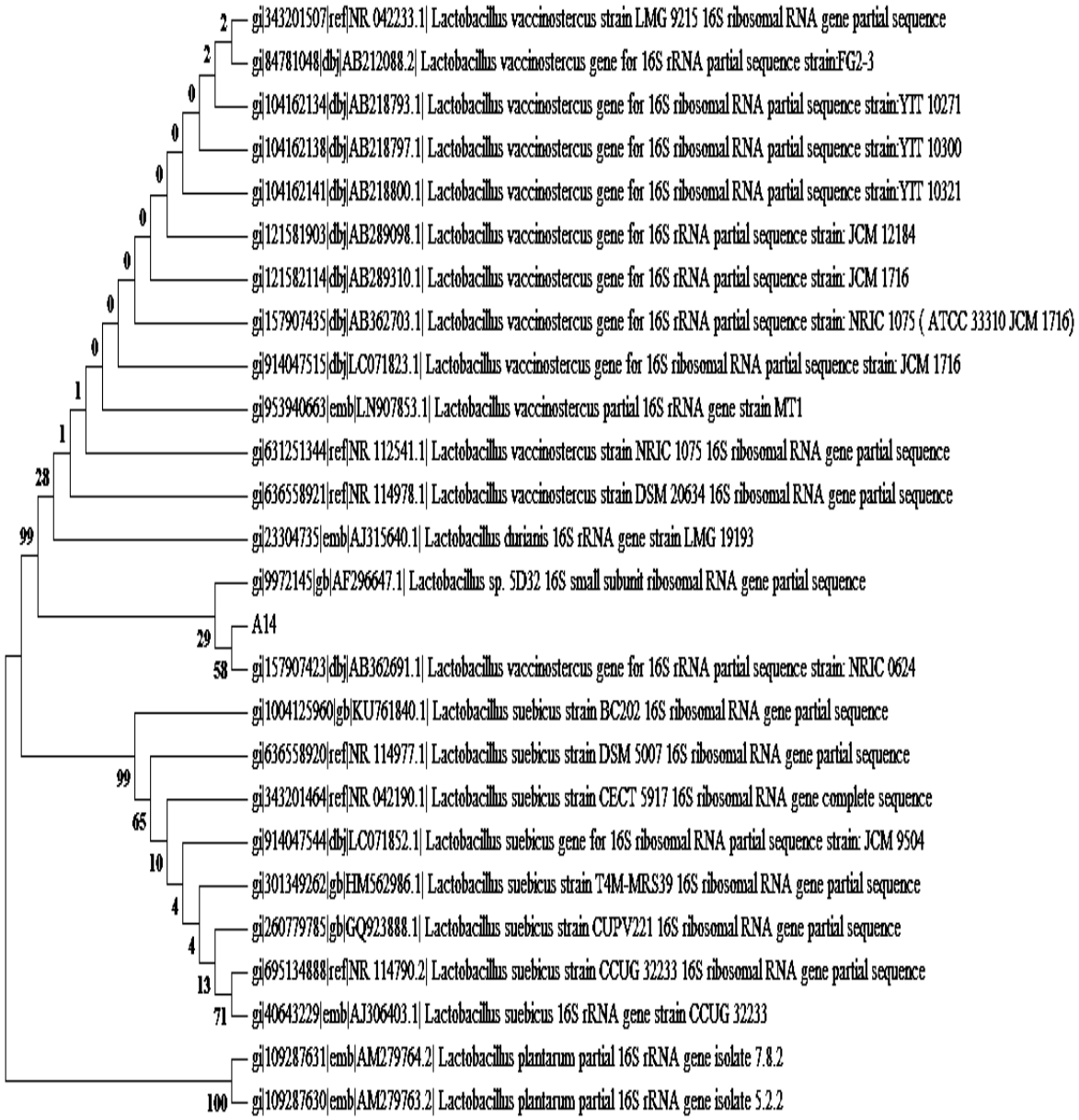

Fig. 1 The neighbor-joining tree showing the phylogenetic position of isolate A14 in the genus Lactobacillus based on 16S rRNA partial gene sequences. Bar, 0.005 K¬nuc.

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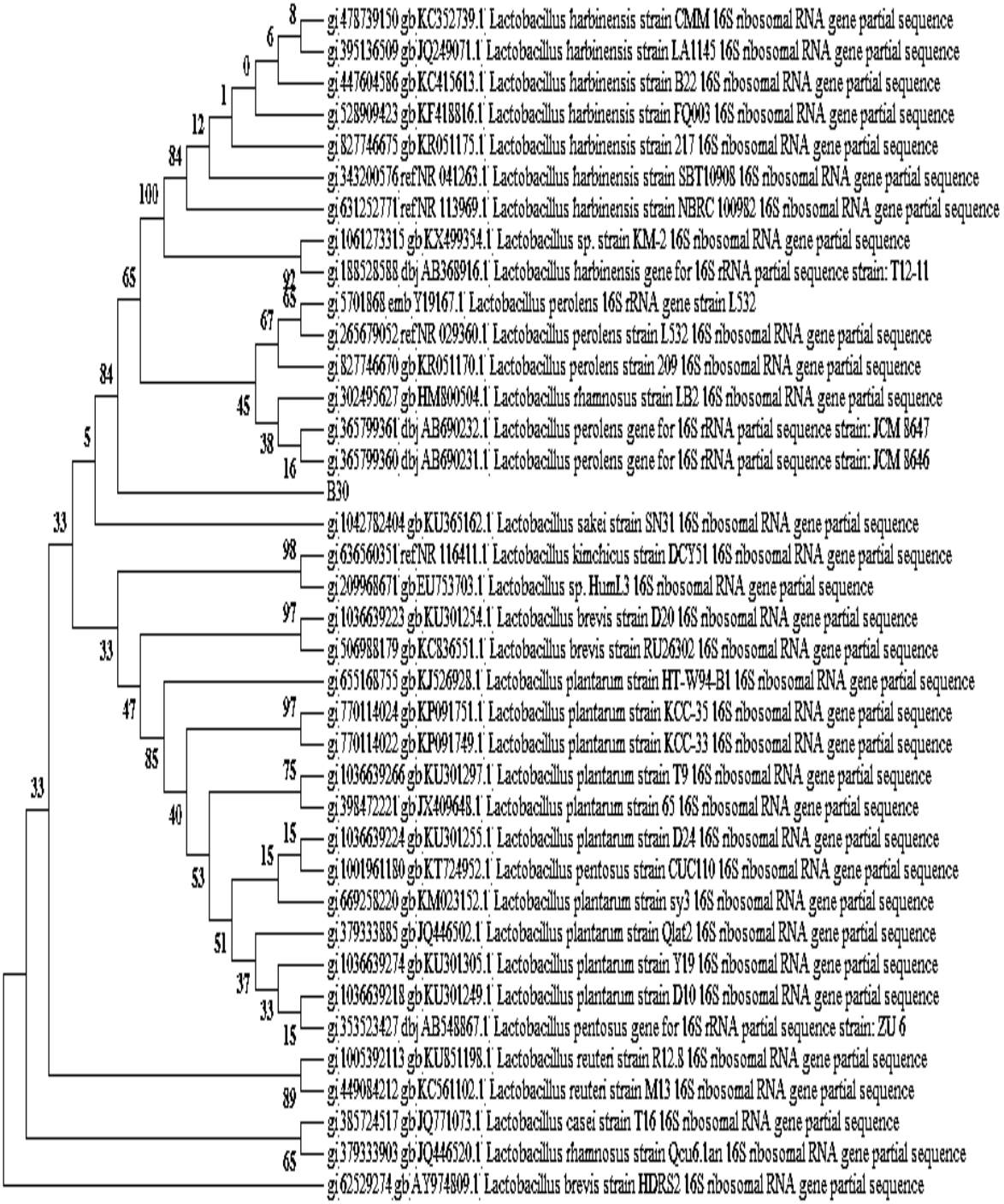

Fig. 2 The neighbor-joining tree showing the phylogenetic position of isolate B30

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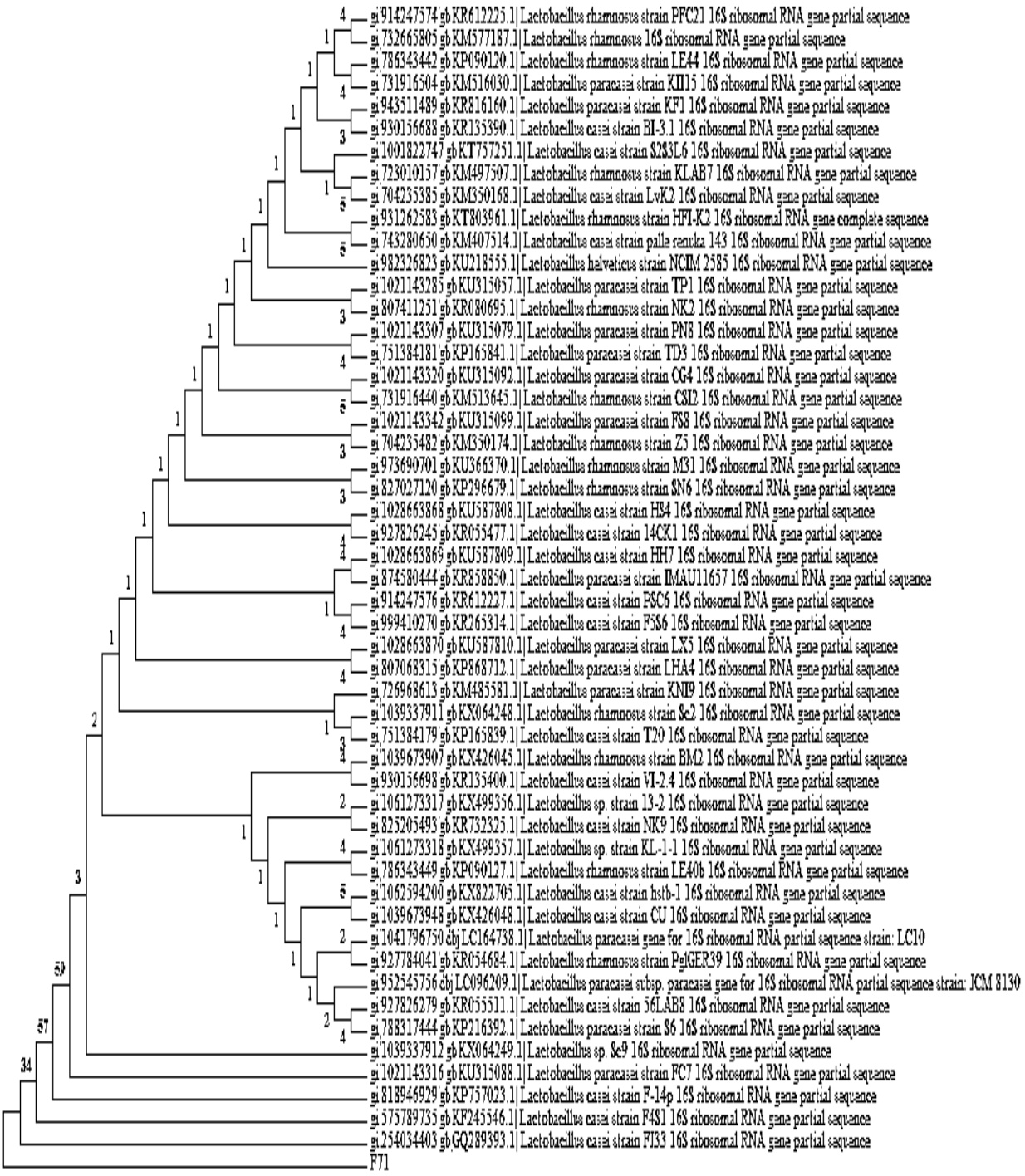

Fig. 3 The neighbor-joining tree showing the phylogenetic position of isolate F71 in the genus Lactobacillus based on 16S rRNA partial gene sequences. Bar, 0.005 K¬nuc

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4 Conclusions

Lactic Acid Bacteria (LAB) from *mandai* which was fermented with 15% w · w^{-1} salt found 17 isolates and 20% w · w^{-1} salt found 24 isolates. Based on screening, 26 isolates from *mandai* were identified as *Lactobacillus* genus. Based on probiotic screening, 26 *Lactobacillus* isolates are categorized as acid tolerance while 17 isolates are categorized as bile salt tolerance. Based on the antimicrobial test, 15 isolates are able to produce antimicrobial substances demonstrated by the ability to inhibit the growth of indicator pathogens (*Listeria monocytogenes*, *Escherichia coli,* and *Salmonella typhimurium*). All genus *Lactobacillus* which screened from *mandai* have probiotic potential which indicated by the ability to survive in stress conditions (acid and bile), and the ability to produce antimicrobial substances that can inhibit the growth of indicator pathogens *in vitro*. Based on partial sequences, all identified genus of *Lactobacillus* were confirmed. Isolate A14 had the most similarity with *Lactobacillus vaccinostercus* NRIC 0624. Isolate B30 had similarities with *Lactobacillus harbinensis* and *Lactobacillus perolens*. Isolate F71 had similarities with *Lactobacillus casei* and *Lactobacillus paracasei.*

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