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Probiotics Potential of Lactic Acid Bacteria from Horse Milk in Tambora, West Nusa Tenggara, Indonesia

(Potensi Probiotik Bakteria Asid Laktik daripada Susu Kuda di Tambora, Barat Nusa Tenggara, Indonesia)

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ABSTRACT

This study explores the probiotic potential of lactic acid bacteria (LAB) in horse milk from Tambora, West Nusa Tenggara by examining its functional and probiotic attributes, including bile salt tolerance, low pH, simulated gastric juice (SGJ), simulated intestinal juice (SIJ), antibacterial activity, as well as bile salt hydrolase (BSH) genes. Genotyping of LAB was performed using restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) analysis and then identified based on the 16S rRNA gene. A total of 25 LAB isolates showed the ability to grow at low pH, tolerant to bile, and survived under SIJ and SGJ conditions. The BSH gene was confirmed in three isolates, namely: SK1-28, SK2-30, and SK2-34. Results of RFLP-PCR analysis showed that the LAB isolates were grouped into three groups based on the number and molecular weight of the differences DNA fragments. The 16S rRNA analysis showed that the first two groups were *Lacticaseibacillus rhamnosus*, whereas the third group was *Lactiplantibacillus plantarum*. In addition, all *Lacticaseibacillus rhamnosus* isolates in group I showed the ability to grow at pH 9.0, but not group II. It can be concluded that *Lacticaseibacillus rhamnosus* and *Lactiplantibacillus plantarum* can be used as the indigenous probiotic bacteria source from Indonesia.

Keywords: Horse's milk; lactic acid bacteria (LAB); *Lacticaseibacillus rhamnosus; Lactiplantibacillus plantarum*; probiotics

ABSTRAK

Penyelidikan ini mengkaji potensi probiotik bakteria asid laktik (LAB) dalam susu kuda dari Tambora, Barat Nusa Tenggara dengan meneliti sifat fungsian dan probiotiknya, termasuk toleransi garam hempedu, pH rendah, jus gastrik disimulasi (SGJ), jus usus disimulasi (SIJ) aktiviti antibakteria serta gen hidrolase garam hempedu (BSH). Genotip LAB dilakukan menggunakan analisis tindak balas rantai polimorfisme-polimerase sekatan panjang serpihan (RFLP-PCR) dan kemudian dikenal pasti berdasarkan gen rRNA 16S. Sebanyak 25 pencilan LAB menunjukkan keupayaan untuk membesar pada pH rendah, bertoleransi dengan hempedu dan bermandiri dalam keadaan SIJ dan SGJ. Gen BSH telah disahkan dalam tiga pencilan iaitu: SK1-28, SK2-30 dan SK2-34. Keputusan analisis RFLP-PCR menunjukkan bahawa pencilan LAB dikelompokkan kepada tiga kumpulan berdasarkan bilangan dan berat molekul perbezaan serpihan DNA. Analisis rRNA 16S menunjukkan bahawa dua kumpulan pertama ialah *Lacticaseibacillus rhamnosus*, manakala kumpulan ketiga ialah *Lactiplantibacillus plantarum*. Di samping itu, semua pencilan *Lacticaseibacillus rhamnosus* dalam kumpulan I menunjukkan keupayaan untuk berkembang pada pH 9.0 tetapi bukan kumpulan II. Dapat disimpulkan bahawa *Lacticaseibacillus rhamnosus* dan *Lactiplantibacillus plantarum* boleh digunakan sebagai sumber bakteria probiotik asli dari Indonesia.

Kata kunci: Bakteria asid laktik (LAB); *Lacticaseibacillus rhamnosus*; *Lactiplantibacillus plantarum*; probiotik; susu kuda

Introduction

The LAB are a group of Gram-positive cocci or rods that do not form spores and produce lactic acid as the main end-product of carbohydrate fermentation. LAB are important industrial microorganism known for its excellent fermentability, health, and nutritional benefits (Rattanachaikunsopon & Phumkhachorn 2010). Among the commonly used microorganisms, LAB are a major group of probiotic bacteria. They are non-pathogenic, technologically suitable for industrial processes, and resistant to acids and bile, as well as produce antibacterial agents (Shehata et al. 2016).

According to FAO/WHO (2002), when given in sufficient quantities, probiotics are microorganisms that can provide health benefits to the host cells. Most LAB can be classified into a large group of probiotics (Pringsulaka et al. 2015). Bhadoria and Mahapatra (2011) reported that probiotic bacteria must meet the criteria of : 1) resistant to stomach acid and bile salts in the intestine, non-pathogenic, and growth in the human intestinal tract; 2) high viability in the small intestine because it can be metabolized quickly; 3) strongly adhesive and able to form colonies in the human small and large intestines; 4) able to produce organic acids, such as lactic, butyric and propionic that have antimicrobial properties against pathogenic bacteria by producing bacteriocins.

Probiotic LAB are different from non-probiotic LAB; the former are superior in their attachment to intestinal epithelial cells because they can produce adhesin proteins (Astawan et al. 2012, 2011). Probiotic bacteria can produce antibacterial bioactive peptides (bacteriocins), such as plantaricin, nisin, and acidophilus that could kill pathogenic bacteria in the intestine (Arief et al. 2014, Sulistiani 2018). Probiotic bacteria demonstrate immunomodulatory benefits by stimulating T lymphocytes, immunoglobulin A, immunoglobulin G, monocytes and macrophages (Arief et al. 2015). Probiotics protect against the invasion of pathogenic bacteria by attaching and colonizing the intestines to compete and inhibit pathogenic bacteria's growth in the intestines (Cook et al. 2012). Swanson et al. (2020) reported that probiotic LAB had high resistance to membrane damage due to a significant decrease in extracellular pH compared to non-probiotic LAB. Bile salt-resistant LAB probiotic cells, when incubated in oxgall solutions, continue to grow and do not undergo lysis (Sulistiani 2018).

Probiotics have been widely used to improve the health of the digestive tract of livestock, fisheries and waters, pets, and humans (Buntin, Chanthachum & Hongpattarakere 2008). Probiotics are very beneficial for health in that they kill pathogenic bacteria; exhibit immunomodulation, immunostimulant, antimutagenic and anticarcinogenic activities; lower serum cholesterol reducing symptoms of lactose intolerance; prevent diarrhea and vaginitis; lower blood pressure; and maintain mucosal integrity (Buntin, Chanthachum & Hongpattarakere 2008; Florou-Paneri, Christaki & Bonos 2013; Klaenhammer & Kullen 1999). The species of LAB which are superior probiotics include *Lactobacillus plantarum, L. johsonii, L. bulgaricus, L. acidophilus, L. rhamnosus, L. casei, L. gasseri, Lactococcus lactis* subsp. *cremoris, Streptococcus thermophilus, Bifidobacterium bifidum, B. breve, B. longum, B. infantis, Enterococcus faecium* and *E. faecalis* (Astawan et al. 2011; Kaur, Chopra & Saini 2002; Savadogo et al. 2006; Zhang et al. 2016).

Their populations were widespread not only in food (vegetables, dairy products, fermented meats, bread dough, beverages), hay, and plant wastes but also in the intestines, respiratory tract, and genital tract of humans and animals (Fhoula et al. 2013; Florou-Paneri, Christaki & Bonos 2013; Savadogo et al. 2006). Shi et al. (2012) reported the isolation of 27 LAB from the milk of Sumbawa horses with 25 strains were *Lactobacillus rhamnosus* and 2 strains were *Lactobacillus fermentum*. Among all isolates, three strains of *Lactobacillus rhamnosus*: FSMM15, FSMM2, and FSMM26, are probiotic. Kusdianawati et al. (2020) reported that Sumbawa horse milk which is a local food of Sumbawa, West Nusa Tenggara is acidic and will not be coagulated when heated due to the presence of LAB. Therefore, this study was carried out to isolate, characterize, and identify the potential probiotic LAB from the milk of Tambora horses in Sumbawa, West Nusa Tenggara. Tambora horse milk was selected because it is Sumbawa's authentic nutritious, healthy drink which is claimed to be rich in probiotic bacteria. This research is targeted to discover new probiotic LAB strains from the mare milk from Tambora, Sumbawa, Indonesia.

Materials and Methods

Research workflow

This was a descriptive and exploratory study, with no specific statistical analysis involved. The research workflow consisted of six steps: (1) exploration of probiotic LAB in the mare's milk from Tambora, West

Nusa Tenggara, (2) determination of functional attributes of probiotic bacteria (bile salt tolerance, low pH, SGJ, SIJ, antibacterial activity and BSH gene), (3) LAB genotype test by restriction fragment length polymorphism analysis RFLP-PCR, (4) identification of LAB based on 16S rRNA gene, (5) characterization of LAB growth at a selected certain pH, and (6) determination of the bacterial species that have the potential to be selected as Indonesian indigenous probiotic bacteria.

HORSE MILK COLLECTION AND ISOLATION OF LAB

Two samples of horse milk (SK1 and SK2) (Figure 1) were obtained from two different horses in a farm in Tambora, Bima, West Nusa Tenggara Province. The samples were stored in an ice box at cold temperatures (4 °C) then transported to the Research Center for Applied Microbiology. In the laboratory, the milk pH was measured and the LAB was isolated. LAB was isolated by vortexing the milk with saline water 0.85% (diluting serially from 10°-10^{-7}mL/mL). A total of 100 µL was inoculated into deMan, Rogosa and Sharpe Agar (MRSA) enriched with 0.5% $CaCO_3$ and incubated anaerobically at 37 °C for two days; then colonies were counted. Colonies of isolated LAB were purified twice on Glucose Yeast Extract Peptone Agar (GYPA) + 0.5% $CaCO_3$. The GYPA medium was described by Okada et al. (1986). The isolated LAB was stored in 20% glycerol at -80 °C for further analysis. From SK1 and SK2, 81 isolates of LAB were collected, but only 25 isolates had probiotic properties, namely antibacterial activity, and resistance to low pH (pH 2.5) and 0.3% of oxgall. The 25 selected isolates were used for this research.

FIGURE 1. Horse milk samples SK1 and SK2

ANALYSIS OF LAB RESISTANCE TO BILE SALTS

Bile salt tolerance was determined using a method by Buntin, Chanthachum and Hongpattarakere (2008) with minor alteration. The LAB strains were grown in MRS broth without bile for 24 h at 37 °C. Then, about 20 µL of active LAB culture was inoculated to 1 mL MRS broth containing 0.3% bile salt (oxgall) and incubated at 37 °C for 24 h. The spotted culture on MRSA determined the viable cell of bile salt tolerance LAB. Bacterial growth on media was observed after incubation at 37 °C for 48 h. Three replications were done for this analysis.

ANALYSIS OF LAB RESISTANCE TO LOW pH

The acid tolerance of LAB was determined using Buntin, Chanthachum and Hongpattarakere (2008) and Tokatli et al. (2015) methods with some minor adjustments. LAB culture was grown and collected, then centrifuging the broth for 5 min at 10,000 rpm, followed by the washing stage. After washing the residue, the samples were centrifuged for 5 min at 10,000 rpm to recollect the cell pellet. To test the viability of the cells, we spread three replications of the cell suspension onto the Glucose-Yeast Extract-Peptone (GYP) enriched with 0.5% $CaCO_3$ and incubated for 48 h at 37 °C. The growth of bacteria was then ascertained.

ANALYSIS OF LAB RESISTANCE TO SGJ AND SIJ

SGJ and SIJ resistance were analyzed using the same method as Sulistiani et al. (2020). For both analyses, the viable cell population was determined before and after incubation by spreading the inoculum on GYPA, adding 0.5% of $CaCO_3$, and incubating for 48 h at 37 °C. The bacterial growth was observed using the same methods mentioned in the Resistance LAB to low pH analysis section.

ANTIBACTERIAL ANALYSIS

The antibacterial analysis was conducted using a modified version of the well diffusion test method described by Buntin, Chanthachum and Hongpattarakere (2008) and Schillinger and Lücke (1989). The pathogenic bacteria for the test were *Aeromonas sobria* (AS), *Plesiomonas shigelloides* (PS), *Escherichia coli* B5 (EC), *Aeromonas veronii* N1 (AV), *Klebsiella pneuomonia* (KP), *Pseudomonas aeruginosa* B3 (PA), *Bacillus cereus* (BC), *Staphylococcus aureus* B4 (SA), *Pseudomonas mosselii* N5 (PM), *Aeromonas hydrophila* N2 (AH), and *Edwarsiella ichtalurgi* (EI). The pathogenic bacteria were grown on brain heart infusion broth (BHI) for 24 h at 37 °C for the test. Petri dishes containing 20 mL of Muller Hinton agar (1.8%) covered with 5 mL of Muller Hinton soft agar (0.75%) and contained approximately 10^6 CFU/mL of newly grown pathogenic bacteria. The 5 mm diameter wells were filled with 50 µL of active LAB cultures (grown in MRSB and incubated at 37 °C for 24 h) and then incubated at 37 °C for 24 h. The diameter of inhibition was measured, then the antimicrobial activity was determined by measuring the clear zones around the well. This experiment was three replicated.

BILE-SALT HYDROLASE GENE DETECTION

BSH was encoded by the BSH genes that were present. Primers 5'-CGTATCCAAGTGCTCATGGTTTAA-3' and 5'-ATGTGTACTGCCATAACTTATCAATCTT-3' were used to detect the BSH gene. PCR amplifications were conducted in a Takara thermal cycler. Initial denaturation at 94 °C for 4 min was followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 64 °C for 30 s, and polymerization at 72 °C for 1 min. The final extension at 72 °C for 7 min was performed for cycling conditions. The PCR products were separated on a 2% agarose gel in a 1x Tris-Acetate EDTA buffer (TAE), running for 30 min at 100 V. Ethidium bromide was used to dye the gel. The stained gel was then visualized using the Atta Gel Doc system. The 100 bp plus DNA ladder (Vivantis) was used in electrophoresis to determine the band size of DNA (Jatmiko, Howarth & Barton 2017).

ANALYSIS OF AMPLIFICATION OF 16S-23S rDNA INTERNAL SPACER REGION (ISR) AND THE POLYMERASE CHAIN REACTION-RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP- PCR)

The 16S-23S rDNA ISR amplification was performed in a procedure previously done by Rachman et al. (2003), using the primers 16S/p2 forward and 23S/P7 reversed were used (Sulistiani et al. 2020). The PCR reaction was conducted at 94 °C for 1.5 min, then 95 °C for 30 s, 50 °C for 30 s, 72 °C for 90 s for 30 cycles, and 72 °C for 5 min for the last extension. PCR ended with cooling at 4 °C for 20 min. The 16S-23S rDNA ISR PCR product was restricted using the AfaI restriction enzyme according to the instructions for Thermo Scientific. The PCR products were visualized using agarose gel electrophoresis (2% with 1x TAE). The electrophoresis

was run for 30 min at 100 V. The gel was immersed in 10 g/mL ethidium bromide solution for 30 min, then washed with 1x TAE, and documented using the gel documentation system. The DNA ladder (100 bp, Vivantis) determined the DNA band size, which was then detected and measured using the PhotoCaptMw program (version 99.03, Vilber-Lourmat, Marne-la-Vallée, France). The DNA bands from the RFLP-PCR results were translated into the binary data which later generated a genetic distance matrix using the formula by Nei and Li (1979) in the Unweighted Pair-Group Method Arithmetic (UPGMA) of the Numerical Taxonomy and Multivariate System (NTSys) version 2.02 (Rohlf 1998).

LAB IDENTIFICATION WITH PCR AMPLIFICATION AND SEQUENCING OF 16S rDNA

Following Mechai, Debabza and Kirane (2014), this study used 27F primer pairs (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'), and amplified the 16S rDNA. After carrying out denaturation for 90 s at 95 °C, 30 cycles of annealing for 30 s at 95 °C, extension for 30 s at 50 °C, and denaturation for 90 s at 72 °C, we performed the final extension at 72 °C for 5 m. Electrophoresis (1 % agarose gel in 1× Tris-acetate-EDTA (TAE) buffer (Promega) at 100 V for 20 min and stained by ethidium bromide was done to examine the PCR product. We used 100 bp plus DNA ladder (Vivantis) as the standard size, and a UV transilluminator (Atta) to visualize the gels. The PCR products were shipped for sequencing to the First Base Laboratory, Malaysia. Each template was sequenced in a single pass using primer 27F and 1492 R. The sequence homologies were determined using the Basic Local Alignment Search Tool (BLAST) program Atta by contrasting the obtained sequences to 16S rDNA sequences available in the GenBank nucleotide databases. The multiple sequences of LAB of the horse milk were run using Mega software version 5.2 with sequences of LAB-type strains (Tamura et al. 2011). The tree was built using the neighbour-joining technique (Saitou & Nei 1987), and the tree's stability was evaluated using the bootstrap technique with 1000 replicates (Efron 1979).

THE PHYSIOLOGICAL PROPERTIES

Physiological characteristics were determined by LAB growth assay in MRS broth at varying temperatures (0 °C, 37 °C, and 45 °C), pH (4, 5.5, and 9.0), and salt concentrations (3%, 5%, and 7%) (Agaliya & Jeevaratnam 2012). Three replications were performed for the analysis.

RESULTS AND DISCUSSION

The original test for the acidity level (pH) of horse milk from Sumbawa Regency showed a pH of 6 (Ardiansyah et al. 2021). However, in this study, the pH of SK1 and SK2 was 4.13 and 4.15, respectively. This indicates that the horse's milk has become sour and possibly fermented after sampling and during transport to the laboratory. The total LAB count of SK1 and SK2 was 5.6×10^8 CFU/mL and 4.4×10^8 CFU/mL, respectively. From the isolation SK1 and SK2 (Figures 2 and 3), 81 LAB isolates were obtained but only 25 isolates had probiotic properties, meaning they had antibacterial activity and resistance to low pH (pH 2.5) and to 0.3% oxgall. The selected isolates were used for further research.

All selected LAB were able to grow in an acidic environment (pH 2.5) and contained bile (0.3% bile) (Table 1). For probiotic strains to reach their site of action under physiologically favourable conditions, they must tolerate very low pH and the detergent effect of bile salts (Agaliya & Jeevaratnam 2012; Pais et al. 2020). To gain assurance of selecting highly acid-tolerant strains, we considered tolerance to acidity (pH 2.5) as an essential functional requirement for probiotics to enable them to survive gastrointestinal transit, although it was not the most prevalent pH value in the human stomach (Argyri et al. 2013; Lee, Park & Kim 2022; Tokatli et al. 2015). Interestingly, it was shown that lactobacilli could grow at pH 2.5 (Kyereh & Sathivel 2021; Mangia, Saliba & Deiana 2019). Acid and bile tolerance properties demonstrate the capacity of probiotic bacteria to resist acidic conditions in the stomach and the presence of bile in the small intestine (Buntin, Chanthachum & Hongpattarakere 2008). According to them, bile salt concentration of 0.3% is required and adequate for screening resistant strains. Therefore, the digestive tract was hypothesized to support the analysis of acid and bail tolerance in 25 bacterial strains. Millette et al. (2008) noted that the ability to withstand bile salts is one of the most crucial survival characteristics of LAB in the duodenum and upper small intestine.

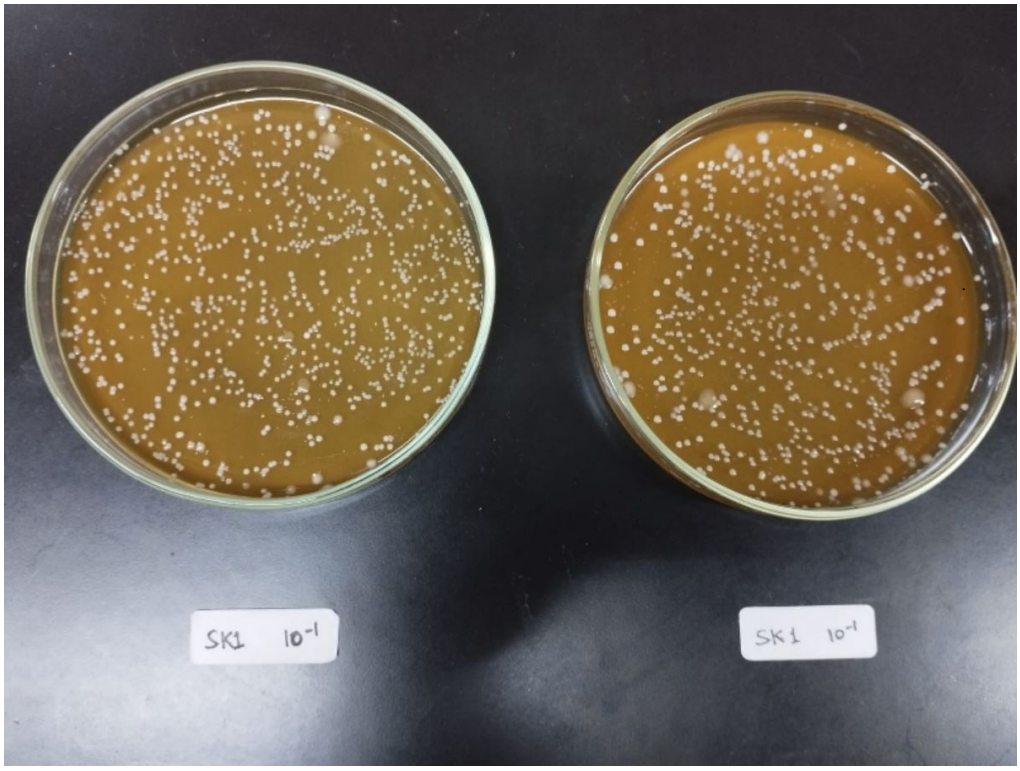


FIGURE 2. Lactic acid bacteria SK1

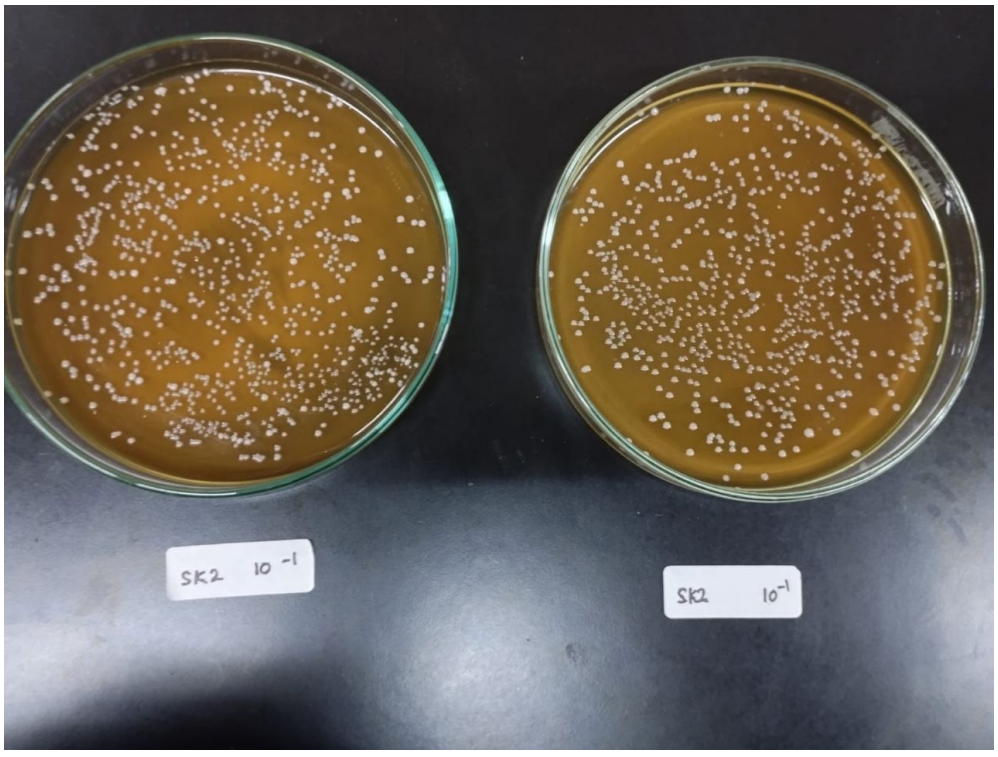


FIGURE 3. Lactic acid bacteria SK2

TABLE 1. The bile and acid resistance, survival of LAB strains in SGJ and SIJ and diameter of inhibition (mm) of selected horse milk LAB against pathogenic bacteria*

No	Strain	pH 2,5	*Oxgall* 0,3%	SIJ	SGJ	Inhibition zone (mm ± 0.0)										
						AS	PS	EC	AV	KP	PA	BC	SA	PM	AH	EI
1	SK1-2	+	+	+	+	20	18	15	18	9	11	18	0	14	13	15
2	SK1-8	+	+	+	+	21	18	15	20	12	13	20	9	14	13	15
3	SK1-13	+	+	+	+	23	18	15	20	11	13	20	0	13	13	15
4	SK1-17	+	+	+	+	23	17	15	20	12	13	20	0	13	13	15
5	SK1-19	+	+	+	+	23	15	15	20	11	12	20	11	14	13	15
6	SK1-20	+	+	+	+	23	18	15	18	12	11	19	0	13	13	14
7	SK1-23	+	+	+	+	23	18	15	20	12	13	19	9	14	13	16
8	SK1-28	+	+	+	+	22	16	15	20	11	12	18	9	13	12	15
9	SK1-42	+	+	+	+	23	18	15	20	10	12	19	9	13	13	15
10	SK1-44	+	+	+	+	22	17	13	20	10	12	19	9	14	14	15
11	SK1-49	+	+	+	+	20	17	13	20	9	12	20	0	14	14	15
12	SK2-2	+	+	+	+	23	17	14	15	5	9	14	0	12	12	14
13	SK2-6	+	+	+	+	23	17	15	13	5	5	14	0	12	12	14
14	SK2-17	+	+	+	+	24	18	15	20	12	12	18	0	14	14	15
15	SK2-28	+	+	+	+	23	18	15	18	10	12	19	0	14	14	15
16	SK2-29	+	+	+	+	24	18	15	15	7	11	18	0	13	13	15
17	SK2-30	+	+	+	+	23	17	14	20	7	11	18	0	13	13	15
18	SK2-32	+	+	+	+	23	17	15	20	11	12	18	0	13	14	15
19	SK2-33	+	+	+	+	23	16	13	20	11	12	20	9	14	14	15
20	SK2-34	+	+	+	+	24	18	15	20	11	13	20	11	15	14	14
21	SK2-36	+	+	+	+	23	18	15	20	11	13	19	0	13	14	15
22	SK2-37	+	+	+	+	24	16	13	20	11	11	20	0	12	13	13
23	SK2-40	+	+	+	+	24	18	15	18	10	11	19	0	14	14	14
24	SK2-41	+	+	+	+	24	17	15	14	5	9	17	0	13	13	14
25	SK2-47	+	+	+	+	24	18	15	20	12	12	19	9	14	14	15
26	MRSB (negative control)	-	-	-	-	0	0	0	0	0	0	0	0	0	0	0

Grow well (+); no growth (-); *Aeromonas sobria* (AS), *Plesiomonas shigelloides* (PS), *Escherichia coli* B5 (EC), *Aeromonas veronii* N1 (AV), *Klebsiella pneuomonia* (KP), *Pseudomonas aeruginosa* B3 (PA), *Bacillus cereus* B9 (BC), *Staphylococcus aureus* B4 (SA), *Pseudomonas mosselii* N5 (PM), *Aeromonas hydrophila* N2 (AH), *Edwarsiella ichtalurgi* (EI).

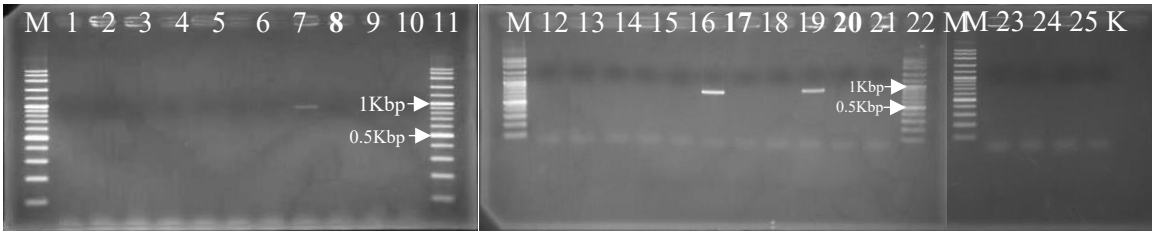
*The experiments were carried out in three replications. The experiment was conducted with three replicates where the inhibition zone was measured in mm, the average of the inhibition zone results was taken (mm ± 0.0)

The results showed all isolates were tolerant SGJ and SIJ. The ability of probiotic bacteria to tolerate stomach and intestinal environments is crucial to their survival, growth, and function in the gut (Ashraf & Smith 2016). This study showed that after 6-hour exposure to SGJ conditions (pH 2.5) and survival in SIJ (pH 8.0), all isolates survived well (Table 1), indicating potential to return to the initial levels throughout the passage through the gastrointestinal tract (Millette et al. 2008).

Antibacterial research showed that every isolate had a variety of inhibitory activities. All lactobacilli prevented the growth of *Aeromonas sobria, Plesiomonas shigelloides, Escherichia coli* B5, *Aeromonas veronii* N1, *Klebsiella pneumonia, Pseudomonas aeruginosa* B3, *Bacillus cereus* B9, *Staphylococcus aureus* B4, *Pseudomonas mosselii* N5, *Aeromonas hydrophila* N2, *Edwarsiella ichtalurgi* (Table 1, Supplementary Figure S1). This indicated that inhibition or antibacterial activity of LAB against the pathogenic bacteria of *Aeromonas hydrophila* N2 (AH), *Bacillus cereus* B9 (BC), *Edwarsiella ichtalurgi* (EI), *Pseudomonas mosselii* N5 (PM), and *Staphylococcus aureus* B4 (SA) was shown by the clear zones around the wells. The wider the clear zone, the higher the antibacterial activity. The production of substances, such as low molecular weight antimicrobial compounds, bacteriocins, carbon dioxide, hydrogen peroxide, organic acids (lactic, acetic, and propionic acids), and diacetyl play a role in the development of LAB's inhibitory properties.

In complicated ecological systems, bacteriocins may improve the survival of LAB. The ability of probiotics to suppress the growth of harmful microbes via secreted products is more essential than the effect of acidic pH (Akinyosoye & Oyetayo 2013; Buntin, Chanthachum & Hongpattarakere 2008; Oluwajoba; Chugh & Kamal-Eldin 2020; Savadogo et al. 2006). In addition to the various basic features, probiotics should display functional health advantages. Furthermore, probiotics should demonstrate their antibacterial properties, notably against GI system infections (Al-Madboly & Abdullah 2015; Santacroce, Charitos & Bottalico 2019). Probiotics improve the host's intestinal homeostasis, and their action method is linked to competition for attachment sites (competitive exclusion). By creating a physical barrier on the gut mucosa, the probiotics inhibit the adherence of harmful bacteria (Shabani et al. 2015). Probiotics also produce antibiotic chemicals that control intestinal pathogens (de F. Reque et al. 2000; Deng et al. 2020). Probiotic bacteria in the gut limit the growth of harmful bacteria by competitive inhibition, the production of organic acids, and other antibacterial compounds (Erkkilä & Petäjä 2000; Hyronimus et al. 2000; Wan, Forsythe & El-Nezami 2019).

Of 25 isolates examined in this study, three isolates (strains SK1-28, SK2-30, and SK2-34) contained the BSH gene with an approximate band size of 900 bp (Figure 4). The presence of BSH gene shows the bacteria's potential to produce BSH enzymes that play a role in hydrolyzing bile salt to reduce the detergent effect (Dong & Lee 2018; Millette et al. 2008). In addition to decreasing bile's detergent properties, BSH enzymes also reduce bactericidal effects on strains. Lilis Nuraida (2015) explained that BSH deconjugates glyco-and tauro-bile acids by hydrolyzing conjugated glycodeoxycholic acid and taurodeoxycholic acid. After deconjugation, bile acids become less soluble and are absorbed by the intestines, resulting in their excretion via stool. In a homeostatic response, cholesterol is utilized to produce new bile acids. This decreases serum cholesterol levels.



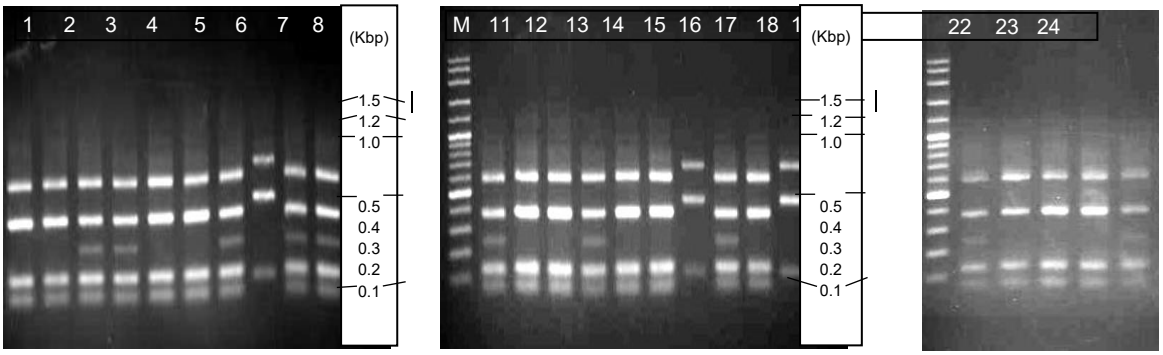
M= 100 bp plus DNA Marker (Vivantis), 1=SK1-2, 2=SK1-8, 3= SK1-13, 4= SK1-17, 5= SK1-19, 6= SK1-20, 7= SK1-23, **8= SK1-28**, 9= SK1-42,10=, SK1-44, 11= SK1-49, 12= SK2-2, 13= SK2-6, 14= SK2-17, 15= SK2-28, 16= SK2-29, **17= SK2-30**, 18= SK2-32, 19= SK2-33, **20= SK2-34**, 21= SK2-36, 22= SK2-37, 23= SK2-40, 24= SK2-41, 25= SK2-47, K= Negative control. The bold indicated the strain had BSH gene.

FIGURE 4. Amplification products of BSH gene on horse milk LA

Discrimination or categorizing strains/genotypes within a species is necessary to identify microorganisms and classify potential microbial isolates according to their physiological activities for further industrial development. The 16S-23S rDNA ISR region has more variable sequences than the 16S and 23S rDNA genes. The region between tRNA Ala and 23S rDNA contains polymorphisms that can be exploited for identification at the level of bacteria species and strains (Ben Belgacem et al. 2009). The variety of strains in a species can be determined by RFLP analysis of 16S-23S rDNA ISR of bacteria and their strain types (Ben Belgacem et al. 2009; Miteva, Boudakov & Ivanova-Stoyancheva 2001; Rachman et al. 2003). The results of RFLP 16S-23S rDNA ISR analysis using the AfaI restriction enzyme in this study produced eight DNA fragments with the lengths of 70, 110, 130, 250, 380, 450, 620, and 700 bp (Figure 5). The UPGM analysis of RFLP- PCR separated the LAB strains into three distinct groups. Group I had four DNA bands with molecular weights of 70, 130, 380, and 620 bp; Group II had five DNA bands with molecular weights of 70, 130, 250, 380, and 620 bp; and Group III had three DNA bands with molecular weights of 110, 450, and 700 bp (Table 2, Figure 6).

We used the 16S rRNA gene sequencing to select one representative strain from each group for further identification. The representative strains from groups I, II, and II were SK1-19, SK1- 23, and SK2-34, respectively. The amplification of 16S rDNA of representative strains produced a single DNA band with a size of 1,500 bp (Figure 7(A)). The NCBI BLAST result of 16S rDNA sequence strains of SK1-19, SK1-23, and SK2-34 showed a maximum identity of 100% near *Lacticaseibacillus rhamnosus* strain LV108 [CP053619], *Lacticaseibacillus rhamnosus* strain 4786 [MT545153], and *Lactiplantibacillus plantarum* strain Heal19 [CP055123]. The phylogenetic analyses based on the neighbour-joining method showed that the sequences have the same clade as their type species with a bootstrap value of 99–100%. Based on data of BLAST results and phylogenetic tree analyses, we finally identified the isolate SK1-19 as *Lacticaseibacillus rhamnosus* (basonym *Lactobacillus rhamnosus*) SK1-23 as *Lacticaseibacillus rhamnosus* (basonym *Lactobacillus rhamnosus*), and SK2-34 as *Lactiplantibacillus plantarum* basonym *Lactobacillus plantarum*) (Zheng et al. 2020) (Figure 7(B)).

All isolates were characterized based on their physiology. The physiological tests showed all isolates could grow well at 37 °C and 45 °C, except for Group III that showed poor growth at 45 °C. All isolates did not grow at 0 °C, indicating that they had mesophilic characteristics. The isolates could tolerate salt concentrations of 3, 6.5%, and 7% (Table 3). It is known that sodium chloride concentration and temperature can influence cell development significantly (Agaliya & Jeevaratnam 2012). *Lacticaseibacillus rhamnosus* groups I and II could be detected with great clarity because of this physiological test. The strains in group II could not develop on media with a pH of 9, thus indicative of a difference between groups I and II (Table 3).



M= 100 bp plus DNA Marker (Vivantis), 1= SK1-2, 2= SK1-8, 3= SK1-13, 4= SK1-17, 5= SK1-19, 6= SK1-20, 7= SK1-23, 8= SK1-28, 9= SK1-42, 10= SK1-44, 11= SK1-49, 12= SK2-2, 13= SK2-6, 14= SK2-17, 15= SK2-28, 16= SK2-29, 17= SK2-30, 18= SK2-32, 19= SK2-33, 20= SK2-34, 21= SK2-36, 22= SK2-37, 23= SK2-40, 24= SK2-41, 25= SK2-47.

FIGURE 5. PCR-RFLP analysis of 16S-23S rDNA Internal spacer region (ISR) on Horse milk LAB using *afa*1

TABLE 2. Clustering RFLP-PCR 16S-23S rDNA ISR analysis using the *Afa*I restriction enzyme

Groups		Strains	RFLP-PCR PCR 16S-23S rDNA ISR - *Afa* I	
			No. DNA bands	bp
I.	1	SK1-2, SK1-8, SK1-19, SK1-20, SK2-2, SK2-6, SK2-28, SK2-29, SK2-33, SK2-37, SK2-40, SK2-41.	4	70, 130, 380, 620
II.	2	SK1-13, SK1-17, SK1-23, SK1-42, SK1-44, SK1-49, SK2-17, SK2-32, SK2-36, SK2-47.	5	70, 130, 250, 380, 620
III.	3	SK1-28, SK2-30, SK2-34.	3	110, 450, 700

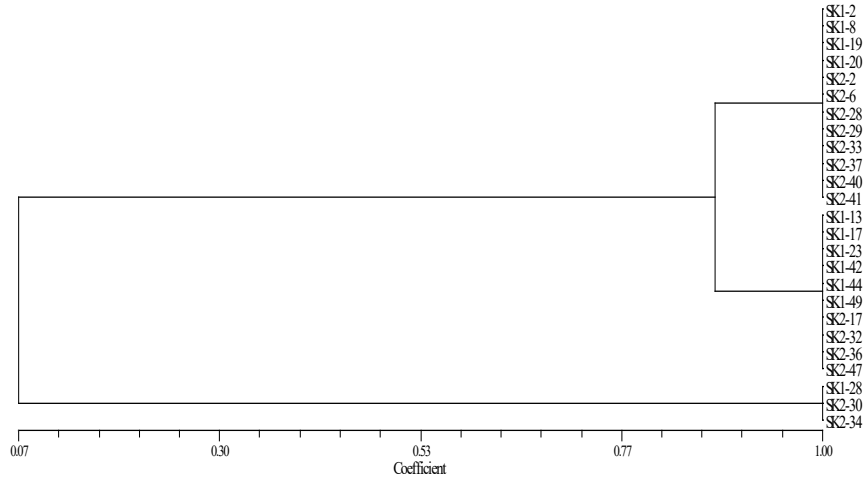


FIGURE 6. Dendogram of RFLP-PCR PCR 16S-23S rDNA ISR horse milk LAB using the UPGMA method

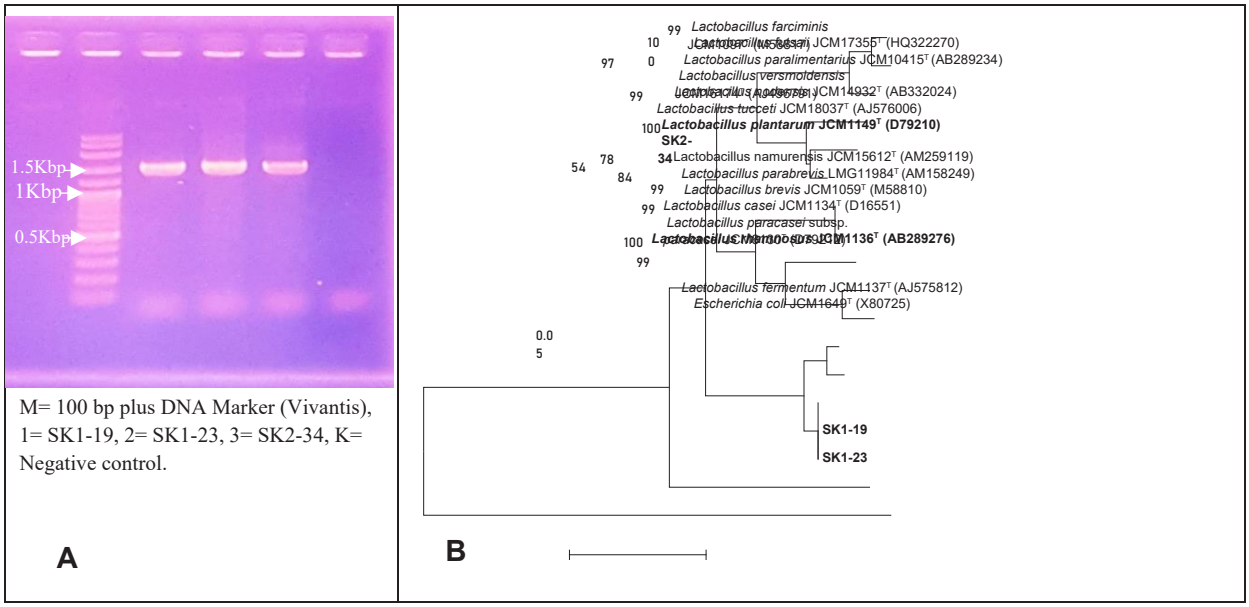


FIGURE 7. Amplification product of 16S rDNA (A) and phylogenetic tree based on 16S rDNA sequence of representative horse milk LAB using neighbor joining tree method and *Escherichia coli* JCM1649^T (X80725) as outgroup (B)

TABLE 3. Physiological analysis of horse milk LAB probiotic candidate

No.	Group	Strain	NaCl			Temperature			pH		
			3%	5%	7%	0 °C	37 °C	45 °C	4	5.5	9.0
1	I	SK1-2	+	+	±	-	+	+	+	+	+
2		SK1-8	+	+	±	-	+	+	+	+	+
3		SK1-19	+	+	±	-	+	+	+	+	+
4		SK1-20	+	+	±	-	+	+	+	+	+
5		SK2-2	+	+	±	-	+	+	+	+	+
6		SK2-6	+	+	±	-	+	+	+	+	+
7		SK2-28	+	+	±	-	+	+	+	+	+
8		SK2-29	+	+	±	-	+	+	+	+	+
9		SK2-33	+	+	±	-	+	+	+	+	+
10		SK2-37	+	+	±	-	+	+	+	+	+
11		SK2-40	+	+	±	-	+	+	+	+	+
12		SK2-41	+	+	±	-	+	+	+	+	+
13	II	SK1-13	+	+	±	-	+	+	+	+	-
14		SK1-17	+	+	±	-	+	+	+	+	-
15		SK1-23	+	+	±	-	+	+	+	+	-
16		SK1-42	+	+	±	-	+	+	+	+	-
17		SK1-44	+	+	±	-	+	+	+	+	-
18		SK1-49	+	+	±	-	+	+	+	+	-
19		SK2-17	+	+	±	-	+	+	+	+	-
20		SK2-32	+	+	±	-	+	+	+	+	-
21		SK2-36	+	+	±	-	+	+	+	+	-
22		SK2-47	+	+	±	-	+	+	+	+	-
23	III	SK1-28	+	+	±	-	+	±	+	+	+
24		SK2-30	+	+	±	-	+	±	+	+	+
25		SK2-34	+	+	±	-	+	±	+	+	+

Grow well (+)*; slightly grow (±)*; (no growth (-)

*The definition of growing well is when the bacteria were grown in a liquid medium showing dense turbidity, while the definition of growing a little is when bacteria were grown in liquid medium, the liquid medium looks clear and the bacteria were only at the bottom of the tube forming spots (collections of bacteria)

Conclusions

This study identified 25 indigenous strains of LAB originating from horse milk which exhibited probiotic characteristics, such as resistance to acid and bile salts, survival under SIJ and SGJ treatments, and antibacterial activity. Bile salt hydrolase activity was confirmed by the detection of BSH gene through PCR technique, in which three strains (SK1-28, SK2-30, and SK2-34) were observed to possess this gene of interest. The result of RFLP-PCR analysis classified the LAB strains into three distinct groups based on their molecular weights of DNA bands, namely Group I with four DNA bands (70, 130, 380, and 620 bp), Group II with five DNA bands (70, 130, 250, 380, and 620 bp), and Group III with three DNA band (110, 450, and 700 bp). Investigation of the 16 rRNA gene sequences showed that strains in Groups I and II were identified as the members of *Lacticaseibacillus rhamnosus* (basonym *Lactobacillus rhamnosus*), while those in Group III were the members of *Lactiplantibacillus plantarum* (basonym *Lactobacillus plantarum*). The *Lacticaseibacillus rhamnosus* strains of Groups I and II could be differentiated based on their physiology where all strains grew at pH 9 (alkaline), but not the case with Group II.

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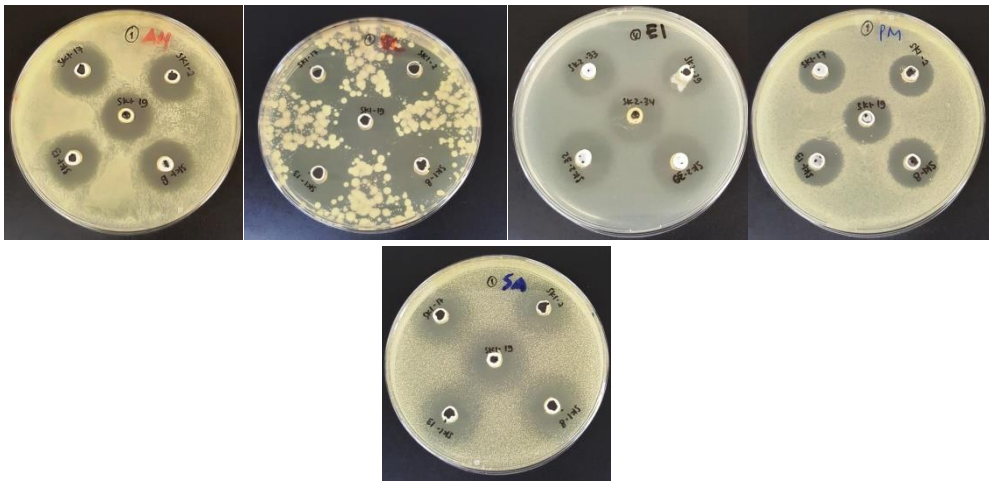

*Supplementary Figure S1 is a supplementary document of Table 1

**Inhibition of LAB against pathogenic bacteria is shown by the clear zone around the well. Figure 4 was supplementary