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RESEARCH ARTICLE

Analysis and characterization of potential probiotic properties of *Lactobacillus* and *Bacillus salmalaya* 139SI

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ABSTRACT

Bacillus salmalaya 139SI and *Lactobacillus sp*. isolated from Kerinci buffalo milk yogurt were investigated for their potential probiotics properties. Three isolates from the milk yogurt were identified as *L. plantarum* 1, *L. plantarum* 2 and *L. delbrueckii*. The three isolates and *B. salmalaya* 139SI exhibited tolerance to gastric acid of pH 3.5, resistance to bile salts and were able to produce lactic acid. All the four strains exhibited antimicrobial activity against *Salmonella* Enteritidis, *Escherichia coli* and *Staphylococcus aureus*. Combination of isolates of *L. plantarum* 1 and *L. plantarum* 2 exhibited the highest coaggregation activity. *L. plantarum* 1 showed the highest hydrophobicity and had no adverse effects for the toxicity test (Lethal Dose 50). Characterization of probiotic properties *in vitro* and *in vivo* indicate that both bacteria *L. plantarum* and *B. salmalaya* 139SI are potential candidates for probiotic therapeutic application against *Salmonella* Enteritidis.

Keywords: Probiotic; *Lactobacillus plantarum; Bacillus salmalaya* 139S

INTRODUCTION

Food of animal products are the main source of the *Salmonella* causes of human disease (Gast, 2003). Diarrhea is one of the biggest health problems in the world in recent years. According to Humprey (1988) the increasing case of diarrhea in humans is usually caused by increasing cases of salmonellosis (Humprey, 1998). Salmonellosis is an infectious zoonotic disease and included in a foodborne disease (Gast, 2003). Salmonellosis is very important in relation to public health because it causes tremendous economical disadvantages. The UK reported on average 4,000 cases of salmonellosis in humans per year. The United States estimated 50% incidence of salmonellosis in humans caused by serovar *Salmonella* Enteritidis, *Salmonella typhimurium* and *Salmonella heidelberg* (Pascual et al., 1999). Center of Disease Control and Prevention (CDC, USA) reports that there are 1.2 million cases of salmonellosis which causes the death of 450 people each year (CDC, 2019). *Salmonella* Enteritidis and *Salmonella typhimurium* were reported as the primary causes of salmonellosis. Therefore, the control of salmonellosis becomes a major problem especially in industrial poultry farming (Mouttotou et al., 2017; Gast, 2003).

In recent years, scientists have paid more attention to the advantages that microbes residing within mammals give. Probiotic bacteria are microbial inhabitants of the gastrointestinal system that are hypothesized to have health advantages through boosting an animal's intestinal microbial balance (Yun et al., 2009). Buffalo fermented milk locally called *dadih*, has been studied extensively in Indonesia for its use as probiotics (Surono, 2015). Studies have been carried out to investigate probiotics' ability to survive conditions in the host digestive tract. According to Chou et al. (2008) probiotics must have the ability to withstand very low pH (around 3.0) during at least 90 minutes of their transition time in the stomach. Once probiotics pass through the stomach, these microorganisms enter the upper intestinal tract where bile salts are secreted. Probiotics must be able to withstand these two extreme transient acidic and basic

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environments prior to colonizing the lower intestinal tract.

The probiotic bacteria used as an alternative treatment against *Salmonellosis* include *Streptococcus lactis* (Sugitha, 1995), *Lactobacillus brevis* (Sirait et al., 1995), *Lactobacillus* sp. strains T6 and B14, *Leucosnostoc paramesentroides* R-62 and *Lactococcus* sp. strain T11, K5 and B9 (Surono, 1999), *Pediococcus pentosaceus* (Yuliawati et al*.,* 2012). The usage of these bacteria can be useful since they have antibacterial activity and boost both immune and anti -tumorigenic activity (Yun et al., 2009). The poultry industry is an important economic activity in many countries. In large-scale rearing facilities, where poultry are exposed to stressful conditions, problem-related to diseases and deterioration of environmental conditions often occur and result in serious economic losses. Prevention and control of diseases have led during recent decades to a substantial increase in the use of veterinary medicines. However, the utility of antimicrobial agents as a preventive measure has been questioned, given the extensive documentation of the evolution of antimicrobial resistance among pathogenic bacteria. This has raised the possibility of antibiotics ceasing to be used as growth stimulants for poultry due to consumers' concern about the side-effects of their use as therapeutic agents, thus putting pressure onto the poultry industry to look for alternatives.

Many farmers administer probiotics to their livestock in place of certain antibiotics to minimize the usage of antibiotics while maintaining or even enhancing production efficiency. Several studies have demonstrated that ingesting fermented foods with probiotics may reduce the number of harmful bacteria in the gastrointestinal system while increasing liveweight growth (Angelakis, 2017; C De et al., 2014; Geary et al., 1999). These factors make probiotics derived from fermented food sources appealing for use in domestic poultry. *Lactobacillus plantarum* and *Bacillus salmalaya* 139SI have the potential to be used as probiotics bacteria against salmonellosis in farm layer chickens. However, little is reported regarding their potential to be used as a probiotic. Therefore, this research work was aimed to isolate and identify *Lactobacillus* sp. isolated from Kerinci buffalo milk yogurt using several screening tests and to analyze and characterize the potential probiotic properties of *Lactobacillus* sp. and *Bacillus salmalaya* 139SI against *Salmonella* Enteritidis.

METHODS

Bacterial isolates

Lactobacillus bacteria used in this study were isolated from buffalo milk purchased from local markets in Kerinci, Jambi, Sumatera Indonesia according to the methods used by Adnan & Tan (2007) and Khedid et al. (2009). The milk was fermented and isolated at the R & D section of Agro Premier Biotech Sdn Bhd, Kuala Lumpur, Malaysia. All lactic acid bacteria (LAB) were further analyzed and maintained at University Malaya Molecular Bacteriology & Toxicology (UMMBTL), Institute of Biological Sciences, Faculty of Science, Universiti Malaya. *Bacillus salmalaya* 139SI, an identified novel species of bacteria strain 139SI (Accession No. JF825470) originated from local agricultural soil maintained at UMMBTL, Institute of Biological Sciences, Faculty of Science, Universiti Malaya. The pathogenic bacterial isolates used in this study included *Salmonella* Enteritidis strain ATCC BAA-711, *Escherichia coli* strain ATCC 35401 and *Staphylococcus aureus* strain ATCC 25923. Also, *Salmonella* Enteritidis local Malaysia (code number: 4301/15) was obtained from the Veterinary Research Institute (VRI), Ipoh Malaysia.

Analysis and characterization of potential probiotic properties of lactobacillus and bacillus salmalaya 139SI

Resistance of Lactobacillus and B. salmalaya 139SI against gastric acid of pH 3.5

The test was performed to determine the ability of isolates to survive in acidic conditions of pH 3.5 (pH of gastric). Isolates were grown in 5 mL MRS/BHI broth media and incubated at 37°C for 24 hours. Inoculum (0.5 mL) was grown in 5 mL of sterile PBS under acidic conditions of pH 3.5 (pH adjustment was performed with the addition of 0.1N HCl or 0.1N NaOH and incubated at 37°C. At every 2 hours interval for a total period of 24 hours the culture was taken and streaked onto MRS/BHI agar. The assays were carried out in triplicates by Duplo, and the average value was calculated. All plates were incubated at 37°C for 24 hours for viability checking against the acidic condition (Conway et al., 1987; Zavaglia et al., 1998; Taheri et al., 2009).

Bile salt resistance

The screening for bile tolerance was carried out by growing the *Lactobacillus* and *B. salmalaya* 139SI, respectively, in MRS/BHI broth containing 0.3% of bile salt (Merck, Germany) for 24 hours at 37°C. The control used was without bile salts. Bacteria from MRS/BHI broth was serially diluted to 10^6 and 100 uL at each dilution was plated on MRS/BHI agar followed by incubation (at 37°C) for 0, 4, 7 and 24 hours. The isolated resistance to bile salts was observed by counting the number of bacteria that could survive at the end of the incubation. The assays were carried out in triplicates by Duplo, and the average value was calculated (Taheri et al., 2009). Tolerance to bile was calculated by the equation of *log Nt/No* as a percentage of the control (without bile, pH 7) which was assigned a value of 100%.

Production of the cell free culture supernatants (CFCS) antimicrobial compounds

Cell free culture supernatants (crude extract) containing antimicrobial compound testing was performed by determining the ability to produce acids and bacteriocin activity. The former was determined by the titration method (Hadiwiyoto, 1994; Nielsen., 2010). The microbes were grown in synthetic medium containing 50 grams/Liter of glucose, 0.4% of urea, 0.1% of KH_2PO_4, 0.05% of $MgSO_4$, 0.01% of $FeSO_4.7H_2O$, 0.05% of KCl dan 0.05% of Yeast extract. *Lactobacillus* sp. and *Bacillus salmalaya* 139SI were revived respectively, in MRS/BHI broth medium for 24 hours at 37°C. The bacterial broth was diluted (1:10) in the synthetic medium and incubated in a shaking water bath (30°C).

The analysis of the resulting acids was performed daily until the fourth day of incubation. The analysis was performed using an aseptic growth synthetic medium and centrifuged at 3,000 rpm for 10 minutes in order to separate the bacteria from the metabolites (soluble solids). The resulting supernatant was diluted 50 times before being titrated with 0.1N NaOH and phenolphthalein (1%) present as an indicator change to light pink. The resulting titration is expressed as % acid (total acid titrated), calculated by the following equations:

$$\% \text{ of acid} = N \times V_1 \times Eq.\ wt.$$

$$V_2 \times 10$$

Where : N NaOH = Normal titrant (mol/mL), V_1 = Volume of titrant (mL), V_2 = Volume of samples (mL), *Eq.wt* = Weight equivalent to acid

The assays were carried out in triplicates by Duplo and the average value was calculated.

For bacteriocin activity a single colony of *Lactobacillus* and *B. salmalaya* 139SI were determined using disc diffusion method (Diop et al*.,* 2008; Abbasiliasi et al*.,* 2009). The assays were carried out in triplicates and the mean diameter of the zone was computed.

Test of coaggregation

Co-aggregation test was conducted using a method by (Vlková et al., 2008). This method uses a mixture of single cultures at a ratio of 1:1, and coaggregation is expressed in relative OD decrease between bacteria mixed with single bacteria. Each single strain of *Lactobacillus* spp. and *B. salmalaya* 139SI was grown in MRS and BHI broth medium, respectively for 24 hours at 37°C and centrifuged at 10,000 r.p.m for 10 minutes (Eppendorf® Centrifuge 5810R, Germany). It was then washed 3 times with sterile phosphate buffer saline solution (PBS) containing 8 g/Litre of NaCl, 0.34 grams/liter KH_2PO_4, and 1.21 grams liter K_2HPO_4.

The cultures were then resuspended and mixed in the same buffer. After mixing, 1.5 mL of suspension was transferred into a cuvette and a decrease of initial OD at 600 nm to a final OD of 0.6 (+0.02), was observed for 4 hours at room temperature using a Spectrophotometer UV-vis 2700 (Shimadzu, Japan). The assays were carried out in triplicates by Duplo, and the average value was calculated.

Percentage of co-aggregation can be calculated using the following equation:

$$\% \text{ of coaggregation} = \frac{(O.D.1 + O.D.2) - 2.\ O.D1.2}{(O.D.1 + O.D.2)} \times 100$$

Where: *O.D.1* = optical density of species 1, *O.D.2* = optical density of species 2, *O.D.1.2* = optical density mixed of species 1 and 2

Test of cell surface hydrophobicity

The tested bacteria (*Lactobacillus* spp. and *Bacillus salmalaya* 139SI) were grown in their respective broth medium (MRS and BHI broth) at 37°C under anaerobic conditions following method outline by Klayraung et al. (2008). The 18-24 hours (stationary phase) test culture was harvested after centrifugation at 6,000 r.p.m for 10 minutes (Eppendorf® Centrifuge 5810R, Germany), washed twice and resuspended in 50mM K_2HPO_4 buffer (pH 6.5) to optical density (OD 560) of 0.8-1.0 (A0) measured spectrophotometrically. A portion of 0.6 ml of n-hexadecane was added to 3 mL of bacterial suspension. The mixture was mixed thoroughly using a vortex mixer for 120s. The tubes were allowed to stand at 37°C for 30 minutes to separate the two phases. The aqueous (phase A) was carefully removed and the OD_{560} determined. Hydrophobicity is calculated from three replicates by duplo as the percentage decrease in the optical density of the initial aqueous bacterial suspension due the cells partitioning into a hydrocarbon layer. The percentage of cell surface hydrophobicity (%H) of the strain adhering to hexadecane is calculated using the equation:

$$\%H = \{(A0-A)/A0 \times 100\}.$$

Where: *A0*: value of $O.D._{600\ nm}$ initial bacterial suspension, *A*: value of $O.D._{600\ nm}$ after suspension mixed with hexadecane.

Toxicity test (Lethal Dose 50)

Acute oral toxicity studies have been approved by the Ethics Committee of the University of Technology

MARA (UiTM) Puncak Alam Campus Selangor and by the guidelines for the care and use of laboratory animals NRC, (2010). In accordance to OECD Test Guidelines 425 (Up and Down Procedure) (Saleem et al., 2017), the rats used were adult female Sprague Dawley (SD) strain (9 weeks old, nulliparous and non-pregnant) weighing between 213-249g. The animals (n=20) were equally and randomly divided into 4 treatment dose levels consisting of (i) 1 mL reverse osmosis water (control), (ii) 175 mg/kg weight/day, (iii) 550 mg/kg weight/day, and 2000 mg/kg weight/day. Feeds and drinking water were provided *ad libitum*. Reverse osmosis water was supplied through a 250 mL water dispenser bottle. The water was changed twice a week. Each animal was individually caged for a minimum of 5 days to allow for acclimatization. Each animal was fasting overnight by withholding food but not water, prior to dosing. Food was returned to the animals approximately 3 hours after dosing. Each animal was individually weighed on Day 0, before administration of the test item (initial). Oral administration on each rat was conducted using a round-headed stainless steel gavage tube measuring 2 inches long and 18G in-diameter, fitted with a syringe.

The observations made include cage-side observation, body weight, and pathology. For cage-side observation the animals were individually observed for mortality and signs of illness, injury or abnormal behavior, once during the first 30 minutes after dosing, and periodically during the first 48 hours (with special attention given during the first 4 hours), and daily thereafter for a total period of 14 days. Each animal was individually weighed on Day 7 and Day 14 (termination) after dosing. All animals were sacrificed on Day 14 and gross necropsies were performed. Microscopic examination was performed on selected vital organs. The histopathological information may be considered for further toxicity studies. After each level was conducted, the short-term and long-term outcomes were input into the Oral Toxicity (Guideline 425) statistical program "(AOT425StatPgm)". When the stopping criteria were engaged, the LD_{50} and 95% confidence intervals were calculated.

RESULTS

Isolation and identification of lactic acid bacteria (LAB)

Isolation of LAB

Twenty-nine (29) colonies were obtained from the isolation, selection, and identification of the Kerinci *dadih*, Jambi with different characteristics. Overall, colonies were milky white or creamy, circular-shaped, with an entire edge and convex elevation. Based on the size, colonies were distinguished into 3 types; consisting of 17 small isolates, 6 medium-sized and 6 large-sized isolates (Table 1). A-Group shows the highest percentage of 58.62% which consist of isolates B1.1, B1.2, B2.1A, B2.1.B, B2.1.C, B4.1.1, B4.2.1, B4.3.1, B4.3.2, B7.B, B8.1.1, B8.2.1, B8.3.1, B8.3.3A. B9.A, B9.B and B11.B, while B Group isolates (B6.1.1.B, B7.A, B8.1.2, B8.3.2, B10.1 and B11.A) and C Group isolates (B2.2, B3.A, B4.3.3, B5.2, B6.1.1.A and B6.2) showed 20.69%, respectively (Fig. 1).

Analysis and characterization of potential probiotic properties of *Lactobacillus* and *Bacillus salmalaya* 139SI

Resistance to gastric acid of pH 3.5

Resistance to low pH is one of the probiotic requirements because the cellular stress experienced by probiotic isolates begins with exposure to gastric acid in the stomach. The results of testing the resistance of isolated Kerinci *dadih* LAB and *Bacillus salmalaya* 139SI to the pH of gastric (pH 3.5) are presented in Table 2. The number of isolates that could survive up to 24 hours of incubation time was expressed in relative resistance (%). Experimental results show that all tested isolates survive pH 3.5 with the relative resistance value range 88.69+ 6.71 to 94.42 + 4.452% with a decrease in the total of bacterial cells that survived after 24 hours of incubation at 10^9 CFU/mL from baseline of 10^{12} CFU/mL (Table 2). *Lactobacillus plantarum* 1 had the highest relative resistance at pH 3.5 around 94.42 + 4.45% followed by *Lactobacillus plantarum* 2, *Lactobacillus delbrueckii* and *Bacillus salmalaya* 139SI.

Bile salt resistance

The ability of bacteria to withstand bile stress is one of the criteria used in probiotic selection. After exposure to acidic pH, the isolates were then tested for resistance to bile salts. The results of testing the isolates resistance to bile salts are shown in Table 2. The number of isolates that could survive up to 24 hours of incubation time was expressed in relative resistance (%). The results of the experiment show that all isolates have high bile salt resistance ranging 86.49+6.98 to 90.36 + 4.94% a decrease in the total of bacterial cells that survived after 24 hours of incubation at 10^9 CFU/mL from baseline of 10^{13} CFU/mL. *Lactobacillus plantarum* 1 had

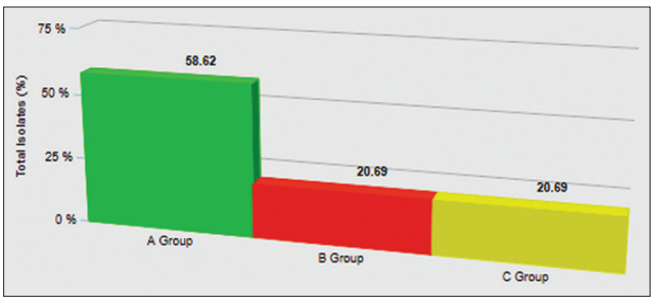

Fig 1. Total Lactobacillus isolates. The colonial morphology group characteristics. A- Small, milky white or cream, circular, entire and convex; B- Medium, milky white or cream, circular, entire and convex; C- Big, milky white or cream, circular, entire and convex.

Table 1: Characteristic morphology of colony of lactic acid bacteria originated from the fermented milk *(dadih).*

Isolate No.	Isolate Code	Size	Pigmentation	Shape	Edge	Elevation	Group
1	B1.1	small	milky white or cream	circular	entire	convex	A
2	B1.2	small	milky white or cream	circular	entire	convex	A
3	B2.1.A	small	milky white or cream	circular	entire	convex	A
4	B2.1.B	small	milky white or cream	circular	entire	convex	A
5	B2.1.C	small	milky white or cream	circular	entire	convex	A
6	B2.2	big	milky white or cream	circular	entire	convex	C
7	B3. A	big	milky white or cream	circular	entire	convex	C
8	B4.1.1	small	milky white or cream	circular	entire	convex	A
9	B4.2.1	small	milky white or cream	circular	entire	convex	A
10	B4.3.1	small	milky white or cream	circular	entire	convex	A
11	B4.3.2	small	milky white or cream	circular	entire	convex	A
12	B4.3.3	big	milky white or cream	circular	entire	convex	C
13	B5.2	big	milky white or cream	circular	entire	convex	C
14	B6.1.1.A	big	milky white or cream	circular	entire	convex	C
15	B6.1.1.B	medium	milky white or cream	circular	entire	convex	B
16	B6.2	big	milky white or cream	circular	entire	convex	C
17	B7.A	medium	milky white or cream	circular	entire	convex	B
18	B7.B	small	milky white or cream	circular	entire	convex	A
19	B8.1.1	small	milky white or cream	circular	entire	convex	A
20	B8.1.2	medium	milky white or cream	circular	entire	convex	B
21	B8.2.1	small	milky white or cream	circular	entire	convex	A
22	B8.3.1	small	milky white or cream	circular	entire	convex	A
23	B8.3.2	medium	milky white or cream	circular	entire	convex	B
24	B8.3.3A	small	milky white or cream	circular	entire	convex	A
25	B9. A	small	milky white or cream	circular	entire	convex	A
26	B9. B	small	milky white or cream	circular	entire	convex	A
27	B10.1	medium	milky white or cream	circular	entire	convex	B
28	B11.A	medium	milky white or cream	circular	entire	convex	B
29	B11.B	small	milky white or cream	circular	entire	convex	A

A Group: Small, milky white or cream, circular, entire and convex. B Group: Medium, milky white or cream, circular, entire and convex. C Group: Big, milky white or cream, circular, entire and convex.

Table 2: Resistance to gastric acid

Resistance to gastric acid.	
Isolate	**Relative resistance (%)**
Lactobacillus plantarum 1	94.42+4.45
Lactobacillus plantarum 2	92.68+5.20
Lactobacillus delbrueckii	92.50+3.54
Bacillus salmalaya 139SI	88.69+6.71
Negative Control (non pH 3.5)	93.05+2.00
Bile salt resistance.	
Lactobacillus plantarum 1	90.36+4.94
Lactobacillus plantarum 2	86.87+3.00
Lactobacillus delbrueckii	86.49+6.98
Bacillus salmalaya 139SI	89.26+2.81
Negative Control (non-salt oxgall)	92.40+2.69

*Total averaged from number of replicates, n = 3

the highest relative resistance followed by *Bacillus salmalaya* 139SI, *Lactobacillus plantarum* 2 and *Lactobacillus delbrueckii*.

Production of the cell-free culture supernatants containing bacteriocin antimicrobial compounds

The ability to produce acid

The ability of bacterial isolates to produce acids is presented in Table 3, where acid production is expressed in % lactic acid. The ability of microbes to produce acids has been proven in this study. The ability strength producing acid among the isolates: *L. plantarum* 1 (3.86+0.70%) > *L. plantarum 2 (*2.04+0.24%) > *L. delbrueckii* (1.25+ 0.11%) > *B. salmalaya* 139SI (0.70+0.05%).

Cell-free supernatant containing Bacitracin activity

The Kirby-Bauer disc diffusion assay was carried out based on recommendations given by the Clinical Laboratory Standards Institute. For *Lactobacillus*, the results are presented in Table 4 and Fig. 2(a). For *Bacillus salmalaya* 139SI, bacteriocin tests against pathogenic bacteria are presented in Table 5 and Fig. 2(b). Bacteriocins test was conducted using the good diffusion test and the test results of antibacterial activity against *Salmonella* Enteritidis local Malaysia (code number 4301/15), *Salmonella* Enteritidis strain ATCC BAA-711, *Escherichia coli* strain ATCC 35401 and *Staphylococcus aureus* strain ATCC 25923 (Table 4 and Table 5). The strength for antibacterial activity against gram-negative bacteria *Salmonella* Enteritidis local Malaysia (code number 4301/15), *Salmonella* Enteritidis strain ATCC BAA-711 and *Escherichia coli* strain ATCC 35401 was sequenced as *L. delbrueckii* > *B. salmalaya* 139SI > *L. plantarum* 2 > *L. plantarum* 1. The three tested

Lactobacillus and *B. salmalaya* strain 139SI showed good antibacterial activity against *Salmonella* Enteritidis.

Test of co-aggregation

Coaggregation between lactic acid bacterial isolates is needed to evaluate the effectiveness of probiotics adhering in the intestine (Handley *et al*., 1987). Coaggregation tests were performed on single and pair isolates, the test results are presented in Table 6. From the test results it was found that the highest coaggregation activity occurred in the combined isolates of *L. plantarum* 1 and *L. plantarum* 2 of 6.03+0.41% with a reduction in OD of 0.600 nm to 0.520 nm after 4 hours of incubation, followed by a combination isolates of *L. plantarum* 1 with *L. delbrueckii* of 3.84+0.24% and combination isolates of *L. plantarum* 2 with *L. delbrueckii* of 2.16+0.18% (Table 6).

Test of cell surface hydrophobicity

Hydrophobicity properties testing was performed to measure cell distribution between a hydrophobic phase and water (hydrophilic). Hydrophobicity properties indicate the tendency of bacteria to form aggregates and further colonize or adhere to the surface of epithelial cells. The principle of this test is that the hydrophobic components are united and separate from the non-hydrophobic ones. The method used in hydrophobicity testing is Microbial adhesion to hydrocarbons (MATH) (Klayraung et al., 2008). The results of the hydrophobicity isolates using the MATH method are presented in Table 7. Overall the *Lactobacillus* tested was classified as the moderate hydrophobic bacteria with hydrophobicity values of 20 to 50%. *L. plantarum* 1 was the highest hydrophobic isolate with a value of 24.76+4.05%, followed by *L. plantarum* 2 at 23.16+2.37% and *L. delbrueckii* at 20.26+3.33%. For *Bacillus salmalaya* 139SI the hydrophobicity value was 11.93+1.79% and it was classified in hydrophilic bacteria because the hydrophobicity value was < 20%.

Toxicity test (Lethal Dose 50) of *L. plantarum*

The results of observing the body weight and necropsy of rats during the *Lactobacillus plantarum* 1 toxicity test were presented in Table 8. In this experiment, the safety assessment of strain *L. plantarum* 1 used an oral toxicity test conducted on the rat. A 14-day oral toxicity trial on Sprague Dawley rats with female sex was conducted to investigate articles of acute toxicity test. Experimental results can provide early toxicological data that are useful in determining the appropriate dose level for future repeated dose oral toxicology studies and for determining possible long-term toxicity studies.

During the experimental period, no change in behavior or activity was observed in the rats. The single oral dosage of

Table 3: Acid production.

Isolate	Acid production (%)			
	1 day	2 days	3 days	4 days
Lactobacillus plantarum 1	0.56+0.05	0.70+0.02	1.62+0.28	3.86+0.70
Lactobacillus plantarum 2	0.41+0.03	0.77+0.04	1.15+0.09	2.04+0.24
Lactobacillus delbrueckii	0.35+0.04	0.47+0.03	0.91+0.06	1.25+0.11
Bacillus salmalaya 139SI	0.51+0.02	0.92+0.04	1.23+0.05	0.70+0.05

Table 4: Inhibition zone of Lactic Acid Bacterial isolates against pathogenic bacteria.

Isolate	*S.* Enteritidis strain ATCC BAA-711	*S.* Enteritidis local Malaysia	*E. coli* strain ATCC 3540	*S. aureus* strain ATCC 2592
***L. plantarum* 1**	8.37+0.47	8.70+0.46	11.64+0.57	13.36+0.23
***L. plantarum* 2**	9.28+0.54	9.24+0.64	11.54+0.11	12.23+0.53
L. delbrueckii	13.11+0.14	13.14+0.28	11.56+0.61	13.13+0.15
Negative control	6.00+0.00	6.00+0.00	6.00+0.00	6.00+0.00
Positive control	18.10+2.21	19.33+1.90	15.24+0.92	14.72+0.59

*Total averaged from number of replicates, n = 3

Table 5: Inhibition zone of *Bacillus salmalaya* 139SI against pathogenic bacteria.

Isolate	*S.* Enteritidis strain ATCC BAA-711	*S.* Enteritidis local Malaysia	*E. coli* strain ATCC 3540	*S. aureus* strain ATCC 2592
Negative control	6.00+0.00	6.00+0.00	6.00+0.00	6.00+0.00
Positive control	19.70+1.02	19.78+0.98	20.82+0.61	13.06+0.13
B. salmalaya 139SI	11.40+0.28	11.66+0.11	13.83+0.12	8.16+0.22

*Total averaged from number of replicates, n = 3

Table 6: Co-aggregation of isolate combinations.

Description	The combination of isolates used in this study					
	AB	AC	AD	BC	BD	CD
% Co-aggregation	6.03+0.41	3.84+0.24	-0.30+0.10	2.16+0.18	-0.45+0.06	-2.45+0.24

*Description: A. *L. plantarum* 1; B. *L. plantarum* 2; C. *L. delbrueckii* and D. *Bacillus salmalaya* 139SI.
*Total averaged from number of replicates, n = 3

175 mg/Kg body weight/day, 550 mg/Kg body weight/day, and 2000 mg/Kg body weight/day did not cause illness or death as well as signs of poisoning related to treatment in any animal. All animals were healthy and survived inoculation after 14 days. From the test results, the probiotic strain of *L. plantarum* 1 acute oral toxicity shows the good status of health, growth and general development of experimental animals. Oral administration of the probiotic *L. plantarum* 1 had no adverse effects and was the effect on an increase in animal weight. Based on the test results of the weight gain of the animal was a dose of 2000 mg/Kg body weight/day > 550 mg/Kg body weight/day > control > 175 mg/Kg body weight/day.

Table 7: Hydrophobicity value of isolates.

Hydrophobicity value	*Hydrophobicity (%)*
Lactobacillus plantarum 1	24.76+4.05
Lactobacillus plantarum 2	23.16+2.37
Lactobacillus delbrueckii	20.26+3.33
Bacillus salmalaya 139SI	11.93+1.79

*Total averaged from number of replicates, n = 3

Table 8: Individual gross necropsy observation.

Dose sequence (mg/Kg)	Animal Number	Tissue/ Organs	Finding
Control	R 100	All tissues/ organs	No gross abnormallities
175	R 102	All tissues/ organs	No gross abnormallities
250	R 102	All tissues/ organs	No gross abnormallities
2,000	R 103	All tissues/ organs	No gross abnormallities

Result of the necropsy test, overall, all of the organs examined in the female rats showed no pathological changes or no abnormalities in the organs (no gross abnormalities) (Table 8). Therefore according to the guidelines of OECD Number 425 (2008), histopathological examination is not required. No evidence of toxicity was detected in the study of acute toxicity in this trial.

DISCUSSION

Based on the results of an *in vitro* investigation as a probiotic potential, *L. plantarum* 1 isolated from fermented buffalo milk (dadih) had the best probiotic characteristic in the current study. Good probiotic characteristics of *L. plantarum* 1 include:, (i) high tolerance to low pH and bile salts, (ii) high acid production capacity, (iii) has a high coaggregation activity with other *Lactobacillus* and (iv) are the most hydrophobic isolates (Heravi *et al*., 2011). Probiotic bacteria should be able to tolerate acid for at least 90 minutes, be able to tolerate bile salts, adhere to the epithelial or intestinal mucosa, and develop in the lower intestinal tract. Probiotic bacteria that successfully overcome these barriers are beneficial to health (Minellia et al., 2004). Elida (2002) claimed 16 isolates were selected as candidates probiotically isolated from *dadih* fermentation that are relatively resistant to pH 3.5 ranged from 80 - 95% after 24 hours of incubation average of 10^7 CFU/mL of the initial amount of 10^8 CFU/mL. He also reported that the strain of *Lactobacillus casei* survived to pH 3.0 but not to pH 1.0. Some researchers have conducted

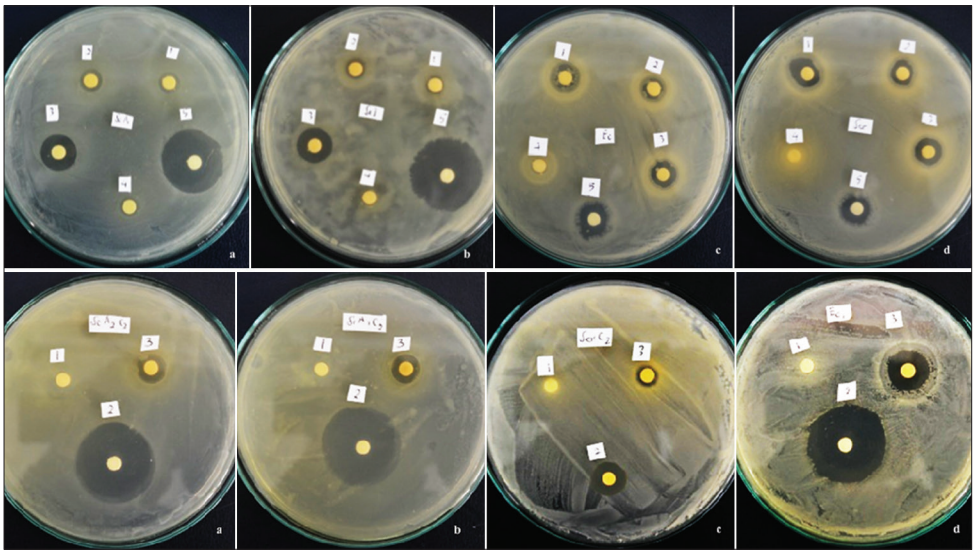

Fig 2. Bacteriocin (cell-free supernatant) test of Lactobacillus isolates against pathogenic bacteria. a. Salmonella Enteritidis strain ATCC BAA-711; b. Salmonella Enteritidis local Malaysia (code number 4301/15); c. Escherichia coli strain ATCC 3540; d. Staphylococcus aureus strain ATCC 2592; 1. Lactobacillus plantarum 1; 2. Lactobacillus plantarum 2; 3. Lactobacillus delbrueckii; 4. Negative control and 5. Positive control. (b) Bacteriocin (cell-free supernatant) test of Bacillus salmalaya 139SI isolates against pathogenic bacteria. a. Salmonella Enteritidis strain ATCC BAA-711; b. Salmonella Enteritidis local Malaysia (code number 4301/15); c. Escherichia coli strain ATCC 3540; d. Staphylococcus aureus strain ATCC 2592; 1. Negative control; 2. Positive control; and 3. Bacillus salmalaya 139SI.

experiments on bacterial survival against bile salts. The results show that variations in species and strains affect their ability to survive in conditions containing bile salts. Pereira et al., (2003) reported that *Lactobacillus fermentum* KC5b had a high tolerance to gastric acid conditions and bile salts and had the high activity of bile salt hydrolase (BSH). Lin et al., (2006) tested yogurt containing *Lactobacillus acidophilus* and *Bifidobacteria* which were previously resistant to pH 2.0 but were able to survive in the condition of 0.3% bile salts.

The acid produced by bacteria can reduce gastric acid expenditure, stimulate peristaltic intestines and prevent gastric infections. Most acids combine with Ca ions to form lactic-calcium acid complexes, making them more easily absorbed by the gut (Mitsuoka, 1978). Ogunbanwo et al., (2003) has reported bacteriocins produced by *Lactobacillus plantarum* F1 and *Lactobacillus brevis* OG1 isolated from Nigerian fermented foods can inhibit *Listeria monocytogenes, Salmonella typhimurium, Staphylococcus aureus*, and *Escherichia coli*. Production of bacteriocins from *Lactobacillus* sp. that isolated from whole milk can inhibit *Escherichia coli, Salmonella typhimurium* and *Listeria monocytogenes* (Usmiati & Marwati, 2007). Khoiriyah & Ardiningsih (2014) also reported that the bacteriocin activity of *Lactobacillus* sp. RED4 inhibited *Bacillus cereus, Bacillus subtilis, Salmonella sp., Aeromonas hydrophila, Escherichia coli, Pseudomonas aeruginosa,* and *Candida albicans*.

Vandevoorde et al., (1992) reported that the isolate pairs of *Lactococcus lactis* and *Lactobacillus crispatus* isolates after 4 hours incubation decreased OD of 0.600 nm to 0.490 nm. Although the treatment reduced the solution pH to 4, the percentage of the coaggregation increased from 65% to 85%. The findings by Syafia (2002) on the properties coaggregation isolates *Lactococcus lactis* from *dadih* relatively weak (+), against both planktonic cells and biofilm cells. The findings Elida (2002) showed the highest coaggregation in the combination that occurred in *Leuconostoc mesenteroides*. 12 pairs of with *Streptococcus raffinolactis* ct4. Kos et al., (2003) in their experiments found that the combination of *Lactobacillus plantarum* L4 and *Lactobacillus acidophilus* M92 isolates produced a coaggregation of 4.36 (+1.81%) after 5 hours incubation at room temperature in PBS solution that had with pH 7.2.

Reid et al., (1992) reported hydrophobicity value of several *Lactobacillus acidophilus* isolates, including strain 68 for 32%, 75 for 13%, RC 14 for 55% and T13 for 29% and *Lactobacillus casei* for 0%. Syafia (2002) reported that strains of *Lactococcus lactis* derived from the *dadih* fermented in brushed bamboo tubes had 16% hydrophobicity and classified in hydrophilic bacteria with a number of cells attached on SS/cm^2 plates of 2.4 x 10^4 CFU/cm^2 from baseline of 10^5 CFU/cm^2. From the experiments, results of Elida (2002) reported that *Streptococcus raffinolactis* Ct4 isolates from bamboo *betung* originated 50 Kota District has hydrophobicity of 15.7%.

The safety of probiotic strains has been the subject of active discussion in recent years and there are still no general guidelines or specific policy requirements on this issue. Acute oral toxicity studies have been advocated as a fundamental test to assess safety (Jia et al., 2011) and have been used before in many safety assessment studies (Laulund et al., 2017). Some researchers have conducted studies regarding the toxicity of *Lactobacillus plantarum* as a probiotic culture. Lastly, Liao et al., (2019) carried out a toxicity study of *Lactobacillus plantarum* PS128™ isolated from spontaneously fermented Mustard Greens. From the results of the study, it can be said that *Lactobacillus plantarum* strain PS128™ is safe for human consumption. Besides, *Lactobacillus plantarum* strains ZS07 and K21 originated from Slovak Bryndza Cheese were also considered safe for use as probiotic cultures (Belicova et al., 2013).

CONCLUSION

The test results of the analysis and characterization of probiotic properties *in vitro* and *in vivo* indicate that both bacteria *Lactobacillus plantarum* and *Bacillus salmalaya* 139SI are potential candidates for probiotic therapeutic application against *Salmonella* Enteritidis. Overall, *Lactobacillus plantarum* has the best activity compared to other bacterial isolates. Further research of the applications of these probiotics in the poultry industry is needed to examine its effectiveness at a larger scale.

Conflicts of interest

The authors declare no conflict interest.

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Author's contributions

This manuscript contains the partial results of Efrizal Ary doctoral degree thesis. The thesis was carried out by Efrizal Ary at the Universiti Malaya (Malaysia), under the direction of Professor Salmah, who designed the research and co-supervised by Dr. Rosazlin Abdullah, and the technical assistance of Dr. Arezoo Dadrasnia. Efrizal Ary carried out the work at laboratory level taking samples, making the physicochemical and microbiological analyses and also the statistical analysis of the data. The manuscript has been reviewed and prepared by Efrizal Ary, Fuad Ameen, Sartaj Ahmad Bhat, Aaronn Avit Ajeng and Nur Nazirah Md. Nasir.

REFERENCES

Abbasiliasi, S., T. I. Azmi, M. D. Hassan, M. Shuhaimi, M. Rosfarizan and A. Ariff. 2009. Antimicrobial activity and antibiotic sensitivity of three isolates of lactic acid bacteria from fermented fish product, Budu. Malaysian J. Microb. 5: 33-37.

Adnan, A. F. M. and I. K. Tan. 2007. Isolation of lactic acid bacteria from Malaysian foods and assessment of the isolates for industrial potential. Bioresour. Tech. 98: 1380-1385.

Angelakis, E. 2017. Weight gain by gut microbiota manipulation in productive animals. Microb. Pathol. 106: 162-170.

Belicova, A., M. Mikulasova and R. Dusinsky. 2013. Probiotic potential and safety properties of *Lactobacillus plantarum* from Slovak Bryndza cheese. Biomed Res. Int. 2013: 760298.

CDC. 2019. *Salmonella*. Available from: https://www.cdc.gov/salmonella/index.html [Last accessed on 2022 Aug 11].

De, B. C., D. Meena, B. Behera, P. Das, P. Das Mohapatra and A. Sharma. 2014. Probiotics in fish and shellfish culture: Immunomodulatory and ecophysiological responses. Fish Phys. Biochem. 40: 921-971.

Chou, H. T., T. Y. Kuo, J. C. Chiang, M. J. Pei, W. T. Yang, H. C. Yu, S. B. Lin and W. J. Chen. 2008. Design and synthesis of cationic antimicrobial peptides with improved activity and selectivity against *Vibrio* spp. Int. J. Antimicrob. Agents. 32: 130-138.

Conway, P. L., S. L. Gorbarch and G. R. Goldin. 1987. Survival of lactic acid bacteria in human stomach and adhesion to intestinal cells. J. Dairy Sci. 70: 1-12.

Elida, M. 2002. Profile of Lactic Acid Bacteria from Dadih Fermented in Various Types of Bamboo and its Potential as Probiotic. [Thesis]. Bogor Agricultural University, Bogor.

Gast, R. K. 2003. Salmonella infections: Introduction. In: Calnek, Barnes, Beard, McDougald and Y. M. Saif (Eds.), Disease of Poultry. 10th ed. Iowa State University Press, New York, pp. 81-82.

Geary, T. M., P. H. Brooks, J. D. Beal and A. Campbell. 1999. Effect on weaner pig performance and diet microbiology of feeding a liquid diet acidified to pH 4 with either lactic acid or through fermentation with *Pediococcus acidilactici*. J. Sci. Food Agric. 79: 633-640.

Hadiwiyoto, S. 1994. Theories and Procedures for Testing the Quality of Milk and Processed Products. 2nd ed. Liberty Publishers, Yogyakarta.

Handley, P. S., D. W. S. Harty, J. E. Wyatt, C. R. Brown, J. P. Doran and A. C. C. Gibbs. 1987. A comparison of the adhesion, coaggregation and cell-surface hydrophobicity properties of fibrillar and fimbriate strains of *Streptococcus salivarius*. J. General Microb. 133: 3207-3217.

Heravi, R. M., H. Kermanshahi, M. Sankian, M. R. Nassiri, A. H. Moussavi, L. R. Nasiraii and A. R. Varasteh. 2011. Screening of lactobacilli bacteria isolated from gastrointestinal tract of broiler chickens for their use as probiotic. Afr. J. Microb. Res. 5: 1858-1868.

Humprey, T. 1998. Important and Relevant Attributes of the *Salmonella* Organism. Proceeding of the International Symposium on Food Borne *Salmonella* in Poultry, Baltimore, Maryland. July 25-26, American Association of Avian Pathologists, pp. 43-48.

Jia, X., W. Wang, Y. Song and N. Li. 2011. A 90-day oral toxicity study on a new strain of *Lactobacillus paracasei* in rats. Food Chem. Toxic. 49: 1148-1151.

Khedid, K., M. Faid, A. Mokhtari, A. Soulaymani and A. Zinedine. 2009. Characterization of lactic acid bacteria isolated from the one humped camel milk produced in Morocco. Microb. Res. 164: 81-91.

Khoiriyah, H. and P. Ardiningsih. 2014. Determination of the optimum incubation time for the bacteriocin activity of *Lactobacillus* sp. RED4. J. Equatorial Chem. 3: 52-56.

Klayraung, S., H. Viernstein, J. Sirithunyalug and S. Okonogi. 2008. Probiotic properties of lactobacilli isolated from Thai traditional food. Sci. Pharm. 76: 485-503.

Kos, B., J. Suskovic, S. Vukovic, M. Simpraga, J. Frece and S. Matosic. 2003. Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. J. Appl. Microb. 94: 981-987.

Laulund, S., A. Wind, P. M. F. Derkx and V. Zuliani. 2017. Regulatory and safety requirements for food cultures (Review). Microorganisms. 5: 28.

Lin, W. H., C. F. Hwang, L. W. Chen and H. Y. Tsen. 2006. Viable counts, characteristic evaluation for commercial lactic acid bacteria products. Food Microb. 23: 78-81.

Minellia, E. B., A. Beninia, M. Marzottob, A. Sbarbatic, O. Ruzzenented, R. Ferrarioe, H. Hendriksf and F. Dellaglio. 2004. Assessment of novel probiotic *Lactobacillus casei* strains for the production of functional dairy foods. Int. Dairy J. 14: 723-736.

Mitsuoka, T. 1978. Intestinal Bacteria and Health. Harcourt Brace Javanovich Japan Inc., Tokyo.

Nielsen, S. S. 2010. Food Analyses. 4th ed. Springer, New York.

Mouttotou, N., S. Ahmad, Z. Kamran and K. C. Koutoulis. 2017. Prevalence, Risks and antibiotic resistance of *Salmonella* in poultry production chain. In: Current Topics in *Salmonella* and Salmonellosis. 1, pp. 215-234.

Ogunbanwo, S. T., A. I. Sanni and A. A. Onilude. 2003. Characterization of bacteriosin produced by *Lactobacillus plantarum* F1 and *Lactobacillus brevis* OG1. Afr. J. Biotech. 2: 219-227.

Pascual, M., M. Hugas, J. I. Baidola, J. M. Monfort and M. Garriga. 1999. *Lactobacilus salivartius* CTC2197 prevents *Salmonella* Enteritidis colonization in chickens. Appl. Environ. Microb. 65: 4981-4986.

Pereira, D. I. A., A. L. McCartney and G. R. Gibson. 2003. An *in vitro* study of the probiotic potential of a bile-salt-hydrolyzing *Lactobacillus fermentum* strain, and determination of its cholesterol-lowering properties. Appl. Environ. Microb. 69: 4743-4752.

Reid, G., P. L. Cuperus, A. W. Bruce, H. C. Mei, L. Tomeczek, A. H. Khoury and H. J. Busscher. 1992. Comparison of contact angels and adhesion to hexadecane of urogenital, dairy, a poultry lactobacilli: Effect of serial culture passage. Appl. Environ. Microb. 58: 1549-1553.

Saleem, U., S. Amin, B. Ahmad, H. Azeem, F. Anwar and S. Mary. 2017. Acute oral toxicity evaluation of aqueous ethanolic extract of *Saccharum munja* Roxb. Roots in albino mice as per OECD 425 TG. Toxic. Rep. 4: 580-585.

Sirait, C. H., N. Cahyadi, T. Pangabean and I. G. Putu. 1995. Identification and Cultivation of Dadih Processing Bacterial Cultures. Research Report. Livestock Research Institute, Ciawi, Bogor.

Sugitha, I. M. 1995. Dadih is a traditional Minang food. Benefits and uses. In: National Widyakarya: The Advantages of Traditional Food. Office Minister of State for Food Matters, Jakarta, pp. 532-540.

Surono, I. S. 2015. Indonesian dadih. Fer. Milk Dairy Prod. 1: 377-399.

Surono, I. S. 1999. The Effect of Freezing Methods and Frozen Storage on Viability and ß-Galaktosidase Activit of Lactic Acid Culture Isolate from Dadih. In: Proceedings of the Food Technology Seminar. PATPA, Jakarta.

Syafia, R. 2002. Adhesi dan Pembentukan Biofilm Bakteri Asam Laktat Pada Permukaan Stainless Steel dan Uji Sifat Hidrofobiknya. Institut Pertanian Bogor, Bogor.

Taheri, H. R., H. Moravej, F. Tabandeh, M. Zaghari and M. Shivazad. 2009. Screening of lactic acid bacteria toward their selection as a source of chicken probiotic. Poult. Sci. 88: 1586-1593.

Usmiati, S. and T. Marwati. 2007. Selection and optimization of bacteriocin production process from *Lactobacillus* sp. J. Postharvest. 4: 27-37.

Vandevoorde, L., H. Christiaens and W. Verstraete. 1992. Prevalence of coaggregation reactions among chicken lactobacilli. J. Appl. Bact. 72: 214-219.

Vlková, E., Rada, V., Šmehilová, M. and Killer, J. 2008. Auto-aggregation and co-aggregation ability in bifidobacteria and *Clostridia*. Fol. Microb. 53: 263-269.

Yuliawati, Y. D. Jurnalis, E. Purwati and G. Lubis. 2012. The effect of *Pediococcus pentosaceus* on stool frequency, TNF-α-level, gut microflora balance in diarrhea-induced mice. Indones. J. Gastroenter. Hepatol. Digest. Endosc. 13: 97-102.

Yun, J. H., K. B. Lee, Y. K. Sung, E. B. Kim, H. Lee and Y. J. Choi. 2009. Isolation and characterization of potential probiotic lactobacilli from pig feces. J. Basic Microb. 49: 220-226.

Zavaglia, A. G., G. Kociubinski, P. Perez and G. D. Antoni. 1998. Isolation and characterization of *Bifidobacterium* strains for probiotic formulation. J. Food Protec. 61: 865-873.