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Potential Probiotic Characteristics and Safety Assessment of *Lactobacillus rhamnosus* SKG34 Isolated from Sumbawa Mare's Milk

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Lactobacillus rhamnosus **SKG34 (LrSKG34), a potential probiotic strain, was successfully isolated from Sumbawa Mare's milk. Our previous studies showed that the strain is resistant to gastrointestinal conditions, possesses antioxidant activity, and lowers blood cholesterol levels. Further clarification of the potential probiotic characteristics and safety assessment are necessary. This study aimed to evaluate the adhesion of LrSKG34 to Caco-2 cell monolayers and its effect on mucosal integrity *in vitro*. We also examined the LrSKG34 safety profile based on antimicrobial susceptibility testing, haemolytic activity determination, Caco-2 cell monolayer translocation evaluation, and *in vivo* investigation of the effect of LrSKG34 on the physiology, biochemical markers, and histopathological appearance of major organs in an animal model. LrSKG34 attached to Caco-2 cell monolayers and maintained mucosal integrity *in vitro*. The typical resistance of lactobacilli to ciprofloxacin, gentamicin, vancomycin, trimethoprim-sulfamethoxazole, and metronidazole was confirmed for LrSKG34. No haemolytic activity was observed on blood agar plates, and no LrSKG34 translocation was observed in Caco-2 cell monolayers. Administration of LrSKG34 to Sprague-Dawley rats did not adversely affect body weight. No abnormalities in hematological parameters, serum biochemistry levels, or histopathological structures of major organs were observed in LrSKG34-treated rats. Collectively, the results implicate LrSKG34 as a promising and potentially safe probiotic candidate for further development.**

Keywords: ***Lactobacillus rhamnosus*** **SKG34, adhesion, mucosal integrity, safety *in vitro*, safety *in vivo***

Introduction

Lactic acid bacteria (LAB) are commonly found in foods, dairy products, fermented fresh vegetables (*i.e.*, sauerkraut, Korean *kimchi*), animal milk, sourdough, yogurt, cheese, *etc.* Along with their long-intertwined history in human food and beverages, LAB also gained wide attention from researchers and the commercial food industry for their promising health benefits, as they are the most common bacteria used as probiotics [1]. The health benefit of probiotics containing product depends on the specific bacterial strain that is used. *Lactobacillus rhamnosus* GG and *Lactobacillus reuteri* are effective for

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treating children's diarrhoea due to rotavirus infection [2]. *Lactobacillus salivarius* UCC118 is capable of producing bacteriocin to inhibit the growth of *Listeria monocytogenes* in an animal model [3]. The multistrain probiotic VSL#3 consists of three bifidobacteria, four lactobacilli, and *Streptococcus thermophilus* and can prevent recurrent pouchitis and improve the quality of life of patients with inflammatory bowel disease (IBD) [4, 5]. Although the mechanism by which probiotics induce health benefits is strain-dependent, expert consensus indicates that all probiotics correct dysbiosis and create a more favourable gut environment [6].

Probiotics are living microorganisms that, when administered in adequate amounts, confer a health benefit on the host [6, 7]. Based on this definition, a bacterial strain should be named a probiotic if it has been identified down to the species and strain level, is viable in the gastrointestinal environment, and is beneficial for the host's health [6]. Our group has successfully isolated and identified a potential probiotic candidate, *L. rhamnosus* SKG34 (accession number MW073405.1), from Sumbawa mare's milk [8, 9]. *L. rhamnosus* SKG34 (LrSKG34) showed good adaptation to the gastrointestinal milieu, did not transform cholic acid into deoxycholic acid [9], and lowered the lipid profile of model animals [10]. Although LAB, particularly *L. rhamnosus,* are generally recognized as safe (GRAS) by USA authorities and designated qualified presumption of safety (QPS) by EU authorities, the safety of these strains must be examined because some cases of systemic infection as associated with the consumption of probiotics [11, 12]. In this study, we conducted safety assessments of LrSKG34 both *in vitro* and *in vivo,* including its resistance to antibiotics, haemolytic activity, possibility of translocation, and effects on model animals, all of which are critically important to investigate. We also determined LrSKG34's adhesion to intestinal epithelial cells and its effect on mucosal integrity *in vitro*, which may be important for the functionality of this strain.

Materials and Methods

Caco-2 cell monolayer

The caco-2 cell line was kindly provided by Professor Yukako Fujinaga from Department of Bacteriology, Graduate School of Medical Sciences, Kanazawa University, Japan and maintained using protocols as described previously [13, 14].

Potential probiotic property assessment *in vitro*

Adhesion to Caco-2 cell monolayers. The adhesive activity of LrSKG34 was examined qualitatively using Caco-2 cell monolayers (4×10^4 cells/cm^2 until confluence) as described previously, with slight modification [13]. The strain (10^8 CFU/ml) was applied to Caco-2 cell lines grown on flexiPERM® (SARSTEDT AG & Co. KG, Germany) that coated with type 1 collagen. The cells were then incubated for 3 h at 37℃ anaerobically. The cells were then fixed with paraformaldehyde (4% PFA) and then stained with Giemsa. Microbial attachment was evaluated with a microscope (Biozero BZ-9000 Series, Keyence, Japan) at 30× magnification.

Effect on intestinal barrier *in vitro*: TER and ZO-1 protein immunofluorescence. The differentiated-Caco-2 cell monolayers grown on Transwell inserts (Corning® Inc., USA) was used to evaluate the effect of LrSKG34 on mucosal integrity *in vitro*, as previously described [13]. The differentiation of Caco-2 cell lines was shown as transepithelial resistance (TER) (Millicell ERS2 voltohmmeter (Merck, Millipore, USA)). The TER measurement of differentiated Caco-2 monolayers was performed based on the manufacturer's instructions (Merck Millipore). All TER measurements were conducted in triplicate. Formation of tight junctions was also evaluated as one of indicators of the differentiation of Caco-2 cell monolayers. ZO-1 protein, one of tight junction proteins, was detected using immunofluorescence in this study. Immunofluorescence assays were conducted as described in a previous study [13, 15]. For disrupting the tight junction, hydrogen peroxide in DMEM cell culture media without FBS was used in this study. Fluorescence was evaluated using fluorescence microscope (Biozero, Keyence, Japan). More than 10 images (30 ×) were taken for each condition in more than three experiments.

Safety assessment *in vitro*

Haemolytic activity. The haemolytic activity was determined by streaking a fresh LrSKG34 culture on 5% defibrinated sheep blood agar plates (TSA agar, Oxoid Ltd., UK) and incubated for 24–48 h at 37℃ under

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anaerobic conditions. Haemolysis was classified as β-haemolysis (clear zones around colonies), α-haemolysis (green-hued zones around colonies) or γ-haemolysis (no zones around colonies). *Staphylococcus aureus* ATCC 25923 and *Streptococcus pyogenes* ATCC 19615 were used as positive controls. The assay was repeated in triplicate.

Antimicrobial susceptibility testing. The antibiotic susceptibility profiles of LrSKG34 were determined by the agar overlay diffusion method, as mentioned elsewhere [16]. An MRS agar plate was used and overlaid with 4 ml of soft agar containing 20 µl of the strain culture (10^6–10^7 CFU/ml). Antibiotic disks were placed onto the agar. The antibiotics used in this study were penicillin (10 µg), amoxycillin-clavulanic acid (30 µg), clindamycin (10 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), erythromycin (10 µg), amoxycillin (10 µg), gentamicin (10 µg), vancomycin (30 µg), trimethoprim-sulfamethoxazole (25 µg), metronidazole (50 µg), tetracycline (30 µg) and cefotaxime (30 µg) (Oxoid Ltd., Basingstoke, Hampshire, UK). The plates were then incubated anaerobically at 37℃ for 48 h. The inhibition zone diameter were measured, and the results were determined as resistance, moderate susceptibility, or susceptibility based on standard mentioned previously [16]. The assay was repeated in triplicate.

Translocation *in vitro*. The capacity of LrSKG34 to translocate through epithelial cells was measured *in vitro* using Caco-2 cell monolayers as described in a previous study [15]. LrSKG34 (1×10^8 CFU/ml) was added to the apical chamber of the *in vitro* cell model for translocation used 24-well 8 µm-pore size Transwell® polycarbonate membrane inserts (Corning® Inc.). After 1 h of incubation, the basolateral medium was collected and streaked on MRS agar plates. The plates were incubated in anaerobic condition at 37℃ for 48 h. Any single colony grown on MRS agar was determined to be a translocation event.

Safety assessment *in vivo*

***Lactobacillus* cultures.** LrSKG34 was cultured in MRS broth (Oxoid Ltd.) and incubated anaerobically at 37℃ for 24 h. Bacterial cells were prepared by washing 1 ml of the bacterial cells (10^9 CFU/ml) with PBS twice and then resuspending in sterile saline.

Animal model. The animal experiments protocol has been carefully reviewed by the Research Ethic Commission of Faculty of Medicine, Udayana University, Bali, Indonesia, and the ethical clearance was issued with number 1675/UN14.2.2/PD/KEP/2018. The protocol used in this study in accordance with the Animal Research: Reporting In vivo Experiments (ARRIVE) guidelines [17].

Five-week-old male SD rats (11 rats for each group) were used for *in vivo* safety assessment experiments (Laboratorium Penelitian dan Pengujian Terpadu/ Integrated Research and Testing Laboratory, Universitas Gajah Mada, Indonesia). The animals were maintained in appropriate cages with appropriate space. Tap water and a standard rodent diet *ad libitum* were provided for the rats. The animals were randomized and grouped into LrSKG34-treated and control groups. After 1 week of acclimatization, the treated group received LrSKG34 (1×10^9 CFU/kg body weight) in saline via oral gavage for 28 consecutive days. The weights of the rats were measured at day 0 and day 29. Blood samples and major organs of the model animals were collected on day 29.

Physical parameters. Monthly weight gain was observed in both animal model groups.

Haematological and serum biochemistry analyses. Blood was collected from the eye for haematology and serum biochemistry analyses as previously described [18]. Animals were anaesthetized with ketamine and xylazine and euthanized by cervical dislocation. Blood was collected in tubes containing EDTA and analysed (iCell-800Vet, Auto Haematology Analyzer, Shenzen iCubio Biomedical Technology Co., Ltd., China) for red blood cell count, haemoglobin, thrombocytes and WBC count (including lymphocytes, monocytes, neutrophils and eosinophils).

Non-heparinized blood was allowed to clot at room temperature, and centrifuged at 3000 *g* for 10 min at 4℃, after which serum was collected. This serum was used for biochemistry analysis (iCubio, iChem-535Vet, Shenzen iCubio Biomedical Technology Co., Ltd.) including alanine aminotransferase, total cholesterol, triglyceride, high-density lipoprotein (HDL) cholesterol,

low-density lipoprotein (LDL) cholesterol, and glucose levels.

Relative organ weight and histopathological examination. To measure the relative organ weight and the histopathological examination, liver, spleen, kidney, and colon of rats were removed after sacrificing the animal models. The gross pathological examination was performed on all organs, including determining the weights, macroscopic and presence of lesions of the organs. The relative weight of each organ was calculated and presented as a percentage (%). All organs were subjected to histopathological examination. The tissues of organs were fixed in 10% formalin, then the slides were dehydrated in an automatic tissue processor and embedded in paraffin wax (Leica EG1150H, Leica EG1150C, Germany). Each paraffin-embedded sample was then cut into 4 μm-thick sections (Leica RM2245, Germany). The sections were fixed on a glass slide, heated until dried (LeicaHI 1220, Germany), stained with haematoxylin and eosin (Leica Autostainer XL, Germany). The slides were then examined under a light microscope (Olympus CX31; Olympus, Japan).

Statistical analysis. All experiments were performed in triplicate, except where otherwise indicated. Values are presented as the mean values and standard deviations of triplicate experiments. One-way ANOVA was used to compare mean differences in mucosal integrity assays, continued with post-hoc analysis (LSD), while Independent T test was used to compare the mean differences of animal model experiments. The differences were considered statistically significant if $p < 0.05$.

Results

Caco-2 cell monolayer adhesion assay

LrSKG34 attachment was evaluated on a Caco-2 cell monolayer stained with Giemsa. As expected, the bacterial strain attached to Caco-2 cells (Fig. 1). This result suggested the possibility of persistence or colonization of LrSKG34 on the surface of intestinal cells.

Effect on mucosal integrity *in vitro*

The effect of LrSKG34 on mucosal integrity was evaluated in this study using an *in vitro* model. A one-way ANOVA was performed to compare the effect of control, hydrogen peroxide and addition of LrSKG34 to hydrogen peroxide-treated Caco-2 cell monolayer on percentage of decrease of trans epithelial resistance (TER). There was a statistically significant difference in percentage of decrease of TER between at least two groups ($F = 79.614$, $df = 2,24$, $p = 0.000$). LSD Test for multiple comparisons found that the mean value of percentage of decrease of trans epithelial resistance (TER) was significantly different between control and hydrogen peroxide-treated group ($p = 0.000$, 95% C.I. = [-74.60 - -53.49]), and hydrogen peroxide-treated group with LrSKG34 in hydrogen peroxide-treated Caco-2 cell lines ($p = 0.000$, 95% C.I. = [-49.54 ~ -28.43]). It could be concluded that LrSKG34 could maintain the transepithelial resistance in hydrogen peroxide-treated Caco-2 cells monolayer as shown in Fig. 2. Furthermore, immunofluorescence staining of ZO-1 tight junction protein showed that the strain could maintain the regularity of tight junctions better than that of hydrogen peroxide-treated cells (Fig. 2), which implied that the strain significantly maintained the mucosal integrity of the confluent Caco-2 cell monolayers, in contrast with that of cells treated with hydrogen peroxide. It was assumed that this potential probiotic strain has a good effect on mucosal integrity maintenance, which is a probiotic criterion, and has a positive effect on health.

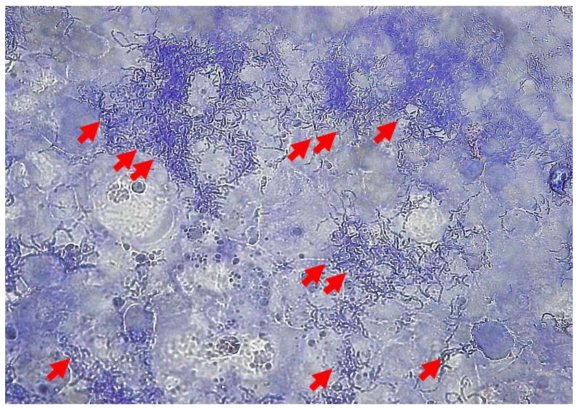

Fig. 1. Attachment of LrSKG34 on Caco-2 cell monolayer stained with Giemsa. As shown that LrSKG34 attached to the cells (red arrowhead). (Figure magnification 40×, Keyence Microscope).

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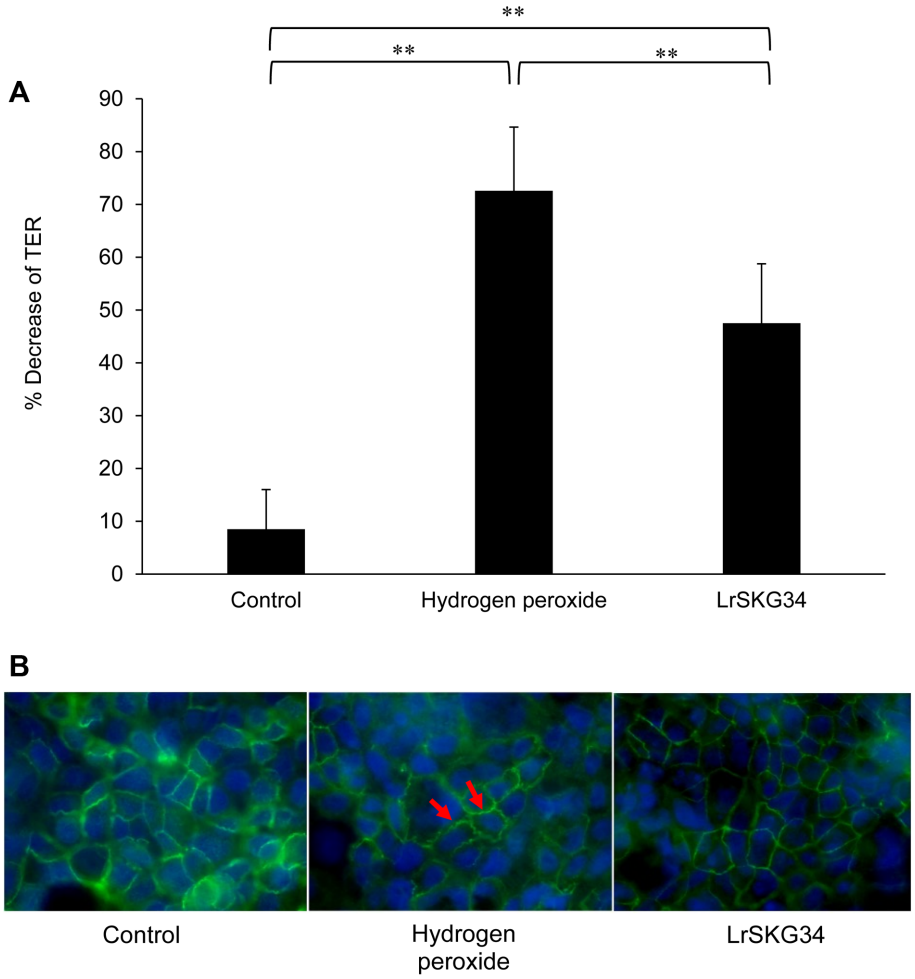

Fig. 2. Effect of LrSKG34 on mucosal integrity. The transepithelial resistance (TER) of Caco-2 cell monolayers treated with LrSKG34 was better than that of monolayers treated with hydrogen peroxide (control for membrane disruption) ($p < 0.0001$) (A). ZO-1 protein immunofluorescence assay (B) showed that the Caco-2 cells monolayer that treated with the strain had more regular border of tight junction than that treated with disruptive agent (red arrows) (magnification 30×, Keyence Microscope).

In vitro and *in vivo* safety assessment

Haemolytic activity. Haemolytic activity was evaluated by streaking LrSKG34 on 5% defibrinated sheep blood agar plates and incubating anaerobically for 24–48 h. No haemolytic zone was observed around colonies at 24 h of incubation, while a slightly brownish zone was observed around the colonies after 48 h of incubation. However, no obvious clear zone around the colonies (haemolytic activity) was found, as compared with positive control of haemolysis (Fig. 3). This result confirmed that the strain does not possess haemolytic activity.

Determination of antibiotic resistance. Resistance to antibiotics is a safety prerequisite for probiotic candidates. In this study, determination of the antibiotic resistance of LrSKG34 was performed by disk diffusion. The results showed that the strain is resistant to ciprofloxacin, gentamicin, vancomycin, trimethoprim-sulfamethoxazole and metronidazole. In addition, LrSKG34 was susceptible to the other antibiotics used in this study (Table 1).

Translocation *in vitro*. Translocation was assayed using an *in vitro* model of Caco-2 cell monolayers grown on insert Transwells. The results showed no translocation of the strain into the lower chamber of the system. On the other hand, some colonies grew on de Mann Rogosa Sharpe (MRS) agar plates when the plate was streaked

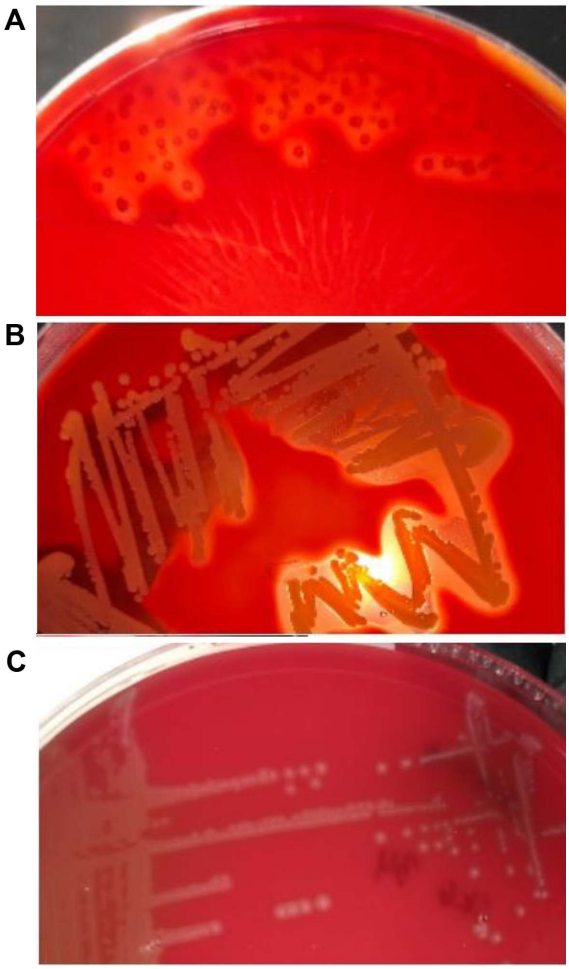

Fig. 3. Haemolysis activity assay for LrSKG34. This study used *S. pyogenes* ATCC 19615 (A), *S. aureus* ATCC 25923 (B) for haemolysis positive control. As shown in figure (C), LrSKG34 did not produce haemolysis, as compared with controls.

with a loopful of lower chamber content of hydrogen peroxide-treated Caco-2 cells. These results confirmed no translocation of LrSKG34 through the intact Caco-2 cell monolayer.

Body weight and relative organ weight. Safety assessment of LrSKG34 was conducted in Sprague-Dawley (SD) rats administered the bacterial strain orally for 28 consecutive days. The weight gain per month for both groups was not different. Furthermore, the relative organ weight was generally similar for both groups, except the kidney relative weight of LrSKG34-treated group was higher than that of the control groups; however, there was no histopathological difference observed for either group (Table 2 and Fig. 4).

Table 1. Antibiotics susceptibility of LrSKG34.

Antibiotic	Susceptibility results
Penicillin	S
Amoxycillin Clavulanic acid	S
Amoxycillin	S
Cefotaxime	S
Vancomycin	R
Clindamycin	S
Ciprofloxacin	R
Chloramphenicol	S
Erythromycin	S
Gentamicin	R
Trimethoprim-Sulfamethoxazole	R
Metronidazole	R
Tetracycline	S

*S = susceptible; **R = resistant.

Histopathological examination. There were no macroscopic pathological changes found in the rats of any of the groups. No histopathological abnormalities, including necrosis, fibrosis, loss of normal architecture, atrophy or inflammation, were observed in any of the examined organs (Figs. 4–7).

Haematology and serum biochemistry analysis. The haematology parameters, *i.e.*, haemoglobin, white blood cells (WBCs), and WBC cell counts (granulocytes), showed no difference between the control and treated groups. Although the number of platelets in the treated group was found to be significantly lower than that in the control group, the platelet values of both groups were still in the normal range [19, 20]. No difference in serum biochemistry results was observed between the two groups, indicating no harmful effect of the strain on the physiology of the model animals (Table 2).

Discussion

Our group had successfully isolated LrSKG34 and reported its viability at the acid condition as low as pH 3 or 4 for three hours, followed by being exposed to pancreatin and sodium deoxycholate for four hours to mimic the gastrointestinal environment [8]. LrSKG34 also

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Table 2. Monthly body weight gain, relative organ weights, hematological parameters and serum biochemical parameters of rats.

Variables	Mean ± SD		Mean difference	95% confidence interval of the difference	*p* value
	Control (water) (n = 11)	LrSKG34 (n = 11)			
Monthly weight gain (g)	33.56 ± 7.73	29.25 ± 5.82	4.32	-1.77 - 10.41	0.155
Liver relative weight (%)	3.07 ± 0.32	3.26 ± 0.27	-0.19	-0.45 - 0.07	0.141
Spleen relative weight (%)	0.23 ± 0.026	0.22 ± 0.03	0.01	-0.01 - 0.04	0.356
Kidney relative weight (%)	0.73 ± 0.05	0.78 ± 0.063	-0.05	-0.11 - -0.00	0.040*
Hb (g/dl)	13.40 ± 2.48	13.51 ± 1.21	-0.11	-1.84 - 1.63	0.897
White blood cells (10^9/L)	7.34 ± 1.58	7.43 ± 2.81	-0.09	-2.12 - 1.94	0.926
Granulocytes (10^9/L)	2.24 ± 0.70	2.53 ± 2.24	-0.29	-1.77 - 1.18	0.685
Granulocytes (%)	30.43 ± 7.27	31.27 ± 12.31	-0.85	-9.84 - 8.15	0.846
Lymphocytes (10^9/L)	4.60 ± 1.05	4.39 ± 1.24	0.20	-0.82 - 1.22	0.688
Lymphocytes (%)	63.0 ± 7.51	61.80 ± 12.19	1.18	-7.82 - 10.19	0.787
Platelets (10^9/L)	726.91 ± 134.01	621.0 ± 88.29	105.91	4.98 - 206.84	0.041*
ALT (IU/l)	60.41 ± 15.48	48.46 ± 14.96	-11.95	-1.59 - 25.48	0.081
Cholesterol (mg/dl)	111.23 ± 18.62	121.95 ± 22.92	-10.73	-29.30 - 7.84	0.242
Glucose (mg/dl)	132.06 ± 26.90	125.15 ± 40.54	6.92	-23.68 - 37.52	0.642

*p value < 0.05.

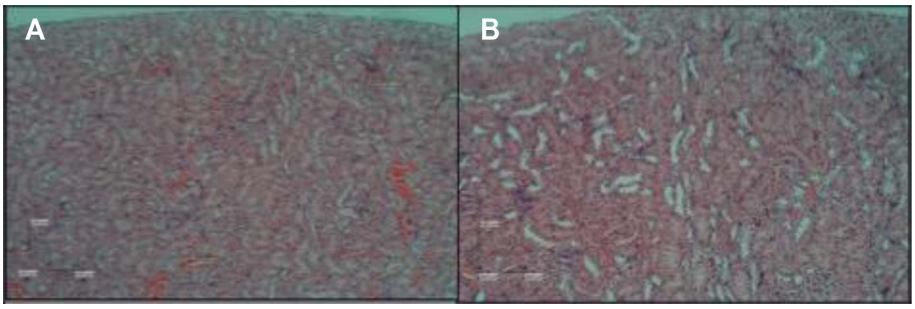

Fig. 4. Light micrograph of the kidney of (A) a control rat and (B) the LrSK34-treated rat, showing normal histological structure of kidney. Glomerulus, tubule and interstitial of the kidney were normal (haematoxylin eosin staining, 100×).

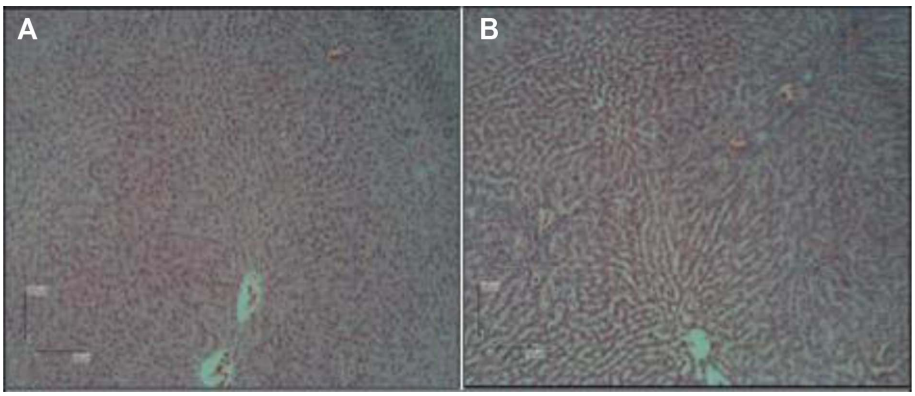

Fig. 5. Light micrograph of the liver of (A) a control rat and (B) the LrSK34-treated rat. It showed that normal histological structure of hepatocytes and sinusoids. Hepatocytes of the liver was arranged in lobules (haematoxylin eosin staining, 100×).

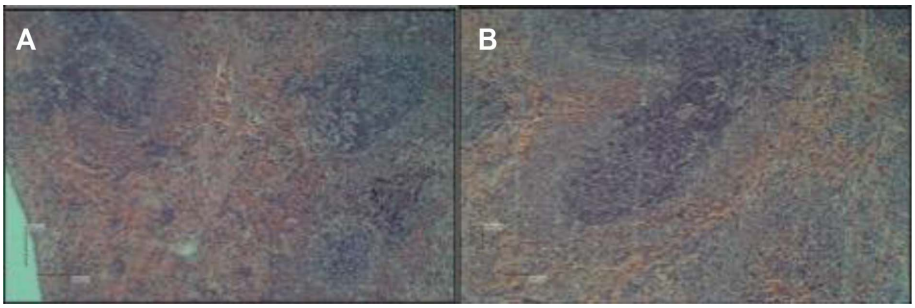

Fig. 6. Light micrograph of the spleen of (A) a control rat and (B) the LrSK34-treated rat. As showed that normal histological structure of spleen comprising of sinusoids, lymphocytes and red blood cells was observed. There was no inflammatory or cellular changes observed in the spleen of the treated rat. The spleen showed normal white pulp and red pulp. (haematoxylin eosin staining, 100×).

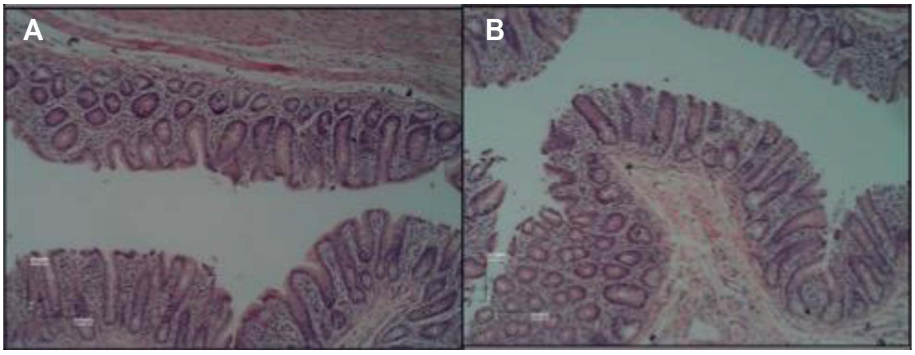

Fig. 7. Light micrograph of the colon mucosa of (A) a control rat and (B) the LrSK34-treated rat, showing normal histological structure of colon mucosa. Surface epithelium of the colon mucosa was intact. None of necrosis or inflammation was observed (haematoxylin eosin staining, 100×).

inhibited the growth of several pathogenic bacteria such as *Escherichia coli*, *Shigella flexneri*, *Salmonella* typhimurium, and *S. aureus* on *in vitro* experiment [9]. Administration of LrSKG34 on mice fed with the high-fat diet increases their HDL level but also reduces their blood cholesterol, LDL, and triglyceride level, compared to the control group [10]. The current study is the continuation of our inquiry regarding the potential of LrSKG34 as a novel probiotic candidate isolated from Sumbawa Mare Milk.

This study evaluated the adhesion activity, functional effect on mucosal barrier integrity *in vitro*, and safety of LrSKG34.

The LrSKG34 strain showed good attachment to Caco-2 cell monolayers, although this *Lactobacillus* strain was not of human origin. This result is partly in line with the studies conducted by Rajoka *et al.* and Bhat *et al.*, which showed that almost all investigated strains of non-intestinal origin of *L. rhamnosus* had high potential to attach to the Caco-2 cell line [21, 22]. Furthermore, Bhat *et al.* also elaborated the expression of the genes encoding mucus-binding (Mub) protein and mucus adhesion-promoting (Map-A) protein of *L. rhamnosus* MTCC-5897 that was isolated from household curd. Their study showed overexpression of the Mub gene of *L. rhamnosus* MTCC-5897 after induction by porcine gastric mucin, but it was reduced significantly if the strain was grown on MRS broth with the addition of mucin, bile, and pancreatin (pH 6.5) to simulate the gut environment. However, the expression of the Map-A gene increased after induction by mucin or a combination of mucin, bile, and pancreatin compared to that in the control group [22]. In addition to the possible role of mucus binding-related proteins, the adherence of probiotic bacteria to Caco-2 cells might be facilitated by pili on their surface. An intensely studied probiotic from the human intestine,

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L. rhamnosus GG, was found to have a spaCBA gene cluster to produce heterotrimeric SpaCBA pili [23]. This extended protrusion structure allows the bacterium to increase its mucus-binding avidity and significantly enhances the competitiveness of *L. rhamnosus* GG in the ecological niche of mucosal surfaces in the gastrointestinal tract. Another study by Jose *et al.* also found that *L. rhamnosus* MI13 (isolated from dairy food) could adhere to Caco-2 cells, but this adherence ability was lower than that of *L. plantarum* RC2 (bovine rumen origin) and human gut commensal bacteria such as *E. coli* [14]. In addition, the adherence ability of *L. rhamnosus* MI13 was greatly diminished by the presence of *E. coli*. Moreover, the presence of *E. coli* did not change the adherence ability of *L. plantarum* RC2 to Caco-2 cells. We plan to further elaborate on the adherence properties of LrSKG34, including the possible mechanism by which this strain attaches to the mucosal surface and its competitiveness with commensal and pathogenic bacteria in the human intestine.

LrSKG34 maintained intestinal barrier integrity *in vitro*, which was in accordance with previous studies using other strains of *L. rhamnosus* [24, 25]. A study conducted by Bhat *et al*. found that *L. rhamnosus* MTCC-5897 ameliorated the hyperpermeability state of Caco-2 cells after induction by *E. coli* ATCC 14948. Moreover, administration of *L. rhamnosus* MTCC-5897 stimulated the expression of numerous key tight junction genes, such as zonula occludens-1 (ZO-1), Claudin-1, Occludin, and Cingulin [25]. Another study by Sultana *et al*. compared the ability of five different probiotic lysates to stimulate tight junction barrier function in human keratinocytes. Their study found that *Bifidobacterium longum* and *L. rhamnosus* GG (LGG) were able to increase keratinocyte TER significantly more than *Lactobacillus plantarum*, *L. reuteri*, and *Lactobacillus fermentum* [26]. Furthermore, this study also differentiated the possible mechanism by which *B. longum* and LGG augment tight junction barrier function based on their dependency on Toll-like receptor 2 (TLR-2) activation. TLR-2 recognizes peptidoglycan (PGN) from Gram-positive bacteria [27], such as *B. longum* and LGG. When TLR-2 was inactivated by an antibody against TLR2, *B. longum* failed to increase tight junction barrier function, whereas this phenomenon did not occur for LGG, suggesting its independence from TLR-2 activation [26]. Administration of two soluble proteins from LGG (p40 and p75) prevented a significant decrease in TER after induction by hydrogen peroxide. However, the protective effects of p40 and p75 were diminished after the introduction of protein kinase-C (PKC) and mitogen-activated protein kinase (MAPK) inhibitors [24]. Several isoforms of PKC have been found in human gastrointestinal cells; most notably, PKCβ and PKCε promote intracellular signalling pathways and barrier integrity by inducing the production of epithelial growth factor (EGF) and suppressing the activation of NF-κβ and the proinflammatory cytokine TNF-α [28]. Moreover, activation of the MAPK pathway attenuated hydrogen peroxide-induced hyperpermeability by stabilizing ZO-1 and occludin in Caco-2 cells [29]. This study did not clearly investigate the mechanism of barrier integrity maintenance elicited by LrSKG34; however, further study of the mechanism of the LrSKG34 effect on mucosal integrity maintenance is necessary for further understanding.

In this study, LrSKG34 was shown to be resistant to vancomycin. Vancomycin resistant trait of most lactobacillus species is an intrinsic resistant. It is related to modification of cell wall peptidoglycan precursors synthesis, in which the terminal D-alanine residue of the muramyl pentapeptide is replaced by D-lactate or D-serine [30–35]. This strain is also resistant to ciprofloxacin, gentamicin, and metronidazole. Lactobacilli have natural resistance to ciprofloxacin, trimethoprim/sulfamethoxazole, gentamicin and metronidazole [36, 37]. Furthermore, this type of resistance in probiotics is different from that in Enterococci, which are inducible and transferrable to other bacteria. The antibiotic resistance pattern shown by LrSKG34 may give an advantage for probiotic bacteria, especially related to restoration the intestinal microbiota after antibiotic treatment by probiotic bacteria [38].

In the selection of probiotic candidates, the safety profile of the strain must be considered [11, 39], including haemolytic activity. There was a brownish area around the LrSKG34 colony incubated for 48 h, suggesting that partial haemolysis may have occurred. Partial haemolysis produced by lactobacilli is due to the production of hydrogen peroxide, which oxidizes haemoglobin in the blood agar plate, thus resulting in the formation of greenish methaemoglobin without any complete destruction of the erythrocytes [39]. Furthermore, haemolysis

may result in iron availability for bacteria. Lactobacilli can grow without the presence of iron, which means that lactobacilli do not perform haemolysis. This trait provides benefits for lactobacilli, especially those related to competition with bacterial pathogens [40]. Bacterial translocation occurs when viable bacteria pass through the gastrointestinal tract to the extraintestinal sites. This event happen when there is an intestinal barrier disruption or in immunocompromised [41, 42]. In this study, translocation *in vitro* was evaluated using differentiated Caco-2 cell monolayers grown on Transwells [15]. No translocation of this strain was found with an intact Caco-2 cell monolayer, indicating that translocation does not occur under normal barrier gut conditions, which is in agreement with previous work [43, 44]. Taken together, the promising results of *in-vitro* safety profiles from LrSKG34 need further elaboration using genomic approaches such PCR, as well as whole-genome analysis, to specifically detect the existence of antibiotic resistance genes or virulence factors to conclusively qualify this strain as safe as stated in several international probiotics regulations.

The safety aspect in an animal model was examined by evaluating the effect of the probiotic candidate on physical performance (weight gain), relative organ weight, and haematological and serum biochemistry parameters. In this study, no harmful effect was found on physical performance, organ relative weight, or haematological and serum biochemistry parameters. Previous studies showed that the strain is not toxic and has no deleterious effects on the animals' health status, growth, or development [24, 44, 45].

In summary, it was clearly observed that LrSKG34 showed probiotic properties and had no deleterious effect *in vitro* and in model animals. Thus, it can be concluded that the strain has the potential to be a safe candidate for probiotics.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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