Resistance of *Lactobacillus* Sp. SKG 34 in The Digestive Tract of Rats Detected Using Random Amplified Polymorphic DNA Technique

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**Abstract.** In recent years, interest in probiotics, particularly *Lactobacillus* strains like *Lactobacillus* sp. SKG34, isolated from Sumbawa mare milk, has surged due to their potential health benefits. This strain holds promise as a probiotic agent, but understanding its behavior in the gastrointestinal (GI) tract is essential for effective application. This study aimed to investigate the resistance and persistence of *Lactobacillus* sp. SKG34 in the GI tract of rats. Rats were orally administered the strain at a concentration of $10^8$ cells for 30 days. The probiotic's ability to modulate gut microbiota and improve host health is a key area of interest. By utilizing techniques like PCR-RAPD, this research sought to elucidate the fate of Lactobacillus sp. SKG34 in the complex environment of the rat GI tract. Results revealed a significant increase in LAB population in cecal contents, from $3.06 \times 10^9$ cfu/g in the control group to $1.25 \times 10^{10}$ cfu/g in the probiotic group. RAPD analysis also detected Lactobacillus sp. SKG34 in approximately 23% of the total population of lactic acid bacteria in cecal contents, indicating its resistance and proliferation within the GI tract. Further investigation is warranted to fully understand the potential of Lactobacillus sp. SKG34 as a beneficial probiotic strain for human application.

1 Introduction

Nowadays, functional food is rapidly evolving, where consumed food is expected not only to fulfil nutritional needs but also to stimulate specific functions in individual health. Lactic acid bacteria (LAB) have been extensively utilized by the food industry in creating functional food products to maintain human digestive health, known as probiotics. Probiotics are live microorganisms that, when administered in adequate amounts, can benefit digestive health [1]. Probiotics are beneficial to health because they can enhance the balance of beneficial microbes in the digestive tract, helps to prevent pathogenic pathogenic microflora from exceeding their number limit and perform many other vital
activities [2]. Food containing probiotics is also classified as functional food if it significantly affects one or more body functions, thereby providing health or therapeutic effects to humans beyond their nutritional value [3].

Before being declared as probiotic bacteria, probiotic candidates must undergo in vitro and in vivo tests [1]. For LAB to be considered as probiotics, they must meet several criteria: (1) withstand low pH of gastric acid, (2) stable against bile salts and able to survive in the small intestine, (3) produce antimicrobial compounds, (4) adhere to the intestine, (5) grow and thrive in the digestive tract, (6) interact between cultures to adhere to each other, and (7) safe for human consumption [4].

Sumbawa mare milk has been claimed to have various health benefits and can cure several diseases. Lactobacillus sp. SKG34 is a potential probiotic strain isolated from Sumbawa mare milk [5,6]. In vitro, Lactobacillus sp. SKG34 was found to have resilience in digestive conditions and has bile-salt hydrolase enzymes that play a role in breaking down bile salts in the digestive tract, contributing to lowering blood cholesterol levels. The probiotic potential of Lactobacillus sp. SGK34 needs to be validated in vivo to determine whether Lactobacillus sp. SKG34 can reach the digestive tract alive and can proliferate in the digestive tract of rats or humans. This is a primary requirement for the development of probiotics related to the functional effects of Lactobacillus sp. SKG34 due to its metabolic activity in the digestive tract.

Analysis of intestinal bacteria is sometimes very difficult to perform due to the high species diversity and oxygen stress factors that affect bacterial growth. Nevertheless, many techniques are used to analyze intestinal bacteria based on DNA fingerprinting sensitivity. One of the most common and easily performed techniques with reliable results is using random amplified polymorphic DNA (RAPD). By using specific primers, characteristic amplification results can be obtained to differentiate detected strains [7,8].

2 Materials and methods

2.1 Bacterial strain and growth condition

Lactobacillus sp. SKG34 stored in 30% glycerol at -20°C in the Udayana University Culture Collection was taken as one loopful and inoculated into 5 mL of MRS broth media. The reaction tubes were incubated anaerobically for 24 hours at 37°C.

2.2 Preparation of Lactobacillus sp. SKG 34 suspensions

Lactobacillus sp. SKG34 culture grown in 5 mL of MRS Broth media was vortexed (to obtain a homogeneous culture), then 1 mL was taken and placed in an Eppendorf tube and centrifuged at 5000 rpm for 5 minutes to separate the cell mass and supernatant. The supernatant was discarded, and the cell mass was washed twice with saline solution (0.85% NaCl) to remove residual media. Washing was done by adding 1 mL of saline to the cell mass, vortexed until homogeneous, and centrifuged at 5000 rpm for 5 minutes. At the final stage, the cell mass was suspended in sterile water to obtain a concentration of 10⁸ cells/ml [9].
2.3. Administration of *Lactobacillus* sp. SKG34 to white rats (*Rattus norvegicus*)

This study used 20 male wistar white rats (*Rattus norvegicus*) aged 5 weeks with a weight of 40-50 grams obtained from BPPH Denpasar, Bali. The rats were placed in plastic rack cages measuring 45cm x 30cm x 10cm. The racks were covered with wire mesh, and rice husks were placed on the bottom of the racks as urine and feces absorbents for the rats [10]. The rats were acclimatized for 1 week before treatment. During this acclimatization phase, the rats were fed a standard diet consisting of corn flour with the addition of 10% 3-day-old mung bean sprouts. After the acclimatization phase, the rats were divided into two groups (each group consisting of 10 rats), each given: standard diet (AOAC, 1990) and standard diet with 0.5 ml cell *Lactobacillus* sp. SKG34. The composition of the standard diet can be seen in Table 1. The *Lactobacillus* sp. SKG34 bacterial suspension was administered to the white rats by oral gavage, with 0.5 ml suspension (equivalent to 10^8 cells) administered orally using a gavage needle. The treatment was given once a day (12.00-13.00 WITA) after feeding for 30 days. The weight of the rats and the weight of the feed were measured daily.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Quantity</th>
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<tbody>
<tr>
<td>Casein</td>
<td>100</td>
</tr>
<tr>
<td>Corn Oil</td>
<td>80</td>
</tr>
<tr>
<td>Mineral Mix</td>
<td>50</td>
</tr>
<tr>
<td>Vitamin Mix</td>
<td>10</td>
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<tr>
<td>CMC</td>
<td>10</td>
</tr>
<tr>
<td>Water</td>
<td>50</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>700</td>
</tr>
</tbody>
</table>

Table 1. The composition of the standard diet [11]

2.4. Total lactic acid bacteria and anaerobic bacteria in the cecum

Total lactic acid bacteria and anaerobic bacteria in the cecum of white rats treated for 30 days were obtained by surgical procedure under chloroform 10% anesthesia. This research followed the regulations and procedures for the use of experimental animals applicable at Udayana University. The cecum obtained was placed on a sterile Petri dish, its contents were extracted and collected in sterile tubes, and physiological saline solution (0.85% NaCl) was added according to the weight of the cecal contents (1:1 dilution) and homogenized. Next, 0.05ml of cecal content suspension was added to dilution tubes containing 0.45 ml saline, resulting in a 10^1 dilution, vortexed until homogeneous, then further diluted to a 10^7 dilution. For the purpose of counting the total population of LAB, 0.1ml of the diluted sample (dilution 10-3-10-6) was spread on the surface of MRS Agar media supplemented with Bromo Cresol Purple (BCP), then incubated aerobically for 24 hours at 37°C. The same method was used for counting anaerobic LAB, but the inoculation was done on anaerobic agar media and incubated anaerobically using an Anaerobic gas pouch within an anaerobic chamber. After incubation, colony counts were performed. Bacterial colonies were counted using dilution method assuming that one colony originated from one cell. Therefore, the total bacterial...
population was obtained by multiplying the number of colonies counted by the dilution factor [12].

2.5 Colonization of *Lactobacillus* sp. SKG 34 in the rat digestive tract

Colonization of *Lactobacillus* sp. SKG 34 in the rat digestive tract was determined in two stages: PCR-RAPD of *Lactobacillus* sp. SKG34 colonies grown on MRS-agar media. Colonies of sufficient size (diameter around 0.5-1 mm) were randomly picked from Petri dishes using sterile toothpicks or pipette tips (crystal tip, 10 μL). The cells were then placed in Eppendorf tubes, added with 50 μl of Tris buffer pH 7.0, and 5 μl of lysozyme solution 0.05 g/mL and triton X 100 [9]. Eppendorf tubes were incubated at 37°C for 30 minutes and centrifuged at 13,000 rpm for 3 minutes. The supernatant was discarded, and the cells were washed with 50 μl of Tris buffer pH 9 then heated in boiling water for 5 minutes followed by freezing for 15 minutes. Heat freezing was done twice and the lysate was used as DNA in the PCR-RAPD reaction.

2.6 PCR-RAPD of *Lactobacillus* sp. SKG34

Random Amplified Polymorphic DNA (RAPD) was performed to specifically determine *Lactobacillus* sp. SKG34 administered. RAPD was performed using the M13F primer. PCR reaction mixtures were prepared in a total volume of 12.5 μl containing: 10 mM each dNTPs, 25 pmol M13F primer, 1X PCR Buffer II, 75 mM MgCl2, 0.45 U AmpliTaq, 1 μL DNA. Amplification reaction was performed as follows: one cycle at 94°C for 5 minutes, followed by 40 cycles at 94°C for 20 seconds, 40°C for 2 minutes, and 72°C for 30 seconds. The final stage was followed by one cycle at 72°C for 5 minutes. The PCR products were then electrophoresed using 1% agarose in 1X TAE buffer, stained with EtBr (50 ng/mL), visualized on a UV illuminator, and photographed. To determine the population of *Lactobacillus* sp. SKG34, the band patterns of isolates were compared with *Lactobacillus* sp. SKG34 controls.

2.7 Fermentation activity

Fermentation activity was observed by measuring the pH of the contents of the white rat cecum. Acidity level (pH) was measured using a pH meter. The cecal contents were measured for pH using a pH meter (TOA ion meter IM 40S) that had been previously calibrated with pH 4 and pH 7 buffers. The cecal contents, diluted once (1:1), were then homogenized by vortexing. Subsequently, the pH of the cecal contents was measured by dipping the pH meter electrode into the sample, and the results were recorded.

3 Results and Discussion

Sumbawa mare milk has become the focus of public attention, as it is believed to have beneficial effects on consumers' health. Considering that Sumbawa mare milk is a good habitat for Lactic Acid Bacteria (LAB), the suspected health aspects of Sumbawa mare milk may be caused by the LAB present in it or the metabolic products of LAB from the components of Sumbawa mare milk. *Lactobacillus* sp. SKG34 is a LAB isolated from Sumbawa mare milk which in vitro has good resistance under digestive conditions such as low pH (pH 2, 3, and 4) and bile (deoxycholic acid). *Lactobacillus* sp. SKG34 is able to pass
through intestinal conditions with a content of 0.4 mM sodium deoxycholate and pancreatin, so both isolates have the potential as probiotics [6].

One of the requirements for LAB to be considered as probiotics is to grow and thrive in the digestive tract. The potential of *Lactobacillus* sp. SKG34 as a probiotic in vivo was evaluated by testing the population of this bacterium in the cecum of white rats. In Fig. 1, it can be seen that the administration of *Lactobacillus* sp. SKG34 stimulates the growth of LAB in the rat cecum. This is closely related to the environment created due to the activity of *Lactobacillus* sp. SKG34 such as the decrease in cecal pH (Fig. 2) and other microaerophilic environments. However, Figure 1 also shows a slight increase in the total anaerobic bacteria. This is closely related to the possibility of LAB being counted in anaerobic plates, causing an increase in the total anaerobic population.

![Graph showing population of lactic acid bacteria and total anaerobic bacteria](image)

**Fig 1.** The effect of administering *Lactobacillus* sp. SKG34 on the population of lactic acid bacteria (1); and Total anaerobic bacteria (2)

In Fig. 2, it can be observed that the cecal pH in rats given *Lactobacillus* sp. SKG34 is lower than in the control group. This indicates better fermentation activity in the administration of *Lactobacillus* sp. SKG34 compared to the control treatment. This may also be due to the proliferation of *Lactobacillus* sp. SKG34 in the rat cecum, as shown in Figure 3.

![Graph showing cecal pH](image)

**Fig 2.** Effect of administering *Lactobacillus* sp SKG34 on cecum pH
Fig. 3. Images of bacterial cell consortium from the cecum of rats administered with *Lactobacillus* sp. SKG34 (A) and RAPD photo of isolates obtained from the same cecal sample (B). M; 100 bp ladder; C+ ; positive control (*Lactobacillus* sp. SKG34). The arrow indicates positive results.

Results of PCR-RAPD on lactic acid bacteria (LAB) isolates obtained from the rat cecum showed that 23% of the colonies tested positive in PCR (Figure 3B). This indicates that the population of *Lactobacillus* sp. SKG34 comprises 23% of the total LAB in the rat cecum. *Lactobacillus* sp. SKG34 has demonstrated good resilience in passing through the rat digestive tract and proliferating in the rat cecum. These findings validate previous in vitro research, where *Lactobacillus* sp. SKG34 exhibited good resilience under digestive conditions such as low pH (pH 2, 3, and 4) and bile (deoxycholic acid).

PCR-RAPD is a molecular technique involving the use of specific markers to study genetic diversity. This technique involves the attachment of specific primers designed as needed. The use of RAPD technique allows for the detection of DNA fragment polymorphisms selected using an arbitrary primer, primarily because DNA amplification in vitro can be performed well and quickly with PCR. The use of RAPD markers is relatively simple and easy in terms of preparation. RAPD technique provides faster results compared to other molecular techniques. This technique is also capable of producing a relatively unlimited number of characters, making it very helpful for analyzing the diversity of organisms with unknown genomic backgrounds.

4 Conclusions

*Lactobacillus* sp. SKG34 can reach the digestive tract alive and proliferate in the rat digestive tract. This is a primary requirement for the development of probiotics related to the functional effects resulting from the metabolic activities of *Lactobacillus* in the digestive tract.

References

2. A.A. Amara and A, Shibi. Role of Probiotics in health improvement, infection control and disease treatment and management, Saudi Pharm 23, 7 (2015)


