JPBIO (Jurnal Pendidikan Biologi)
Vol. 10, No. 2, November 2025, 478 – 487 //e-ISSN 2540-802X
http://jurnal.stkippersada.ac.id/jurnal/index.php/JBIO/index

Exploration of lactic acid bacteria from husk and black rice (Oryza sativa L) of toraja origin

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Article Info

Article History:
Received 20 October 2025
Revised 31 October 2025
Accepted 03 November 2025
Published 30 November 2025

Keywords:
Black rice
Lactic acid bacteria
Lactococcus taiwanensis

ABSTRACT

Lactic acid (LAB) is a bacteria involved in food fermentation and can be used as a probiotic to provide health benefits for those who consume it. This research aims to bring innovation and added value, and improve the community's utilization of probiotics (LAB) from husks and black rice from Toraja. The method used is collecting and processing samples, isolation and purification of LAB, microscopic and macroscopic identification, testing the activity/potential of LAB (Kirby Bauer), and molecular identification of 16S rRNA (sequencing). This study has confirmed that fermented rice has *Lactococcus taiwanensis* type lactic acid bacteria, while fermented husk is not a type of lactic acid bacteria. *Lactococcus taiwanensis* bacteria have probiotic potential and can produce antibacterial substances.

Citation: Hapiwaty, S., Zainal, T.H., Abidin, H.L., & Utami, Y.P. (2025). Exploration of lactic acid bacteria from husk and black rice (Oryza sativa L) of toraja origin. *JPBIO (Jurnal Pendidikan Biologi), 10*(2), 478-487. DOI: https://doi.org/10.31932/jpbio.v10i2.5529

INTRODUCTION

Lactic Acid Bacteria (LAB) are involved in food fermentation and can be used as probiotics (Fauziyah et al., 2023). According to Prof. Dr. Ir. Tyas Utami, M.Sc., in her speech, she stated that increasing public awareness of health has led to higher demand for healthy food products, including probiotic products. However, most probiotic supplements and foods available in the domestic market use imported probiotics. This challenges Indonesian researchers to produce indigenous probiotic products from Indonesia (Ekaptiningrum, 2023).

The National Research and Innovation Agency (BRIN, 2022) reported that Indonesia is experiencing an increasing market demand for probiotic products. According to Research and Markets (2020), the demand for probiotics in Indonesia is projected to grow by 11.29% between 2019 and 2025.

Several data related to using food sources as probiotics include the fermentation of traditional food such as starch, which has antimicrobial potential showing significant growth reduction, and highlighting the broad-spectrum antimicrobial potential of probiotic isolates (Anumudu et al., 2024; Paul et al., 2023). Another data point is the utilization of tapas, a traditional Indonesian fermented food that contains various microorganisms, including Lactic Acid Bacteria (LAB) such as *Lactiplantibacillus plantarum* and *Lactobacillus acidophilus*. This species produces antimicrobial compounds (bacteriocins) against test bacteria, as observed under Scanning Electron Microscope (SEM) imaging (Liu et al., 2024; Santoso et al., 2023).

The development of indigenous probiotic products in Indonesia, beyond starch and tape, includes the exploration of local resources such as rice husks and black rice from Toraja, a highland region in South Sulawesi known for its sustainable farming practices and strong cultural heritage. Empirically, black rice has long held symbolic value in Toraja—formerly used as offerings in ancestral rituals and prepared as pa'piong (rice cooked in bamboo) (Jainuddin et al., 2022) during post-harvest thanksgiving ceremonies following the spread of Christianity. This continuity reflects the community's deep connection to their agricultural products and traditional eco-friendly cultivation methods, which may contribute to the unique microbial diversity associated with black rice. Such aspects of local wisdom make Toraja a promising site for the development of indigenous probiotic innovations. Supporting data further show that black rice extract can be developed into an antibacterial gel with clear inhibition zones against test bacteria (Khairi, Hapiwaty, Indrisari, et al., 2023). Additionally, the standardization of specific and non-specific parameter measurements has been conducted to maintain quality, safety, and efficacy, thereby increasing public trust in the benefits of natural ingredients (Khairi, Hapiwaty, Yusuf, et al., 2023).

On the other hand, Lactic Acid Bacteria (LAB) can demonstrate positive results as a potential food preservative by producing bacteriocins. Bacteriocins are ribosomally synthesized peptides or proteins produced by bacterial strains that can inhibit pathogenic bacteria. Many factors influence the possible activity of bacteriocins in the food matrix, such as food additives, chemical composition, physical conditions, and sensitivity to proteolytic enzymes (Shafique et al., 2023).

RESEARCH METHODS

Research Design

This study employed an experimental laboratory design to explore, isolate, and identify LAB from husk and black rice (*Oryza sativa* L.) of Toraja origin. The research workflow consisted of several sequential stages: Sample collection and fermentation, isolation and purification of LAB, macroscopic and microscopic identification, antibacterial activity testing, and molecular characterization through 16S rRNA gene amplification and sequencing.

Population and Samples

The research population consisted of black rice plants (Oryza sativa L. indica var. black) from the Toraja region, South Sulawesi, Indonesia. Approximately 1 kg of mature black rice grains and rice husks were collected aseptically from local farmers in Belau Village, Masanda District, Tana Toraja Regency, during the 2024 harvest season. The samples represented traditionally cultivated black rice with a dark-purple to black pericarp and minimal synthetic fertilizers or pesticides. Both grains and husks were placed in sterile containers and immediately transported to the microbiology laboratory for further processing and isolation of potential LAB.

Instruments

The instruments used in this study consisted of both general microbiological laboratory equipment and molecular biology instruments. Analytical balances, beakers, Erlenmeyer flasks, sterile containers, and a vortex mixer were used to weigh and homogenize the rice husk and black rice samples. A laminar airflow cabinet, micropipettes, sterile Petri dishes, test tubes, inoculating loops, an incubator maintained at 37°C, a refrigerator, and an autoclave were used to ensure aseptic handling, inoculation, and cultivation of LAB on MRS agar medium. LAB colonies were observed and identified using a compound light microscope and a Gram staining set consisting of crystal violet, iodine, ethanol, and safranin, along with glass slides and cover slips for microscopic examination. The antagonistic (antibacterial) test utilized inoculating loops, sterile cotton swabs, micropipettes, a cork borer or well cutter, an incubator, and a digital caliper or ruler for measuring inhibition zones using the agar diffusion (Kirby–Bauer) method. For molecular characterization, instruments such as a microcentrifuge (14,000–16,000 × g), a heating block or water bath set at 60°C, a vortex mixer, micropipettes, and 1.5 mL microcentrifuge tubes were used for DNA extraction and lysis. To prepare the reaction mixture, PCR amplification of the 16S rRNA gene was performed using a thermal cycler (PCR machine), supported by vortex mixers and micropipettes. A gel electrophoresis apparatus, power supply, agarose gel mold, UV transilluminator, and gel documentation system were used to detect and visualize amplified DNA fragments. Sequencing and data analysis were conducted using commercial DNA sequencing services (1st BASE Singapore), while sequence alignment and species identification were performed using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information.

Procedures

1. Sample Preparation

Two hundred grams (200g) of rice husks and black rice were washed and rinsed thoroughly to remove dirt and dust. Fermentation was carried out by soaking each of the rice husks and black rice in sterile distilled water (rice: water (1:3)) for 72 hours (3 days) at room temperature (Makut et al., 2022).

2. Isolation of Lactic Acid Bacteria

Fermentation liquid from rice husks and black rice was taken in the amount of 10 ml and placed in a test tube, then vortexed. Dilutions were made for each sample from 10-1 to 10-8. Using the spread method, 1 ml was taken from each dilution level and put into Petri dishes containing MRS agar + CaCO3. Subsequently, they were incubated at 37°C for 48 hours. A clear zone around the colonies indicated the growth of lactic acid bacteria. Bacteria showing a clear zone were purified using the quadrant streak method. The growing colony isolates were inoculated using slanted MRS agar media.

3. Macroscopic and Microscopic Identification of Lactic Acid Bacteria

Macroscopic identification was carried out by observing LAB's shape, color, and specific characteristics. Meanwhile, microscopic identification was done using Gram staining.

4. Antagonistic Test of LAB Isolates

The antibacterial capability test was conducted on the fermentation results of lactic acid bacteria (LAB) cultures using the agar diffusion method (Kirby-Bauer) against a group of test microbes (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, and Candida albicans*). The formation of a clear zone indicates a positive result.

5. Molecular Characterization

a. DNA Extraction

Genomic DNA was extracted from the bacterial isolates using a silica column-based protocol with slight modifications. Briefly, bacterial cells (approximately 10^7 CFU/mL) were centrifuged and resuspended in PBS. Proteinase K was added to the suspension and incubated at 60°C to promote cell lysis. Lysis buffer was added, and the mixture was incubated at 60°C to ensure complete cell disruption. The lysate was mixed with ethanol and transferred to a GD spin column for DNA binding. Then, the column was washed sequentially with Buffer W1 and Wash Buffer to remove impurities. After a final centrifugation to dry the column, DNA was eluted using 100 µL of elution buffer. Purified DNA was stored at 20°C until further analysis (Harini et al., 2019).

b. PCR Amplification of 16S rRNA Gene

The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the universal primers 63F (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 1387R (5′-GGG CGG WGT GTA CAA GGC-3′). The PCR reaction mixture (50 µL total volume) contained GoTag® Green Master Mix, primers, template DNA, and nuclease-free water. Amplification was performed for 35 cycles under the following conditions: pre-denaturation at 95°C for 2 min, denaturation at 95°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 45 s, and final extension at 72°C for 5 min (Promega, 2019).

c. Electrophoresis and Visualization

The PCR products were analyzed by electrophoresis on a 2% agarose gel prepared with 1× TAE buffer and stained with ethidium bromide. Electrophoresis was conducted at 100 V for approximately 50 min, and a UV transilluminator and gel documentation system was used to visualize and document the DNA bands.

d. Sequencing and Data Analysis

Purified PCR products were sent for sequencing at the 1st BASE Singapore. We analyzed the obtained nucleotide sequences using the Basic Local Alignment Search Tool available at the National Center for Biotechnology Information (NCBI) to determine the closest known bacterial species based on 16S rRNA gene similarity.

Data Analysis

Morphological, microscopic, biochemical, and molecular identification data were descriptively analyzed. Morphological and Gram-staining observations were used to classify the isolates. The antagonistic activity was evaluated based on the inhibition zone diameter (mm), measured using a digital caliper after 24 h of incubation at 37 °C. Each test was performed in triplicate, and the mean values were recorded (Table 2). The 16S rRNA gene sequences obtained were aligned and compared using Basic Local Alignment Search Tool (BLAST) to identify the closest bacterial species. Tables and figures summarize the characteristics and diversity of LAB isolated from husk and black rice samples.

RESULTS

The isolation of LAB from black rice and rice husk produced three distinct isolates, namely, BL (from rice), SB, and ST (from husk), as shown in Figure 1.

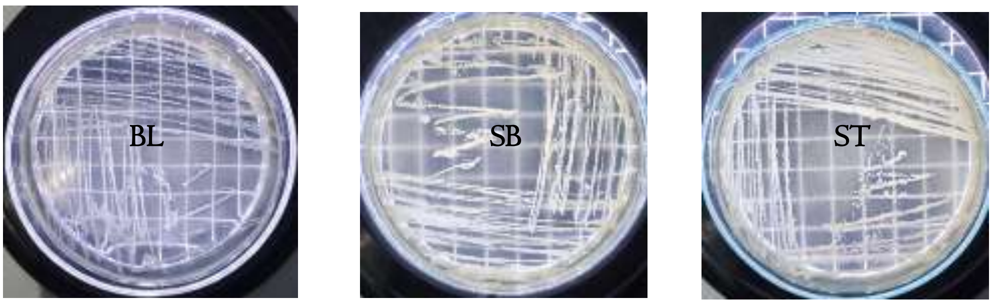

Figure 1. Colony morphology of BL isolate from rice and ST, SB isolates from rice husk

Table 1 presents the macroscopic and microscopic characteristics of the isolates. All isolates exhibited round, smooth, and convex colonies with a white to cream coloration. Microscopic observation revealed that all isolates were gram-positive and round-shaped, indicating their potential as lactic acid bacteria candidates.

Table 1. Presents the macroscopic and microscopic characteristics of the isolates

	Macroscopic				Microscopic	
Isolate	Shape	Edge	Color	Surface	Cell Shape	Gram
BL	Round	Smooth	White	Convex	Round	Positive
SB	Round	Smooth	Cream	Convex	Round	Positive
ST	Round	Smooth	Cream	Convex	Round	Positive

The antagonistic activity test showed that all isolates inhibited the growth of pathogenic bacteria but did not exhibit antifungal activity against *C. albicans* (Table 2). Among them, isolate BL demonstrated the strongest inhibitory effect, particularly against *E. coli* (8.52 mm).

Table 2. Inhibition zone of antagonistic test

	Inhibition of antagonistic test (mm)				
Isolate	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. thypi*	*C. albicans*
BL	7.1	8.52	8.35	8.12	6.00
SB	6.87	7.39	7.79	6.93	6.00
ST	6.79	7.51	6.72	7.19	6.00

Molecular identification was performed to confirm the presence of the isolate species. The amplified DNA fragments were visualized through electrophoresis (Figure 2) and sequenced using 1st Base Singapore services. Sequences were analyzed using the BLAST tool of GenBank. The BL isolate showed 98.72% similarity with *L. taiwanensis*, whereas the SB and ST isolates exhibited 98.55% and 98.76% similarities with *S. hominis*, respectively.

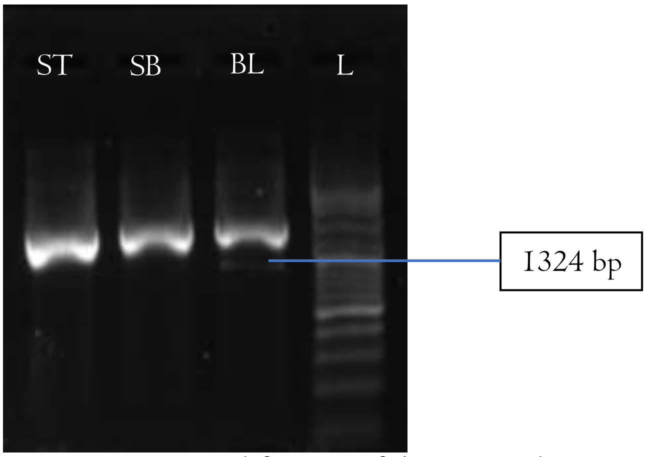

Figure 2. PCR amplification of the LAB isolates

These results indicate that isolate BL, identified as *L. taiwanensis*, exhibited the strongest antibacterial potential and can be considered a promising candidate for further study.

DISCUSSION

Three bacterial isolates were successfully obtained from Toraja-origin black rice and its husk, consisting of one isolate from the rice (BL) and two isolates from the husk (SB and ST). The successful isolation demonstrates that both substrates provide favorable microenvironments for LAB growth. Black rice and its husk are rich in carbohydrates, proteins, and phenolic compounds that can serve as nutritional sources and stress modulators, supporting acid-tolerant microbial species' metabolism and selection (Lestari, 2024). Black rice, particularly the Pare Ambo cultivar from South Sulawesi, contains high levels of essential nutrients, such as carbohydrates (85%), protein (1.04%), and fat (1.9%), and mineral components, including calcium, magnesium, and potassium, which contribute to its potential as a microbial growth substrate (Mangiri et al., 2016). These nutritional properties, coupled with antioxidant anthocyanin pigments, create a unique ecological niche for the colonization of indigenous LAB.

The findings of this study are consistent with those of other studies on BR and its derivatives' functional potential. Fermentation studies using *Pleurotus* spp. on Pare Ambo black rice reported enhanced protein, lipid, and fiber contents, along with increased antioxidant and antidiabetic activity, demonstrating the beneficial microbial activity of this biochemically rich substrate. Similarly, the successful isolation of LAB from fermented sawi ansabi (Dayak kimchi) using MRS agar enriched with 1% $CaCO_3$ confirmed that this medium selectively promotes the growth of LAB, particularly L. plantarum and P. cerevisiae (Teul et al., 2023). This parallel strengthens the methodological rationale for using MRS agar, which is nutritionally balanced and provides the acidic and carbon-rich environment required for LAB recovery from complex plant matrices.

These results imply that Toraja-origin black rice and its husk not only act as nutritional sources but also as natural reservoirs of potentially probiotic LAB strains adapted to phenolic-rich environments. The combination of phenolic antioxidants and LAB activity could synergistically contribute to the health-promoting potential of fermented BR products. Nevertheless, the limitation of using standard MRS medium must be acknowledged; while effective for general LAB isolation, it may not fully recover species requiring specific micronutrients or cofactors inherent to the black rice substrate. Therefore, future work may incorporate modified MRS formulations or black rice extract supplementation to enhance the recovery of substrate-adapted LAB and to better explore their biotechnological potential.

Morphological and microscopic characterization, all isolates were gram-positive and formed convex, circular, smooth-edged colonies with white to cream pigmentation. These characteristics are consistent with the general morphological profile of LAB, which are typically non-spore-forming and Gram-positive due to their thick peptidoglycan layer that retains crystal violet during staining. Although further biochemical tests, such as catalase or spore formation assays, were not performed in this study, the observed morphological traits are consistent with similar LAB characteristics reported in previous studies.

Comparable findings were reported in LAB isolated from pakatikng rape, a traditional Dayak fermented food made from kelampai fruit, where nine isolates exhibited cream-colored, circular, convex colonies with smooth surfaces and Gram-positive coccoid cells (Rahayu & Setiadi, 2023). Similarly, LAB isolates obtained from sarobuong, a fermented bamboo shoot product from Riau, also showed round, convex colonies with gram-positive cocci and bacilli forms (Saryono et al., 2023). The consistency of these features across different plant-based fermentation sources suggests that LAB from various natural substrates share conserved morphological patterns, likely reflecting adaptation to carbohydrate-rich and mildly acidic environments.

These observations support the assumption that the isolates obtained from black rice of Toraja origin and its husk belong to the LAB group. However, morphological and staining data alone are insufficient for accurate taxonomic identification. Therefore, additional biochemical and molecular analyses, such as carbohydrate fermentation profiles or 16S rRNA sequencing, are needed to confirm the species' identity and explore their potential probiotic or biopreservative properties.

All isolates exhibited antibacterial activity against pathogenic bacteria, including *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and Salmonella typhi*, but none inhibited *Candida albicans* growth. This inhibitory response is most likely attributed to the production of antimicrobial metabolites, such as organic acids, hydrogen peroxide, and bacteriocin-like compounds, which can disrupt bacterial membranes and acidify the surrounding environment.

A comparative analysis among isolates revealed that BL displayed the strongest antibacterial activity, producing inhibition zones ranging from 7.1 to 8.52 mm, whereas ST and SB exhibited slightly lower activities. This variation likely reflects strain-specific differences in bacteriocin synthesis and organic acid production. LAB species produce different types and quantities of antimicrobial substances. Previous studies have shown similar variability among Lactococcus strains isolated from PBFs. The superior inhibitory effect of BL suggests that this isolate may produce more potent antimicrobial compounds or maintain better metabolic activity under the test conditions. Identifying such a strong isolate is valuable for developing functional cultures with enhanced antimicrobial potential. However, this study did not quantify acid or bacteriocin production, which limits mechanistic interpretation.

Previous studies have reported comparable results on LAB isolated from traditional fermented foods. For instance, Lactobacillus fermentum isolated from Dengke Naniura (a Batak Toba traditional fermented fish) demonstrated strong antibacterial activity against diarrhea-causing pathogens through membrane disruption mechanisms, as evidenced by increased DNA and protein leakage at 260 and 280 nm absorbance, respectively (Nasri et al., 2021). Similarly, LAB isolates from fermented Ethiopian dairy products exhibited broad-spectrum antibacterial activity with inhibition zones ranging from 5 to 16 mm and minimum inhibitory concentrations (MIC) between 0.10–0.30 μg/μL. These effects were attributed to the production of metabolites typical of LAB species such as *Lactobacillus plantarum, L. rhamnosus*, and *Leuconostoc mesenteroides*, which secrete bacteriocins and acids capable of inhibiting both gram-positive and gram-negative pathogens (Girma & Aemiro, 2021).

The current findings on LAB isolates from Toraja-origin black rice and its husk are in line with these observations, suggesting that plant- or cereal-based substrates can yield LAB with potent antibacterial capabilities similar to those found in animal-based fermented foods. However, the absence of antifungal activity against *C. albicans* in this study may reflect the specificity of secondary metabolite production or the inherent tolerance of fungi to acidic environments.

Overall, the demonstrated antibacterial properties highlight the potential of these isolates as natural antimicrobial agents or probiotic candidates for food preservation. Nonetheless, as the present study was limited to in vitro assessments, further investigation using food matrix systems or in vivo models is required to validate the practical effectiveness and stability of the antibacterial activity under real conditions.

Molecular characterization confirmed the success of DNA extraction and 16S rRNA gene amplification. Clear bands observed in electrophoresis indicated that the extraction procedure effectively yielded pure DNA suitable for PCR. The successful amplification validates the molecular workflow and ensures the reliability of downstream sequencing for taxonomic identification. Similar studies employing comparable extraction methods have reported consistent results in LAB identification from cereal-based sources. However, the present study utilized only partial 16S rRNA sequencing; therefore, full-length sequencing or multilocus sequence analysis would provide greater taxonomic resolution.

Sequence analysis using the BLAST algorithm revealed that isolate BL shared 98.72% similarity with *L. taiwanensis*, while isolates SB and ST exhibited approximately 98.6%–98.7% similarity with *S. hominis*. The successful identification of *L. taiwanensis* from Toraja black rice indicates that this substrate provides a favorable environment for LAB growth. LABs are well known for their acid tolerance, metabolic flexibility, and antimicrobial potential. Although unexpected, the presence of *Staphylococcus hominis* in the husk samples suggests possible environmental adaptation or co-occurrence with LAB in natural matrices where microbial interactions are shaped by complex ecological factors.

The nutrient composition of this substrate, which is rich in carbohydrates, proteins, and phenolic compounds that selectively favor LAB proliferation, supports the growth of *L. taiwanensis* in black rice. Previous studies have demonstrated that the natural niches for LAB colonization are cereal-based fermentations, including rice and grains. For example, a study on fermented rice water reported that Lactococcus taiwanensis was among the dominant probiotic species thriving under simple overnight fermentation, producing postbiotics that promoted colonocyte health and intestinal barrier integrity (Anbalagan et al., 2024). These findings confirm that rice and its derivatives are not only cultural staples but also biologically supportive environments for LAB development, aligning with the ecological pattern observed in Toraja black rice.

The isolation of *L. taiwanensis* from black rice in this study is consistent with the findings of a grain-based research in South Korea, where L. taiwanensis was one of several LAB species identified via 16S rRNA sequencing, including *Lactiplantibacillus plantarum, Levilactobacillus brevis*, and *Pediococcus pentosaceus*. These strains demonstrated acid and bile tolerance, intestinal epithelial cell adhesion, and anti-adipogenic effects, thereby emphasizing their probiotic potential (Seo et al., 2023). The *L. taiwanensis* strain identified from Toraja black rice may share similar physiological characteristics, suggesting that region-specific cereal ecosystems could yield distinct yet functionally convergent LAB communities. Meanwhile, the detection of *S. hominis* from husk samples diverges from most LAB-based studies but is not entirely unprecedented; some S. hominis isolates from food and environmental sources have shown antimicrobial activity, implying a potential ecological interplay between LAB and Staphylococcus species in natural substrates.

The presence of *L. taiwanensis* in Toraja black rice supports the concept that traditional and region-specific food materials can serve as functionally promising LAB reservoirs. Given the known bioactivities of *L. taiwanensis* and its relatives—including antioxidant, antimicrobial, and anti-adipogenic effects—these isolates may represent valuable local resources for probiotic or postbiotic development. The findings reinforce the notion proposed in studies on fermented rice water, which demonstrated that regionally adapted probiotic species and their metabolites contribute to host health through gut–organ axis modulation. Thus, the black rice ecosystem could provide new LAB strains suitable for functional food innovation and community-based microbial resource development.

Nevertheless, the molecular identification results in this study should be interpreted with caution. Similarity levels of 99% suggest tentative identification, requiring further confirmation through whole-genome sequencing, multilocus sequence typing, or comprehensive biochemical assays. In addition, the use of general MRS agar may have limited the recovery of more fastidious or nutritionally specialized LAB, potentially underestimating microbial diversity. Environmental variables—such as pH, humidity, and sampling conditions—could also influence the presence of *S. hominis* as a secondary contaminant or cohabiting species. Therefore, future research should employ culture-independent approaches (e.g., metagenomic sequencing) and metabolomic profiling to elucidate the full microbial diversity and functional capacity of LAB from Toraja black rice and its husk.

CONCLUSION

This study successfully isolated and identified three bacterial strains, including Lactococcus taiwanensis and Staphylococcus hominis, from Toraja-origin black rice and its husk, demonstrating that these substrates harbor diverse microbial communities with potential functional value. The detection of L. taiwanensis confirms that black rice provides a favorable niche for lactic acid bacteria known for their acid tolerance and antimicrobial potential, consistent with previous findings from grain- and rice-based fermentation. The antibacterial activities observed in all isolates further highlight their potential application as natural antimicrobial or probiotic candidates. Although the molecular identification achieved up to 98.7% similarity suggests strong species-level proximity, comprehensive genomic and biochemical validation remains necessary to confirm taxonomic identity and functional properties. In the future, these findings may contribute to the development of Indonesia's indigenous probiotic formulations derived from local agricultural resources, thereby supporting food security and functional food innovation. Further exploration of their probiotic efficacy, safety, and stability in food matrices is recommended to realize their potential application in the local functional food industry.

ACKNOWLEDGMENT

This research was funded by Kementerian Pendidikan, Kebudayaan, Riset dan Teknologi Indonesia, Direktorat Jenderal Pendidikan Tinggi, Riset dan Teknologi through a Novice Lecturer Research Grant 2024. The authors would like to thank the Kementerian Pendidikan, Kebudayaan, Riset dan Teknologi Indonesia, LLDIKTI Wilayah IX Indonesia, and Lembaga Penelitian dan Pengabdian Kepada Masyarakat Universitas Almarisah Madani Indonesia for providing support.

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