

CHARACTERIZATION OF LACTIC ACID BACTERIA AND ANTIMICROBIAL ACTIVITY IN SUI WU'U FROM BAJAWA DISTRICT, NUSA TENGGARA TIMUR, INDONESIA

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ABSTRACT

Objective: This research aimed to isolate, determine the characteristics of lactic acid bacteria (LAB) of Sui Wu'u from Bajawa, Nusa Tenggara Timur and identify LAB using 16S rRNA potential as antimicrobial activity against pathogenic bacteria.

Methods: Sui Wu'u which has been stored for 6 months was obtained from Bajawa district, inoculated on de Man Rogosa-Sharpe Agar (Merck) + 0.5% CaCO₃, purification of LAB, characterization of selected isolates, biochemical test, tolerance test for pH, viability to test temperature, and content NaCl, determination of antimicrobial action by the agar well disk diffusion method using antibiotic (Amoxicillin) as a control and as indicator bacteria (*Staphylococcus aureus* and *Escherichia coli*) and isolation of genomic 16S rRNA; molecular identification.

Results: Based on research results obtained five isolates of LAB, Gram staining the LAB isolated from Sui Wu'u showed that the isolated bacteria (bacilli and coccus) are Gram-positive, catalase-negative and the isolates have tolerance of viability at temperatures of 10°C, 45°C, and 50°C and to salinitas of 4% and 6.5%. The inhibitory zone LAB isolates (2PKT) against *E. coli* bacteria (20 mm) and *S. aureus* (12 mm), and (2PKB) against *E. coli* bacteria (17 mm) and *S. aureus* (10 mm). The two selected isolates were identified as *Lactobacillus fermentum* strain HB bacteria with 100% identification value and 98.93% query cover and *L. fermentum* strain HT with 100% identification value and 99.23% query cover.

Conclusion: *L. fermentum* from Sui Wu'u has antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*.

Keywords: Antimicrobial, Fermented pork, Lactic acid bacteria, Sui Wu'u.

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INTRODUCTION

Sui Wu'u is a traditional food from Flores, East Nusa Tenggara, which is spontaneously fermented pork well known in Bajawa. Sui Wu'u is a form of the skill of the ancients to preserve pork. Preservation of pork is done by mixing cornstarch and salt in bamboo (Tuku). Ideally eaten after being stored for 6 months, the length of time it stores can affect the taste and the longer the time it stores the tastes better but does not damage the texture of the meat.

Fermented foods have the potential to be developed as functional foods. Functional food is now important for the human body due to its benefits in the health field with the content of compounds contained in it [1]. Fermented food is a food product that involves microorganisms in the manufacturing process. The fermentation process was originally a technique conducted for the preservation of food products, but the development of technology in the field of fermentation has enabled humans to produce various products that cannot be synthesized in the body and are difficult to produce through chemical processes [2].

Several *in vitro* studies have been conducted to lactic acid bacteria (LAB), *Lactobacillus fermentum* [3], *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus salivarius*, *Lactobacillus futsaii* [4], *Pediococcus pentosaceus*, and *Pediococcus acidilactici* [5] are LAB isolated from various traditional Indonesian fermented foods. *Lactobacillus* is one of the most important genera of LAB [5,6]. These organisms are also known to produce various compounds such as bacteriocin which can antagonize the growth of some pathogenic bacteria in foods [7,8]. LAB are regarded as a major group of probiotic bacteria and have been used successfully to treat acute infantile diarrhea and various diarrheal illnesses [9,10].

The LAB are conventionally used to improve immune system also used in pharmaceutical as an alternative of antibiotic [11], antimicrobial [12], anticancer [13], antidiabetic [14], anthelmintic [15], immunomodulatory [16], lactose intolerance, as well as bio preservatives in food [16,17], and improvement of gut microflora or to manage gut-related problems [18]. One product that can be produced by fermentation is an antibacterial compound. One of Indonesia's fermented foods whose manufacturing processes involve LAB is Sui Wu'u. This study aimed to investigate the antimicrobial activity LAB isolated from fermented food (Sui Wu'u) from Bajawa, Flores, Nusa Tenggara Timur has yet to be studied.

METHODS

Sample and materials

Sui Wu'u has been fermented for 6 months and was obtained from Bajawa, Flores, East Nusa Tenggara, Indonesia. The indicator bacteria *Staphylococcus aureus* and *Escherichia coli* were kindly supplied from the Department of Biology, Faculty of Science and Mathematics, Universitas Diponegoro.

LAB isolation and purification

About 1 g of each Sui Wu'u sample was mixed with 9 ml of sterile NaCl 0.85% [19]. An appropriate dilution (10^{-6} - 10^{-7}) was made and inoculated on de Man Rogosa-Sharpe (MRS) Agar (Merck) + 0.5% CaCO₃ [20] medium by spread plate and incubated at 37°C for 48 h [19]. Colonies with the surrounding clear zone were randomly selected on each plate. A single colony was then transferred by an ose needle to MRS agar to isolate the colony by the streak method and incubated for 24 h at 37°C.

Isolate characterization

Isolates were examined by Gram staining and catalase reaction tests and the cell shape was evaluated microscopically. Gas production from

glucose was tested using a Durham tube and MRS broth to determine the fermentation type. Then, the isolates will be tested for tolerance to pH, viability to temperature, and NaCl. Phenotype characterization was based on Bergey's Manual of Determinative Bacteriology [21]. Isolates were stored in medium MRS agar at 4°C and were for further analyzes [21].

Preparation of bacterial suspension test (*E. coli* and *S. aureus*)

The growing stock of bacterial cultures of *E. coli* and *S. aureus* was taken with sterile ose wires and then suspended in a test tube containing 9 ml of sterile distilled water to obtain the turbidity of the bacterial suspension equal to the turbidity of the standard solution McFarland 0.5. Turbidity standard is intended to replace bacterial calculations one at a time and to estimate cell density to be used in antimicrobial testing procedures, which means the concentration of bacterial suspension is 10^8 CFU/mL. Preparation of standard solution by McFarland preparation 9% H_2SO_4 solution of 9.95 mL was mixed with 1% $BaCl_2$ solution of 0.05 mL in a test tube. Then, shaker until homogen [22].

Preparation of LAB suspension

LAB isolates from the selection taken 1 ose were grown on MRS agar medium, incubated at 37°C for 24 h. Then taken with a sterile ose wire and then suspended in a test tube containing 9 mL of sterile distilled water to obtain the turbidity suspension of bacteria equal to the turbidity of the standard solution McFarland 0.5 [23].

Antimicrobial assay

The testing of antibacterial activity by LAB from the Sui Wu'u fermented food on the growth of pathogenic bacteria was conducted by the disk diffusion method (Kirby-Bauer) using the smear technique and amoxicillin 100 µg/mL as a positive control [24-26]. Pathogenic bacteria were etched on the surface of MRS media with sterile cotton buds left for ± 5 min. Sterile disc paper is soaked in each vial bottle containing a LAB suspension, soaking is done for ± 30 min. Disc paper is placed on the media using tweezers according to the pattern of Hudzicki [27]. The size of the inhibition zone indicating antibacterial activity of the isolate was measured after 24 h.

Molecular identification

DNA isolation was conducted using the Chelex method [27]. Identification of LAB by 16S rRNA was done using 27F (5'TACGGYTACCTTGGTACGACTT-3') and 1492R (5'-AGAGTTTGGATCCTGGCTCAG-3'). Amplification was conducted using a thermal cycler chain reaction polymerase chain reaction (PCR). The PCR conditions were as follows: Initial denaturation at 95°C for 1 min, followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 15 s, and a final extension at 72°C for 4 min. The PCR products were analyzed on 1.0% (w/v) agarose gel electrophoresis (Mupid-exU submarine electrophoresis system, Advance) in ×1 tris-acetate-EDTA buffer at 100 V for 30 min. It was visualized on a gel documentation system (Biodoc Analyze, Biometra, USA). Purified PCR products were sequenced with 16S rRNA primers. Sequences of the whole gene fragment were used for similarity search against NCBI GenBank database using the Basic Local Alignment Search Tool (BLAST) program available at website <https://blast.ncbi.nlm.nih.gov/Blast.cgi>. The phylogenetic tree of LAB was constructed with MEGA 10 software (Proprietary Freeware, Pennsylvania State University) and neighbor-joining methods were performed to test confidence with bootstrap data set of 1000 times [28].

RESULTS

Isolation and characterization of LAB

LAB were isolated by MRS agar medium with the addition of 0.5% $CaCO_3$ by dilution method. Isolates were selected that there is a clear zone around the colonies. Isolates that form a single colony and have a clear zone are inoculated on the MRS agar medium to obtain a pure single colony. Reinoculation was conducted 5 times until pure bacteria culture was discovered. The isolation results obtained were five

isolates, namely, 2PKB, 2PKT, 2KH, 2ST, and 3SP. Colony morphology obtained by rounded shape, punctiform, flat, and convex surface and milky white (Table 1).

Five isolates that characteristic morphological cell categorized as Gram-positive bacteria. The Gram stain results revealed that the LAB isolated of Sui Wu'u from Bajawa was rods (bacilli and coccus) (Fig. 1).

Morphological characterization consists of Gram staining, observation of cell shape, and formation of endospores. The physiological test performed is the motility test. Biochemical tests are catalase tests. Based on Bergey's manual of determinative bacteriology [21], LAB have characteristics of Gram positive, non-porous, negative catalase, and non-motile. The results of the identification test of LAB can be seen in Table 2.

All isolates obtained were Gram positive, rod shaped, coccus, non-spore-forming, gave a negative reaction to the catalase test, and non-motile. In this study, LAB isolates Sui Wu'u was able to survive at pH 4.4, tolerance to NaCl concentration and can grow well at various temperatures.

Antimicrobial assay

Based on the results of research conducted by LAB from Sui Wu'u can be antagonistic to pathogenic bacteria. Two isolates showed a zone of inhibition against pathogenic bacteria, namely, 2PKB and 2PKT (Fig. 2).

Inhibition zone diameter against pathogenic bacteria *E. coli* (2PKB: 17 mm) (2PKT: 20 mm) and *S. aureus* (2PKB: 10 mm) (2PKT: 12 mm). Amoxicillin antibiotic inhibition zones against *E. coli* (40 mm) and *S. aureus* (20 mm) (Table 3). The antimicrobial activity of LAB competes favorably with standard antibiotics used as control. Isolate LAB Sui Wu'u showed the highest zone of inhibition for *E. coli* while the antimicrobial activity decreased with time.

Molecular identify

LAB isolates were selected to identify 16S rRNA gene sequences and be analyzed phylogenetically (Fig. 3). The 16S rRNA encoding gene can be

Table 1: Colony morphology and cell morphology lactic acid bacteria

| Isolate | Colony morphology | Cell morphology |
|---------|--------------------------------|-----------------|
| 2KH | Punctiform creamy white convex | Coccus |
| 2ST | Punctiform creamy white flat | Coccus |
| 2PKB | Circular creamy white flat | Bacilli |
| 2PKT | Circular creamy white convex | Bacilli |
| 3SP | Circular creamy white convex | Bacilli |

Table 2: Characteristic of Isolate LAB Sui Wu'u

| Variables | Isolate code | | | | |
|-------------------|--------------|-----|------|------|-----|
| | 2KH | 2ST | 2PKB | 2PKT | 3SP |
| Cell morphology | | | | | |
| Motility | - | - | - | - | - |
| Biochemical | | | | | |
| Catalase | - | - | - | - | - |
| Endospore | - | - | - | - | - |
| Fermentation type | Ho | Ho | Ho | Ho | Ho |
| Physiological | | | | | |
| NaCl 4% | + | + | + | + | + |
| NaCl 6.5% | + | + | + | + | + |
| Temp. 10°C | - | - | - | - | - |
| Temp. 45°C | + | + | + | + | + |
| Temp. 50°C | + | + | + | + | + |
| pH 4.4 | + | + | + | + | + |
| pH 9.6 | - | - | - | - | - |

(+): Positive/tolerance, (-): Negative/intolerance/nonmotile, Ho: Homofermentatif

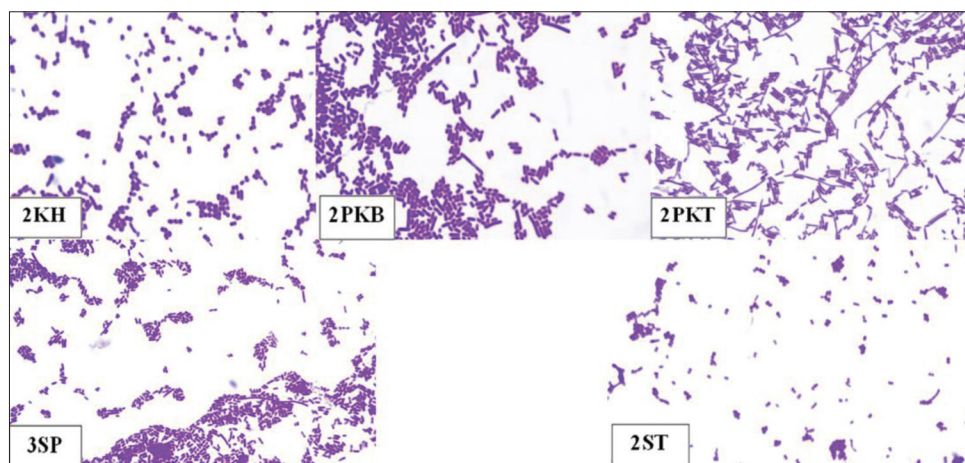


Fig. 1: Gram staining lactic acid bacteria isolated of Sui Wu'u

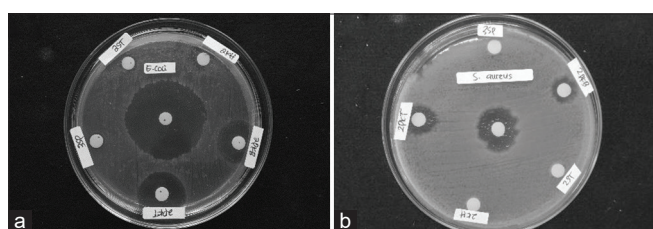


Fig. 2: Zones of inhibition lactic acid bacteria against *Escherichia coli* and *Staphylococcus aureus*. *(a) *E. coli*, (b) *S. aureus*

Table 3: Clear zone diameter indicating antimicrobial activity

| Lactic acid bacteria | Inhibition zone (mm) | |
|----------------------|------------------------------|-------------------------|
| | <i>Staphylococcus aureus</i> | <i>Escherichia coli</i> |
| 2KH | - | - |
| 2ST | - | - |
| 2PKB | 10 | 17 |
| 2PKT | 12 | 20 |
| 3SP | - | - |
| Antibiotics | 20 | 40 |

used to determine taxonomies, phylogeny (evolutionary relationships), and estimate bacterial rates of species divergence [29]. The results of the electrophoresis of pure Polymerase Chain Reaction CR products (Fig. 4).

DISCUSSION

Isolation and characterization of LAB

Based on research conducted, obtained five isolates from the results of the isolation of LAB from the source of Sui Wu'u. Differences of isolates were seen based on morphology of isolate colonies consisting of shape, edges, elevation, color, and diameter of the isolates. Colonies obtained from isolation in the form of circular and punctiform. The edge of the colony is entire or slippery. Convex colony elevation, flat colony elevation is only owned by 2PKB and 2ST. The color of the colony is milky white and the diameter of the colony is in the range of 0.2–0.5 cm. Differences in each colony isolate based on morphological characters can be seen in Table 1.

LAB have the characteristics of Gram positive, non-spore, negative catalase, and nonmotile. The results of the identification test of LAB can be seen in Table 2. All isolates obtained were Gram positive, rod shaped, and non-spore-forming, gave a negative reaction in the catalase test, and non-motile. Based on the results of Gram staining, the results obtained are that all isolates have a positive gram character. According to Hardy [30], LAB are Gram-positive bacteria because they do not undergo decolorization and still bind to the violet crystal violet color at

the final stage of staining. Based on the results of Gram staining can be observed also the form of the bacterial cell. The shape of the bacterial cell is based on observations of stem and round.

Catalase test results, all isolates have non-catalase characteristics or give negative results on catalase testing. According to Alfonzo [31], LAB are negative catalase bacteria because they do not produce catalase enzymes that can break down hydrogen peroxide. Siti [32] added that LAB are generally microaerophilic to obligate anaerobic, which means that if there is O_2 during growth, it will be toxic and can inhibit the growth of LAB.

The endospora staining results, in all the isolates observed, were nonspore characteristics or did not produce spores. According to Axelsson [33], LAB are bacteria that do not form spores, so when an endospore is stained, vegetative cells appear to produce a pink color at the end of the staining stage. Fardiaz [34] added, in staining endospores, endospores will be seen on a microscope in the form of green dots.

Based on the results of the motility test, all isolates were nonmotile or immobile. Cell movement is observed visually by inoculating on semi-solid upright media. According to Fardiaz [34], LAB have immovable properties. Characterization based on the phenotypic characteristics included isolate growth test at different pH, temperature, and salinity, as well as observations on isolate type fermentation. Phenotype characteristics can also be performed to determine the genera of the isolates observed.

The observation of the characterization of selected isolates is shown in Table 2. The selected isolates were observed growth in the different salt concentrations, conducted by growing isolates in the variation of the NaCl concentration of 4% and 6.5%. Based on the results of testing, the growth of isolates at different salt concentrations, isolates can grow salinitas concentrations of 4% and 6.5%. According to Axelsson [33], LAB were able to grow at salinitas concentration of 3–7% if they are rod shaped; they belong to the *Lactobacillus* genera. Selected bacteria growth observed at different pH conditions have variations in pH 4.4 and 9.6, respectively, represented for acid and alkaline pH based on test results isolates growth at different pH showed that isolates were able to grow at pH 4.4 and unable to grow at pH 9.6.

According to Axelsson [33], LAB were able to grow at pH 4.4 and cannot to grow at pH 9.6 if it is in the form of a stem, then it belongs to the *Lactobacillus* genera. Selected isolates were observed to grow at different temperatures, conducted by growing isolates at 10°C and 45°C. Based on the results of testing, the growth of isolates at different temperatures, it was found that the isolates were able to grow at temperatures of 10°C and 45°C. According to Axelsson [33], LAB were able to grow at temperatures of 10°C and 45°C when they are rod shaped, are included in the *Lactobacillus* genera.

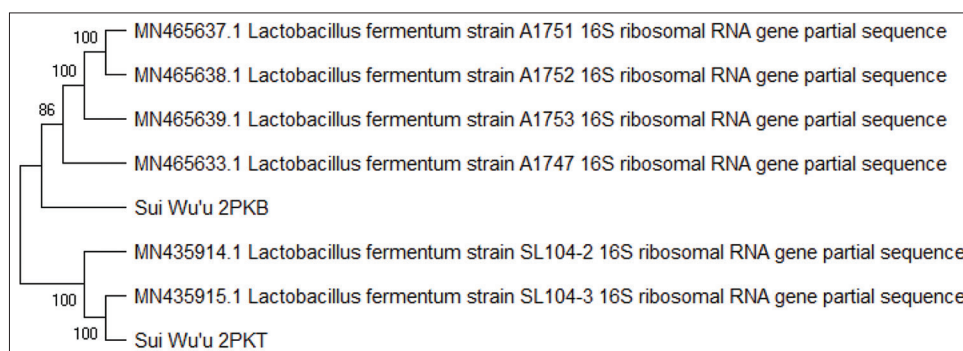


Fig. 3: Neighbor-joining tree of LAB Sui Wu'u showing phylogenetic relationship by MEGA 10

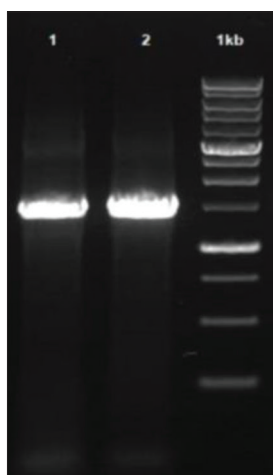


Fig. 4: Polymerase chain reactions product lactic acid bacteria isolates in 1% agarose gel. *1=2PKB, 2= 2PKT, 1kb= DNA marker

Antimicrobial assay

To select and obtain the most potent LAB isolates of to suppress the growth of pathogenic bacteria such as *S. aureus* and *E. coli*, the isolates were screened by using the disk diffusion well method. This study uses positive controls, such as antibiotics, because the tests were performed to determine the ability of the isolates to inhibit the growth of pathogenic bacteria.

The test results showed a zone of inhibition marked by the emergence of a clear zone around the selected LAB isolate colonies after being incubated for 24–48 h, both in *S. aureus* and *E. coli* cultures (Fig. 2). Inhibition zone diameters formed by selected LAB isolates against *S. aureus* and *E. coli* vary the results of the measurement of clear zone diameters can be seen in Table 3. The highest inhibition of *S. aureus* was shown by 2PKT isolates which were 12 mm, while the lowest measured inhibition shown by 2PKB isolates which is 10 mm. The highest inhibition of *E. coli* was shown by 2PKT isolate that was equal to 20 mm, while the lowest inhibition was shown by 2PKB isolate which was 17 mm.

Clear zone of inhibition was thought to be caused by antimicrobial activity produced by the LAB isolates. The inhibition shown by the LAB isolates was thought to be caused by changes in pH due to the production of organic acids during fermentation. As reported by Lee *et al.* [35], the ability of LAB to inhibit the growth of pathogenic bacteria is shown by the wide clear zone produced during antimicrobial testing and is related to the ability of LAB to produce secondary metabolites such as lactic acid, acetic acid, and bacteriocin. The main inhibitory effect is speculated on the main metabolic pathway of LAB, which means the fermentation pathway. LAB use fermentation pathways to produce cellular energy and produce organic acids such as lactic acid as they grow [19].

Lactic acid produced by LAB isolates diffuses into the growing media of test bacteria so that it can interfere with the integrity of the pathogenic bacterial cell membranes. Damage to the cell membrane causes the nutrients needed by the test bacteria to grow cannot be absorbed so that the metabolic process does not work as it should and causes its growth to be inhibited. Lactic acid is one of the inhibitor compounds produced by LAB and is the main end product of carbohydrate catabolism because from the process of converting this carbon source produced at least 50% lactic acid, so this group of bacteria is called LAB [36].

Acid produced during the metabolic process by LAB will cause a decrease in pH and cause pathogenic microbes and food destroyers that generally cannot stand the acidic atmosphere will be inhibited [37]. Accumulation of acidic end products results in a decrease in pH and will inhibit the growth of Gram-positive and Gram-negative bacteria. The activity of lipophilic acids such as lactic acid in an undissociated form can penetrate microbial cells, and at higher intracellular pH, dissociates to produce hydrogen ions, and interferes with the function of essential metabolites, translocation of substrate, and oxidative phosphorylation, thereby reducing intracellular pH. The difference in LAB antimicrobial activity against several test microbes is based on differences in the structure of the test microbial cell walls, different concentrations of antimicrobial compounds can also produce different inhibitory zones [28].

Molecular identify

Genomic isolation and 16S rRNA reaction of LAB: The electrophoresis results in Fig. 4 indicate that the 16S rRNA gene region of the fermented pork (Sui Wu'u) of Bajawa was successfully amplified. Successful amplification of the 16S rRNA gene was indicated by the appearance of a 1.5 kb PCR product, which was the expected fragment size when using the 27F forward primer AGAGTTTGATCCTGGCTGAG with the reverse primer 1492 R GTTACCTTACGACTT. A phylogenetic tree based on 16S rRNA gene sequence analysis is shown in Fig. 4.

Based on the BLAST search result, the bacterial isolate 2PKB and 2PKT have confirmed *L. fermentum* strain HB bacteria with 100% identification value and 98.93% query cover and *L. fermentum* strain HT with 100% identification value and 99.23% query cover. The phylogenetic tree shows that the nearest distant neighbor is the *L. fermentum* strain HB and HT. This indicates that the LAB isolated from Sui Wu'u were *L. fermentum* strain HB and HT. Hagstrom *et al.* [38] suggested that isolates with a 16S rRNA sequence similarity over 97% may represent the same species, while sequence similarity between 93 and 97% indicates the same genus but different species.

Based on previous research Bao *et al.* [39] suggested that cell-free supernatant from *L. fermentum* can significantly inhibit the growth of Gram-positive bacteria (*Listeria monocytogenes* C53-3, *S. aureus* AC1.2456) and Gram-negative bacteria (*E. coli* O157 882365, *Shigella flexneri* CMCC (B) 51592, *Salmonella typhimurium* S50333). In pozol (corn) fermentation, 40% of LAB are found that have amyolytic ability. *L. fermentum* UN01 is able to produce bacteriocin with the highest activity

at 37°C and pH 2.0 [17]. *L. fermentum* SBS001 isolated from seawater showed inhibitory activity against ten pathogenic bacteria, namely, *S. aureus* (12 mm), *Pseudomonas aeruginosa* (12 mm), *Salmonella* Typhi (10 mm), *Salmonella paratyphi* (8 mm), *Klebsiella oxytoca*, (8 mm) *E. coli* (8 mm), *Lactobacillus bulgaricus* (8 mm), *Vibrio cholerae* (8 mm), *Proteus mirabilis* (7 mm), and *Klebsiella pneumoniae* (7 mm) [40]. *L. fermentum* and *L. plantarum* isolates in Dangke [21], *L. fermentum* B111K from cow's milk can produce bacteriocin and are antagonistic [41].

CONCLUSION

Based on the results of the study, five LAB were successfully isolated, have tolerance of viability at temperatures, NaCl concentration, and homofermentative. Categorized as Gram-positive bacteria and in the form of coccus, rod. Are antagonistic because they can inhibit the growth of Gram negative and positive pathogenic bacteria. Identified based on the 16S rRNA gene is *L. fermentum*. Therefore, LAB from Sui Wu'u have the potential as a bio preservative is recommended to food processing industries to enhance the extension of shelf life of food products and reduction in food contamination which causes illness to human beings.

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AUTHORS' CONTRIBUTIONS

Rosalina Yuliana Ayen designed, conducted, and wrote up the search and Endang Kusdiyantini and Sri Pujiyanto provided guidance and helped with manuscript revision.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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