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LACTIC ACID BACTERIA FROM TOFU WASTEWATER AGAINST *Staphylococcus aureus*

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Abstract

Background: *Staphylococcus aureus* is a pathogenic bacterium that causes skin infections and has been reported to develop resistance to antibiotics. Lactic acid bacteria, which can be isolated from tofu wastewater, have the potential to serve as natural antimicrobial agents through the production of bacteriocins, organic acids, hydrogen peroxide, and carbon dioxide.
Objective: To evaluate the antibacterial activity of lactic acid bacteria isolates from tofu wastewater against *Staphylococcus aureus*.
Methods: This descriptive analytic study used purposive sampling. Tofu wastewater samples were collected from 20 tofu factories in Klungkung District. Lactic acid bacteria were isolated on de Man, Rogosa, and Sharpe media, identified through catalase test, Gram staining, and fermentation type test, and tested for antibacterial activity against *Staphylococcus aureus* using the well diffusion method.
Results: A total of 22 isolates of lactic acid bacteria were obtained, characterized as catalase-negative, Gram-positive, with bacilli and cocci morphology, and homofermentative properties. All isolates exhibited antibacterial activity against *Staphylococcus aureus* with an average inhibition zone diameter of 8.97 mm, categorized as moderate to strong.
Conclusions: Lactic acid bacteria isolated from tofu wastewater demonstrated antibacterial activity against *Staphylococcus aureus* and have potential as natural antimicrobial agents.

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INTRODUCTION

Infectious diseases remain a major public health problem in developing countries, including Indonesia. One of the most common types is skin infection, often caused by *Staphylococcus aureus*, a bacterium that normally exists as part of the body's flora but can become pathogenic when the immune system is compromised (1,2). Treatment of skin infections generally relies on antibiotics, yet irrational and prolonged use may contribute to antibiotic resistance.

Some strains of *Staphylococcus aureus* are resistant to isoxazolyl penicillin antibiotics such as methicillin, flucloxacillin, and oxacillin, and are known as Methicillin-Resistant *Staphylococcus aureus* (MRSA). In addition, this bacterium can produce defense molecules that help it evade the host immune response (3). These resistance issues emphasize the need for alternative therapeutic agents, including natural antimicrobials such as lactic acid bacteria (4).

Lactic acid bacteria are known to ferment carbohydrates and produce antimicrobial compounds including lactic acid, bacteriocins, hydrogen peroxide, diacetyl, reuterin, and other organic substances (5). Previous studies have reported that LAB isolated from fermented foods, dairy products, and various agro-industrial by-products exhibit antibacterial activity against pathogenic microorganisms, including *Staphylococcus aureus* (6,7). Furthermore, nutrient-rich industrial waste substrates have been shown to support LAB growth and enhance the production of antimicrobial metabolites. These findings suggest that agro-industrial waste may serve not only as a growth medium but also as a valuable source of beneficial microorganisms with antibacterial potential.

Tofu wastewater, particularly the whey produced during tofu manufacturing, contains abundant organic matter, including approximately 40%–60% protein, carbohydrates, vitamins, and minerals, making it a favorable substrate for LAB growth (8). Compared with LAB isolated from fermented foods or dairy products, LAB originating from tofu wastewater inhabit a unique ecological environment characterized by high concentrations of soybean-derived nutrients and distinct microbial interactions. These conditions may influence the diversity of indigenous LAB and their capacity to produce antimicrobial compounds, potentially resulting in different antibacterial activities (9). This study differs from previous reports by focusing on indigenous LAB isolated from tofu wastewater generated by local tofu industries in Klungkung District, where environmental conditions and production practices may influence microbial diversity and antibacterial activity.

Although several studies have reported the isolation and antibacterial activity of LAB from fermented foods and other agro-industrial wastes, studies focusing specifically on LAB isolated from tofu wastewater remain limited. Furthermore, no previous study has evaluated the antibacterial activity of indigenous LAB isolated from tofu wastewater generated by tofu industries in Klungkung District, Bali. Considering that environmental conditions, raw materials, and production practices may affect the characteristics of indigenous microbial communities, data obtained from other geographical regions cannot necessarily be generalized to this area.

Therefore, the novelty of this study lies in the isolation and phenotypic characterization of indigenous LAB from tofu wastewater generated by tofu industries in Klungkung District, Bali, followed by the evaluation of their antibacterial activity against *Staphylococcus aureus*. This study provides new regional data regarding the antibacterial potential of tofu wastewater-derived LAB and contributes to the exploration of agro-industrial waste as a sustainable source of natural antimicrobial microorganisms while supporting future development of alternative antimicrobial agents.

MATERIALS AND METHODS

This study employed a laboratory-based descriptive design to examine the isolation, phenotypic characterization, and antibacterial activity of lactic acid bacteria (LAB) obtained from tofu wastewater. The study was conducted from October 2024 to April 2025. Samples were collected by purposive sampling from four tofu factories in Klungkung District, which were selected based on active production status, accessibility, and willingness to participate in the study. Each factory contributed independent samples of post-coagulation tofu wastewater. The inclusion criterion was wastewater that appeared whitish-yellow and turbid, while samples showing changes in colour or texture after collection were excluded. A total of 20 samples were collected, consisting of five independent samples from each factory. All samples were aseptically transferred into sterile containers, transported in cool boxes, and processed immediately in the laboratory. Lactic acid bacteria were isolated using standard microbiological methods, and presumptive isolates were selected based on colony morphology, Gram staining, and catalase test results, where Gram-positive and catalase-negative isolates were considered potential LAB. The antibacterial activity of LAB isolates against *Staphylococcus aureus* was evaluated using the agar well diffusion method, and each assay was performed in triplicate (n = 3). The inhibition zone diameter was measured in millimeters using a caliper, and the mean value was calculated by averaging the results of three replicates. The results are presented as mean ± standard deviation (SD). No inferential statistical analysis was performed, as this study focused on descriptive evaluation of antibacterial activity, and the activity of isolates was categorized based on the magnitude of inhibition zones.

The instruments used included sterile containers (Biologix, China), analytical balance (ACIS, China), Erlenmeyer flasks (Iwaki, Japan), graduated cylinders (Iwaki, Japan), hotplate stirrer (JISICO, South Korea), autoclave (Tomy Sx-500, Japan), test tubes (Iwaki, Japan), Petri dishes, pipettes, biosafety cabinet (Biobase, China), anaerobic jar, inoculating loop, Bunsen burner, microscope (Leica, Germany), Durham tubes, vortex, McFarland densitometer (Biosan, Latvia), micropipettes with tips (Socorex, Swiss), incubator (Esco, Singapore), vernier caliper (Kenmaster, Indonesia), and other supporting tools. Materials consisted of tofu wastewater, De Man Rogosa and Sharpe (MRS) Broth and Agar (Merck, Germany), sterile distilled water, *Staphylococcus aureus* ATCC 29213, Mueller Hinton Agar (MHA) (Oxoid, United Kingdom), Gram staining reagents (Merck, Germany), immersion oil (Merck, Germany), 3% hydrogen peroxide (Merck, Germany), 0.9% NaCl solution (Merck, Germany), gentamicin as the positive control (Merck, Germany), and sterile MRS Broth as the negative control. Culture media were prepared according to standard microbiological procedures: MRS Broth (26.1 g/500 mL; Merck, Germany), MRS Agar (34.1 g/500 mL; Merck, Germany), and MHA (19 g/500 mL; Merck, Germany) were dissolved in

distilled water, homogenized, sterilized at 121 °C for 15 minutes using an autoclave, and aseptically poured into sterile tubes or Petri dishes.

For isolation, 1 mL of tofu wastewater was inoculated into 9 mL MRS Broth and incubated anaerobically at 37 °C for 48 hours. Serial dilutions (10^{-1}–10^{-5}) were prepared using sterile 0.9% NaCl, and 0.1 mL of the final dilution was streaked onto MRS Agar, followed by anaerobic incubation at 37 °C for 48 hours. Distinct colonies were purified by re-streaking and phenotypically characterized through colony morphology, catalase testing, Gram staining, and fermentation type testing using Durham tubes. Presumptive LAB were defined as catalase-negative, Gram-positive bacilli or cocci and further classified as homofermentative or heterofermentative based on gas production. No molecular identification or species-level confirmation was performed. Therefore, the isolates were reported as presumptive lactic acid bacteria based solely on phenotypic characteristics.

A suspension of *Staphylococcus aureus* ATCC 29213 was prepared by culturing pure colonies on Nutrient Agar for 24 hours at 37 °C. Fresh colonies were then suspended in 0.9% NaCl and adjusted to match the 0.5 McFarland standard using a densitometer. Antibacterial activity was evaluated using the agar well diffusion method on Mueller Hinton Agar (MHA) plates inoculated with the *Staphylococcus aureus* suspension. Wells with a diameter of 6 mm were prepared on the agar surface. Lactic acid bacteria cultures were grown in MRS Broth for 24 hours at 37 °C, and the whole LAB culture was used as the test sample to evaluate the overall antibacterial activity of LAB under conditions that resemble their natural state. Therefore, the inhibition zones observed in this study reflect the combined effects of viable bacterial cells and the antimicrobial metabolites produced during cultivation, rather than the activity of extracellular metabolites alone. A volume of 40 µL of LAB culture was added into each well. The LAB inoculum was standardized based on 24-hour cultivation under the same conditions for all isolates. Gentamicin (10 µg/mL, 40 µL per well) was used as the positive control, while sterile MRS Broth (40 µL) served as the negative control. Each test was performed in triplicate plates for each isolate.

After incubation at 37 °C for 24 hours, inhibition zones were measured using a vernier caliper. The diameter of the inhibition zone was measured in two perpendicular directions, and the mean value was recorded. The inhibition zone measurement included the well diameter. The antibacterial activity was categorized based on standard inhibition zone classification from previous literature sources commonly used for antimicrobial susceptibility testing. Ethical approval was not required for this study because it involved environmental samples (tofu wastewater) and a reference bacterial strain (*Staphylococcus aureus* ATCC 29213) without the involvement of human participants, animals, or identifiable personal data.

RESULTS AND DISCUSSIONS

Results

Characteristics of Tofu Wastewater

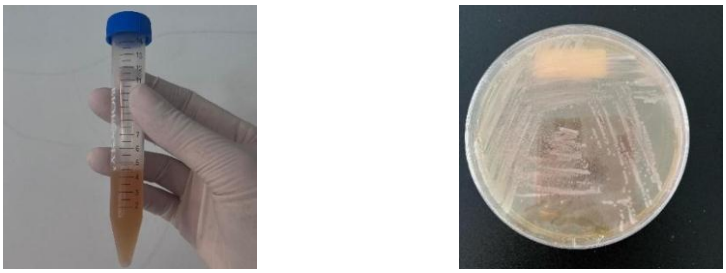
Tofu wastewater is a liquid byproduct of tofu production. In this study, samples were obtained from a tofu factory in Klungkung District, Klungkung Regency. The wastewater was characterized by a whitish-yellow and turbid appearance, slightly acidic odor, and moderately viscous texture, as observed during sample collection and isolation. It was

generally stored in open tanks or containers near the production site as temporary daily waste storage. Most wastewater was discharged into the environment without specific treatment, although some factories utilized it for biogas production.

Tofu wastewater generally contains organic compounds such as proteins, carbohydrates, and lipids (10), which provide a suitable medium for the growth of fermentative microorganisms, including lactic acid bacteria. In this study, lactic acid bacteria were successfully isolated from tofu wastewater and showed good growth on selective media, indicating that tofu wastewater naturally supports their proliferation

Figure 1. Tofu Wastewater Used As The Source Of Lactic Acid Bacteria Isolates Isolation of Lactic Acid Bacteria from Tofu Wastewater
Source: Authors' documentation.

Isolation of lactic acid bacteria was carried out on 20 tofu wastewater samples, all of which exhibited bacterial growth as indicated by the formation of colonies on MRS Agar medium. From the 20 positive samples, a total of 22 isolates of lactic acid bacteria were obtained, which were selected based on morphological differences in the colonies and subsequently subjected to further identification.



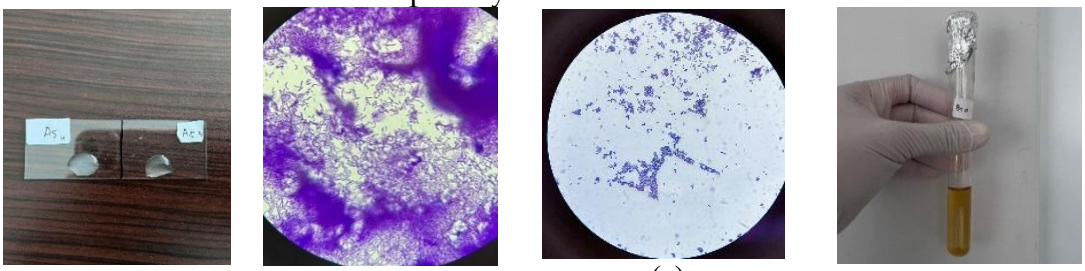
(a) (b)

Figure 2. (a) Lactic Acid Bacteria Culture in MRS Broth, (b) Lactic Acid Bacteria Colonies on MRS Agar
Source: Authors' documentation.

Identification of Lactic Acid Bacteria from Tofu Wastewater

Catalase testing showed that all 22 isolates were catalase-negative, as indicated by the absence of bubble formation upon exposure to 3% H_2O_2 solution. This result suggests that none of the isolates produced the catalase enzyme. Gram staining revealed that all isolates were Gram-positive, with 21 isolates exhibiting rod-shaped (bacillus) morphology and only one isolate showing a spherical (coccus) form. Fermentation tests indicated that all isolates were homofermentative, as evidenced by the absence of gas production in the Durham tubes, suggesting that the fermentation process produced only acid without gas formation.

Based on these three assays, a total of 22 isolates were confirmed as lactic acid bacteria. These isolates were presumed to belong to two genera, namely *Lactobacillus* and *Streptococcus*. The isolates were subsequently tested to determine their antibacterial activity.



(a) (b) (c) (d)

Figure 3. (a) Catalase-Negative Result, (b) Gram-Positive Bacillus, (c) Gram-Positive Coccus, (d) Homofermentative Result
Source: Authors' documentation.

Antibacterial Activity of Lactic Acid Bacteria Isolates

The inhibition zone diameter produced by the positive control against the growth of *Staphylococcus aureus* had an average of 25.20 mm, while the negative control showed an average of 0 mm. The largest inhibition zone in the positive control was 25.80 mm, whereas the smallest was 24.70 mm. The inhibition zone of the positive control was categorized as very strong, while the negative control exhibited no inhibitory effect.

No	Isolation Code	Zone Diameter (mm)			Average±SD	Antibacterial Activity
		I	II	III		
1	K+	25,10	24,70	25,80	25,20 ± 0,56	Very powerful
2	K-	0	0	0	0 ± 0	None
3	A1	8,76	8,50	8,30	8,52 ± 0,23	Medium
4	A2	8,50	8,40	9,50	8,80 ± 0,61	Medium
5	A3	9,41	10,70	12,30	10,80 ± 1,45	Medium
6	A4	7,50	9,50	8,10	8,37 ± 1,03	Medium
7	A5a	7,80	7,10	8,00	7,63 ± 0,47	Medium
8	A5b	7,56	9,42	8,54	8,51 ± 0,93	Medium
9	B1a	8,20	9,20	10,90	9,43 ± 1,37	Medium
10	B1b	8,38	9,98	8,90	9,09 ± 0,82	Medium
11	B2	9,10	10,78	9,24	9,71 ± 0,93	Medium
12	B3	8,12	9,10	7,30	8,17 ± 0,90	Medium
13	B4	10,70	9,10	8,44	9,41 ± 1,16	Medium
14	B5	12,40	10,20	11,20	11,27 ± 1,10	Strong
15	C1	8,20	9,60	10,10	9,30 ± 0,98	Medium
16	C2	8,40	7,22	7,12	7,58 ± 0,71	Medium
17	C3	7,10	8,50	8,14	7,91 ± 0,73	Medium
18	C4	7,60	10,10	7,80	8,50 ± 1,39	Medium
19	C5	9,22	8,30	7,80	8,44 ± 0,72	Medium
20	D1	9,74	10,10	11,10	10,31 ± 0,70	Medium
21	D2	9,40	7,40	7,20	8,00 ± 1,22	Medium
22	D3	8,60	9,60	9,80	9,33 ± 0,64	Medium
23	D4	10,18	10,68	8,30	9,72 ± 1,25	Medium
24	D5	9,90	8,30	7,50	8,57 ± 1,22	Medium

Table 1. The Result of Antibacterial Activity

The antibacterial activity of the 22 presumptive LAB isolates against *Staphylococcus aureus* is presented in Table 1. Each inhibition zone diameter represents the mean ± standard deviation (SD) of three independent measurements (n = 3). All isolates inhibited the growth of *S. aureus*, producing mean inhibition zone diameters ranging from 7.58 ± 0.73 mm (isolate C2) to 11.27 ± 1.10 mm (isolate B5), with an overall mean inhibition zone diameter of 8.97 mm. The inhibition zone diameters were measured as the total diameter of the clear

inhibition zone, including the 6-mm agar well, in accordance with the agar well diffusion method used in this study.

Based on the inhibition-zone classification adopted in this study, 21 isolates exhibited moderate antibacterial activity, whereas one isolate (B5) demonstrated strong antibacterial activity. These findings indicate variability in the antibacterial potential among the isolates recovered from tofu wastewater. The greater inhibition exhibited by isolate B5 may reflect differences in the overall antibacterial activity of the isolate under the conditions tested. However, the factors responsible for this variation were not investigated in the present study and therefore cannot be confirmed. No isolate showed weak antibacterial activity or an inhibition zone comparable to that of the positive control. The positive control consistently produced a substantially larger inhibition zone than all LAB isolates, while the negative control showed no inhibitory effect, confirming that the observed antibacterial activity was associated with the tested LAB cultures.

Figure 4. (a)

(a) (b)

Inhibition Zone in Negative Control (Sterile MRSB) and Positive Control (Gentamicin), (b) Inhibition Zone of Lactic Acid Bacteria Isolates
Source: Authors' documentation.

Discussion

Isolation of Lactic Acid Bacteria from Tofu Wastewater

The isolation of lactic acid bacteria (LAB) was carried out to obtain pure isolates from tofu wastewater, which contains organic compounds such as proteins, carbohydrates, and other nutrients that support the growth of microorganisms, including LAB (10). The process began with enrichment using de Man, Rogosa, and Sharpe Broth (MRS Broth) for 48 hours at 37°C under anaerobic conditions in an anaerobic jar. Following enrichment, serial dilutions from 10^{-1} to 10^{-5} were prepared using 0.9% NaCl solution, and then inoculated onto de Man, Rogosa, and Sharpe Agar (MRS Agar) using the streak plate method. The cultures were incubated anaerobically at 37°C for 24–48 hours.

The isolation resulted in 22 isolates with colony morphologies characterized as round, white, with varying sizes, convex and flat elevations, and margins that were entire, undulate, or irregular. These findings are consistent with previous studies (6, 19). The characteristics and growth of LAB can be influenced by several environmental factors, including pH, temperature, and bile salt concentration (11).

The obtained isolates were further purified to obtain single colonies. A single sample could yield more than one isolate due to microbial diversity. The selection of isolates was

based on the typical morphological features of LAB colonies, namely round, white to cream-colored colonies (12). A total of 22 isolates were successfully obtained.

Identification of Lactic Acid Bacteria from Tofu Wastewater

The identification of lactic acid bacteria from tofu wastewater was carried out through preliminary characterization consisting of the catalase test, Gram staining, and fermentation type test. These three assays were used to obtain a presumptive identification of lactic acid bacteria based on typical phenotypic characteristics, namely catalase-negative reaction, Gram-positive staining, and homofermentative metabolic profile.

The catalase test showed that all isolates were catalase negative, as indicated by the absence of bubble formation after addition of 3% hydrogen peroxide. This result is consistent with lactic acid bacteria, which generally lack catalase activity and may utilize alternative oxidative stress mechanisms. This finding may support their presumptive classification as lactic acid bacteria (13).

Gram staining revealed that all twenty-two isolates were Gram positive. Morphologically, twenty-one isolates were rod-shaped (bacilli) and one was spherical (coccus), indicating morphological diversity commonly reported among lactic acid bacteria. These results are consistent with previous studies (12, 19). Gram-positive cells retain crystal violet due to their thick peptidoglycan layer, which reduces permeability during decolorization (13).

The fermentation type test showed that all isolates did not produce gas in Durham tubes, indicating a homofermentative metabolic pattern, characterized by lactic acid as the primary fermentation product (16).

Based on the catalase-negative reaction, Gram-positive morphology, and homofermentative characteristics, the isolates were classified as presumptive lactic acid bacteria according to their phenotypic characteristics. However, this identification was limited to phenotypic characterization and did not provide genus- or species-level confirmation. Therefore, further identification using biochemical profiling or molecular methods, such as 16S rRNA gene sequencing, is recommended to accurately determine the taxonomic identity of the isolates

Antibacterial Activity of Lactic Acid Bacteria Isolates

The antibacterial activity assay aimed to evaluate the overall antibacterial potential of presumptive lactic acid bacteria (LAB) isolates against *Staphylococcus aureus* using the agar well diffusion method. In this study, whole LAB cultures were used as the test samples. Therefore, the observed inhibition zones represent the combined effects of viable bacterial cells and antimicrobial metabolites produced during cultivation. Consequently, the antibacterial mechanism could not be distinguished. Although previous studies have associated clear inhibition zones with bacteriocin-related activity and diffuse inhibition zones with inhibition by organic acids (17). These mechanisms were not directly evaluated in the present study and should therefore be regarded only as possible explanations.

The antibacterial activity test was performed using gentamicin as the positive control and sterile de Man, Rogosa, and Sharpe (MRS) broth as the negative control. Gentamicin produced a mean inhibition zone diameter of 25.20 ± 0.56 mm, whereas no inhibition was observed in the negative control. All 22 presumptive LAB isolates inhibited the growth of *Staphylococcus aureus*, producing inhibition zone diameters ranging from 7.58 ± 0.73 mm to 11.27 ± 1.10 mm, with an overall mean inhibition zone diameter of 8.97 mm. The inhibition

zone diameters represent the total diameter of the clear zone, including the 6-mm agar well, as measured in this study. Larger inhibition zones may indicate higher apparent activity, however smaller zones do not necessarily reflect lower effectiveness due to differences in diffusion rates or concentrations of inhibitory substances (18). Based on the inhibition-zone classification adopted, one isolate exhibited strong antibacterial activity, whereas the remaining twenty-one isolates exhibited moderate antibacterial activity (17).

Differences in inhibition zone diameters among isolates may be influenced by several factors, including variations in bacterial growth, the diffusion characteristics of inhibitory substances, and differences in the production of antimicrobial metabolites during cultivation (17). In addition, molecular size, concentration, and physicochemical properties of the compounds may influence their diffusion through the agar medium. However, because these factors were not directly evaluated in the present study, they should be regarded only as possible explanations for the observed differences among isolates.

Previous literature has reported that lactic acid bacteria may produce Previous studies have demonstrated that lactic acid bacteria are capable of producing antimicrobial metabolites, including organic acids, bacteriocins, hydrogen peroxide, and carbon dioxide, which have been reported to inhibit the growth of pathogenic bacteria (13). Nevertheless, the present study did not evaluate the production or concentration of these compounds. Therefore, the antibacterial activity observed against *Staphylococcus aureus* cannot be attributed to any specific antimicrobial substance, and the involvement of these metabolites should be considered as a possible mechanism rather than a confirmed explanation.

Similarly, factors influencing antimicrobial compound production, including pH, temperature, carbon source, and bacterial growth phase, have been reported in previous studies (13). Therefore, their contribution to the antibacterial activity observed remains uncertain and warrants further investigation.

CLINICAL IMPLICATION

These findings suggest that tofu wastewater may be a source of presumptive lactic acid bacteria with in vitro antibacterial activity against Staphylococcus aureus. However, further studies are required, including bacterial identification at the species level, metabolite characterization, safety evaluation, and application testing, before any potential clinical or industrial use can be considered.

LIMITATIONS

This study has several limitations. First, the identification of lactic acid bacteria was only carried out through basic characterization, such as catalase test, Gram staining, and fermentation type test, which does not allow for accurate identification at the species level. Second, the antibacterial activity testing focused only on one pathogenic bacterium, which is *Staphylococcus aureus*, so the antibacterial spectrum of the isolates remains unclear. Third, this study did not include an analysis of the bioactive compounds that are suspected to play a role in inhibiting the growth of pathogenic bacteria. These limitations may serve as a basis for designing more comprehensive future research.

CONCLUSIONS

This study successfully isolated 22 presumptive lactic acid bacteria (LAB) from tofu wastewater. Based on phenotypic characterization, all isolates were Gram-positive, catalase-negative bacteria with bacilli or cocci morphology and homofermentative metabolic characteristics. All presumptive LAB isolates exhibited in vitro antibacterial activity against *Staphylococcus aureus*, producing inhibition zone diameters ranging from 7.58 mm to 11.27 mm, with a mean diameter of 8.97 mm, corresponding to moderate to strong antibacterial activity. These findings indicate that phenotypically characterized presumptive LAB isolated from tofu wastewater possess in vitro antibacterial activity against *Staphylococcus aureus*. However, molecular identification and further studies on the antimicrobial compounds responsible for the observed activity are required before drawing conclusions regarding their taxonomic identity or potential practical applications.

CONFLICT OF INTEREST

The authors declare that the research was carried out solely for academic purposes and that no financial, commercial, or personal relationships exist that could be interpreted as a potential conflict of interest.

AUTOR CONTRIBUTIONS

All authors made substantial contributions to the preparation of this manuscript. Ni Made Anggun Ciptasari designed the study, conducted the research, analyzed the data, and drafted the manuscript. Ni Nyoman Astika Dewi supervised the study, provided guidance, and offered critical revisions. I Gusti Agung Dewi Sarihati assisted in supervising the research process, contributed to data interpretation, and reviewed the manuscript for important intellectual content.

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DECLARATION OF ARTIFICIAL INTELLIGENCE USE

The authors used ChatGPT (OpenAI, version GPT-5) solely for language editing and formatting purposes. The authors take full responsibility for the content of the manuscript and have verified its accuracy.

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