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Isolation and identification of indigenous lactic acid bacteria with inhibitory activity against *Aeromonas hydrophila* in Vinh Long province

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ABSTRACT

Aeromonas hydrophila is a common cause of hemorrhagic disease that seriously harms several economically valuable farmed fish species in Vietnam and worldwide. Therefore, the objective of this investigation was to isolate and identify indigenous lactic acid bacteria (LAB) strains that inhibit the growth of *A. hydrophila*, causing hemorrhagic illness in farmed striped catfish in Vinh Long province. This finding yielded a total of 45 isolates of LAB from the intestine of healthy pangasius catfish. Using the well diffusion agar method, the results showed that 30 out of 45 isolated LAB strains (66.67%) had antibacterial activity against *A. hydrophila*. Among them, 10 out of 30 strains (33.33%) had strong activity, 15 out of 30 strains (50%) showed moderate activity, and 5 out of 30 strains (16.67%) exhibited weak activity, with mean inhibition diameters of 18.8 ± 3.52, 12.02 ± 1.32, and 5.89 ± 0.73 mm, respectively. With the exception of lipase activity, the enzyme activities of proteases and amylases were found in strain TMT1. Phylogenetic analysis using the 16S rRNA gene sequence showed that strain TMT1 was identified as *Lactobacillus casei*. The results of the research indicate that isolate TMT1 may be used to produce probiotics that help prevent pangasius hemorrhagic sickness.

1. INTRODUCTION

In Vinh Long province, in the Mekong Delta, the striped catfish (*Pangasianodon hypophthalmus*) is one of the major farmed fish species. According to the report of the Vinh Long Statistics Department, the province's pangasius farming area reached 7,973 hectares in 2023 (personal information). In recent years, the high densities of this fish farming have been one of the reasons for more and more disease outbreaks and significant damage to the pangasius farming industry. Among the diseases caused by bacterial pathogens in aquatic animals, hemorrhagic disease caused by *Aeromonas hydrophila* is one of the diseases that appears frequently. This disease causes a lot of serious loss to many farmed fish species in the world and in Vietnam, including pangasius in the Mekong Delta provinces such as Vinh Long, Tien Giang, and An Giang [1]. In addition to pangasius, many recent studies show that *A. hydrophila* also causes disease in other farmed fish species, such as clown knife fish (*Chitala chitala*) [2], rohu (*Labeo rohita*) [3], tilapia (*Oreochromis niloticus*) [4], red tilapia (*Oreochromis* spp.) [5], grass carp (*Ctenopharyngodon idellus*) [6], climbing perch (*Anabas testudineus*) [7], channel catfish (*Ictalurus punctatus*) [8], and many other fish species [9].

Currently, industrial fish farmers in the world and in Vietnam have used different measures to control *A. hydrophila*, such as using immune stimulants [10], herbs [11,12], or phage therapy [13–15]. Recently, various vaccines have been developed to prevent diseases caused by *A. hydrophila*. The need to replace antibiotics has also received attention, but there are still many difficulties when applying them [16,17]. Many studies have shown that the vaccine is effective in protecting striped catfish with a high relative percent survival [18,19]. However, to date, for Vietnamese striped catfish, there is no proven commercial vaccination that can stop the hemorrhagic sickness caused by *A. hydrophila* [20]. To reduce the usage of antibiotics, numerous domestic and international investigations have worked to develop novel approaches for the prevention of illnesses in aquatic animals. This includes the research and application of probiotics supplemented with beneficial bacteria groups, such as lactic acid bacteria (LAB) [21,22], *Bacillus* [23,24], and *Streptomycetes* [25,26].

LAB are gram-positive bacteria, have negative oxidase and catalase reactions, are non-spore-forming, and are non-motile [27]. LAB has been used as probiotics due to their beneficial characteristics for fish and shrimp [22,28]. Previous reports showed that LAB has the ability to

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suppress the growth of pathogenic bacteria [29,30]. Besides, LAB also has the ability to improve the farming environment [31] and increase the growth and survival rate of aquatic animals [32,33]. Generally, the above studies show the potential application of LAB in aquaculture. However, little information is still available on the research and application of LAB to control hemorrhagic illnesses caused by *A. hydrophila* in farmed striped catfish in Vinh Long. Therefore, the aim of the work was to isolate and identify indigenous LAB strains with antibacterial activity against *A. hydrophila*. Potential LAB strains may be used in the manufacture of probiotics to control bacterial infectious diseases.

2. MATERIALS AND METHODS

2.1. Fish Source for LAB Isolation

LAB isolates were isolated from 80 fish samples. Fish were collected from ponds in two districts (Long Ho and Mang Thit) of Vinh Long province (Fig. 1). At each sampling location, 3–5 fish were randomly collected per pond, with the weight of the fish samples ranging from 300 to 500 g. The fish samples were healthy and alive, and they were brought to the laboratory for LAB isolation. The healthy fish samples were determined by observing external signs (recorded in fish ponds), while internal signs and isolating pathogenic bacteria were performed in the laboratory.

2.2. LAB Isolation

LAB was obtained from striped fish's intestines, according to Muthukumar and Kandeepan [34], with some minor modifications. In brief, the fish were sterilized externally with 70% alcohol. The fish were then cleaned of slime and dissected. Next, the intestine of the fish was cut into small pieces, about 1–2 cm, and placed in 100 ml of a 0.85% NaCl solution. The sample was ground to homogeneity, allowed to settle for about 10 minutes, and the upper solution was collected. The solution (10 ml) was added to 90 ml of de Man, Rogosa and Sharpe (MRS) broth medium (Himedia, India) and incubated at 37°C for 48 hours. Finally, 100 µl of the sample was spread onto a plate containing MRS agar medium supplied with 0.5% $CaCO_3$ and incubated at 37°C [35]. After 48 hours, the LAB isolates with a clear zone around the colony were chosen and subcultured several times on MRS agar medium until the colony was homogeneous. Bacterial isolates were tested for morphological and biochemical characteristics such as Gram and spore staining, motility, oxidase, and catalase activity [36,37].

2.3. Antibacterial Activity of Isolated LAB Strains

The antibacterial ability of isolated LAB strains was checked using the well diffusion agar method [38]. The *A. hydrophila* bacterial suspension of strain 1A3, which originated from hemorrhagic disease-infected striped catfish [39], was prepared at a bacterial concentration of 10^8 CFU/ml (the bacterial suspension's turbidity corresponds to a 0.5 McFarland standard) by dissolving the bacterial colonies into a 0.85% NaCl saline solution. The bacterial solution (100 ml) was spread onto a TSA (Himedia, India) medium plate. Then, wells with a diameter of 6 mm were done with a sterile col tip. In parallel, LAB was cultured in an MRS broth medium and incubated at 37°C for 48 hours. Then, 2 ml of bacterial culture was centrifuged for 5 minutes at 4°C at 10,000 rpm. Finally, 80 µl of the supernatant was injected into the wells and incubated at 30°C. The antibacterial activity of LAB

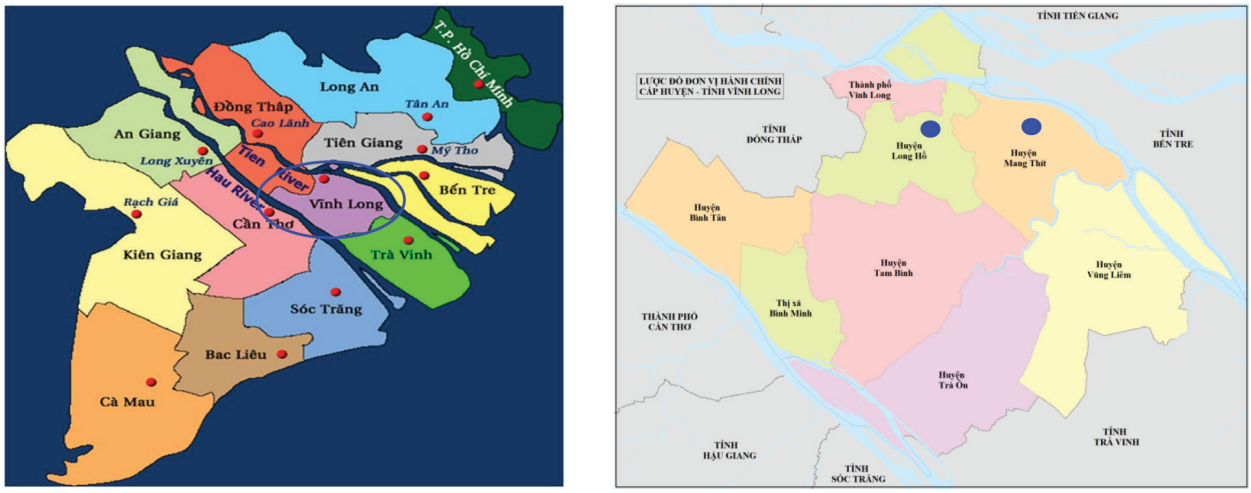

Figure 1. Striped catfish samples were collected in different intensive ponds in Vinh Long province (blue circle).

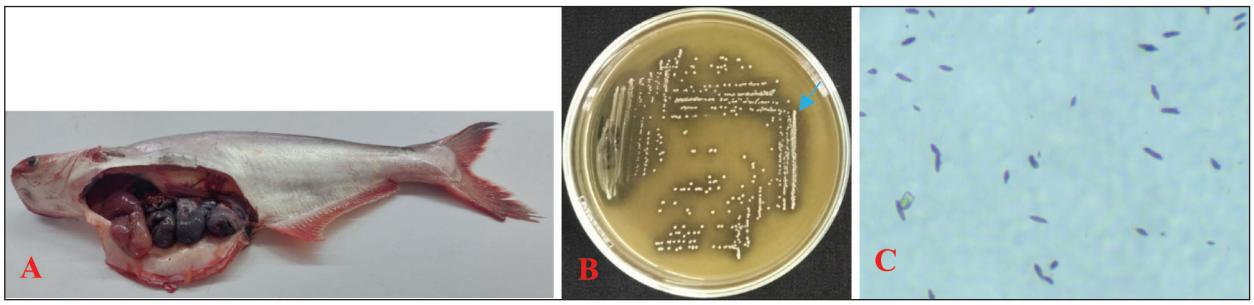

Figure 2. LAB isolated on MRS agar medium. (A) Striped catfish sample; (B) LAB grown on MRS agar medium; (C) Gram staining.

Table 1. The colony morphology and biochemical characteristics of isolated bacterial strains.

Colony morphology	Characteristics	Number of bacterial isolates	Rate (%)
Motility	Non-motile	45	100
Cell shape	Long-rod	40	88.89
	Short-rod	5	11.11
Colony morphology			
Form	Circular	40	88.89
	Irregular	5	11.11
Edge	Entire	45	100
Color	Opaque white	35	77.78
	Clear white	10	22.22
Elevation	Convex	40	88.89
	Flat	5	11.11
Biochemical features			
Gram staining	Gram-positive	45	100
Spore staining	Non-spore	45	100
Oxidase reaction	Negative-oxidase	45	100
Catalase reaction	Negative-catalase	45	100

is determined when the inhibition halo around the well is detected after 24–48 hours of incubation. The diameter of the inhibition zone is calculated according to d = d1–d2 (d: diameter of the clear zone, d1: total diameter of the inhibition halo, d2: diameter of the well [equivalent to 6 mm)]. LAB are considered to have strong activity when $d > 15$, moderate activity when $10<d<15$, weak activity when $5<d<10$ mm, and not inhibitory activity when $d = 0$ [40].

2.4. Determination of Enzymatic Activity

The protease, amylase, and lipase activity of LAB were assessed according to Guo *et al.* [41], Taheri *et al.* [42], and Moslehishad *et al.* [43], with some minor modifications. To test for proteolytic activity, LAB was spot-inoculated on MRS agar medium supplemented with 1% skimmed milk. Similarly, MRS agar medium supplemented with 2% starch and Tween 80 was used for amylolytic and lipolytic activity, respectively. After a 24-hour incubation period at 37°C, the enzymatic characteristics were evaluated based on the presence of amylolytic halo zones (flooding with Lugol's solution) and lipolytic and proteolytic turbidity zones around the colony growth.

2.5. Identification of LAB Isolates Using the 16S rRNA Gene

2.5.1. Extraction of bacterial DNA

Bacterial DNA was extracted according to the method of Dung [44], with some minor modifications. In brief, LAB strains used for DNA extraction were grown in a TSB medium and shaken at 110 rpm for 24 hours. After centrifuging 2 ml of the bacterial culture for 5 minutes at 13,000 rpm, the biomass was collected. The lysis buffer (0.5 mM EDTA, 1 M Tris-HCl, 10% SDS, 5 M NaCl, pH 8.0) was mixed with 1 ml of bacterial biomass and incubated at room temperature for 10 minutes. For 5 minutes, the solution was centrifuged at 13,000 rpm. The 700 µl of solution was poured into a fresh Eppendorf tube. After adding 700 µl of 95% ethanol, the mixture was centrifuged for 5 minutes at 13,000 rpm. The mixture was centrifuged for 5 minutes at 13,000 rpm after dissolving the precipitated DNA in 500 µl of 70% ethanol. The DNA was dissolved in 100 µl of 0.1 X TE (1 mM EDTA, 10 mM Tris-HCl, pH 8.0) after eliminating all of the ethanol. After extraction, bacterial DNA was measured for optical density at wavelengths of 260 and 280 nm to determine purity and concentration. Extracted DNA was kept at −20 for use in Polymerase chain reaction (PCR) reactions.

Table 2. The antagonistic activity of isolated LAB isolates against *A. hydrophila*.

Bacterial isolates	Antibacterial activity		
	Strong activity	Moderate activity	Weak activity
TMT1	+++		
TMT2	+++		
TMT7		++	
TMT10	+++		
TLH1		++	
TLH2			+
TLH3		++	
TLH7	+++		
TVL2		++	
TVL4		++	
TVL5	+++		
TVL6			+
TTO4	+++		
TTO5		++	
TTO6			+
ĐLH2		++	
ĐVL1		++	
ĐVL6		++	
ĐBM2		++	
ĐBM5	+++		
ĐBM6			+
LVL3		++	
LVL4		++	
LVL8			+
LTO2	+++		
LTO4		++	
LTO6	+++		
LĐTB1	+++		
LĐTB2		++	
LĐTB3		++	
Total	10	15	5

+: Weak activity ($5<d<10$ mm); ++: Moderate activity ($10<d<15$ mm); +++: Strong activity (d >15 mm).

2.6. LAB Identification by PCR Reaction

The bacterial 16S rRNA gene segment was amplified using the primer pairs 27F: 5′-AGATTTGATCCTGGCTCAG-3′ and 1492R: 5′-GGTTACCTTGTTACGACTT-3′ [45]. The ingredients of PCR reactions include: PCR buffer solution (1X), dNTPs (150 µM), $MgCl_2$

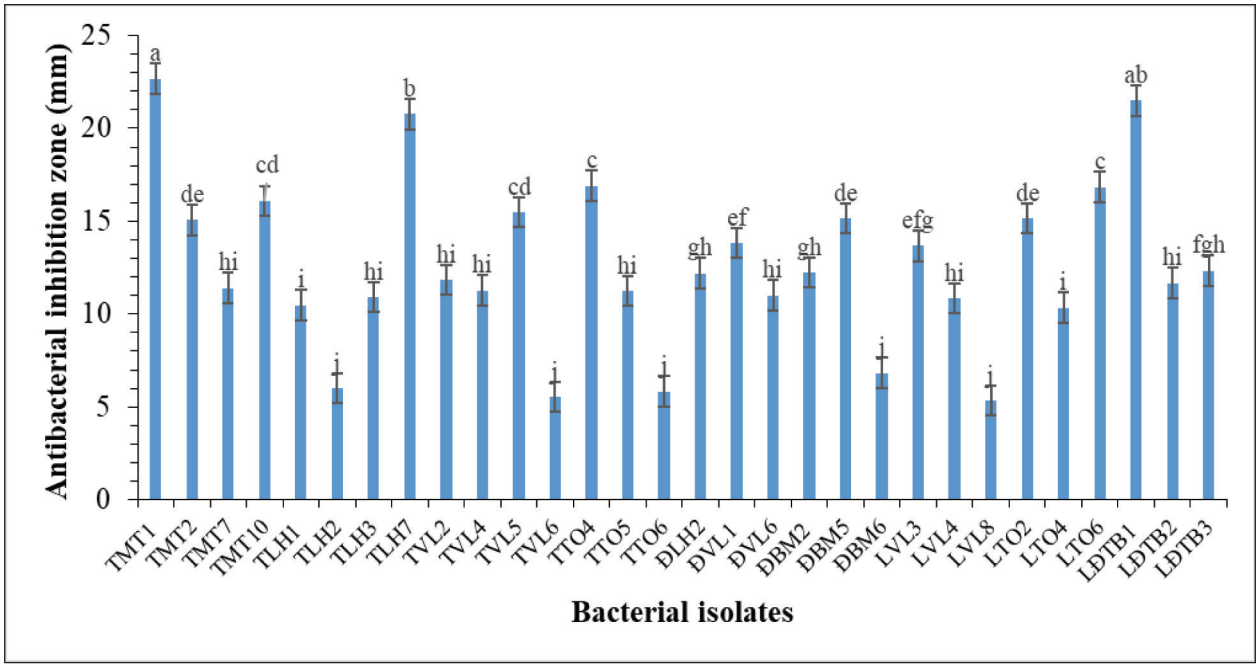

Figure 3. Antagonistic activity of isolated LAB **strains.** The bars with different letters indicate the significant differences ($p \leq 0.5$).

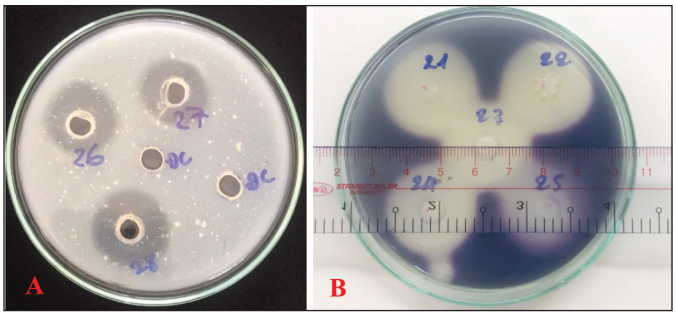

Figure 4. Protease and amylase activity of **isolated** LAB **strains. (**A**)** Protease activity of isolated LAB strains; **(**B**)** Amylase activity of isolated LAB strains.

(2.5 mM), *Taq* DNA polymerase (2U), forward primer (27F, 20 pmol), reverse primer (1492R, 20 pmol), and LAB DNA (40 ng). The thermal cycle for the PCR reaction includes pre-denaturation stages at 94°C for 5 minutes, then 35 cycles including denaturation at 95°C for 1 minute, annealing at 58°C for 1 minute, extension at 72°C for 2 minutes, and final extension at 72°C for 10 minutes. The PCR products (1,500 bp) after amplification were electrophoresed on a 1.5% agarose gel in 1X TAE buffer at 50V for 45 minutes. Electrophoresis results were read and the gel was captured on a BioRad UV 2000 machine (USA). The bacterial strain with the highest level of antibacterial activity was selected for sequencing based on its 1.500 bp PCR products.

2.7. Data Analysis

Descriptive statistical methods were used to calculate mean values and standard deviations. The differences in inhibitory activity between bacterial strains were analyzed by ANOVA using the MiniTab 20 software at the 5% significance level. Sequencing results of bacterial strains were compared for similarity with sequences of reference LAB on the NCBI database using the BLASTn program. The DNA sequences of LAB bacteria were multialigned using the CLUSTAL W [46]. Using MEGA X software and a bootstrap value of 1,000 replications [47], the phylogenetic tree illustrating the genetic links between bacterial strains was built based on the neighbor-joining algorithm [48].

2.8. Ethical Approval

The study protocol was approved by the Ethical Management in Animal Experiments, College of Aquaculture and Fisheries, Can Tho University, Vietnam (Approval no. 3965/QD-DHCT, October 15, 2021).

3. RESULTS

3.1. Isolation of LAB

Based on the characteristics of colony morphology, cell shape, and bacterial physiological characteristics, 45 different strains of LAB were identified from fish samples' intestines in Vinh Long province (Fig. 2A). Among the isolated bacterial strains, bacterial strains originating from Long Ho district accounted for the highest proportion (30 out of 45 strains, accounting for 66.67%).

3.2. Colony Morphology and Biochemical Characteristics of Isolated Bacterial Strains

In general, observation results showed that most colonies of bacterial strains isolated on MRS medium are round, convex, opaque white, or clear white (Fig. 2A). In addition, a clear zone (Fig. 2B, arrow) around the colonies was observed when they grew on MRS agar medium supplemented with $CaCO_3$ after 48 hours of incubation at 37°C. The findings showed that all isolated bacterial strains were non-motile, short- or long-rod, gram-positive bacteria (Fig. 2C). The isolated bacterial strains did not form spores, negative oxidase, or catalase reactions. The colony morphology and biochemical characteristics of isolated bacterial strains are presented in Table 1.

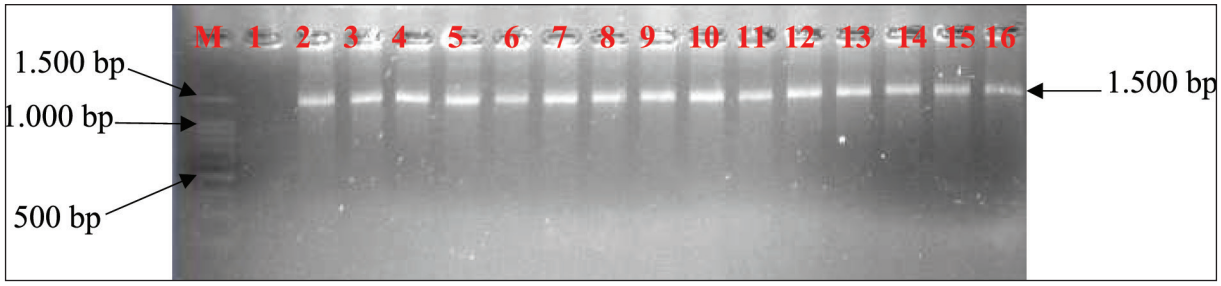

Figure 5. Amplification of the 16S rRNA gene segment of representative LAB strains by PCR reaction. *M: 100 bp-DNA standard marker. Lane 1: Negative control; Lane 2–16: bacterial strains* TMT1, TMT2, TMT10, TLH7, TVL5, TTO4, ĐLH2, ĐVL1, ĐBM2, ĐBM5, LVL3, LT02, LT06, LĐTB1, and LĐTB2, respectively.

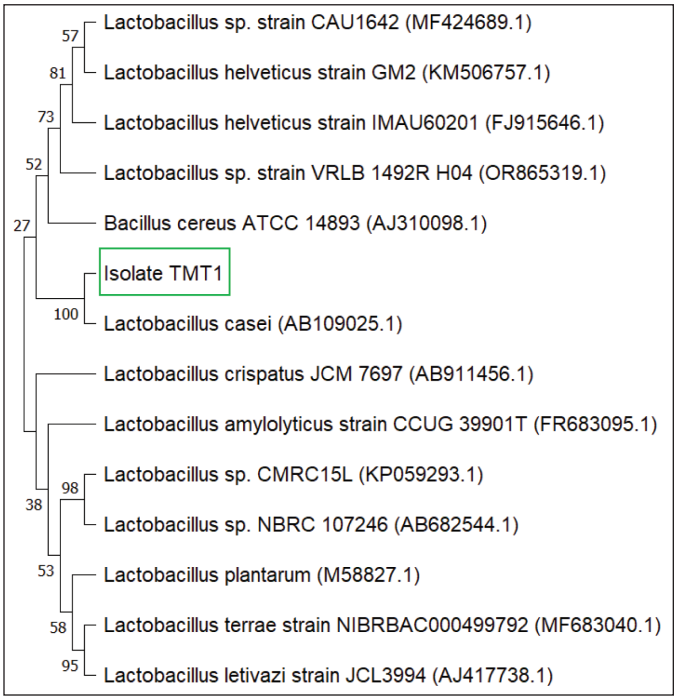

Figure 6. Phylogenetic tree showing isolate TMT1 belonging to the same group as *L. casei* (Numbers on branches are bootstrap values of 1.000 replicates, and *Bacillus cereus* strain ATCC 14893 CIP 5832 [AJ10098.1] is used as an outgroup).

3.3. Antagonistic Abilities of Indigenous LAB Strains

This observation indicated that 30 out of 45 isolated LAB strains (66.67%) had antibacterial activity (Fig. 3). Among them, 10 out of 30 strains (33.33%) had strong activity with clear zone: d >15 mm (average inhibitory halo diameter d = 18.8 ± 3.52 mm), 15 out of 30 strains (50%) exhibited moderate activity with inhibitory diameters of 10<d<15 mm (mean clear zone diameter d = 12.02 ± 1.32 mm), and 5 out of 30 strains (16.67%) in this study showed weak antibacterial activity with the inhibitory halo of 5<d<10 mm (average clear zone diameter d = 5.89 ± 0.73 mm). The antagonistic activity of isolated LAB isolates against *A. hydrophila* was presented in Figure 3 and Table 2.

3.4. Enzymatic Activity

The result showed that the enzymatic activities of proteases and amylases were found in 35 tested strains (Fig. 4A and B). On the other hand, no bacterial strains exhibited lipase activity in the study.

3.5. Identification of LAB Based on the 16S rRNA Gene

Electrophoresis results of PCR products showed that all bacterial strains selected in the study showed a single DNA band at a position of 1.500 bp (Fig. 5).

The sequencing results revealed that isolate TMT1 (selected isolate TMT1 due to its strong antibacterial activity) is 99.01% similar to *Lactobacillus* sp. (MF424689.1). The bacterial strains are grouped into two clusters, in which isolate TMT1 as the same cluster with reference *Lactobacillus casei* on GenBank, according to the phylogenetic tree (Fig. 6). The high bootstrap values illustrate strong support for the clustering of this isolate with known *Lactobacillus* species.

4. DISCUSSION

Numerous aquatic species' intestines were found to contain LAB in earlier investigations [49–51]. In the current study, 45 LAB isolates were obtained from the intestines of striped catfish. This finding is similar to Phuong and Oanh [52], who reported that LAB strains were also obtained from some catfish species, consisting of stripped catfish, *Mystus nemurus*, *Pangasius larnaudii*, *Clarias macrocephalus*, and *P. larnaudii*. In this study, isolate TMT1 was identified as *L. casei* based on 16S rRNA fragment gene sequencing together with morphological and biochemical features. *Lactobacillus* is a gram-positive and catalase-negative rod LAB commonly found in lactic acid fermented products [53] and in the intestines of aquatic animals, including shrimp [54], freshwater fish [55], and marinewater fish [56,57]. *Lactobacillus* gives multiple benefits to fish, such as growth promotion [58], inhibition of bacterial pathogens [59], improvement of nutrient digestion [60], water quality [61], stress tolerance [62], and enhancement of reproduction [63].

In the present study, 30 out of 45 LAB strains exhibited inhibitory activity against *A. hydrophila*. This finding is in accordance with many earlier studies that proved LAB inhibited the growth of pathogenic bacteria in aquaculture [64,38]. In the present finding, isolated LAB strains showed different inhibitory abilities against *A. hydropila*, such as strong activity (33.33%), moderate activity (50%), and weak activity (16.67%), with an average clear zone diameter of 18.8 ± 3.52, 12.02 ± 1.32, and 5.89 ± 0.73 mm, respectively. This investigation is supported by the findings of Meidong *et al.* [65], who demonstrated that the investigated fish pathogens were inhibited by LAB isolates generated from tilapia fish, sediment and water surrounding the culture fish cages, and a variety of traditional fermented foods, with inhibition zones *A. hydrophila*, *Aeromonas caviae*, and *Streptococcus agalactiae* ranging from 8.6–16, 9.8–16, and 8.2–18 mm, respectively. Similarly, LAB isolates obtained from the digestive tract of eels (*Monopterus albus*) were discovered to stop the spread of harmful bacteria like *Staphylococcus aureus*, *A. hydrophila*, and *Vibrio harveyi*, with the

clear zone ranging from 11.33–12.67, 9.00–16.67, and 18.67–25.33 mm, respectively [37]. According to Zhou *et al.* [66], the inhibitory capacity against *A. hydrophila* of *Lactococcus lactis* RQ516 was 7.43 ± 0.47 mm at 6 hours and 14.77 ± 1.17 mm at 24 hours. In the present study, the inhibitory mechanism of LAB isolates was not studied. However, prior research has indicated that the synthesis of organic acids, hydrogen peroxide, or bacteriocins may be the cause of the inhibitory actions of LAB [67,68]. Moreover, *L. lactis* strain A5, which was isolated from the gastro-intestinal tissues of broadhead catfish, produced a nisin-like bacteriocin that was effective against a variety of gram-positive and gram-negative bacterial pathogens, including *Salmonella thyphimurium*, *B. cereus*, and *S. aureus* [69]. The study by Loh *et al.* [70] also showed that bacteriocin-like substances from *L. lactis* subsp. *lactis* CF4MRS were found to be antagonistic to various fish pathogens, including *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Escherichia coli*, *A. hydrophila*, *Edwardsiella tarda*, and *Serratia marcescens*.

Numerous studies proved that LAB was able to produce different digestive enzymes like amylases, cellulases, proteases, and lipases [71,72]. The presence of extracellular enzymes such as amylase and protease are criteria that probiotics must possess to increase the ability of fish to digest various food ingredients. In this study, the enzymatic activities of amylases and proteases were detected in strain TMT1, except for lipase activity. This finding is similar to the research of Marchwińska and Gwiazdowska [73], who also revealed that all tested LAB strains originated from the suckling piglets' feces and the weaned piglets' feces caused the degradation of milk casein, 46% showed starch degradation, and LAB strains were incapable of lipolytic activities. In another study by Agustina *et al.* [74], it was detected that isolates of LAB from the intestines of *P. waandersi* displayed amylolytic, proteolytic, and lipolytic activities. Similar findings by Konkit and Kim [75] demonstrated the presence of enzymes such as lipase, proteinase, and amylase in *L. chungangensis* CAU 28T. Balcázar *et al.* [76] found that probiotics' capacity to secrete extracellular enzymes improves the host's ability to digest food. Recently, according to Ringø *et al.* [77], LAB probiotics have been shown to enhance feed consumption and absorption by releasing a variety of digestive enzymes and nutrients that can aid in feed utilization and digestion. Additionally, the absorption of diet components has been linked to improved host health.

5. CONCLUSION

In this study, LAB isolates isolated from the intestines of striped catfish inhibited the growth of *A. hydrophila*, which causes hemorrhagic illness in intensively farmed striped catfish. The strain TMT1 with strong antibacterial activity was identified as *L. casei* based on traditional biochemical characteristics and molecular biology techniques in combination with 16S rRNA fragment sequencing results. Furthermore, strain TMT1 exhibited protease and amylase enzyme activities without lipase activity.

6. ACKNOWLEDGMENTS

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7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

8. FUNDING

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

The study protocol was approved by the Ethical Management in Animal Experiments, College of Aquaculture and Fisheries, Can Tho University, Vietnam (Approval no. 3965/QD-DHCT, October 15, 2021).

11. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

12. PUBLISHER'S NOTE

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13. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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