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Probiotic properties of lactic acid bacteria obtained from intensive snakehead fish (*Channa striata*) culture ponds

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Abstract. *Chi NTY, Minh NLK, Thi QVC. 2025. Probiotic properties of lactic acid bacteria obtained from intensive snakehead fish (*Channa striata*) culture ponds. Biodiversitas 26: 1171-1179.* Intensively farmed snakehead fish (*Channa striata*, hereinafter referred to as *C. striata*) in Vinh Long Province of the Mekong Delta, Vietnam, have suffered significant harm from *Aeromonas schubertii* (hereinafter can be written as *A. schubertii*), the causative agent of visceral white spot disease. Hence, the purpose of the present study was to collect and select Lactic Acid Bacteria (LAB) with potential probiotic properties. The finding revealed that twenty-three LAB strains were yielded from pond water samples and fish guts. Regarding probiotic features, eleven of twenty-three LAB strains exhibited antagonistic activity against *A. schubertii*. Among them, strain LABB, which displayed the highest antibacterial activity, was classified as *Lactiplantibacillus* based on morphological, physiological, biochemical, and phylogenetic analysis results in the investigation. After 24 hours of incubation, strain LABB was able to survive in media with a pH between 2.0 and 4.0. Strain LABB, in particular, showed the ability to withstand bile salt at contents of 0.3% and 0.5% after being inoculated for 24 and 3 hours, respectively. Besides, the findings also revealed that the strain LABB could produce extracellular enzymes such as protease, amylase, and cellulase. Additionally, this bacterial strain was susceptible to penicillin, levofloxacin, ofloxacin, erythromycin, clindamycin, doxycycline, ampicillin, ampicillin/sulbactam, and chloramphenicol. According to research findings, LABB may be used to manage *A. schubertii*, which causes visceral white spot disease in farmed snakehead fish.

Keywords: *Aeromonas schubertii,* antibacterial ability, lactic acid bacteria, Mekong Delta, snakehead fish

INTRODUCTION

Aeromonas are commonly present in freshwater as well as saltwater aquaculture environments (Chenia and Duma 2017; Fernández-Bravo and Figueras 2020), in which *A. schubertii* has been determined as the causative agent of many aquatic animals, including humans (Peepim et al. 2016; Dong et al. 2017; Emeish et al. 2018; Ren et al. 2018; Fernández-Bravo and Figueras 2020). To date, most reports showed that *A. schubertii* mainly causes disease in fish, consisting of Nile tilapia, *Oreochromis niloticus* (*O. niloticus*) (Ren et al. 2018), *Garra rufa* (Yu et al. 2009), *Channa maculata* (Chen et al. 2012), *Ophiocephalus argus* (*O. argus*) (Liu and Li 2012), the hybrid fish between *O. argus* and *C. maculata* (Liu et al. 2012), and snakehead fish, *C. striata* (Tahir et al. 2024). Additionally, the study by Latif-Eugenin et al. (2016) showed that this bacterium is also found in other aquatic animals such as frogs and mussels. More recently, this bacterium has been reported as the cause of the prevalence of *A. schubertii* infections in shrimp (Cao et al. 2015; Sangpo et al. 2020). In Vietnam, all of the provinces in the Mekong Delta, like Dong Thap, An Giang, Dong Nai, and Tra Vinh, have reported snakehead fish with visceral white spot disease caused by *A. schubertii* (Tu et al. 2019).

Vinh Long is one of the provinces with a strong aquaculture sector in the Mekong Delta. In addition to striped catfish (*Pangasianodon hypophthalmus*) and red tilapia (*Oreochromis* spp.), which are the two main farming species, recently, the province's agricultural sector has promoted the development of several high-value aquatic products to increase production value and reduce risks, including snakehead fish. Currently, snakehead fish are commonly raised in some provinces of the Mekong Delta due to their fast growth characteristics, good adaptation to harsh environmental conditions, and the high nutritional value of their meat. However, the expansion of farming areas, coupled with intensive farming practices, high stocking densities, and poor environmental management of ponds, has contributed to the emergence of numerous pathogens in farmed snakehead fish. This has caused losses to farmers as well as made disease prevention and treatment increasingly challenging. The survey findings of Nguyen et al. (2020) revealed that many diseases caused by microbial agents commonly appear on snakehead fish raised in ponds of Tra Vinh and An Giang Provinces, in which visceral white spot disease due to *A. schubertii* has a particularly high incidence, ranging from 82% to 88%.

Until now, antibiotics are still used to treat bacterial infectious diseases in aquatic animals (Chandra et al. 2023). Drug resistance in *A. schubertii* is caused by improper use of antibiotics (Eid et al. 2022). Therefore, the use of biological measures like phage therapy as well as potential probiotic bacteria, such as *Bacillus* and Lactic

Acid Bacteria (LAB), is of interest to numerous countries (Luo et al. 2024; Rahayu et al. 2024). LAB is commonly present in traditional fermented foods and is widely used in the field of food technology (Azam et al. 2017; Sionek et al. 2023). In the aquaculture field, LAB is attracting significant attention due to their potential probiotic properties (Chizhayeva et al. 2022; Rahayu et al. 2024). In Vietnam, the previous study reported the presence of LAB in various aquatic species and has many probiotic properties that can be applied in aquaculture (Nguyen et al. 2022a,b). According to the review of Rahayu et al. (2024), probiotics' primary advantages include improving immunity, promoting growth and reproduction, improving digestion, enhancing water microbial composition, enhancing illness and stress resistance, and improving digestion. To date, however, there has been no research on the probiotic properties of LAB on snakehead fish. Hence, this investigation was done to isolate and select LAB isolates with probiotic properties. The findings provide a scientific basis for further research in the production of probiotics to control *A. schubertii* in snakehead fish and aim to develop a sustainable aquaculture industry in the future.

MATERIALS AND METHODS

Sample sources for lactic acid bacteria isolation

Intensively farmed snakehead fish samples, pond water, and sediments were collected to isolate LAB strains. Fish samples (weight 300-600 g) were collected from ponds (the area ranges from 500-1,000 m^2) in the Tra On District, Vinh Long Province. Only healthy fish (Figure 1.A and B) that were swimming normally were selected. A total of 38 fishes were collected (three to five fish from each pond), placed in an aerated icebox, and transported to the laboratory for bacterial isolation. The pond water and sediment samples were collected from five different positions of the same pond and mixed before isolating.

Lactic Acid Bacteria (LAB) isolation

LAB was isolated from fish bowel according to the method given by Nurhayati et al. (2023). First, the fish was externally sterilized with 70% ethanol after being thoroughly cleaned with sterile distilled water. Fish intestine was then cut into small pieces and homogenized in physiological saline (0.85% NaCl). Then, the homogenate was cultured overnight in MRS broth medium (de Man et al. 1960) at 120 rpm. The enriched solution was then diluted to a 10^{-6} dilution. One hundred microliters of each dilution were spread onto MRS agar medium. Next, the plates were incubated at 37°C for 48 hours. Meanwhile, LAB isolated from pond water, and sludge samples were processed according to Lee et al. (2016). The samples (10 mL) were enriched overnight in 90 mL of MRS broth medium at 120 rpm. Then, the samples were diluted and spread as described above. Finally, pure bacterial strains were named as follows ND5L, ND5S, ND6L, ND6S, ND4S, ND4L, CMB1, CM4.1, CM1, CM3, CM2, and CM4.2 and checked for basic morphological, physiological, and biochemical traits, including Gram stain, motility, oxidase, and catalase reactions (Nofiani et al. 2022), and were stored at -40°C in 20% (v/v) glycerol.

Probiotic characteristics of lactic acid bacteria

Inhibitory activity of isolated lactic acid (LAB) bacteria against Aeromonas schubertii

The isolated strains' antibacterial activity was evaluated using the well diffusion agar method (Govindaraj et al. 2021). First, the colonies of *A. schubertii* (isolate As11) used as the indicator bacteria (Quach et al. 2023) were suspended in 0.9% physiological saline to obtain a bacterial solution with a density of 10^8 CFU/mL (turbidity equivalent to McFarland standard 0.5). The indicator bacterial solution (0.1 mL) was then spread onto TSA plates using a cotton swab. Meanwhile, LAB isolates were cultured in MRS broth medium and incubated for 48 hours at 37˚C. One microliter (1 µL) of bacterial solution was centrifuged at 10,000 rpm for 5 minutes. One hundred microliters of the supernatant were added into the wells. The plates were then incubated at 30˚C, and the results were recorded after 24 hours. The antibacterial properties of LAB were determined by measuring the diameter of the inhibition zone (d) around the wells and classified as follows: weak resistance (+) when $d<5$ mm, moderately resistant (++) when $5 \leq d \leq 10$ mm, strong resistant (+++) when $d>10$ mm, and no resistance (-) when $d \leq 2$ mm. In this study, isolate LABB exhibited the strongest resistance against *A. schubertii* was chosen for hemolytic test, acid resistance, bile salt tolerance, extracellular enzyme activity, antibiotic susceptibility, and gene sequencing.

Hemolytic ability

The hemolytic activity of LAB isolates was tested according to Mangia et al. (2019) by using Blood Agar (BA) medium (Nam Khoa, Vietnam). The BA plates, after supplementing with 5% sheep blood, were streaked with LAB isolates and then incubated at 37°C for 48 hours. The hemolytic activity (alpha, α), beta (β), and gamma (γ) was assessed by observing the clearing zones surrounding the colonies.

Acid resistance

The bacterial strain was tested for acid tolerance following Khochamit et al. (2015). Briefly, bacterial solutions were prepared with a density of 10^8 CFU/mL and then introduced into NB medium that had been adjusted for pH 2.0 and 3.0. After that, the mixture was agitated at 200 rpm and incubated at 37°C. To ascertain the cell counts after one and three hours, the samples were spread out onto TSA medium and cultured for twenty-four hours at 37°C. The amounts of cells that survive after the test period was used to calculate acid tolerance.

Bile salt tolerance

The bile salt tolerance of LAB isolates was assessed according to Menconi et al. (2014). LAB isolates were spread onto MRS agar containing 0%, 0.3%, or 0.5% bile salts to evaluate the presence or absence of growth in both the control (bile salt-free) and experimental cultures at 1, 3, and 24 hours.

Extracellular enzyme activity

As stated by Dogan and Taskin (2021), the capacity to break down cellulose, protein, fat, and starch was demonstrated. After centrifuging the bacterial suspension at 12,000 rpm for 15 minutes, the supernatant was collected. To detect cellulase, protease, amylase, and lipase, 60 microliters of the supernatant were placed into wells (diameter 6 mm) on MRSA enriched with 1% CMC (carboxymethylcellulose), 0.5% casein, 1% Tween 20, and 1% starch. The cellulolytic, proteolytic, amylolytic, and lipolytic activities were assessed by flooding the plates with Congo red, Trichloroacetic Acid (TCA), and Lugol's solution following a 24-hour incubation period at room temperature.

Antibiotic susceptibility

According to Bauer et al. (1966), the Kirby-Bauer disk diffusion method was used to evaluate the antibiotic susceptibility of LAB on MHA (Mueller Hinton Agar, HiMedia, India). Twenty antibiotics (Nam Khoa, Vietnam) were tested, including ceftazidime (CTX/30 µg), ampicillin (AMP/10 µg), chloramphenicol (CHL/30 µg), penicillin (PEN/5 µg), ampicillin/sulbactam (AMS/10/10 µg), clindamycin (CLI/2 µg), doxycycline (DOX/30 µg), tetracycline (TET/30 µg), erythromycin (ERY/15 µg), levofloxacin (LEV/5 µg), sulfamethoxazole/trimethoprim (SXT/1.25/23.75 µg), ciprofloxacin (CIP/5 µg), gentamicin (GEN/10 µg), kanamycin (KAN/30 µg), neomycin (NEO/30 µg), nalidixic acid (NAL/30 µg), norfloxacin (NOR/5 µg), ofloxacin (OFL/5 µg), streptomycin (STR/10 µg), and vancomycin (VAN, 30 µg). Single bacterial colonies suspended in 0.85% saline solution to turbidity matching McFarland 0.5 were used to disseminate the MHA plate. After that, the plates were incubated at 30°C for 24 hours. The isolates were categorized as sensitive (S) and resistant (R) based on the inhibition zone diameter (mm) as per the interpretative standards recommendations of the Clinical and Laboratory Standards Institute (CLSI 2020).

Lactic acid bacteria identification by polymerase chain reaction

16S rRNA fragments of LAB were amplified by PCR reactions with primer pairs 27F: 5'-AGAGTTTGATCMTGCTCAG-3' and 1492R: 5'-TACGGYTACCTTGTTACGACTT-3' (Heuer et al. 1997). In short, bacteria were grown in MRS broth medium at 28°C for 24 hours at 120 rpm. The bacterial biomass was then collected by centrifugation at 13,000 rpm for 5 minutes. Next, bacterial DNA was extracted using the AccuRive Bacteria DNA Prep Kit-EX-DNA04.1 A (Khoa Thuong Biotech, Vietnam) following the manufacturer's instructions. PCR reaction components include 12.5 µL iStandard iVAPCR Master Mix (Thermo Scientific, USA); 9.5 µL double distilled water; 0.5 µL primer 27F (25 pmol); 0.5 µL primer 1492R (25 pmol); and 2 µL of sample DNA. The thermal cycle to perform the PCR reaction includes stages: initial denaturation at 94°C for 5 minutes, then performing 30 cycles including denaturation at 94°C for 1 minute, primer annealing at 60°C for 1 minute, extension at 72°C for 2 minutes, and final extension at 72°C for 10 minutes. Finally, the LAB strain with the strongest resistance to *A. schubertii* was selected for sequencing at the DNA Sequencing Company (Vietnam).

Data analysis

Data are presented as mean±standard deviation (mean±SD). Data were statistically processed using Minitab 16.0 software. The mean value was subjected to one-way ANOVA according to Tukey's test with 95% confidence. The BLASTN tool was used to compare the sequence similarity of LAB strain sequences with bacterial sequences on the NCBI database. The CLUSTAL W program was used to compare bacterial sequences with one another. Using the neighbor-joining algorithm and a bootstrap value of 1,000 replications, the MEGA5 software was used to create the phylogenetic tree that illustrates the genetic links between bacterial strains.

RESULTS AND DISCUSSION

Isolation of lactic acid (LAB) bacteria

The study isolated twenty-three strains of LAB from pond water samples and snakehead fish intestines. No strain was collected from sediments. Of these, ten bacterial strains (43.48%) originated from water samples, and thirteen strains (56.52%) were isolated from snakehead fish intestines.

Basic morphological, physiological, and biochemical characteristics of lactic acid bacteria

The findings revealed that most of the colonies of isolated bacterial strains were round, convex elevations, yellow or opaque white (Figure 1.C and D), with colony size ranging from 0.5-2.0 mm after 48 hours of incubation on MRS agar medium. This investigation revealed that LAB isolates belong to the group of Gram-positive (Figure 1.E), non-spore-forming, non-motile, long or rod-shaped bacteria (Figure 1.E), and negative oxidase and catalase reactions.

Inhibitory activity of lactic acid bacteria against *Aeromonas schubertii*

Research results showed that eleven out of twenty-three isolated bacterial strains displayed the ability to resist *A. schubertii* (Figure 2.A). Among them, five out of eleven strains (45.45%) showed strong resistance (two strains, CMB1 and LABB, exhibited the strongest resistance), six out of eleven strains (54.55%) showed moderate resistance, and no LAB strain displayed weak resistance (Figure 2.B, Table 1).

Hemolytic ability

The results showed that strain LABB did not have a transparent or green area appearing around the colony on the blood agar medium. Therefore, strain LABB in this study exhibited no hemolytic or γ-type hemolytic activity.

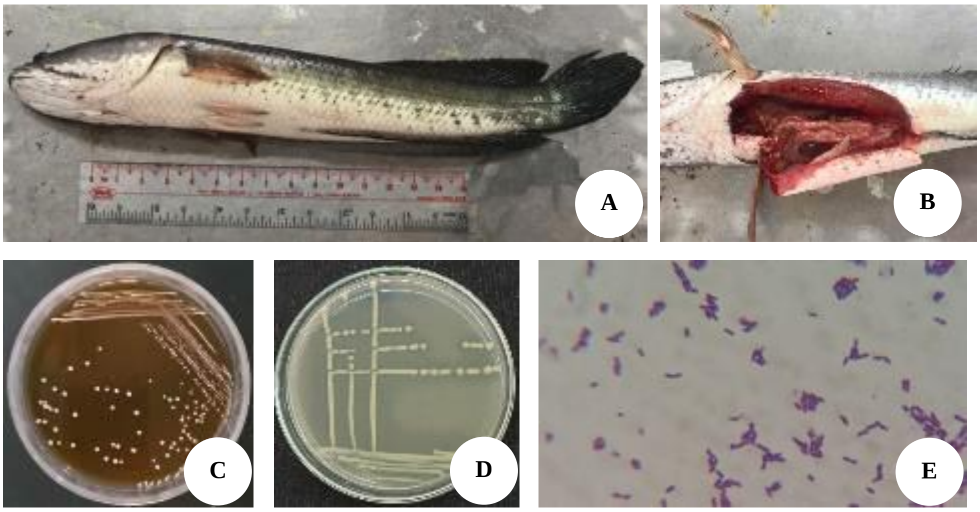

Figure 1. Morphological and biochemical characteristics of isolated lactic acid bacteria A and B: Snakehead fish in good health with no outward or internal symptoms of illness; C. The colony of isolate LABB; D. The colony of isolate CMB1; E: Positive Gram staining (100X)

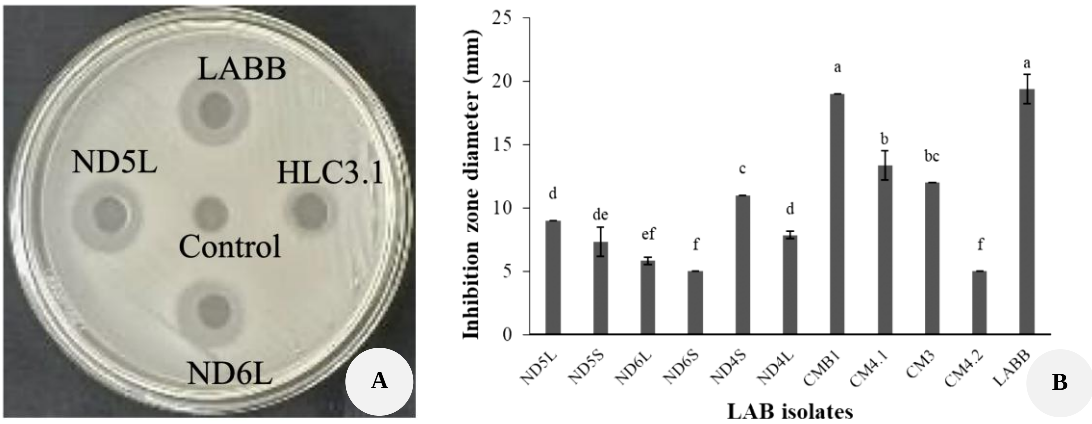

Figure 2. A. Antagonistic activity of lactic acid bacteria strains against *A. schubertii*. B. Bars with different letters indicate significant differences (p<0.5). Weak resistance (+) when d<5 mm, moderately resistant (++) when 5≤d≤10 mm, strong resistant (+++) when d>10 mm, and no resistance (-) when d≤2 mm

Table 1. Antibacterial capacity of lactic acid bacteria strains against *Aeromonas schubertii*

Isolated LAB strains	*A. schubertii* strain As11	Isolated LAB strains	*A. schubertii* strain As11
ND5L	++	CM5	-
ND5S	++	LABB	+++
ND6L	++	CN2.1	-
ND6S	++	CN2.2	-
ND4S	+++	HLC3.1	-
ND4L	++	HLC3.2	-
CMB1	+++	QC1	-
CM4.1	+++	CTC2.1	-
CM1	-	CTC2.2	-
CM3	+++	CTC1.2	-
CM2	-	CTC1.3	-
CM4.2	++		

Note: Weak resistance (+) when d<5 mm, moderately resistant (++) when 5≤d≤10 mm, strong resistant (+++) when d>10 mm, and no resistance (-) when d≤2 mm

Antibiotic susceptibility

The results showed that strain LABB was sensitive to ampicillin, ampicillin/sulbactam, chloramphenicol, clindamycin, doxycycline, erythromycin, levofloxacin, ofloxacin, and penicillin (Table 2). Meanwhile, this bacterial strain was resistant to sulfamethoxazole/trimethoprim, ciprofloxacin, ceftazidime, gentamicin, kanamycin, neomycin, nalidixic acid, norfloxacin, streptomycin, tetracycline, and vancomycin (Table 2).

Acid resistance

The results showed that strain LABB was not able to tolerate acid at pH 1.0 and 1.5. At pH 2.0 and 2.5, this bacterial strain can survive in media after 3 and 6 hours of incubation (Table 3). However, this bacterial strain can survive in media with pH 3.0 to 4.0 after 9 and 24 hours of culture (Table 3).

Bile salts tolerance

The findings demonstrated that strain LABB was capable of tolerating bile salts at concentrations of 0.1% and 0.3% after 24 hours of culture (Table 4). In particular, strain LABB was also capable of resisting bile salt at a concentration of 0.5% after 3 hours of incubation. At a concentration of 1%, strain LABB was also able to tolerate bile salts after 1.0 hours of culture. However, strain LABB was incapable of tolerating bile salts at a concentration of 1.5% and 2.0% (Table 4).

Extracellular enzyme activity

The results showed that strain LABB was capable of producing cellulase, amylase, and protease enzymes (Figure 3), while this bacterial strain did not produce lipase.

Identification of lactic acid bacteria by polymerase chain reaction

Electrophoresis results showed that all representative LAB strains with antagonistic activity against *A. schubertii* had a DNA band at a size of 340 bp (Figure 4).

Identification of lactic acid bacteria by polymerase chain reaction

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Table 2. Antibiotic susceptibility of isolate LABB

Antibiotics	Isolate LABB	Antibiotics	Isolate LABB
Ampicillin	S	Vancomycin	R
Ampicillin/Sulbactam	S	Kanamycin	R
Sulfamethoxazole/ Trimethoprim	R	Levofloxacin	S
Ciprofloxacin	R	Neomycin	R
Chloramphenicol	S	Nalidixic acid	R
Clindamycin	S	Norfloxacin	R
Ceftazidime	R	Ofloxacin	S
Doxycycline	S	Penicillin	S
Erythromycin	S	Streptomycin	R
Gentamicin	R	Tetracycline	R

Note: S: Sensitivity; I: Intermediate; R: Resistance

Table 3. The number of bacterial cells (CFU/mL) that persisted following low pH treatment of strain LABB

Low pH value	1.0	1.5	2.0	2.5	3.0	3.5	4.0
1 hour	0	0	131	>200	>200	>200	>200
3 hours	0	0	8	>200	>200	>200	>200
6 hours	0	0	0	27	>200	>200	>200
9 hours	0	0	0	0	12	>200	>200
12 hours	0	0	0	0	0	>200	>200
24 hours	0	0	0	0	0	>200	>200

Table 4. Number of bacterial cells (CFU/mL) that survived after strain LABB was treated with different bile salts

Bile salt (%)	0.10%	0.30%	0.50%	1%	1.50%	2%
1 hour	>200	>200	>200	>200	0	0
3 hours	>200	>200	>200	0	0	0
6 hours	>200	>200	0	0	0	0
9 hours	>200	>200	0	0	0	0
12 hours	>200	>200	0	0	0	0
24 hours	>200	65	0	0	0	0

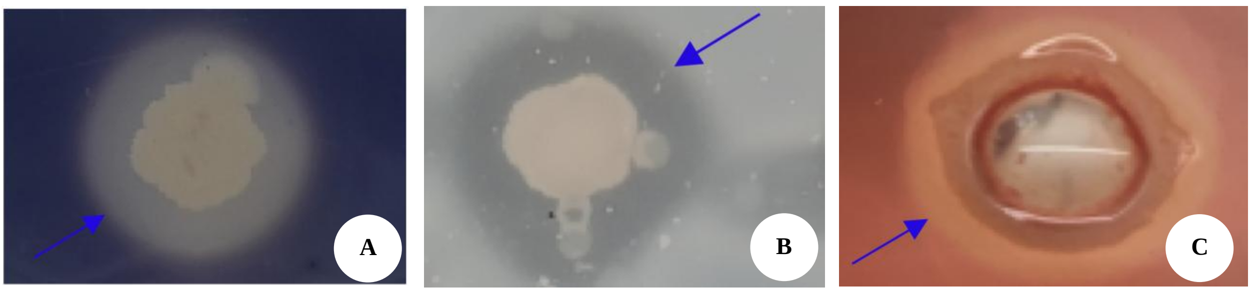

Figure 3. Protease and amylase activity of isolated isolates. A. Amylase activity; B. Protease activity; C. Cellulase

To sequence the 16S rRNA gene, the two LAB strains, CMB1 and LABB, that exhibited the highest resistance to *A. schubertii* were chosen. According to the 16S rRNA gene segment gene sequencing results, strain CMB1 shared 100% of its similarities with *Lactiplantibacillus plantarum* (*L. plantarum*) strain CT6.2 (OQ842246.1), 99.35% with *L. plantarum* strain BL6_Lac1 (OM368499.1), 99.67% with *Lactobacillus plantarum* (*L. plantarum*) strain LBH1064 (KT000588.1), 99.34% with *L. plantarum* isolate LP34C (EF439684.1), and 99.02% with *L. plantarum* strain RU22301 (KC836544.1). In contrast, the LABB strain shared 100% of its similarities with *L. plantarum* strain LBH1063 (KT000587.1), 99.68% with *L. plantarum* strain CT6.2 (OQ842246.1) and *L. plantarum* isolate LP34C (EF439684.1), 99.67% with *L. plantarum* strain BL6 (OM368512.1), and 99.35% with *L. plantarum* strain RU22301 (KC836544.1). According to the phylogenetic tree (Figure 5), strains CMB1 and LABB were closely related to *L. plantarum*. As a result, it helped confirm and precisely identify the isolated bacteria using morphological, physiological, and biochemical traits in conjunction with PCR and gene sequencing.

Discussion

LAB is commonly present in natural environments, and they can exist in many different ecological regions (Holzapfel and Wood 2014; Liu et al. 2024). Many previous studies showed that most of the LAB are present in traditional fermented products such as fermented vegetables (Tamang et al. 2016). In this study, twenty-three strains were obtained from snakehead fish guts and fermented vegetables. The colony morphological, physiological, and biochemical traits of isolated LAB strains were similar to those of described LAB by many previous studies (Goa et al. 2022). Nofiani et al. (2022) showed that these features of LAB strains obtained from traditionally fermented fish were Gram-positive, catalase-negative, and non-motile. Additionally, 16S rRNA gene sequencing and phylogenetic analysis showed that strain LABB, which displayed the strongest inhibitory activity, was classified as *Lactiplantibacillus* (previously known as *Lactobacillus*) (Rocchetti et al. 2021). This research was in line with many earlier studies that showed this genus was obtained from many fish species (Qiu et al. 2024). Without endangering the host, LAB species settle in and establish themselves as the typical microbiota in the intestines of healthy fish (Talwar et al. 2018).

The effect of inhibiting the growth of pathogenic microorganisms, helping to balance the intestinal microflora, and limiting gastrointestinal diseases is one of the important properties of probiotics (Chandra et al. 2024). Research results show that 11 isolated bacterial strains have inhibitory activity against *A. schubertii*. These findings were in line with many studies presenting that LAB displayed antibacterial activity against many Gram-positive and Gram-negative bacteria (Amin et al. 2020; Van Doan et al. 2021). Quach et al. (2023) found that LAB reduced *A. hydrophila* and *Streptococcus agalactiae*, which induce hemorrhagic skin illness and enlarged eyes in caged red tilapia (*Oreochromis* sp.) in Vinh Long Province.

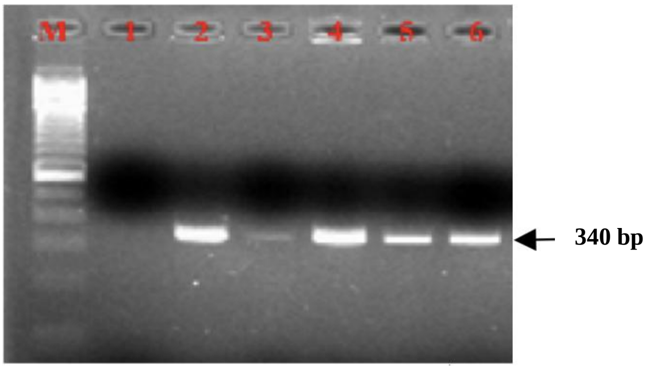

Figure 4. PCR products of presumptive LAB isolates on 1.5% agarose gel. M: 100 bp DNA standard ladder; Lane 1: Negative control; Lanes 2-6: Bacterial strains ND5L, ND4S, CMB1, LABB, and CM4.1, respectively

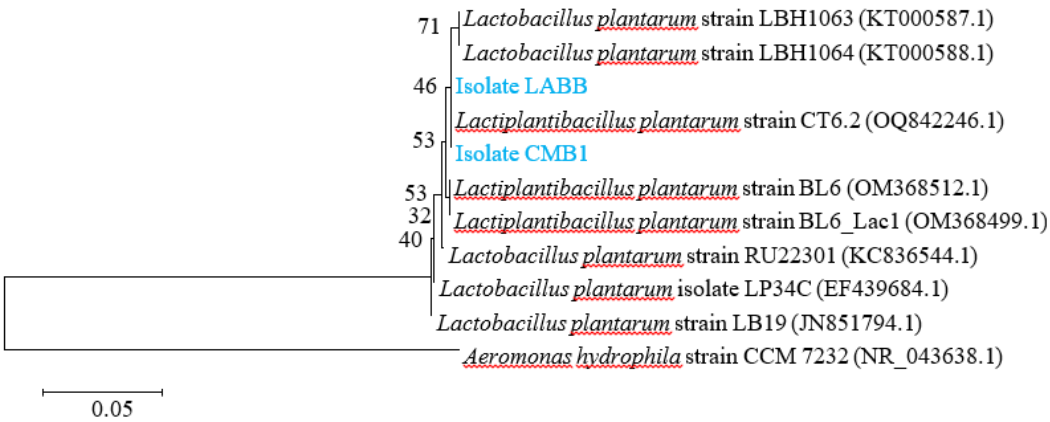

Figure 5. Phylogenetic tree showing genetic relationships between two isolated LAB isolates (isolate CMB1 and LABB) with reference strains on GenBank using *Aeromonas hydrophila* strain CCM 7232 (NR_043638.1) as an outgroup, the neighbor-joining algorithm, and a bootstrap value of 1,000 repetitions

However, the bacterial strains in this study showed different strong and weak inhibitory activities. Ibrahim et al. (2021) demonstrated that LAB generates organic acids, including lactic, acetic, and formic acids, which lower pH and prevent the growth of pathogens. Additionally, LAB produces many antibacterial substances, including bacteriocins that may be due to differences in bacterial strains and environmental conditions between them. Therefore, further studies are needed to clarify this issue, such as evaluating the effects of pH, temperature, nitrogen, and carbon sources on the antibacterial activity of strain LABB.

Hemolytic activity is an important criterion and is recommended to evaluate the safety of a bacterial strain with probiotic characteristics before investigating other probiotic properties to ensure that the bacterial strain does not have toxic potential (Banerjee et al. 2017). Hemolytic-positive bacteria are considered unsuitable for use as probiotics. In the current study, the LABB strain showed no hemolytic activity. This research was similar to earlier reports showing that LAB isolates also did not have hemolytic activity (Halder et al. 2017; Azevedo et al. 2024). In aquaculture, research by Amin et al. (2020) showed that all LAB from the abalone's gastrointestinal tracts produced no hemolytic activity. Similarly, Lingga et al. (2023) showed that all LAB strains from slender walking fish (*Clarias nieuhofii*) exhibited no hemolysis activity.

According to the findings, strain LABB was susceptible to penicillin, levofloxacin, ofloxacin, erythromycin, clindamycin, doxycycline, ampicillin, ampicillin/ sulbactam, and chloramphenicol. Coulibaly et al. (2023), who found that the LAB isolates from *O. niloticus* fish intestines exhibited intermediate resistance or susceptibility to antibiotics, such as chloramphenicol, amoxicillin, cephalothin, kanamycin, imipenem, penicillin, rifampicin, streptomycin, and tetracycline, and resistance to ciprofloxacin, gentamicin, and oxacillin. In the meanwhile, this strain has shown resistance to vancomycin, ciprofloxacin, ceftazidime, gentamicin, neomycin, nalidixic acid, norfloxacin, streptomycin, tetracycline, kanamycin, and ciprofloxacin. In the study of Mazlumi et al. (2022), it was shown that strain F18 was resistant to all the used antibiotics, like ciprofloxacin, ampicillin, vancomycin, erythromycin, azithromycin, gentamicin, penicillin, chloramphenicol, tetracycline, and streptomycin. A study by Mazlumi et al. (2022) also revealed that the LAB isolates exhibited the greatest resistance to ciprofloxacin, penicillin, and gentamycin, which were found in 57.9% of the isolates, while isolate F18 had the least resistance to tetracycline.

Another necessary characteristic in selecting bacterial strains that produce probiotics is the ability of the bacteria to survive under the influence of low pH in the digestive system (Sayes et al. 2018). The pH in the small intestine in the stomach of animals usually has a low pH; for fish, it is usually between 2-3. Hence, the strains of microorganisms that grow in the fish intestine can tolerate low pH. In this study, strain LABB was able to survive at pH levels 2.0 and 3.0 for a longer period (3 hours). These findings were consistent with some earlier studies where LAB isolates with probiotic properties could resist low pH (Bektas et al. 2020). A study by Agustina et al. (2022) demonstrated that LAB isolates from the intestines of repang fish (*Puntioplites waandersi*) could still live in media with an acid-base pH (pH 2-8).

Another challenge to microbial survival in the gastrointestinal tract is the presence of bile salts in the fish's small intestine. Bile has the effect of destroying microorganisms thanks to the action of destroying microbial cell membranes. A bile concentration of 0.3% is often used to select bile-resistant probiotic strains because this concentration is considered the average bile concentration in the fish intestine. The LABB strain in this study showed the ability to grow at 0.3% bile concentration. This investigation was consistent with the finding of Coulibaly et al. (2023), who found that the isolates of LAB from tilapia (*O. niloticus*) fish intestines were capable of growing in a 0.3% bile salt environment. Another research study by Mazlumi et al. (2022) demonstrated that at pH 3.0 with 0.3% bile oxgall, LAB strains F3, F7, F12, and F15 from the gut of saltwater fish demonstrated greater than 90% viability.

The capacity to produce extracellular enzymes is considered as one of the crucial factors to consider while choosing bacterial strains to be probiotics (Sayes et al. 2018). Cellulase, amylase, and protease are examples of extracellular enzymes that enhance food digestion, facilitate easy absorption, and aid in animals' healthy weight gain (Liang et al. 2022). The results showed that strain LABB exhibiting the ability to produce cellulase, amylase, and protease enzymes, while this bacterial strain does not produce lipase. This work was also in line with some earlier studies showing that LAB can produce extracellular enzymes to degrade substrates (Nofiani et al. 2022). According to Govindaraj et al. (2021), LAB strains were obtained from freshwater fishes like *C. striata*, *O. mossambicus*, and *Rasbora daniconius*, displaying extracellular enzyme secretions such as lipase, amylase, and protease. However, according to Coulibaly et al. (2023), the LAB isolates from the intestines of tilapia (*O. niloticus*) fish did not exhibit any activity for the cellulases, amylases, or proteases, respectively, whereas, the nine LAB strains that were examined had good results for β-galactosidase and lipases. Extracellular enzymes like amylase and protease enzymes are necessary for probiotics to have a digestive effect because these enzymes can produce a variety of amino acids, sugars, organic acids, and low molecular compounds (Mokrani and Nabti 2024).

The beneficial effects of using *Lactiplantibacillus* as a probiotic in aquaculture have also been demonstrated previously (Iorizzo et al. 2022; Liu et al. 2024). According to a study by Liu et al. (2024), the growth and survival rates of large yellow croakers following a *Pseudomonas plecoglossicida* PQLYC4 challenge were considerably enhanced by the dietary supplementation of *L. plantarum* E2, which is derived from the intestinal tract of the large yellow croaker, *Larimichthys crocea*. However, the effectiveness of the LABB strain in snakehead fish disease control caused by *A. schubertii* in this study has not been evaluated in the field. Therefore, further studies need to be performed to clarify this issue.

In conclusion, the findings showed that eleven of the twenty-three strains of LAB isolated from samples of fermented vegetables and snakehead fish guts exhibited antagonistic activity against *A. schubertii* in terms of probiotic traits. According to the investigation's morphological, physiological, biochemical, and 16S rRNA gene sequencing data, strain LABB exhibiting the strongest antibacterial activity was identified as *Lactiplantibacillus*. In this experiment, strain LABB was able to survive in media with a pH between 2.0 and 4.0 after three to nine hours of incubation. After incubation for 24 and 3 hours, strain LABB in particular demonstrated the capacity to tolerate bile salts at concentrations of 0.3% and 0.5%. The results showed that strain LABB was capable of producing extracellular enzymes such as cellulase, amylase, and protease. Furthermore, this bacterial strain was sensitive to penicillin, levofloxacin, ofloxacin, erythromycin, clindamycin, doxycycline, ampicillin, ampicillin/ sulbactam, and chloramphenicol.

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