

ISOLATION AND CHARACTERIZATION OF LACTOBACILLUS FROM GRASS CARP (*Ctenopharyngodon idellus*) FOR USE AS PROBIOTIC IN AQUACULTURE

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ABSTRACT

In aquaculture, probiotics are given directly to animals to regulate their gut bacteria, enhance their immune systems, and aid digestion. Additionally, probiotics are introduced into the pond's water to enhance the water quality and increase the chances of survival for aquatic creatures. *Lactobacillus* belongs to the group of lactic acid bacteria (LAB), which are considered beneficial for aquaculture. Incorporating LAB into fish feed can enhance its nutritional quality. LAB produces various digestive enzymes that aid in food breakdown and digestion, promoting growth to the host. In this study, 95 isolates of lactic acid bacteria were recovered in the gut of grass carp in Thai Binh, Bac Giang and Nam Dinh province, Vietnam. Only 9 of 95 isolates had the inhibition effects on *Aeromonas hydrophila* and *Staphylococcus aureus*. The isolates L18 showed the strongest antimicrobial activity to *Aeromonas hydrophila* and *Staphylococcus aureus* with the diameter of antimicrobial zones were 18.22 ± 0.16 mm and 10.14 ± 0.23 mm, respectively. The strain L18 was identified as *Lactobacillus fermentum* based on its morphological, physiological, biochemical characteristics, and 16S rRNA sequences. The *L. fermentum* L18 is capable of withstanding concentrations of bile salts ranging between 0.0% and 0.5%. Moreover, it can tolerate stomach acid with a pH range of 1 to 4. Furthermore, the bacterial *L. fermentum* strain L18 can produce extracellular enzymes that include cellulase and amylase, which are responsible for breaking down starch and cellulose substrates. Thus, the *Lactobacillus* L18 strain could be considered a potential antimicrobial probiotic strain against fish pathogens and should be further studied for its probiotic benefits.

Keywords: Aquaculture, probiotic, *Lactobacillus fermentum*.

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1. INTRODUCTION

The fisheries and aquaculture sectors have been increasingly recognized for their essential contribution to global food security and nutrition in the twenty-first century. In 2020, farmed finfish reached 57.5 million metric tons (USD 146.1 billion), including 49.1 million metric tons (USD 109.8 billion) from inland aquaculture and 8.3 million metric tons (USD 36.2 billion) from mariculture in the sea and coastal aquaculture on the shore [1]. This shows that farmed aquatic animal species have grown steadily over the past

decade. The industry is expected to reach over USD 120 billion in 2022. Efforts to intensify aquatic animal production have led to the excessive use of various antimicrobial agents, the adverse side effects of which have become uncomfortable and apparent to both producers and consumers. To fight those stressors and their negative impacts, probiotics have become recognized as effective substitutes, acting as immunity modulators and increasing resistance to various microbial pathogens [2, 3].

In aquaculture, probiotics confer several benefits and play significant roles in improving growth performance, disease resistance, immunity, health status, intestinal epithelial barrier integrity, gut microbiome, and water

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quality. In addition, probiotics in aquaculture diets could minimize antibiotic side effects. Probiotics are live beneficial bacteria introduced into the gastrointestinal tract through food or water, promoting overall health by enhancing internal microbial balance. Probiotic microbes produce bacteriocins, siderophores, lysozymes, proteases, and hydrogen peroxides, inhibiting harmful pathogens [4]. Probiotics stimulate the immune system, provide essential nutrients, and increase growth rates and feed efficiency. They can also improve water quality and reduce environmental pollution.

The genus *Lactobacillus* is the largest genus among lactic acid bacteria (LAB), consisting of more than 237 species [5], with a continuous diversity of species discoveries. *Lactobacillus* species are among the most widely used probiotics [2]. The mechanisms of action of *Lactobacillus* in aquaculture include the secretion of antagonistic compounds [6, 7], effects on quorum sensing mechanisms [8, 9], inhibition of adhesion and colonization by pathogenic bacteria via competitive exclusion [3], modulation of gut microbiota and immune reactions [10], antiviral effects [11], and the improvement of water quality and modulation of the aquatic microbiota [4, 12]. This research aimed to isolate, characterize, and identify the *Lactobacillus* isolates isolated from the intestine of grass carp to explore their probiotic properties and the potential of using them as probiotic products production.

2. MATERIAL AND METHOD

2.1. Isolation of microorganisms

A total of twenty-nine separate samples of fish guts from grass carp (*Ctenopharyngodon idellus*) were collected from local markets in Thai Binh, Bac Giang, and Nam Dinh, stored at 4°C by ice to avoid contamination and spoilage during transportation to the laboratory of Faculty of Biotechnology, Vietnam National University of Agriculture [13]. For the isolation of microorganisms, we followed the protocol described by Karami *et al.* (2017) [14] with some modifications. Fish gut samples were finely

ground (one gram) and mixed with 99 ml of sterile physiological saline to release the microbiota in each sample. After homogenization, the samples were spread on a plate of MRS agar (g/l: Glucose 20.0, Meat extract 10.0, yeast extract 5.0, peptone 10.0, Tween 80 1 ml, triammonium citrate, 2.0, $K_2HPO_4 \cdot 3H_2O$ 2.0, CH_3COONa 5.0, $MgSO_4 \cdot 7H_2O$ 0.58, $MnSO_4 \cdot 4H_2O$ 0.28, $CaCO_3$ 5.0, agar 15.0, pH = 6.5 ± 0.2) incubated at 30°C for 48 hours under aerobic conditions. From the isolated samples, strains of bacteria with typical shapes for *Lactobacillus* bacteria (small colonies, opaque white or colorless, and capable of decomposing $CaCO_3$) were selected, and primary biochemical characteristics such as Gram staining, oxidase and catalase test were performed according to Karami *et al.* (2017) [14].

2.2. Antimicrobial activity assay

The antibacterial activity was detected against different indicator strains (*Aeromonas hydrophila* and *Staphylococcus aureus*) by the agar-well diffusion method [15]. *Aeromonas hydrophila* and *Staphylococcus aureus*, obtained from diseased carp samples which were collected in Hung Yen province, were preserved, and stored at the laboratory of the Department of Microbial Technology, Faculty of Biotechnology, Vietnam National University of Agriculture. The overnight culture of 95 lactic acid bacteria isolates were centrifuged at 10,000 rpm for 10 minutes at 10°C. 100 μ L of the cell-free supernatants (CFS) of culture broth were added to 5 mm wells on nutrient agar plates with were previously spread with a 100 μ L suspension of *Aeromonas hydrophila* or *Staphylococcus aureus*, the plates were incubated at 30°C for 12 hours. The antimicrobial activity was expressed as the diameter of the inhibition zones (mm) around the wells. The presence of clear zones was an indication of the activity in the tested samples against the two pathogens.

2.3. Growth at different pH concentrations

Lactobacillus strains were grown at 30°C. The culture broth was then centrifuged at 10,000 rpm for 10 minutes to collect the cell pellet. The pellets

were then washed with distilled water to remove the residue and then added into MRS broth with a pH of 1 to 4. After 3 hours, 6 hours, and 9 hours. The OD values of cultures were determined at 620 nm every 3 hours by a UV-vis spectrophotometer [16]. Acid tolerance was determined based on ΔOD value > 0 (the difference between the OD value measured at 9 h and the OD value measured at 3 hours) at each pH concentration. The absorbance values obtained were plotted against the incubation time, and the acid tolerance of the strain was based on the time required for the absorbance value to increase by 0.3 units.

2.4. Growth at different bile concentrations

Lactobacillus strains were incubated in 3 ml of MRS broth supplemented with bile salts (Thermo Fisher Scientific, US) of 0.0%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, and 0.8%. The bile salt tolerance of *Lactobacillus* strains was measured by OD at 600 nm after 0 hour, 12 hours, 24 hours, and 48 hours [17].

2.5. Genetic identification of bacteria

In this current study, the DNA of the *Lactobacillus* strain was extracted using the method of Han *et al.* (2018) [18]. PCR for the amplification of the 16S rRNA gene was carried out using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTGTGTGTGTTACGA CTT-3'). The PCR conditions were: initial denaturation

of 5 min at 95°C, followed by 30 cycles of 1 min at 94°C, 1.5 min at 53°C and 1 min at 72°C, and a final extension at 72°C for 10 min. PCR reactions were stored at 4°C. The PCR products underwent sequencing at the 1st BASE, Singapore. The sequences obtained were compared with the NCBI database through BLAST searches (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The phylogenetic tree was constructed using Mega X software based on the 16S rDNA sequences of the strain curated and the homologous sequences of other strains.

2.6. Determination of microbial extracellular enzyme activity

The extracellular enzyme production of selected bacteria was performed by plate assay according to Hmani *et al.* (2017) [19], with some modifications. Amylase and cellulase production were determined using Luria-Bertani (ThermoFisher Scientific, US) agar plates supplemented with 1% starch and 1% CMC, incubated for 24 hours at 30°C. The clear zone around the agar well revealed enzyme production.

2.7. Statistical analyses

Data were analyzed in Excel version 16.76.1 (Microsoft) using a statistical software package MIX2.0. All statistical analyses were performed using GraphPad Prism 9.0.2.

3. RESULTS AND DISCUSSION

3.1. Isolation of *Lactobacillus* bacteria

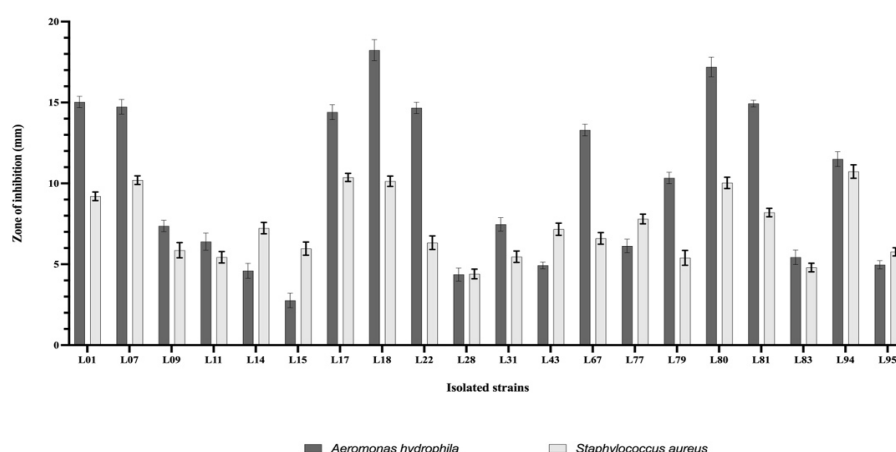


Figure 1. Diameter of inhibition zone (mm) of different *Lactobacillus* isolates against *Aeromonas hydrophila* and *Staphylococcus aureus*

From 29 different collected fish gut samples, 95 bacterial isolates with typical characteristics of lactic acid bacteria, such as milky white color and CaCO_3 resolving rings, were recovered. All isolates were Gram-positive, catalase-negative, and oxidase-negative. In general, all isolates were rod shaped. Those characteristics suggested the isolates corresponded to the LAB group. Thirty-six strains of bacteria with *Lactobacillus* characteristics are bacilli, while the remainder are cocci.

3.2. Screening of *Lactobacillus* strains for antimicrobial activity

Among the isolates that exhibited inhibition properties against targeted microorganisms

(*Aeromonas hydrophila* and *Staphylococcus aureus*), 20 strains showed antimicrobial activity (Figure 1). The *Lactobacillus* strain L18 showed the strongest antibacterial activity with the largest antimicrobial zone diameter to *Aeromonas hydrophila* (18.22 ± 0.16 mm) and *Staphylococcus aureus* (10.14 ± 0.23) mm, respectively (Figure 3). This strain was identified as *Lactobacillus*, and it exhibited a broad spectrum of antibacterial activity. This study suggests that *Lactobacillus* L18 could be a promising natural antimicrobial agent. This finding indicates the potential of *Lactobacillus* L18 to be used in the development of novel and effective antibiotics to combat bacterial infections.

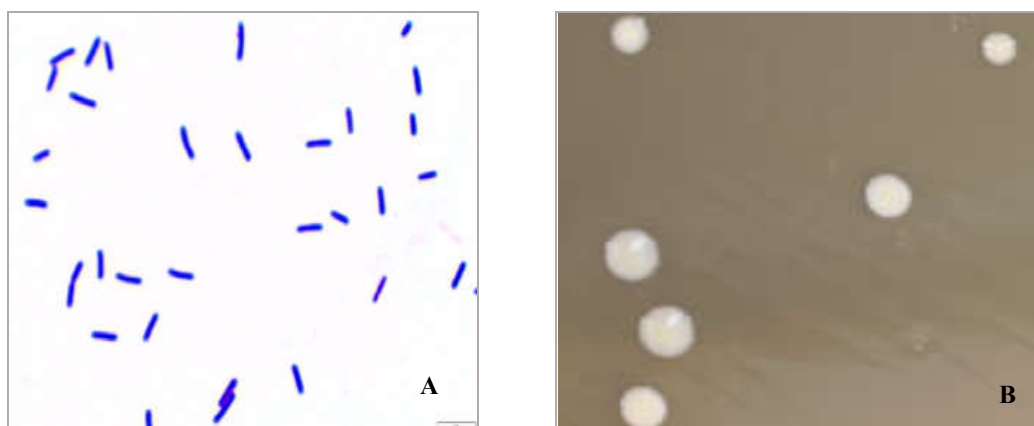


Figure 2. Bacilli shape, Gram positive isolate L18 with 1000-times magnification (A) and white and convex colonies of isolates L18 on MRS agar (B)

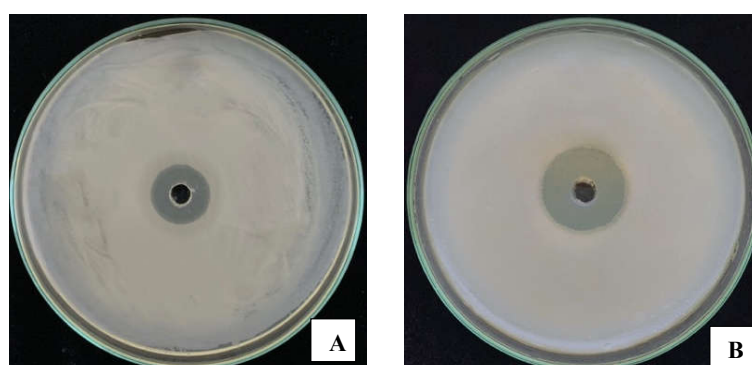


Figure 3. Antagonism of the *Lactobacillus* strain L18 to *Staphylococcus aureus* (3A) and *Aeromonas hydrophila* (3B)

This result is relatively consistent with Koohestani *et al.* (2018) [20] who investigated the resistance to *Staphylococcus aureus* of *Lactobacillus acidophilus* and *Lactobacillus casei*. They showed the ability to inhibit *Staphylococci* of *Lactobacillus acidophilus* with an antimicrobial zone that measures 16 mm, and *Lactobacillus casei* measures 13 mm. Their results indicate that *L. acidophilus* is more effective in inhibiting *Staphylococci* than *L. casei*. Furthermore, it was verified that these two strains of *Lactobacillus* can be used to reduce the presence of *Staphylococcus aureus* in food. In 2020, Ma *et al.* (2020) [21] investigated the resistance to *Staphylococcus aureus* ATCC 25923 of *Lactobacillus casei* strain KLDS 1.0338 with an antimicrobial zone diameter of 10.64 ± 0.15 mm. Additionally, Ma *et al.* (2020) also showed that *Lactobacillus casei* strain KLDS 1.0338 had superior inhibitory activity against *Escherichia coli* ATCC 25922, with an antimicrobial zone diameter of 11.64 ± 0.14 mm [21].

Our findings suggest that *Lactobacillus* strain L18 is a promising probiotic with the potential to improve gut health. However, further experiments need to be done to understand the long-term effects of this probiotic on gut health, how *Lactobacillus* L18 exhibits its antimicrobial activity, what is the dosage and delivery method for this probiotic for optimal gut health.

3.3. Effect of pH on the *Lactobacillus* bacteria

Gastric juice in the stomach is considered one of the first challenges faced by probiotic bacteria due to the low pH and antibacterial effects of pepsin [16, 22]. Probiotic bacteria are also exposed to bile salts and fatty acids that reduce their viability [23]. To survive these conditions, probiotic bacteria must be robust and highly resistant to harsh environmental conditions. Probiotic strains must survive in the gastrointestinal tract at an intestinal pH of 2.5 to 3.5, and they persist in the stomach for 2 - 4 hours or more before being transported to the intestinal tract.

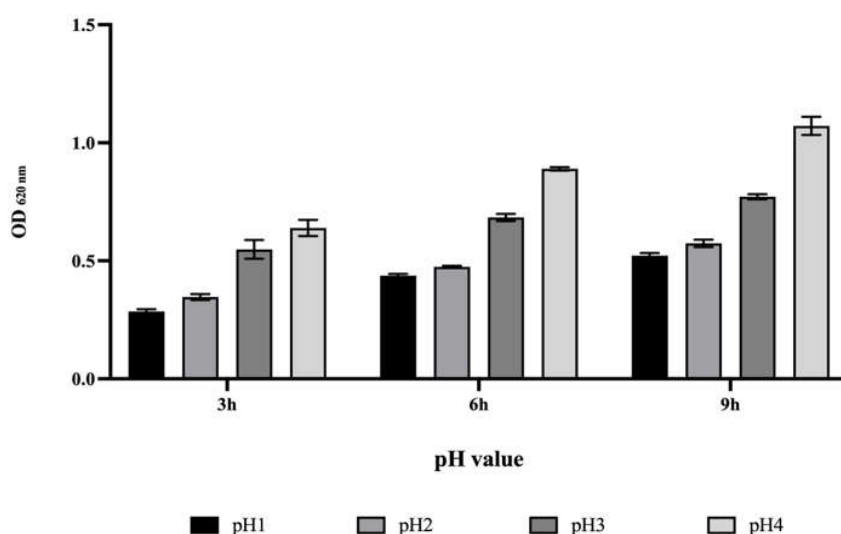


Figure 4. Acid tolerance of *Lactobacillus* strain L18

The bacterial *Lactobacillus* strain L18 was grown in MRS broth for 24 hours at 30°C and centrifuged at 5000 rpm for 5 min. Wash the cell residue with distilled water and then put it in a MRS broth with a pH range from 1 to 4. And monitor the results after 3 hours, 6 hours, and 9 hours.

The experimental results showed that the *Lactobacillus* strain L18 increased steadily in the pH range from 1 to 4 over 3 to 9 hours. At pH 4, the *Lactobacillus* strain L18 grew the fastest (Figure 4). This result is consistent with the author's study [24] that investigated the tolerance to stomach acid with a pH of 2 to 4 for 4 hours of

Lactobacillus plantarum LZ95 and *Lactobacillus plantarum* CY3. The results showed that the gastric acid tolerance of the two strains was not affected in the pH range of 2 to 4 for 2 hours. After 4 hours, 2 strains of *Lactobacillus plantarum* LZ95 and *Lactobacillus plantarum* CY3 showed a significant increase in their viability in the pH range of 2 - 4. The *Lactobacillus plantarum* strain CY2 viability was significantly impaired after 4 hours at pH 2 and pH 3 (60% loss of viability). The *Lactobacillus plantarum* strain CY2 viability was significantly affected after 4 hours at pH 4 (85% loss of viability). However, all three strains showed similar viability at pH 4 after 6 hours. These results suggest that *Lactobacillus plantarum*

strains are moderately acid-tolerant. For example, the *Lactobacillus plantarum* strain CY2 showed 60% viability loss after 4 hours at pH 2, compared to 85% viability loss at pH 4.

According to the study of gastric acid tolerance, *Lactobacillus* strain L18 showed excellent stomach survival. Furthermore, *Lactobacillus* L18 displayed a higher ability to tolerate simulated gastric acidity than other *Lactobacillus* strains. It also has strong anti-inflammatory activity. This suggests that *Lactobacillus* strain L18 could be a promising probiotic for gastrointestinal diseases.

3.4. Effect of bile on the *Lactobacillus* bacteria

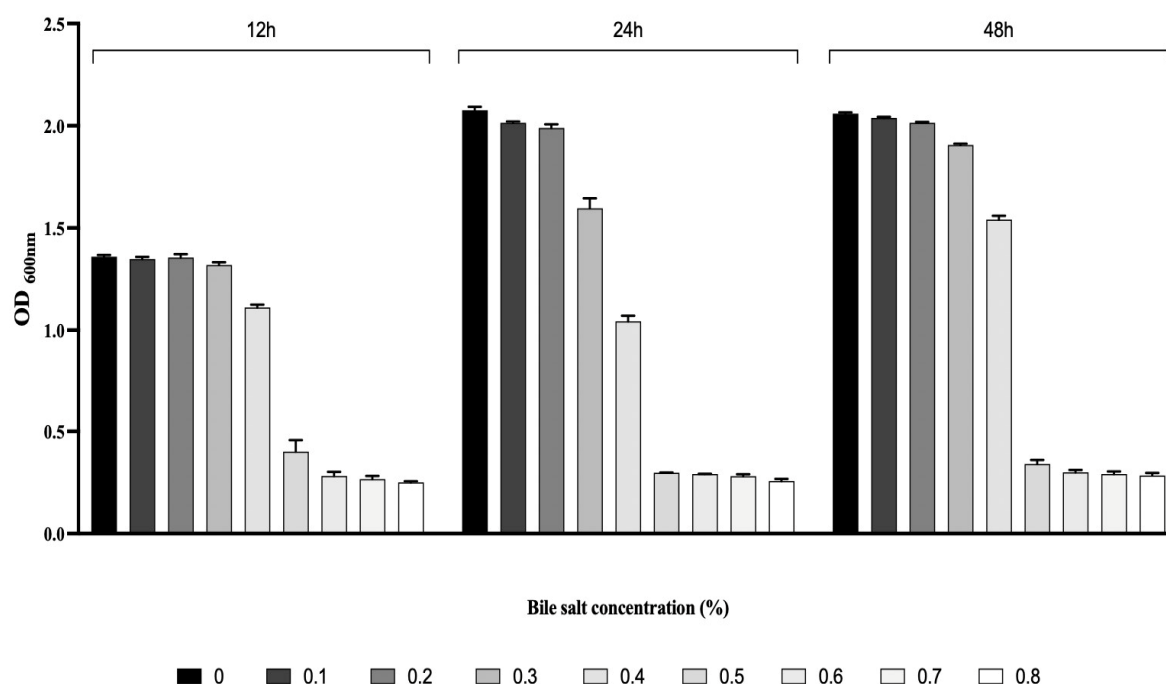


Figure 5. Bile tolerance of *Lactobacillus* strain L18

To exert beneficial effects in the gastrointestinal tract, probiotic bacteria must be able to pass through the stomach and tolerate concentrations of bile salts in the small intestine [25]. Bile salts play an essential role in lipid emulsification and also have antibacterial activity. Therefore, probiotic bacteria's ability to withstand bile salts is essential for survival, adhesion, and colonization in the small intestine. Some probiotic strains possess bile salt hydrolase activity, which

allows them to break down bile salts and survive in the intestine.

The results showed that strain L18 grew well under bile salt conditions ranging from 0% to 0.2%. The OD values decreased with increasing bile salt concentrations between 0.5% and 0.8%. Thereby, it can be seen that high bile salt concentrations inhibit the growth and development of strain L18. Experimental results showed that *Lactobacillus* strain L18 can resist bile salts in the

range of 0% to 0.4%. The tolerance to bile salts gradually increased from 0.1% to 0.3% after 12 to 48 hours of culture. At 0.5%, there was growth from 12 to 24 hours but a decline at 48 hours. In addition, bacterial *Lactobacillus* strain L18 has poor tolerance to bile salts in the concentration range of 0.6 - 0.8%. At higher concentrations, strain L18 growth was inhibited (Figure 5).

The results showed that the optimal concentration of bile salts for strain L18 was 0.3 - 0.5%. The results provide insight into strain L18 bile salt tolerance. The results indicate that the *Lactobacillus* strain L18 is a promising probiotic candidate for use in bile-containing environments. Further studies should be conducted to

understand the mechanisms underlying its bile resistance.

This result is consistent with Hassan *et al.* (2020) [6], which investigated *Lactobacillus* salt tolerance in the range of 0 - 1%. The results showed that the bile salt tolerance of the bacterial strains was highest in the range of 0.05–0.3%. However, bacterial strains are less tolerant at high concentrations of 0.6 to 1%. This suggests that the bacteria can better survive in saline environments with lower concentrations of bile salts. This could have implications for the use of *Lactobacillus* in food preservation and other industrial applications.

3.5. Genetic identification of bacteria

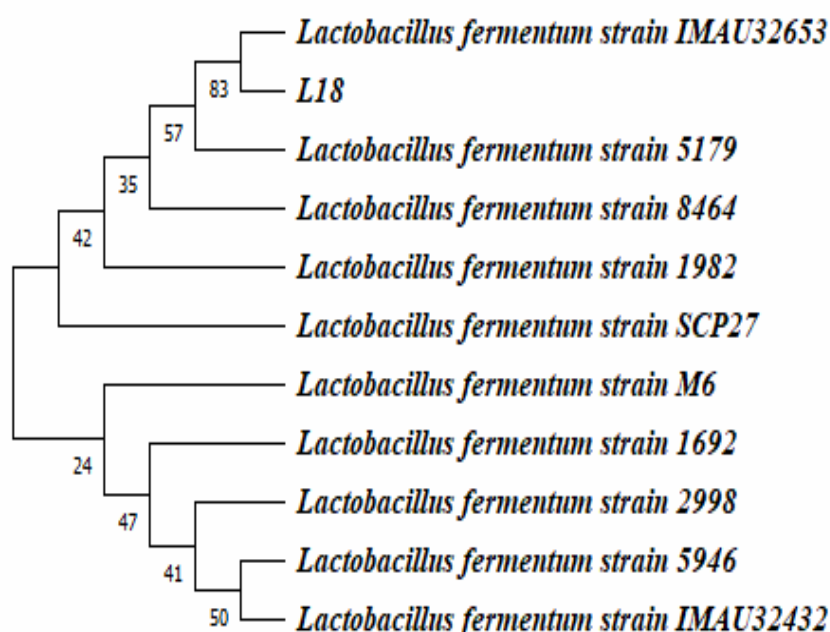


Figure 6. Phylogenetic tree based on 16S rRNA gene sequences representing *Lactobacillus* isolates. Numbers of nodes representing levels of bootstrap support (%) from a 1,000-record resample dataset

Based on the above characteristics and biochemical tests, L18 strain could be a species in *Lactobacillus* genus and therefore the information of 16S rRNA sequences could be contributed to the species identification of L18 strain. Here we used the primer pairs 27F and 1492R to amplify the 16S rRNA gene fragment and resulted in a single band of about 1500 bp. This is consistent with the theoretical size when multiplying the gene fragment with this primer pair. The results of

sequence and phylogenetic tree comparison showed that strain L18 had 99.43% similarity to *Lactobacillus fermentum* strain IMAU32653 and was in the same branch as *Lactobacillus fermentum* strain IMAU32653 with the same value of Bootstrap 83% (Figure 6). In terms of reliability and similarity, strain L18 and *Lactobacillus fermentum* strain IMAU32653 are similar. The combination of biological and molecular characteristics shows that strain L18 is closely

related to *Lactobacillus fermentum* and is named *Lactobacillus fermentum* strain L18.

3.6. Extracellular enzyme production

Extracellular enzymes are biologically active substances that directly or indirectly inhibit and destroy pathogenic microorganisms in probiotics [26]. The results of this study showed that bacterial

strain L18 can produce amylase and cellulase enzymes with a resolution ring diameter of 13.96 ± 0.35 mm and 10.07 ± 0.25 mm, respectively (Figure 7). These two enzymes degrade complex carbohydrates, which improves fish absorption. In addition, strain L18 enzyme can also inhibit pathogenic bacterial growth and help maintain intestinal micro-ecosystem balance. Therefore, L18 is a potential probiotic candidate for fish farming.

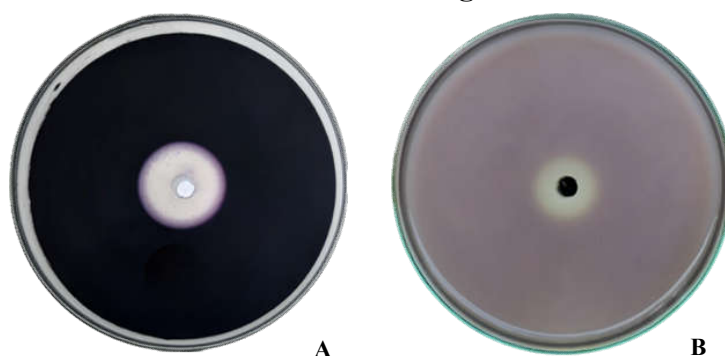


Figure 7. The amylase (A) and cellulase (B) enzyme activities of *Lactobacillus fermentum* strain L18

4. CONCLUSIONS

LAB strains isolated from tropical freshwater fish intestines have probiotic properties that make them potential candidates for probiotic applications. Among the isolates, *Lactobacillus fermentum* strain L18 exhibited significant probiotic activity along with significant antimicrobial activity against *Staphylococcus aureus* and *Aeromonas hydrophila*. Additionally, *L. fermentum* strain L18 was found to tolerate a wide range of pH and bile salt conditions. Further studies of *in vivo* assay and safety profiles are needed to determine their practical applications.

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