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RESEARCH ARTICLE
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Isolation and Characterization of Gut-derived Probiotic Bacteria from *Clarias batrachus* with Antagonistic Activity against Aquaculture Pathogens

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ABSTRACT

Diseases caused by opportunistic bacteria such as *Aeromonas hydrophila* and *Vibrio alginolyticus* remain a major constraint in intensive aquaculture, while the use of antibiotics raises concerns about resistance and environmental risks. This research aimed to isolate and characterize gut-derived probiotic bacteria from *Clarias batrachus* and to evaluate their antagonistic activity against major aquaculture pathogens. Intestinal bacterial isolates were obtained through selective culturing, followed by biochemical and phenotypic characterization, antagonistic testing, agar-diffusion antibacterial assays, pH tolerance evaluation, and pathogenicity testing using the brine shrimp lethality test (BSLT). Six isolates were obtained, of which four (BP-1, BP-3, BP-4, and BP-6) exhibited significant inhibitory activity ($P<0.05$) against both pathogens, whereas two (BP-2 and BP-5) were inactive. The BP-3 isolate showed the best antibacterial activity against *A. hydrophila* and *V. alginolyticus*, with inhibition zone diameters of 14.33 mm and 13.67 mm, respectively. The active isolates were predominantly Gram-positive, catalase-variable, and tolerant of acidic conditions (pH 2-6), indicating suitability for survival in the gastrointestinal tract. Pathogenicity testing confirmed all active isolates as non-toxic ($LC_{50}>35\times10^3$ppm). These findings demonstrate that the intestine of *C. batrachus* harbors host-associated probiotic candidates with strong antibacterial potential and promising biosafety attributes. Based on biochemical and microscopic characterization, all four isolates were suspected to be *Lactobacillus, Bacillus,* and *Eubacterium*. Further molecular identification and in vivo validation are recommended to confirm functional stability and the feasibility of application.

Keywords: *Aeromonas hydrophila*, Antibacterial, Cat fish, Probiotic bacteria, *Vibrio alginolyticus*.

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INTRODUCTION

Freshwater fish farming is a crucial subsector in providing high-protein food and supporting global food security (FAO/WHO, 2023). Among the various freshwater fish species farmed, catfish (*Clarias batrachus*) occupies a strategic position due to its rapid growth, tolerance of extreme environmental conditions, and high demand in local and export markets, making it a leading commodity in aquaculture (Caesar et al., 2021; Saselah & Indriani, 2025). However, intensification of aquaculture has led to greater environmental stress and declining water quality, which has triggered infections by opportunistic pathogens such as *Aeromonas hydrophila* and *Vibrio alginolyticus* (Bandeira-Junior & Baldisserotto, 2020; Kim et al., 2021). These two bacteria are the primary causes of motile *Aeromonas septicemia* (MAS) and *vibriosis*, which negatively affect growth, feed efficiency, and fish survival rates (Azhar et al., 2022; Jumina et al., 2024).

For decades, disease control in aquaculture systems has relied heavily on antibiotics and synthetic chemicals. While initially effective, this approach has led to serious problems, including antimicrobial resistance, accumulation of residues in fish, and environmental pollution (Elgendy et al., 2024; Pelic et al., 2024; Srinivasan et al., 2025). This situation has prompted the need for more environmentally

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friendly, sustainable strategies to maintain the health of farmed fish, including the use of probiotic microorganisms. Probiotics are defined as live microorganisms that, when administered in adequate amounts, provide health benefits to their host. In aquaculture, probiotics function to stabilize microbial communities, increase feed efficiency, stimulate the non-specific immune system, and produce bioactive compounds such as organic acids, bacteriocins, and hydrogen peroxide that can inhibit the growth of pathogenic microbes (Rad et al., 2020; Anee et al., 2021; Usman et al., 2024). Several studies have reported that the genera *Bacillus, Lactobacillus*, and *Pediococcus* are effective in improving fish growth performance and reducing pathogen levels (Shija et al., 2023; Fachri et al., 2024a).

However, most probiotic isolates used in the aquaculture industry still originate from non-organism sources such as sediment, seawater, or fermented milk products, often resulting in poor adaptation to fish physiological conditions (Kuebutornye et al., 2019). Recent approaches have demonstrated that isolating probiotics from the digestive tract of host species known as host-associated probiotics yields more effective results due to their higher colonization capacity, better metabolic compatibility, and higher biosafety compared to foreign isolates (Lingga et al., 2023; Jilani et al., 2024). Although several studies have reported isolating lactic acid bacteria from the intestines of catfish, most studies still focus on identifying morphological and basic biochemical characteristics without specific testing against dominant pathogens such as *A. hydrophila* and *V. alginolyticus*. Furthermore, very few studies have comprehensively assessed the antibacterial potential of endogenous isolates against these two pathogens, thereby limiting the development of safe and applicable natural biocontrols for intensive aquaculture systems. This situation leaves a research gap for a holistic assessment that not only characterizes endogenous isolates but also evaluates their antibacterial activity, physiological tolerance, and biosafety.

The novelty of this study lies in its comprehensive exploration of endogenous probiotic bacteria derived from the digestive tract of the catfish *C. batrachus*, a species that has rarely been studied. Unlike previous studies that focused solely on basic identification, this study integrates biochemical characterization, antagonistic testing, agar diffusion-based antibacterial activity measurements, extreme pH resistance testing, and pathogenicity testing using BLST to comprehensively assess the probiotic's viability. Furthermore, this study evaluates the ability of endogenous isolates to simultaneously inhibit two major aquaculture pathogens, namely *A. hydrophila* (freshwater) and *V. alginolyticus* (brackish water), providing a more realistic picture of real-world cultivation conditions. Through this approach, this study successfully identified a superior isolate (BP-3) with strong antibacterial activity, high physiological resistance, and 211good biosafety, thus providing a new scientific contribution to the development of more adaptive, effective, and environmentally friendly host-specific probiotics to support the sustainability of fresh - and brackish water fish farming systems.

Based on this background, this study aims to isolate and characterize candidate probiotic bacteria from the intestines of catfish (*C. batrachus*) and evaluate their antagonistic activity against pathogenic bacteria *Aeromonas hydrophila* and *Vibrio alginolyticus*.

MATERIALS & METHODS

Research Design

This research was conducted at the Marine Microbiology Laboratory, Fish Pest and Disease Laboratory, and Water Quality Laboratory of the Faculty of Marine Sciences and Fisheries, Hasanuddin University, Makassar, South Sulawesi, Indonesia. The samples used were catfish (*Clarias batrachus*) weighing 163g and 39cm long, obtained from the Fisheries Hatchery, Faculty of Marine Sciences and Fisheries, Hasanuddin University. The pathogenic bacteria used were *A. hydrophila* and *V. alginolyticus,* isolates from the South Sulawesi Provincial Fish Quarantine Office collection that had been tested for pathogenicity. To test the cytotoxicity of probiotic bacterial isolates against test organisms, Artemia salina was used as a test animal. The media used for the isolation and identification of probiotic bacteria were MRSA (de Man Rogosa and Sharpe agar), GYPA (glucose yeast peptone agar), and TSA (tryptic soy agar). All media were sterilized by autoclaving at 121°C for 15min.

Research Procedure

This research procedure consists of several stages: isolation of probiotic bacteria, antagonistic testing and antibacterial activity using the agar diffusion method, bacterial characterization, and pathogenicity testing of the probiotic bacteria.

Stage-1: Isolation of Probiotic Bacteria

Isolation of probiotic bacteria using catfish (*Clarias batrachus*) samples that were aseptically dissected and the intestines were removed. A total of 1g of intestinal sample was ground and placed into a test tube containing 10mL of sterile 0.9% NaCl solution to prepare a stock solution, then homogenized with a vortex. To prepare a 10^{-1} dilution, 1mL of the stock solution was transferred to a test tube containing 9mL of 0.9% NaCl. For a 10^{-2} dilution, 1mL of the 10^{-1} solution was taken and vortexed for 5min; the process was repeated until a 10-7 dilution was achieved. The results of the 10^{-4} to 10^{-7} dilutions were then taken, 1mL each, and inoculated into each petri dish. Then, 15mL of MRSA, TSA and GYPA media was poured into each dish, which were still warm. The dish containing the inoculum was homogenized by rotating it until the inoculum was evenly distributed, then left to solidify. Then, they were incubated at 37°C for 24 hours. The growing bacterial colonies were identified based on differences in color, border shape, and elevation. Each colony type was purified by streaking until a single bacterial colony was obtained. Some bacterial isolates were used as stock cultures, while others were used as inoculum in subsequent tests.

Stage-2: Antagonistic Testing and Antibacterial Activity with Agar Diffusion Method

An antagonist test was conducted to determine the effectiveness of probiotic bacteria against test pathogenic bacteria by dividing the petri dish into two areas. The first area was grown with probiotic bacterial isolates using the streak method and then incubated for 48 hours at 37°C to allow bacterial growth and the production of antibacterial compounds. The second area was used to streak the test organisms, and then it was incubated for an additional 24 hours at 37°C. A positive result was indicated by the absence of bacterial growth, except in the streak.

Antibacterial activity of probiotic candidates against pathogenic bacteria was tested using the agar diffusion method with paper discs (Zainuddin, 2006). The test pathogens, *A. hydrophila* and *V. alginolyticus*, were pure isolates obtained from the Makassar Fish Quarantine Center. The rejuvenated test pathogenic bacteria were dissolved in 3mL of 0.9% NaCl solution. Afterward, 200µL was taken and placed into a bottle containing 20mL of warm TSA medium, then homogenized. Then the mixture was poured into a Petri dish and allowed to solidify before being used in the antibacterial activity test. One loop of bacterial isolate culture on agar was taken and inoculated into 6 mL of TSB, incubated for 72 hours at 37°C. The incubation mixture was centrifuged for 15min at 6000rpm to separate the bacterial cells from the supernatant (a solution containing antibacterial compounds), and then filtered through Whatman filter paper. A 50µL supernatant was dropped onto a paper disc and allowed to dry. Once dry, it was placed on the solidified agar medium. Next, the dish was pre-incubated in a refrigerator for 2 hours, then incubated at 37°C for 24 hours. As a positive control, 50µL of Yakult solution was used. Bacteria that produce antibacterial compounds inhibit pathogenic bacteria, as evidenced by an inhibition zone. The diameter of the inhibition zone was measured using a vernier caliper. This test was performed with 3 replicates.

Stage-3: Bacterial Characterization

Characterization of isolated bacteria by morphological observation and biochemical testing. Morphological observations include observations of the colony and color forms. Biochemical tests include Gram staining, catalase testing, motility, and pH. The determination of the genus from the characterization results is done by referring to the Manual for the Identification of Medical Bacteria (Barrow & Feltham, 1993).

Stage-4: Pathogenicity Testing of Probiotic Bacteria

This test employs the *brine shrimp lethality test* (BSLT), using Artemia salina larvae cultured for 48 hours as test animals. Ten *Artemia salina* larvae were placed in a vial containing 5 ml of seawater with a test solution concentration of 40×10^3, 20×10^3, 10×10^3 and 5×10^3ppm. As a control, seawater is used without bacteria. Each treatment and control was repeated 2 times. Observations were made after 24 hours by counting the number of dead *Artemia salina* at each concentration. The LC_{50} concentration (ppm) was determined using Probit analysis and regression equations (Zainuddin, 2010).

Data Analysis

Antibacterial activity data were analyzed using one-way ANOVA. If an effect was observed, a Tukey test was used to determine differences in activity or inhibition against pathogenic bacteria. The analysis program used was SPSS version 16.0. For the pathogenicity test, the LC_{50} value was determined by Probit analysis using a Probit table, and the regression equation was generated in Microsoft Excel.

RESULTS

Isolation and Identification of Probiotic Bacterial Isolates

Six isolates (BP-1, BP-2, BP-3, BP-4, BP-5, and BP-6) were obtained with different shapes, colors, elevations, edges, and media. Four of the isolates were *Lactobacillus* and had irregular, white, convex colonies (Fig. 1).

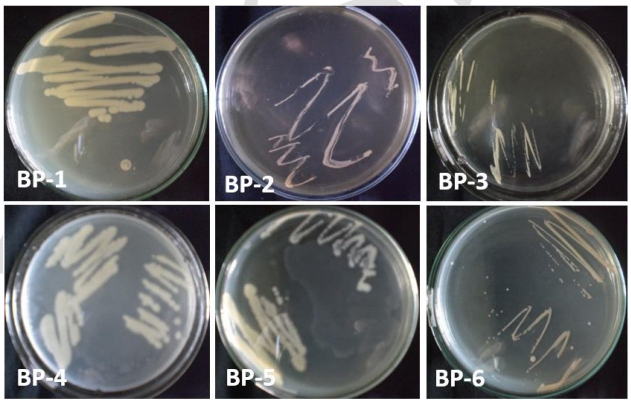

Fig. 1: Results of purification of bacterial isolates from the intestines of catfish (*Clarias batrachus*).

To obtain pure isolates, the 6 isolates were purified using different media. Macroscopic observations of isolated colonies are presented in Table 1. Table 1 shows that there are 4 isolates (BP-1, BP-2, BP-5, and BP-6) that grow on TSA media, 1 isolate (BP-3) grows on GYPA media and 1 isolate (BP-4) grows on MRSA media. 2 isolates (BP-3 and BP-4) have irregular colony shapes and jagged edges, while 4 isolates (BP-1, BP-2, BP-5 and BP-6) have circular colony shapes and flat edges. Isolates BP-1, BP-3, BP-4, BP-5, and BP-6 were milky white, while isolate BP-2 is yellow.

Table 1: Macroscopic observation of probiotic bacteria

Isolated Bacteria	Characteristic of Isolated Bacteria				
	Colony Form	Colony Colour	Elevation	Edges	Medium
BP-1	Spherical	Yellow	Convex	Jagged	TSA
BP-2	Spherical	White	Flat	Flat	TSA
BP-3	Irregular	White	Flat	Flat	GYPA
BP-4	Irregular	White	Convex	Jagged	MRSA
BP-5	Spherical	White	Convex	Jagged	TSA
BP-6	Spherical	White	Flat	Flat	TSA

Note: de Man Rogosa Sharpe Agar (MRSA), Tryptic Soya Agar (TSA), Glucose Yeast Pepton Agar (GYPA).

The results in Table 2 indicate significant differences ($P<0.05$) among the isolates in their ability to inhibit the growth of both pathogens. Different superscript letters within the same column denote statistically significant differences according to Tukey's HSD test. Isolate BP-2

exhibited the smallest inhibition zones against both tested bacteria, indicating that its antagonistic activity was relatively weaker than that of the other isolates.

Table 2: The average (n=3) of the inhibition zone diameter of candidate probiotic bacterial isolates against *Aeromonas hydrophila* and *Vibrio alginolyticus*

Isolates	Inhibition Zone (mm) of *Aeromonas hydrophila*	Inhibition Zone (mm) of *Vibrio alginolyticus*
BP-1	11.33±0.58^{bc}	11.67±0.50^{bc}
BP-2	6.00±0.00^{a}	6.00±0.00^{a}
BP-3	14.33±1.15^{c}	13.67±1.15^{c}
BP-4	12.33±2.08^{c}	7.00±1.73^{a}
BP-5	7.00±1.73^{ab}	6.67±1.15^{a}
BP-6	11.33±1.15^{bc}	9.00±0.00^{ab}

Values (mean±SD) bearing different superscript letters in a column indicate differences (P<0.05) according to Tukey's HSD test.

Antagonistic Testing and Antibacterial Activity Test

Results of antagonistic testing and antibacterial activity test (Fig. 2).

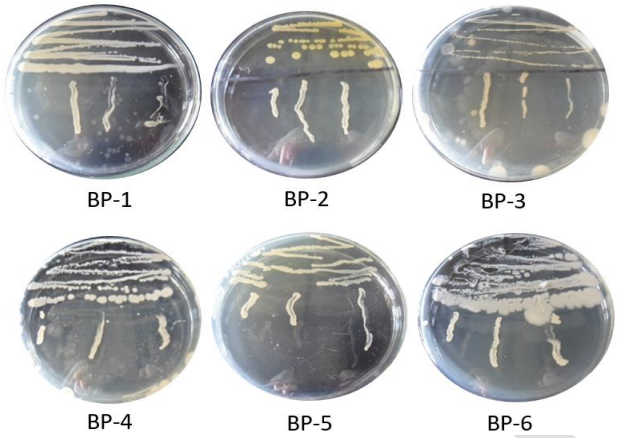

Fig. 2: Results of antagonistic testing of six bacterial isolates.

Antagonist testing was conducted to determine the ability of candidate probiotic bacteria to inhibit the growth of pathogenic bacteria. Based on the results of the antagonist test, the six isolates (BP-1, BP-2, BP-3, BP-4, BP-5, and BP-6) were able to fight pathogenic bacteria. To assess the antibacterial activity of candidate probiotic bacteria against the pathogenic bacteria *A. hydrophila* and *V. alginolyticus*, an agar diffusion test using paper discs was conducted (Fig. 3).

To examine the differences between the six isolates, a further Tukey test was conducted. The results showed that isolate BP-1 was not significantly different from isolates BP-3, BP-4, and BP-6, but 2 isolates (BP-3 and BP-4) were significantly different from isolates BP-2 and BP-5 in inhibiting the pathogenic bacteria *A. hydrophila*. Meanwhile, isolate BP-1 was not significantly different from isolates BP-3 and BP-6; however, the three isolates (BP-1, BP-3, and BP-6) were significantly different from isolates BP-2, BP-4 and BP-5 in their ability to inhibit the pathogenic bacterium *V. alginolyticus*.

Biochemical Tests of Bacterial Isolates, Gram Staining Tests, Catalase Tests, Motility and pH Resistance Tests

Biochemical tests were performed to characterize the bacterial isolates, including Gram staining, catalase, motility, and pH resistance, as shown in Table 3.

Based on Gram staining, five bacterial isolates were Gram-positive (BP-1, BP-3, BP-4, BP-5, and BP-6), and one was Gram-negative (BP-2) (Fig. 4).

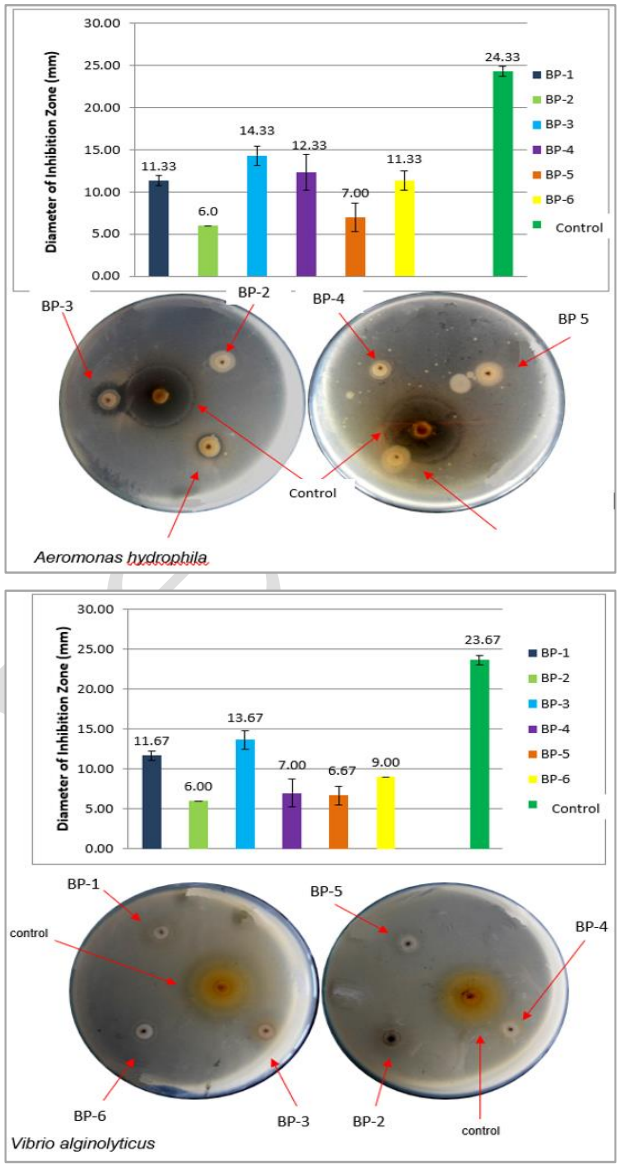

Fig. 3: Antibacterial activity of six bacterial isolates against *Aeromonas hydrophila* and *Vibrio alginolyticus*.

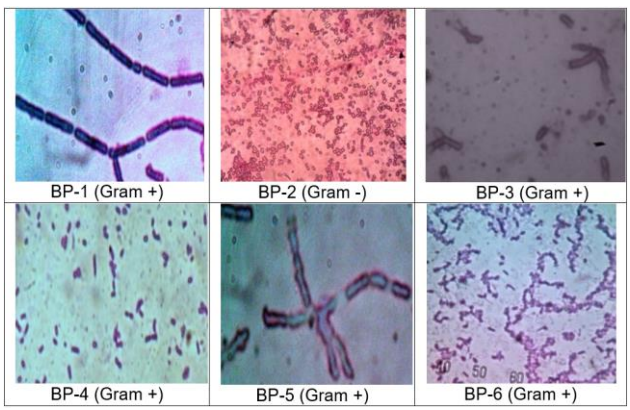

Fig. 4: Performance of Gram-staining bacterial isolates from the intestines of catfish (*Clarias batrachus*).

Table 3: Characterization of candidate probiotic bacterial isolates from catfish (*Clarias batrachus*)

Observation	Isolated Bacteria					
	BP-1	BP-2	BP-3	BP-4	BP-5	BP-6
Cell Form	Basil	Basil	Basil	Rod	Basil	Basil
Gram Stain	+	-	+	+	+	+
Catalase	+	+	-	-	+	+
Motility	+	+	+	-	-	+
pH	+	-	+	+	-	+

Note: + (positive result), - (negative result).

The catalase test showed that 4 isolates (BP-1, BP-2, BP-5, and BP-6) were catalase-positive, while 2 isolates (BP-3 and BP-4) were catalase-negative (Table 3; Fig. 5).

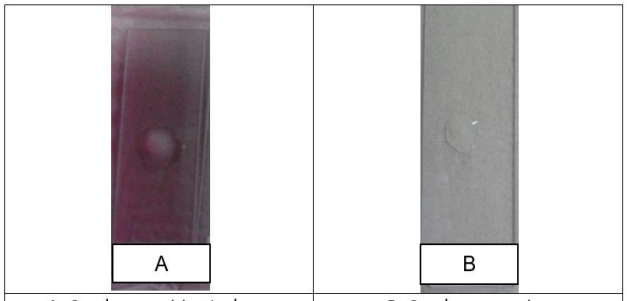

Fig. 5: Catalase activity of bacterial isolates from the intestines of catfish (*Clarias batrachus*).

The results of the motility test showed that 2 isolates (BP-3 and BP-4) were non-motile, while 4 isolates (BP-1, BP-2, BP-5, and BP-6) were motile (Table 3 and Fig. 6).

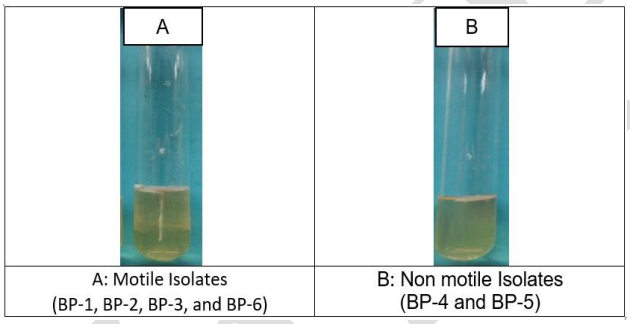

Fig 6: Motile activity of bacterial isolates from the intestines of catfish (*Clarias batrachus*).

A pH tolerance test was conducted to determine the resistance of candidate probiotic bacteria to low pH. This test was performed by adjusting the medium pH to 2, 4, and 6 for four potential isolates: BP-1, BP-3, BP-4, and BP-6 (Table 3). The test results showed that all four isolates survived at pH 2, with an average optical density (OD) of 0.1-0.2 (Table 4).

Table 4: Average OD (*Optical Density*) value of probiotic bacterial isolates

Isolates	pH 2	pH 4	pH 6	Control (pH 8)
BP-1	0.1	0.2	0.6	1.2
BP-3	0.1	0.1	1.4	0.6
BP-4	0.2	0.4	1.3	1.5
BP-6	0.1	0.2	1.0	1.2

These four bacterial isolates are potential probiotic candidates because one requirement for a microbe to be used as a probiotic is its ability to grow in acidic conditions. Isolate BP-4 grew optimally at pH 6 with a higher OD value than the control.

Test Results of Cytotoxicity Isolate Probiotic Bacteria against Salt Water Shrimp (*Artemia salina*)

Pathogenicity activity testing was carried out based on the *brine shrimp lethality test* (BSLT) method using Artemia salina shrimp larvae cultured for 48 hours (Table 5).

Table 5: LC_{50} of each probiotic bacterial isolate from the intestine of catfish

No.	Isolates	Concentration of LC_{50} (ppm)
1.	BP-1	$50x10^3$
2.	BP-3	$85x10^3$
3.	BP-4	$55x10^3$
4.	BP-6	$35x10^3$

The concentrations used were 50×10^3, 10×10^3, 20×10^3, and 40×10^3ppm. Based on the test results of the four probiotic bacterial isolates, BP-1 had an LC50 of 50×10^3ppm, BP-3 had an LC50 of 85×10^3ppm, BP-4 had an LC50 value of 55×10^3ppm, and BP-6 had an LC50 value of 35×10^3ppm. Based on Table 5, the highest concentration that resulted in the death of 50% of the test animal population was shown by isolate BP-6 (35×10^3ppm), while the lowest concentration was shown by isolate BP-3 (85×10^3ppm).

DISCUSSION

The results of this study indicate that the intestines of catfish are a source of commensal bacteria that have the potential to act as probiotics, exhibiting significant antibacterial activity against two important pathogens in aquaculture: *A. hydrophila* and *V. alginolyticus*. Of the six isolates obtained, four (BP-1, BP-3, BP-4 and BP-6) produced significant inhibition zones (Table 2), indicating antimicrobial activity through the production of metabolites such as organic acids, hydrogen peroxide, or bacteriocins. This finding aligns with reports that lactic acid bacteria and *Bacillus* originating from the digestive tract of fish possess natural antagonistic abilities against opportunistic pathogens (Chizhayeva et al., 2022; Figueras et al., 2022; Ringø et al., 2022; Kumalasari et al., 2025).

The gastrointestinal tract of fish is increasingly recognized as a complex ecological niche harboring diverse microbial communities that play essential roles in host nutrition, immune modulation, and disease resistance. Recent studies emphasize that endogenous gut bacteria exhibit better host compatibility and colonization efficiency than exogenous or commercial probiotics, making them more effective in aquaculture applications (Zhang et al., 2023; Hoseinifar et al., 2024). Therefore, isolating probiotic candidates directly from *C. batrachus* intestines offers an important advantage for developing host-specific probiotic formulations tailored to local aquaculture systems.

Isolate BP-3 was the most promising candidate, with inhibition zones of 14.33 mm and 13.67 mm against *A. hydrophila* and *V. alginolyticus*, respectively. This demonstrates a consistent and strong inhibitory capacity, as also reported for *Lactococcus* and *Lactobacillus*-based aquatic probiotics in recent studies (Saadony et al., 2021; Li

et al., 2022). Differences in inhibitory strength between isolates reflect variations in the ability to produce bioactive metabolites common in fish gut bacterial communities.

Recent molecular investigations have shown that probiotic efficacy is often associated with the presence of functional genes responsible for bacteriocin synthesis, organic acid production, quorum-sensing interference, and competitive exclusion mechanisms (Wang et al., 2023; Al-Sadi et al., 2024). The superior performance of BP-3 suggests that this isolate may harbor a broader spectrum of antimicrobial genes or exhibit higher metabolic activity compared to other isolates. Further genomic and metabolomic analyses are therefore recommended to confirm the molecular basis of its antagonistic behavior.

The inactive isolates BP-2 and BP-5 likely lack genes or physiological conditions that support antimicrobial production, supporting the concept that only a small subset of gut microbiota are effective as probiotics (Lee et al., 2021; Chen et al., 2022). This selective functionality reinforces the importance of rigorous screening when developing probiotic candidates for aquaculture use.

The more consistent inhibitory response to *A. hydrophila* compared to *V. alginolyticus* also aligns with the physiological characteristics of both pathogens, with *Aeromonas* being more sensitive to pH changes and bacteriocin compounds, while *Vibrio* generally exhibits higher tolerance to acidic conditions and environmental stress (Thompson et al., 2022; Tayyab et al., 2025).

This observation is particularly relevant for freshwater aquaculture, where *A. hydrophila* remains one of the most destructive pathogens causing motile aeromonad septicemia. Its susceptibility to probiotic-derived antimicrobial compounds underscores the strategic potential of probiotic-based biocontrol to reduce disease outbreaks without relying on antibiotics (Austin & Austin, 2024). Meanwhile, the moderate resistance of *Vibrio alginolyticus* underscores the need for multi-strain or synergistic probiotic formulations to achieve broader-spectrum disease protection.

Biochemical characterization revealed that the active isolates were predominantly Gram-positive bacteria with good pH tolerance, particularly at pH levels of 4-6, a basic requirement for orally administered probiotics. This tolerance is essential for bacterial survival as they pass through the stomach before reaching the intestines, where they function as probiotic agents (Fachri et al., 2024b).

Tolerance to acidic conditions and bile salts is widely considered a prerequisite for probiotic functionality in aquatic organisms. Studies conducted between 2022 and 2025 have shown that Gram-positive bacteria, particularly from the genera *Lactobacillus* and *Bacillus*, possess thick peptidoglycan cell walls and adaptive stress-response mechanisms that enhance their survival in harsh gastrointestinal environments (Kuebutornye et al., 2022; Dawood et al., 2023). This physiological robustness further supports the suitability of the active isolates as probiotic candidates. These characteristics suggest that the isolates in this study belong to the genera *Lactobacillus*, *Bacillus*, and *Eubacterium*, which are widely reported as natural probiotics in freshwater fish and are known to enhance innate immunity, feed digestibility, and disease resistance (Calcagnile et al., 2024).

Recent experimental trials demonstrate that probiotics from these genera can significantly improve growth performance, antioxidant capacity, and immune-related gene expression in catfish and other freshwater species (El-Haroun et al., 2023; Rahman et al., 2025). Additionally, *Bacillus*-based probiotics produce extracellular enzymes such as proteases, amylases, and cellulases, which enhance nutrient utilization and reduce the accumulation of organic waste in aquaculture systems, thereby contributing to environmental sustainability.

Pathogenicity testing using the Brine Shrimp Lethality Test demonstrated that all active isolates were non-toxic, indicating they are safe for use in aquaculture systems. This safety evaluation is crucial because probiotics must be pathogen-free, non-toxic, and non-invasive to the host organism (Irianto & Austin, 2020). In recent regulatory and scientific frameworks, safety assessment has become a mandatory step in probiotic development, including hemolytic activity tests, antibiotic resistance profiling, and in vivo challenge trials (FAO/WHO, 2023; EFSA, 2024). BP-3's non-toxicity strengthens its eligibility for further clinical trials and eventual commercialization.

The combination of BP-3's strong antibacterial properties and its non-pathogenicity confirms its potential as a superior probiotic for biocontrol-based disease control efforts in aquaculture. This study makes an important contribution because probiotic isolates from *C. batrachus* have been relatively understudied compared to those from other catfish species, such as *C. gariepinus*. The identification of indigenous probiotic strains from *C. batrachus* is particularly valuable for small-scale and local aquaculture operations, where access to imported commercial probiotics may be limited or economically unfeasible. Locally sourced probiotics are also better adapted to regional environmental conditions, increasing their effectiveness and acceptance among farmers (Hidayat et al., 2023).

By demonstrating that the gut of *C. batrachus* also contains probiotic bacteria with high antibacterial activity, this study fills the knowledge gap regarding potential local probiotic sources for freshwater aquaculture. Amid increasing antibiotic resistance and the need for environmentally friendly disease control strategies, isolate BP-3, in particular, offers exciting prospects as a natural probiotic agent that can be integrated into modern aquaculture practices (Srisapoome & Areechon, 2020).

Overall, this study reinforces the global shift toward sustainable aquaculture practices that emphasize microbial management rather than chemical intervention. The utilization of host-derived probiotics such as BP-3 aligns with the principles of eco-friendly aquaculture, supporting fish health, environmental protection, and long-term productivity. Future studies should focus on molecular identification, whole-genome sequencing, in vivo performance evaluation, and formulation optimization to fully realize the commercial and ecological potential of this probiotic candidate (Hoseinifar et al., 2024; Nguyen et al., 2026).

Conclusion

Based on the research conducted, it can be concluded that bacterial isolation from the intestines of catfish (*Clarias batrachus*) yielded 6 isolates. The selection results showed that 4 isolates (BP-1, BP-3, BP-4, and BP-6) inhibited the pathogenic bacteria *Aeromonas hydrophila* and *Vibrio alginolyticus*. The results of the pathogenicity test showed that the 4 isolates (BP-1, BP-3, BP-4 and BP-6 were not pathogenic. The results of biochemical and microscopic characterization of the probiotic bacterial candidates indicated that the 4 isolates were likely members of the genera *Lactobacillus, Bacillus* and *Eubacterium*.

DECLARATIONS

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Conflict of Interest: The authors declare no conflict of interest

Data Availability: Upon reasonable request, the datasets of this study can be made available from the corresponding author.

Ethics Statement: This study did not involve human or animal subjects. Therefore, no ethical approval or ethics code from an ethics committee was required.

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REFERENCES

Al-Sadi, A.M., Al-Saadi, A.M., & Al-Mahmooli, I.H. (2024). Genomic insights into bacteriocin-producing probiotics for aquaculture. *Microbial Pathogenesis, 184*, 106314. https://doi.org/10.1016/j.micpath.2024.106314

Anee, I.J., Alam, S., Begum, R.A., Shahjahan, R.M., & Khandaker, A.M. (2021). The role of probiotics on animal health and nutrition. *Journal of Basic and Applied Zoology, 82*, 51. https://doi.org/10.1186/s41936-021-00239-8

Austin, B., & Austin, D.A. (2024). *Bacterial fish pathogens: Disease of farmed and wild fish* (7th ed.). Springer. https://doi.org/10.1007/978-3-031-36145-5

Azhar, F., Andre, R.S., & Dewi, P.L. (2022). Control of bacterial diseases in Nile fish using betel leaf extract (*Piper betle* L.) in Gontoran Village, West Lombok. *Pepadu, 8*(2), 287–291.

Bandeira-Junior, G., & Baldisserotto, B. (2020). Fish infections associated with the genus *Aeromonas*: A review of the effects on oxidative status. *Journal of Applied Microbiology, 131*(3), 1083–1101. https://doi.org/10.1111/jam.14986

Barrow, G. I., & Feltham, R. K. A. (Eds.). (1993). *Cowan and Steel's manual for the identification of medical bacteria* (3rd ed.). Cambridge University Press

Caesar, N.R., Yanuhar, U., Raharjo, D.K.W.P., & Junirahma, N.S. (2021). Monitoring of water quality in the catfish (*Clarias* sp.) farming in Tuban Regency. *IOP Conference Series: Earth and Environmental Science, 718*, 012061. https://doi.org/10.1088/1755-1315/718/1/012061

Calcagnile, M., Tredici, S.M., & Alifano, P. (2024). A comprehensive review on probiotics and their use in aquaculture: Biological control, efficacy, and safety through the genomics and wet methods. *Heliyon, 10*(1), e24124. https://doi.org/10.1016/j.heliyon.2024.e24124

Chen, H., Wang, W., & Li, X. (2022). Functional diversity of gut microbiota in freshwater fish. *Microorganisms, 10*(4), 815. https://doi.org/10.3390/microorganisms10040815

Chizhayeva, A., Ringø, E., & Chikindas, M.L. (2022). Probiotic bacteria from fish gut microbiota: Antagonistic activity and applications in aquaculture. *Aquaculture Reports, 24*, 101123. https://doi.org/10.1016/j.aqrep.2022.101123

Dawood, M.A.O., Koshio, S., & Esteban, M.Á. (2023). Beneficial roles of probiotics in fish health and production. *Aquaculture, 557*, 738344. https://doi.org/10.1016/j.aquaculture.2022.738344

EFSA Panel on Additives and Products or Substances used in Animal Feed (2024). Safety assessment of microorganisms used as feed additives. *EFSA Journal, 22*(1), e8581. https://doi.org/10.2903/j.efsa.2024.8581

Elgendy, M.Y., Ali, S.E., Dayem, A.A., Khalil, R.H., Moustafa, M.M., & Abdelsalam, M. (2024). Alternative therapies recently applied in controlling farmed fish diseases: Mechanisms, challenges, and prospects. *Aquaculture International, 32*, 9017–9078. https://doi.org/10.1007/s10499-024-01309-3

El-Haroun, E., Amer, S. A., Rizk, S. A. A., Dawood, M. A. O., Zaineldin, A. I., Ahmed, S. A. A., & Mahboub, H. H. (2023). Effects of varying levels of dietary multi-strain probiotic (*Pediococcus acidilactici, Lactobacillus acidophilus* and *Bifidobacterium bifidum*) on growth performance, antioxidant capacity, immune response, and gene expression of African catfish (*Clarias gariepinus*). *Animals, 13*(21), 3311. https://doi.org/10.3390/ani13213311

Fachri, B.A., Yuliana, S., & Putra, A.A. (2024b). Acid and bile tolerance of probiotic bacteria isolated from freshwater fish. *Biodiversitas, 25*(2), 789–796. https://doi.org/10.13057/biodiv/d250234

Fachri, M., Amoah, K., Huang, Y., Cai, J., Alfatat, A., Ndandala, C.B., Shija, V.M., Jin, X., Bissih, F., & Chen, H. (2024a). Probiotics and paraprobiotics in aquaculture: A sustainable strategy for enhancing fish growth, health, and disease prevention. *Frontiers in Marine Science, 11*, 1335469. https://doi.org/10.3389/fmars.2024.1335469

FAO/WHO (2023). *Guidelines for the evaluation of probiotics in food and feed*. FAO. https://doi.org/10.4060/cc3916en

Figueras, M.J., Beaz-Hidalgo, R., & Austin, B. (2022). Aeromonas infections in fish: Epidemiology, virulence, and control strategies. *Journal of Fish Diseases, 45*(9), 1241–1261. https://doi.org/10.1111/jfd.13642

Hidayat, R., Nurhayati, T., & Lestari, D. (2023). Indigenous probiotics for sustainable freshwater aquaculture. *Sustainability, 15*(7), 6032. https://doi.org/10.3390/su15076032

Hoseinifar, S.H., Dadar, M., Van Doan, H., Harikrishnan, R., & Esteban, M.Á. (2024). Probiotics in aquaculture: A review of functional roles and future perspectives. *Reviews in Aquaculture, 16*(1), 198–222. https://doi.org/10.1111/raq.12782

Irianto, A., & Austin, B. (2020). Probiotics in aquaculture. *Aquaculture Research, 51*(4), 1701–1711. https://doi.org/10.1111/are.14406

Jilani, A.K., Haider, M.N., Hasan, A., Mahfuz, M.A., Rifat, M.N.I., Hossain, M.M., & Hasan, M.M. (2024). Isolation, identification, and potentiality of gut-derived probiotic bacteria from *Heteropneustes fossilis*. *Journal of Advanced Veterinary and Animal Research, 11*(3), 560–572. https://doi.org/10.5455/javar.2024.k828

Jumina, M., Salosso, Y., & Djonu, A. (2024). *Pencegahan infeksi bakteri Aeromonas hydrophila pada ikan lele dumbo (Clarias gariepinus) dengan kombinasi madu dan Patikan Kerbau (Euphorbia hirta)*. *JFMR*

(Journal of Fisheries and Marine Research), 8(1), 83–91. https://doi.org/10.21776/ub.jfmr.2024.008.01.10

Kim, K.T., Lee, S.H., Lee, K.K., Han, J.E., & Kwak, D. (2021). Enhanced virulence of *Aeromonas hydrophila* induced by stress and serial passaging in mice. *Animals, 11*(2), 508. https://doi.org/10.3390/ani11020508

Kuebutornye, F.K.A., Abarike, E.D., & Lu, Y. (2019). A review on the application of *Bacillus* as probiotics in aquaculture. *Fish & Shellfish Immunology, 87*, 820–828. https://doi.org/10.1016/j.fsi.2019.02.010

Kuebutornye, F.K.A., Abarike, E.D., & Lu, Y. (2022). A review on the application of Bacillus as probiotics in aquaculture. *Fish & Shellfish Immunology, 128*, 238–251. https://doi.org/10.1016/j.fsi.2022.07.027

Kumalasari, C., Asmara, I.Y., Nayan, N., & Adriani, L. (2025). Characteristics of *Bacillus subtilis* and *Bacillus licheniformis* consortium as probiotics for late-phase laying hens. *International Journal of Agriculture and Biosciences*, 14(2), 238-243. https://doi.org/10.47278/journal.ijab/2024.218

Lee, S.Y., Kim, S.H., & Lee, J.H. (2021). Screening and characterization of probiotic bacteria from fish intestine. *Journal of Applied Microbiology, 131*(1), 312–324. https://doi.org/10.1111/jam.15038

Li, X., Liu, Y., Yang, Y., Zhang, Y., & Wang, Q. (2022). Antibacterial mechanisms of Lactobacillus probiotics against aquatic pathogens. *Fish & Shellfish Immunology, 120*, 126–134. https://doi.org/10.1016/j.fsi.2021.12.006

Lingga, R., Adibrata, S., Roanisca, O., Sipriyadi, Wibowo, R.H., & Arsyadi, A. (2023). Probiotic potential of lactic acid bacteria isolated from slender walking catfish (*Clarias nieuhofii*). *Biodiversitas, 24*(8), 4572–4580. https://doi.org/10.13057/biodiv/d240832

Nguyen, T.H., Hoseinifar, S.H., & Dawood, M.A.O. (2026). Next-generation probiotics in aquaculture: From genomics to application. *Reviews in Aquaculture*. Advance online publication. https://doi.org/10.1111/raq.12945

Pelic, D.L., Radosavljevic, V., Pelic, M., Balos, M.Z., Puvaca, N., Dujakovic, J.J., & Gavrilovic, A. (2024). Antibiotic residues in cultured fish: Implications for food safety and regulatory concerns. *Fishes, 9*(12), 484. https://doi.org/10.3390/fishes9120484

Rad, J. S., Rodrigues, C. F., Dimitrijevic, M., Aleksic, A., Vasiljevic, P., Nikolic, M., & Sokovic, M. (2020). Probiotics: Versatile bioactive components in promoting human health. *Medicina, 56*(9), 433. https://doi.org/10.3390/medicina56090433

Rahman, M., Islam, S. M. M., Nuruzzaman, M., & Rafiquzzaman, S. M. (2025). Efficacy of host-associated *Bacillus* probiotic RBT-7 in improving red tilapia (*Oreochromis* spp.) growth, health, and resistance to *Streptococcus agalactiae* infection at various salinities. *Veterinary Research Communications, 49*(2), 71. https://doi.org/10.1007/s11259-024-10628-x

Ringø, E., Hoseinifar, S.H., Ghosh, K., Doan, H.V., Beck, B.R., & Song, S.K. (2022). Lactic acid bacteria in finfish An update. *Frontiers in Microbiology, 13*, 893327. https://doi.org/10.3389/fmicb.2022.893327

Saadony, F.M.A., Osman, G.H., Desouky, S.E., & Sitohy, M.Z. (2021). Antimicrobial activity of probiotics against fish pathogens. *Aquaculture Research, 52*(12), 5881–5892. https://doi.org/10.1111/are.15458

Saselah, J. T., & Indriani, Y. (2025). Pengaruh padat penebaran berbeda terhadap peningkatan produksi budidaya ikan lele (*Clarias gariepinus*) di kolam terpal. *E-Journal Budidaya Perairan, 13*(1). https://doi.org/10.35800/bdp.v13i1.59374

Shija, V. M., Amoah, K., & Cai, J. (2023). Effect of *Bacillus* Probiotics on the Immunological Responses of Nile Tilapia (*Oreochromis niloticus*): A Review. *Fishes*, 8(7), 366. https://doi.org/10.3390/fishes8070366

Srinivasan, R., Jin, X., Lin, X., & Zhao, Z. (2025). Essential Oil Compounds as Antibiotic Alternatives: A Comprehensive Review of Antibacterial, Anti-Quorum Sensing, and Antibiofilm Effects Against Vibrio spp. in Aquaculture. *Reviews in Aquaculture*, 17. https://doi.org/10.1111/raq.70065

Srisapoome, P., & Areechon, N. (2020). Probiotic strategies in freshwater aquaculture. *Aquaculture International, 28*(5), 1733–1755. https://doi.org/10.1007/s10499-020-00562-4

Tayyab, M., Zhang, C., Islam, W., & Lin, W. (2025). Environmental stress tolerance of Vibrio species in aquaculture systems. *Aquaculture Environment Interactions, 17*, 1–14. https://doi.org/10.3354/aei00456

Thompson, F.L., Austin, B., & Swings, J. (2022). The biology of Vibrio species in aquatic environments. *Microbiology Spectrum, 10*(4), e01918-21. https://doi.org/10.1128/spectrum.01918-21

Usman, U., Khaliq, A., Akram, F., Afzal, H., & Aziz, S. (2024). A review on the role of bacillus spp. as probiotics in tilapia culture. *Agrobiological Records*, 18, 80-95. https://doi.org/10.47278/journal.abr/2024.040

Wang, Y., Li, J., Zhang, M., & Xu, H. (2023). Functional genes associated with probiotic efficacy in aquaculture. *Aquaculture Reports, 29*, 101459. https://doi.org/10.1016/j.aqrep.2023.101459

Zainuddin, E.N. (2006). *Chemical and biological investigation of selected cyanobacteria (blue green algae)* (Doctoral dissertation). University of Greifswald.

Zainuddin, E.N. (2010). Preliminary screening of marine algae from South Sulawesi coast for cytotoxic activity using brine shrimp Artemia salina lethality test. Proceedings of International Conference on Medicinal Plants. Surabaya, Indonesia, 2(3-6): 622-632.

Zhang, Z., Li, D., Refaey, M.M., Xu, W., & Wang, Y. (2023). Host-associated probiotics in aquaculture: Advantages and future perspectives. *Aquaculture, 563*, 738929. https://doi.org/10.1016/j.aquaculture.2022.738929