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ISOLATION, DETECTION, AND CHARACTERIZATION OF LACTIC ACID BACTERIA (LAB) FROM TILAPIA *SPECIES AND THEIR PROBIOTIC ACTIVITY AGAINST* AEROMONAS HYDROPHILA

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1. INTRODUCTION

Lactic acid bacteria (LAB) are beneficial microorganisms naturally present in the gastrointestinal tract of fish and other animals. These bacteria improve host health by producing organic acids and antimicrobial compounds known as bacteriocins, which inhibit the growth of pathogenic microorganisms. In aquaculture, the use of LAB as probiotics has gained significant attention as an eco-friendly alternative to antibiotics for controlling bacterial diseases. Among the common fish pathogens, *Aeromonas hydrophila* is responsible for severe infections such as hemorrhagic septicemia, leading to high mortality and economic losses. Therefore, isolating probiotic LAB from fish and evaluating their antibacterial activity against *A. hydrophila* is important for sustainable fish health management.

2. MATERIALS AND METHODS

Healthy tilapia fish were collected, and their gut samples were aseptically removed for bacterial isolation. The samples were serially diluted and cultured on de Man, Rogosa, and Sharpe (MRS) agar to selectively isolate LAB. After incubation, individual colonies were purified and maintained for further analysis. The isolates were screened for bacteriocin production, and their antimicrobial activity against *Aeromonas hydrophila* was evaluated using the agar well diffusion method. Morphological, physiological, and biochemical characteristics, including Gram staining, catalase test, carbohydrate fermentation, temperature

tolerance, pH tolerance, and salt tolerance, were used for bacterial identification. Antibiotic susceptibility of *A. hydrophila* was determined using the Kirby–Bauer disc diffusion method.

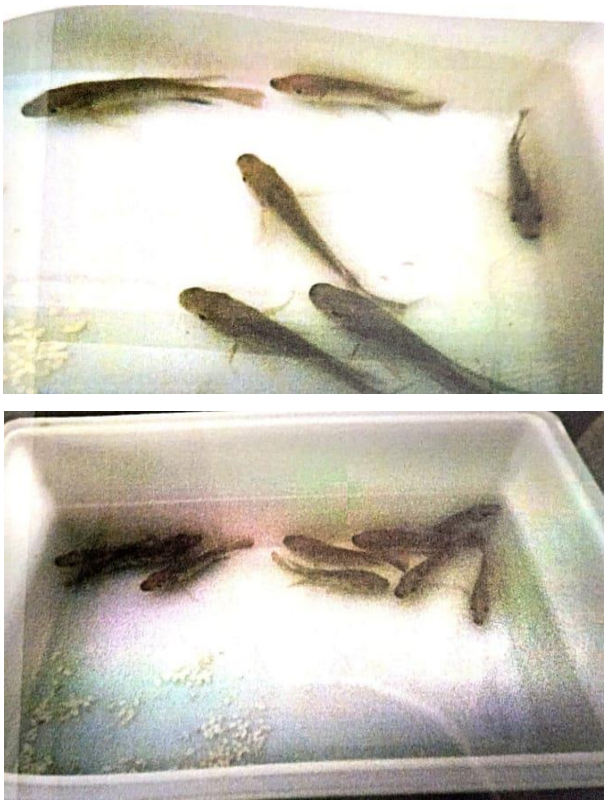

Fig: 1 Fish Samples Maintained For The Bacterial Isolation.

Fig: 2 Fish Samples Placed Over A Board After Killing For The Bacterial Isolation.

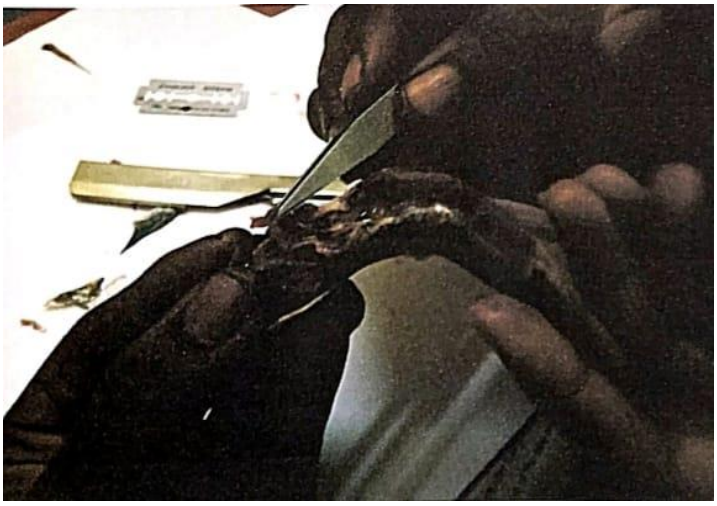

Fig: 3 Fish Samples Dissected For The Bacterial Isolation.

2.1 Chemicals and Microorganisms

All chemicals, reagents, and culture media used in this study were of analytical grade. Chemicals were procured from SRL and SD Fine Chemicals, India, while bacteriological media and enzymes were obtained from HiMedia Laboratories, India. The reference strain of *Aeromonas hydrophila* was obtained from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. Lactic acid bacteria (LAB) were isolated from the intestinal tract of healthy tilapia fish for probiotic evaluation.

2.2 Isolation and Screening of Lactic Acid Bacteria

The intestinal contents of tilapia fish were aseptically collected and serially diluted using sterile normal saline. The diluted samples were spread onto de Man, Rogosa and Sharpe (MRS) agar plates and incubated anaerobically at 37°C for 48–72 hours. Twenty-five well-isolated colonies were selected, purified by repeated streaking on MRS agar, and maintained in MRS broth containing glycerol at −20°C. The isolates were cultured in MRS broth, and the cell-free supernatant was obtained by filtration. The antimicrobial activity of the bacteriocin-containing supernatant against *Aeromonas hydrophila* was evaluated using the agar well diffusion method.

2.3 Characterization of LAB Isolates

The selected LAB isolates were characterized based on their morphological, physiological, and biochemical properties. Gram staining and microscopic examination were performed to determine cell morphology and Gram reaction. Catalase activity, growth at different temperatures (10–45°C), pH values (4.4–9.0), and sodium chloride concentrations (6.5–15%) were evaluated. Additional biochemical tests, including carbohydrate fermentation, nitrate

reduction, gelatin hydrolysis, ammonia production, and homo- or heterofermentative metabolism, were carried out for species identification.

2.4 Isolation and Identification of Bacteria

Fish samples were processed aseptically, and swab samples from the skin, shell, and intestinal regions were inoculated onto Nutrient Agar, Blood Agar, MacConkey Agar, and TCBS agar. After incubation at 37°C for 24 hours, bacterial colonies were examined for colony morphology. Gram staining and motility tests were performed, followed by biochemical identification using IMViC, oxidase, catalase, urease, nitrate reduction, indole, citrate utilization, and sugar fermentation tests to confirm bacterial identity.

2.5 Antibiotic Sensitivity Test

The antibiotic susceptibility of *Aeromonas hydrophila* was determined using the modified Kirby–Bauer disc diffusion method. A standardized bacterial suspension was spread uniformly on Mueller–Hinton agar plates, and antibiotic discs were placed on the surface under sterile conditions. Following incubation at 37°C for 24 hours, the diameter of the inhibition zones around each antibiotic disc was measured and recorded to determine the sensitivity or resistance pattern of the isolate.

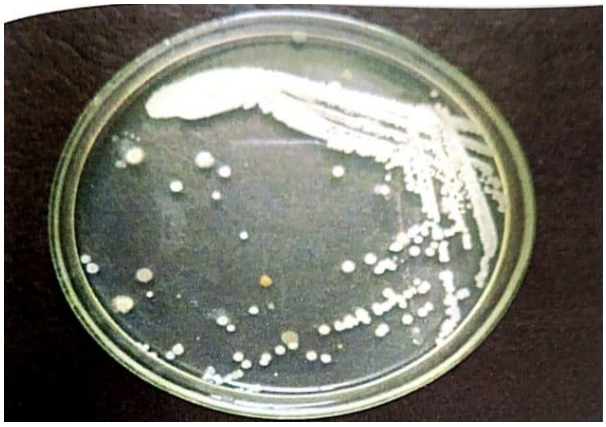

Fig: 4 Lactobacillus Plantarum Isolated From Fish Gut.

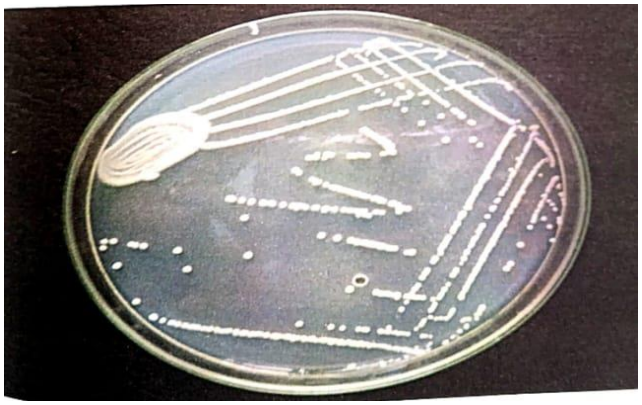

Fig: 5 Aeramanas Hydrophila Culture Used In Bacteriocin Activity.

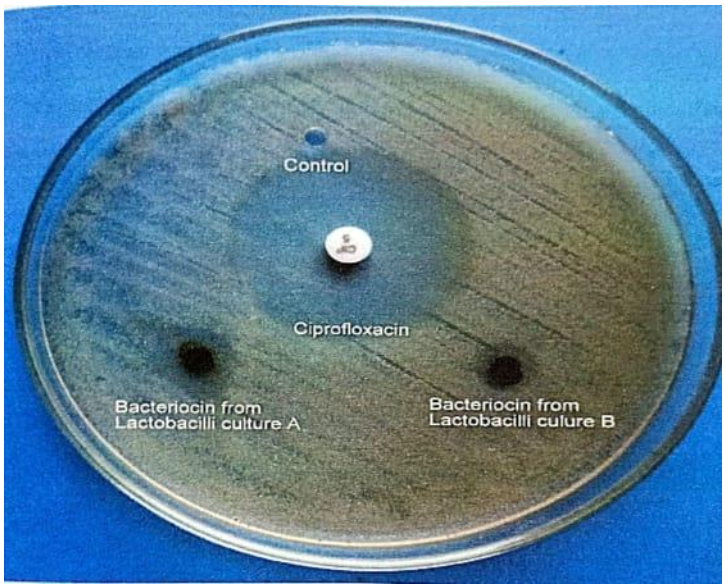

Fig: 6 Anti- Aeromonas Hydrophila Activity Of Bacteriocin Produced By Lactobacillus Isolates.

From Fish Samples

2.6 Chemicals

All analytical-grade chemicals and dyes used in this study were purchased from SRL Laboratories, India, and SD Fine Chemicals, India. Proteolytic enzymes and bacteriological media were obtained from HiMedia Laboratories, India.

2.7 Microorganisms

The indicator bacterium Aeromonas hydrophila was obtained from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. Lactic acid bacteria (LAB) were isolated from the gut of fish.

2.8 Isolation and Screening of LAB

Fish gut samples were diluted with sterile saline and spread on MRS (de Man, Rogosa, and Sharpe) agar plates. The plates were incubated anaerobically at 37°C for 48–72 hours. Twenty-five well-isolated colonies were selected and transferred to MRS broth. The cultures were purified by repeated subculturing and stored in 0.5% MRS soft agar containing 50% glycerol at –20°C. The isolates were screened for bacteriocin production by growing them in MRS broth for 48 hours at 37°C. Cell-free supernatants were adjusted to pH 5.0, concentrated, sterilized using a 0.22 µm membrane filter, and tested for antimicrobial activity against Aeromonas hydrophila using the agar well diffusion method.

2.9 Characterization of Bacteriocin-Producing LAB

The selected LAB isolates were characterized by Gram staining, microscopic examination, and catalase testing. Growth was studied at different temperatures (10, 15, 37, and 45°C), different pH values (4.4, 5.0, 8.6, and 9.0), and different sodium chloride concentrations (6.5%, 10%, and 15% NaCl). Acid and gas production from glucose, ammonia production, carbohydrate fermentation, gelatin hydrolysis, nitrate reduction, and homo- or heterofermentative properties were evaluated using standard microbiological methods. The type of lactic acid produced was determined using D- and L-lactate dehydrogenase enzymes.

2.10 Processing of Samples

Shrimp samples were collected using sterile cotton swabs from the shell, skin, and gut. The swabs were inoculated onto Nutrient Agar, Blood Agar, MacConkey Agar, and TCBS Agar for the isolation of different bacteria, including Vibrio species. The inoculated plates were incubated at 37°C overnight.

2.11 Culture Media

The bacterial isolates were grown using standard culture media:

- Nutrient Agar (pH 7.4 ± 0.2)
- Blood Agar containing 5% defibrinated blood (pH 7.6 ± 0.2)
- MacConkey Agar (pH 7.1 ± 0.2)

Each medium was prepared using standard microbiological ingredients such as peptone, sodium chloride, agar, beef extract, yeast extract, lactose, bile salts, neutral red, and distilled water.

2.12 Identification of Bacteria

The bacterial colonies obtained from different media were identified based on their morphological, cultural, and biochemical characteristics**.**

2.13 Morphological Study

The isolates were examined by Gram staining. A thin smear of each bacterial culture was prepared on a glass slide, heat-fixed, stained, and observed under an oil immersion microscope (100× objective).

2.14 Gram Staining Procedure

The heat-fixed smear was stained with crystal violet for 1 minute, rinsed with water, and treated with Gram's iodine for 1 minute. The smear was then decolorized using ethyl alcohol or acetone and counterstained with safranin for 1 minute. After washing and drying, the

stained bacteria were observed under a microscope to determine whether they were Gram-positive or Gram-negative.

2.15 Motility Test:

Bacterial motility was determined using the hanging drop method. A drop of bacterial suspension was placed on a coverslip, and a concave glass slide was inverted over it. The preparation was examined under a light microscope to observe the movement of bacterial cells.

2.16 Gram Staining Reagents

The Gram staining reagents included:

- **Crystal Violet:** Prepared by mixing crystal violet, ethanol, ammonium oxalate, and distilled water.
- **Gram's Iodine (Lugol's Iodine):** Prepared using iodine, potassium iodide, and distilled water.
- **Decolorizer:** 95% ethyl alcohol diluted with distilled water.
- **Safranin:** Prepared by dissolving safranin in distilled water.

3. SIMPLIFIED BIOCHEMICAL IDENTIFICATION AND ANTIBIOTIC SENSITIVITY TESTING

3.1 Biochemical Identification

The bacterial isolates obtained from Nutrient Agar and Blood Agar were identified using standard biochemical tests, including IMViC tests (Indole, Methyl Red, Voges-Proskauer, Citrate), nitrate reduction, urease, sugar fermentation, catalase, oxidase, and string tests**.**

3.2 Sugar Fermentation Test

The ability of bacteria to ferment different sugars was determined using peptone water containing 1% sugar and a pH indicator with an inverted Durham's tube for gas detection. The sugars tested included glucose, lactose, maltose, sucrose, and arabinose. After incubation at 37°C for 24 hours, acid production was indicated by a colour change, while gas production was observed in the Durham's tube.

3.3 Indole Test

Bacterial colonies were inoculated into indole broth and incubated at 37°C for 24 hours. After adding Kovac's reagent, the appearance of a red ring indicated a positive result for indole production.

3.4 Methyl Red (MR) Test

The isolates were inoculated into MR-VP broth and incubated at 37°C for 24 hours. After adding methyl red indicator, the development of a red colour indicated a positive result, showing mixed acid fermentation.

3.5 Voges-Proskauer (VP) Test

The isolates were cultured in MR-VP broth for 24 hours at 37°C. After adding Barritt's reagent, the appearance of a pink to bright red colour indicated a positive VP reaction.

3.6 Citrate Utilization Test

The bacterial isolates were streaked on Simmons Citrate Agar slants and incubated at 37°C for 24 hours. A green-to-blue colour change indicated the ability of the organism to utilize citrate as the sole carbon source.

3.7 Oxidase Test

The bacterial colonies were tested using oxidase reagent on filter paper. The appearance of a purple colour within a few seconds indicated a positive oxidase reaction.

3.8 Urease Test

The isolates were inoculated into Christensen's Urea Agar or urea broth and incubated at 37°C overnight. A yellow-to-pink or red colour change indicated urease enzyme production due to ammonia formation.

3.9 Catalase Test

A loopful of bacterial culture was mixed with 3–10% hydrogen peroxide (H_2O_2)**.** The formation of bubbles (effervescence) indicated a positive catalase test.

3.10 Nitroso-Indole Test

The bacterial culture was grown in peptone water at 37°C overnight. After adding concentrated sulfuric acid (H_2SO_4)**,** the development of a red colour indicated simultaneous production of nitrate reductase and indole.

3.11 Nitrate Reductase Test

The bacterial culture was inoculated into nitrate broth and incubated at 45°C for 10 minutes. After adding N-naphthylamine and sulphanilic acid reagents, the appearance of a red colour indicated a positive nitrate reduction test.

3.12 Culture Media and Reagents

Standard microbiological media and reagents were used for biochemical identification, including:

- **Indole Medium**

- **MR-VP Broth**
- **Kovac's Reagent**
- **Methyl Red Indicator**
- **Barritt's Reagent**
- **Simmons Citrate Agar**
- **Christensen's Urea Agar**
- **Trypticase Soy Agar (TSA)**
- **DNase Toluidine Blue Ampicillin (DNTA) Agar**

All media were prepared according to standard microbiological formulations with the required pH.

3.13 Antibiotic Sensitivity Test

The antibiotic susceptibility of the bacterial isolates was determined by the Kirby–Bauer disc diffusion method using Mueller-Hinton Agar. A bacterial lawn was prepared from nutrient broth cultures, and antibiotic discs were placed on the agar surface using sterile forceps. The plates were incubated at 37°C for 24 hours, after which the zone of inhibition around each antibiotic disc was measured. The diameter of the inhibition zone was used to determine the susceptibility or resistance of the bacterial isolates.

3.14 Mueller-Hinton Agar Composition

Mueller-Hinton Agar was prepared using standard ingredients including:

- Beef infusion
- Casein acid hydrolysate
- Starch
- Agar
- Distilled water

The final medium was adjusted to pH 7.3 ± 0.2 before sterilization and use.

4. RESULTS AND DISCUSSION

4.1 Microbiological Quality of Fish Samples

The microbiological quality of the fish samples was evaluated using the Standard Plate Count (SPC) method to determine the total aerobic mesophilic bacterial count. Different bacterial colonies were isolated from the fish samples, and their morphological, cultural, and biochemical characteristics were studied for identification.

4.2 Morphological and Cultural Characteristics of Bacterial Isolates:

The bacterial isolates showed differences in Gram staining, motility, colony appearance, and growth on different culture media. Most isolates were Gram-positive cocci, while some were Gram-positive rods and Gram-negative rods. Gram-positive cocci were generally non-motile and produced golden yellow colonies on nutrient agar with beta-hemolysis on blood agar and small pink colonies on MacConkey agar. Gram-negative rods were motile, produced large transparent or mucoid colonies, and showed variable hemolytic activity. These observations helped in the preliminary identification of the bacterial species.

Table: 1 Morphological and Cultural Characteristics of Bacterial Isolates.

Isolate	Gram Staining	Motility	Nutrient Agar	Blood Agar	MacConkey Agar
1	Gram-positive rod	Non-motile	White rhizoidal colonies	Non-hemolytic	Pink colonies
2	Gram-positive cocci	Non-motile	Golden yellow colonies	β-Hemolytic	Small pink colonies
3	Gram-positive cocci	Non-motile	Golden yellow colonies	β-Hemolytic	Small pink colonies
4	Gram-negative rod	Motile	Large transparent/mucoid colonies	Hemolytic	Colourless colonies
5	Gram-positive cocci & Gram-negative rod	Non-motile / Motile	Yellow and mucoid colonies	Alpha/non-hemolytic	Small pink colonies

Table: 2 Biochemical Characteristics of Bacterial Isolates.

Test	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5
Indole	–	–	Nil	–	–
Methyl Red	–	–	Nil	–	–
Voges-Proskauer	–	–	Nil	–	–
Citrate Utilization	ND	+	Nil	ND	–
Catalase	+	+	Nil	+	+
Oxidase	–	–	Nil	–	–

4.3 Biochemical Characteristics

The bacterial isolates were identified using standard biochemical tests, including Indole, Methyl Red, Voges-Proskauer, Citrate Utilization, Catalase, Coagulase, and Oxidase tests. Most isolates were catalase-positive, while the reactions for other biochemical tests varied among the isolates, enabling their differentiation and identification.

Table: 3 Biochemical Characteristics.

Isolate	Glucose	Lactose	Sucrose	Mannitol
1	Acid	Acid	Acid	Acid
2	Acid	Acid	Acid + Gas	Acid
3	Acid + Gas	–	Acid	Acid
4	Acid	Acid	Acid	Acid
5	Acid	Acid + Gas	Acid + Gas	Acid + Gas

4.4 Sugar Fermentation Analysis:

The isolates were tested for their ability to ferment different carbohydrates. All isolates fermented glucose, producing acid, while several isolates also produced gas. Most bacteria were able to ferment lactose, sucrose, and mannitol, indicating their carbohydrate utilization capability.

Table: 4 Morphological Characteristics.

Parameter	Observation
Gram Staining	Gram-negative rod
Motility	Motile

Table: 5 Cultural Characteristics.

Medium	Observation
Nutrient Agar	White colourless colonies
TCBS Agar	Yellow colonies
Trypticase Soy Agar	Yellow colonies
DNTA Agar	Yellow colonies
Blood Agar	β-Hemolytic colonies
Bile Esculin Agar	Black colonies (Positive)
MacConkey Agar	Pink colonies

Table: 5 Biochemical Characteristics.

Test	Result
Catalase	Positive
Oxidase	Positive
Indole	Positive
Methyl Red	Positive
Voges-Proskauer	Positive
Citrate Utilization	Negative
Triple Sugar Iron (TSI)	H_2S Positive (A/AS)
Nitrate Reduction	Negative
Urease	Negative
String Test	Negative

Table: 6 Sugar Fermentation.

Sugar	Result
Glucose	Positive with gas
Sucrose	Positive
Mannitol	Positive

Arabinose	Positive
Sorbitol	Negative

Table: 7 Amino Acid Decarboxylase Test.

Test	Result
Ornithine Decarboxylase	Negative

4.5 Identification of *Aeromonas hydrophila*

The isolated organism was identified as *Aeromonas hydrophila* based on its morphological, cultural, biochemical, and sugar fermentation characteristics.

Table: 8 Antibiotic Sensitivity Pattern of *Aeromonas hydrophila.*

S. No.	Antibiotic	Zone of Inhibition (mm)	Interpretation
1	Chloramphenicol	16	Sensitive
2	Gentamicin	25	Sensitive
3	Tetracycline	22	Sensitive
4	Amikacin	20	Sensitive
5	Ampicillin	No Zone	Resistant
6	Ceftriaxone	21	Sensitive
7	Cefotaxime	21	Sensitive
8	Meropenem	9	Sensitive
9	Imipenem	20	Sensitive
10	Penicillin-G	No Zone	Resistant

DISCUSSION

The present study successfully isolated lactic acid bacteria (LAB) from the intestinal tract of healthy fish and evaluated their antagonistic activity against the fish pathogen *Aeromonas hydrophila*. LAB are recognized as important probiotic microorganisms because they produce antimicrobial compounds such as organic acids, hydrogen peroxide, and bacteriocins that suppress the growth of pathogenic bacteria. The inhibitory activity observed against *A. hydrophila* suggests that the isolated LAB possess probiotic potential and may serve as a biological alternative to antibiotics in aquaculture.

The microbiological examination of fish samples revealed the presence of diverse bacterial populations, including Gram-positive cocci, Gram-positive rods, and Gram-negative rods. Differences in colony morphology, hemolytic activity, and growth on selective media demonstrated the microbial diversity naturally associated with fish samples. Similar observations have been reported by several researchers, who found that aquatic environments harbor a mixture of beneficial and opportunistic microorganisms.

Biochemical characterization played a vital role in differentiating the bacterial isolates. The use of Gram staining, catalase, oxidase, IMViC, urease, citrate utilization, and carbohydrate fermentation tests enabled preliminary identification of the isolates. Most isolates showed positive catalase activity, whereas variations in the remaining biochemical reactions confirmed the presence of different bacterial species. These findings agree with standard microbiological identification procedures commonly employed for aquatic bacterial isolates.

Sugar fermentation studies demonstrated that all isolates were capable of fermenting glucose, while several isolates also fermented lactose, sucrose, and mannitol. Acid production, with or without gas formation, indicated active carbohydrate metabolism. These metabolic characteristics are useful taxonomic markers and support differentiation of bacterial species isolated from fish. Similar carbohydrate utilization patterns have been reported for both lactic acid bacteria and *Aeromonas* species.

The pathogenic isolate was identified as *Aeromonas hydrophila* based on morphological, cultural, biochemical, and sugar fermentation characteristics. The isolate was Gram-negative, motile, β-hemolytic, oxidase-positive, catalase-positive, indole-positive, and methyl red-positive, while citrate utilization, urease, nitrate reduction, and string tests were negative. These characteristics correspond closely with published descriptions of *A. hydrophila*, confirming the reliability of the identification methods used in this study.

The antibiotic susceptibility profile showed that the isolate was sensitive to chloramphenicol, gentamicin, tetracycline, amikacin, ceftriaxone, cefotaxime, meropenem, and imipenem, whereas resistance was observed against ampicillin and penicillin-G. Resistance to β-lactam antibiotics among *Aeromonas* species has been widely documented and is mainly attributed to the production of β-lactamase enzymes. The high susceptibility to aminoglycosides, carbapenems, and third-generation cephalosporins suggests that these antibiotics remain effective therapeutic options for treating *Aeromonas* infections.

The observed antibacterial activity of LAB against *A. hydrophila* further supports the use of probiotics as a sustainable alternative to antibiotics in aquaculture. Continuous use of antibiotics often results in antimicrobial resistance, environmental contamination, and drug residues in aquatic products. In contrast, probiotic LAB enhance gut health, improve immune responses, inhibit pathogenic microorganisms, and promote growth performance without contributing to antibiotic resistance. Therefore, the present findings highlight the potential application of bacteriocin-producing LAB as natural biocontrol agents for disease management in aquaculture.

Overall, the results demonstrate that fish gut serves as a valuable source of probiotic LAB with significant antimicrobial properties. The combined use of bacteriocin-producing LAB and appropriate antibiotic therapy may provide an effective strategy for controlling *Aeromonas hydrophila* infections while reducing dependence on conventional antibiotics.

5. CONCLUSION

The present investigation successfully isolated and characterized lactic acid bacteria from the intestinal tract of healthy fish and demonstrated their antibacterial activity against *Aeromonas hydrophila*. Morphological, cultural, biochemical, and carbohydrate fermentation studies confirmed the identification of the bacterial isolates, while antibiotic susceptibility testing showed that *A. hydrophila* remained susceptible to gentamicin, tetracycline, amikacin, ceftriaxone, cefotaxime, meropenem, imipenem, and chloramphenicol but was resistant to ampicillin and penicillin-G. The inhibitory activity exhibited by LAB indicates their ability to produce antimicrobial compounds such as bacteriocins, making them promising probiotic candidates for disease prevention in aquaculture. The use of probiotic LAB may reduce antibiotic dependence, improve fish health, minimize antimicrobial resistance, and contribute to sustainable aquaculture practices. Further molecular characterization, purification of bacteriocins, and in vivo evaluation are recommended before commercial application.

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