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Isolation and screening of probiotics candidates from the intestinal tracts of Nile tilapia (*Oreochromis niloticus*)

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Abstract

Probiotics play a vital role in aquaculture by maintaining digestive tract balance, enhancing enzymatic digestion, synthesizing essential biomolecules, increasing nutrient availability, strengthening intestinal function, detoxifying harmful compounds, modulating immune responses, and improving resistance to pathogens in fish farming. Notably, probiotics derived from the gastrointestinal tract of the same host species exhibit higher colonization efficiency and provide greater benefits compared to those from external sources. Thus, this study aimed to isolate, identify, and evaluate the probiotic potential of bacteria obtained from the digestive tracts of Nile tilapia (*Oreochromis niloticus*) cultured in a rice-fish farming system. The research followed a systematic approach, including bacterial isolation, phenotypic and molecular identification, and an assessment of probiotic properties. A total of 27 bacterial isolates were successfully obtained, and five of which had a capacity to produce digestive enzyme (protease), exhibited strong antimicrobial activity against *Aeromonas hydrophila*. Based on their 16S rRNA gene sequences, two isolates were identified as *Kurthia gibsonii* (99.43% and 100% similarity), one isolate as *Bacillus thuringiensis* (91.41% similarity), one isolate as *Bacillus cereus* (85.01% similarity), and the other isolate as *Lactococcus lactis* (100% similarity). Further analysis confirmed that all isolates were non-pathogenic and susceptible to four antibiotics (Tetracycline, Amoxicillin, Chloramphenicol, and Gentamicin). Additionally, all isolates demonstrated high tolerance to variations in pH and temperature. Among the five isolates, *L. lactis* exhibited the strongest pathogen inhibition and the highest tolerance to environmental fluctuations, suggesting its superior probiotic potential. Overall, these findings indicate that the isolated bacteria, particularly *L. lactis*, hold promise as probiotic agents and dietary supplements for improving fish health and enhancing aquaculture sustainability.

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Introduction

Freshwater fisheries production is a well-developed aquaculture activity in Indonesia. According to the Directorate General of Fisheries of the Indonesian Ministry of Maritime Affairs and Fisheries, aquaculture production in 2020 yielded 18.44 million tons of fish and non-fish commodities, with a target to increase that figure to 22.65 million tons by 2024. A popular fish commodity in Indonesia is tilapia, primarily due to its high demand. Additionally, previous reports indicated that production in 2022 reached 401 thousand tons, reflecting a 9.01% increase from the

previous year. Forecasts also predict an annual increase in the production value of this commodity. To meet the growing market demand, various cultivation techniques have been developed, including the minapadi system. This system has been successfully implemented in tilapia farming across several regions of Indonesia, such as Yogyakarta and Banyumas. However, the application of the minapadi system tends to differ from region to region, providing more opportunities for enhancement. According to previous reports, intensive tilapia cultivation using the Minapadi system is currently facing significant challenges, such as slow fish growth (1-6). These challenges primarily stem from a lack of fish feed absorption, disease outbreaks caused by pathogenic microorganisms (7), and the use of chemicals from pesticides employed in rice cultivation activities (8). This situation has led many cultivators to resort to synthetic chemicals, including antibiotics, growth hormones, and vaccinations. However, consistent use can result in the emergence of antibiotic-resistant microorganisms, mutations in microorganisms, and residues in fish flesh, which pose significant risks to food safety (9,10). To address these limitations, previous reports recommend using probiotic bacteria due to their safe and environmentally friendly characteristics. Fish farming that involves probiotic bacteria presents both positive and negative impacts on the environment and the host (11). Various reports on the use of probiotics in tilapia have shown significant positive effects on growth and health. These bacteria also play crucial roles in the aquaculture industry by enhancing growth, feed efficiency, microbiota balance, and the quality of cultivation media (12-14). Thus, the development of probiotic products in tilapia farming has become a focal point for many studies and cultivators. Despite their widespread availability, the effectiveness of commercial probiotic products remains uncertain due to the varying origins of the bacteria used. This highlights the need to develop indigenous probiotics isolated from the environment, specifically from the host (blood, liver, kidney, spleen, and intestines) and the environment (water and sediment). Indigenous bacteria are reported to be more effective because of their high adaptability to the environment and stable growth patterns (15,16). Some commonly used indigenous bacteria as probiotics include species such as *Bacillus* sp., *Lactobacillus* sp., *Enterococcus* sp., and *Carnobacterium* sp. (17). According to several studies, the intestine is a highly complex organ due to its direct interaction with the environment through feed consumption, leading to the development of unique mechanisms (18). These mechanisms, including digestive enzymes secreted by the host, defensive chemical molecules, and bacterial communities, assist in various metabolic functions (19). Moreover, certain bacterial communities in the intestine have demonstrated beneficial potential that can be isolated and developed into probiotics. A previous report indicated that several groups of bacteria can hydrolyze organic feed compounds in the intestine, such as protein and starch. The potential of these bacteria is also evidenced by their ability to inhibit pathogens, including *Bacillus* sp. and *lactic acid* bacteria (LAB). While *Bacillus* group bacteria are commonly found in various environments, such as tilapia digestion (20-22), LAB is comparatively rare due to their non-native status. Nevertheless, LAB has shown significant potential for fish health by promoting the growth and well-being of farmed fish and thriving in acidic environments (23,24). Certain bacteria in the intestine are also capable of stimulating the development of the gastrointestinal (GI) tract and digestive functions, as well as enhancing immune responses and disease resistance in farmed fish. Given the sensitivity of tilapia fish culture to environmental factors and the intricacy of digestive processes, there is a critical need for testing and developing probiotics. Previous research on isolating bacteria from the digestive tract of tilapia has been reported. For example, a study by Reda *et al.* (25) investigated the selection and characterization of potential probiotic bacteria from the digestive tract of tilapia. The results of this study successfully isolated bacteria as probiotic candidates, including the following species: *Lactococcus lactis*, *Enterococcus faecalis*, *Lysinibacillus* sp., and *Citrobacter freundii*. This is further supported by research conducted by Huang *et al.* (26), who isolated bacteria from the digestive tract of farmed fish. This study tested several characteristics, including enzyme production, antagonistic ability, hemolytic activity, drug sensitivity assessment, and in vivo safety evaluation. The isolated bacteria included *Bacillus tequilensis*, *B. aryabhattai*, *B. megaterium*, *B. velezensis*, *B. licheniformis*, *B. aryabhattai*, and *Lactococcus lactis* LL0306-15 (LL). However, the number of isolated probiotic strains was limited, and the function of probiotics was not thoroughly investigated in these prior studies. Therefore, additional studies on probiotic strains and comprehensive probiotic characteristics are needed. This study successfully isolated bacteria from the digestive tract of tilapia cultivated in the rice-fish system. Based on the isolation obtained, probiotic products can be produced and applied to the rice-fish cultivation of tilapia. The digestive characteristics and dietary habits of Nile tilapia, derived from both animal and plant sources, significantly influence intestinal environmental conditions, necessitating additional probiotic support to facilitate the digestion process (27).

This indicates that characterizing potential bacteria from the intestine of Nile fish is essential for obtaining bacteria that can enhance productivity and health. Therefore, this study aims to isolate, identify, and test the capabilities of potential bacteria from Nile fish cultivated in the rice-fish farming system as probiotics.

Materials and methods

Ethical approval

This study was conducted with the approval of Medical/Health Research Bioethics Commission Faculty of Medicine Sultan Agung Islamic University of Semarang, under protocol number 332/VIII/2024.

Sampling

Six Nile tilapia (*Oreochromis niloticus*) at different life stages (three juveniles and three adults) were collected from the rice-fish farming system pond. The fish were killed, placed in a cool box, and transported to the Biology Education Laboratory, Muhamadiyah University, Purwokerto, Indonesia.

Bacterial isolation

The fish were dissected aseptically, and the intestines were placed individually in 1.5 mL sterile microtubes. Subsequently, the samples were homogenized and serially diluted up to 10^{-3} with a sterile physiological solution (0.9% NaCl). A total of 1 ml of each dilution was then inoculated into the De Man Rogosa and Sharpe Agar (MRSA) media, which had previously been added with 1% CaCO3 as an indicator of acid-producing bacteria. Then, the media was inoculated at 28°C for 24 hours (28).

Biochemical identification

Colonies that grew and produced clear zones were selected and purified. To confirm LAB bacteria, the characteristics of colonies' morphology and biochemistry, such as gram KOH testing, catalase, and endospore staining, were observed. Simple biochemical tests were also carried out, such as oxidative fermentative, MR-VP, Simmon citrate, and enzymatic activity (proteolytic, amylolytic).

Bacterial motility test

The motility test was carried out following the modified procedure. For each isolate tested, Sulfide Indole Motility Agar (SIM-A) was prepared in a test tube. The test bacterial isolates were taken using an inoculant puncture needle and poked into the SIM-A medium to a depth of 2/3 of the prepared media. Subsequently, the cultured bacteria were incubated for 48 hours at 28°C. Positive motility was indicated by the growth of bacteria that spread like roots around the stabbing area of the test isolates.

Carbohydrate fermentation test

Bacteria that can ferment carbohydrates can be detected using triple sugar iron agar (TSI) (28). The carbohydrates detectable included glucose, lactose, and sucrose, while the TSI agar was prepared in sterile 8 ml tubes. The test isolates were cultured by using an inoculant needle to pick the samples from the bottom of the tube and then incubated for 48 hours at 28ºC. The color change in the agar from red to yellow indicated acid production due to fermentation reactions, and the formation of gas in the media indicated CO_2 production.

Molecular identification

Identification of bacteria was conducted based on the similarity of the *16S rRNA* gene sequence to data in GenBank. The bacterial genomic DNA was extracted using the Presto™ Mini gDNA Bacteria Kit, following the procedure outlined in https://www.geneaid.com/data/download/attached/160274590882278432 7.pdf, to obtain a pure DNA solution. The results of each bacterial gDNA extraction were combined with mastermix, which included a pair of primers, nuclease-free water, and Mytaq HS Redmix 2x (Bioline) (Table 1). After mixing with mastermix, PCR amplification was performed using the Primers 25 Thermocycler PCR (Peqlab). The primers used in amplification were based on the study by Marchesi *et al.* (29), specifically 27f (5'- AGA GTT TGA TCC TGG CTC AG -3') and 1392r (5'- GGT TAC CTT GTT ACG ACT T -3'), yielding a 1500bp amplification product. The annealing temperature was set at 55ºC for 30 seconds. The PCR program for amplification included pre-denaturation at 94ºC for 2 minutes, followed by 35 cycles of denaturation at 94ºC for 20 seconds, annealing at 55ºC for 30 seconds, extension at 72ºC for 20 seconds, followed by a final extension at 72ºC for 5 minutes and storage at 25ºC for 1 minute. The complete sequence results were analyzed using the Basic Local Alignment Search Tool (BLAST) via the online program provided by NCBI (http://ncbi.nlm.nih.gov/).

Table 1: PCR amplification reaction components

Component	Volume (µL)
DNA template	1
Primer 27f	1
Primer 1392r	1
Taq HS RedMix	25
Nucleus Free Water	22
Volume Reaction	50

Selection of probiotic candidate lab isolates

The standard for determining probiotic bacterial isolates is (30,31) that isolates that can be used as probiotic bacteria are tolerant to temperature and pH environments, not pathogenic, can inhibit pathogens, have the potential to help digestion with extracellular enzymes, and are compatible with common bacteria or other strains.

pH and temperature tolerance test

This test aimed to determine the ability of bacteria to thrive under various environmental conditions, specifically differing temperatures and pH levels. The testing involved taking bacterial isolates with loops and placing them in

DeMan Rogosa Sharpe Broth (MRSB) media with varying pH concentrations of 2, 4, and 7. The samples were incubated at different temperatures, including 20, 30, and 40ºC. Bacterial growth was observed every 12 hours during incubation using a spectrophotometer. Subsequently, phenotypic characteristics were assessed based on growth ability and cell numbers relative to the control. The cell count was measured with a spectrophotometer and analyzed through an online program.

Hemolytic test

The hemolytic test was conducted using blood agar supplemented with 5% fish blood. Furthermore, to prevent contamination of the mixed media, ultraviolet irradiation was applied. The hemolytic testing process involved scraping the probiotic bacterial isolates onto the blood agar media, which had been supplemented with 5% fish blood, and incubating it at 30°C for 24 hours. The resulting indicators were a green zone around the colony, indicating α-hemolysis (imperfect hemolytic zone), the presence of a clear zone indicating β-hemolysis (perfect hemolytic zone), and the absence of any zone around the colony, referred to as no- or γ-hemolysis (32).

Antibacterial activity test

The method for testing the presence of antibacterial activity produced by the test was determined using the agar diffusion method (33,34). A colony of *Aeromonas* (*A.*) *hydrophila* bacteria, which had been incubated for 24 hours in Tryptone Soy Broth (TSB) media, was pipetted out in a volume of 100 µl, cultured using the spread plate method, and allowed to stand for 5-10 minutes to enable the suspension to seep into the media. Paper disks with a diameter of 5 mm were placed on top of the media and were added with 0.5 µl of the tested bacterial isolate suspension that had been cultured on MRSB. The MRSA media was then incubated at 28°C for 24 hours, and the diameter of the inhibition zone was measured to assess the antibacterial activity against *A. hydrophila*.

Enzyme producing test

Analyses were conducted using protease and amylase enzymes. The testing mediums employed skim milk agar for protease enzymes and starch agar for amylolytic enzymes. Each LAB was cultured on both media and incubated for 48 hours at 28°C. The formation of a clear zone around the colony indicated enzyme production. The clear zone was measured to determine the index of enzyme activity (35,36).

Synergism test

Tests were conducted on TSA media using the cross-streak method between test isolates. Bacterial isolates were incubated for 24 hours at 28ºC and observed for the presence of an inhibition zone between the two intersecting isolates. The two isolates were considered synergistic when their inhibition zones did not overlap, and antagonistic when an inhibition zone formed between their respective inhibition zones (37).

Antibiotic sensitivity test

The levels of sensitivity and resistance to antibiotics were tested using the Kirby-Bauer disk diffusion test method (38,39), by creating a direct colony suspension of bacteria. The bacterial isolates cultured on MRSB media were taken with a volume of 100 µl, spread over the surface of TSA, and allowed to stand for 5-10 minutes to enable the suspension to seep into the media. Tetracycline, Amoxicillin, Chloramphenicol, and Gentamicin antibiotic discs were placed on the TSA media and incubated at 28ºC for 24 hours. Subsequently, sensitivity to antibiotics was indicated by the presence of a zone of inhibition of bacterial growth around the paper disc (40).

Results

Morphological and Physiological Characteristics of bacteruak Isolates

A total of 27 intestinal bacterial isolates were obtained from six fish samples, including 10 from 3 jevenile tilapia and 17 from 3 adult tilapia. Morphological and biochemical characteristics of the bacterial isolates are presented in Table 2 and Figure 1. The morphological observations in Table 1 indicated that five bacteria could develop into LAB. This was evident from the biochemical characteristics of LAB, specifically from the Gram stain, catalase test, and motility, as shown in Table 3.

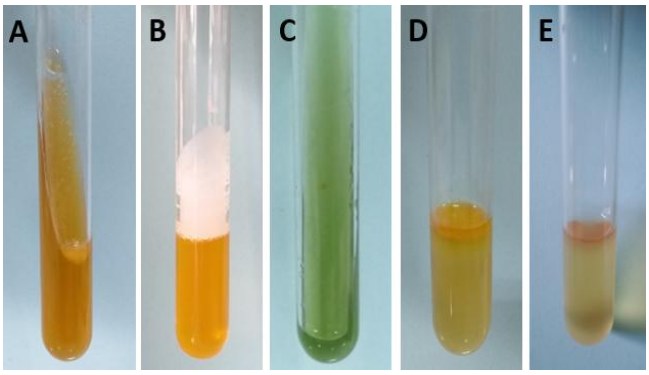

Figure 1: Biochemical testing results of bacteria: (A) Triple Sugar Iron Agar test (TSIA); (B) Oxidation-Fermentation (O/F) test; (C) Simmons Citrate test; (D) Sulphite Indole Motility (SIM) test; (E) Methyl Red-Voges Proskauer (MR/VP) test.

Molecular identification

Molecular identification strengthens the bacterial species obtained from biochemical tests. Selected bacterial isolates were identified molecularly based on the *16S rRNA* gene.

Identification was carried out using the *16S rRNA* gene amplification with the help of a PCR machine to obtain a 1500-bp amplicon product (Figure 2 and 3). The resulting amplicon products were sequenced to obtain complete sequences, which were then blast analyzed in GenBank. The results of bacterial blast analysis are shown in Table 4.

Table 2: Morphological characteristics of bacterial isolates

Fish	Isolate code	Colony Morphology				
		Form	Elevation	Edge	Colour	Size
Intestines of Seed Fish	BU1	Rhizoid	Flat	Filamentous	Clear	Medium
	BU2	Punctiform	Flat	Undulate	Clear	Medium
	BU3	Circular	Flat	Entire	Deep White	Big
	BU4	Circular	Flat	Lobate	Deep White	Small
	BU5	Circular	Flat	Lobate	Yellowish White	Medium
	BU6	Circular	Flat	Lobate	Brownish white	Small
	BU7	Irregular	Flat	Lobate	Brownish white	Big
	BU8	Circular	Flat	Lobate	Clear	Small
	BU9	Irregular	Flat	Lobate	Clear	Big
	BU10	Irregular	Flat	Lobate	Clear	Small
Intestines of Adult Fish	UIS 2	Circular	Umbonate	Entire	Shiny white	Big
	UIS 3	Circular	Umbonate	Entire	White	Big
	UIS 5	Circular	Umbonate	Entire	Shiny white	Medium
	UIS 6	Circular	Umbonate	Entire	Yellowish white	Medium
	UIS 7	Circular	Umbonate	Entire	Yellowish white	Small
	UIS 8	Circular	Umbonate	Entire	Yellowish white	Small
	UIS 9	Circular	Umbonate	Entire	Yellow	Big
	UIS 10	Circular	Pulvinate	Entire	Shiny white	Medium
	UIT 1	Circular	Pulvinate	Entire	Shiny white	Small
	UIT 2	Circular	Pulvinate	Entire	White	Medium
	UIE 1	Circular	Pulvinate	Entire	Yellow	Medium
	UIE 2	Circular	Pulvinate	Entire	Yellow	Small
	UIE 4	Circular	Pulvinate	Entire	White	Small
	UIE 5	Circular	Pulvinate	Entire	Fading white	Medium
	UIE 6	Circular	Pulvinate	Entire	Fading white	Medium
	UIL 1	Circular	Pulvinate	Entire	Fading white	Medium
	UIS 4	Circular	Pulvinate	Entire	Fading white	Medium

Table 3: Biochemical characteristics of potential bacterial isolates

	UIS 6	UIS 7	UIE 1	UIE 5	BU 7
Motile	-	-	-	-	-
Grams	+	+	+	+	+
Morphology	coccus	basil	basil	basil	basil
Catalase	-	+	+	-	-
Endospore	-	-	-	-	-
Glucose	+	+	+	+	+
Lactose	+	+	+	-	-
Sucrose	+	+	+	-	-
OF	F	F	F	F	F
Oxidase	-	-	-	-	-
Indole	-	-	-	-	-
MR	+	-	+	+	-
VP	-	-	-	-	-
SC	-	-	+	-	-

OF: Oxidative/fermentative. MR: Methyl red. VP: Voges Proskauer. SC: Simmon citrate.

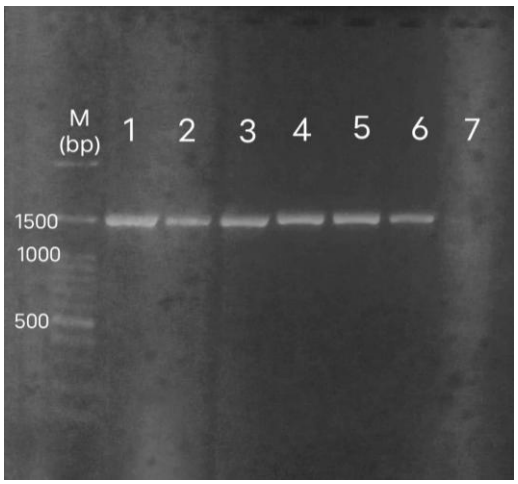

Figure 2: Visualization of the electrophoretic gel amplification results. Note: 1: M= Marker DNA 100bp, 1= BU7, 2=UIS6, 3= UIS7, 4=UIE1, 5=UIE5, 6= Positive Control, 7= Negative Control.

Table 4: Identification based on 16S rRNA gene of LAB isolates candidate

Isolate	Blast Result	Query cover	Identic (%)	Order	Accession number
BU7	*Kurthia gibsonii* strain B3	100	99.43	Bacillales	KM391941.1
UIS6	*Lactococcus lactis* strain 1881	100	100.00	Lactobacillales	MT597705.1
UIS7	*Bacillus thuringiensis* strain LDC 507	100	91.41	Bacillales	KF779471.1
UIE1	*Bacillus cereus* strain TBMAX58	99	85.01	Bacillales	MK83469.7
UIE5	*Kurthia gibsonii* strain KH2	100	100.00	Bacillales	MN453416.1

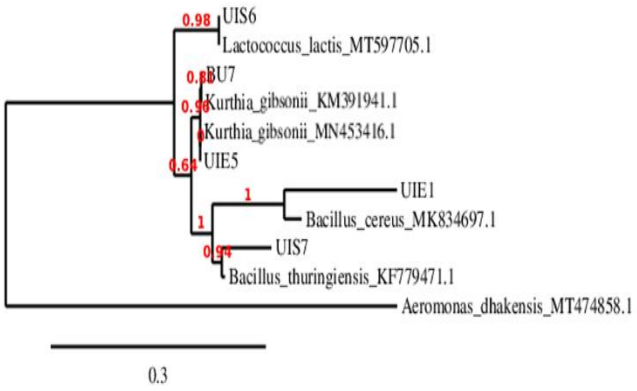

Figure 3: Phylogenetic tree of bacterial probiotic agents and closely related strains based on 16S rDNA sequences. NCBI accession numbers are displayed in parentheses. Tree construction using evolutionary techniques was calculated based on neighbour-joining and bootstrap values applied to 1,000 replications. Bacterial strains without an accession number represent sample bacteria. Branching on Aeromonas_dhakensis (MT474858.1) serves as the out-group.

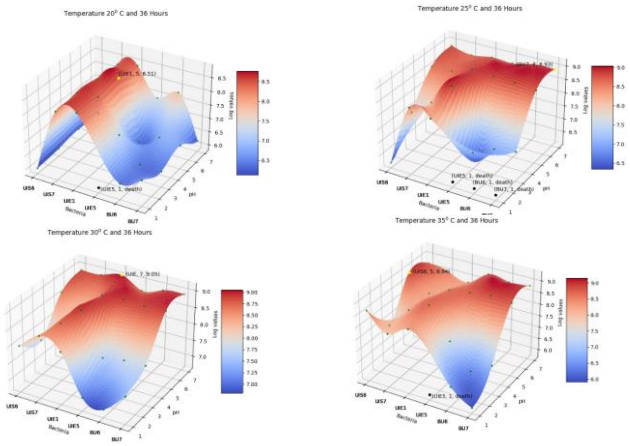

Figure 4: The effect of differences in pH and temperature on the growth of LAB, Bacterial density data is displayed in log 10 form.

pH and temperature tolerance test

Based on Figure 4, a total of five bacterial isolates demonstrated good tolerance to the environment. This indicates that the test bacteria could grow in a low pH environment and at low temperatures (pH 1, 20ºC), although in varying amounts. Furthermore, the test results revealed that at high pH and temperature (pH 7, 35ºC), the bacteria still grew. The results of the tests suggested that these bacteria could tolerate a wide variety of environmental conditions.

Hemolytic test

The hemolytic test results from the bacterial candidate showed a negative hemolytic based on the absence of a clear zone around the scratch. In addition, the results revealed that the bacteria were safe and non-pathogenic in fish.

Antibacterial test

The candidate LAB isolates were tested for their antibacterial activity against the growth of *Aeromonas hydrophila*, a known pathogen for fish. The inhibition zone results were observed in some of the isolates. The antibacterial activity was categorized into four levels: weak (≤5.0 mm), moderate (6-10 mm), strong (11-20 mm), and very strong (≥20 mm). The findings of the antibacterial test are presented in Table 5.

Table 5: Antibacterial activity test results

Strain	Inhibition zone diameter (mm)			Inhibition zone category
	12 hours	24 hours	36 hours	
K. gibsonii	0	0	0	-
L. lactis	0	0	6	Medium
B. thuringiensis	0	0	0	-
B. cereus	0	6	7	Medium
K. gibsonii	0	7,6	0	Medium

Extracellular enzyme activity test and synergism

The results of testing the extracellular enzyme activity of the obtained bacteria were necessary to ascertain their potential for breaking down various organic materials in the environment and facilitating the digestive processes of fish. Some bacteria exhibited proteolytic activity, suggesting that these microbes could contribute to protein degradation in the environment and the breakdown of protein in fish intestines. The results of the enzymatic activity tests can be found in Table 6. The synergistic test results show that the five isolates formed a consortium, indicating their inability to inhibit one another's growth. The absence of an inhibition zone in the cross-scratches between the bacteria demonstrated that they could coexist and interact. The results of the bacterial synergism test are presented in Table 7.

Table 6: The results of the candidate LAB producer extracellular enzyme test

Kode sampel	Proteolytic	Amylolytic
K. gibsonii	+	-
L. lactis	+	-
B. thuringiensis	+	-
B. cereus	-	-
K. gibsonii	-	-

Description: Positive (+) Negative (-).

Table 7: Results of candidate LAB isolates synergism test

Strain	*K. huakuii*	*L. lactis*	*B. thuringiensis*	*B. cereus*	*K. gibsonii*
K. gibsonii	+				
L. lactis	+	+			
B. thuringiensis	+	+	+		
B. cereus	+	+	+	+	
K. gibsonii	+	+	+	+	+

Description: Positive (+) Negative (-).

Antibiotic test

It has been found that there are differences in the levels of antibiotic sensitivity among the candidate probiotic bacteria. The diameter of the inhibition zone (mm) for bacteria tested with standard antibiotics is shown in Figure 5. In our study, we discovered that *K. gibsonii* exhibited sensitivity in the intermediate and susceptible categories to the antibiotic's amoxicillin, tetracycline, chloramphenicol, and gentamicin. Meanwhile, *L. lactis* displays a sensitivity profile that is resistant to the antibiotic's amoxicillin and gentamicin but intermediate to tetracycline and chloramphenicol. On the other hand, the two *Bacillus* strains, namely *B. thuringiensis* and *B. cereus*, demonstrate sensitivity in the intermediate and susceptible categories to the antibiotic's tetracycline, chloramphenicol, and gentamicin, but are resistant to amoxicillin. The results of the antibiotic resistance testing can be seen in Table 8.

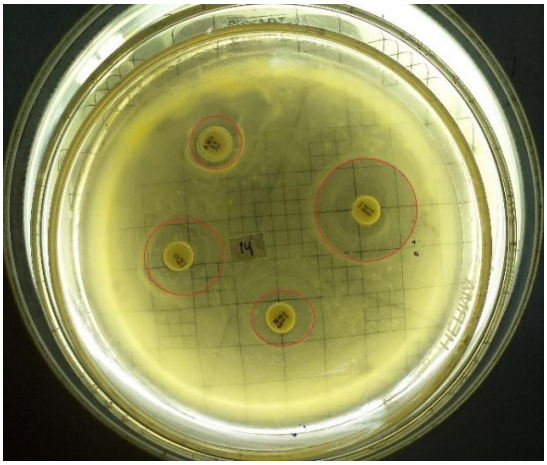

Figure 5: Results of antibiotic resistance testing.

Table 8: The results of the antibiotic activity test

Strain	Tetracycline (30 mcg)	Amoxicillin (25 mcg)	Chloramphenicol (30 mcg)	Gentamicin (10 mcg)
K. gibsonii	S	S	S	S
L. lactis	I	R	I	R
B. thuringiensis	S	R	S	I
B. cereus	S	R	S	1
K. gibsonii	S	I	I	I

Description: Resistant (R), Intermediate (I), and Susceptible (S).

Discussion

Providing probiotics in aquaculture can be accomplished through feed or maintenance media. Probiotics often confer beneficial effects, such as inhibiting pathogen growth, improving growth, enhancing feed efficiency, and promoting water quality along with the immune system by boosting immunity (12). This study identified 27 isolates, with five classified as lactic acid-producing bacteria based on biochemical testing methods. Several LAB strains employed as probiotics include *Lactobacillus plantarum*, *Pediococcus*, *Lactococcus*, and *Enterococcus* (12,41-45). Numerous LAB strains have been reported to significantly affect the growth and health of fish. In this study, five promising bacterial isolates showed strong potential for probiotics based on their properties. The capability to isolate bacteria was indicated by their broad tolerance to environmental conditions, such as temperature and pH. The best performance was observed in the strain *L. lactis* as LAB based on the 16S rRNA gene. Figure 2 illustrates that LAB from low pH and temperature conditions (pH 1, 20ºC) demonstrated growth and a consistent increase until reaching neutral conditions (pH 7, 30ºC).

Due to Indonesia's diverse geography and acidic digestion conditions, this broad environmental tolerance facilitated the application of probiotics. This tolerance has been reported to significantly impact the bacteria's ability, as lactic acid bacteria (LAB) can thrive and effectively perform their role as probiotics. Additionally, the LAB obtained in this study exhibited the characteristics of probiotic bacteria, including non-pathogenicity, the ability to suppress pathogen growth, and the capacity to adhere to the intestinal epithelium, thereby outcompeting pathogenic bacteria. This was indicated by the presence of antibacterial activity against *A. hydrophila*, a common freshwater pathogen. The LAB strain isolated in this study was *L. lactis*, known for its various beneficial properties. According to sourcesNguyen *et al.* (46), *L. lactis demonstrate*d the most effective prevention against pathogenic bacteria compared to other strains. *L. lactis* could also prevent diseases caused by bacteria such as Streptococcus parauberis, *S. iniae, S. parauberis*, *E. viikkiensis*, and *L. garviae*.

The group of *Lactococcus* sp. and *Bacillus* sp. can produce bacteriocin, inhibiting the growth of pathogenic bacteria (47,48). The results of Gonçalves *et al.* (49) and Lin *et al.* (50) indicated that LAB had advantages in producing various types of extracellular substances, including bacteriocins (51), organic acids, enzymes, and hydrogen peroxide, which contributed to mitigating the effects or toxicity of pathogenic bacteria. Furthermore, LAB and potential bacteria in this study produced protease enzymes that support the bacteria's ability to function as probiotics. These enzymes assist in breaking down feed particles and absorbing nutrients during metabolic processes. Huddy & Coyne (52) also noted that probiotic bacteria enhanced protease activity, protein absorption, and growth in abalone by 14.5%. Gómez & Shen, (53) study demonstrated that adding probiotics, specifically *Bacillus* sp. bacteria, to the diet of vannamei shrimp (*Litopenaeus vannamei*) can increase their survival growth rate by 12.34%. This study identified three bacterial strains with proteolytic activity that supports the digestive processes within the intestines of fish.

This study aimed to obtain bacterial strains capable of suppressing pathogens and biocontrolling the environment of the rice-fish farming system. Assessing the potential of LAB and conducting antibiotic testing on various bacteria was crucial in selecting probiotic candidates while considering the associated risks. Most antibiotic tests in this study exhibited suspect properties, suggesting that the isolates could serve as probiotics against pathogens. Several scenarios could arise when probiotic isolates displayed antibiotic-resistant properties. Probiotic isolates carried resistance genes, enabling the transfer of these genes to other bacteria through a gene transduction process. Furthermore, when other present bacteria were pathogenic, the microbe could acquire resistance genes, potentially leading to new challenges in the cultivation environment. The five strains identified in this study met these criteria, particularly strain *L. lactis*, a type of LAB known for its excellent probiotic properties (54).

Conclusion

Five bacterial isolates were obtained from the intestines of Nile tilapia and demonstrated the ability to produce protease and antimicrobial compounds against *Aeromonas hydrophila*. Based on 16S rRNA gene sequencing, these isolates were identified as *Kurthia gibsonii* (two isolates), *Bacillus thuringiensis*, *Bacillus cereus*, and *Lactococcus lactis*. All five isolates exhibited broad tolerance to environmental conditions and were confirmed to be safe, as no beta-hemolytic activity was detected. Among them, *L. lactis* demonstrated the most promising probiotic characteristics due to its high tolerance to pH and temperature fluctuations, as well as its strong antimicrobial activity. Given these properties, *L. lactis* holds potential for further evaluation as a biocontrol agent in aquaculture practices.

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Conflict of interest

The author should declare any conflict of interest.

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عزل وفحص البروبيوتيك المرشحة من القناة المعوية لـ Nile tilapia

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الخلاصة

تلعب البروبيوتيك دورا حيويا في تربية الأحياء المائية من خلال الحفاظ على توازن الجهاز الهضمي، وتعزيز الهضم الأنزيمي، وتوليف الجزيئات الحيوية الأساسية، وزيادة توافر المغذيات، وتقوية وظيفة الأمعاء، وإزالة السموم من المركبات الضارة، وتعديل الاستجابات المناعية، وتحسين مقاومة مسببات الأمراض في تربية الأسماك. والجدير بالذكر أن البروبيوتيك المشتق من الجهاز الهضمي لنفس الأنواع المضيفة يظهر كفاءة أعلى ويوفر فوائد أكبر مقارنة بتلك من مصادر خارجية. وهكذا، هدفت هذه الدراسة إلى عزل وتحديد وتقييم إمكانات البروبيوتيك للبكتيريا التي تم الحصول عليها من القناة الهضمية للـ Nile tilapia المستزرعة في نظام استزراع الأرز والأسماك. اتبع البحث نهجا منهجيا، بما في ذلك العزلة البكتيرية، وتحديد النمط الظاهري والجزيئي، وتقييم خصائص البروبيوتيك. تم الحصول على ما مجموعه ٢٧ عزلة بكتيرية بنجاح، وخمسة منها لديها القدرة على إنتاج إنزيم هضمي

(بروتياز)، وأظهرت نشاطا قويا مضادا للميكروبات ضد الأيروموناس هيدروفيلا. بناء على تسلسلات جين الرنا الريباسي ١٦ ثانية، تم تحديد عزلتين على أنهما Kurthia gibsonii (تشابه ٩٩,٤٣ ٪ و ١٠٠٪) ، عزل واحد مثل Bacillus thuringiensis (تشابه ٩١,٤١٪)، عزل واحد مثل Bacillus cereus (تشابه ٨٥,٠١٪) ، والعزل الآخر مثل Lactococcus lactis (تشابه ١٠٠٪). أكد مزيد من التحليل أن جميع العزلات كانت غير مسببة للأمراض وعرضة لأربعة مضادات حيوية (التتراسيكلين، الأموكسيسيلين، الكلورامفينيكول، والجنتاميسين). بالإضافة إلى ذلك، أظهرت جميع العزلات تحملا عاليا للتغيرات في درجة الحموضة ودرجة الحرارة. من بين العزلات الخمس، أظهر L. lactis أقوى تثبيط للممرض وأعلى تحمل للتقلبات البيئية، مما يشير إلى إمكاناته الفائقة للبروبيوتيك. بشكل عام، تشير هذه النتائج إلى أن البكتيريا المعزولة، وخاصة L. lactis، تبشر بالخير كعوامل بروبيوتيك ومكملات غذائية لتحسين صحة الأسماك وتعزيز استدامة تربية الأحياء المائية.