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Isolation of lactic acid bacteria as potential probiotic candidates from the digestive tract of *Gobiopterus* sp.

MIKKA HAKKINEN[1], ABDUL RAHEM FAQIH[2,♥], ASEP AWALUDIN PRIHANTO[2], SEPTI ANITASARI[2]

[1]Program of Aquaculture, Faculty of Fisheries and Marine Science, Universitas Brawijaya. Jl. Veteran, Malang 65145, East Java, Indonesia.
Tel.: +62-341-553-512, Fax.: +62-341-557-837, ♥email: ar.faqih@ub.ac.id

[2]Faculty of Fisheries and Marine Science, Universitas Brawijaya. Jl. Veteran, Malang 65145, East Java, Indonesia

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Abstract. *Hakkinen M, Faqih AR, Prihanto AA, Anitasari S. 2025. Isolation of lactic acid bacteria as potential probiotic candidates from the digestive tract of* Gobiopterus *sp. Biodiversitas 26: 1008-1017.* The distinctive ecological niche presented by the volcanic Lake Ranu Grati offers a fascinating setting for the exploration of microbial diversity, with a particular focus on lactic acid bacteria (LAB), which are renowned for their advantageous characteristics and hold significant promise as potential probiotics. The fish species *Gobiopterus* sp., which resides within this lake, presents a compelling opportunity for the isolation of novel strains of lactic acid bacteria (LAB). The objective of this study was to isolate and characterize probiotic bacteria from the digestive system of *Gobiopterus* sp. and to find bacterial strains that can improve aquaculture cultivation, health, and disease resistance. This study obtained 30 bacterial isolates originating from the digestive tract of *Gobiopterus* sp., of which 8 isolates were Gram-positive and catalase-negative. These isolates showed the ability to tolerate acid and bile salts which were one of the properties for probiotic candidates. Antibacterial activity varied among the isolates, with some showing significant inhibition against *Aeromonas hydrophila, Vibrio alginolyticus,* and *Vibrio harveyi*. Enzyme activity test found that the selected isolates can produce protease and cellulase enzymes. The selected isolates had sufficient hydrophobicity properties ranging from 23-41% and the DNA sequencing results identified the 3 isolates as the member of genus *Enterococcus*. The results of this study yielded isolates that have the potential as probiotics in the field of aquaculture. Therefore, further research should be conducted for the comprehensive characterization and safety assessment required for the use of probiotic bacteria.

Keywords: Digestive tract, *Enterococcus*, *Gobiopterus*, lactic acid bacteria, probiotics

INTRODUCTION

Bacteria are microorganisms that are spread throughout the world, from the depths of the deep sea to the highest mountain peaks. These microscopic entities are very capable of collaborating or interacting with various hosts that can affect the lives of humans, plants, and animals. Such associations may manifest as mutually advantageous, neutral, or, in certain cases, detrimental to the host organism. Bacteria are broadly categorized into two principal groups, Gram-positive and Gram-negative, based on their cellular structure and reaction to the Gram staining technique (Savitri et al. 2019). The characteristic of bacteria with purple color are gram-positive bacteria is attributed to their thick, peptidoglycan-dense cell walls, which hold onto the crystal violet dye used in the staining procedure, and Gram-negative bacteria are unable to hold onto the dye due to their outer membrane and comparatively thin peptidoglycan layer, which causes it to be washed away and give them a pink or red color. Bacteria with Gram-positive and Gram-negative had different compositions in their cell walls and had significantly different effects on their physiological characteristics and different levels of pathogenicity.

Bacteria can be determined as pathogenic or probiotic in several ways according to their function and characteristics. Probiotic bacteria are microorganisms that are introduced to aquaculture systems with the aim of improving water quality, enhancing feed efficiency, bolstering immune responses, and inhibiting the proliferation of harmful pathogens. Using probiotics to modulate intestinal microbiota is an alternative method of reducing pathogen adhesion and colonization in the digestive tract. Therefore, to increase the proportion of healthy bacteria in the gut, these probiotic species can be added to a fish diet or water (Amenyogbe 2023).

Probiotic bacteria residing within the gastrointestinal tract of fish play an essential role in enhancing digestive efficiency, thereby optimizing the utilization of feed. The beneficial impact of probiotic supplementation on reproductive performance is likely linked to the increased availability of critical nutrients, such as fatty acids and amino acids, which serve as vital components in the processes of oocyte formation and maturation, as well as in embryo development and fry growth. These nutrients can influence the expression of genes related to lipid metabolism. It has been observed that the feeding approach, where probiotics are given at different times has better benefits on the regulation of the immune system so that it can increase activity in response to microbial infections. Probiotics are given periodically to prevent the immune system from being overstimulated and provide high levels of protection against bacterial infections (Dias et al. 2020). The digestive tracts of many fish species naturally contain probiotic bacteria, which are vital for maintaining general health. Probiotics, often known as helpful microorganisms, promote a variety of physiological processes, such as enhancing

food absorption, facilitating digestion, bolstering the immune system, and offering defense against pathogenic bacteria. For example, other studies have successfully identified specific probiotic strains uniquely associated with particular fish species, further underscoring their significance in aquaculture as well as Catfish (*Clarias gariepinus*): *Lactococcus lactis*, *Enterococcus hirae*, and *Weissella confusas* (Nurhayati et al. 2023), and Rhynchocypris fish (*Rhynchocypris lagowskii*): *Bacillus velezensis* (Elsadek et al. 2023)

Gobiopterus sp., commonly known as dart gobies, are native Indonesian fish found in Ranu Grati Lake, Pasuruan Regency, East Java (Anitasari et al. 2024). The presence of *Gobiopterus* sp. underscores the importance of Ranu Grati as a unique and crucial habitat for the survival of this species (Anitasari et al. 2024). The high sulfur content in the volcanic Ranu Grati Lake water can range from 0.5 to 10 mg/L, which makes it different from other lakes. This unique environmental factor may have a major impact on the structure of probiotic bacteria, especially in the digestive tract of *Gobiopterus* sp. The microbiota in the digestive tract of *Gobiopterus* sp. may be a candidate for probiotics with beneficial qualities. The objective of this study was to isolate and characterize probiotic bacteria from the digestive system of *Gobiopterus* sp. and to find bacterial strains that can improve aquaculture cultivation, health, and disease resistance.

This study helps in the preservation of regional biodiversity and understanding the microbial ecology form this fish species and more effectively establishes a foundation for future conservation actions and offers important insights into the adaptation processes of creatures that flourish in unique conditions.

MATERIALS AND METHODS

Study area

The research was undertaken at Universitas Brawijaya, with the sampling of *Gobiopterus* sp. conducted at Ranu Grati Lake in Pasuruan, East Java, Indonesia. Fish specimens were collected from three distinct locations at Point A (on the edge of the lake), Point B (middle of the lake, between points Point A and Point C) and Point C (in the middle of the lake) to ensure a representative and evenly distributed sampling process. The sample were taken to the laboratory and then stored in the refrigerator, until preparation for taking digestive tract samples was carried out **(**Figure 1).

Procedures

Isolation of gut bacteria

For the isolation of total heterotrophic bacteria (THB), the gut was done by surgery on the fish's stomach and taking the digestive tract. The digestive tract used for the sample was the entire digestive tract until weighing 1 g. Then 1 g sample was suspended in 9 mL of sterile distilled water. The mixture was subjected to agitation for several minutes using a shaker to ensure homogeneity. Subsequently, the sample underwent serial dilution, ranging from a 10^{-1} to a 10^{-6} dilution, to facilitate the bacterial isolation process (Ramanathan and Alagesan 2012). 0.1 mL aliquot of each dilution was plated onto Man Rogosa Sharpe (MRS) agar (Ji et al. 2015), and modified agar medium, supplemented with 0.5% $CaCO_3$, and then petri plates were incubated at 37°C for 24 hours.

Macroscopic analysis

Macroscopic examination involved three distinct observational approaches: viewing the colonies from above to ascertain their overall shape, closely inspecting the edges to determine the morphology of the colony margins, and examining the colonies from the side to evaluate their height (Irawan 2024).

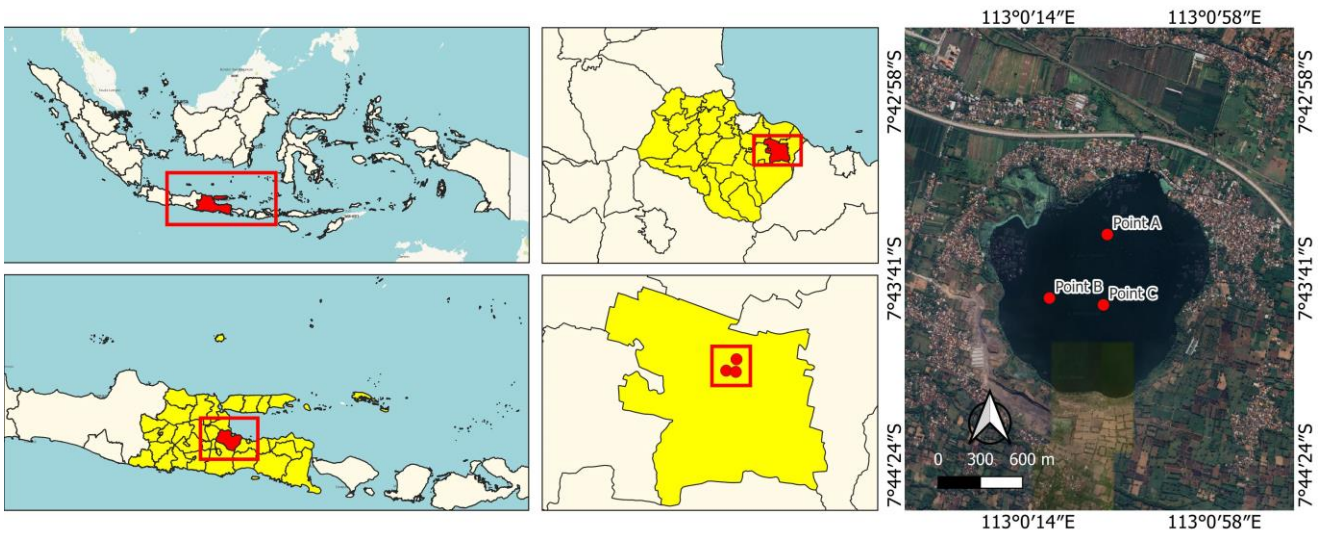

Figure 1. Ranu Grati Lake, Pasuruan, East Java, Indonesia shows the location of sampling of *Gobiopterus* sp.: Point A (7°43'30.5"S 113°00'32.8"E), Point B (7°43'45.2"S 113°00'19.4"E); and Point C (7°43'46.8"S 113°00'31.9"E)

Microscopic analysis and Gram staining

Microscopic identification used modified methods of (Lainjong 2024), while colony morphology was observed using Gram staining technique. Initially, the bacterial sample was prepared on a glass slide, followed by fixation. After fixation, 2-3 drops of crystal violet were added onto the bacterial colony and left undisturbed for two minutes, then washed with sterile distilled water and allowed to air dry. Once dried, 2-3 drops of Lugol's iodine solution were added, left for another two minutes, and subsequently rinsed again with sterile distilled water before air- drying. The dried slide was then treated with 2-3 drops of 96% ethanol, left for 30 seconds, washed with sterile distilled water, and air-dried. Next, the slide was stained with 2-3 drops of 0.5% safranin solution, left for one minute, rinsed, and air-dried. Finally, the prepared slide was examined under a microscope. The purpose of Gram staining was to observe the morphology of the bacterial colonies microscopically and to differentiate between Gram-positive and Gram-negative bacteria.

Bile tolerance

The bile salt tolerance was determined as (Gou et al. 2021) with slight modifications. Initially, the inoculant was aerobically cultured in MRS broth at 38°C for 18 hours. Subsequently, 1 mL samples of fermentation broth were collected and subjected to centrifugation at 2,235 g for 10 minutes to separate the pellet. The resulting pellet was then resuspended uniformly in 1 mL of inoculant medium containing 2.5-10% (w/v) bovine bile salts. The prepared mixtures were then incubated aerobically on MRS agar at 38°C for 24 hours to facilitate further analysis.

Gastric juice tolerance

The acid resistance test was conducted by inoculating bacterial cultures into an MRSB medium adjusted to a pH range of 4 to 7, followed by incubation for 24 hours at 37°C. The ability of bacteria to grow within this pH range was indicated by the medium becoming turbid, reflecting the presence of bacterial particles. Bacterial growth was further confirmed by streaking the medium onto the surface of de Man Rogosa Sharpe agar (MRSA). The presence of bacterial colonies on the MRSA streaks after 24 hours of incubation confirmed the survival and growth of the bacteria at pH levels between 4 and 7 (Istiqomah et al. 2019).

Antibacterial test

The isolate was cultured in 1 mL of MRSB medium within a microtube and incubated for 24 hours. Following incubation, the culture was subjected to centrifugation at 10,000 rpm for 5 minutes at a temperature of 4°C to separate the supernatant, which was subsequently utilized for antibacterial testing. The pathogenic bacterial strains included *Aeromonas hydrophila* (30×10^8 CFU/mL), *Vibrio harveyi* ($<3\times10^8$ CFU/mL), and *Vibrio alginolyticus* ($<3\times10^8$ CFU/mL). The challenge test was carried out with pathogenic bacteria smeared on the surface of the de Man Rogosa Sharpe agar (MRSA) media, and a paper disc containing supernatant isolate was placed on the surface of the MRSA media, then incubated for 24-48 hours and observed the clear zone. The diameter of inhibition zone was measured using the formula (Ngamsurach and Praipipat 2022):

$$\text{Diameter of Inhibition zone} = \frac{(Dv - Dc) + (DH - DC)}{2}$$

Where:
DV: Diameter of clear zone vertical
DC: Diameter of blank disc (control)
DH: Diameter of clear zone of horizontal

Catalase analysis

The catalase test was employed to ascertain the presence of the catalase enzyme in bacterial samples. For this, a bacterial sample from an agar slant was collected using a sterile loop and smeared onto a glass slide pre-treated with alcohol. A drop of 3% H_2O_2 solution was then applied to the sample. The presence of gas bubbles indicates a positive catalase reaction, signifying the presence of the enzyme (Fevria and Hartanto 2020)

Protease analysis

The bacterial isolates were rejuvenated in MRSB medium through 24 hours of incubation at 37°C. Subsequently, the isolates were assessed by culturing them on milk agar, a screening medium designed to evaluate protease activity. The presence of protein decomposition on the agar plates following incubation served as an indicator of effective protease activity, suggesting that the isolates may be classified as potential protease producers (Salam Khattab and Al-Nazzal 2024). The diameter of inhibition zone was measured using the formula of Ngamsurach and Praipipat (2022):

$$\text{Diameter of Inhibition zone} = \frac{(Dv - Dc) + (DH - DC)}{2}$$

Where:
DV: Diameter of clear zone vertical
DC: Diameter of blank disc (control)
DH: Diameter of clear zone of horizontal

Cellulase analysis

The screening for cellulase-producing bacteria was carried out using CMC agar, prepared with the following composition: 1.0 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L NaCl, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 0.01 g/L $MnSO_4 \cdot H_2O$, 0.3 g/L NH_4NO_3, 10 g/L carboxymethyl cellulose, and 12 g/L agar, as described by Teather and Wood (1982). Following incubation at 25°C for three days, the agar medium was treated with a 0.1% Congo red solution for 15 to 20 minutes, after which it was washed with 1 M NaCl for a similar duration to visualize zones of cellulose degradation. After staining, agar plates were observed for zones around the colonies. The diameter of the inhibition zone was measured using the formula (Ngamsurach and Praipipat 2022):

Diameter of inhibition zone = $\frac{(Dv - Dc) + (DH - DC)}{2}$

Where:
DV: Diameter of clear zone vertical
DC: Diameter of blank disc (control)
DH: Diameter of clear zone of horizontal

Cell surface hydrophobicity assay

The cell surface hydrophobicity of selected isolates was determined according to the method of (Thapa et al. 2004). Toluene, chloroform, and ethyl acetate were employed to assess the surface hydrophobicity of the bacterial isolates. Cells grown overnight were harvested via centrifugation at 6,000 g, washed thrice with PBS (Phosphate-Buffered Saline), and subsequently resuspended in 10 mL of Ringer's solution. The optical density (OD) at 600 nm (A_0) was recorded as the control value. For the test samples, an equal volume of solvent was added to the cell suspension, followed by a vortex for 2 minutes. The mixture was then allowed to stand at room temperature for 30 minutes. The aqueous phase was carefully separated, and its absorbance was measured at 600 nm (A_1). Bacterial adhesion to solvents was classified as hydrophilic if the calculated percentage was below 50% and hydrophobic if it exceeded 50%, thereby reflecting the nature of the cell surface. These hydrophobicity values were compared against those of a positive control. Among the selected isolates, those demonstrating superior survival capability during digestive transit and exhibiting significant hydrophobicity were further screened to evaluate their adhesion and invasion properties. The bacterial surface hydrophobicity was determined using the formula:

$(1-A_1/A_0) \times 100$.
Where:
A0: Control
A1: Value of the remaining turbidity after the addition of solvent

Detection of biofilm-capability

This method relied on the distinctive morphological characteristics exhibited by biofilm-forming bacteria when cultured on Congo red medium. The bacterial isolates were streaked onto Mueller-Hinton agar (HIMEDIA) enriched with 0.8 g/L of Congo red dye and incubated at 37°C for 48 hours. The presence of black colonies with a dry, crystalline texture was indicative of biofilm formation, whereas the development of red colonies identified non-biofilm-forming strains (Kavitha et al. 2018)

Hemolytic activity

The hemolytic activity of bacterial isolates was assessed using nutrient agar supplemented with a 5% (v/v) erythrocyte suspension derived from Labeo rohita and human blood. The isolates were spot-inoculated onto the hemolysis plates and subsequently incubated at 37°C for 24 hours. Following incubation, the plates were examined for hemolytic reactions. Isolates that caused no discernible alteration in the agar surrounding the colonies were classified as non-hemolytic. In contrast, those producing a clear zone of hemolysis around the colonies were identified as hemolytic, indicative of β-hemolysis (Ramesh et al. 2015).

Molecular identification

The bacterial isolates were cultured overnight in nutrient broth at 37°C under shaking conditions to promote growth. Genomic DNA was subsequently extracted using the conventional phenol-chloroform method, and its purity was evaluated through spectrophotometric analysis. The 16S rRNA gene, serving as a molecular marker for bacterial identification, was amplified using specific primers via polymerase chain reaction (PCR). The resulting DNA fragments were resolved through agarose gel electrophoresis and subsequently purified. The purified DNA underwent sequencing to ascertain its nucleotide composition. The obtained sequences were then compared against established bacterial sequences in the highly reliable NCBI database using BLAST analysis. To further elucidate evolutionary relationships, a phylogenetic tree was constructed, illustrating the connections between the isolates and reference strains. Finally, the newly generated sequences were submitted to GenBank for public access (Kavitha et al. 2018)

RESULTS AND DISCUSSION

Isolation of bacteria originating from the digestive tract

A total of 30 bacterial isolates were obtained from the digestive tract of *Gobiopterus* sp. Observations regarding colony characteristics, including size, shape, color, elevation, and margin morphology, as well as Gram staining results and catalase enzyme activity, were recorded. Among these isolates, only eight were identified as Gram-positive and catalase-negative, as summarized in Table 1. The results showed that isolates that displayed Gram-positive and catalase-negative characteristics were labeled as codes 1, 17, 18, 19, 27, 28, 29, and 30. Consequently, these specific samples were selected for subsequent testing and analysis.

Bile juice tolerance

The findings revealed that all eight selected bacterial isolates, identified as potential probiotic candidates, demonstrated continued growth in media with pH levels of 4, 5, 6, and 7, yielding positive results across all conditions. These results indicate the ability of eight candidate isolates to thrive in acidic environments, as summarized in Table 2. Table 2 presented eight bacterial isolates identified as potential probiotic candidates with sample codes 1, 17, 18, 19, 27, 28, 29, and 30 showed one of the characteristics of probiotics, especially in their acid resistance tolerance.

Bile salt tolerance

The observational results regarding bile salt resistance indicated that all eight isolates demonstrated growth on solid media across a range of bile salt concentrations, specifically 2.5%, 5%, 7.5%, and 10%. This finding suggests that the selected isolates possess the ability to tolerate varying bile salt levels, thereby fulfilling one of the essential criteria for lactic acid bacteria to qualify as potential probiotic candidates, as summarized in Table 3.

The eight bacterial isolates tested demonstrated growth on solid media containing bile salt concentrations of 2.5%, 5.0%, 7.5%, and 10.0%, suggesting that these isolates possess the capacity to endure environments with high bile salt concentrations. These findings imply that these isolates could be utilized as probiotics within the digestive system. As a result, eight probiotic bacterial isolate candidates, labeled as 1, 17, 18, 19, 27, 28, 29, and 30, exhibited the essential bile salt tolerance.

Antibacterial test

Antibacterial results on 8 candidate bacterial isolates tested with the challenge bacteria *Aeromonas hydrophila* (30×10^8), *Vibrio harveyi* ($<3\times10^8$), and *Vibrio alginolyticus* ($<3\times10^8$) produced a clear zone; the clear zone of each challenge test was different (Table 4).

Table 1. Morphological characteristics of isolated bacteria

Isolates	Colony size	Form	Color	Elevation	Edge	G (Gram)	C (Catalase)
1	Punctiform	Circular	Milky White	Raised	Entire	+	X
2	Small	Circular	Cream	Flat	Entire	-	
3	Small	Circular	Milky White	Raised	Entire	-	
4	Small	Circular	Milky White	Raised	Entire	-	
5	Punctiform	Circular	Milky White	Raised	Entire	-	
6	Small	Circular	Cream	Raised	Entire	-	
7	Small	Irregular	Milky White	Raised	Undulate	-	
8	Small	Irregular	Milky White	Raised	Undulate	-	
9	Punctiform	Circular	Milky White	Raised	Entire	-	
10	Punctiform	Circular	Milky White	Raised	Entire	-	
11	Moderate	Irregular	White	Flat	Undulate	-	
12	Punctiform	Circular	Milky White	Raised	Entire	-	
13	Punctiform	Circular	Milky White	Raised	Entire	-	
14	Punctiform	Circular	Milky White	Raised	Entire	-	
15	Moderate	Irregular	Cream	Raised	Undulate	-	
16	Small	Circular	Milky White	Raised	Entire	-	
17	Punctiform	Circular	Milky White	Raised	Entire	+	X
18	Punctiform	Circular	Milky White	Raised	Entire	+	X
19	Moderate	Irregular	Cream	Flat	Undulate	+	X
20	Small	Irregular	Cream	Flat	Undulate	-	
21	Punctiform	Circular	Milky White	Raised	Entire	-	
22	Small	Circular	Milky White	Raised	Entire	-	
23	Small	Irregular	White	Flat	Undulate	-	
24	Punctiform	Circular	Milky White	Raised	Entire	-	
25	Punctiform	Circular	Milky White	Raised	Entire	-	
26	Punctiform	Circular	Milky White	Raised	Entire	-	
27	Big	Irregular	Cream	Flat	Undulate	+	X
28	Big	Irregular	Cream	Flat	Undulate	+	X
29	Big	Irregular	Cream	Flat	Undulate	+	X
30	Small	Circular	Cream	Raised	Entire	+	X

Notes: +: Gram-positive, -: Gram-negative, X: Not Produce (catalase-negative)

Table 2. Result of bacterial growth on different pH

Isolates	pH			
	4	5	6	7
1	+	+	+	+
17	+	+	+	+
18	+	+	+	+
19	+	+	+	+
27	+	+	+	+
28	+	+	+	+
29	+	+	+	+
30	+	+	+	+

Table 3. The result from bacterial growth in different concentrations of bile salt

Isolates	Bile salt concentrations			
	2.5%	5.0%	7.5%	10.0%
1	+	+	+	+
17	+	+	+	+
18	+	+	+	+
19	+	+	+	+
27	+	+	+	+
28	+	+	+	+
29	+	+	+	+
30	+	+	+	+

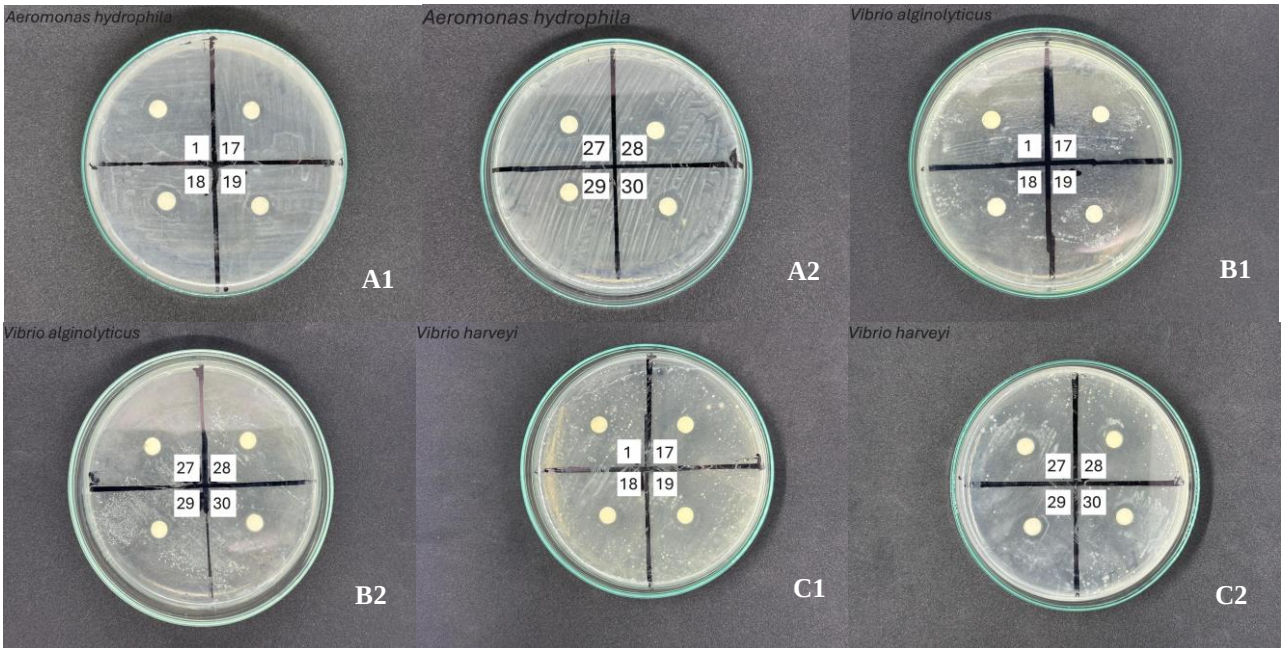

Figure 2. Result of antibacterial. A: *Aeromonas hydrophila*, B: *Vibrio alginolyticus*, C: *Vibrio harveyi*. 1: 1, 17, 18, 19; 2: 27, 28, 29, 30

The results revealed the formation of clear zones on the pathogen growth media, indicating the antibacterial activity of the isolates. The size of these clear zones was found to be different when tested against the three pathogenic bacteria, suggesting differences in their antibacterial efficacy. The average antibacterial activity is summarized in Table 4. In the challenge test with *A. hydrophila*, isolate 28 produced the largest clear zone, measuring 7.03 mm, demonstrating the most significant activity against this pathogen. Similarly, for *V. alginolyticus*, isolate 28 again displayed the largest clear zone, measuring 4.30 mm, while against *V. harveyi*, isolate 30 exhibited the largest clear zone, which was 4.96 mm.

This study revealed that all eight probiotic bacterial isolate candidates exhibited antibacterial activity against the tested pathogens, although the extent of their antibacterial effectiveness varied across different pathogen species. The average data, as presented in Table 4, provides a basis for comparing the overall antibacterial activity of the isolates. Consequently, the isolates identified by codes 1, 17, 18, 19, 27, 28, 29, and 30 were selected for further evaluation.

Enzyme activity

The protease enzyme test was evaluated based on the proteolytic activity exhibited by the isolates. The results were assessed after 24 hours of incubation, with observations made at 16, 20, and 24 hours. The formation of clear zone around the bacterial colony signifies the production of protease enzyme.

The cellulase enzyme activity was assessed based on the breakdown of cellulose. The results were recorded following 72 hours of incubation. The presence of a clear zone surrounding the colony indicated a positive outcome. The formation of clear zone around the bacterial colony confirmed the production of cellulase enzyme (Table 5).

Table 4. Result of antibacterial bacterial activity of selected isolates

Isolates	Average (mm)		
	A. hydrophila	***V. alginolyticus***	***V. harveyi***
	24 hours	**24 hours**	**24 hours**
1	6.30 ± 1.64	4.05 ± 0.98	1.47 ± 1.01
17	3.16 ± 1.64	2.19 ± 0.98	3.75 ± 1.01
18	4.99 ± 1.64	2.53 ± 0.98	2.86 ± 1.01
19	2.19 ± 1.64	1.30 ± 0.98	3.36 ± 1.01
27	4.55 ± 1.64	3.17 ± 0.98	3.95 ± 1.01
28	7.03 ± 1.64	4.30 ± 0.98	3.56 ± 1.01
29	3.25 ± 1.64	3.12 ± 0.98	4.00 ± 1.01
30	5.05 ± 1.64	3.50 ± 0.98	4.96 ± 1.01

Table 5. Protease and cellulose enzyme activity of selected isolates

Isolates	Average (mm)	
	Protease	**Cellulase**
1	26.70 ± 5.28	0.00
17	19.95 ± 5.28	3.46 ± 3.62
18	16.65 ± 5.28	14.76 ± 3.62
19	15.75 ± 5.28	7.54 ± 3.62
27	15.50 ± 5.28	7.58 ± 3.62
28	16.50 ± 5.28	11.71 ± 3.62
29	10.40 ± 5.28	7.74 ± 3.62
30	24.60 ± 5.28	10.36 ± 3.62

The data presented in Table 5 highlighted significant variability in the protease and cellulase enzyme activities across the tested bacterial isolates. All isolates, with the exception of isolate 1, were capable of producing both enzymes. However, isolate 1 only produced protease, which means it did not meet the ideal criteria for a probiotic candidate. Despite this, isolate 1 exhibited the highest protease activity, with a clear zone of 26.7 mm, however isolate 1 did not produce cellulose so isolate 1 did not proceed to the next test. Isolate 18, on the other hand, demonstrated the highest cellulase activity, with an average clear zone of 14.76 mm, while also producing protease. Isolates 18, 19, 27, 28, 29, and 30 were able to produce both protease and cellulase enzymes. Based on their superior enzyme activities, particularly in protease and cellulase production, isolates 18, 19, 28, and 30 were selected for further testing.

Hydrophobicity assay

The hydrophobicity of bacteria was influenced by the components of their cell surfaces, which in turn affect their ability to adhere to host cell surfaces. A higher hydrophobicity value generally correlates with improved bacterial adhesion to both the cell surface and various solvents, including xylene, toluene, chloroform, and ethyl acetate. These solvents were used in the test to assess the bacterial cell surface's adhesion properties, as shown in Figure 3.

Biofilm and hemolytic activity

The results from four samples tested for biofilm production were negative, as no black colonies or dry crystalline formations were observed around the isolates in the media. Additionally, the hemolytic activity test revealed that only isolate 18 was capable of hydrolyzing blood agar. This was evident from the blackened media surrounding the isolate and the formation of a clear zone. In contrast, the remaining three isolates did not exhibit any hemolytic activity, as no hydrolysis of the blood agar was observed (Table 6).

DNA extraction, PCR amplification, sequence identification, and phylogenetic analysis

The results of DNA quality analysis at the extraction and isolation stages showed good purity values, ranging from 1.8 to 1.9. The electrophoresis profile of the sample exhibited a single, distinct band with no smearing, as shown in Figure 4, suggesting that DNA possesses high integrity. The sample can proceed to the DNA sequencing stage, and the result of 3 samples was *Enterococcus* sp. (19), *Enterococcus* sp. (28) *Enterococcus* sp. (30). The obtained DNA sequence was analyzed using the BLAST algorithm to compare it with the database of known sequences. The results obtained from this comparison serve as the foundation for constructing a phylogenetic tree, which shows illustrate the evolutionary relationships between the organism under study and other identified organisms, as depicted in Figure 5.

Table 6. Biofilm and hemolytic activity of selected isolates

Isolates	Biofilm	Hemolytic
18	-	+
19	-	-
28	-	-
30	-	-

Notes: -: Not produce, +: Produce

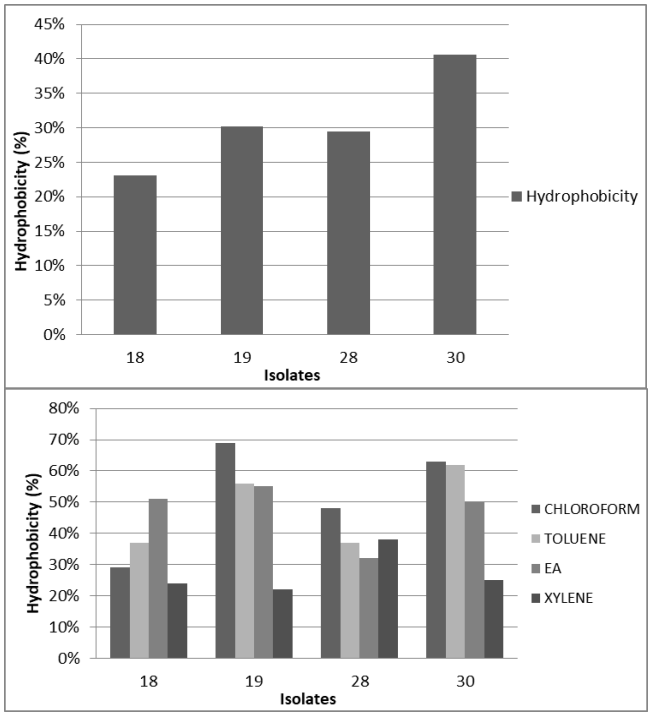

Figure 3. Graph of hydrophobicity

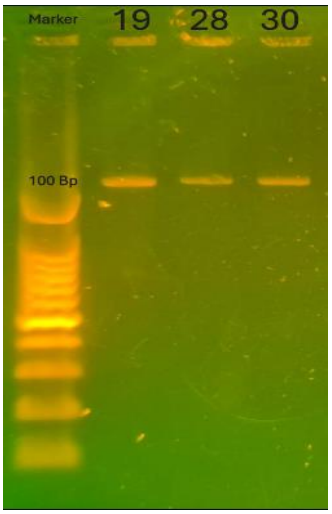

Figure 4. Electrophoresis profile of isolates 19, 28, 30

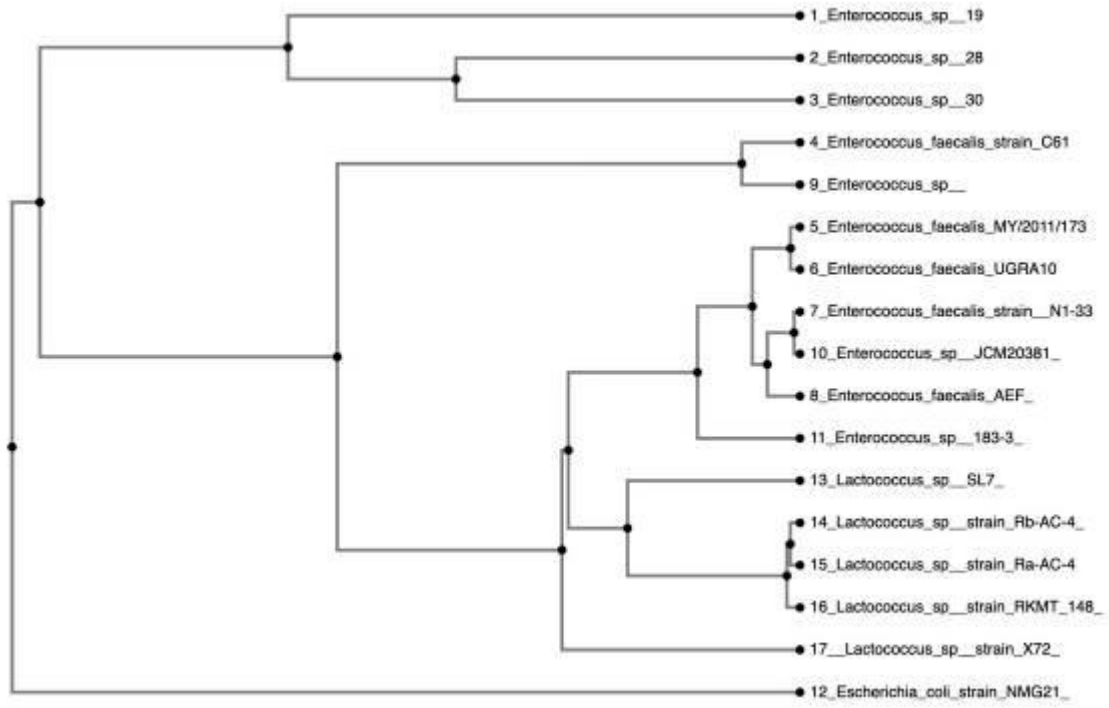

Figure 5. Phylogenetic tree analysis of *Enterococcus* sp.

Discussion

Probiotics can influence fish development, lifespan, physiology, and immunity and provide a barrier against pathogens by enhancing the intestinal microbiota (Yan et al. 2016; Uma et al. 2020). Consequently, components that facilitate microbial colonization in the fish gut offer significant advances in preventing and treating fish diseases (Xiong et al. 2019; Uma et al. 2020). The isolation and characterization of probiotic microbes are crucial for their development. Adequate provision of probiotics can enhance the host's health by regulating and stimulating the fish's immune system (Verschuere et al. 2000; Kavitha et al. 2018). Therefore, a healthy fish digestive tract can be considered a primary source for isolating beneficial bacteria that help prevent fish diseases. In this study, 30 bacterial isolates were successfully obtained from the digestive tract of *Gobiopterus* sp., of which only 8 colonies were identified as Gram-positive and catalase-negative. These characteristics align with those of lactic acid bacteria (LAB), which were typically Gram-positive, non-sporulating, catalase-negative, acid-tolerant, and facultatively anaerobic. The eight isolates demonstrated the ability to survive in media with pH levels ranging from 4 to 7. However, Yang et al. (2018) reported that LAB generally thrives within a pH range of 6.2 to 8.5. Their survival in low pH environments can be attributed to their capacity to maintain internal homeostasis in response to external acidic conditions. Furthermore, acid resistance may be further enhanced by the natural protective compounds, such as proteins and fats, present in the consumed products, which act as buffers against the acidic environment (Priadi et al. 2020).

Furthermore, these isolates demonstrated the ability to tolerate bile salt concentrations ranging from 2.5% to 10%, a finding consistent with Okfrianti et al. (2018), who reported that LAB isolated from Lemea exhibited resistance to salt concentrations between 0.30% and 0.90%. The ability of LAB to withstand bile salts is crucial, as it directly affects their viability and functionality within the digestive tract, particularly in the upper intestine where bile is secreted. The results of this study indicate that these eight bacterial isolates possess probiotic potential, as they are capable of surviving both in low pH environments and under high bile salt concentrations.

In antibacterial activity using three challenge bacteria, consisting of *A. hydrophila* (30×10^8), *V. harveyi* ($<3\times10^8$), and *V. alginolyticus* ($<3\times10^8$) had inhibition zone activity from 8 bacterial samples tested. Gharib (2020) said lactic acid bacteria (LAB) produce variant antimicrobial compounds that are considered necessary for the food and feed bio-preservation. The antimicrobial activity of LAB was closely associated with the production of various substances during lactic fermentation, including organic acids, hydrogen peroxide (H_2O_2), and bacteriocins. Illustrates that the eight strains proved to have significant antibacterial activity against the pathogens mentioned above. For instance, *P. aeruginosa* was the most sensitive bacterial pathogen to the probiotic strains, where the ZDI ranged from (1.43±0.03 to 1.83±0.27), the highest effective strain was *L. plantarum* from LAB, and *B. adolescentis* from Bifidobacteria.

In 8 candidate probiotic bacteria, protease and cellulase enzymes were produced. Hassaan et al. (2020) reported that the inclusion of protease in diet has been demonstrated to

significantly improve several growth parameters in fish, such as final body weight, weight gain, specific growth rate, feed conversion ratio, and protein efficiency ratio. These results suggest that protease supplementation can enhance both growth performance and feed utilization efficiency. Proteases play a crucial role in digestion by hydrolyzing the peptide bonds in dietary proteins, thereby releasing the amino acids essential for the body's needs. The application of probiotics can improve protein digestibility in fish, which explains the better performance of fish-fed probiotic-supplemented diets that the protease activity of shrimp (*Penaeus vannamei*) fed probiotic *Bacillus* sp. supplemented diet was significantly higher (about 78.38%) compared with the control (Assan et al. 2022). According to the literature, in addition to the primary digestive enzymes-amylase, protease, and lipase-subclasses such as cellulase (related to amylase) and alginase are also modulated by probiotics. These enzymes are particularly important, as omnivorous and herbivorous fish, which predominantly consume plant matter, including algae, rely on them to break down cellulose and algin. Since certain fish species either do not synthesize the enzymes required for the hydrolysis of cellulose (cellulase) and algin (alginase) or produce them in only small quantities, supplementation of these enzymes becomes necessary. Despite cellulose being strictly from plants, a higher population of cellulase-producing bacterial strains was observed in herbivorous fish (Kar and Ghosh 2008; Assan et al. 2022) due to the presence of microbial flora in the digestive tract, however, only a few researchers have mentioned the modulation of cellulase in fish with regard to probiotic activities.

The hydrophobicity results of the isolates obtained did not reach 50%. The hydrophobicity of the cell surface was assessed by measuring the percentage of cells adhering to a hydrophobic surface. According to Kavitha et al. (2018) percentage with a value below 50% indicates a hydrophilic surface, while for percentage value above 50% indicates a hydrophobic surface; if a percentage value above 50% indicates higher hydrophobicity and a higher survival rate under simulated digestion. Nevertheless, additional testing is necessary to evaluate their potential as viable probiotic candidates thoroughly. Biofilms can function as a shield that protects probiotic bacteria from attacks by white blood cells. Rendueles et al. (2013) stated biofilms consist of thickly packed microbial cells surrounded by a self-synthesized extracellular polymeric matrix mainly comprising polysaccharides attached to a surface, including biological tissues. The formation of biofilms imparts several advantageous properties to bacteria, including increased tolerance to antimicrobial peptides and other stressful conditions within the body. Numerous studies have demonstrated that biofilm formation can enhance probiotic activity by improving immunomodulatory properties and intestinal permeability, among other beneficial effects (Chamignon et al. 2020).

The genus *Enterococcus* consists of lactic acid bacteria (LAB) found predominantly in the gut of humans and animals. Additionally, certain species of *Enterococcus* are utilized as probiotics to maintain healthy gastrointestinal microbiota and reduce gastrointestinal inflammation. They also demonstrate the ability to produce bacteriocins, which are proteins produced by bacteria to inhibit growth or kill other competing bacteria (Im et al. 2023). In another study Liu et al. (2021) revealed that tilapia raised for 42 days by adding *B. subtilis* and *E. faecalis* was improved the most, supporting the view that specific use of probiotics can lead to better results instead of diversifying probiotics. The digestive enzyme and antioxidant enzyme activities, as well as immune indicators, are positively correlated with the combined probiotics *B. subtilis* and *E. faecalis*. Also, supplementation of *B. subtilis* and *E. faecalis* in the diet can promote the good development of the gut mucosal structure of tilapia, thereby providing a good gut microecological environment to improve body health.

In conclusion, isolation and molecular identification results revealed three bacterial candidates, namely isolates 18, 28, and 30, were identified as potential probiotics of of the genus *Enterococcus*. These isolates demonstrated Gram-positive staining, acid tolerance, bile salt resistance, antibacterial activity, as well as production of both protease and cellulase enzymes. Additionally, they exhibited approximately 23-41% hydrophobicity and showed negative results in blood hemolysis tests. Due to these characteristics, all three isolates can be considered as promising candidates for probiotic development.

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