



Acid-Producing *Enterococcus* Sp. from Grati Lake's Endemic *Gobiopterus* Sp.: A Novel Probiotic Candidate

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Abstract | *Gobiopterus* sp., inhabiting the extreme volcanic lake Ranu Grati, offers a potential source of unique lactic acid bacteria (LAB) with probiotic properties. This study aimed to isolate, characterize, and evaluate LAB from *Gobiopterus* sp. digestive tract as potential probiotic candidates. Isolates underwent tests for Gram staining, gastric juice and bile salt tolerance, antibacterial activity, extracellular enzyme production, and hydrophobicity. A total of 16 bacterial isolates were obtained, of which 9 were Gram-positive and catalase-negative. These isolates exhibited tolerance to acidic pH conditions and bile salts. Antibacterial activity varied among the isolates, with some showing significant inhibition against common fish pathogens. Enzyme activity assays revealed the production of protease and cellulase enzymes, aiding in nutrient digestion. DNA sequencing identified four isolates as belonging to the genus *Enterococcus*, a well-established probiotic genus. These findings suggest that *Enterococcus* spp., identified as a lactic acid bacteria isolated from *Gobiopterus* sp., is a promising candidate for use as a probiotic.

Keywords | Biofilm, Enzymes, *Gobiopterus* sp., Grati lake, Lempuk fish, Probiotics

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INTRODUCTION

The intestinal microbiota, a complex community of bacteria, is essential for the health and development of aquatic animals. It aids in digestion, nutrient absorption, and disease resistance, a vital “multifunctional organ,” significantly influences the growth, size, and overall health of

aquatic animals. These microbial communities play crucial roles in nutrient processing, immune function, and development (Zhao *et al.*, 2024).

Probiotics, recognized as viable alternatives to conventional therapeutic agents, have the potential to bolster fish health and mitigate the risk of opportunistic bacterial infections.

By combining probiotics with prebiotics, a synergistic approach known as synbiotics can be employed to further enhance the beneficial effects on aquatic organisms (Yousefi *et al.*, 2023). Numerous probiotic species are efficiently utilized in aquaculture nutrition, greatly enhancing nutrient availability, feed efficiency, growth, and feed conversion rates, while also improving immune response, disease management, and overall health promotion.

Probiotics must fulfill specific criteria both in vitro and in vivo, including adherence to gut epithelial tissue, non-pathogenicity, synthesis of antimicrobial compounds, tolerance to gastric acid and bile, and compatibility with technological procedures (Akinyemi *et al.*, 2024).

Gobiopertus sp., commonly known as dart gobies, are native to Indonesia and are found in Ranu Grati Lake, Pasuruan Regency, East Java. This fish is known for its excellent nutritional value, making it a valuable resource (Faqih *et al.*, 2021; Prihanto *et al.*, 2022). The existence of *Gobiopertus* sp. highlights the significance of Ranu Grati as a unique and essential habitat for the survival of this species (Anitasari *et al.*, 2024). This volcanic lake is characterized by high sulfur levels in its water, ranging from 0.5 to 10 mg/l. These unique environmental conditions, particularly within the digestive tracts of *Gobiopertus* sp., likely influence the composition of probiotic bacteria. Consequently, the gut microbiome of *Gobiopertus* sp. may harbor distinct probiotic candidates with beneficial properties.

This research aims to obtain probiotics from the digestive tract of *Gobiopertus* sp. The discovered probiotics can be used in aquaculture to improve fish health, growth, and disease resistance.

MATERIALS AND METHODS

STUDY AREA AND FISH SAMPLE

The research was conducted at Universitas Brawijaya, and sampling of *Gobiopertus* sp. fish was carried out at Ranu Grati Lake in Pasuruan. The fish used in this study complied with ethical guidelines and received approval from the Ethical Clearance Committee of Brawijaya University. The fish samples were collected from three different areas (Figure 1).

ISOLATION OF GUT BACTERIA

For isolation of THB (Total heterotrophic bacteria), 1g of gut sample was suspended in 9ml sterile distilled water, agitated for few a minutes in a shaker and the sample was serially diluted on 10^{-1} to 10^{-6} dilutions (Ramanathan *et al.*, 2012). Technique for gut bacteria isolation was adopted with a few modifications. The intestinal stock solution was serially diluted before being dispersed over MRS (Man

Rogossa Sharp) Agar plates (Chattaraj *et al.*, 2024). Following inoculation and MRS (Man Rogossa Sharp) agar medium which had been supplemented with 0.5% CaCO_3 and then the petri was incubated at 37°C for 24 hours.

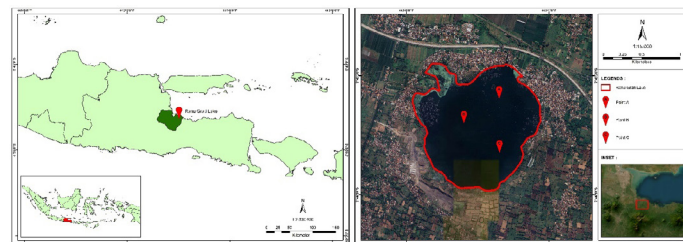


Figure 1: Ranu Grati Lake which shows the location of sampling of *Gobiopertus* sp.: Point A ($7^\circ43'30.5''\text{S}$ $113^\circ00'32.8''\text{E}$), Point B ($7^\circ43'45.2''\text{S}$ $113^\circ00'19.4''\text{E}$); and Point C ($7^\circ43'46.8''\text{S}$ $113^\circ00'31.9''\text{E}$).

MACROSCOPIC ANALYSIS TEST

Macroscopic colony characteristics were evaluated by a visual examination of colony shape, margin, and elevation. Top-down observations were conducted to assess overall colony shape and edge morphology, while side-view observations were performed to determine colony height (Irawan *et al.*, 2024).

MACROSCOPIC ANALYSIS TEST AND GRAM STAINING

Microscopic identification uses research methods from (Fitri and Yasmin, 2011). Observation of colony morphology is carried out using the Gram staining technique. First, a review of the bacteria is made on the glass object and fixation is carried out. Fixation is carried out with 2-3 drops of violet dripped on the bacterial colony and left for 2 minutes then washed using sterile distilled water and dried. After drying, 2-3 drops of Lugol's solution are added which are dripped again over the preparation and left for 2 minutes and washed again with sterile distilled water and then air-dried. The dried preparations are then dripped again with 2-3 drops of 96% solution and left for 30 seconds then washed again with sterile distilled water and dried. Next, drip it again with 0.5% safranin solution as much as 2-3 drops and leave it for 1 minute, then wash it and air dry it. The final step is to observe the dry preparation using a microscope. Gram staining aims to see the morphology of the colony microscopically and to differentiate gram-positive or negative bacteria.

CATALASE ANALYSIS

The catalase test is used to determine whether or not catalase is present in bacteria. Most bacteria produce the enzyme catalase which can break down H_2O_2 into H_2O and O_2 . The catalase enzyme is thought to be important for growth because H_2O_2 which is formed with the help of various respiratory enzymes is toxic to microbial cells. Bacteria that grow on the agar slant are taken in one lap, then

smeared on a glass object that has been treated with alcohol. The glass object was dripped with 3% H₂O₂ solution. Observe the formation of gas bubbles in the preparation. If there are gas bubbles, it means the catalase test is positive (Fevria and Hartanto, 2020).

BILE TOLERANCE ASSAY

The bile salt resistance of the isolates was assessed using overnight cultures of the bacterial strains inoculated in MSB broth containing 2.5%, 5.0%, 7.5%, and 10% bile salts. The bile salt tolerance was assessed as (Gou *et al.*, 2021) with slight modifications. The inoculant was first aerobically cultivated in MRS Broth and then cultured for eighteen hours at 38°C. Subsequently, 1 ml samples of the fermentation broths were collected, centrifuged at 2,235 g for 10 minutes to form a pellet, and then uniformly resuspended in 1 mL of inoculant medium containing 2.5%-10% (w/v) cow bile salt. The combinations were incubated aerobically on MRS Agar for 24 hours at 38°C.

GASTRIC JUICE TOLERANCE

The acid resistance test was carried out by inoculating bacteria in MRSB medium which has a pH of 4 - 7 and incubating for 24 hours at a temperature of 37°C. Bacteria that can grow in pH 4 - 7 media and cause the media to become cloudy. Bacterial growth in the medium is determined by the presence of turbidity in the medium (there are bacterial particles) as well as by inoculating the medium on the surface of *de Man Rogosa Sharpe agar* (MRSA). If after 24 hours there are bacterial colonies on *de Man Rogosa Sharpe agar* (MRSA) scratch marks, the bacteria live at pH 4 - 7 (Istiqomah *et al.*, 2019).

ANTI-BACTERIAL TEST

The isolate was cultured in 1 mL of MRSB media in a microtube and incubated for 24 hours, the isolate was centrifuged at 10,000 rpm for 5 minutes at a temperature of 4°C to obtain the supernatant. The supernatant was used for antibacterial testing. The pathogenic bacteria used were *Aeromonas hydrophila* (30 x 10⁸), *Vibrio harveyi* (< 3 x 10⁸), and *Vibrio Alginolyticus* (< 3 x 10⁸). The challenge test is 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 is placed on the surface of the MRSA media. Incubate for 24 - 48 hours and observe the resulting clear zone. Calculation of diameter using the formula (Ngamsurach and Praipipat, 2022). The diameter of the inhibition zone is measured using the formula;

$$\frac{(Dv - Dc) + (DH - DC)}{2}$$

PROTEASE ANALYSIS

Isolates were rejuvenated in MRSB media for 24 hours at 37°C. The isolated bacteria were examined by growing

them on a milk agar screening medium. The decomposition of protein materials on agar plates after incubation indicates that they have effective protease activity and can be considered protease producers (Khattab and Al-Nazzal, 2024). Calculation of diameter using the formula (Ngamsurach and Praipipat, 2022). The diameter of the inhibition zone is measured using the formula;

$$\frac{(Dv - Dc) + (DH - DC)}{2}$$

CELLULASE ANALYSIS

The screening was performed on CMC agar composed of 1.0 g/l KH₂PO₄, 0.5 g/l MgSO₄·7H₂O, 0.5 g/l NaCl, 0.01 g/l FeSO₄·7H₂O, 0.01 g/l MnSO₄·H₂O, 0.3 g NH₄NO₃, 10 g/l carboxymethyl cellulose, and 12 g/l agar (Teather and Wood, 1982). Following a 3-day incubation at 25°C, the agar was inundated with 0.1% Congo red for 15 to 20 minutes, subsequently treated with 1 M NaCl for an additional 15 to 20 minutes. Agar plates were examined for zones surrounding the colonies following staining. Determination of diameter via the formula (Ngamsurach and Praipipat, 2022). The diameter of the inhibition zone is measured using the formula;

$$\frac{(Dv - Dc) + (DH - DC)}{2}$$

CELL SURFACE HYDROPHOBICITY ASSAY

The cell surface hydrophobicity of chosen isolates was assessed using the method described by (Thapa *et al.*, 2004). Toluene, chloroform, and ethyl acetate were employed to assess the surface hydrophobicity of the isolates. The cells cultured overnight were harvested via centrifugation at 6000g, washed thrice with PBS, resuspended in 10 ml of Ringer's solution, and the optical density at 600 nm was recorded (A0) as a control measurement. The cell suspension was combined with an equivalent amount of solvent using vortexing for 2 minutes and thereafter maintained at room temperature for 30 minutes. The aqueous phase was discarded, and absorbance was measured at 600 nm (A1). The hydrophobicity of bacterial adherence to solvent was determined using the formula: (1 - A1/A0) × 100. Values below 50% were classified as hydrophilic, while values beyond 50% were classified as hydrophobic, reflecting the characteristics of the cell surface. The hydrophobicity percentage was compared to that of the positive control. The isolates exhibiting superior survival throughout intestinal transit and hydrophobicity were evaluated for their adhesion and invasion capabilities.

DETECTION OF BIOFILM-CAPABILITY

This technique relies on the distinctive shape of biofilm-forming bacteria on Congo red media. The isolates

were streaked over Muller Hinton agar (HIMEDIA) enriched with 0.8 g/l of Congo red dye and incubated for 48 hours at 37 °C. The emergence of black colonies with a dry crystalline texture signifies biofilm development, while non-biofilm-producing bacteria yield red colonies (Kavitha *et al.*, 2018).

HEMOLYTIC ACTIVITY

The haemolytic test of the isolates was conducted on nutrient agar enriched with 5% (v/v) erythrocyte suspension from *L. rohita* and human blood. The isolates were inoculated into haemolytic plates and incubated at 37°C for 24 hours. The plates were examined for hemolytic response. Strains that exhibited no alteration on the agar plates surrounding the colonies were classified as non-haemolytic, whereas strains demonstrating a clear haemolytic zone around the colonies were categorized as haemolytic (behaemolysis) (Ramesh *et al.*, 2015).

MOLECULAR IDENTIFICATION

The isolates were cultivated in nutrient broth in a rotary shaker at 37 °C overnight. The DNA was isolated via the phenol-chloroform technique. The DNA purity was assessed by measuring the absorbance ratio at 260 nm and 280 nm. Values ranging from 1.7 to 1.9 typically signify 85% purity. The 16S ribosomal RNA (rRNA) gene was amplified with universal primers (Bioserve, India) 295367F (5'-GTGCTGCAGAG AGTTTGATCCTGGCT-CAG-3') and 295268R (5'-CACGGATCCTACGGG TACCTTGTTACGACTT-3'). The DNA products were amplified using PCR utilizing Applied Biosystems under the subsequent conditions: initial denaturation at 94 °C for 5 minutes, succeeded by 25 cycles comprising 94 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 1 minute and 30 seconds, culminating in a final extension at 72 °C for 7 minutes. The PCR results were examined using agarose gel electrophoresis (1.2% w/v). The amplicons were eluted, and the pure DNA products were sequenced at Eurofins Genomics in Bangalore, India. The acquired nucleotide sequences were compared with the existing sequences of *Bacillus* species in the National Center for Biotechnology Information (NCBI) database utilizing the Basic Local Alignment Search Tool (BLAST). The NJ tree was generated using MEGA 7. All strain sequences were submitted to NCBI GenBank (Kavitha *et al.*, 2018).

RESULTS AND DISCUSSION

The probiotic mechanism of action in the digestive system includes modifying the metabolism of pathological bacteria by changing their paths, and stimulating the body's cellular immunity and disease resistance, by occupying receptors, which leads to the inhibition of colonization by harmful organisms (Al-Noor *et al.*, 2023).

Isolation of Bacteria Originating from the Digestive Tract
A result of the bacterial diversity within the digestive tract of *Gobiopertus* sp. yielded 16 isolates. The characterization, including Gram staining and catalase testing, revealed that only 9 of these isolates displayed a Gram-positive, catalase-negative (Table 1).

Table 1: Morphology colony.

No	Colony Size	Form	Color	Elevation	Edge	G Gram	C Catalase
1	Moderate	Irregular	Cream	Flat	Undulate	+	-
2	Moderate	Irregular	Cream	Flat	Undulate	-	-
3	Small	Circular	Milky White	Raised	Entire	+	-
4	Moderate	Irregular	Cream	Flat	Undulate	+	-
5	Moderate	Irregular	Cream	Flat	Undulate	+	-
6	Small	Circular	Cream	Raised	Entire	+	-
7	Punctiform	Circular	Milky White	Raised	Entire	+	-
8	Punctiform	Circular	Milky White	Raised	Entire	+	-
9	Moderate	Irregular	Cream	Flat	Undulate	-	-
10	Small	Circular	Cream	Raised	Undulate	+	-
11	Small	Circular	Cream	Raised	Entire	+	-
12	Small	Circular	Cream	Flat	Entire	-	-
13	Small	Circular	Milky White	Raised	Entire	-	-
14	Small	Circular	Milky White	Raised	Entire	-	-
15	Punctiform	Circular	Milky White	Raised	Entire	-	-
16	Small	Circular	Cream	Raised	Entire	-	-

Based on the morphological colony, Gram staining, and catalase results in Table 1, it can be observed that inoculants or samples with Gram-positive and catalase-negative characteristics were found in samples coded 1, 3, 4, 5, 6, 7, 8, 10, and 11. Therefore, these samples were selected for further testing. Probiotics in aquaculture mostly derive from strains such as Lactic Acid Bacteria (LAB) and *Bacillus* spp., which are isolated from natural aquatic habitats or particular host species (Rahayu *et al.*, 2024). A multitude of studies has explored the efficacy of probiotics in aquaculture. Research by Ramesh *et al.* (2015) underscores the significance of in vitro antagonism studies in the selection of probiotic strains capable of efficiently inhibiting pathogenic organisms and investigates the usefulness of probiotics, especially in the management of illnesses in aquaculture.

The majority of probiotic strains are inefficient in delivering the necessary benefits to aquatic creatures, as they

originate from non-fish sources. There have not been many investigations on the potential of probiotics isolated from the host. A prior study examined bacterial species isolated from the digestive tract of *Labeo calbasu*, focusing on the isolates' survival in high bile salt concentrations, low pH, elevated temperatures, adhesion capabilities (auto-aggregation and cell surface hydrophobicity), antimicrobial efficacy, safety, compatibility among the three isolates for multispecies application, hemolytic activity, and antibiotic susceptibility (Kavitha *et al.*, 2018).

In this study, 16 bacterial isolates were successfully obtained from the digestive tract of *Gobiopertus* sp., with only 9 colonies identified as Gram-positive and catalase-negative. These characteristics are consistent with Lactic Acid Bacteria (LAB). Similar to (Chavez *et al.*, 2024), Lactic Acid Bacteria (LAB) are a diverse and varied collection of Gram-positive, acid-tolerant, non-sporulating bacteria that are characterized by being catalase-negative.

GASTRIC JUICE TOLERANCE

The nine selected probiotic candidate isolates will be subjected to gastric juice tolerance tests using media adjusted to pH levels of 4.5, 6, and 7. Results from this study indicate that all nine isolates were capable of surviving in medium with pH levels from acidic to neutral. The isolates that were successfully obtained can live in pH 4, 5, 6 and 7 media with the aim that these isolates can survive after passing through the stomach which has quite high acidic conditions, so that these isolates can reach the fish's intestines and can develop and increase the number of probiotic bacteria in the fish's intestines (Table 2).

Table 2: Result of bacterial growth on different pH.

No.	pH			
	4	5	6	7
1	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5	+	+	+	+
6	+	+	+	+
7	+	+	+	+
8	+	+	+	+
10	+	+	+	+
11	+	+	+	+

The results of the nine candidate probiotic bacterial isolates with code 1, 3, 4, 5, 6, 7, 8, 10, and 11 were identified as promising probiotic candidates, demonstrating significant acid tolerance. The ability of these isolates to withstand the acidic conditions of the stomach suggests their potential to exert beneficial effects in the intestinal tract, warranting further testing.

BILE SALT TOLERANCE

The results of the bile salt resistance test of 9 selected isolates, showed growth in media that had been modified with bile salts at concentrations of 2.5%, 5%, 7.5% and 10%, so that the selected isolates could show that could tolerate bile salt with different levels concentration (Table 3).

Table 3: Result bacterial growth in different concentration of bile salt.

No.	Bile Salt			
	2.5 %	5.0 %	7.5 %	10.0 %
1	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5	+	+	+	+
6	+	+	+	+
7	+	+	+	+
8	+	+	+	+
10	+	+	+	+
11	+	+	+	+

This essential probiotic quality ensures their survival in the harsh conditions of the gastrointestinal tract and this is one of the requirements for lactic acid bacteria as good probiotic candidates with the aim that these isolates can survive the bile salt fluid, so that these isolates can reach the intestines of the fish and play a role in helping to increase the number of probiotic bacteria in the intestines of the fish.. Based on their impressive bile salt tolerance, isolates 1, 3, 4, 5, 6, 7, 8, 10, and 11 were chosen for further evaluation as potential probiotic candidates.

ANTIBACTERIAL

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

The bacterial isolates significantly suppressed the proliferation of the three harmful bacteria, as demonstrated by the distinct zones observed on the growth media. The dimensions of these clear zones differed among isolates and pathogens, suggesting variations in antibacterial efficacy. Isolate 4 proved to be the most effective against *Aeromonas hydrophila*, with a clear zone of 5.08 mm. Isolate 5 exhibited the strongest inhibition against *Vibrio alginolyticus* (4.91 mm), while isolate 1 was most effective against *Vibrio harveyi* (4.60 mm).

Based on these results, All nine candidate probiotic isolates demonstrated varying degrees of antibacterial activity

against the tested pathogens. A comparative analysis of the average antibacterial activity (Table 4) revealed that isolates 1, 3, 4, 5, 6, 7, 8, 10, and 11 exhibited the most promising antibacterial properties, warranting further investigation.

Table 4: Result of antibacterial zone.

No	Aeromonas hydrophila	Vibrio alginolyticus	Vibrio harveyi
	Average (mm)		
	24 Hours	24 Hours	24 Hours
1	3.56	3.97	4.60
3	4.16	2.70	3.50
4	5.08	3.91	3.10
5	2.45	4.91	3.30
6	1.70	4.30	3.60
7	1.34	2.60	3.47
8	2.37	3.75	2.63
10	2.25	3.60	3.13
11	1.91	4.25	1.84

Table 5: Result of protease and cellulase enzyme activity.

No	Protease	Cellulase
	Average (mm)	
1	13.05	7.23
3	29.85	7.14
4	20.05	3.97
5	22.05	4.41
6	17.60	3.73
7	19.00	6.03
8	15.40	4.29
10	7.85	4.63
11	29.75	3.64

In antibacterial activity using three challenge bacteria, consisting of *Aeromonas hydrophila* (30×10^8), *Vibrio harveyi* ($< 3 \times 10^8$), and *Vibrio Alginolyticus* ($< 3 \times 10^8$) had inhibition zone activity from 9 bacterial samples tested. Lactic acid bacteria (LAB) generate diverse antibacterial chemicals deemed essential for the bio-preservation of food and feed. The antibacterial efficacy of LAB is associated with the synthesis of several metabolites during lactic fermentation, including organic acids, hydrogen peroxide (H₂O₂), and bacteriocins (Smith *et al.*, 2020) and according to (Barenji *et al.*, 2024) demonstrated that the MRS-based media of *L. plantarum*, *L. brevis*, and *L. rhamnosus* (LAB) had a substantial antibacterial activity against pathogenic bacteria.

ENZYME ACTIVITY

Proteolytic activity can be seen from isolates grown on Skim Milk Agar media producing a clear zone around the isolate. The results were obtained after incubation for 24

hours and observed at 24 hours. Positive results are indicated by the presence of a clear zone around the colony. The clear zone formed around the bacterial colony indicates that the protease enzyme (Table 5).

The cellulase enzyme assay is indicated by cellulase activity. The isolate cultivated on CMC media has cellulase activity, evidenced by the formation of a clear zone with the application of Congo red dye surrounding the isolate. The data were acquired over a 72-hour incubation period. The existence of a clear zone surrounding the colony indicates positive outcomes. The formation of a clear zone formed around the bacterial colony indicates that the cellulase enzyme (Table 5).

Data presented in Table 5 revealed variability in protease and cellulase enzyme activities among the tested bacterial isolates. Isolate 12 exhibited the highest protease activity with a clear zone of 29.85 mm, while isolate 1 displayed the highest cellulase activity with an average clear zone of 7.23 mm. All Isolate can produce both protease and cellulase enzymes. These results indicate that isolates 3, 4, 5, 6, 7, and 8. These six isolates were chosen for subsequent testing based on their highest recorded protease and cellulase enzyme activities among all tested samples.

In 9 candidate probiotic bacteria, protease enzymes and cellulase enzymes were produced, the study (Jin and Zheng, 2024) said Proteases are ubiquitous enzymes that catalyze hydrolytic processes, leading to the degradation of protein molecules into peptides and amino acids. Dietary protease can mitigate the shortage of endogenous enzymes, particularly in young animals, and facilitate the digestion of macromolecular proteins that are challenging to assimilate. There are opportunities to enhance the protein digestibility of conventional aquafeed components by using proteases. The research demonstrated that the inclusion of HuPro protease in low-protein diets enhances growth performance, feed efficiency, apparent digestibility coefficients, certain antioxidant indices, protease activities, and intestinal flora diversity. Consequently, it was concluded that the incorporation of HuPro protease in the low-protein diet was appropriate for this study.

In addition to the well-known digestive enzymes like amylase, protease, and lipase, probiotics can also influence the activity of lesser-known enzymes such as cellulase (related to carbohydrate digestion) and alginase (involved in algal polysaccharide breakdown). These enzymes are particularly important for omnivorous and herbivorous fish, which rely heavily on plant-based diets, including algae. Since many fish species either lack or produce insufficient amounts of these enzymes, supplementation is often necessary. While cellulase is exclusively plant-derived, herbivorous fish have

a higher abundance of cellulase-producing bacteria in their gut microbiota (Assan *et al.*, 2022). However, the specific role of probiotics in modulating cellulase activity in fish remains relatively understudied.

HYDROPHOBICITY ASSAY

The results of the peripheral hydrolysis test with high OD (Optical Density) indicate that there is significant hydrolysis activity in the tested sample. Optical Density is a measure of how much light is absorbed by a sample. The hydrophobicity properties of bacteria depend on the components of the bacterial cell surface that affect the adhesion of bacteria to the host cell surface. The higher the hydrophobicity value, the efficacy of bacteria for adherence to cell surfaces and to xylene, toluene, chloroform, and ethyl acetate was evaluated to determine the adhesion capability of the bacterial cell surfaces (Figure 2).

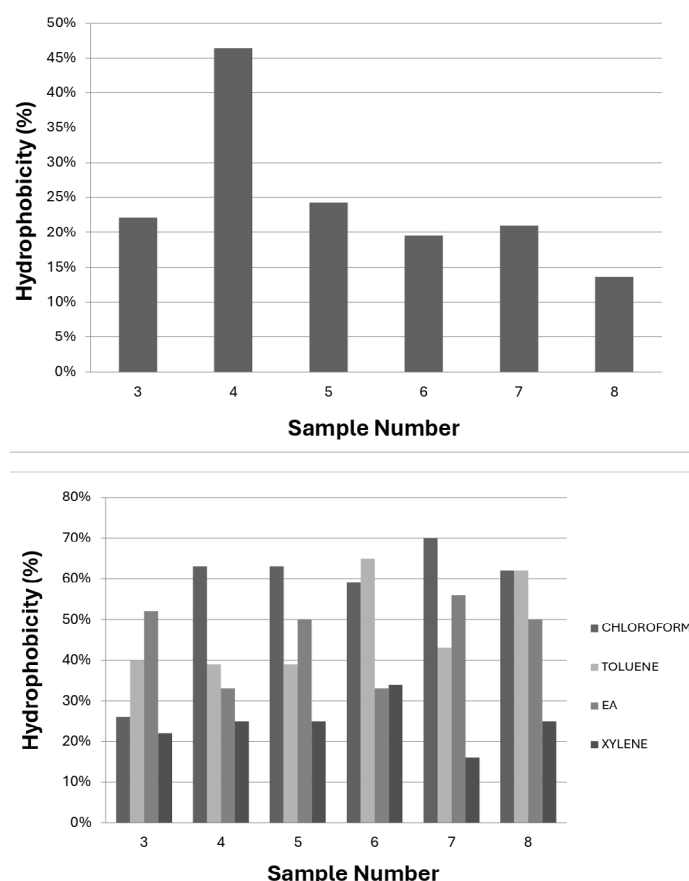


Figure 2: Graph of hydrophobicity.

The isolates did not exhibit sufficient hydrophobicity, as their values were below the 50% threshold proposed by (Kavitha *et al.*, 2018). This indicates a hydrophilic nature of the cell surface. Despite this, isolates with better digestive transit survival were selected for further analysis of adhesion and invasion capabilities. (Ji *et al.*, 2015) suggest that cell hydrophobicity can influence bacterial adhesion to host tissues. However, additional testing is necessary to fully assess the potential of these isolates.

BIOFILM AND HEMOLYTIC ACTIVITY

The result of six samples tested to observe the biofilm produced showed negative results. Because around the isolate in the media did not produce black colonies and did not produce dry crystals, the isolates that do not form biofilms will reduce the level of bacterial resistance and the balance of their life levels, so that bacteria that do not form biofilms will reduce their level of effectiveness as probiotics. Then the results of hemolytic showed that out of six selected samples, there were 2 isolate samples that could hydrolyze blood agar, namely isolates code 7 and 8. This can be seen from the results on the isolate media which were black and produced a clear zone and 4 samples that did not hydrolyze blood agar Table 6.

Table 6: Result of biofilm and hemolytic activity.

No	Biofilm	Hemolytic
3	-	-
4	-	-
5	-	-
6	-	-
7	-	+
8	-	+

Biofilms, intricate microbial communities encased within a self-produced extracellular matrix, function as protective shields, shielding probiotic bacteria from the immune system's assault. This extracellular matrix, primarily composed of polysaccharides, adheres to surfaces, including biological tissues, providing a fortified environment for bacterial proliferation. This biofilm architecture confers several advantages to the resident microorganisms, including heightened tolerance to antimicrobial peptides and other physiological stressors. Moreover, numerous studies have demonstrated that biofilm formation can potentiate probiotic activity by augmenting immunomodulatory effects, improving intestinal permeability, and eliciting other beneficial responses (Chamignon *et al.*, 2020). However, unfortunately, biofilm formation was not observed in the samples analyzed in this study.

DNA EXTRACTION, PCR AMPLIFICATION, SEQUENCE IDENTIFICATION AND PHYLOGENETIC

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 also showed a single, clear band without smears (Figure 3), indicating high DNA integrity. The sample can proceed to the DNA sequencing stage and the result of 4 sample is *Enterococcus* sp. (3) *Enterococcus* sp. (4) *Enterococcus* sp. (5) and *Enterococcus* sp. (6). The DNA sequence obtained will then be analyzed using the BLAST algorithm to compare it with a database of known sequences. The results of the

BLAST comparison will be used as a basis for building a phylogenetic tree, which will describe the evolutionary relationship between the studied organism and other organisms that have been identified (Figure 4).

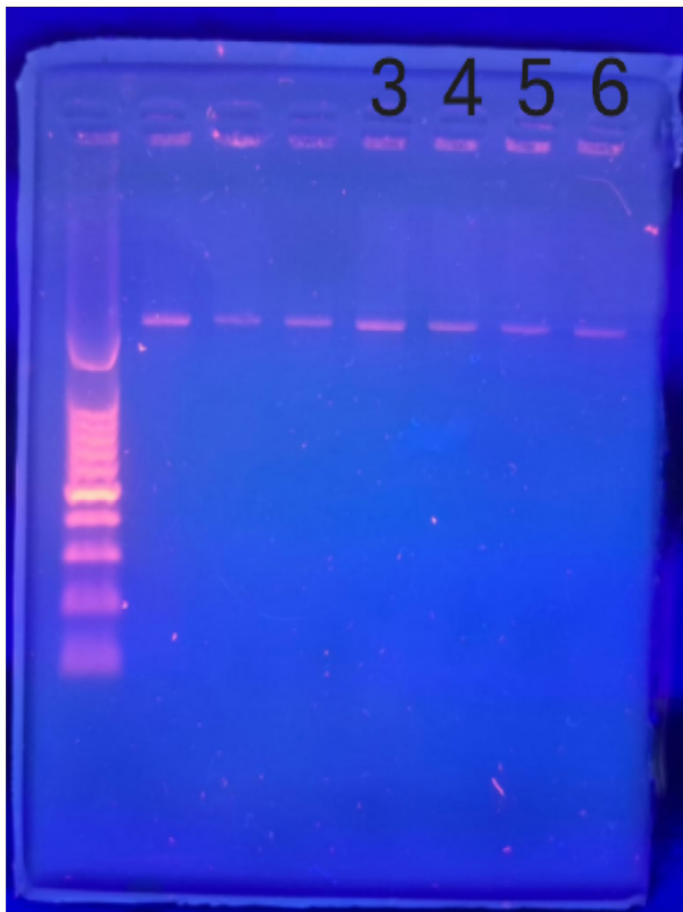


Figure 3: Electrophoresis profile.

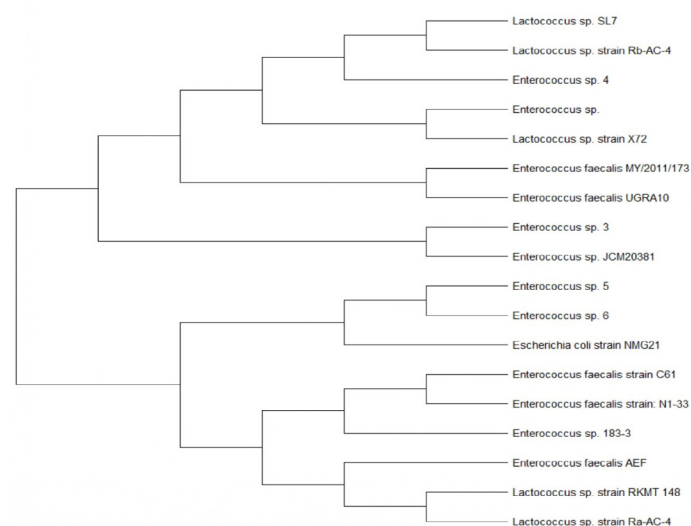


Figure 4: Phylogenetic tree.

The genus *Enterococcus* comprises lactic acid bacteria (LAB) mostly found in the gastrointestinal tract of humans and animals. Moreover, certain *Enterococcus* species are employed as probiotics to preserve healthy gut micro-

biota and mitigate gastrointestinal inflammation. They also have the capacity to synthesize bacteriocins, which are proteins generated by bacteria to suppress the growth or eliminate rival bacterial strains (Im *et al.*, 2023) and according to (Heng and Chou, 2024) in their research has demonstrated that dietary supplementation with double-layer-coated *Enterococcus faecium* can enhance digestive processes, nutrient absorption, intestinal health, immune function, and liver antioxidant capacity in crucian carp. Furthermore, it can assist in preserving gut microbial equilibrium and alleviating intestinal injury induced by LPS exposure. The 1.0×10^8 CFU/g double-layer-coated *E. faecium* dose is notably effective among the evaluated dosages. These findings suggest that LAB, such as *E. faecium*, may offer a promising alternative to antibiotics in aquaculture.

After a rigorous selection process involving isolation, molecular identification and in vitro, only four bacterial strains, all identified as *Enterococcus* species (isolates 3, 4, 5, and 6), emerged as potential probiotic candidates. These strains exhibited several desirable probiotic traits, including Gram-positive morphology, acid and bile salt tolerance, antimicrobial activity, production of protease and cellulase enzymes, moderate hydrophobicity, and non-hemolytic behavior. These characteristics collectively suggest that these isolates possess the potential to be developed into effective probiotic formulations.

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, LAB isolated from the *Gobiopertus* sp. digestive tract isolates revealed that they have a great prospect as aquaculture probiotic in Lake Ranu Grati. Among these 16 isolates, 9 recorded favorable probiotic properties such as relative resistance to acids and bile salts obtained in in vitro assays adapted to the conditions in the gastrointestinal tract. The isolates were also found to possess antibacterial activities towards common fish pathogens and were able to produce extracellular protease and cellulase enzymes, important for nutrient digestion. Four of these isolates were confirmed by DNA analysis to belong to the probiotic genus *Enterococcus*. Such intriguing features come up with a potentiality of *Gobiopertus* isolated LAB as a natural probiotic for improving the health and productivity of fish in aquaculture. This research will be continued in vivo and further to fully explore the benefits of bacteria obtained from *Gobiopertus* sp. for cultivation and research.

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This study presents the discovery of *Enterococcus* species from the digestive tract of *Gobiopterus* sp. in Ranu Grati Lake, demonstrating their potential as probiotics for aquaculture. The isolates showed resistance to gastric acidity and bile salts, antibacterial activity against fish pathogens, and the production of digestive enzymes, offering a sustainable approach to improving fish health.

AUTHOR'S CONTRIBUTIONS

MH perform the experiment, AAP, ARF designed and directed the project; AAP, SA, HN, RLH, HSY provide analysis, image and Table. HMH, AAP, wrote the manuscript with input from all authors.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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