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Probiotics potential of lactic acid bacteria isolated from Slender Walking Catfish (*Clarias nieuhofii*)

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Abstract. *Lingga R, Adibrata S, Roanisca O, Sipriyadi, Wibowo RH, Arsyadi. 2023. Probiotics potential of lactic acid bacteria isolated from Slender Walking Catfish (*Clarias nieuhofii*). Biodiversitas 24: 4572-4580.* Probiotics are living microorganisms or bioactive agents that positively impact animal digestion. They have successfully isolated from various sources. Recently, we isolated and characterized lactic acid bacteria (LAB) from slender walking fish (*Clarias nieuhofii* Valenciennes, 1840). The fish sample was obtained from Batu Rusa and Paya Benua Rivers in Bangka Island, Indonesia. LAB was isolated from the fish intestine using the pour plate method. The isolated LAB was then characterized based on their phenotypic traits, biochemical properties, and the 16S rRNA gene identification. The chosen isolates were tested to determine their ability to produce lactic acid, hemolytic and antibacterial activity, and antibiotic resistance. All isolates have the characteristic of rod-shaped and short rod-shaped cells with Gram-positive properties. Isolate KP1 showed the number of populations (2.89 x 107 CFU/mL) and lactic acid production with a concentration of 1.85%. All isolates exhibited no hemolysis activity and displayed sensitivity to antibiotics. The twelve lactic acid bacteria formed clear zones against *Staphylococcus aureus* and *Escherichia coli*. The results of the 16S rRNA gene identification indicated that isolates KB4, KB7, KB8, and KP1 belong to *Lactobacillus vaginalis, Lactobacillus fermentum, Limosilactobacillus fermentum*, and *Levilactobacillus brevis*, respectively. Lactic acid bacteria isolated from slender walking fish exhibited potential probiotic traits.

Keywords: *Clarias nieuhofii*, lactic acid bacteria, prebiotic, probiotic, slender walking catfish

INTRODUCTION

Probiotics have become a popular dietary supplement for promoting the health and growth of fish and livestock (Wanka et al. 2018). These are defined as living microorganisms or bioactive agents that have a positive impact on animal digestion. They can alter the microflora in the gut to enhance nutrition and disease resistance (Hoseinifar et al. 2016; Lazado and Caipang 2014; Nayak 2010). The crucial characteristics of probiotics among others have a beneficial impact; non-toxic, non-allergic, and nonpathogenic, large population of viable cells; adapted to the conditions of the intestinal environment; able to be stored and maintained in a stable condition (Amara and Shibl 2013). Besides that, candidates of probiotics should possess resistance to damage caused by bile salts and proteases; the ability to lower intestinal pH by producing lactic acid, thereby preventing the growth of harmful bacteria; reduction in the production of toxic and carcinogenic metabolites; facilitation of mineral absorption like calcium due to increased intestinal acidity; the production of compounds such as bacteriocins, organic acids, and hydrogen peroxide that inhibit the growth of pathogenic microorganisms, as well as vitamin B and K. Lactic acid bacteria (LAB), which are abundantly found in nature, exhibit these beneficial properties (Perez-Sanchez et al. 2011; Corcionivoschi et al. 2010).

In aquaculture, improving feed quality is crucial for optimizing growth and feed efficiency (Mulyasari et al. 2016), and probiotics have been shown to reduce the risk of infection from pathogenic bacteria (Kim et al. 2011). Additionally, supplementing feed with probiotics may reduce stress on the digestive system, leading to stronger villi and microvilli and a decreased likelihood of pathogenic bacteria colonization (Munir et al. 2017; Kim et al. 2011). The digestive tract of fish is a natural habitat for beneficial microbes found in aquatic environments, including probiotics (Llewellyn et al. 2015). These probiotics are commonly found in the digestive tracts of various fish species, such as *Catla catla, Labeo rohita, Cirrhinus mrigala, Cyprinus carpio, Channa striatus, and Oreochromis niloticus*, as well as in shrimp and mollusks. Studies have confirmed the presence of probiotics in healthy digestive tracts of these aquatic animals (Muthukumar and Kandeepan 2015; Allameh et al. 2014; Lara-Flores and Olvera-Novoa 2013; Widanarni et al. 2015; Sánchez-Ortiz et al. 2015). Commonly, the existence of lactic acid bacteria or probiotics in the digestive tract of

fish comes from the aquatic environment through water and food that are populated with bacteria. The digestive tract of fish, being abundant in nutrients, provides a favorable environment for the growth of microorganisms (Muthukumar and Kandeepan 2015).

The use of antibiotics in aquaculture is currently prohibited under international regulations. To replace antibiotics, the FAO has approved the use of alternative substances such as prebiotics, probiotics, symbiotics, phytobiotics, and other similar substances (Denev 2008). The future potential for probiotics in aquaculture is expected to be significant. So far, various efforts to control diseases have extensively employed the utilization of probiotics, particularly pathogenic bacterial infections (Merrifield and Ringo 2014; Lee et al. 2015; Caipang et al. 2020; Hasan and Banerjee 2020). In this particular study, probiotics were isolated from the digestive tracts of Slender Walking Fish (*Clarias nieuhofii* Valenciennes, 1840) living in natural aquatic habitats. This approach was taken because previous studies did not always take into account the physiological differences between the isolates obtained, even though isolates from fish hosts were expected to perform better than those from terrestrial hosts (Van Doan et al. 2018). In addition, several factors must be taken into consideration when exploring potential probiotic candidates. These include the ability to colonize the digestive tract, survive and reproduce in that environment, produce extracellular enzymes, possess nonpathogenic traits, and exhibit sensitivity to antibiotics treatments (Pundir et al. 2013; Loh et al. 2014). This study conducted to isolated and characterized lactic acid bacteria (LAB) from slender walking fish (*Clarias nieuhofii*) as potential probiotics.

MATERIALS AND METHODS

Sample collection

The Slender Walking Catfish (*Clarias nieuhofii*) were collected from Batu Rusa and Paya Benua River in Bangka Regency, Bangka Belitung Islands, Indonesia immediately transported to the laboratory with proper aeration for further analysis.

Materials sterilization

The equipment was initially washed and subsequently rinsed with distilled water. Subsequently, the materials were sterilized by placing them in a dry oven set to 140°C for 2 hours. The materials i.e. Man Ragosa Sharpe Agar (MRSA), Man Ragosa Sharpe Broth (MRSB), NaCl, and $CaCO_3$ were dissolved using heat from a stove. The equipment and materials were then sterilized using an autoclave at a pressure of 121°C for 2 hours.

Isolation and selection of lactic acid bacteria from the intestine of slender walking catfish

Three fish samples from each water resource were used for bacterial isolation through the following steps: at first, fish were anesthetized before undergoing surgery. *Clarias nieuhofii* were then carefully removed by dissection and placed in sterile plastic containers. A portion of the intestine was collected and placed in a plastic sample for isolation. Approximately 1 cm of the fish intestine was taken and weighed. Then, the digestive tract was added to a test tube containing 30 mL of selective media called MRS Broth and incubated in an incubator at 37°C for 48 hours. Furthermore, 350 mL of NaCl 0.85% solution was prepared and divided into serial test tubes up to 9 mL. Additionally, 350 mL of MRS Agar was made and all media were sterilized using an autoclave at a pressure of 121°C for 2 hours. Dilution was carried out by taking up 1 mL of the incubated MRSB and serially diluting up to 10^{-9}. From a 10^{-9} dilution, 1 mL of the samples was then cultured on MRS Agar supplemented with 1% $CaCO_3$ using the pour plate method and incubated for 48 hours at 37°C. The colonies with clear zone were evaluated and selected for further analysis.

Morphological characterization, purification, and Gram staining

The bacterial isolates were identified by observing their morphological characteristics such as shape, edge, elevation, margin, color, optical character, and surface. Following the characterization of colonies, pure cultures were subjected to the Gram's staining procedure. A clean object glass was prepared by rinsing it with 95% alcohol and adding drops of distilled water. The bacterial culture was then prepared and the inoculating loop was heated using a Bunsen burner. Next, the bacterial culture was applied to the slide and fixed. The bacterial smear was treated with violet dye for 1 minute and then rinsed with distilled water. Iodine was added and left for 1 minute before rinsing with distilled water. Decolorizer was added and left for 30 seconds before rinsing with distilled water. Safranin was then added and left for 30 seconds before rinsing with distilled water. The slide was then observed under a microscope using a 100x objective lens. The staining results were interpreted as purple for Gram-positive and red for Gram-negative bacteria.

Biochemical properties test

Biochemical or metabolic activity refers to the different chemical reactions occurring in the microbes cells to maintain life. Several tests were used to characterize these activities. The Catalase Test, where a pure isolate was taken using an inoculation loop and placed on an object glass. H_2O_2 (3%) was then dripped onto the isolate, and the presence or absence of gas bubbles was observed. Positive results indicated the presence of bubbles, while negative absence of bubbles. The Motility *Test* involved taking a pure isolate using an inoculation loop and putting it into a test tube containing SIM media, which was then incubated at 37°C for 24 hours to observe the motile or non-motile nature of the microbe. The Methyl Red (MR) Test required taking a pure isolate using an inoculation loop and putting it into a test tube containing MRVP, which was then homogenized and incubated for 72 hours at 37°C. After this, 3-4 drops of methyl red were added, and any color change was observed. Finally, the Triple Sugar Iron Agar (TSIA) Test involved streaking one inoculation loop of

pure isolate onto the surface of the agar upright and the agar slanted, followed by incubation at 37°C for 72 hours (Facklam and Elliott 1995; Cowan 1953; Harley 2005).

Production of lactic acid

This assay was carried out to evaluate the potency of isolates in producing lactic acid. During the experiment, the bacteria were cultured on MRS Broth and prebiotic medium which contained substrate medium (solid powder purchased from Jambi University), molasses, and well water. For inoculum preparation, two loopful of each isolate were cultured into 25 mL of MRSB and incubated for 48 hours at 37°C. Afterward, about 1.25 mL (5% v/v) of culture was inoculated into 25 mL of sterile prebiotic medium, incubated for 48 hours at 37°C, and used for further analysis.

Lactic acid bacteria enumeration was performed by Total Plate Count (TPC) method. About 1 mL of culture was serially diluted in NaCl 0.85% solution in the test tubes up to 10^{-7}. From 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} dilutions, 1 mL of the samples were then cultured on MRS Agar using the pour plate method and incubated for 24 hours at 37°C. The lactic acid percentage was calculated using the acid-base titration method (Kurnia et al. 2020). Approximately 5 mL of culture in a prebiotic medium was used for centrifugation at 500 rpm for 10 minutes. Furthermore, 2.5 mL of supernatant was added onto a conical flask containing 22.5 mL of sterile distilled water, dripped with 2 drops of phenolphthalein 1%, and titrated with NaOH 0.1 N to form a pink-color complex. The total of titrated acid was expressed and calculated as a lactic acid percentage:

$$\%\ lactic\ acid = \frac{Vol\ NaOH\ x\ N\ NaOH\ x\ 90.08}{Vol\ Sample\ x\ 1000}\ x\ dilution\ factor\ x\ 100\%$$

Temperature and acid resistance test

The objective of the experiment was to investigate how bacterial isolates would grow under different temperatures and acidic conditions. To achieve this, 500 µL of LAB culture was inserted into a test tube containing 4.5 mL MRSB media and incubated at four different temperatures: low temperature (15°C), room temperature (27°C), incubator temperature (37°C), and high temperature (45°C) for 48 hours. On the other hand, the isolates were cultured in MRS Broth with several drops of HCl to get pH 2.5 and 3 as the pH of stomach acid. The bacterial growth in each tube was then closely observed and recorded.

Antimicrobial test

The experiment was conducted by pouring 15 mL of Nutrient Agar (NA) into a petri dish and letting it solidify. Then, a sterilized loop was used to scrape the surface of the agar and streak *Staphylococcus aureus* and *Escherichia coli* onto the nutrient agar (NA) medium. A disc paper that was soaked in lactic acid bacteria was placed on the medium that had been inoculated with bacteria. The petri dish was incubated at a temperature of 30°C for 48 hours.

Hemolysis test

The bacterial colonies were grown in a blood agar solid medium, then incubated at 37°C for 24 hours. After incubation, the hemolytic reaction on a blood agar plate was observed by holding up the plate to a light source and observed with the light coming from behind (transmitted light). The colonies with Beta hemolysis (β) activity showed complete or true lysis of red blood cells. A clear zone, approaching the color and transparency of the base medium, surrounds the colony. Alpha hemolysis (α) activity showed a reduction of the red blood cell hemoglobin to methemoglobin in the medium surrounding the colony. This causes a green or brown discoloration in the medium. The colonies with no clear zone are considered as Gamma hemolysis (γ) group (ASM 2016).

Isolation of the DNA genomic of a bacterial isolate

A total of 1.5 mL of bacterial culture was placed into a 5.0 mL Eppendorf tube and centrifuged at 8000 rpm for 10 minutes. The resulting pellet was washed with STE buffer and centrifuged again at 8000 rpm for 10 minutes. This process was repeated three times. The pellet was then resuspended in 200 µL of STE buffer, mixed with 45 µL of lysozyme, and incubated at 55ºC for 1 hour to form protoplasts. Proteinase-K was added and incubated at 55ºC for 60 minutes. 400 µL of 10% CTAB in a 0.7 M NaCl solution was added and incubated at 65ºC for 30 minutes. The mixture was centrifuged and the clear phase was transferred to a new tube. Isopropanol and sodium acetate were added, then incubated at -20ºC overnight. The resulting pellet was washed with 70% alcohol, air-dried for 1 hour, and dissolved in 50 µL of sterile ddH2O. The isolated DNA can be stored at 4ºC or -20ºC (modified from Sambrook and Russell 2001).

Identification of 16S rRNA gene from bacterial isolate

The Polymerase Chain Reaction (PCR) technique was used to amplify the 16S rRNA gene from bacterial genomic DNA, using specific prokaryotic primers (forward primer 63F and reverse primer 1387R). The PCR reaction composition included GoTag Green master mix enzyme, specific primers, Nuclease Free Water, and DNA template. The PCR reaction was performed using pre-denaturation, denaturation, annealing, elongation, and post-PCR stages, with a total of 30 cycles. The PCR product DNA was observed using mini-gel electrophoresis with 1% agarose, followed by Ethidium Bromide stain visualization on a UV transilluminator. Afterward, the amplicon of the 16S rRNA gene was delivered to 1st BASE DNA Sequencing Service, Malaysia to identify the gene sequence. The raw sequencing data was trimmed and assembled using ChromasPro version 1.5 program and then blasted against the genomic data registered with NCBI. The sequences were analyzed using MEGA 5.0 program to construct a phylogenetic tree to show the relationship level of the LAB isolates with other bacteria using the Neighbor-Joining Tree method with a 1000 bootstrap.

RESULTS AND DISCUSSION

Isolation and selection of lactic acid bacteria from *Clarias nieuhofii*

Lactic acid bacteria from the intestines of *Clarias nieuhofii* were isolated using MRS Agar medium supplemented with 1% $CaCO_3$. After incubation, about 12 different isolates were grown with a clear zone around the colonies representing the production of lactic acid qualitatively. All of these isolates were Gram-positive bacteria with the shape of bacilli and coccobacilli cells. The characteristics of the bacterial cell and biochemical properties of selected isolates were shown in Table 1. Based on the catalase and motility test, LAB collected from *Clarias nieuhofii* digestive tracts were negative in catalase production and motility. Furthermore, all 12 isolates were able to produce stable acid through glucose fermentation in the methyl-red test, did not produce hydrogen sulfide (H_2S) gas, and carried out triple sugar fermentation including dextrose, sucrose, and lactose (except KB5 and KB6 mainly fermented glucose), also exhibited homofermentative type during fermentation which observed by negative gas bubble production in TSIA test.

Production of lactic acid

The viability of lactic acid bacteria in the prebiotic medium was in the range of 1.1 x 10^7 to 2.89 x 10^7 CFU/mL with the isolate KP1 as the highest number of populations after 48 hours of incubation. In addition, during the fermentation stage, all the isolates excreted lactic acid in the range of 1.23% to 1.85% which changes the pH medium to acidic conditions around 4.08 to 4.93. Based on Table 2, isolate KP1 and KB8 exhibited the highest lactic acid production with a concentration of 1.85% and 1.74% respectively.

Growth characteristics and environmental resistance

The characterization of lactic acid bacteria to resist and grow in different environmental conditions is important to evaluate the potencies of isolates as good probiotics. In this study, LAB was cultured in a temperature range of 15°C to 45°C and pH around 2.5 and 3. After 48 hours of incubation, the results suggested that all bacteria were resistant in all temperature and pH conditions except 15°C and pH 2.5 (Table 3). However, some isolates showed a slight growth at a pH of about 3 such as KK1, KK3, KB6, KP2, and KP3 indicating their sensitivity to highly acidic conditions. The latter isolate KP3 was the most sensitive bacteria observed in this study.

Table 1. Characteristics of LAB isolated from *Clarias nieuhofii*

LAB isolate	TSIA test				MR test	Motility	Catalase production	Cell shape	Gram
	Slant	*Butt*	Gas	H_2S					
KK1	Y	Y	-	-	+	-	-	Coccobacillus	+
KK3	Y	Y	-	-	+	-	-	Bacillus	+
KK7	Y	Y	-	-	+	-	-	Coccobacillus	+
KB4	Y	Y	-	-	+	-	-	Bacillus	+
KB5	R	Y	-	-	+	-	-	Bacillus	+
KB6	R	Y	-	-	+	-	-	Bacillus	+
KB7	Y	Y	-	-	+	-	-	Bacillus	+
KB8	Y	Y	-	-	+	-	-	Coccobacillus	+
KB10	Y	Y	-	-	+	-	-	Coccobacillus	+
KP1	Y	Y	-	-	+	-	-	Bacillus	+
KP2	Y	Y	-	-	+	-	-	Bacillus	+
KP3	Y	Y	-	-	+	-	-	Bacillus	+

Table 2. The growth and lactic acid production and of LAB isolated from *Clarias nieuhofii* in prebiotic medium

LAB Isolate	Concentration of lactic acid (%)	pH		Number of colonies (CFU/mL)
		Pre-incubation	Post-incubation	
KK1	1.23	5.9	4.82	1.1 x 10^7
KK3	0.88	5.9	4.93	1.3 x 10^7
KK7	1.13	5.9	4.76	1.4 x 10^7
KB4-IK	1.42	5.9	4.24	1.4 x 10^7
KB5	1.65	5.9	4.20	1.8 x 10^7
KB6	1.27	5.9	4.82	1.69 x 10^7
KB7-AM	1.50	5.9	4.29	1.1 x 10^7
KB8-C2	1.74	5.9	4.10	1.82 x 10^7
KB10	1.38	5.9	4.30	1.41 x 10^7
KP1-LS	1.85	5.9	4.08	2.89 x 10^7
KP2	1.33	5.9	4.46	1.61 x 10^7
KP3	1.44	5.9	4.39	1.90 x 10^7

Antimicrobial and hemolysis activity of lactic acid bacteria

The examination of resistance to antibiotics and pathogenic bacteria as well as the hemolysis activity of lactic acid bacteria were necessary to understand the health risks of their application as probiotic for human or other animals. All isolates exhibited the absence of hemolysis or discoloration indicating them as nonpathogenic bacteria. Furthermore, the isolates of this study were sensitive to both antibiotics with isolates KB4 and KB8 being the most sensitive bacteria to ampicillin and chloramphenicol respectively. On the other hand, the twelve lactic acid bacteria had a clear zone against *Staphylococcus aureus* and *Escherichia coli.* The former was the most sensitive pathogenic bacteria to KB7 with a clear zone of 19 mm and the latter was to isolate KB5 with a clear zone of 17.55 mm (Table 4).

Molecular identification using 16S rRNA gene

Identification of lactic acid bacteria as probiotic candidates was carried out using the 16S rRNA gene. Based on the lactic acid production, the viability in different temperature and pH conditions, as well as hemolysis activity and clear zone diameter of LAB against antibiotics and pathogenic bacteria, we selected four potential isolates for molecular identification that were KB4, KB7, KB8, and KP1. Amplification of the 16S rRNA gene was carried out using the PCR method with the 63f (5'-CAGGCCTAACACATGCAAGTC-3') and 1387r (5'-GGGCGGWGTGTACAAGGC-3') primers which produced a DNA fragment approximately of 1300 bp in size (Figure 1). After sequencing, the DNA amplicons, the edited sequences were compared to GenBank data using the BLAST program on the NCBI website. The results were analyzed to construct the phylogenetic tree and demonstrated that isolates KB4, KB7, KB8, and KP1 belong to *Lactobacillus vaginalis, Lactobacillus fermentum, Limosilactobacillus fermentum,* and *Levilactobacillus brevis* respectively (Figure 2).

Table 3. The viability of LAB isolated from *Clarias nieuhofii* at different temperatures and acidic conditions

LAB Isolate	Temperature				pH	
	15°C	27°C	37°C	45°C	2.5	3
KK1	+	+++	+++	+++	++	++
KK3	+	+++	+++	+++	++	++
KK7	+	+++	+++	+++	++	+++
KB4	+	+++	+++	+++	++	+++
KB5	+	+++	+++	+++	++	+++
KB6	+	+++	+++	+++	++	++
KB7	+	+++	+++	+++	++	+++
KB8	+	+++	+++	+++	++	+++
KB10	+	+++	+++	+++	++	+++
KP1	+	+++	+++	+++	++	+++
KP2	+	+++	+++	+++	++	++
KP3	+	++	++	+++	++	++

Notes: +++: A lot of bacterial growth; ++: Some bacterial growth; +: Less bacterial growth

Table 4. The hemolysis activity and clear zone diameter of LAB against antibiotics and pathogenic bacteria

LAB Isolate	Hemolysis activity	The diameter of clear zone (mm) against antibiotics		The diameter of inhibition (mm) against pathogenic bacteria	
		Ampicillin	Chloramphenicol	*S. aureus*	*E. coli*
KK1	Gamma	15.87	3.65	5.45	3.65
KK3	Gamma	16.86	4.17	6.02	4.17
KK7	Gamma	22.26	4.69	13.43	10.21
KB4	Gamma	30.43	18.98	14.74	16.37
KB5	Gamma	15.04	5.38	16.21	17.55
KB6	Gamma	13.02	5.38	13.09	11.87
KB7	Gamma	20.18	14.01	19	16.42
KB8	Gamma	16.64	20.21	16.76	15.88
KB10	Gamma	14.9	9.04	12.52	11.99
KP1	Gamma	13.27	13.37	15.44	15.06
KP2	Gamma	20.43	18.61	15.8	15.35
KP3	Gamma	27.08	14.15	14.12	13.08

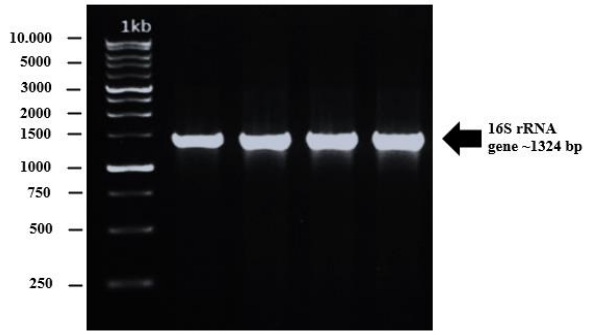

Figure 1. PCR amplification of the 16S rRNA gene using 63f and 1387r primers; M: 1 Kb ladder marker (Fermentas); lanes 1-6: PCR products of LAB samples

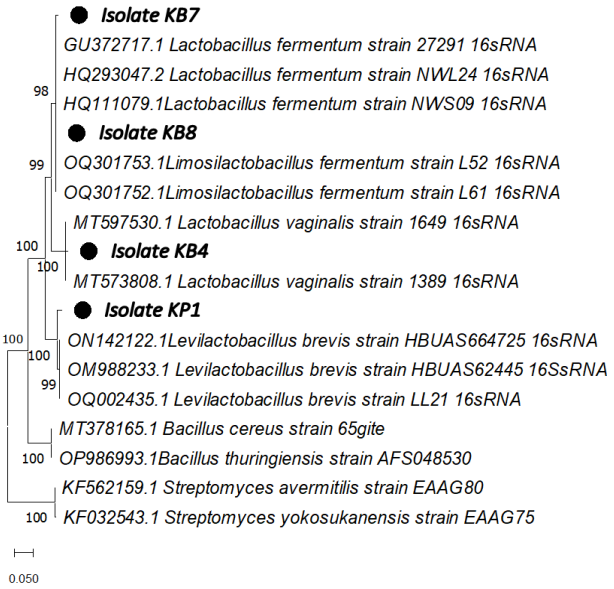

Figure 2. Phylogenetic tree construction of Isolates KB4, KB7, KB8, and KP1, based on Neighbor joining Tree, Tamura 2 parameter method with 1000 bootstrap replicates

Discussion

Fisheries are crucial to sustaining the global demand for a nutritional diet of a wide community, especially in Indonesia with innumerable islands. Fish consumption has grown in recent decades so the improvement in culture, feed quality, and reduction of antibiotic usage is necessary for producing high-quality fisheries products to meet the great demand. Probiotics isolated from indigenous species exhibited more advantageous traits which effectively improved the growth of farmed species (Husain et al. 2022; Wanka et al. 2018). However, only limited studies revealed the novel lactic acid bacteria from native fishes including *Clarias* species. In the present study, we obtained 12 lactic acid bacteria isolated from slender walking fish that produced a clear zone around the colonies. MRS Agar supplemented with 1% CaCO3 is usually used as selective media for LAB isolation. The latter compound reacts with lactic acid from the colonies to form calcium lactate which dissolves in the media and forms a transparent zone around the bacterial colonies (Haro et al. 2020).

The type and density of culturable LAB isolates vary among fish species. Several studies have reported less LAB obtained from *Channa* sp., *Puntioplites waandersi*, and farmed *Clarias* sp. with only eight, five, and nine bacterial isolates respectively (Agustina et al. 2022; Bayu et al. 2023; Prihanto et al. 2020). Physiological and environmental factors may play a vital role in the LAB diversity among fish species particularly water and feed quality. The morphological characterization of isolates showed the similarity between the LAB to those of the *Lactobacillus* sp. which were rod-shaped, Gram-positive and non-producing H_2S bacteria, catalase-negative, producing acid through carbohydrate fermentation, and non-motile (Ghanbari et al. 2009; Nasri et al. 2021; Vijayaram et al. 2016). However, lactic acid bacteria mostly have the characteristics of bacilli-shaped or coccus, Gram-positive, nonsporulating bacteria, produce lactic acid as the main product of sugar fermentation, and are facultatively anaerobic (Salminen et al. 2004).

Inoculation of LAB to a prebiotic medium was carried out to examine the viability and potency of isolates for producing lactic acid in formulated medium. The number of lactic acid bacterial populations with potential probiotics must be > 10^6 CFU/gram after incubation at pH 3.0. Despite the dissimilarity of pH conditions, all of the isolates have a density of more than 10^7 CFU/mL indicating their normal growth in the prebiotic medium. Compared with other studies, the lactic acid production by the isolates was slightly similar with *Lactobacillus plantarum* and other lactic acid bacteria of 1.2-1.92% in MRSB medium after 24 hours' incubation (Vamanu et al. 2005; Kurnia et al. 2020). Isolate KP1 and KB8 excreted the greatest lactic acid that showed their potencies as probiotic candidates. Probiotic ability can be demonstrated through various mechanisms by producing lactic acid to create an acid condition then inhibit the growth of pathogenic bacteria (Lokapirnasari et al. 2018). However, the capacity of LAB in lactic acid production mainly depends on nutrients and fermentation states including pH and temperature (Abedi and Hashemi 2020).

Probiotics are defined as living microorganisms when acquired in adequate amounts provide a health benefit to the host by reshaping the gut microenvironment (Ayivi and Ibrahim et al. 2022). Nevertheless, to carry out the beneficial activities as probiotics on the host, the LAB must be survived against stomach gastric juice (pH of 2-3) to reach and colonize in the intestines (Husain et al. 2022). Furthermore, for scaling up probiotic production and distribution in the future, the living bacteria have to overcome temperature fluctuations during the transportation and preservation of products. Therefore, all the selected isolates were cultured on various environmental stress conditions. We observed qualitatively the resistance of bacteria toward the range of temperature and pH (except 15°C and pH 2.5) for instance on isolates KB4, KB7, KB8, and KP1. On the other hand, isolate KK1, KK3, KB6, KP2, and KP3 showed the lowest growth in both acid conditions (pH 2.5 and 3). This result is

concurrent with several previous research reports that found the different ability of LAB to survive at pH 2.5 and 3 with the viability of 55.27-98.18% and 45.1-84.7% respectively (Hernentis et al. 2020); Govindaraj et al. 2021). In addition to the impact of temperatures on LAB growth, a recent study has demonstrated that *Lactobacillus* can grow at temperatures ranging from 5-53°C and optimally grow at 30-40°C depending on the species (Ahmed et al. 2006).

In recent years, researchers have explored the LAB potencies through phenotypic, physicochemical, and genotypic characterization to select the ideal probiotics. Commonly, the potential candidates are underscored by the following parameters including acid and bile tolerance, intestinal epithelial adhesion properties, sugar and protein utilization patterns, phenotype and genotype stability, immunogenicity and influence metabolic activities of the host, pathogenicity, as well as antibiotic resistance patterns and the production of antimicrobial substances to inhibit microbial pathogens (Harzallah and Belhadj 2013). Among twelve isolates, none of them were pathogenic bacteria or produce a clear halo zone in blood agar (gamma hemolysis) hence, they are safe for probiotics. Moreover, the LAB in this work were susceptible to β-lactams (ampicillin) and inhibitors of protein synthesis (chloramphenicol) that were shown by isolate KB4 and KB8 respectively. Antibiotic susceptibility profiling is necessary to examine the genetic instability and possible distribution of antibiotic resistance determinants or genes (Anisimova et al. 2022). Further, the presently isolated LAB exhibited antimicrobial activities against *Staphylococcus aureus* with an inhibition zone diameter of 5.45-19 mm and *Escherichia coli* ranging from 3.65-17.55 mm. Isolate KB7 and KB5 were the most antagonistic bacteria to each pathogen. There are three classes of antimicrobial activity: high (> 6 mm), moderate (> 3-6 mm), and low (0-3 mm) (Pan et al. 2009). The difference in clear zone diameter depends on the types and concentration of antimicrobial compounds excreted by the LAB as well as the response of pathogenic bacteria toward the substances (Ouchari et al. 2019).

Four of the most promising LAB isolates, KB4, KB7, KB8, and KP1 were selected for identification based on the 16S rRNA gene. The nucleotide sequences of bacteria were compared with the database in GenBank and collected for phylogenetic tree analysis. The results confirmed these isolates were closely related to *Lactobacillus vaginalis*, *Lactobacillus fermentum*, *Limosilactobacillus fermentum*, and *Levilactobacillus brevis* respectively. *Lactobacillus vaginalis*. Lactobacilli are Gram-positive bacteria with rod-shaped cells and belong to the group of lactic acid bacteria with *Leuconostoc*, *Pediococcus*, and *Streptococcus*. They are found in highly sugar-containing substances available, including the mucosal membranes of humans and animals (intestine, oral cavity, and vagina), on plants, and in fermenting or spoiling foods. For many years, these bacteria have been widely found in raw milk and used to produce dairy products such as fermented milk, cheese, and yoghurt (Bernardeau et al. 2008). *Limosilactobacillus* and *Levilactobacillus* are two of the twenty-three novel genera that were reported after the reclassification of the genus *Lactobacillus* (Ayivi and Ibrahim 2022). All of these genera have been widely reported and can be used as probiotics in aquaculture. For instance, the feeding of fishes by probiotic *Levilactobacillus brevis* isolated from *Sander lucioperca* and *Limosilactobacillus fermentum* increased the immune function toward pathogenic bacteria *Aeromonas hydrophila* and reduce the adverse effects of toxic metal cadmium (Cd) respectively (Adeli et al. 2022; Faeed et al. 2020).

In conclusion, we reported twelve lactic acid bacteria isolated from *Clarias nieuhofii* defined as Gram-positive bacteria with rod and coccobacilli cells, catalase-negative, non-motile, and able to produce lactic acid from sugar fermentation. Of these bacteria, we select four potential candidates as probiotics including isolate KB4, KB7, KB8, and KP1 which showed the greatest production of lactic acid, high resistance to temperature and acid pH, non-pathogens, susceptible to antibiotics, and excreted antimicrobial substances against pathogenic bacteria. Based on the identification of the 16S rRNA gene and phylogenetic tree results demonstrated that these LAB belong to the genera *Lactobacillus*, *Limosilactobacillus*, and *Levilactobacillus*. Further studies on the effect of probiotic candidates on growth performance and the immune system of slender walking fish toward pathogenic bacteria need to be carried out.

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