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Antibacterial activity of lactic acid bacteria from various freshwater fish species against pathogenic bacteria in caged red tilapia (*Oreochromis* sp.)

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Abstract. *Quach VCT, Tran QD, Ha NH, Nguyen BT, Tu TD, Nguyen PT. 2023. Antibacterial activity of lactic acid bacteria from various freshwater fish species against pathogenic bacteria in caged red tilapia* (Oreochromis *sp*.). *Biodiversitas 24: 3373-3383.* Antibiotic-resistant bacteria are a big challenge for many aquaculture countries around the world. The search for new solutions, such as probiotics to replace antibiotics in aquaculture, attracts the interest of many scientists. The investigation was accomplished by isolating Lactic Acid Bacteria (LAB) inhibiting *Streptococcus agalactiae* and *Aeromonas hydrophila*, which cause swollen eyes and hemorrhagic skin disease in caged red tilapia (*Oreochromis* sp.) in Vinh Long province. The findings isolated 37 isolates of LAB from the intestines of catfish, red tilapia, and tilapia. Using the diffusion well method, the results showed that 13/37 (35.14%) and 18/37 (48.65%) isolates were inhibitory against *S. agalactiae* and *A. hydrophila*, respectively. The research found that eight isolates were inhibitory to two species of *S. agalactiae* and *A. hydrophila*, especially isolate CT6.2, which has the strongest inhibition (10.5 mm inhibition zone diameter). Our finding revealed that the isolates CT6.2 and RP6 demonstrate the highest antagonistic activity against *S. agalactiae* and *A. hydrophila* at a density of 10^8 CFU/mL after incubation at 35°C, pH = 5-6 for 48-60 h. Using PCR techniques and 16S rRNA gene sequencing combined with morphological and biochemical characteristics, the LAB isolates in the findings belong to the genera *Lactobacillus* and *Pediococcus*, being 99.35% and 99.02% similar to *Lactobacillus plantarum* and *Pediococcus pentosaceus*, respectively. This is the first report on the antimicrobial activity of LAB against *S. agalactiae* and *A. hydrophila* in caged red tilapia in the Mekong Delta.

Keywords: Antimicrobial activity, aquaculture, *Lactobacillus*, probiotics, red tilapia, *Streptococcus agalactiae*

INTRODUCTION

Red tilapia (*Oreochromis* sp.) is one of the many freshwater species intensively cultivated in Vinh Long Province of the Mekong Delta due to its easy-to-raise, fast-growing, good meat quality, and potential for future exports. However, the rapid expansion of farming areas and increasing intensification have caused adverse effects on the environment and increased the risk of disease outbreaks. One of the diseases causing great damage to intensive red tilapia farming is popeye (swollen eye) and skin hemorrhage caused by *Streptococcus agalactiae* (Oanh and Phuong 2012). According to Phú et al. (2017), the disease occurs during the rainy season and the changing seasons, with high mortality rates of up to 81.8-100%.

Aeromonas hydrophila, a Gram-negative bacteria ubiquitous in both marine and freshwater environments, is recognized as an important pathogen that can have an enormous impact on the fish farming industry (Harikrishnan and Balasundaram 2005). Many aquatic animal species have been reported with *A. hydrophila* infection, including red hybrid tilapia (Lee and Wendy 2017; Pauzi et al. 2020), Nile tilapia (*Oreochromis niloticus* Linnaeus, 1758) (Vaz Rodrigues et al. 2019), and channel catfish (*Ictaluras punctatus* Rafinesque, 1818) (Zhang et al. 2016). In Vietnam, *A. hydrophila* has been found to affect many fish species, such as tilapia, channel catfish, and carp (common carp and grass carp), which are the main culture species in Northern Vietnam (Nhinh et al. 2021). In Pangasius, previous studies revealed that hemorrhagic disease caused by *A. hydrophila* (Ly et al. 2009; Crumlish et al. 2010) make great damage to fish farming. This is a disease that can occur in Pangasius at all stages of culture with a loss rate of up to 90%. A recent study by Hoa et al. (2021) showed that *A. hydrophila* was detected in 11% of larvae, 30% of fry, and 30% of apparently healthy striped catfish in the Mekong Delta. Farmers in the Mekong Delta of Vietnam have used antibiotics to treat infected red hybrid tilapia fish with high doses and unscientific mixing (Phu et al. 2017), which increases the resistance of bacteria and reduces the effectiveness of treatment (Pauzi et al. 2020; Fauzi et al. 2021; Alazab et al. 2022). Therefore, finding a product that is both safe and economical in the prevention and treatment of aquatic diseases and that can limit the use of antibiotics

will be a big challenge for the industry.

Lactic Acid Bacteria (LAB) are one of the beneficial bacteria that are widely used in natural environments, such as milk and dairy fermented products (Widyastuti et al. 2014; Ağagündüz et al. 2021), fermented vegetables (Sáez et al. 2018), and the gut Tmicrobiota of fish (Ringø et al. 2018; Iorizzo et al. 2021). The use of LAB was the safest microorganism in the development of probiotics for aquaculture due to their antagonistic activity against opportunistic pathogens, fungi, and viruses that cause microbiological spoilage of feed, pollute water bodies, and cause diseases in aquatic animals (Chizhayeva et al. 2022). Karthikeyan and Santosh (2009) isolated *Lactobacillus plantarum* from the intestines of black tiger shrimp (*Penaeus monodon* Fabricius, 1798) with the ability to produce bacteriocin against some pathogenic bacteria such as *Salmonella typhimurium*, *Vibrio cholera*, and *Staphylococcus aureus*. On the other hand, LAB strains were also isolated from the fish intestine, and some fermentation products were inhibitory to pathogenic bacteria in seawater and freshwater aquatic animals (Alonso et al. 2018; Lim et al. 2020; Amin et al. 2023). Until now, there have been many studies and review articles showing that LAB has the ability to produce antibacterial substances such as diacetyl, lactic acid, H_2O_2, and bacteriocin against some pathogenic bacteria on shrimp and fish in aquaculture (Zacharof and Lovitt 2012; Ringø et al. 2018). Previous studies concentrated on the isolation and characterization of LAB from striped catfish (*Pangasius hypophthalmus* Sauvage, 1878) (Phương and Oanh 2016), in white leg shrimp (*Penaeus vannamei* Boone, 1931) (Linh et al. 2017). However, there are not many studies on LAB that are inhibitory to *Streptococcus agalactiae* and *A. hydrophila*, causing popeye and hemorrhagic skin disease in red tilapia. Therefore, this investigation was carried out to isolate and characterize LAB from the intestine of fish for use as probiotics in red tilapia (*Oreochromis* sp.). This is the first study on the inhibitory activity of LAB against *S. agalactiae* and *A. hydrophila*, which affect caged red tilapia in the Mekong Delta.

MATERIALS AND METHODS

Isolation of lactic acid bacteria from fish intestines

A total of 60 farmed fish samples, including *Pangasius* catfish (n = 21), red tilapia (n = 18), and tilapia (n = 25), intensively cultured in three provinces of Mekong Delta, consisting of Vinh Long, Dong Thap, and An Giang (Figure 1), were collected for LAB isolation. All fish samples are healthy fish (Figure 1), with the following average weights: *Pangasius* catfish (400-800 g), red tilapia (200-350 g), and tilapia (180-400 g). The fish collection was carried out from January 2022 to May 2022, and the fish samples were preserved in an aerated plastic box during transportation to the laboratory for bacterial isolation.

Lactic acid bacterial isolates were isolated from the fish intestine, according to Amin et al. (2020) with some modifications. Briefly, the fish body surface was sterilized with 70% alcohol. The intestines were cut into small pieces, and they were put into a test tube containing 5 mL of 0.9% sterile physiological solution. Let the test tube stand for 10 min to allow sedimentation. Then, 1 mL of supernatant was placed into 9 mL of 0.9% sterile physiological solution, which was serialized and spread in de Man Rogosa and Sharpe media (De Man et al. 1960) supplemented with 0.5% $CaCO_3$ (Meidong et al. 2017). Finally, the plate was anaerobically incubated at a temperature of 37°C for 48 h. Lactic acid bacterial isolates were preliminarily identified when there was a formation of clearance zones around the colony. Besides, the colony with Gram-positive, oxidase-, and catalase-negative results accomplished according to Buller (2014) was selected for the determination of antibacterial activity.

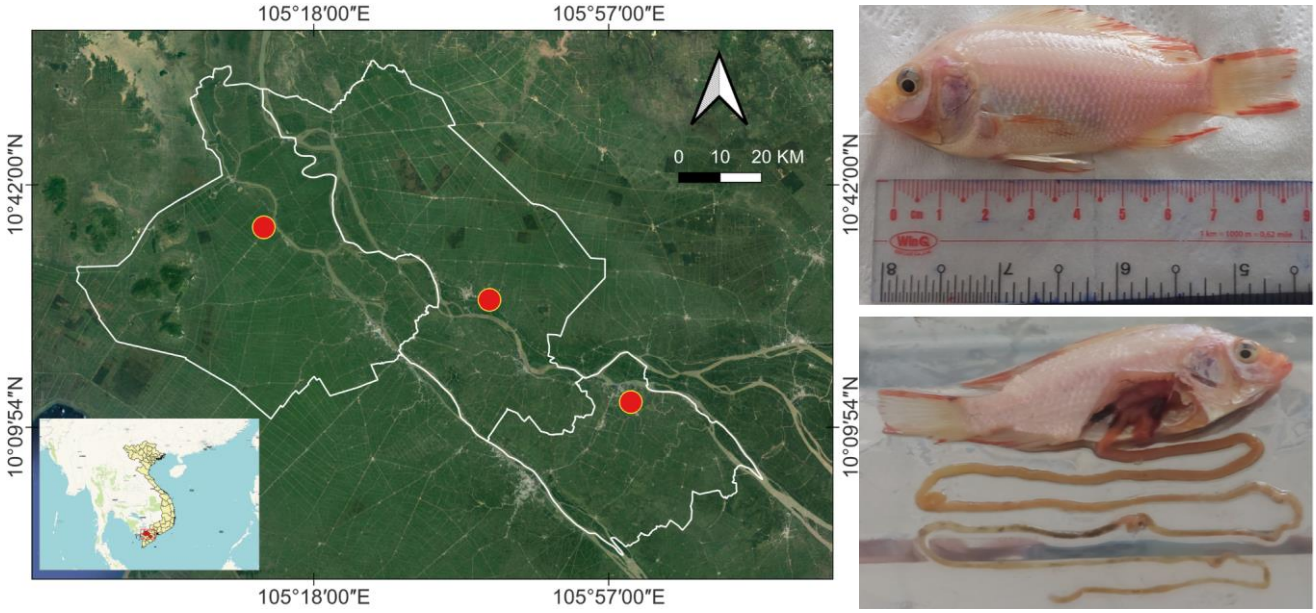

Figure 1. Sampling sites (red circle) and fish samples (healthy red tilapia) for LAB bacterial isolation

Antagonistic activity test

Lactic acid bacteria were performed for inhibitory activity by the modified well diffusion agar method of Amin et al. (2023).

Preparation of indicator bacteria

The indicator bacteria *A. hydrophila* and *S. agalactiae*, isolated as causing hemorrhagic disease in red tilapia (Oanh and Phuong 2012; Dang et al. 2021), were recovered on TSA medium (Tryptone Soya Agar) with 24-48 h of incubation at 28-30°C. The pathogenic bacteria were added to 0.9% physiological saline to have a cell concentration of 10^8 CFU/mL and spread over the TSA surface. Then, using the base of a sterile pipette tip, 6 mm-diameter wells were punched on the agar plate. The plate was then incubated aerobically at 30°C for 24 h, and the clearance zone around the wells was measured in mm.

Preparation of LAB

LAB was enriched in an MRS medium and incubated at 37°C for 48 h. The enriched culture was centrifuged at 10.000 rpm for 5 min at 4°C, and 80 µL of the supernatant from each LAB isolate was added to each well of the indicator bacterial agar plate. Then, the plate incubates at 28-30°C for the indicator bacteria to grow. The antibacterial activity of LAB was achieved based on clear zones around the well and calculated by the formula: d = d1 - d2 (d is the zone of inhibition diameter; d1 is the total diameter of the inhibition zone; d2 is the diameter of the well (6 mm). Inhibition was classified as weak antibacterial activity (+) when d<5 mm, moderate (++) when 5≤d≤10 mm, strong inhibition (+++) when d>10 mm, and no inhibition (-) when d≤2mm (Hernández et al. 2005).

Effect of culture conditions on lactic acid bacteria's inhibitory activity

In this study, two isolates, namely CT6.2 and PR6, were selected to evaluate the effect of culture conditions, such as bacterial densities, pH, incubation temperature, and time, on their inhibitory activity for *A. hydrophila* and *S. agalactiae*.

Effect of lactic acid bacterial densities

Each bacterial isolate was prepared with bacterial densities of 10^4, 10^5, 10^6, 10^7, and 10^8 CFU/mL, respectively. The culture corresponding to the above LAB densities is evaluated for its ability to inhibit *A. hydrophila* and *S. agalactiae* after being incubated at 30°C for 24 h. The antibacterial activity of LAB against *A. hydrophila* and *S. agalactiae* was tested as described above by Amin et al. (2023). The experiments were performed by a Completely Randomized Design (CRD) in triplicate, with the control treatment having no bacteria.

Effect of pH

The chosen pH values for study in this experiment were 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0. Use NaOH 0.01N or HCl 0.01N to adjust the bacterial culture to the required pH values. The antibacterial activity of LAB against *A. hydrophila* and *S. agalactiae* was tested as described above by Amin et al. (2023). The experiments were performed by a Completely Randomized Design (CRD) in triplicate, with the control treatment having no bacteria.

Effect of incubation temperature

The selected temperature values for the investigation include 15°C, 20°C, 25°C, 30°C, and 35°C. The antibacterial activity of LAB against *A. hydrophila* and *S. agalactiae* was tested as described above by Amin et al. (2023). The experiments were performed by a Completely Randomized Design (CRD) in triplicate, with the control treatment having no bacteria.

Effect of incubation time

The *incubation* times chosen for the study include 24, 36, 48, 60, 72, and 84 h at 30°C. The antibacterial activity of LAB against *A. hydrophila* and *S. agalactiae* was tested as described above by Amin et al. (2023). The experiments were performed by a Completely Randomized Design (CRD) in triplicate, with the control treatment having no bacteria.

Molecular identification of lactic acid bacteria

Bacterial DNA extraction

Lactic acid bacteria DNA was extracted according to standard techniques (Sambrook and Russell 1989). In brief, bacterial isolates were grown for 24 h in an LB medium (Luria-Bertani). The culture was added to 100 µL of 0.1X TE solution (Tris-HCl 10 mM, EDTA 1 mM, pH = 8.0) and incubated at 95°C for 15 min. Finally, the solution cooled rapidly in ice water and was centrifuged at 14,000 rpm for 2 min. The collected bacterial DNA was checked for purity and stored at -20°C for further analysis.

Lactic acid bacteria identification

The study used primer pairs Lac1: 5'-AGCATAGGGAATCTTCCA-3' and Lac2: 5'-ATTCCACCGCTACCATG-3' (Walter et al. 2001) to identify LAB. PCR reaction components include 1X PCR buffer, 2.5 mM $MgCl_2$, 200 µM dNTPs, 2U *Taq* DNA polymerase, 10 pmol of forward primer (Lac1) and reverse primer (Lac2), and 20-40 ng of a bacterial DNA sample. Thermal cycle performed, including initial denaturation at 95°C for 5 min, then 30 cycles of denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min, extension at 72°C for 2 min, and the final elongation step at 72°C for 10 min. PCR products were electrophoresed on a 1.5% agarose gel in 1X TBE buffer (Tris-borate-EDTA) at 95 volts for 30 min and captured on a BioRad UV 2000 machine (USA). The length of the amplification products is 340 bp. *L. plantarum* (strain RP11-1) was used as a positive control (provided by the Faculty of Aquatic Pathology, College of Aquaculture and Fisheries, Can Tho University). Two LAB isolates with the strongest activity toward *A. hydrophila* and *S. agalactiae* were selected for sequencing at Macrogen Company, Korea (www.macrogen.com).

Statistical analysis

The antibacterial activity of LAB isolates is presented as the mean±standard deviation. Significant differences between mean values ($P < 0.05$) were determined by Analysis of one-way Variance (ANOVA) by Tukey's test

with the Minitab 20.0 version. The sequence similarity of bacterial isolates was compared with sequences on the National Center for Biotechnology Information (NCBI) data bank using the BLASTn program. The sequences of the isolated bacteria and the sequences of the LAB in the NCBI database will be compared with each other (multi-alignment) using the CLUSTAL W program (Thompson et al. 1997). The phylogenetic tree showing the genetic relationships between bacterial strains was constructed using MEGA6 (Molecular Evolutionary Genetics Analysis) software based on the neighbor-joining algorithm (Saitou and Nei 1987) with bootstrap values of 1,000 replications (Tamura 2013).

RESULTS AND DISCUSSION

Isolation of lactic acid bacteria from the intestines

A total of 37 isolates of LAB were isolated from 60 fish samples, of which 13 isolates (35.14%) originated from the intestines of *Pangasius* catfish, 14 isolates (37.84%) derived from the intestines of red tilapia, and 10 isolates (27.02%) originated from the intestines of tilapia (Table 1).

Morphological and biochemical characteristics of isolated lactic acid bacteria

The results showed that all colonies of the isolates were round, convex, opaque white, or clear white, and the small bacterial colony ranged from 1 to 2.5 mm after 48 h of incubation on an MRS medium. On the other hand, the research indicated these bacterial isolates were capable of producing acid to dissolve $CaCO_3$ due to the appearance of clearance zones around the colony. All 37 isolates in this study were rod-shaped (34/37 isolates, 91.89%) or cocci-shaped (3/37 isolates, 8.11%), Gram-positive bacteria, non-spore-forming, oxidase- and catalase-negative, and homofermentative. Morphological and biochemical characteristics of representative LAB isolates are presented (Figure 2).

The antibacterial ability of isolated lactic acid bacteria

The results showed that 18/37 (48.65%) isolates were antagonistic to *A. hydrophila*, and 13/37 (35.14%) isolates were inhibitory to *S. agalactiae*. In general, there is a difference in the prohibitory activity of LAB bacterial isolates against *A. hydrophila* and *S. agalactiae*, according to our findings (Table 2).

There were 2 of the 13 isolates (15.39%) that had strong inhibition against *S. agalactiae*, 5 of the 13 isolates (38.46%) that had moderate inhibition, and 6 of the 13 isolates (46.15%) that had weak inhibition against *S. agalactiae*, including CT7.2, H1, H2, H3, RP2, and RP3. The antagonistic activities of LAB bacterial isolates against *S. agalactiae* are detailed (Figure 3). Especially, the findings also revealed that twelve isolates, consisting of CT4.1, CT6.2, CT6.3, CT7.2, CT8.1, ĐH1, ĐH3, ĐH4, ĐH10, RP2, RP3, and RP6, were inhibitory to both *A. hydrophila* and *S. agalactiae*. Meanwhile, among 18 isolates of LAB with inhibition to *A. hydrophila*, there are 3/18 (16.67%) isolates, namely CT6.2, CT8.2, and RP6, with strong inhibition activity. Four isolates (22.22%), consisting of CT5.2, CT6.3, CT7.3, and DH10, were moderately inhibitory, and eleven isolates (61.11%), comprising of CT2.2, CT3.2, CT4.1, CT5.1, CT7.2, ĐH1, ĐH3, ĐH4, ĐH10, RP2, and RP3, were weakly inhibitory (Figure 4).

Effect of culture conditions on lactic acid bacteria's inhibitory activity

Effect of bacterial densities on antibacterial activity

The findings revealed that the antibacterial activity of isolates CT6.2 and RP6 increased with bacterial density (Figure 5). Two bacterial isolates showed the maximum antibacterial activity in the treatment with a bacterial density of 10^8 CFU/mL. The analysis results revealed a statistically significant difference ($P<0.05$) in bacterial densities on the antibacterial activity of two isolates, CT6.2 and RP6, against *S. agalactiae* and *A. hydrophila* at the 5% significance level.

Effect of pH on antibacterial activity

The research demonstrated that the suitable pH for the highest antibacterial activity and the isolates CT6.2 and RP6 was at pH = 5.0 (isolate CT6.2) and pH = 6.0 (isolate RP6) (Figure 6). In general, the higher the pH value, the lower the antibacterial activity of the two bacterial isolates, with the lowest antibacterial activity at pH = 9.0 (two bacterial isolates have weak or no antibacterial activity at this pH value). While at pH = 4.0, the bacterial isolates exhibited moderate inhibition activity. The analysis results revealed a statistically significant difference ($P<0.05$) in pH values on the antibacterial activity of two isolates, CT6.2 and RP6, against *S. agalactiae* and *A. hydrophila* at the 5% significance level.

The influence of incubation time on the antibacterial activity

The findings demonstrated that isolate RP6 had the highest inhibitory activity at 48 h after inoculation, while isolate CT6.2 showed the highest antagonistic activity at 60 h (Figure 7). In addition, the results also showed that isolates CT6.2 and RP6 showed weak antibacterial activity at 24 and 36 h after inoculation. Besides, the results also recorded that isolates CT6.2 and RP6, had a decrease in antibacterial activity after 60 h of culture, and at 84 h, isolates CT6.2 and RP6 had the weakest antibacterial activity. At the 5% significance level, the analysis results revealed a statistically significant difference ($P<0.05$) in incubation time for the antibacterial action of isolates CT6.2 and RP6.

Effect of incubation temperature on antibacterial activity

In general, the research revealed that the antibacterial activity of isolates CT6.2 and RP6 increased sharply at temperatures of 25-35°C (Figure 8). However, the antibacterial activity of isolates CT6.2 and RP6 was weak or showed no inhibitory activity at low temperatures of 15-20°C. The prohibitory activity of isolate CT6.2 was highest at 35°C. However, isolate RP6 was the highest inhibitory against *S. agalactiae* at 35°C, while this isolate has the highest inhibitory activity at 30°C for *A. hydrophila*. The analysis results showed a statistically significant difference ($P<0.05$) in the impact of incubation temperature on the antibacterial activity of isolates CT6.2 and RP6 at the 5% significance level (Figure 8).

Identification of lactic acid bacteria

All isolates that displayed inhibitory activity with *A. hydrophila* and *S. agalactiae* were PCR-positive with an amplification product size of 340 bp (Figure 9).

Two isolates, CT6.2 and RP6, representing two groups of rod- and cocci-shaped bacteria and also inhibitory to *A. hydrophila* and *S. agalactiae*, were selected for 16S rRNA gene sequencing. The BLAST analysis results after 16S rRNA gene sequencing showed isolate RP6 (Accession number OQ842244) having a 99.02% similarity to *Pediococcus pentosaceus* strain CILSWE5 (MF967224.1), *P. pentosaceus* strain PB2 (KP883297.1), and *P. pentosaceus* strain GB5 (JN851779.1). Meanwhile, isolate CT6.2 (Accession number OQ842246) showed a 99.67% similarity to *Lactobacillus plantarum* strain LBH1063 (KT000587.1) and *L. plantarum* strain LBH1064 (KT000588.1), and a 99.35% similarity to *L. plantarum* isolate LP34C (EF439684.1) in GenBank. The phylogenetic tree showed that *Lactobacillus* and *Pediococcus* isolates were distributed into two separate clusters (Figure 10).

Many reports have shown the role of LAB in aquaculture (Ringø et al. 2020; Chizhayeva et al. 2022). The current research isolated 37 LAB isolates from the digestive tracts of fish with morphological and biochemical characteristics (Figure 2) similar to those previously described (Khagwal et al. 2019). According to Pundir et al. (2013), LAB isolated from food samples had cultural, morphological, and biochemical characteristics, such as colonies that were round, creamy, smooth, opaque, flat, and entire, mostly rod-shaped cells, Gram-positive, catalase-negative, non-motility, and non-endospore-forming. In this study, 34/37 (91.89%) isolates have long rod-shaped cells. Meanwhile, only 3/37 (8.11%) isolates were isolated from the intestines of catfish, red tilapia, and tilapia. These results might be due to the isolation of LAB from the MRS medium. According to Lankaputhra et al. (1996), the MRS medium, created by de Man, Rogosa, and Sharpe (De Man et al. 1960), was intended to promote the development of the LAB, which includes species of the genera *Lactobacillus*, *Streptococcus*, *Pediococcus*, and *Leuconostoc*. Therefore, most of the bacterial isolation research showed that the genus *Lactobacillus* was dominantly detected in fermented foods and the guts of mammals (Dowarah et al. 2017), fish (He et al. 2017; Gupta et al. 2019), and poultry (Rocha et al. 2014). Bucio et al. (2006) reported that MRS is an effective medium for enhancing the recovery of LAB from fish gut microbiota. By this investigation, it was shown that several species of lactobacilli, including those found in wild European eels, perch, rudd, ruffe, bleak, silver bream, chub, somnul, and farmed African catfish, were presented in large numbers in the intestines of edible freshwater fish from the river.

Table 2. Antibacterial activity of lactic acid bacterial isolates against *Streptococcus agalactiae* and *Aeromonas hydrophila*

Bacterial isolate	Zone of inhibition diameter (mm)			
	A. hydrophila		*S. agalactiae*	
	Diameter (mm)	Antibacterial activity	Diameter (mm)	Antibacterial activity
CT2.2	$2.20^{i} \pm 0.00$	+	0	-
CT3.2	$5.17^{f} \pm 0.28$	+	0	-
CT4.1	$3.17^{h} \pm 0.28$	+	$5.17^{e} \pm 0.28$	++
CT5.2	$5.67^{ef} \pm 0.57$	++	0	-
CT6.2	$10.33^{c} \pm 0.28$	+++	$10.50^{b} \pm 0.00$	+++
CT6.3	$8.00^{d} \pm 0.00$	++	$17.17^{a} \pm 0.28$	+++
CT7.2	$2.33^{i} \pm 0.28$	+	$4.17^{f} \pm 0.28$	+
CT7.3	$5.50^{f} \pm 0.00$	++	0	-
CT8.1	$2.60^{ghi} \pm 0.28$	+	$7.17^{d} \pm 0.28$	++
CT8.2	$11.00^{b} \pm 0.00$	+++	0	-
ĐH1	$4.00^{g} \pm 0.00$	+	$3.00^{g} \pm 0.00$	+
ĐH2	$6.17^{e} \pm 0.28$	+	$5.17^{e} \pm 0.28$	+
ĐH3	0	-	$2.83^{g} \pm 0.28$	+
ĐH4	$2.30^{i} \pm 0.28$	+	$3.00^{g} \pm 0.00$	++
ĐH5	$4.00^{g} \pm 0.00$	+	$7.00^{d} \pm 0.00$	++
ĐH10	$3.00^{gh} \pm 0.00$	++	0	-
RP2	$2.40^{hi} \pm 0.00$	+	$4.00^{f} \pm 0.00$	+
RP3	$2.33^{i} \pm 0.28$	+	$2.50^{g} \pm 0.00$	+
RP6	$17.17^{a} \pm 0.28$	+++	$8.17^{c} \pm 0.28$	++

Note: Results are shown as mean ± SE. Means within the same column with different superscripts are significantly different ($P<0.05$). Weak antibacterial activity (+) when $d<5$ mm, moderate (++) when $5 \leq d \leq 10$ mm, strong inhibition (+++) when $d>10$ mm, and no inhibition (-) when $d \leq 2$mm

Table 1. The isolation of lactic acid bacteria from three fish species

Bacterial source	Vinh Long			Dong Thap			Can Thao		
	Striped catfish	Red tilapia	Tilapia	Striped catfish	Red tilapia	Tilapia	Striped catfish	Red tilapia	Tilapia
No of fish samples	6	5	6	7	5	6	8	8	9
No of bacterial isolates	5	4	4	4	5	3	4	5	3

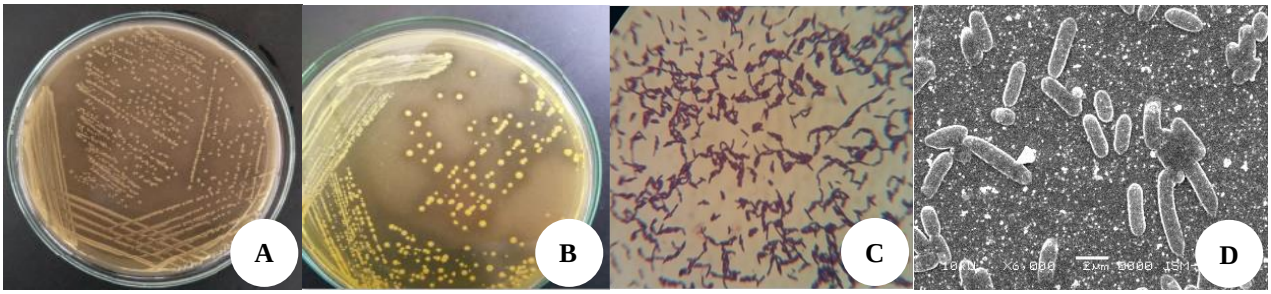

Figure 2. Morphological and biochemical characteristics of LAB isolates isolated from the fish' intestine. A. Bacterial colonies (isolate CT6.2) with opaque white on MRS medium, B. Clearance zones around the colony of isolate RP2 on the MRS medium supplemented with 0.5% CaCO3, C. Long rod-shaped bacteria (isolate CT6.2) with Gram stain (100X), D. Scanning electron microscope image of isolate CT6.2

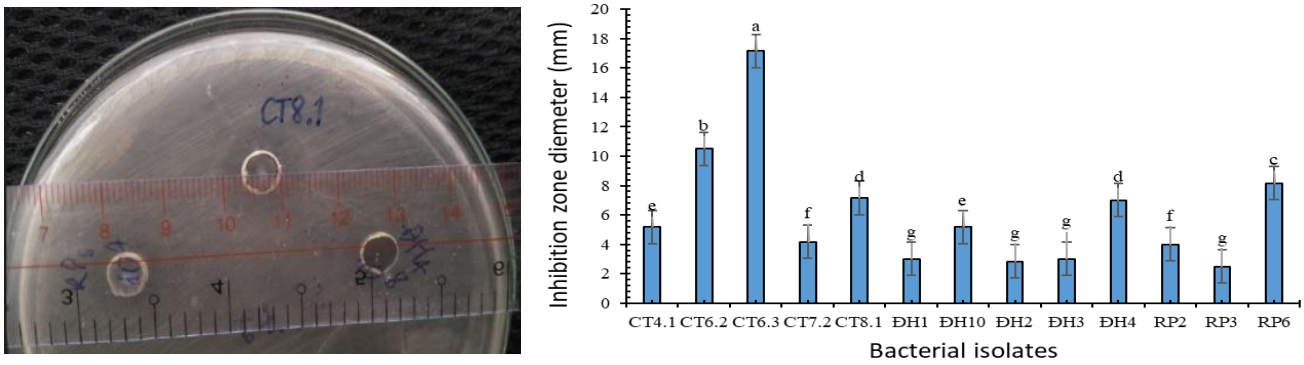

Figure 3. Antagonistic activity of the isolated lactic acid bacterial isolates against *Streptococcus agalactiae*

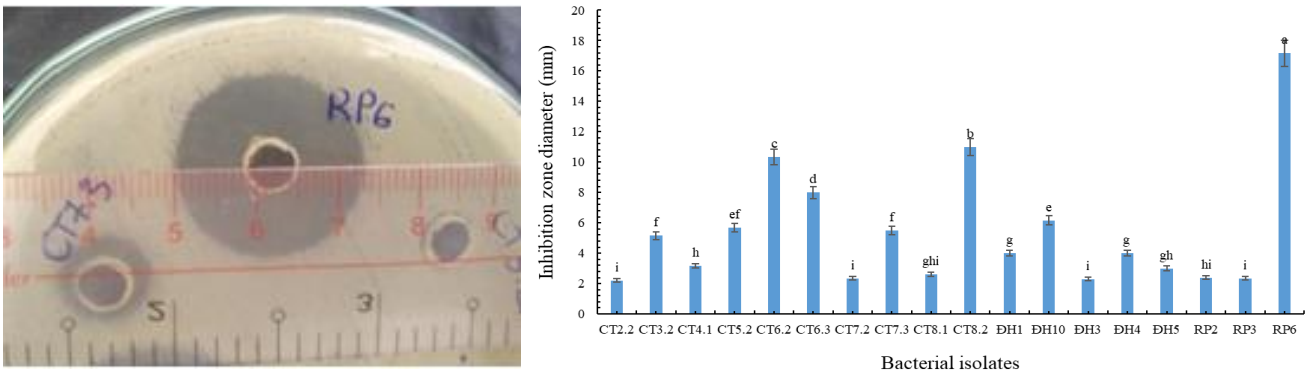

Figure 4. Antagonistic activity of the isolated lactic acid bacterial isolates against *Aeromonas hydrophila*

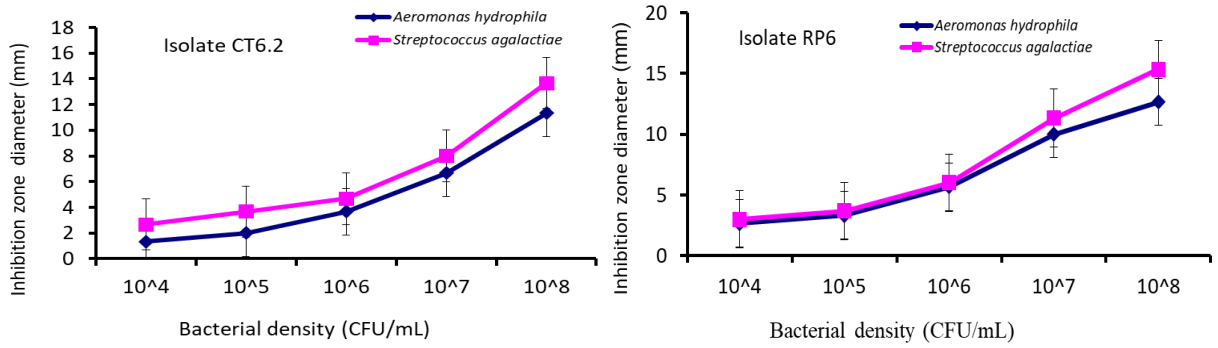

Figure 5. Effect of lactic acid bacterial densities on prohibitory activity against *Streptococcus agalactiae* and *Aeromonas hydrophila*

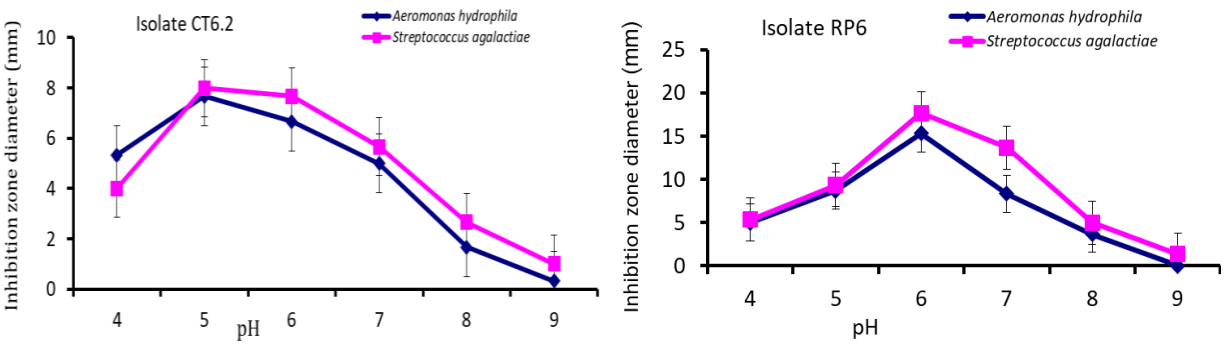

Figure 6. Effect of pH on lactic acid bacterial inhibitory activity against *Streptococcus agalactiae* and *Aeromonas hydrophila*

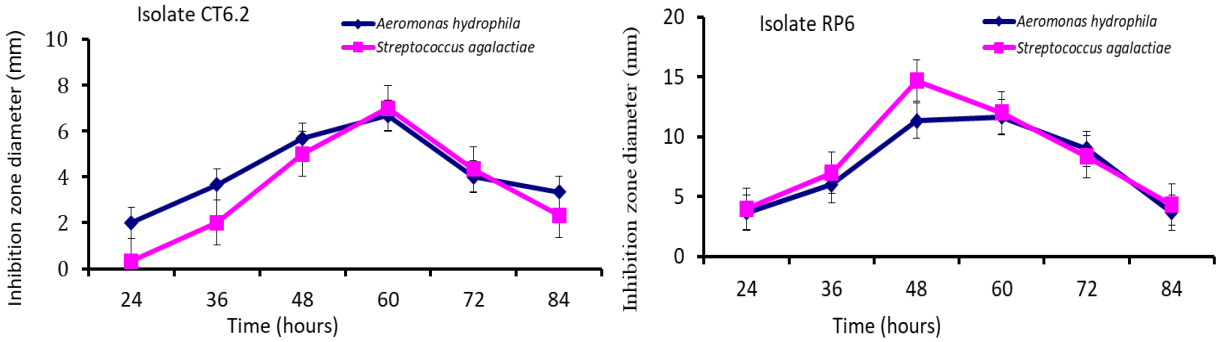

Figure 7. Effect of incubation time on prohibitory activity against *Streptococcus agalactiae* and *Aeromonas hydrophila*

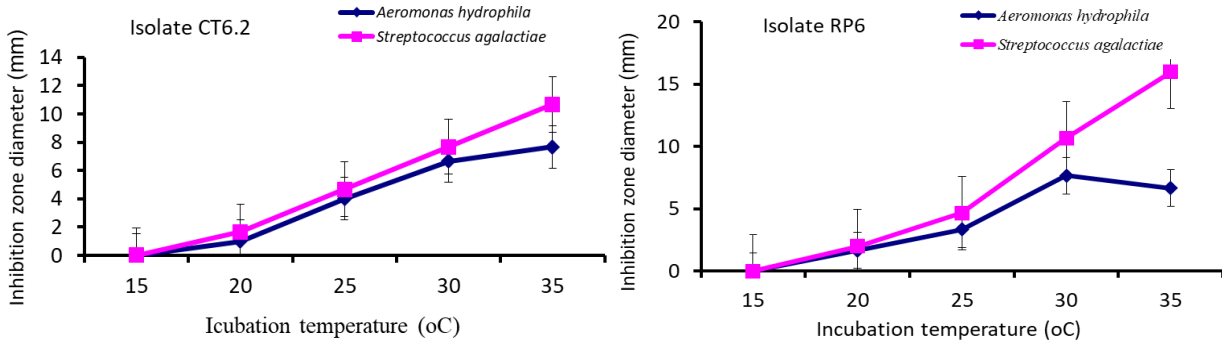

Figure 8. Effect of incubation temperature on prohibitory activity against *Streptococcus agalactiae* and *Aeromonas hydrophila*

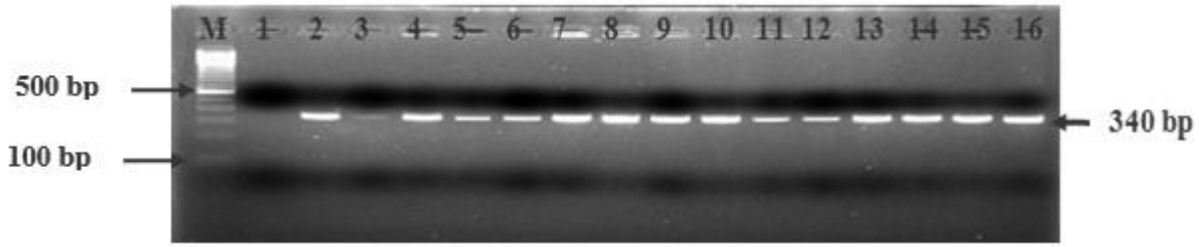

Figure 9. Electrophoresis results of PCR products to detect lactic acid bacterial isolates. M: 100 bp (Fermentas), Lane 1: Negative control, Lane 2: Positive control (*L. plantarum*, strain RP11-1), Lane 3-16: isolates CT4.1, CT6.2, CT7.2, CT6.3, DH1, DH4, DH10, RP6, CT3.2, CT5.2, CT7.3, CT8.1, RP2, and isolate RP4, respectively

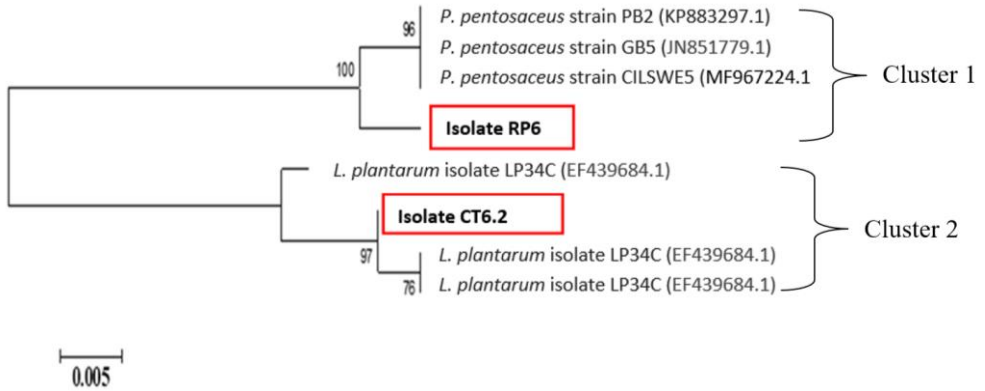

Figure 10. Phylogenetic tree of three lactic acid bacterial isolates based on partial 16S rRNA gene sequences (bootstrap values are given at branching points)

In this study, 34/37 (91.89%) isolates were long rod-shaped cells showed that these bacterial isolates belong to *Lactobacillus* (Musikasang et al. 2012). In addition, the study also identified species levels by using PCR (Figure 9) and gene sequencing. These findings indicated that the isolates of CT6.2 (Table 2) had inhibitory effects on *A. hydrophila* and *S. agalactiae* were *L. plantarum.* According to Walter et al. (2001), in addition to the genus *Lactobacillus*, the primer pairs Lac1 and Lac2 also detected other strains of LAB such as *Pediococcus*, *Weisella*, and *Leuconostoc*. Meanwhile, 3/37 (8.11%) isolates, for instance, have a spherical shape under the microscope. Therefore, this isolate can be classified as one of the genera *Pediococcus*, *Streptococcus*, *Leuconostoc*, *Lactococcus*, *Weissella*, *Oenococcus*, *Vagococcus*, and *Tetragenococcus*. However, the result of gene sequencing showed that strain RP6 was 99.02% homologous with *P. pentosaceus.* The phylogenetic tree in Figure 10 also shows that strain RP6 and other strains of *P. pentosaceus* in the NCBI database form a separate cluster. Thereby, it can be concluded that the strain RP6 isolated in the study is *P. pentosaceus*. Similar to *L. plantarum*, *P. pentosaceus* is a homofermentative LAB, which is a Gram-positive, non-motile, and non-spore-forming bacterium that gives a negative catalase reaction (Tanasupawat et al. 1993). Cells have a twinning spherical shape and are highly acidic because the end product of metabolism is lactic acid (Cai et al. 1999). In addition, *P. pentosaceus* has the ability to produce bacteriocins that inhibit some Gram-positive and Gram-negative bacteria (Osmanagaoglu et al. 2011). To date, most studies have shown that bacterial species of the genus *Pediococcus* are abundantly present in dairy products, fermented foods, and beverages (Leroy and De Vuyst 2004; Ho et al. 2009). In the future, in addition to its antimicrobial abilities, *P. pentosaceus* might be a worthwhile LAB strain for both the food industry and biological applications due to its anti-inflammation, anti-cancer, antioxidant, detoxification, and lipid-lowering abilities (Jiang et al. 2021).

Many previous studies have reported about the existence of LAB in the guts of aquatic animals, including fish (Hanol et al. 2020), shrimp (Thompson et al. 2022), and invertebrates, for instance, abalone (Amin et al. 2020). Most research demonstrated that the composition of LAB bacterial genera and species in the gut of fish changed with their host species (Iorizzo et al. 2021; Sequeiros et al. 2022). Lara-Flores et al. (2013) illustrated that five LAB species, consisting of *Enterococcus faecium*, *E. durans*, *Leuconostoc* sp., and *Streptococcus* sp. type I, and *Streptococcus* sp. type II, were recovered from the digestive tract of Nile tilapia (*O. niloticus*). Another study by Alonso et al. (2018) identified LAB strains, consisting of *Lactococcus lactis* subsp. *lactis*, *Enterococcus* spp., *L. plantarum*, and *Leuconostoc mesenteroides* subsp. *mesenteroides*, from the gut microbiota of marine fish. Amin et al. (2023) discovered and identified anti-Yersinia activity in LAB derived from aquatic animals' Gastrointestinal Tracts (GIT). By this investigation, the three bacterial strains were identified as *Enterococci*, related to *Enterococcus* sp. (99%), *Enterococcus thailandicus* (99%), and *Enterococcus durans* (99%), according to the analysis of the 16S rRNA gene sequences. However, most of the research showed that *Lactobacillus* is the most commonly present bacterial genus of the LAB in the gastrointestinal tract of aquatic animals (Talpur et al. 2012). Seema (2000) isolated *Lactobacillus* strains from frozen fish, crustaceans, and fish that were inhibitory to indicator bacteria such as *Vibrio cholerae, Bacillus cereus*, and *E. coli*. Iman et al. (2013) evaluated the impact of the probiotic *L. plantarum* on the immunity of tilapia by feeding the fish with *L. plantarum* (10^6 CFU/g) feed, followed by 30 days of exposure to the pathogen *Pseudomonas fluorescens.* The results showed that the growth and inhibition of pathogens in fish-fed diets containing *L. plantarum* were significantly higher than those of the control group (the weight of fish fed-diets containing *L. plantarum* increased by 69.6% after the experimental period, while the control only increased by 39.6%, increasing the survival rate of those exposed to the pathogens).

Antibacterial activity is the most important criterion by which LAB isolates are selected for probiotics. In general, the antimicrobial activity of different LABs varied against test pathogens. In this study, the results showed that 18/37 isolates (48.65%) were antagonistic to *A. hydrophila*, and 13/37 isolates (35.14%) were inhibitory to *S. agalactiae* (Figures 3 and 4). Especially, this finding revealed that twelve LAB isolates were inhibitory to both *A. hydrophila* and *S. agalactiae*. Nanasombat et al. (2012) demonstrated that fifty-two of 222 LAB isolates (24.3%) from raw seafood and Thai fermented seafood products were able to inhibit the growth of at least one of the eight indicator strains. Vanniyasingam et al. (2019) reported that three isolates (C1, Y1, and M6), isolated from cheddar cheese, yogurt, and cow milk, showed wide-spectrum antimicrobial activity against the maximum number of pathogenic organisms, such as *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella* sp., and *Staphylococcus aureus.* According to Pereira et al. (2022), LAB bacterial strains originating from aquatic environments were capable of inhibiting the growth of food and fish pathogens, such as *Listeria monocytogenes*, *Salmonella* Choleraesuis and *Salmonella* Typhimurium. Another recent study by Mazlumi et al. (2022) revealed that LAB isolates F15 and F12 were able to inhibit the growth of all eight indicator bacteria, including *V. harveyi*, *V. cholera*, *S. iniae*, *V. alginolyticus*, *V. fluvialis*, *V. parahaemolyticus*, *S. agalactiae*, and *Clostridium cochlearium*. Meanwhile, isolates F11, F14, and F18 were able to inhibit the growth of two Gram-positive indicators named *S. iniae* and *S. agalactiae*. Antimicrobial effects of LAB due to their antibacterial substances such as reuterin, reutericyctin, 2-pyrrolidone-5-carboxylic acid, diacetyl, CO_2, H_2O_2, lactic acid, and bacteriocins (İspirli et al. 2015). However, bacteriocins are considered one of the main antibacterial compounds of LAB besides the substances described above (Ekhay et al. 2013; Elyas et al. 2015).

Many reports have shown that the antibacterial activity of LAB strains likely influenced by culture elements consisting of temperature, pH, density, and incubation time

(Kumar and Arumugam 2012). The results of the study showed that density (Figure 5), pH values (Figure 6), incubation temperature (Figure 7), and incubation time (Figure 8) affected the inhibitory ability of the two isolates CT6.2 and PR6 against *S. agalactiae* and *A. hydrophila*. These results are consistent with the reports of many previous authors (Singh et al. 2014). Balasubramanyam and Varadaraj (1998) showed that in milk medium, *L. delbruecki* ssp. *bulgaricus* CFR 2028 had the highest antibacterial activity at 48 h and an incubation temperature of 37°C. In addition, the research results also showed that the antibacterial activity of three isolates was stable at low pH of 3.8-5.0 when incubated at 75°C for 30 min. Similarly, the study by Assefa et al. (2008) showed that LAB strains isolated from Ergo (fermented milk) in Ethiopia had inhibitory activity at 30°C, 60°C and 80°C for 30 min incubation period but no inhibitory activity at 121°C during incubation time of 15 min. In addition, bacterial isolates had stable inhibitory activity at pH ranging from 2 to 10. However, at pH = 12, the antibacterial activity decreased significantly ($P > 0.05$). The other investigation by Ogunbanwo et al. (2003) revealed that *Lactobacillus brevis* OG1 had the highest inhibitory activity at pH = 5.5 at temperature and incubation time of 30-37°C and 48 h. Abbasiliasi et al. (2011) reported that antibacterial substances produced by *L. paracasei* LA07 were highest at optimum temperature and pH of 30°C and 8.5. Meanwhile, the report of Malheiros et al. (2015) showed that *L. sakei* subsp. *sakei* 2a isolated from beef sausage in Brazil produced the highest bacteriocin at pH = 5.0 or 5.5 with an incubation temperature of 25°C or 30°C. The findings of the research by Zalán et al. (2005) showed that the concentration of H_2O_2 produced during the cultivation of five strains of *L. plantarum* 2142, *L. curvatus* 2770, *L. curvatus* 2775, *L. casei* subsp. *pseudoplantarum* 2750 and *L. casei* Shirota in MRS medium increased linearly with time and reached maximum concentrations at 72 hours. In addition, the study of Çadirci and Çitak (2005) when examining the antibacterial ability of three strains of *L. casei*, *L. plantarum* and *L. helveticus* against 10 bacterial strains as indicator *P. aeruginosa*, *E. coli* ATCC 25927, *Enterococcus faecalis*, *Bacillus subtilis*, *Enterobacter cloaceae*, *E. coli* O157:H7 DraftBS150-16654, *Staphylococcus aureus*, *E. coli* ATCC 25922 and *Proteus mirabilis* by drip method at different culture times (24, 48 and 72 h) and with different densities (10^3 and 10^6 cells/mL) showed that the diameter of sterile ring produced by the treatment at 48 h and 72 h was higher than at 24 h. However, the zone of inhibition diameter produced at the density of 10^3 cells/mL was not statistically different from the density of 10^6 cells/mL.

In conclusion, the LAB were isolated from the intestine of catfish, red tilapia, and tilapia were inhibitory against *A. hydrophila* and *S. agalactiae*. The isolates CT6.2 and RP6 exhibit the highest antagonistic activity against *S. agalactiae* and *A. hydrophila* at a density of 10^8 CFU/mL after incubation at 35°C with a pH of 5-6 for 48-60 h. By combining 16S rRNA gene sequencing, PCR, and morphological and biochemical traits, the LAB isolates in the findings were classified into the genera *Lactobacillus* and *Pediococcus*. The results show that two LAB isolates, CT6.2 and RP6, are potential candidates for probiotics in the prevention and treatment of popeye and hemorrhagic skin disease in red tilapia. In the future, it will be necessary to continue to study the effectiveness of LAB in controlling *S. agalactiae* and *A. hydrophila* in ponds in order to reduce the use of antibiotics in aquaculture.

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