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In-vitro Inhibitory Activities of Potential Probiotic Isolated from *Pangasius nasutus* against *Aeromonas hydrophila* and *Streptococcus agalactiae*

(Aktiviti Perencatan *In-vitro* Probiotik Berpotensi Diasingkan daripada *Pangasius nasutus* terhadap *Aeromonas hydrophila* dan *Streptococcus agalactiae*)

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ABSTRACT

In aquaculture, using probiotics is crucial for strengthening the immune system and encouraging the growth and survival of many aquatic organisms, including the *Pangasius* species. This approach is particularly significant given the impact of bacterial diseases on *Pangasius* survival. This study aimed to assess the effectiveness of probiotics isolated from *Pangasius nasutus* as alternatives to antibiotics for combating infections caused by *Aeromonas hydrophila* and *Streptococcus agalactiae.* Potential bacteria were isolated from the intestine and stomach of healthy *P. nasutus.* Seventy probiotic strains were successfully isolated and further screened using *A. hydrophila* and *S. agalactiae* as pathogens in an *in vitro* disc diffusion assay. Preliminary screenings indicated that five probiotic strains inhibited the growth of *A. hydrophila.* Stomach-derived strain S1 and intestine-derived strain L1 suppressed *A. hydrophila* growth with inhibition zones of 10.5±1 mm and 8.5±1 mm, respectively. Likewise, strains L2, L8, and L12 from the intestine showed inhibitory zones of 6.0±1 mm, 6.5±1 mm, and 6.0±1 mm, respectively. Of these, only L12 inhibited the growth of *S. agalactiae* with a clear zone of 5.0±1 mm. In the elimination of pathogenic strains, potential strains S1 and L1 did not grow on the *Aeromonas* isolation medium. Co-culture assays demonstrated that both potential strains significantly inhibited *Aeromonas hydrophila* growth at concentrations of 10^6 and 10^8 CFU mL^{-1} over 48- and 96-hour periods, respectively. The potential bacterial strains were identified using 16s rRNA gene sequencing and were classified as follows: S1 - *Lactococcus lactis,* L1 - *Weissella confusa,* L2 - *Cosenzaea myxofaciens,* L8 - *Lactococcus garvieae,* and L12 - *Plesiomonas shigelloides.* Strain S1 *L. lactis* and strain L1 *W. confusa* are suggested for further evaluation and acquired additional research to fully elucidate their mechanisms and potential as probiotics.

Keywords: Antagonistic activity; *in vitro* screening; *Pangasius* species; pathogens; potential probiotics

ABSTRAK

Dalam akuakultur, penggunaan probiotik adalah penting untuk menguatkan sistem keimunan dan menggalakkan pertumbuhan dan kemandirian hidup pelbagai organisma akuatik, termasuk spesies *Pangasius*. Pendekatan ini amat penting memandangkan kesan buruk penyakit bakteria terhadap kelangsungan hidup spesies *Pangasius*. Kajian ini bertujuan untuk menilai keberkesanan probiotik yang dipencilkan daripada *Pangasius nasutus* sebagai alternatif kepada antibiotik untuk melawan jangkitan penyakit yang disebabkan oleh *Aeromonas hydrophila* dan *Streptococcus agalactiae*. Bakteria berpotensi sebagai probiotik telah diasingkan daripada organ usus dan perut *P. nasutus* yang

sihat. Tujuh puluh *strain* probiotik berjaya diasingkan dan seterusnya disaring dengan menggunakan *A. hydrophila* dan *S. agalactiae* sebagai patogen dalam ujian penyebaran cakera *in vitro*. Pemeriksaan awal menunjukkan bahawa lima *strain* probiotik dapat merencat pertumbuhan *A. hydrophila*. *Strain* S1 yang dipencilkan daripada perut dan strain L1 daripada usus dapat merencat pertumbuhan *A. hydrophila* dengan zon perencatan masing-masing sebanyak 10.5±1 mm dan 8.5±1 mm. Begitu juga, *strain* L2, L8 dan L12 daripada usus menunjukkan zon perencatan ke atas *A. hydrophila* masing-masing sebanyak 6.0±1 mm, 6.5±1 mm dan 6.0±1 mm. Manakala, hanya L12 merencat pertumbuhan *S. agalactiae* dengan zon yang jelas 5.0±1 mm. Dalam pengasingan *strain* patogen, S1 dan L1 tidak hidup di atas medium pengasingan *Aeromonas*. Ujian kultur bersama menunjukkan bahawa kedua-dua SI dan LI dengan ketara merencat pertumbuhan *A. hydrophila* pada kepekatan 10^6 dan 10^8 CFU mL^{-1} dalam tempoh 48 dan 96 jam. Semua *strain* bakteria dikenal pasti menggunakan penjujukan gen 16s rRNA dan dikelaskan seperti berikut: S1 - *Lactococcus lactis*, L1 - *Weissella confusa*, L2 - *Cosenzaea myxofaciens*, L8 - *Lactococcus garvieae* dan L12 - *Plesiomonas shigelloides*. *Strain* SI *L. lactis* dan L1 *W. confusa* dicadangkan untuk penilaian lanjut dan memerlukan penyelidikan tambahan untuk menjelaskan sepenuhnya mekanisme dan potensi sebagai probiotik.

Kata kunci: Aktiviti antagonis; kajian *in vitro*; patogen; probiotik berpotensi; spesies *Pangasius*

Introduction

Pangasiidae is a family of catfish species recognized physically by having 5 to 6 pelvic fin rays, a laterally compressed body, two pairs of barbels which were maxillary and mandibular barbels, a long anal fin, a short dorsal fin, and a small adipose fin with a free posterior margin (Fitri & Christianus 2019). *Pangasius* species is generally a hardy and fast-growing fish that has been commercially cultured in various Asian countries (China, Malaysia, Thailand, Vietnam, Indonesia, Bangladesh, India, Philippines, and Myanmar) (FAO 2022; Soni et al. 2018). Besides, the *Pangasius* species is also regarded as one of the world's largest and most important freshwater fish, with Malaysia producing more than 17 million tons in 2019 (DOF 2019). *Pangasius nasutus* (Bleeker, 1863) has high market demand for its sweet and firm white flesh, and its market price is three times higher (RM 70–300/kg) than other commercial *Pangasius* species, such as *Pangasius hypophthalmus* (Yusof & Nakajima 2019).

Pangasius nasutus is one of the popular finfish in the Malaysian aquaculture industry due to its high profit. However, the major constraint in the farming of *P. nasutus* is disease outbreaks. *Pangasius* species are susceptible to diseases such as motile *Aeromonas* septicemia (MAS) (Mamun et al. 2022), bacillary necrosis of Pangasius (BNP) (Parven et al. 2020), channel catfish virus disease (CCVD) (Siti-Zahrah et al. 2013), and some parasitic infestations (endo- and ectoparasites) (Sharma et al. 2020). *Aeromonas* species were major pathogens causing red mouth, swollen abdomen, and hemorrhages on the anus of *Pangasius* species (Nahar et al. 2016). Cultured fish become vulnerable to diseases due to high stocking density under stressful conditions that weaken their immunity (Vargas-Chacoff et al. 2014). The mortality in farmed *P. nasutus* is commonly caused by *Aeromonas* spp. (Chuah et al. 2016) and *Streptococcus* spp. (Dong et al. 2015). The combination of bacterial and viral infections had previously caused a 30% mortality of cultured *P. nasutus* in Sungai Pahang (Mahmud et al. 2019).

Probiotics have become a great alternative to antibiotics in controlling bacterial diseases in aquaculture. Probiotics are live microorganisms that provide health benefits to the host when administered in adequate amounts (Fuller & Fuller 1992; Meidong et al. 2021). The application of probiotics in fish disease management continues to increase in an effort to tackle the looming health problems while restricting the extensive use of chemotherapeutics (Banerjee & Ray 2017). Trung and Dung (2023) reported that the misused of antibiotics led to antimicrobial resistance that presents health risks to humans and animals. Therefore, the present study aimed to isolate, screen, and evaluate potential bacteria isolated from *P. nasutus* as probiotics that able to reduce the risk of *A. hydrophila* and *S. agalactiae* infections.

Materials and Methods

Isolation and Purification of Bacterial Strains

Six healthy *P. nasutus,* length 4 inches were obtained from the Three Ocean Fish Pond & Trading Sdn. Bhd.,

Rawang, Selangor. The fish were dissected to obtain their internal organs including the stomach, intestine, and liver. Each organ was mashed using a sterile mortar and homogenized by using vortex mixer (Benchmixer, Benchmark Scientific, USA). The homogenized samples were then serially diluted (10^1 to 10^8) and plated (0.1 mL) on the tryptic soy agar (TSA, Millipore, Germany). The plates were then incubated at 30 °C for 24 h. After incubation, a single colony was isolated into a new plate for purification to obtain a pure culture and stored at 4 °C until use (Fulbright, Chisholm & Reardon 2016).

ELIMINATION OF PATHOGENIC STRAINS

The elimination of pathogenic strains was performed on the *Aeromonas* isolation medium (Shotts Jr. & Rimler 1973). All isolated bacterial strains were streaked on the *Aeromonas* isolation medium (Himedia, Mumbai). The plates were then incubated overnight at 30 °C. Any bacteria that grow on the medium were eliminated and not used for further evaluation.

IN VITRO SCREENING FOR THE ANTAGONISTIC POTENTIAL OF PROBIOTICS

DISC DIFFUSION ASSAY

Twelve isolates were screened for their antagonistic activity against *A. hydrophila* and *S. agalactiae* via the disc diffusion assay. All potential probiotics were grown overnight in Tryptic Soy Broth (TSB, Millipore, Germany). Overnight cultures were then swabbed onto TSA plates using sterile cotton swabs at the adjusted concentrations of 10^5, 10^6, and 10^7 CFU mL^{-1}. A sterile filter paper (4 mm diameter) was dipped into potential probiotics. All plates were incubated overnight for 16 h at 30 °C. The presence of any inhibition zones was finally observed and measured (Grossart et al. 2004).

CO-CULTURE ASSAY

The potential probiotics that showed inhibition zones in the *in vitro* assay were cultured overnight in the TSB with shaking at 30 °C. The concentration of the potential probiotic strains was adjusted to 10^4, 10^6, and 10^8 CFU mL^{-1}, whereas the concentration of *A. hydrophila* was adjusted to 10^6 CFU mL^{-1}. Both the bacterial strain and pathogen were added into a 15 mL tube and agitated overnight on an incubator shaker (Innova 42, News Brunswick Scientific) at 30 °C. The treatment without the probiotic was used as the control. After that, 0.1 mL of the mixture was taken (during 6-, 12-, 24-, 48-, and 96-h) and serially diluted (for ease in colony counting) before plating on the *Aeromonas* isolation medium (Himedia, Mumbai) and incubated for 16 h at 30 °C. After 16 to 24 h, the colony forming unit per mL (CFU mL^{-1}) was calculated using the formula herewith:

$$\text{CFU mL}^{-1} = \frac{(\text{No.of colonies X dilution factor})}{\text{Volume of culture plate}}$$

The co-culture testing for *S. agalactiae* was not conducted because the strain inhibited the growth of *S. agalactiae* in the disc diffusion method was identified as a pathogenic strain.

IDENTIFICATION OF POTENTIAL PROBIOTICS

GRAM STAINING

The Gram staining method was performed to differentiate potential bacteria (either Gram-positive or Gram-negative). Briefly, the bacteria were smeared thinly and evenly on a clean glass slide, air-dried and heat-fixing properly. Firstly, the sample was stained with 1% crystal violet solution for one minute and then rinsed briefly with distilled water for 30 s. Secondly, the glass slide was fixed with Lugol's iodine and idled for one minute before being rinsed again. After that, the sample was decolorized with 95% ethanol for 30 s and rinsed again with distilled water for 30 s. Safranin was then used to counterstain the bacteria for one minute before rinsing again with distilled water. The glass slide was air-dried for 30 to 60 s and mounted with a coverslip. Lastly, the glass slide with immersion oil were examined under the optical microscope (Primo Star, Zeiss, Germany) with 100x magnification for bacterial classification.

MOLECULAR IDENTIFICATION USING 16 RRNA GENE ANALYSIS

The molecular identification was done following Alonso et al. (2012). The total genomic DNA of potential bacteria was isolated using a Genomic DNA Mini Kit (Genaid, Taiwan). The universal primers used to amplify the 16S rRNA gene sequences for each DNA template consist of 8F (5' AGA GTT TGA TCC TGG CTC AG 3') and 1482R (5' ACG GCT CCT TGT TAC GAC TT 3') (Barman et al. 2011). The 16S rRNA amplification of DNA was performed using a gradient thermal cycler (Eppendorf, Germany). The amplification was performed by initial denaturation at 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing of the primers at 55 °C for 15 s, and extension at 72 °C for 90

s. The detection and separation of DNA fragments were performed using gel electrophoresis, where 12 µL of the PCR amplicons was run on a 1% agarose gel matrix with the addition of the RedSafe Nucleic Acid Staining Solution (20,000x) (Labotaq, Spain). A 1 kb DNA ladder was used as the molecular marker (GeneDireX, USA). The gel was then viewed under a UV transilluminator for gel documentation. The purification of PCR products and DNA sequencing were done by 1st BASE Laboratories Sdn. Bhd., Malaysia. The determine sequences were aligned and compared using the NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast/).

STATISTICAL ANALYSIS

Data were presented as mean ± standard error of the mean (SEM). All data obtained were analysed using one-way analysis of variance (ANOVA). Tukey's test was applied for a pairwise comparison of the means. All differences were considered statistically significant at $p<0.05$. Statistical analyses were performed using the IBM SPSS Statistic 20 software.

RESULTS

BACTERIAL ISOLATION AND PURIFICATION

A total of 70 isolates were isolated from the internal organs of *P. nasutus* (stomach and intestine). Twenty-one strains were isolated from the stomach while 49 strains were from the intestine (Table 1).

ELIMINATION OF PATHOGENIC STRAINS

The elimination test distinguished the pathogenic from non-pathogenic strains. During the process, 12 potential strains did not grow on the *Aeromonas* isolation medium and were considered safe to be used in the *in vitro* test.

DISC DIFFUSION ASSAY

Five strains of isolated probiotics showed the ability to inhibit the growth of *A. hydrophila* (Table 2 & Figures 1 & 2). The largest inhibition zone against *A. hydrophila* was observed for isolate S1 (10.5 ± 1.0 mm), while isolates L2 and L12 produced the smallest inhibition zone of 6.0 ± 1.0 mm. Meanwhile, isolate L12 produced an inhibition zone of 5.0 ± 1.0 mm against *S. agalactiae*.

TABLE 1. Bacterial isolates from the internal organs of *Pangasius nasutus*

Organ	Number of different isolates
Stomach	21
Intestine	49

TABLE 2. Diameter of clear zone of probiotics against *Aeromonas hydrophila* and *Streptococcus agalactiae* in the disc diffusion assay

Isolate	Size of the inhibition zone (mm)	
	Aeromonas hydrophila	*Streptococcus agalactiae*
S1	10.5 ± 1	-
L1	8.5 ± 1	-
L2	6.0 ± 1	-
L8	6.5 ± 1	-
L12	6.0 ± 1	5.0 ± 1

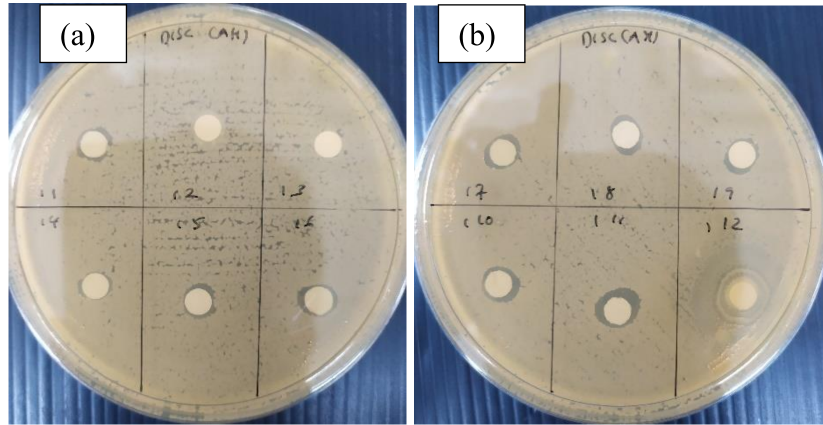

FIGURE 1. (a) and (b). Inhibition zones of probiotics against *Aeromonas hydrophila* in the disc diffusion assay

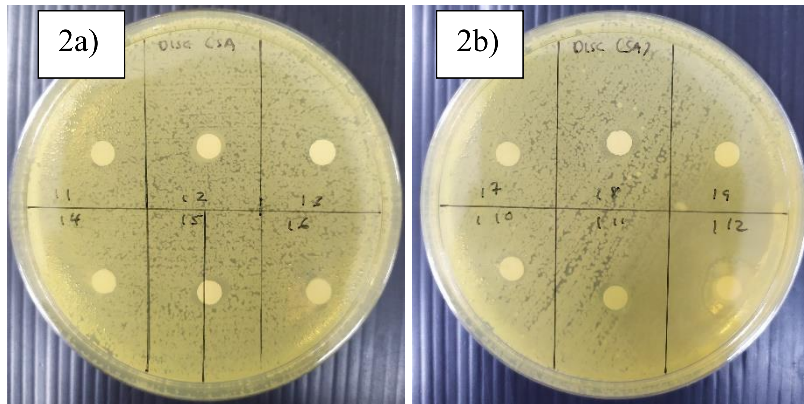

FIGURE 2. (a) and (b) Inhibition zones of probiotics against *Streptococcus agalactiae* in the disc diffusion assay

CO-CULTURE ASSAY

The isolated strain S1, which had the highest inhibitory activity against *A. hydrophila* in the previous screening assay was tested in a co-culture assay to identify its optimum concentration that could inhibit the growth of the pathogen. The growth of *A. hydrophila* was significantly inhibited (p<0.05) by the strain S1 at the tested concentrations of 10^6 and 10^8 CFU mL^{-1} during 24-, 48-, and 96-h of incubation (Figure 5). Meanwhile, the growth of *A. hydrophila* increased during 24- and 96-h when incubated with S1 at 10^4 CFU mL^{-1}. Furthermore, results were not significantly different (p>0.05) from the control treatment during those observation hours. Concentrations at 10^6 and 10^8 CFU mL^{-1} showed the greatest inhibitions on the growth of *A. hydrophila*.

Moreover, strain L1, also inhibited the growth of *A. hydrophila* (Figure 6). Strain L1 significantly reduced (p<0.05) the growth of *A. hydrophila* at the concentration of 10^6 CFU mL^{-1} during 48-h. On the contrary, a significant inhibition (p<0.05) was observed for the concentration of 10^8 during 96-h. Concentrations of 10^6 and 10^8 showed the greatest inhibition against the pathogen during 48- and 96-h, respectively.

IDENTIFICATION OF POTENTIAL PROBIOTICS

GRAM STAINING

Results showed that three bacterial strains were gram-positive (Figure 3(a)-3(c)) while two strains were gram-negative (Figure 3(d)-3(e)).

MOLECULAR IDENTIFICATION USING 16 RRNA GENE ANALYSIS

In brief, PCR products with an approximate size of 1500 bp were successfully amplified (Figure 4).

Table 3 shows the potential probiotics isolated from the stomach and intestine of *P. nasutus*. The potential bacteria strain S1 isolated from stomach was identified as *Lactococcus lactis*. Meanwhile, strains L1, L2, l8 and L12 isolated from intestine were identified as *Weisella confusa, Cosenzae myxofaciencs, Lactococcus garvieae,* and *Plesiomonas shigelliodes,* respectively.

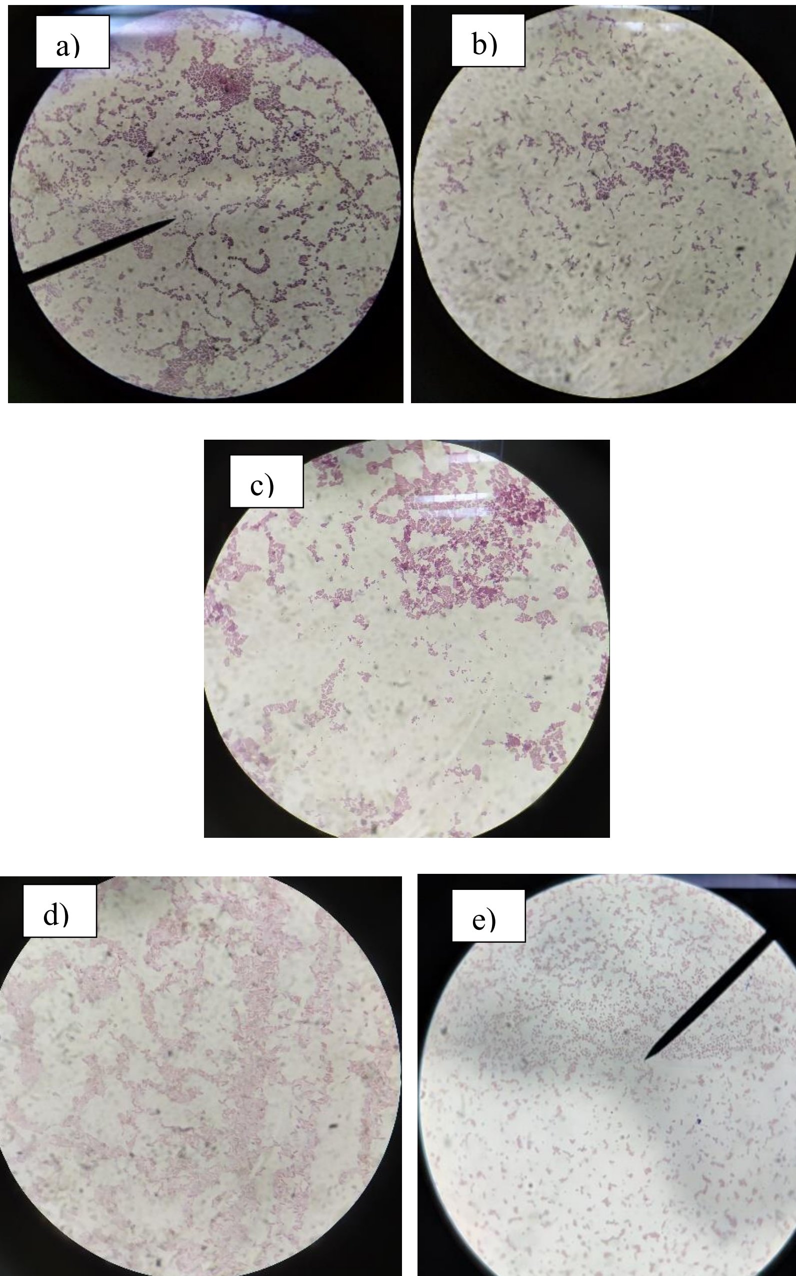

FIGURE 3. Gram staining for (a) S1 – positive, coccus, (b) L1 – positive, coccus, (c) L8 – positive, coccus, (d) L2 – negative, bacillus, (e) L12 – negative, bacillus

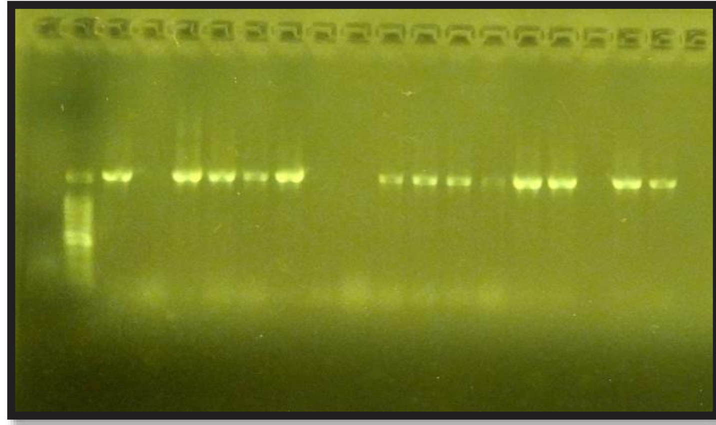

FIGURE 4. Agarose gel electroporesis of bacterial DNA fragments (1) 100 bp ladder, (2) Isolates S1, (3) Isolates L1, (4) Isolates L2, (5) Isolates L8, (6) Isolates L12, (7) Control

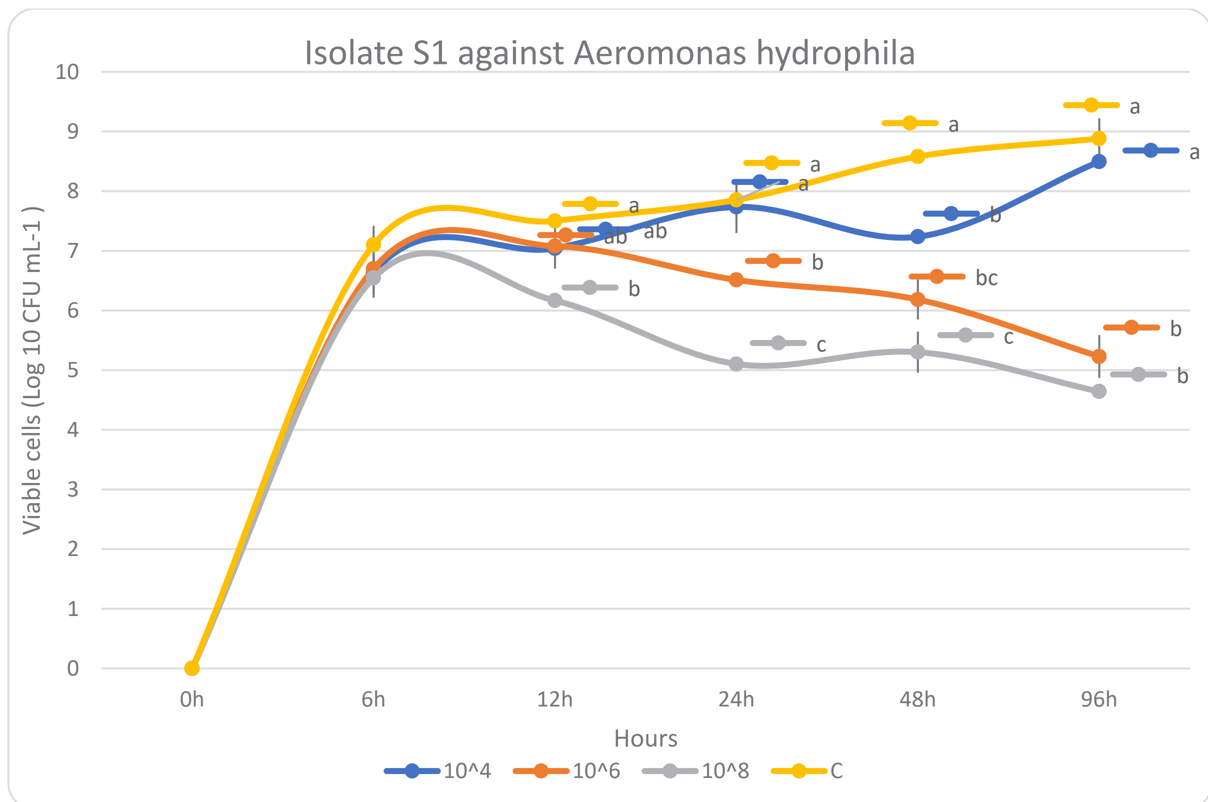

FIGURE 5. The growth pattern of *Aeromonas hydrophila* incubated with different concentrations of the isolate S1 (at 10^4, 10^6, and 10^8 CFU mL^{-1}) and C as control group. Error bars indicate standard error of the mean (SEM). Different alphabets indicate significant differences among treatments (p<0.05)

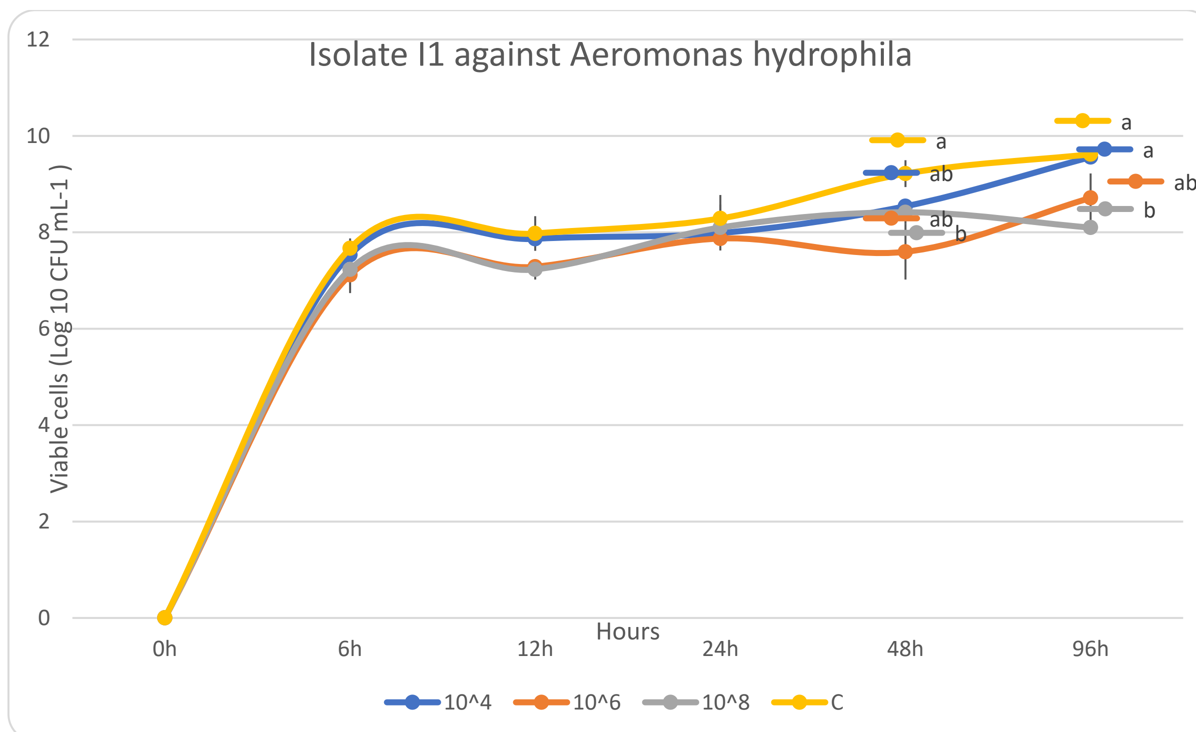

FIGURE 6. The growth pattern of *Aeromonas hydrophila* incubated with different concentrations of the isolate L1 (at 10^4, 10^6, and 10^8 CFU mL^{-1}) and C as control group. Error bars indicate standard error of the mean (SEM). Different alphabets indicate significant differences among treatments (p<0.05)

TABLE 3. Identification of potential probiotics isolated from the internal organs of *Pangasius nasutus*

Isolates	Descriptions	Query Cover (%)	Percent Identity (%)	Accession No.
S1	*Lactococcus lactis* strain NBRC 100933	99	99	NR_113960.1
L1	*Weissella confusa* strain JCM 1093	99	98.81	NR_040816.1
L2	*Cosenzaea myxofaciens* ATCC 19692	97	96.86	NR_043999.1
L8	*Lactococcus garvieae* strain JCM 10343	99	99.66	NR_113268.1
L12	*Plesiomonas shigelloides* strain DSM 8224	99	96.56	NR_117763.1

DISCUSSION

The sustainable development of the aquaculture industry is facing many challenges, such as disease outbreaks, poor disease resistance, and sluggish growth performance of farmed species under stressful culture conditions. Stressful environmental conditions lead to disease outbreaks in fish farming (due to poor water quality) (Mahmud et al. 2019). Infectious diseases are the biggest threat to the aquaculture industry, which causes significant economic losses globally (Assefa & Abunna 2018).

Besides, the combination of environmental factors, such as intensive culture practices and high microbial load, predisposes cultured animals to infections. Therefore, the application of probiotics in aquatic health management is the best approach against infectious diseases while restricting the usage of antimicrobials (Maqsood et al. 2011).

Probiotics are live microorganisms (bacteria and yeasts) that confer health benefits and support the presence of healthy bacteria in the host. These beneficial microorganisms are often found in dietary supplements as well as fermented foods and beverages (Martinez-Villaluenga, Peñas & Frias 2017). Probiotics provide health benefits to the host through stimulation of growth and the activity of the gut microbiota (Ayisi, Apraku & Afriyie 2017). Probiotics could promote the health of living hosts (through a healthy digestive tract and immune system) when administered adequately (La Fata, Weber & Mohajeri 2018). The application of probiotics in aquaculture has drawn considerable attention worldwide. Many aquatic microbes have been proven to enhance the disease resistance in fish and shellfish against numerous pathogens (Newaj-Fyzul & Austin 2015). Most importantly, the bacteria selected must not harm the host because of toxin secretion (Lim, Webster & Lee 2015). In addition, probiotic isolated must be able to prevent the colonization of the pathogenic bacteria in the distinct tract of the diverse microbial of the host (Lee et al. 2022). Many probiotics have been isolated, evaluated, and regarded as having high potential for use in aquaculture to prevent and control infectious diseases (Hai 2015).

The strains that demonstrated inhibitory activity against the pathogens tested were considered potential probiotics at a preliminary stage. Probiotic inclusion into the diet of *Pangasianodon hypophthalmus* exhibited significantly higher yield, growth performance, survival, and better feed usage with a profitable economic return (Chowdhury, Roy & Chowdhury 2020).

In this study, strains S1 and L8 were identified as *L. lactis* and *L. garvieae*, respectively. Both were lactic acid bacteria (LAB) that produce lactic acid as a metabolic end product of carbohydrate fermentation (Vinderola et al. 2019). Several strains of LAB have been reported to synthesize essential free amino acids, short-chain fatty acids (SCFA), and water-soluble vitamins, which are indispensable for animal growth (Masuda et al. 2012). The genus *Lactococcus* is known as non-pathogenic to humans and animals (Devirgiliis, Zinno & Perozzi 2013). However, *L. garvieae* is the only pathogenic species from the genus (Chapela et al. 2018). It is an aetiologic agent for Lactococcosis, an emerging disease that affects rainbow trout in Japan (Vendrell et al. 2006).

In contrast, *L. lactis* has been used as a probiotic in aquaculture. *L. lactis* was used as a probiotic to the enhance immune response in African catfish (El-Bouhy et al. 2021). Sun et al. (2012) reported that *L. lactis* (strain MM1) have increased the serum lysozyme activity, serum protease activity, and improved feed utilization in juvenile grouper (*Ephinephelus coioides*). The suggested supplemental dose of *L. lactis* was 1.0 × 10^8 CFU g^{-1} for 60 days. The probiotic re-isolated from the guts of juvenile grouper inhibited the growth of *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Vibrio harveyi,* and *Vibrio metschnikovi*. Additionally, *L. lactis* RQ516 which were isolated from fresh milk also increased the concentrations of serum protein and globulin, respiratory burst activity, serum lysozyme content and SOD activity in Tilapia (*Oreochromis niloticus*) against *A. hydrophila* when fed with 1 × 10^7 CFU mL^{-1} every 2 days for 40 days (Zhou et al. 2010). Apart from that, *L. lactis* LH8 isolated from marine fishes (*Mugil cephalus, Sebastes schlegelii, Hexagrammos otakii,* and *Cleisthenes herzensteini*) also increased the survival rate and enhanced the immune responses of the sea cucumber (*Apostichopus japonicus*). The recommended supplemental dose was 1.0 × 10^9 CFU g^{-1} for 30 days (Li et al. 2018).

The strain L1 identified as *W. confuse* has been previously used as a probiotic (Li et al. 2018). Furthermore, *W. confusa* isolated from marine animals (no specification given) showed an inhibitory effect against some fish pathogens (including *Vibrio splendius*, *Vibrio anguillarum*, *V. parahaemolyticus*, and *S. aureus*), but showed no effect on growth performance, serum alkaline phosphatase (AKP), serum lysozyme activity, and superoxide dismutase (SOD) activity in the sea cucumber (Wang et al. 2019). The three bacterial strains acquired were not evaluated because of their potential pathogenicity. Specifically, strain L8 (*Lactococcus garviae*) poses a risk as an etiological agent for Lactococcosis in fish (Meyburgh, Bragg & Boucher 2017). In addition, there is limited literature available on the strain L2 (*Cosenzae myxofaciens*), impeding a thorough evaluation for prospective research. Moreover, L12 (*Pleisomonas shigelloides)* is the causative agent of diarrhea in humans (Janda, Abbott & McIver 2016).

A co-culture assay exposes a pathogen directly to the probiotic by culturing it in a liquid medium (broth) (Knipe et al. 2021). A beneficial bacterium that exhibits a significant inhibitory effect against a pathogen has a high

potential to act as a probiotic. In the co-culture assay, each probiotic inhibited the growth of *A. hydrophila* at different concentrations and observation hours. The isolate S1 inhibited the growth of *A. hydrophila* at all tested concentrations (10^4, 10^6, and 10^8 CFU mL^{-1}) during 6- and 12-h and declined during 24-h. At the concentration of 10^8 CFU mL^{-1}, it inhibited the growth of the pathogenic bacteria at all observation hours. For the isolate L1, all tested concentrations were most effective during the 48-h. Jasmin et al. (2016) reported that increasing the amount of the probiotic at a specific time might improve the effectiveness of inhibiting pathogens. Current findings laid the foundation for developing potential probiotics that could benefit the local aquaculture industry.

Conclusions

In conclusion, two strains, *L. lactis* strain SI and *W. confuse* strain L1, are recommended for in-depth assessment due to their safety for both fish and human consumption. In contrast, other bacterial strains were not extensively tested. For instance, *L. garviae* could lead to Lactococcosis in fish, while *P. shigelloides* is associated with various infections in humans, such as diarrhea, septicemia, central nervous system diseases, and eye infections. Additionally, there is limited information on *C. myxofaciens* regarding its potential as a probiotic. Thus, deeper investigations are required to fully understand the effects and potentials of these two potential strains in aquaculture.

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