PROBIOTICS BACTERIA AS QUORUM SENSING DEGRADER CONTROL Aeromonas hydrophila PATHOGENICITY IN CULTURED RED HYBRID TILAPIA

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ABSTRACT

Quorum sensing (QS) is the interaction of bacteria cell-to-cell to regulate various bacterial functions, including bacterial virulence. It has been suggested that QS interruption is an anti-infective strategy to combat diseases in aquaculture. This research was conducted to isolate the potential probiotics bacteria as QS degrader from the fish gut and investigate its efficiency in reducing Aeromonas hydrophila pathogenicity by in vitro and in vivo assay. In this study, three isolates from the fish gut were able to degrade N-acyl homoserine lactone (AHL), one of the QS signals of A. hydrophila. Based on 16S rDNA sequence analysis, isolate CPI12 was identified as Klebsiella sp., CBa5 and CBa7 were identified as Enterobacter tabaci. These isolates were co-cultured with A. hydrophila for five days. Results revealed that all isolates could decrease the AHL production of A. hydrophila but did not affect the growth of the pathogen. In vivo assay results showed that isolate CPI12 reduced the pathogenicity of A. hydrophila against tilapia with a significantly higher survival rate (p<0.05) of fish in the CPI12-fed group than the control group. Meanwhile, isolate CBa7 was significant (p<0.05) for growth performance, including specific growth rate (SGR), weight gain (WG), feed conversion ratio (FCR), mean weight gain (MWG), and average daily growth (ADG) among whole treatment and control group. These results displayed that the probiotics bacteria as QS degrader isolated from the fish gut could control the pathogenicity of Aeromonas hydrophila. It has been proposed that QS degrader bacteria might be an alternative solution for disease control.

KEYWORDS: Growth performance; N-acyl homoserine lactone; pathogen; quorum sensing

INTRODUCTION

Tilapia aquaculture is the second most crucial group of cultured fish after carp (FAO, 2020) with 70.4% global tilapia products is contributed by Asian region (El-Sayed, 2020). Red hybrid tilapia (O. mossambicus × O. niloticus) has become the dominant species (>90%) in Malaysia due to customer preference and fast-growing features (Lim et al., 2016). Due to their rapid growth, an intensive tilapia culture system has developed (Siti-Zahrah et al., 2008; Low et al., 2015). However, this system often contributes to the occurrence of diseases because of high stocking densities and high organic matter content (Najiah et al., 2012; Torres et al., 2016). Though tilapias are more resistant to bacterial, parasitic, and viral infections than other cultivated fish, there are numerous reports of pathogenic infections in tilapias (Al-Harbi & Uddin, 2005).

Common pathogens that infect tilapia are Flavobacterium columnare, Streptococcus sp., Aeromonas hydrophila, Edwardsiella tarda, Gyrodactylus niloticus, Vibrio sp., Ichthyophthirius multifiliis, and Trichodina sp. (Al-Harbi & Uddin, 2005; Musa et al., 2009; Lim et al., 2016; Lee et al., 2016). A. hydrophila infection was recorded as significant mortalities in South Asia and South-East Asia fish farm (Laith & Najiah, 2013). A. hydrophila is an opportunistic pathogen that can enter the body through the fish gills and damage skin, which could induce macrophage apoptosis and destroy the intestinal mucus layer. Besides, due to the high-stress levels in intensive culture conditions, intestinal inflammation caused by A. hydrophila is emerging as a significant cause of high mortality in aquaculture that could lead to enormous economic losses (Song et al., 2014). Hence, traditional anti-bi-
otics and chemical drugs have been used to handle the outbreak of diseases in aquaculture (Torres et al., 2016). However, a lot of farmers use antibiotics in a prophylactic way, some on daily although there is no evident of the presence of pathogen (Defoirdt et al., 2011). The frequent usage of antibiotics along with the overuse of the chemical drugs, lead to chemical accumulation in the food chain, antibiotics resistant and multi-drug resistant in fish pathogens (Chu et al., 2014; Ramesh & Souissi, 2018). These antibiotic-resistant pathogenic bacteria cause the antibiotics to become ineffective, infections become more difficult to treat, and thus new antibiotics is needed to overcome the fish disease outbreak (Chu et al., 2014). Thus, in order to protect aquaculture animals from pathogenic bacteria, strategies on fish diseases management without the worry of emerging antibiotic-resistant bacteria are developed.

Quorum sensing (QS) can regulate the gene expression of different phenotypes that are vital for bacteria growth, survival, and pathogenicity (Chu et al., 2014). QS is a communication process of bacteria. It depends on the cell population density that comprises the action of detection, creation and release of small-signal molecules recognized as autoinducers (Torres et al., 2016). As a population of bacteria reaches a high cell density, the autoinducers accumulate and reach a certain threshold level that can affect the population gene expression by re-entering the cells that finally induces differential gene expression (Sakr et al., 2014). Autoinducers that are frequently utilized by Gram-negative pathogenic bacteria such as A. hydrophila is N-acyl homoserine lactones (AHLs) (Grandclément et al., 2016). Anti-virulence aims at targeting the virulence functions and behaviours rather than the viability of the pathogens. The disruption of the QS strategies belong to these anti-virulence approaches (Jiang et al., 2019).

For example, research by Novita et al. (2015) displayed that the probiotics bacterium, Bacillus sp., which inhibits the QS of A. hydrophila capable of increasing catfish growth and survival rate. Besides, it has been discovered that the weakening of QS in pathogenic Aeromonas spp. significantly decreased the mortality towards burbot and zebrafish (Zhang & Li, 2016; Torres et al., 2016).

Since QS is vital in virulence towards the aquatic host, degradation of QS could be an alternative solution for disease control (Noorashikin et al., 2016). Therefore, the objectives of this study were to isolate and identify the potential QS degrading probiotics bacteria from the fish gut and investigate its efficiency in controlling tilapia disease by breaking down the signal molecules (AHL) produced by the pathogenic bacteria, A. hydrophila via in vitro and in vivo assay.

**MATERIALS AND METHODS**

**Bacterial strains**

An opportunistic pathogen, Aeromonas hydrophila ATCC® 19570™ was purchased from American Type Culture Collection. Chromobacterium violaceum strain CV026 was gained from the Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, Selangor, Malaysia. It was used as a biosensor to induce the production of the purple pigment violacein in the existence of AHL molecules (Tinh et al., 2007).

**Culture conditions**

Aeromonas Isolation Medium Base, Luria Bertani (LB) agar and broth were used as growth media for bacteria. Aeromonas hydrophila was grown at 30°C on LB media with shaking at 100 rpm (Chu et al., 2010). A. hydrophila culture was kept as stock at -80°C in 100%glycerol.

CV026 was grown at 30°C on LB media supplemented 20 ppm kanamycin with shaking at 100 rpm for 48 hours. The addition of kanamycin was to protect the plasmid carrying the gene responsible for violacein production (Torabi Delshad et al., 2018) and prevent the mutation in CV026 strain (Noorashikin et al., 2016).

**Sample collection**

Three hundred specimen of juvenile red hybrid tilapia were obtained from local farmers at Kampung Ulu Sat, Kuala Berang, Terengganu, Malaysia. The fish abdomen were wiped with alcohol (70%ethanol) and dissected aseptically to remove the gut. The fish gut was transferred into a sterile Petri dish and carefully washed with sterilized distilled water. The fish guts were homogenized with sterilized 10 ml of 0.75%NaCl solution to one part by weight of the dissected tissue (10:1; v/w) in a 50 ml tube. Homogenates were used as inoculum for microbial culture.

**Isolation of quorum sensing degrader bacteria from juvenile tilapia gut**

**Enrichment of bacteria from fish guts as QS degraders**

Method was following Tinh et al. (2007) with slight modification. One hundred microliters of the fish gut homogenate were inoculated into a tube contained 10 ml of sterilized 0.75%NaCl solution and 80 µl N-hexanoyl-L-Homoserine lactone (C6-HSL) with a con-
centrations of 20 ppm. This was done in three replicates. For control, NaCl solution was without the addition of C6-HSL. These tubes were incubated at 30°C (100 rpm). The enrichment was done in three consecutive cycles: 1st cycle, three days of incubation period; 2nd to 3rd cycle, two days of the incubation period, respectively.

At the end of each cycle, 1 ml of each bacteria culture was serially diluted up to 10⁴ and 100 µl of each dilution was spread on LB agar. Plates were incubated at 30°C (24 hours). At the same time, 10% of each bacteria culture was inoculated into fresh 10 ml of sterilized 0.75% NaCl solution and 80 µl of C6-HSL of 20 ppm for the next cycle. For control, there were no additions of C6-HSL. This cycle was repeated until there was no bacterial growth in the control tubes. The colonies grew on the LB agar were randomly selected, pure culture, and stored in 100% glycerol in the freezer at -80°C (Tinh et al., 2007; Chan et al., 2009).

**AHL degradation assay**

Method was based on Torres et al. (2013) with modification. The colonies from the enrichment culture were re-grown overnight in LB broth. After that, 50 µl of the enrichment culture was transferred into 5 ml LB broth supplemented with 10 ppm AHL and incubated at 30°C for 24 hours (100 rpm). 1 ml of each bacteria culture was then filtered with a 0.2-micron filter sterilized. Subsequently, 10 µl of the filtrate was dropped on the centre of LB agar that had been spread-plated with 100 µl of CV026 culture. LB broth with 10 ppm AHL without bacteria was used as control. The QS degradation activity was observed through the inhibition of violacein formation after 24 h of incubation.

**Identification of potential isolates as QS degraders**

Distinct colonies on culture media were examined for phenotypic characteristics such as colony morphology (shape, size, color, and opacity). Primary identification was by using classical Gram staining technique and observed microscopically. The chosen isolates were further identified molecularly by sequence data analysis.

**DNA extraction**

The bacterial genomic DNA of isolates was extracted by using a direct boiling method following Peng et al. (2013) with slight alterations. First, the isolates were cultivated in LB broth. After 24 hours, 1 ml of each bacteria culture was centrifuged for 2 minutes at 14,000 rpm. Supernatants were thrown away and 100 µl of sterile distilled water was added, followed by vortexing for 30 s. Subsequently, DNA from each bacterial culture was extracted by boiling the bacterial suspensions for 10 minutes at 100°C in Eppendorf Master Cycler with an initial denaturation of 4 min at 95°C, 30 cycles of 95°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 1 min 30 seconds and final extension at 72°C for 7 min (Iehata et al., 2013).

The amplified product was confirmed in 1.7% agarose gel electrophoresis in 1X TAE Buffer. Then, PCR products of 16S rDNA were sequenced by 1st BASE, Selangor, Malaysia. The raw sequence data obtained were edited using Chromas and nucleotide sequences were searched in the GenBank DNA database using the Basic Local Alignment Search Tool (BLAST) program (Altschul et al., 1997) accessible at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) on 2018, March 31.

**Hemolytic activity**

The potential probiotics bacteria as QS degraders and A. hydrophila were streaked on the blood agar. The plates were incubated at 28°C for 24 to 48 hour (Laith & Najiah, 2013). The zone forms around the colonies were observed. The existence of clear zone around the colony signifies β-hemolysis. Green or brown discoloration around colony signifies α-hemolysis. The green or brown zone indicates the reduction of haemoglobin to methemoglobin in red blood cells. The absence of hemolysis is known as y-hemolysis (Laith & Najiah, 2013).

**In vitro assay**

Method was following Torabi Delshad et al. (2018) with modification. All potential QS degrader isolates and A. hydrophila were cultured separately in LB broth. After 24 hours, each isolate and A. hydrophila were centrifuged for 2 minutes at 14,000 rpm. Supernatants were thrown away and 0.75% of NaCl solution was added and mixed by using pipette. Afterwards, each isolates were co-culture with A. hydrophila into 0.75% NaCl solution, both with an initial concentration of 10⁴ CFU/ml. Sole culture of A. hydrophila was used as control. Those co-cultures were incubated...
Probitoic bacteria as quorum sensing degrader control .... (Noor Syazwani Omar)

at 30°C (100rpm). Samples were withdrawn every 24 hours to determine the colony count of each isolate by using the drop method on Aeromonas Isolation Medium Base agar. Colonies on agar plates were counted after 24-hour incubation.

Following Chu et al. (2014) with slight modification, concurrently, AHL degradation assay was done at 0, 8, 24, 32, 48, and 72 h by using agar well diffusion method to observe the capability of the isolates to degrade the AHL produced by A. hydrophila.

100 µl of CV026 culture was spread on LB agar by using a sterile hockey stick. A hole with a diameter of 6mm was made and 100 µl of each sample was inoculated into the well. The diameter of purple violacein production was measured (in cm) after incubation. The co-culture was done for five days and all assays were done in triplicate. Isolates that able to degrade the AHL, which produced the diameter of purple zone less than one centimeter (< 1 cm) were chosen for in vivo assay.

In vivo assay

Experimental fish and rearing condition

The experiment was held in Challenge Test Laboratory, Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, Malaysia. Healthy juvenile red hybrid tilapia with an average weight of 3.68 ± 0.96 g and body length of 5.30 ± 0.48 cm were bought from local farmer at Kuala Ping, Terengganu and transferred into 60-gallon poly oval tank. For acclimatization, the tank was continuously aerated for two weeks before use. The fish were fed twice daily with a commercial diet (crude protein ≥ 32% based on 4% of fish body weight) (Torabi Delshad et al., 2018). In order to remove feed remains and feces, the tank was siphorned and about 75% of water was exchanged daily.

Determination of pathogenicity and LC<sub>50</sub> in A. hydrophila

Method was following Torabi Delshad et al. (2018) with slight modification. Before in vivo assay, three different doses of A. hydrophila (10<sup>6</sup>, 10<sup>7</sup> and 10<sup>8</sup> CFU/ml) were prepared to estimate the pathogenicity by using an immersion bath method and LC<sub>50</sub> value for fish with an average weight of 4.97 ± 0.96 g. The immersion bath was set by adding a full-grown A. hydrophila in LB broth (OD<sub>600</sub>) into an aerated aquarium of 15 L freshwater and juvenile tilapia fish for 30 minutes. The assay was done in twelve groups of ten fish (three different doses of A. hydrophila and one negative control without the bacterium). Each group was done in triplicate. Fish mortalities and clinical signs were monitored and recorded for four days.

Preparation of test feed

Commercial diet pellet with a minimum protein content of 32% was used in this study. The chosen potential QS degrader isolates were cultivated separately in LB broth and incubated for 24 hours at 30°C (100 rpm). Then, each isolate with concentration of 10<sup>8</sup> CFU/ml was separately sprayed on pellet following ratio 10<sup>8</sup> CFU per ml: 1 gram of fish pellet and air-dried (Torabi Delshad et al., 2018). For negative control group, sterile phosphate buffer saline (PBS) solution without bacterial culture was sprayed on the pellet and used as feed. All prepared feed was stored at 4°C in airtight plastic zip-lock bags for further used. Fresh diets were prepared every week to assure high isolate level in the pellet.

Experimental design

Feeding trial

Aquarium with size of 15 L were used with each of aquarium contained of ten fish. Fish were given diets supplemented with potential QS degrader isolates for eight weeks. For control, fish were fed with basal pellets without the bacteria. Each treatment was run in triplicate. Fish health was recorded by visual observation of behavior (Torabi Delshad et al., 2018). The fish weight and length were measured weekly. Based on Panase & Mengumphan (2015), for growth performance, mean weight gain (MWG), weight gains (WG), specific growth rates (SGR), average daily growth (ADG), feed conversion ratio (FCR) and survival rate (%) were calculated by using formulae:

\[
\text{MWG} = \frac{\text{Mean final weight (g)} - \text{Mean initial weight (g)}}{\text{Time (days)}}
\]

\[
\text{WG (%) = } \frac{\text{Final weight (g)} - \text{Initial weight (g)}}{\text{Initial weight (g)}} \times 100
\]

\[
\text{SGR ( % day}^{-1}) = \frac{\ln \text{mean final weight (g)} - \ln \text{mean initial weight (g)}}{\text{Time (days)}} \times 100
\]

\[
\text{ADG} = \frac{\text{Mean final weight (g)} - \text{Mean initial weight (g)}}{\text{Time (days)}}
\]
Challenge test

After eight weeks of the feeding trial, all fish were challenged with A. hydrophila. Before the challenge test, all fish were clinically healthy, showing no signs of A. hydrophila infection or any mortality due to the disease. Fish were kept fasting for 24 h before the experiment. All fish were exposed to A. hydrophila (10^{7} CFU/ml) by immersion bath method for 30 minutes. Feed was not given on the first day of challenge test. Then, the fish were monitored for up to two weeks and fed daily with a commercial diet. Fish was observed for mortalities and clinical signs of A. hydrophila infection (Chu et al., 2014; Torabi Delshad et al., 2018).

Data analysis

Data presented as mean± standard deviation (SD). Independent sample t-test was used to determine any significant difference between the growth of A. hydrophila in monoculture and in each co-culture, comparison of each AHL treatment with control in the AHL degradation assay and survival rate of fish. The difference between pairs of treatments was considered to be significant if the p-value< 0.05. All statistical analysis was performed using Microsoft Excel 2010 version 14.0.

RESULTS AND DISCUSSION

Selection of QS degrader bacteria from juvenile tilapia gut

A total of 150 bacteria were isolated from the fish gut. The isolation of QS degraders bacteria was based on the capability of the microbial communities to develop in a minimal medium contained a mixture of 0.75%NaCl solution supplemented with medium-chain AHL (C6-HSL). The AHL molecules were used as the only source of nitrogen and carbon (Noorashikin et al., 2016).

For screening, CV026 was used as the biosensor strain to detect the existence of exogenous AHLs with N-acyl side chains between C4 and C8 (Haridas & Pillai, 2019). The potential QS degrader isolates were inoculated at 10^{6} CFU ml^{-1} into LB broth supplemented with 10 ppm C6-HSL to determine if they can break down AHLs in a nutrient-rich context, imitating the existence of high amounts of other nutrients as in the gastrointestinal environment (Defoirdt et al., 2011). Standard N-hexanoyl-L-homoserine lactone (HHL) is one of the exogenous AHLs. HHL was utilized as a trial molecule because it is produced by most aquatic animal pathogens like E. tarda, A. salmonicida, A. hydrophila, and V. salmonicida (Vinoj et al., 2014).

Based on the AHL degradation test, three out of 150 isolates showed the best degradation rate after 24 hours of incubation. Isolate CPi12 was completely degrading the AHL (Figure 1). CBa5 and CBa7 showed the same result. This revealed that the isolates could degrade the AHL with N-acyl side chains between C4 and C8 and might be the potential of QS degraders.

For colony morphology on LB agar, for isolate CPi12, it was circular, white and opaque. Meanwhile, for isolates CBa5 and CBa7, both were circular, white and translucent. The isolates were Gram-negative and rod shape. By molecular identification, these isolates belong to the genus of Klebsiella, and Enterobacter (Table 1).

These three isolates were under Proteobacteria. Similar with other study by Wanka et al. (2018), in isolation of native probiotics from the fish gut, results showed that 89.7%of 195 isolates that isolated from intestinal tract of healthy flatfish were members of the Proteobacteria. Other study by Tyagi et al. (2019) also found that more than three-fourths of total bacteria population in the gut of freshwater carp (Labeo rohita) was Proteobacteria.

According to Torres et al. (2016), the microorganisms under the phyla of Bacteroidetes, Proteobacteria, Firmicutes, and Actinobacteria were able to produce enzymes that could degrade the AHLs. Other than that, different bacteria that capable in degrading the AHLs are Bacillus, Agrobacterium, Rhodococcus, Streptomyces, Arthrobacter, Pseudomonas, Enterobacter & Klebsiella (Chan et al., 2016; Noorashikin et al., 2016).

According to LaPatra et al. (2014), Enterobacter sp. that isolated from rainbow trout, Onchorhynchus mykiss gut and used as probiotics in dietary administration capable of decreasing the mortality in rainbow trout. Besides, the effect of nonpathogenic Kleb-
Probiotic bacteria as quorum sensing degrader control ..... (Noor Syazwani Omar)

Klebsiella pneumoniae was proved in the mouse model, where it could not cause lung infection in the infected mouse (Rajkumari et al., 2021).

In this study, CPI12, CBA5 and CBA7 were under the phyla of Proteobacteria. These isolates might have at least one type of AHL degrading enzymes which yet to be identified. However, Klebsiella pneumoniae that detected in fish gut is potentially pathogenic member of family Enterobacteriaceae (Tyagi et al., 2019). Enterobacter also has been reported to induce severe infection to ornamental fish (Preena et al., 2021).

Enterobacter also has been reported to induce severe infection to ornamental fish (Preena et al., 2021). Hemolytic activity on blood agar plate is an essential initial step in identifying pathogenic bacteria (Yeh et al., 2009). For hemolytic activity, isolates CPI12, CBA5, and CBA7 showed no hemolysis on blood agar (Figure 2). Previous studies stated that rainbow trout and other fish intestinal microbiota are conquered by phyla Proteobacteria including a variety of nonpathogenic bacteria such as Shewanella, Enterobacter, and Acinetobacter (Yilmaz et al., 2018).

For A. hydrophila, complete lysis of the red blood cells was observed as a clear and colorless zone surrounding colony on the blood agar medium. This study was in line with other studies that the pathogenic bacterium, A. hydrophila was β-hemolysis (aerolysin) on the blood agar (Laith & Najiah, 2013; Singh et al., 2008). This may be used as indicator of enterotoxicity and it is lethal to the fish (Najiah & Laith, 2013). Several extracellular toxins and enzymes are responsible for the virulence of A. hydrophila such as hemolysins, cytotoxins, enterotoxins and proteases (Novoa et al., 2008). These virulence factors could provide the ability of A. hydrophila to bind with host cells during

![Figure 1. AHL degradation test on LB agar. A: LB broth with 10 ppm AHL served as control. B: No purple violacein production on the LB agar after 24 hours of incubation indicates the ability of the isolates to completely degrade the AHL molecules.](image)

Table 1. Identification of potential probiotics bacteria as QS degrader based on 16S rDNA sequencing

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Identification</th>
<th>Taxonomy</th>
<th>Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPI12</td>
<td>Klebsiella sp.</td>
<td>Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Klebsiella (MT197248)</td>
<td>100%</td>
</tr>
<tr>
<td>CBA5</td>
<td>Enterobacter tabaci</td>
<td>Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Enterobacter (MN733344)</td>
<td>99%</td>
</tr>
<tr>
<td>CBA7</td>
<td>Enterobacter tabaci</td>
<td>Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Enterobacter (MN733344)</td>
<td>96%</td>
</tr>
</tbody>
</table>

Copyright @ 2023, Indonesian Aquaculture Journal, p-ISSN 0215-0883; e-ISSN 2502-6577
disease development (Singh et al., 2008). These three isolates (CPi12, CBa5, and CBa7) might be the potential probiotics bacteria as QS degrader because the general selection criterion to be considered as a probiotics candidate is non-pathogenic microorganisms (Feckaninová et al., 2017; Simón et al., 2021).

**Effect of potential QS degrader isolates to pathogen in in vitro assay**

Aeromonas agar was used to distinguish the isolates and \( A. \) hydrophila during in vitro assay. All isolates were cream and opaque, while \( A. \) hydrophila was dark green and opaque with dark center on the agar.

For co-culture of CPi12, CBa5, and CBa7 with \( A. \) hydrophila, respectively, results revealed that the growth of \( A. \) hydrophila was lower than the isolates. There was a significant difference in bacterial growth between \( A. \) hydrophila with CPi12 on days one, three, and four (Figure 3). In AHL inactivation assay, there was a significant difference on the degradation of AHL by CPi12 on 8H, 24H, 48H, and 72H of co-culture period (Table 2).

For CBa5, there was a significant difference in the bacterial growth that started from day one until day four of co-culture (Figure 4). For AHL inactivation assay, there was significant different on AHL degradation at 0H, 24H and 72H (Table 3).

For CBa7, the result showed a significant difference in the growth of both bacteria from day two until day four (Figure 5). For AHL degradation test, CBa7 was able to degrade the AHL and there was significant different at 24H and 48H of co-culture incubation period (Table 4).

In this study, results proved that the potential QS degrader isolates (CPi12, CBa5, and CBa7) could suppress the growth of the \( A. \) hydrophila and at the same time able to degrade the AHL that produce by \( A. \) hydrophila.

![Hemolytic test of CPi12, CBa5, CBa7, and \( A. \)hydrophila on blood agar. The presence of clear zones around the \( A. \) hydrophila indicates \( \beta \)-hemolysis. CPi12, CBa5, and CBa7 showed no hemolysis.](image)

![Growth of bacterial co-culture between \( A. \) hydrophila with CPi12 in 0.75%NaCl solution. Symbol * indicate significant difference p-value<0.05. The error bars indicate the standard error of the three replicates.](image)
Probiotic bacteria as quorum sensing degrader control ..... (Noor Syazwani Omar)

Proibotic bacteria as quorum sensing degrader control experiments. AHLs and QS-associated virulence factors produced by A. hydrophila were significantly modified because A. hydrophila exposed to QQ enzymes secreted by CPI12, CBA5, and CBA7 in co-culture. According to Roche et al. (2004), co-culturing of AHL-inactivating bacteria with AHL-producing bacteria has been shown to inhibit the QS-related activities, both in liquid culture and on plant tissue, dramatically reduce the accumulation of AHL. Besides, in mixed culture, the interaction between the bacteria involves the degradation or alteration of QS factors produced by other community members (Conway et al., 2012).

Results in this study were contradicted by the study of Chan et al. (2011), which isolate SE14 belong to the genera Klebsiella sp., capable of weakening the virulence factor production in the plant pathogen, Pseudomonas aeruginosa in co-culture without inhibiting the pathogen growth. A study performed by Chu et al. (2014), the Bacillus sp. as the QS degrader gave no effect on the growth of A. hydrophila in co-culture but could decrease the quorum sensing molecules produced by the pathogen. Other than that, Novita et al. (2015) also proved that the co-culturing of isolate QSI-1, that identified as Bacillus sp., with A. hydrophila showed that both isolates were able to

Table 2. AHL degradation assay of co-culture A. hydrophila with CPI12

<table>
<thead>
<tr>
<th>Type of culture</th>
<th>Isolate</th>
<th>Average diameter (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0h</td>
</tr>
<tr>
<td>Single culture</td>
<td>A. hydrophila</td>
<td>2.9±0.2</td>
</tr>
<tr>
<td>Co-culture</td>
<td>CPI12 - A. hydrophila</td>
<td>2.6±0.1</td>
</tr>
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Data represent mean± SD. n=3. Symbol * indicate significant difference p-value<0.05

Figure 4. Growth of bacterial co-culture between A. hydrophila with CBA5 in 0.72%NaCl solution. Symbol * indicate significant difference p-value<0.05. The error bars indicate the standard error of the three replicates.

Table 3. AHL degradation assay of co-culture A. hydrophila with CBA5

<table>
<thead>
<tr>
<th>Type of culture</th>
<th>Isolate</th>
<th>Average diameter (cm)</th>
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<tr>
<td></td>
<td></td>
<td>0h</td>
</tr>
<tr>
<td>Single culture</td>
<td>A. hydrophila</td>
<td>2.9±0.2</td>
</tr>
<tr>
<td>Co-culture</td>
<td>CBA5 - A. hydrophila</td>
<td>2.4±0.1*</td>
</tr>
</tbody>
</table>

Data represent mean± SD. n=3. Symbol * indicate significant difference p-value<0.05
grow together and do not inhibit each other growth because there was no antagonistic activity occur.

Therefore, in this study, results revealed that isolates CPi12, CBa5, and CBa7 might be a new finding as these isolates could degrade the AHL produced by pathogen A. hydrophila, and at the same time can decrease the development of A. hydrophila in the co-culture experiment.

**Pathogenicity test of A. hydrophila on juvenile tilapia**

Immersion bath method was used as it is more natural and closely mimicking the infection route under aquatic environments (Zhang et al., 2016). The LC50 of A. hydrophila was 10^7 CFU/ml (Figure 7). The lethal dosage of A. hydrophila was comparable to that reported for juvenile rohu, Labeo rohita and channel catfish, Clarias gariepinus (Das et al., 2014; Hussein et al., 2017).

In this study, the mortality rate increased at 10^6 - 10^7 CFU/ml. This is because the fish mortality depends on the bacteria concentration and the virulence factor (production of extracellular product) of A. hydrophila (Dias et al., 2016). However, at 10^8 CFU/ml, a low mortality rate was observed because some strains of Aeromonas could produce exotoxins and express the toxin genes in specific growth conditions (Tomás, 2012). According to Tomás (2012),

![Figure 5. Growth of bacterial co-culture between A. hydrophila with CBa7 in 0.75%NaCl solution. Symbol * indicate significant difference p-value<0.05.](image)

**Table 4. AHL degradation assay of co-culture A. hydrophila with CBa7**

<table>
<thead>
<tr>
<th>Type of culture</th>
<th>Isolate</th>
<th>Average diameter (cm)</th>
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<tr>
<td></td>
<td></td>
<td>0H</td>
</tr>
<tr>
<td>Single culture</td>
<td>A. hydrophila</td>
<td>2.9±0.2</td>
</tr>
<tr>
<td>Co-culture</td>
<td>CBa7 - A. hydrophila</td>
<td>2.8±0.1</td>
</tr>
</tbody>
</table>

Data represent mean± SD. n=3. Symbol * indicate significant difference p-value<0.05

According to Julinta et al. (2017), the degree of virulence and fatal dosage of A. hydrophila at 10^7 was moderately pathogenic to juvenile tilapia with the potential for systemic infection. The variances in the fatal toxicity and/or LC50 of A. hydrophila is related to the culture conditions, experiment conditions and the pathogenicity vary between fish size and species (Hussein et al., 2017).

**Feeding trial and challenge test: in vivo assay**

During feeding trials, there were no mortalities of fish. For all growth performance, group of fish fed with CPI12 showed no significant different to the control group (Table 5). For CBa5, there was signifi-
Probiotic bacteria as quorum sensing degrader control .... (Noor Syazwani Omar)

...cant higher for growth parameter MWG and ADG compare to the control group. Meanwhile, for group of fish fed with CBa7, there was significant improvement in all growth performance compare to the control group.

For all the growth performance, CBa7 was the best isolate compared to CPI12 and CBa5. Similar results were demonstrated by Novita et al. (2015) that there was a significant difference in FCR and SGR for a group of fish fed with QQ bacteria, and it was proved that the QQ bacteria was able to increase the African catfish (Clarias gariepinus) growth. Besides, a study conducted by Wee et al. (2018) showed that juvenile giant freshwater prawns, Macrobrachium rosenbergii that fed with B. cereus BP-MBRG/1b as QS degrader has significantly increased the growth performance of the juvenile prawn compared to an untreated diet. It also has been reported that the inclusion of Bacillus sp. as feed additives help in increasing the digestive activities, metabolic activity and assimilation in fish and crustacean by improving the production of digestive enzyme that could increase nutrient absorption and growth performance (Novita et al., 2015; Han et al., 2015).

After being challenged with A. hydrophila, there was no significant difference in the survival rate of fish fed with CBa5 and CBa7, respectively, with the control group (Figure 6). This might be due to the fact that the stability of enzymes in in vivo is hard to maintain as compared to in vitro (Jiang et al., 2019).

Meanwhile, the survival rate of fish fed with CPI12 was 100% and significantly higher (p<0.05) than the control group. Results demonstrated that CPI12 have the strongest ability in decreasing or weaken the virulence factor of A. hydrophila so that the A. hydrophila was not pathogenic to the tilapia. As in in vitro assay, CPI12 also was able to completely degrade the AHL after 24 hours of co-culture with A. hydrophila. According to Paluch et al. (2020), some bacterial species such as K. pneumonia could produce enzymes that could break down the AHL molecules, thus constraining virulence in various pathogenic bacteria.

Table 5. Growth performance of juvenile tilapia cultured for eight weeks

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MWG (g)</th>
<th>WG (%)</th>
<th>SGR(% day⁻¹)</th>
<th>ADG (g)</th>
<th>FCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPI12</td>
<td>8±0</td>
<td>271.75±0</td>
<td>2.18±0</td>
<td>0.13±0</td>
<td>2.58±0</td>
</tr>
<tr>
<td>CBa5</td>
<td>9.67±0.17*</td>
<td>276.19±4.76</td>
<td>2.21±0.02</td>
<td>0.16±0.02*</td>
<td>2.45±0.01</td>
</tr>
<tr>
<td>CBa7</td>
<td>13.58±0.08*</td>
<td>430±20*</td>
<td>2.78±0.06*</td>
<td>0.23±0.00*</td>
<td>2.22±0.01*</td>
</tr>
<tr>
<td>Control</td>
<td>8.5±0</td>
<td>263.1±20.28</td>
<td>2.15±0.09</td>
<td>0.14±0</td>
<td>2.49±0</td>
</tr>
</tbody>
</table>

Data represent mean± SD. deviation. n=3. Symbol * indicate significant different p-value<0.05. MWG= Mean Weight Gain, WG = Weight Gain, SGR = Specific Growth Rate, ADG = Average Daily Growth, and FCR = Feed Conversion Ratio

![Figure 6. Survival rate of juvenile tilapia after challenge with A. hydrophila. Symbol * indicate significant different p-value<0.05. The error bars indicate standard error for the three replicates.](image-url)
pneumonia also was shown to produce AHL lactonase enzyme under metallo-ß-lactamase superfamily (Romero et al., 2015). Therefore, this result indicated that CPi12 could totally decrease the AHL signal of the pathogen and interfere with the QS function, which significantly reducing fish mortality caused by A. hydrophila infection.

Similar results were obtained from other bacteria as QS degrader, which could improve the survival rate of other aquatic organisms against pathogenic bacteria: Bacillus sp. as anti-quorum sensing (AQS) was able to increase the survival rate of zebrafish and catfish against A. hydrophila (Novita et al., 2015; Torres et al., 2016), oral administration of B. licheniformis was significantly increase the survival rate of juvenile tilapia against S. iniae (Han et al., 2015) and the usage of PQQ-42 as QS degrader was able to reduce the cumulative mortality of corals against pathogenic Vibrio species (Torres et al., 2016).

The inclusion of probiotics through feed could enable the colonization of the probiotics bacteria in the gastrointestinal tract and could modulate the genes expression in the digestive tract (Feèkaninová et al., 2017). This situation could create a favorable habitat for them and could prevent the invasion of other bacteria introduced later in the environment (Feèkaninová et al., 2017).

The fish mortalities can be observed after 24 hours of fish infection. Most dying fish exhibited clinical signs of A. hydrophila infection such as rot of tails and fins, fin hemorrhages, losses of scales, dropsy, and septicemia (Figure 7). Behavioral changes that can be seen were lethargic, loss of appetite and slow in movement.

Besides, mucus excess also was observed. This study was in line with a study conducted by Novoa et al. (2008), where it was observed that there were

![Figure 7. Clinical signs of red hybrid tilapia following A. hydrophila infection. A. Healthy control fish showed no clinical signs; B. Hemorrhages at base of dorsal fin (arrow) and loss of scales at the abdomen (circle); C: Fin rot at the caudal and pectoral fin (circle).](image)
external symptoms (abdominal and visceral hemorrhages and abdominal distention) on zebrafish after infected with A. hydrophila. Other than that, a study by Pauzi et al. (2020) demonstrated that depigmentation on operculum areas and body, hemorrhagic foci, fin and tail erosion were also observed on red hybrid tilapia after being infected with A. hydrophila. The clinical symptoms were comparable to catfish affected with A. hydrophila (Laith & Najiah, 2013). Aeromonas bacteria were able to rupture the blood vessels, causing ulcerative lesions intoegument with a hemorrhagic feature that causes a reddish color on the body (Oliveira et al., 2011). The high explosion of Aeromonas bacteria in the fish gut resulted in excessive mucus secretion (Oliveira et al., 2011). A. hydrophila has been recognized to cause numerous distinct pathological conditions including fin/tail rot and hemorrhagic septicemias (Austin & Austin, 2016). The secretion of aerolysin toxin by the A. hydrophila that cause the lysis of the red blood cells could result in signs of hemorrhage on the fish’s skin and internal organs (Singh et al., 2008).

For isolate CBa5, it displayed a positive result in in vitro which it able to degrade the AHL production by A. hydrophila and suppress the growth of pathogen. However, in in vivo, it did not display positive result in either disease resistance or growth performance of the host. This is because positive results in in vitro occasionally fail to determine an in vivo effect (Hai, 2015).

CONCLUSION

Isolate CPI12 identified as Klebsiella sp. can act as QS degrader probiotics bacteria. It was able to degrade the AHL molecules completely in in vitro assay and could promote 100% survival rate of the juvenile tilapia that infected with pathogen A. hydrophila. Meanwhile, CBa7 identified as Enterobacter tabaci used in fish feed promotes greater growth performance for fish. This study demonstrated that probiotics bacteria as QS degrader could be isolated from freshwater fish and could be used against pathogenic bacterial QS systems as well as combat the bacterial diseases. It can be used as a replacement of using antibiotics and could help in reducing the risen of antibiotics resistant bacteria and also at the same time promote better growth performance for fish.

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