DOI: http://dx.doi.org/10.15578/iaj.18.1.2023.1-15 *Indonesian Aquaculture Journal, 18 (1), 2023, 1-15*

Available online at: http://ejournal-balitbang.kkp.go.id/index.php/iaj

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

Noor Syazwani Omar[*)], Sharifah Noor Emilia[*)#], Muhd Danish-Daniel[**)], Shumpei Iehata[*)], and Natrah Fatin Mohd Ikhsan[***)]

[*)] Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia

[**)] Institute of Marine Biotechnology, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia

[***)] Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

(Received: November 28, 2022; Final revision: May 24, 2023; Accepted: May 26, 2023)

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., *Ichthyophitirius multifilis,* 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 antibi-

[#] Correspondence: Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu

E-mail: emilia@umt.edu.my

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 30ºC on LB media with shaking at 100 rpm (Chu *et al*., 2010). *A. hydrophila* culture was kept as stock at -80ºC in 100% glycerol.

CV026 was grown at 30ºC 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-

centration 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 30ºC (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 10^{-4} and 100 µl of each dilution was spread on LB agar. Plates were incubated at 30ºC (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 -80ºC (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 30ºC 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 served 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 100ºC in Thermo-shaker and centrifuged for 10 minutes at 10,000 rpm. The supernatants were transferred into 500 µl micro tube and used in PCR amplification.

PCR amplification

The PCR amplification of bacterial 16S rDNA was performed by using eubacterial universal primer pair 8F and universal reverse primer 1492R (Mohana *et al*., 2007). PCR amplification was performed by using Eppendorf Master Cycler with an initial denaturation of 4 min at 95ºC, 30 cycles of 95ºC for 30 seconds, annealing at 56ºC for 30 seconds, extension at 72ºC for 1 min 30 seconds and final extension at 72ºC 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 28ºC 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 ß-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 10^5 CFU/ml. Solo culture of *A. hydrophila* was used as control. Those co-cultures were incubated

at 30ºC (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 proteine ≥32%) based on 4% of fish body weight (Torabi Delshad *et al*., 2018). In order to remove feed remains and feces, the tank was siphoned and about 75% of water was exchanged daily.

Determination of pathogenicity and LC_{50} in A. hydrophila

Method was following Torabi Delshad *et al.* (2018) with slight modification. Before *in vivo* assay, three different doses of *A. hydrophila* (10^6, 10^7 and 10^8 CFU/ml) were prepared to estimate the pathogenicity by using an immersion bath method and LC_{50} 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_{600}) 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 30ºC (100 rpm). Then, each isolate with concentration of 10^8 CFU/ml was separately sprayed on pellet following ratio 10^8 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 4ºC 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:

$$\text{MWG} = \text{Mean final weight (g)} - \text{Mean initial weight (g)}$$

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

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

$$\text{ADG} = \frac{\text{Mean final weight (g)} - \text{Mean initial weight (g)}}{\text{Time (days)}}$$

$$FCR = \frac{4\% \times \text{fish weight (g) x days feed}}{\text{Final fish weight - Initial fish weight}}$$

$$\text{Survival rate } (\%) = \frac{\text{Final no. of live fish}}{\text{Initial no of live fish}} \times 100$$

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 10^8 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, *Oncorhynchus mykiss* gut and used as probiotics in dietary administration capable of decreasing the mortality in rainbow trout. Besides, the effect of nonpathogenic *Kleb-*

siella 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).

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

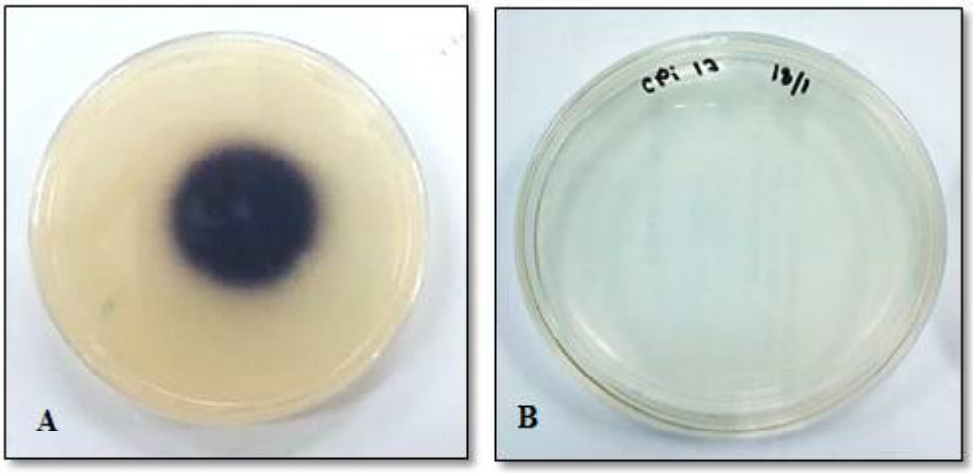

Figure 1. AHL degradation test on LB agar. **A:** LB broth with 10 ppm AHL served as control. B: No purple violacein production on the LB agar after 24 hours of incubation indicates the ability of the isolates to completely degrade the AHL molecules.

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

Isolate	Identification	Taxonomy	Similarity
CPi12	*Klebsiella* sp.	Proteobacteria; Gammaproteobacteria; Enterobacterales; Enterobacteriaceae; *Klebsiella* (MT197248)	100%
CBa5	*Enterobacter tabaci*	Proteobacteria;Gammaproteobacteria; Enterobacterales; Enterobacteriaceae*;* *Enterobacter* (MN733344)	99%
CBa7	*Enterobacter tabaci*	Proteobacteria; Gammaproteobacteria; Enterobacterales; Enterobacteriaceae; *Enterobacter* (MN733344)	96%

was in line with other studies that the pathogenic bacterium, *A. hydrophila* was ß-hemolysis (aerolysin) on the blood agar (Laith & Najiah, 2013; Singh *et al*., 2008). This may be used as indicator of enteroxicity 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

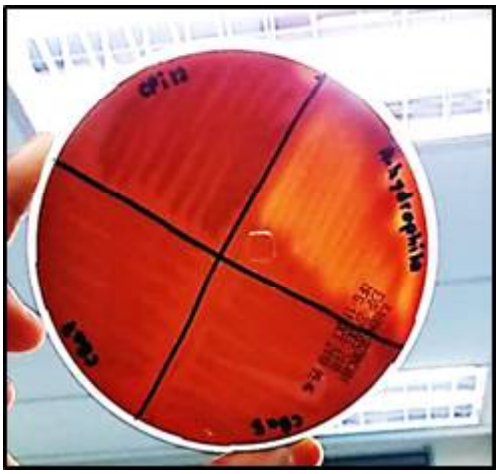

Figure 2. Hemolytic test of CPi12, CBa5, CBa7, and *A.hydrophila* on blood agar. The presence of clear zones around the *A. hydrophila* indicates ß-hemolysis. CPi12, CBa5, and CBa7 showed no hemolysis.

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.*

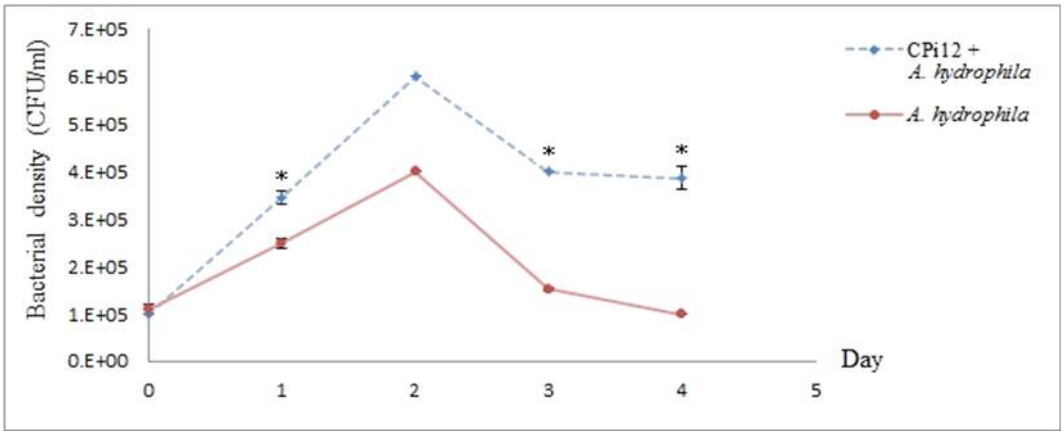

Figure 3. Growth of bacterial co-culture between *A. hydrophila* with CPi12 in 0.75% NaCl solution. Symbol * indicate significant difference *p*-value < 0.05. The error bars indicate the standard error of the three replicates.

hydrophila in co-culture experiment. 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

Type of culture	Isolate	Average diameter (cm)					
		0h	8h	24h	32h	48h	72h
Single culture	*A. hydrophila*	2.9±0.2	2.7±0.2	2.7±0.1	2.5 ± 0.2	2.5 ± 0.1	1.9 ± 0.2
Co-culture	CPi12 - *A. hydrophila*	2.6±0.1	2.0±0.2*	2.0±0.2*	1.7 ± 0.9	0 ± 0*	0 ± 0*

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

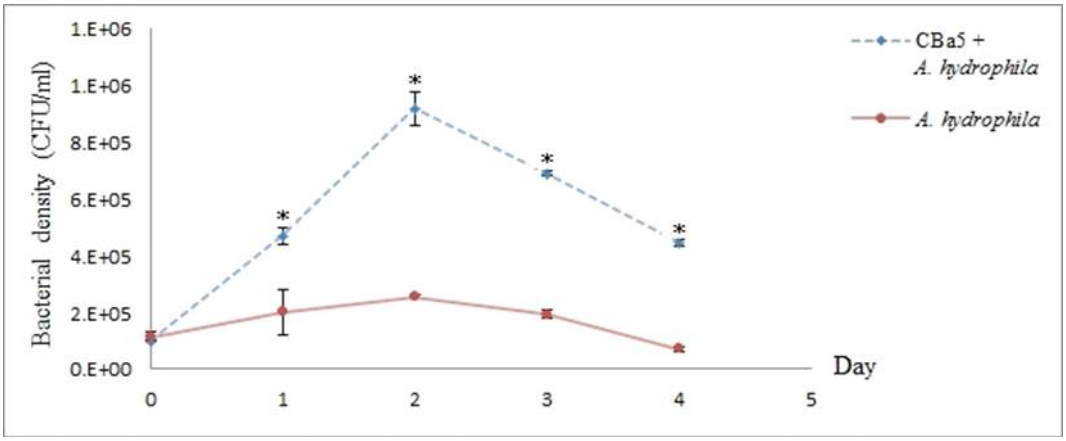

Figure 4. Growth of bacterial co-culture between *A. hydrophila* with CBa5 in 0.72% NaCl solution. Symbol * indicate significant difference *p*-value < 0.05. The error bars indicate the standard error of the three replicates.

Table 3. AHL degradation assay of co-culture *A. hydrophila* with CBa5

Type of culture	Isolate	Average diameter (cm)					
		0h	8h	24h	32h	48h	72h
Single culture	*A. hydrophila*	2.9± 0.2	2.7± 0.2	2.7± 0.1	2.3± 0.3	2.5± 0.1	1.9± 0.2
Co-culture	CBa5 - *A. hydrophila*	2.4±0.1*	2.2± 0.3	2.1±0.2*	1.9± 0.3	1.1± 1.0	0.5±0.9*

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 LC_{50} 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),

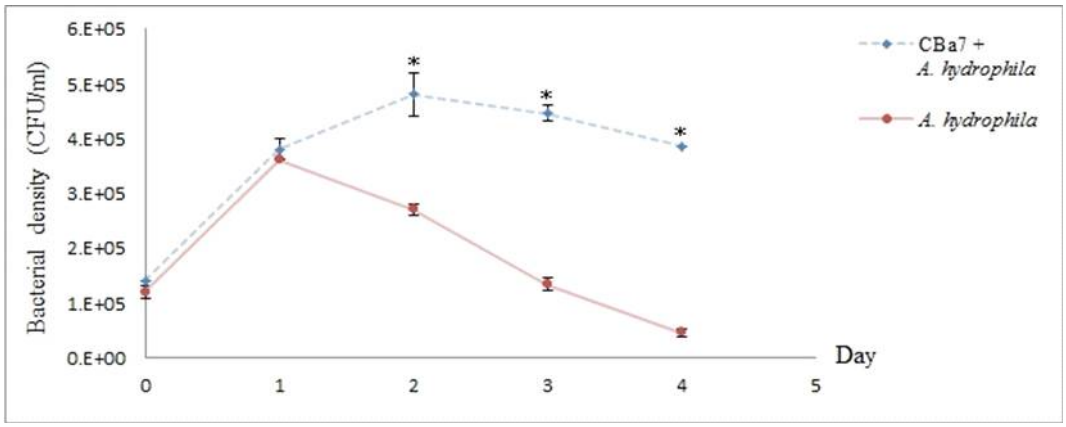

Figure 5. Growth of bacterial co-culture between *A. hydrophila* with CBa7 in 0.75% NaCl solution. Symbol * indicate significant difference *p*-value < 0.05.

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

Type of culture	Isolate	Average diameter (cm)					
		0H	8H	24H	32H	48H	72H
Single culture	*A. hydrophila*	2.9 ± 0.2	2.7± 0.2	2.7± 0.1	2.3 ± 0.1	2.5 ± 0.1	1.9 ± 0.2
Co-culture	CBa7 - *A. hydrophila*	2.8 ± 0.1	2.1 ± 0.3	2.1 ± 0.1*	1.9 ± 0.3	1.0 ± 0.8*	1.1 ± 1.0

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

Aeromonas could produce up to two different types of hemolysins without enterotoxic properties: α-hemolysins and ß-hemolysins. The α-hemolysins are produced in the stationary growth phase, leading to reversible cytotoxic effects and incomplete erythrocytes lysis. Meanwhile, the ß-hemolysins are commonly produced during the exponential growth phase. It is a pore-forming toxin that totally destroys the erythrocytes and osmotic lysis. As in this study, low mortality rate at 10^8 CFU/ml might be due to the fact that the bacteria have reached stationary growth phase and thus resulted in decrease of *A. hydrophila* pathogenicity.

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 LC_{50} 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-

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. *K.*

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

Treatment	MWG (g)	WG (%)	SGR(% day^{-1})	ADG (g)	FCR
CPi12	8 ± 0	271.75 ± 0	2.18 ± 0	0.13 ± 0	2.58 ± 0
CBa5	9.67 ± 0.17*	276.19 ± 4.76	2.21 ± 0.02	0.16 ± 0.02*	2.45 ± 0.01
CBa7	13.58 ± 0.08*	430 ± 20*	2.78 ± 0.06*	0.23 ± 0.00*	2.22 ± 0.01*
Control	8.5 ± 0	263.1 ± 20.28	2.15 ± 0.09	0.14 ± 0	2.49 ± 0

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

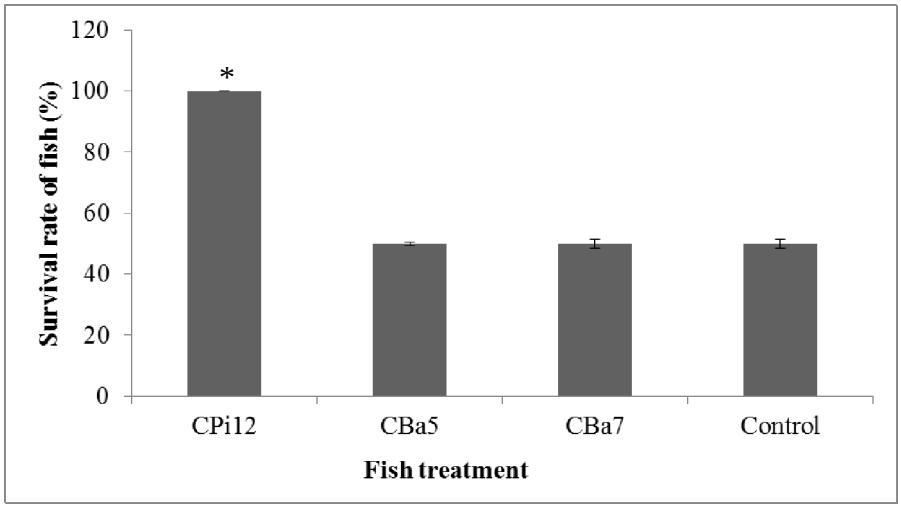

Figure 6. Survival rate of juvenile tilapia after challenge with *A. hydrophila*. Symbol * indicate significant different p-value < 0.05. The error bars indicate standard error for the three replicates.

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

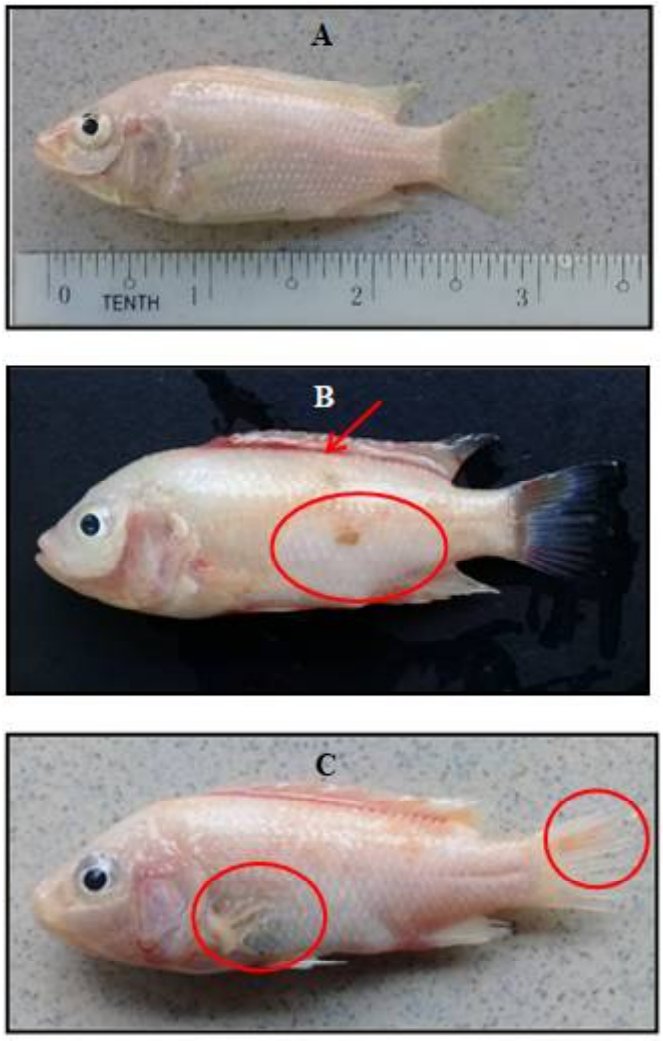

Figure 7. Clinical signs of red hybrid tilapia following *A. hydrophila* infection. **A.** Healthy control fish showed no clinical signs; B. Hemorrhages at base of dorsal fin (arrow) and loss of scales at the abdomen (circle); C: Fin rot at the caudal and pectoral fin (circle).

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 integument 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 literation (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.

ACKNOWLEDGMENTS

This work was financially supported by Malaysian Ministry of Higher Education through Fundamental Research Grant Scheme (FRGS) 59404 awarded to the corresponding author. The MyBrain 15 scholarship to the first author is gratefully acknowledged.

REFERENCES

Al-Harbi, A. H. & Uddin, N. (2005). Bacterial diversity of tilapia (*Oreochromis niloticus*) cultured in brackish water in Saudi Arabia. *Aquaculture,* 250(3-4), 566-572.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research,* 25(17), 3389-3402.

Austin, B. & Austin, D. A. (2016). Aeromonadaceae Representatives (Motile Aeromonads). In: Bacterial Fish Pathogens (pp. 25-30). Springer, Cham. Retrieved from https://doi.org/10.1007/978-3-319-32674-0_4

Chan, K. G., Yin, W. F., Sam, C. K., & Koh, C. L. (2009). A novel medium for the isolation of N-acylhomoserine lactone-degrading bacteria. *Journal of Industrial Microbiology and Biotechnology*, 36(2): 247-251.

Chan, K. G, Atkinson, S., Mathee, K., Sam, C. K., Chhabra, S.R., Camara, M., & Williams, P. (2011). Characterization of N-acylhomoserine lactone-degrading bacteria associated with the *Zingiber officinale* (ginger) rhizosphere: co-existence of quorum quenching and quorum sensing in *Acinetobacter* and *Burkholderia. BMC Microbiology,* 11(1), 1-14.

Chan, X. Y., How, K. Y., Yin, W. F., & Chan, K. G. (2016). N-Acyl homoserine lactone-mediated quorum sensing in *Aeromonas veronii* biovar sobria strain 159: Identification of LuxRI homologs. *Frontiers in Cellular and Infection Microbiology,* 6, 7.

Chu, W., Lu, F., Zhu, W., & Kang, C. (2010). Isolation and characterization of new potential probiotics bacteria based on quorum sensing system. *Journal of Applied Microbiology,* 110(1), 202-208.

Chu, W., Zhou, S., Zhu, W., & Zhuang, X. (2014). Quorum quenching bacteria *Bacillus* sp. QSI-1 protect zebrafish (*Danio rerio*) from *Aeromonas hydrophila* infection. *Scientific Reports,* 4, 5446.

Conway, C.A., Esiobu, N., & Lopez, J. V. (2012). Co-cultures of *Pseudomonas aeruginosa* and *Roseobacter denitrificans* reveal shifts in gene expression levels compared to solo cultures. *Science World Journal,* vol. 2012, 1-5.

Das, S., Mishra, J., Mishra, A., Mahapatra, K. D., Saha, J. N., & Sahoo, P. K. (2014). Establishment of route of challenge and tissue level persistence study of *Aeromonas hydrophila* infection in rohu, *Labeo rohita* for running a selection programme. *International Journal of Fisheries & Aquatic Studies* 1(5), 216-220.

Dias, M. K. R., Sampaio, L. S., Proietti-Junior, A. A., Yoshioka, E. T. O., Rodrigues, D. P., Rodriguez, A. F. R., Ribeiro, R. A., ...Tavares-Dias, M. (2016). Lethal dose and clinical signs of *Aeromonas hydrophila* in *Arapaima gigas* (Arapaimidae), the giant fish from Amazon. *Veterinary Microbiology,* 188, 12-15.

Defoirdt, T., Sorgeloos, P., & Bossier, P. (2011). Alternatives to antibiotics for the control of bacterial disease in aquaculture. *Current Opinion in Microbiology,* 14(3), 251-258.

El-Sayed, A. F. M. (2020). Tilapia Culture (2nd ed.). London, United Kingdom

FAO (2020, June 22) *Oreochromis niloticus.* Retrieved from http://www.fao.org/fishery/culturedspecies/Oreochromis_niloticus/en.

Feèkaninová, A, Košèová, J, Mudroòová, D, Popelka, P, & Toropilová, J. (2017). The use of probiotics bacteria against *Aeromonas* infections in salmonid aquaculture. *Aquaculture,* 469, 1-8.

Grandclément, C., Tannières, M, Moréra, S, Dessaux, Y, & Faure, D. (2016). Quorum quenching: role in nature and applied developments. *FEMS Microbiology Review,* 40(1), 86-116.

Hai, NV. (2015). The use of probiotics in aquaculture. *Journal of Applied Microbiology,* 119, 917-935.

Han, B, Long, W. Q., He, J. Y., Liu, Y. J., Si, Y. Q., & Tian, L. X. (2015). Effects of dietary *Bacillus licheniformis* on growth performance, immunological parameters, intestinal morphology and resistance of juvenile Nile tilapia (*Oreochromis niloticus*) to challenge infections. *Fish & Shellfish Immunology,* 46(2), 225-231.

Haridas, D. V. & Pillai, D. (2019). Evaluation of quorum quenching and probiotics activity of *Bacillus thuringiensis* QQ17 isolated from fish culture pond. *International Journal of Current Microbiology & Applied Science,* 8(5), 1634-1649.

Hussein, M. M. A., Hassan, W. H., & Kamel, M. O. (2017). Pathogenicity of *Aeromonas hydrophila* isolated from diseased sharp teeth catfish, *Clarias gariepinus,* with special reference to the lethality of its extracellular products. *Journal of Veterinary Medical Research,* 24(1), 114-123.

Iehata, S., Valenzuela, F., & Riquelme, C. (2013). Analysis of bacterial community and bacterial nutritional enzyme activity associated with the digestive tract of wild Chilean octopus (*Octopus mimus* Gould, 1852). *Aquaculture Research*, 46(4), 861-873.

Jiang, Q., Chen, J., Yang, C., Yin, Y., & Yao, K. (2019). Quorum sensing: a prospective therapeutic target for bacterial diseases. *BioMed Research International*, 2019, 1-15. https://doi.org/10.1155/2019/2015978

Julinta, R. B., Roy, A., Singha, J., Abraham, T. J., & Patil, P. K. (2017). Evaluation of efficacy of oxytetracycline oral and bath therapies in nile tilapia, *Oreochromis niloticus* against *Aeromonas hydrophila* infection. *International Journal of Current Microbiology & Applied Science,* 6(7), 62-76.

Laith, A. R. & Najiah, M. (2013). *Aeromonas hydrophila*: antimicrobial susceptibility and histopathology of isolates from diseased catfish, *Clarias gariepinus* (Burchell). *Journal of Aquaculture Research & Development,* 5(2), 1-7.

LaPatra, S. E., Fehringer, T. R., & Cain, K. D. (2014). A probiotics *Enterobacter* sp. provides significant protection against *Flavobacterium psychrophilum* in rainbow trout (*Oncorhynchus mykiss*) after injection by two different routes. *Aquaculture,* 433(2014), 361-366.

Lee, S. W., Sim, K. Y., Wendy, W., & Zulhisyam, A. K. (2016). *Peperomia pellucida* leaf extract as immunostimulator in controlling motile aeromonad septicemia due to *Aeromonas hydrophila* in red hybrid tilapia, *Oreochromis* spp. farming. *Veterinary World,* 9(3), 231.

Lim, S. Y., Ooi, A. L., & Wong, W. L.. (2016). Gill monogeneans of Nile tilapia (*Oreochromis niloticus*) and red hybrid tilapia *(Oreochromis* spp.) from the wild and fish farms in Perak, Malaysia: infection dynamics and spatial distribution. *Springerplus,* 5(1), 1609.

Low, K. H., Zain, S. M., Abas, M. R., Salleh, KM, & Teo, Y. Y. (2015). Distribution and health risk assessment of trace metals in freshwater tilapia from three different aquaculture sites in Jelebu Region (Malaysia). *Food Chemistry,* 177, 390-396.

Mohana, S,. Desai, C., & Madamwar, D. (2007). Biodegradation and decolourization of anaerobically treated distillery spent wash by a novel bacterial consortium. *Bioresource Technology,* 98(2), 333-339.

Musa, N., Wei, L. S., Musa, N., Hamdan, R. H., Leong, L. K., Wee, W., & Abdullah, S. Z. (2009). Streptococcosis in red hybrid tilapia (*Oreochromis niloticus*) commercial farms in Malaysia. *Aquaculture Research,* 40(5), 630-632.

Najiah, M., Aqilah, N. I., Lee, K. L., Khairulbariyyah, Z., Mithun, S., Jalal, K. C. A., & Nadirah, M. (2012). Massive mortality associated with *Streptococcus agalactiae* infection in cage-cultured red hybrid tilapia *Oreochromis niloticus* in Como River, Kenyir Lake, Malaysia. *Online Journal of Biological Sciences,* 12(8), 438-442.

Noorashikin, M. N., Li, L. Y., Karim, M., Daud, H. M., & Natrah, FMI. (2016). Screening and identification of quorum sensing degraders from live feed Artemia. *Journal of Environmental Biology,* 37(4), 811-6.

Novita, H., Rusmana, I., Yuhana, M., & Pasaribu, F. H. (2015). Potential of *Bacillus* sp., as a Producer of AHL Lactonase and its Application as a Probiotics for the Prevention of Mas in Catfish (*Clarias gariepinus*). *Journal of Fisheries & Aquatic Sciences, 10*(6), 464-476.

Novoa, B, Rodríguez, I., & Figueras, A. (2008) Immune response of zebrafish (*Danio rerio*) against a newly isolated bacterial pathogen *Aeromonas hydrophila*. *Fish & Shellfish Immunology,* 23(3), 239-249.

Oliveira, S. R., de Souza, R. T. Y. B., Brasil, E., Andrade, J. I. A., Nunes, É. D. S. S., Ono, E. A., & Affonso, E. G. (2011). LD_{50} of the bacteria *Aeromonas hydrophila* to matrinxã, *Brycon amazonicus*. *Acta Amazonica,* 41(2), 321-326.

Paluch, E., Rewak-Soroczyñska, J., Jêdrusik, I., Mazurkiewicz, E., & Jermakow, K. (2020). Prevention of biofilm formation by quorum quenching. *Applied Microbiology & Biotechnology,* 104(5), 1871-1881.

Panase, P. & Mengumphan, K. (2015). Growth performance, length-weight relationship and condition factor of backcross and reciprocal hybrid catfish reared in net cages. *International Journal of Zoological Research,* 11(2), 57-64

Pauzi, N. A., Mohamad, N., Azzam-Sayuti, M., Yasin, I. S. M., Saad, M. Z., Nasruddin, N. S., & Azmai, M. N. A. (2020). Antibiotic susceptibility and pathogenicity of *Aeromonas hydrophila* isolated from red hybrid tilapia (*Oreochromis niloticus* × *Oreochromis mossambicus*) in Malaysia. *Veterinary World,* 13(10), 2166-2171.

Peng, X, Yu, K. Q., Deng, G. H., Jiang, Y. X., Wang, Y., Zhang, G. X., & Zhou, H. W. (2013). Comparison of direct boiling method with commercial kits for extracting fecal microbiome DNA by Illumina sequencing of 16S rRNA tags. *Journal of Mirobiology Methods,* 95(3), 455-462.

Preena, P. G., Dharmaratnam, A., Raj, N. S., Raja, S. A., Nair, R. R., & Swaminathan, T. R. (2021). Antibiotic-resistant Enterobacteriaceae from diseased freshwater goldfish. *Archieves of Microbiology,* 203, 219-231. doi: 10.1007/s00203-020-02021-8.

Rajkumari, J., Choudhury, Y., Bhattacharjee, K., & Pandey, P. (2021). Rhizodegradation of pyrene by a non-pathogenic *Klebsiella pneumoniae* isolate applied with *Tagetes erecta L*. and changes in the rhizobacterial community. *Frontiers Microbiology*, 12, 593023. doi: 10.3389/fmicb.2021.593023

Ramesh, D., & Souissi, S. (2018). Antibiotic resistance and virulence traits of bacterial pathogens from infected freshwater fish, *Labeo rohita*. *Microbial Pathogenesis,* 116, 113-119.

Roche, D. M., Byers, J. T., Smith, D. S., Glansdorp, F. G., Spring, D. R., & Welch, M. (2004). Communications blackout? Do N-acylhomoserine-lactone-degrading enzymes have any role in quorum sensing? *Microbiology,* 150(7), 2023-2028.

Romero, M., Mayer, C., Muras, A., & Otero, A. (2015). Silencing Bacterial Communication Through Enzymatic Quorum-Sensing Inhibition. In: Kalia V. (eds) Quorum Sensing vs Quorum Quenching: A Battle with No End in Sight (pp. 219-236). Springer, New Delhi. https://doi.org/10.1007/978-81-322-1982-8_19

Sakr, M., Aboulwafa, M., Aboshanab, K. M., & Hassouna, N. (2014). Screening and preliminary characterization of quenching activities of soil *Bacillus* isolates against acyl homoserine lactones of clinically isolated *Pseudomonas aeruginosa*. *Malaysian Journal of Microbiology,* 10, 80-91.

Simón, R., Docando, F., Nuñez-Ortiz, N., Tafalla, C., & Díaz-Rosales, P. (2021). Mechanisms used by probiotics to confer pathogen resistance to teleost fish. *Frontiers Immunology,* 12, 653025.

Singh, V., Rathore, G., Kapoor, D., Mishra, B. N., & Lakra, W. S. (2008). Detection of aerolycin gene in *Aeromonas hydrophila* isolated from fish and pond water. *Indian Journal of Microbiology,* 48, 453-458.

Siti-Zahrah, A., Padilah, B., Azila, A., Rimatulhana, R., & Shahidan, H. (2008). Multiple streptococcal species infection in cage-cultured red tilapia but showing similar clinical signs. Diseases in Asian Aquaculture VI. Manila: Fish Health Section, Asian Fisheries Society, vol 2008. pp. 313-320

Song, X., Zhao, J., Bo, Y., Liu, Z., Wu, K., & Gong, C. (2014). *Aeromonas hydrophila* induces intestinal inflammation in grass carp (*Ctenopharyngodon idella*): an experimental model. *Aquaculture,* 434, 171-178

Tinh, N. T. N., Asanka Gunasekara, R. A. Y. S., Boon, N., Dierckens, K., Sorgeloos, P., & Bossier, P. (2007). N-acyl homoserine lactone-degrading microbial enrichment cultures isolated from *Penaeus vannamei* shrimp gut and their probiotics properties in *Brachionus plicatilis* cultures. *FEMS Microbiology Ecology,* 62(1), 45-53.

Tomás, J. M. (2012). The main *Aeromonas* pathogenic factors. *International Scholarly Research Notices,* vol. 2012, 1-22. doi:10.5402/2012/256261

Torabi Delshad, S., Soltanian, S., Sharifiyazdi, H., & Bossier, P. (2018). Effect of quorum quenching bacteria on growth, virulence factors and biofilm formation of *Yersinia ruckeri in vitro* and an *in vivo* evaluation of their probiotics effect in rainbow trout. *Journal of Fish Diseases,* 41(9), 1429-1438.

Torres, M., Romero, M., Prado, S., Dubert, J., Tahrioui, A., Otero, A., & Llamas, I. (2013). N-acylhomoserine lactone-degrading bacteria isolated from hatchery bivalve larval cultures. *Microbiological Research,* 168(9), 547-554.

Torres, M., Rubio-Portillo, E., Antón, J., Ramos-Esplá, A. A., Quesada, E., & Llamas, I. (2016). Selection of the N-acylhomoserine lactone-degrading bacterium *Alteromonas stellipolaris* PQQ-42 and of its potential for biocontrol in aquaculture. *Frontiers Microbiology,* 7, 646.

Tyagi, A., Singh, B., Billekallu, N. K., & Sing, N. K. (2019). Shotgun metagenomics offers novel insights into taxonomic compositions, metabolic pathways and antibiotic resistance genes in fish gut microbiome. *Archieves of Micrbiology,* 201, 295-303.

Vinoj, G., Vaseeharan, B., Thomas, S., Spiers, A. J., & Shanthi, S. (2014). Quorum- quenching activity of the AHL-lactonase from *Bacillus licheniformis* DAHB1 inhibits *Vibrio* biofilm formation in vitro and reduces shrimp intestinal colonisation and mortality. *Journal of Marine Biotechnology,* 16(6), 707-715.

Wanka, K. M., Damerau, T., Costas, B., Krueger, A., Schulz, C., & Wuertz, S. (2018). Isolation and characterization of native probiotics for fissh farming. *BMC Microbiology,* 18(2018), 119.

Wee, W. C., Mok, C. H., Romano, N., Ebrahimi, M., & Natrah, I. (2018). Dietary supplementation use of *Bacillus cereus* as quorum sensing degrader and their effects on growth performance and response of Malaysian giant river prawn *Macrobrachium rosenbergii* juvenile towards *Aeromonas hydrophila*. *Aquaculture Nutrition,* 24(6), 1804-1812.

Yeh, E., Pinsky, B. A., Banaei, N., & Baron, E. J. (2009). Hair sheep blood, citrated or defibrinated, fulfills all requirements of blood agar for diagnostics microbiology laboratory tests. *PloS One,* 4(7), 1-8.

Yilmaz, S., Sova, M., & Ergün, S. (2018). Antimicrobial activity of trans-cinnamic acid and commonly used antibiotics against important fish pathogens and nonpathogenic isolates. *Journal of Applied Microbiology* 125(6), 1714-1727.

Zhang, W., & Li, C. (2016). Exploiting quorum sensing interfering strategies in gram-negative bacteria for the enhancement of environmental applications. *Frontiers in Microbiology*, 6, 1-15.

Zhang, D., Xu., D H., & Shoemaker, C. (2016). Experimental induction of motile *Aeromonas septicemia* in channel catfish (*Ictalurus punctatus*) by waterborne challenge with virulent *Aeromonaas hydrophila. Aquaculture Republic*, 3, 18-23.

