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Potential of *Bacillus* sp., as a Producer of AHL Lactonase and its Application as a Probiotic for the Prevention of Mas in Catfish (*Clarias gariepinus*)

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ABSTRACT

Motile Aeromonad Septicaemia (MAS) disease in catfish (*Clarias gariepinus*) is caused by infection with *Aeromonas hydrophila*. One alternative way to control infection by this bacteria is applying probiotics, which inhibit quorum sensing, also known as Anti-Quorum Sensing (AQS), using *Bacillus* sp. administered by feeding. *Bacillus* sp. product AHL (Acyl Homoserine Lactone) Lactonase, which is speculated to be able to inhibit quorum sensing in pathogenic bacteria. *In vivo* test of probiotics was set up in triplicate and consisted of five treatments. Effect of AQS probiotic bacteria as feed supplement was set up in improving survival rate, Feed Conversion Ratio (FCR), Specific Growth Rate (SGR) and non specific immune response of catfish (*Clarias gariepinus*). The result showed that the fish feed with AQS had a high percentage of survival rate. The AQS mix of treatment ($TS1Rif^R$, $TS2Rif^R$ and $TA23Rif^R$) had the highest survival rate (93%). The AQS treatments were also had values of FCR and SGR better than that of control. However, the control treatments had survival rate only 31%. The AQS isolates could also improve a non specific immune response. Result indicated that AHLs degrading bacteria could be considered as better alternative to replace application of antibiotics in aquaculture as biocontrol of bacterial fish disease and in reducing the pathogenicity of *A. hydrophila* in catfish (*Clarias gariepinus*).

Key words: Probiotic, anti-quorum sensing, lactonase, *Bacillus* sp., *Aeromonas hydrophila*

INTRODUCTION

Clarias gariepinus, more commonly known as catfish, is an important commercial fish in freshwater fish cultivation in Indonesia. Disease is one of the obstacles faced in catfish cultivation in the intensive culture system. For example, the Motile Aeromonad Septicaemia (MAS), which is caused by infection with *Aeromonas hydrophila*. Efforts to control this disease include using antibiotics, however, the continuous use of antibiotics resulted in bacterial resistance to that particular antibiotic. Application of probiotics through feed could improve fish immunity and therefore protecting the fish from bacterial infection. Many studies have been conducted regarding, the use of probiotics to supplement feed, to improve resistance to disease and to improve growth performance (Nayak, 2010). Probiotics can also function as an immunostimulant, improve the feed

conversion rate, inhibit the growth of pathogenic bacteria, produce antibiotics and improve water quality (Kesarcodi-Watson *et al*., 2008).

In order to inhibit the growth of pathogenic bacteria in catfish cultivation, bacteria that can function as a probiotic with a virulence-inhibiting mechanism, which disrupts the quorum sensing system known as Anti Quorum Sensing (AQS) or Quorum Quenching (QQ) mechanism can be utilized. The disruption of the communication system could be developed into a novel strategy to inhibit the expression of virulence and to prevent bacterial infection. The biocontrol strategy based on the QQ mechanism is a sustainable method because it is a selective pressure which is limited to the survival of the microbes; superior to treatments using biocides (Uroz *et al.,* 2009).

The quorum sensing system controls the bacteria's behaviour through the alteration in the expression of genes by the signal molecule. This gene expression is regulated by a communication signal between bacterial cells which is known as Quorum Sensing (QS) (Dong *et al*., 2007; Defoirdt *et al*., 2004). The quorum sensing activity in bacteria is the bacteria's response to the environment which often changes quickly. This response is very useful in ensuring the bacteria's survival. The response could be an adaptation to the presence of nutrition, defence against other microorganisms which also use the same source of nutrition, or avoidance of toxic substances which are dangerous to the bacteria (Miller and Bassler, 2001). Quorum sensing in pathogens improve the chance of infecting a host by postponing the production of their virulence factor until the population is dense enough to affect the host's immune system (Donabedian, 2003).

The application of bacteria which produce QQ effects have often been studied in aquaculture (Chu *et al*., 2011; Defoirdt *et al*., 2006, 2008), such as *Halogenated furanones* from the red algae *Delisea pulchra* (Manefield *et al*., 1999) to protect rotifers and protecting artemia and rainbow trout from *Vibrio* (Defoirdt *et al*., 2006; Rasch *et al*., 2004). Furanone, which has a similar structure, as the AHL molecule (acyl homoserine lactone), which binds the LuxR protein (Manefield *et al*., 1999) as in *Vibrio harveyi* (Henke and Bassler, 2004). Recombinant AHL-lactonase from *Bacillus* sp., which is injected together with the fish pathogen *A. hydrophila* to carp, could decrease the fish mortality rate (Chen *et al*., 2010) is another examples. The actinobacteria *Streptomyces albus* is able to reduce the formation of biofilm and inhibit the quorum-sensing system of *V. harveyi, V. vulnificus* and *V. anguillarum* (You *et al*., 2007).

Quorum-sensing bacteria can be isolated from catfish cultivation environment, meaning that the pathogen and the fish have similar environments. One of the diseases that often attack catfish is MAS or red spot disease caused by *A. hydrophila*. In this study, bacterial isolates were tested for their potential as AQS to inhibit the pathogenic bacteria's virulence factor which causes disease in catfish. The AQS bacteria isolates can be applied as a probiotic for catfish.

MATERIALS AND METHODS

Culturing bacteria: The AQS bacteria used in this study consisted of 3 isolates of *Bacillus* sp., TS1 (*Bacillus amyloliquefaciens*), TS2 (*Lysinnibacillus sphaericus*) and TA 23 (*B. cereus*). The pathogenic bacterium used was *A. hydrophila*, a collection of the Freshwater Fish Cultivation Research and Development Research Station, Bogor. The three *Bacillus* sp. isolates, *A. hydrophila* and *Chromobacterium violaceum,* they were cultured on Triptone Soya Agar (TSA) media and were propagated in Triptone Soya Broth (TSB). The *Bacillus* sp. cultures were incubated for 48 h, whereas *A. hydrophila* and *C. violaceum* were incubated for 24 h.

AQS isolate verification: Selection of AQS bacteria isolates was done using the *C. violaceum* biosensor as the AQS marker on Luria Agar (LA) medium (McClean *et al*., 1997). The purified AQS

bacteria isolates were cultured on Luria Broth (LB) medium. After 48 h, the culture was centrifuged at 10000×g for 10 min. One hundred micorlitre of the supernatant was dripped on to a paper disc placed on the LA medium on a dish containing the *C. violaceum* biosensor (aged 16-18 h). Sterile LB medium was used as the negative control. The Petri dishes were incubated at 28°C for 24 h to see the inhibition of the production of the *C. violaceum* purple coloring; QS inhibition was indicated by the presence of a non-purple ring around the disc.

Rifampicin-resistant marking of AQS bacterial isolate: One milliliter of suspension of wild-type AQS bacteria cells which were sensitive to the antibiotic rifampicin was concentrated and then diluted with 100 μL physiological sodium solution and spread on a LB medium which had been enhanced with 50 and 100 μg mL^{-1} rifampicin to obtain mutant AQS RFR bacteria. The stability of the AQS Rf^R mutant bacteria was tested by sub-culturing the mutant bacteria on an LA medium which contained 100 μg mL^{-1} rifampicin after 48 h three times consecutively. In order to obtain the growth ratio between the mutant and the wild type, the growth curve was determined for both the AQS Rf^R bacteria and the wild type. The bacteria were cultured on LB medium, which contained 100 μg mL^{-1} rifampicin for the mutants and LB medium without Rifampicin for the wild type. The growth curve was measured through the cells' optic density at 620 nm every two hours for 24 h until the stationary phase was reached.

***In vitro* testing of the AQS Rf^R bacterial isolates against *A. hydrophila*:** This test was conducted to observe the ability of each AQS bacterial isolate to inhibit *A. hydrophila's* quorum sensing *in vitro*. Each of the AQS Rf^R bacterial isolates were cultured on TSA media. On the petri dish containing TSA medium, 100 μL of *A. hydrophila* 10^8 CFU mL^{-1} was spread and each AQS Rf^R isolate was dotted onto the TSA medium that had *A. hydrophila* spread on it.

Preparation of the test feed: The feed used in this study was commercial feed with a protein content of 28.60%. The feed was prepared by adding AQS Rf^R bacteria based on the 1% treatment dosage (Wang, 2007) and 2% (V/w) egg white, which were then sprayed on and air-dried. The control pellets were sprayed with PBS and 2% (V/w) egg white and air-dried. Daily rations were administered at a Feeding Rate (FR) of 3% of the experimental animal's biomass weight. The feed was administered three times a day (morning, noon and afternoon) for 21 days.

***In vivo* testing of AQS Rf^R bacteria:** The experimental animals were African catfish (*Clarias gariepinus*) with a body length of 10.75±0.5 cm (Mean±SD) and weighed 10.685± 0.2 g (Mean±SD). The experiments were conducted in plastic holding tanks measuring 60×50×40 cm^3 filled up with 40 L of waters and 25 catfish per tank. Feed was given *ad satiation* (until the fish were full) every morning, noon and afternoon. During the course of the experiment (21 days), the aquariums were not siphoned nor was the water changed.

Three hundred and seventy five catfish were distributed equally into 15 tanks which clustered into 5 groups. Group A, B and C received 1% (1 mL per 100 g feed) of TS1 Rif^R, TS2 Rif^R and TA23 Rif^R, respectively, whereas group D received 1% of equal combination of TS1 Rif^R, TS2 Rif^R and TA23 Rif^R and group K received commercial feed only as a control. At 14 days post-treatment (dpt), the fish were challenged with *A. hydrophila* by immersion at a dose of 4×10^8 CFU per tank. All fish were monitored for SR, SGR and FCR for another 7 days. At 0, 4, 7, 14 and 21 dpt, blood was collected from three of fish from each tank and used for measuring fish non-specific immune responses.

The survival rate of fish was calculated equation developed by Boujard *et al.* (2002).

To obtain Specific Growth Rate (SGR), the fish were weighed every 7 days and SGR was calculated (Huisman and Richter, 1987). The Feed Conversion Ratio (FCR) was calculated from the amount of fish biomass which was produced compared to the amount of feed given. The feed conversion ratio was calculated (Takeuchi, 1988). Phagocytic activity was measured according to Anderson and Siwicki (1995). Lysozyme activity was tested using catfish serum according to a method by Ellis (1990).

Statistical analysis: A one way ANOVA and Duncan's post hoc test were run for each experiment using, the statistical program SPSS 16.0. Significant differences between groups were accepted at $p<0.05$.

RESULTS

The isolates that have AHL degradation activity are signified by the formation of a non-purple zone around the paper disc that had culture supernatant of the bacterial isolate dripped onto it. This demonstrates that the bacterial isolate produces an extracellular substance which inhibits *C. violaceum's* production of violacein pigment, a product of the quorum sensing process. Verification results of the AQS isolates demonstrated that the three *Bacillus* sp. isolates were able to inhibit the production of the violacein pigment by *C. violaceum* (Fig. 1).

Marking of rifampicin resistant AQS bacterial isolates: Mutant AQS isolates that are resistant to rifampicin (Rif^R) can be obtained, using the spontaneous resistance method for TS1, TS2 and TA23. Mutant AQS-Rif^R can grow in a LA medium containing 100 μg mL^{-1} rifampicin. The growth curves of wild type TS1, TS2 and TA23 are almost the same with those of the mutant. These demonstrates that spontaneous rifampicin resistance does not inhibit the growth of the bacteria in a medium containing rifampicin. Mutant AQS Rif^R isolates can grow normally and can proliferate as easily as the wild types in media not containing rifampicin (Fig. 2).

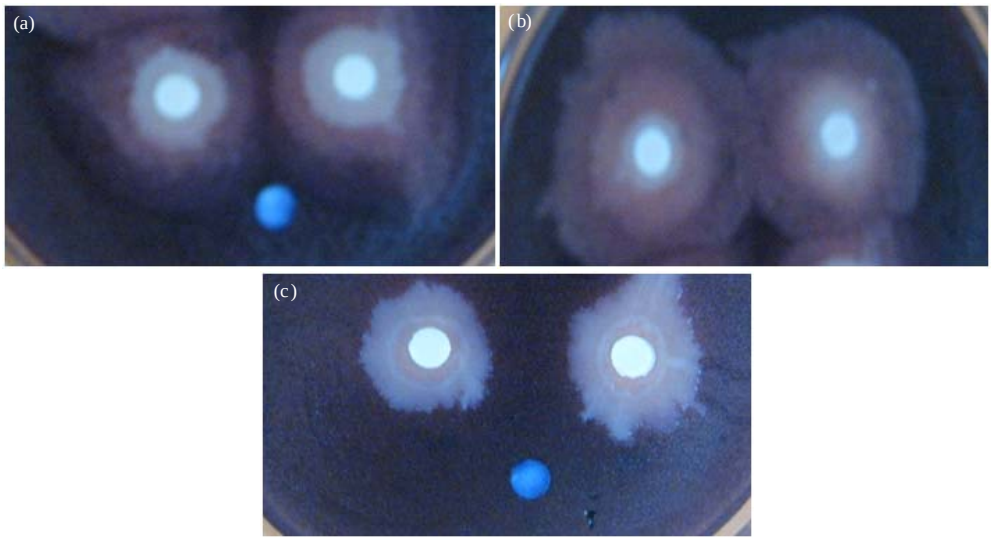

Fig. 1(a-c): Antiquorum sensing activity of isolates (a) TS2, (b) TS1 and (c) TA 23

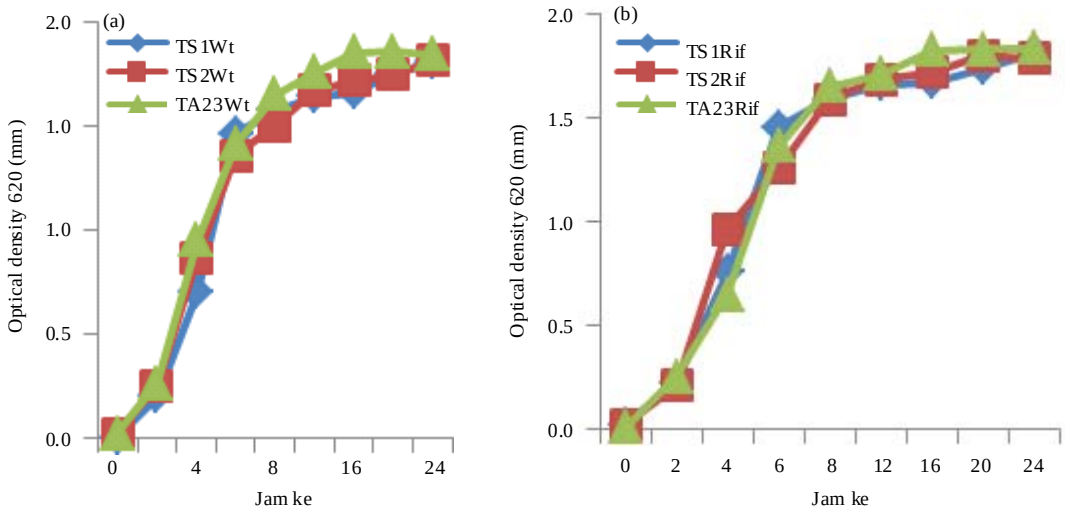

Fig. 2(a-b): Comparison between the growth curves of wild-type (a) AQS isolates and (b) Rifampicin mutants (WT= wild type and Rif = rifampicin mutant)

***In vitro* testing of QQ Rif^R bacterial isolates against *A. hydrophila*:** Co-culturing of AQS Rif^R and *A. hydrophila* was done to evaluate the interaction between two organisms through the process of antagonism and competition in an environment with limited nutrition in order to discover the interaction process between *A. hydrophila* as the pathogen and AQS Rif^R isolate as the biocontrol candidate. The results of the experimental co-culturing of *A. hydrophila* and AQSRif^R bacteria demonstrate that the two isolates do not inhibit each others growth and that they could grow side-by-side because there were no antagonistic activity, as there was no inhibition zone exhibited (Fig. 3). Therefore, the interaction process between the three AQS Rif^R isolates and *A. hydrophila* as the pathogen demonstrated that the AQS Rif^R isolates could be used to inhibit *A. hydrophila*'s pathogenicity without inhibiting its growth.

Catfish survival rate: This *in vivo* testing was conducted to test the potential of the AQS bacteria as a probiotic in inhibiting the growth of *A. hydrophila* and preventing MAS in catfish. The SR of fish for each treatment is presented in Fig. 4. The results demonstrated that the survival rate of fish in AQS bacteria treated groups (A, B, C and D) were significantly higher ($p<0.05$) than group K (control), indicating that AQS bacteria provide protection of fish against *A. hydrophila* infection. Moreover, group D has significantly higher survival rate of catfish ($p<0.05$) than other AQS bacteria treated groups, suggesting that the combination of three AQS bacteria is more effective in protecting catfish than single AQS bacterium.

Specific Growth Rate (SGR): The SGR of fish for each treatment can be seen in Fig. 5. As shown in Fig. 5, the growth rate of catfish in all treated groups (A, B, C and D) was significantly higher ($p<0.05$) than K (control group). However, there was no statistically difference ($p>0.05$) in the SGR between treatment A, B, C and D, suggesting that AQS Rif^R strengthened immune system which in turn increased the growth rate. This was in line with the results of the study by Sun *et al.* (2010), who stated that the probiotics *Bacillus pumilus* and *Bacillus clausii* could improve the

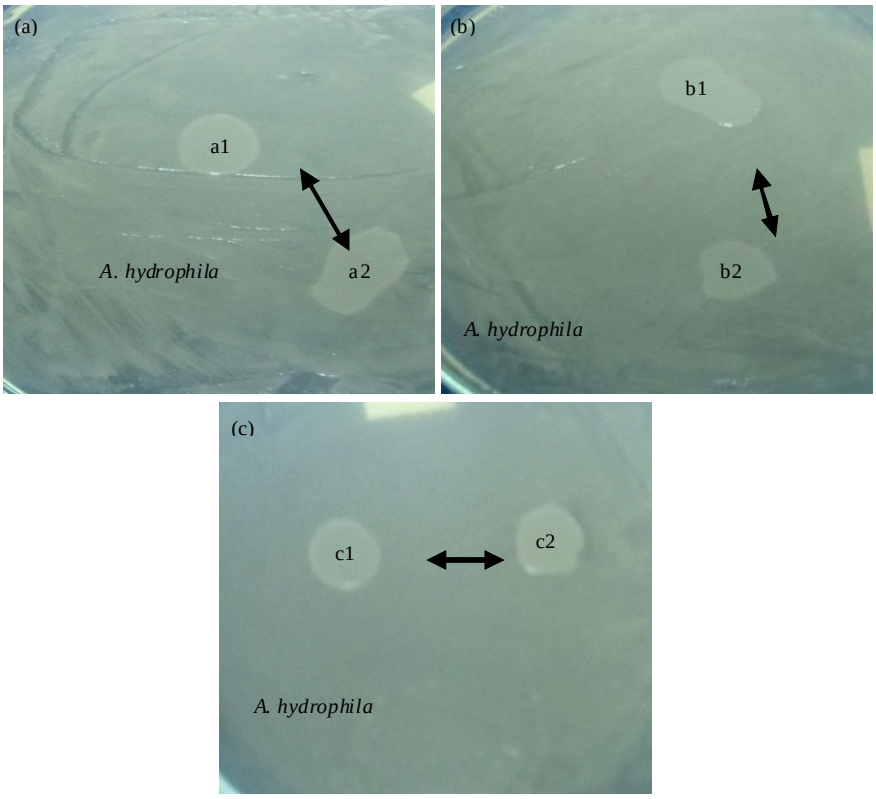

Fig. 3(a-c): Co-culturing of AQS Rif^R isolates, (a) TS1, (b) TS2 and (c) TA23 with *A. hydrophila*

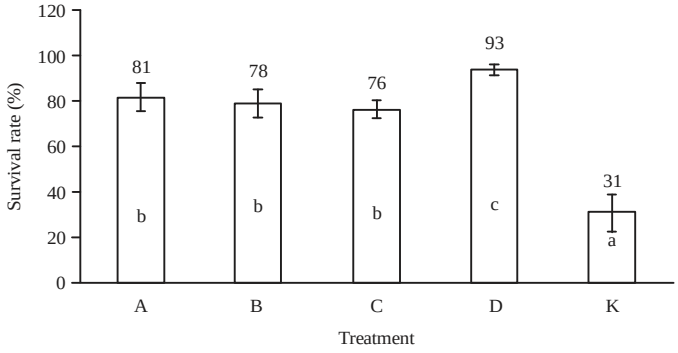

Fig. 4: Survival rate of catfish (*C. gariepinus*) following treatment with AQS-Rif^R bacteria as probiotic and challenged with *A. hydrophila* A (TS1Rif^R), B (TS2Rif^R), C (TA23Rif^R), D (combination of TS1Rif^R, TS2Rif^R and TA23Rif^R) and K (control). Data is Means±SD of 3 tanks per group. Different letters on the bars indicate a statistical difference ($p<0.05$) between the groups

performance and immune responses in orange-spotted grouper, *Epinephelus coloides*. The probiotic *Bacillus* spp. applied via water could improve the growth and survival rate of Indian white shrimp (*Fenneropenaeus indicus*) (Ziaei-Nejad *et al*., 2006).

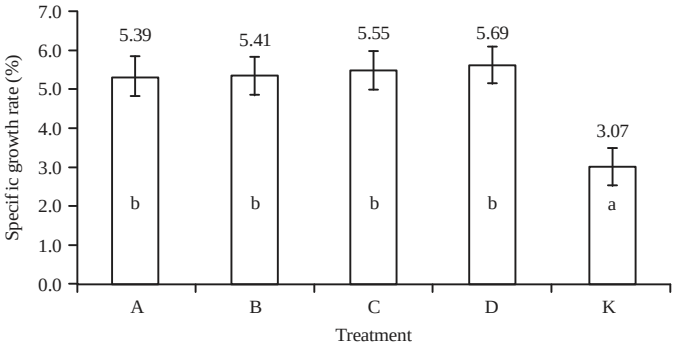

Fig. 5: Specific Growth Rate of catfish (*C. gariepinus*) treated with AQS bacteria as a probiotic and challenged with *A. hydrophila*. A(TS1Rif^R), B(TS2Rif^R), C(TA23Rif^R) and D (combination of TS1Rif^R, TS2Rif^R and TA23Rif^R) and K (control). Data is Means±SD of 3 tanks per group. Different letters on the bars indicate a statistical difference ($p<0.05$) between the groups

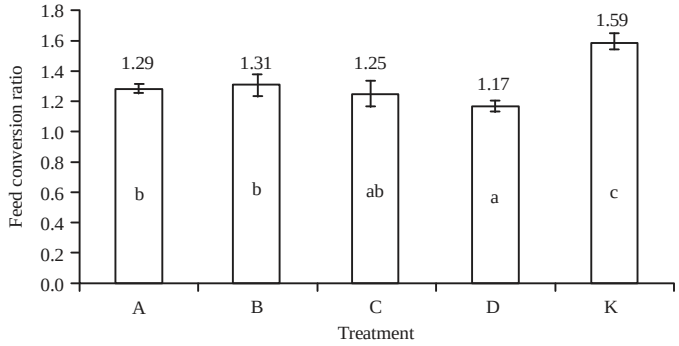

Fig. 6: Feed Conversion Ratio for African catfish (*C. gariepinus*) treated with AQS Rif^R and challenged with *A. hydrophila*. A(TS1Rif^R), B(TS2Rif^R), C(TA23Rif^R), D (combination of TS1Rif^R, TS2Rif^R and TA23Rif^R) and K (control), Data is Means±SD of 3 tanks per group. Different letters on the bars indicate a statistical difference ($p<0.05$) between the groups

Feed Conversion Ratio (FCR): The feed conversion ratio for each treatment is presented in Fig. 6. The results demonstrate that the FCR for treatment A (1.29), B (1.31), C (1.25) and D (1.17) were significantly lower ($p<0.05$) than K (control;1.59). There was no significant difference in FCR between treatments A, B and C but treatment D was significantly lower than the other treatments, indicating that the combination of TS1Rif^R, TS2Rif^{R}and TA23Rif^{R}provided more efficient FCR than single use of either of TS1Rif^R, TS2Rif^R and TA23Rif^R. The fish in each treatment responded well to the feed. The addition of a probiotic to the feed had an effect on the FCR and this is in line with Gatesoupe (1999) that the FCR is not only affected by the amount of feed given but also by several other factors, such as; density, individual weight, the animal's age group, water temperature and feed administration method (feed quality, feed placement and feeding frequency).

Phagocytic index: The phagocytic index for each treatment is presented in Fig. 7. The results show that the phagocytic index prior to the probiotic treatment in each group (0 dpt) was not significantly different ($p>0.05$), indicating that all the fish used for the experiment have the same

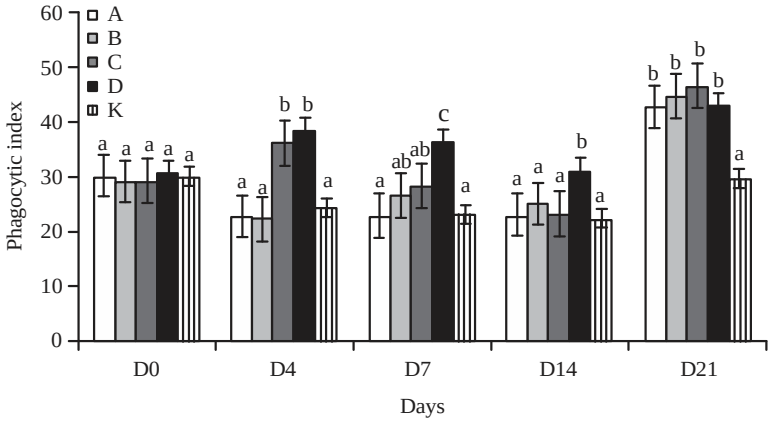

Fig. 7: Phagocytic index for catfish (*C. gariepinus*) treated with AQSRif^R as probiotic and challenged with *A. hydrophila.* A(TS1Rif^R), B(TS2Rif^R), C(TA23Rif^R), D (combination of TS1Rif^R, TS2Rif^R and TA23Rif^R) and K (control). Data is Means±SD of 3 tanks per group. Different letters on the bars indicate a statistical difference ($p<0.05$) between the groups

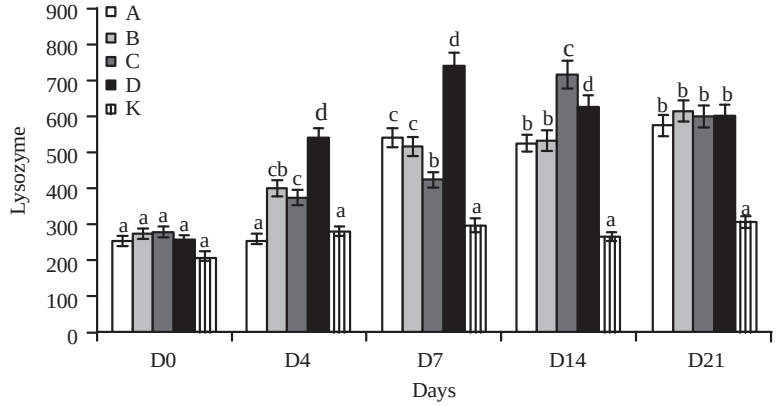

Fig. 8: Lysozyme levels in catfish (*C. gariepinus*) treated with AQS Rif^R as probiotic and challenged with *A. hydrophila* A(TS1Rif^R), B(TS2Rif^R), C(TA23Rif^R), D (combination of TS1Rif^R, TS2Rif^R and TA23Rif^R) and K (control). Data is Means±SD of 3 tanks per group. Different letters on the bars indicate a statistical difference ($p<0.05$) between the groups

phagocytic index and that the differences observed during the course of the experiment are due to the treatment. Subsequent to the administration of the probiotic, the phagocytic index increased at 4, 7 and 14 dpt and continued until after the challenge test (21 dpt). After being challenged with *A. hydrophila,* there was a significant difference ($p<0.05$) in the phagocytic index in treatments (A, B, C and D) and the control (K), whereas among the treatments themselves they were not significantly different. The addition of AQS the probiotic to feed had an effect on the phagocytic index, particularly after the treated catfish were challenged with *A. hydrophila*.

Lysozyme: Lysozyme levels for each treatment is presented in Fig. 8. The results demonstrated that the lysozyme levels in the catfish before the probiotic treatment 0 dpt for each treatment were not significantly different ($p>0.05$). The lysozyme level in the fish after the probiotic treatment

4, 7 and 14 dpt for treatments A, B, C and D were significantly different ($p<0.05$) from that of the control (K). Subsequent to the challenge test with *A. hydrophila,* the levels for treatments A, B, C and D, were significantly higher ($p<0.05$) than that of the control (K).

DISCUSSION

The bioassay conducted on the three *Bacillus* isolates (TS1, TS2 and TA23) demonstrate that the isolates are able to inhibit the formation of the purple pigment in *C. violaceum*. The purple pigment is an antimicrobial substance called violacein (McClean *et al.,* 1997). The production of the pigment violacein in *C. violaceum* is regulated by the QS process. The non-purple zone around the paper disc that had been saturated with the supernatant of the Bacillus TS1, TS2 and TA23 cultures demonstrate the inhibition of QS which regulates the production of violacein. QS in *C. violaceum* involves AHL which functions as a signal molecule. The inhibition of the QS is caused by the lactonase enzyme (an AHL molecule hydrolyzer) produced by the three *Bacillus* isolates.

The higher survival rates in both the singular treatments A ($TS1Rif^R$, 81%), B ($TS2Rif^R$, 78%) and C ($TA23Rif^R$, 76%) and the multiple treatment D (combination of $TS1Rif^R$, $STS2Rif^R$ and $TA23Rif^R$, 93%) compared to that of the control (K, 31%) may be due to the *A. hydrophila* in the water was not able to cause illness in the catfish because the AQS Rif^R bacteria could weaken the bacterium virulence factor so that the *A. hydrophila* was not pathogenic to the catfish and that the role of the bacteria AQS Rif^R as a probiotic, was significant in the prevention of MAS and in reducing the mortality of the catfish.

Based on the in vivo test, the application of the AHL-degrading bacteria or the AQS had a positive effect on the survival rate of fish. A similar result was obtained from other AHL-degrading bacteria; they could increase the survival rate of aquatic organisms against pathogens, for example turbot larvae *Scophthalmus maximus* (Tinh *et al.,* 2008) and freshwater prawns *Macrobrachium rosenbergii* (Nhan *et al*., 2010) against *V. harveyi* and zebrafish against *A. hydrophila* (Nhan *et al*., 2010). In catfish cultivation system, especially in fish seed production, improvement of the survival rate and larvae quality are very important in order to improve the economic efficiency of the production line. The abuse of antibiotics in aquaculture has made it no longer effective in controlling *A. hydrophila*. The application of AHL-degrading or AQS bacteria could increase aquaculture production in a more sustainable way by replacing antibiotics for disease control (Nhan *et al*., 2010).

The QQ or AQS enzyme produced by microorganisms could reduce AHLs and prevent pathogenic bacteria from producing their virulence factor or from forming biofilm and inhibit virulence. Therefore, QQ microorganisms could be utilized as quorum sensing quenchers against pathogenic bacteria. Bacteria, which have quorum sensing activity, *Bacillus* sp. QSI-125, could reduce the AHL signal and disrupt QS function of *A. hydrophila*, which would reduce its pathogenicity, thus significantly, reducing the zebrafish mortality of zebra fish due to *A. hydrophila* infection (Chu *et al.,* 2014). This study demonstrates the potential of AQS bacteria as an environmentally friendly alternative to antibiotics in aquaculture.

The QS inhibition does not interfere with the growth of *A. hydrophila* (Manefield *et al*., 2002); therefore, the development of resistance is less likely compared to using conventional antibiotics. Because the AQS substance weakens the virulence of pathogenic bacteria without affecting its growth, it can be used as an antipathogenic as opposed to an antibacterial (Hentzer and Givskov, 2003). Antipathogenics target regulation system of the pathogenic bacteria which controls the expression of the virulence factor. This substance does not exert selective pressure for the

development of resistance and can be applied as a sustainable biocontrol strategy. Thus AQS is useful for controlling MAS in catfish cultivation system. The AQS substance only targets signal production, signal detection, or transduction signals. Bacteria that have the ability to degrade QS molecules can be applied as biocontrol agents in aquaculture. Some studies that are related to AHL by *Bacillus* spp. degradation (Dong *et al.,* 2000, 2005; Molina *et al*., 2003) demonstrate the role of *Bacillus* spp. role as a probiotic in fish cultivation system (Moriarty, 1998; Rengpipat *et al*., 2003) by inactivating the quorum sensing molecule in producing a substance that inhibits bacterial growth.

A significant increase observed in the growth parameters (SR and SGR) and low FCR in fish fed with AQS bacteria demonstrate the ability of the AQS isolate to increase fish growth. The increase in the fish body demonstrates the fish ability to utilize the feed. Feed conversion is the amount of feed needed to produce one kilogram of body weight. The lower FCR is, the more efficient the feed is utilized in growth. According to Rahiman *et al.* (2010), the FCR is inverse to the fish weight gain; the lower the ratio is, the better the quality of the feed and the more efficient the fish are in utilizing the feed consumed for growth. Similar results were reported by Rahiman *et al.* (2010), who described an increase in weight and SGR in catfish fed with AQS-laced feed without a significant improvement in the SR. An increased in SGR and FCR in *Penaeus monodon* and *Penaeus indicus* when fed with feed containing *Bacillus* spp. which increased digestive activities involving vitamin and co-factor synthesis and enzymatic activity (Gatesoupe, 1999; Zimmer and Gibson, 1998; Ziaei-Nejad *et al*., 2006; Wang, 2007). This could increase the absorption of nutrients, resulting in better growth.

In aquatic environments, the animal intestinal microbiota is dominated by Gram negatives from the *Aeromonas* and *Pseudomonas* group (Vine *et al*., 2006) and some studies have demonstrated that the microbiota could be suppressed by adding Gram positive bacteria (Ziaei-Nejad *et al*., 2006; Vieira *et al.,* 2008, 2010). The administration of AQS Rif^R significantly reduced the catfish mortality rate and improved growth. These results offered a new explanation of how probiotics suppress the bacteria QS system; a new non-antibiotic method of preventing bacterial diseases in fish cultivation system. The results of this study demonstrate a significant decrease in the QS expression of pathogenic bacteria without interfering with its growth. In this study, AQS bacteria administered through feed could control the pathogenic bacteria *A. hydrophila*. The effect of AQS bacteria on the survival rate and growth rate of catfish in general was quite high after the challenge test with *A. hydrophila*. The high survival rate is possibly caused by the good quality of the environment which in turn would improve fish immunity. In this study, the survival rate and growth rate of fish after the challenge test were improved by the administration of the AQS isolates through feed compared to those of the control.

Increased immunity of fish can be seen by the increased activity of phagocytic cells. Phagocytic cells perform phagocytosis of foreign objects entering the fish body (Treves-Brown, 2009). Phagocytosis is the first line of defence of cellular responses performed by monocytes (macrophages) and granulocytes (neutrophils) by Tizard (1988). Phagocytosis is one of the most important elements in the immune system. This process provides immediate and effective protection against infection. Immunity mechanisms in the body consist of three important stages: (1) Identification of the adversary. In this case the adversary is antigens (microorganisms), either bacteria or viruses, (2) Destruction of antigens by the immune system and (3) Restoration of the normal condition (Roth *et al*., 1988).

Probiotics in feed have a profound in the lysozyme level of fish following challenge with pathogenic bacteria. Based on results, the administration of AQS Rif^R is effective in increasing the

lysozyme activity. The level of the lysozyme activity shows that there is an increased in immune response activity, an indicator for non-specific humoral immune responses (Harikrishnan *et al.,* 2011). Lysozyme enzyme works by dissolving bacterial cell walls by hydrolyzing N-acetylglucosamine and N-acetylmuramic acids in the peptidoglycan damaging the cell wall and therefore killing, the bacteria. Lysozyme is the main defense factor of humoral immunity in the cellular defense mechanism and its ability to break down the cellular walls of pathogens enable lysozyme to naturally work against pathogenic microorganisms such as parasites, bacteria and viruses (Harikrishnan *et al.,* 2011). This study demonstrates that increased lysozyme activity protects the catfish from *A. hydrophila* infection, high lighting the important role of lysozyme in the defense mechanism against infectious diseases in fish.

CONCLUSION

Based on the study that has been conducted, infection by *A. hydrophila,* which causes MAS, could be inhibited using AQS bacteria administered through fish feed as indicated by higher survival rates of fish in treatment D (93%) compared to that of the control K (31%). A significant increase in SGR, low FCR and specific immune responses observed in the fish fed with AQS-containing feed demonstrate that AQS.

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