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The Utilization of Native Freshwater Mussel *Pilsbryoconcha exilis* as Biocontrol of Pathogenic Bacteria *Aeromonas hydrophila* in Tilapia Aquaculture

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ABSTRACT

This research aims to evaluate the capacity of freshwater mussel *Pilsbryoconcha exilis* as a biocontrol agent to prevent the transmission of *Aeromonas hydrophila* in tilapia cultivation. Briefly, 10 tilapia fish with average bodyweight 7,88±0,25 g were subjected to four treatments in the 8-liter aquarium with three replications in a randomized design trial. The treatments were M1 (one mussel for a two-liter of water), M2 (two mussels for a two-liter of water), and two control treatments without mussel (M+ and M-). All treatments, except the M-, then challenged by adding *A. hydrophila* live culture to obtain a final density of 10^5 CFU mL^{-1} into the aquarium for 7 days duration. The final survival rate of fish, the water-total bacterial count, and the blood profile of animals were assessed. The research revealed that there is a significant impact from the presence of freshwater mussel on tilapia cultivation. Generally, the M2 treatment showed better results with a significant different ($P<0,05$) according to the survival rate of fish (100±0,00%), water-total bacterial count (4,53±0,03 log CFU mL^{-1}), and fish leucocytes (4,30±0,70x10^4 cell mm^{-3}). Nonetheless, there was no different ($P>0,05$) effect on fish erythrocytes among the treatments. Therefore, the feeding activity of freshwater mussel in the water column able to deplete pathogenic bacteria abundance and prevent pathogen transmission along with increasing the survival rate of fish.

Keywords *: native mussel, biocontrol, feeding activity, pathogen depletion*

ABSTRAK

Penelitian ini bertujuan untuk mengevaluasi kapasitas kerang air tawar *Pilsbryoconcha exilis* sebagai agen biokontrol untuk mencegah penularan *Aeromonas hydrophila* dalam budidaya nila. Secara singkat, 10 ikan nila dengan berat badan rata-rata 7,88 ± 0,25 g menjadi sasaran empat perlakuan di akuarium 8 liter dengan tiga ulangan dengan uji coba desain acak. Perlakuannya adalah M1 (satu kerang untuk air dua liter), M2 (dua kerang untuk air dua liter), dan dua perlakuan kontrol tanpa kerang (M + dan M-). Semua perlakuan, kecuali M-, kemudian diuji dengan menambahkan *A. hydrophila* untuk mendapatkan kepadatan akhir 105 CFU mL^{-1} ke dalam akuarium selama 7 hari. Penilaian dilakukan untuk tingkat kelangsungan hidup akhir ikan, jumlah bakteri total air, dan profil darah hewan. Penelitian ini mengungkapkan bahwa ada dampak yang signifikan dari keberadaan kerang air tawar pada budidaya nila. Secara umum, perlakuan M2 menunjukkan hasil yang lebih baik dengan perbedaan yang signifikan ($P <0,05$)dengan tingkat kelangsungan hidup ikan (100 ± 0,00%), jumlah bakteri total di dalam air (4,53 ± 0,03 log CFU mL^{-1}), dan leukosit ikan (4,30 ± 0,70x104 sel mm^{-3}). Meskipun demikian, tidak ada perbedaan ($P> 0,05$) efek pada eritrosit ikan di antara perlakuan. Oleh karena itu, aktivitas makan kerang air tawar di kolom air mampu menekan kelimpahan bakteri patogen dan mencegah penularan patogen bersamaan dengan meningkatkan tingkat kelangsungan hidup ikan.

Kata Kunci *: native mussel, biokontrol, aktivitas makan, patogen*

1. Introduction

Tilapia *O. niloticus* is one of the major aquaculture commodities in Indonesia wherein 2016 there was an increasing domestic production as 14,73% reaching 1.187.812 mt compared to the previous year's production. However, this rate was not able to achieve a proposed target in 2017 where the domestic production had expected reaching 1.246.278 mt (KKP 2018). Environmental alteration and disease outbreak are somewhat relevant to production failure because most of the tilapia production in Indonesia is outdoor activities and often experienced unpredictable outcomes when these factors interacted. The dynamic of the environment able to shape the disease severity when the pathogenic agents are present in the surrounding of a susceptible population (Raiha *et al.* 2019).

Motile aeromonad septicemia (MAS) which caused by *A. hydrophila* infection was responsible for the death of 60% of tilapia in Kutai Kartanegara, East Kalimantan (Hardi *et al.* 2014) and infected as many as 22.22% of tilapia in the Lake Tondano (Tantu *et al.* 2013). Common symptoms of fish infected with MAS are hemorrhagic, melanization to the fish body, dropsy, and exophthalmia (Rosidah *et al*. 2018). Consequently, this infection often results in lower fish growth rates and significant economic losses in the national aquaculture industry.

Infections in aquaculture industries usually prevented and treated with chemicals, antibiotics and vaccinations (Austin and Austin 2007), which is relatively expensive or often followed by secondary problems such as increasing the antimicrobial resistance (AMR) incidence in aquaculture (Henriksson *et al*. 2017) and spill-over of the residues into the environment (Martinez, 2009). The indispensable exploration of ecologically and sustainable methods for preventing diseases in aquaculture production is necessary.

The ecological approach in aquaculture disease management is gaining attention among the scientist since it is offering an environmental friendly practice and propose the aquaculture environment health. The feeding activities of various bivalve in reducing or removing waterborne pathogen from the aquatic environment are notable and have growing literature in many publications. The presence of blue mussel *Mytilus edulis* proved to lead to the rapid removal of parasitic *Paramoeba perurans* the causative agent of salmonid amoebic gill disease (AGD) from the water column in an experimental nature (Rolin *et al*. 2016), and also ingested the infective stage of sea lice *Lepeoptheirus salmonis* in a lab study (Molloy *et al.* 2011).

Freshwater mussel *Pilsbryoconcha exilis* is a native bivalve in Indonesia that naturally dwelling in the aquaculture environment. Dissimilar to the marine mussels that gained considerable studies, this edible-freshwater mussel is in lack of attention and lack of utilization to be incorporated in aquaculture management by the local institution or by fish farmers. Since the feeding activity of mussel able to deplete the organic suspension in the water column, preventive measures in managing disease is promising. This research will evaluate the biological feasibility and utilization of *P. exilis* in the reduction of pathogenic bacteria abundance *A. hydrophila* in the tilapia cultivation system.

2. Material and Methods

2.1. Fish and freshwater mussels stocking

The juvenile of tilapia (*Nirwana strain*) with average bodyweight 7,88±0,25 g obtained from a local fish farms in the Sukabumi district were pooled and reared in 3x2x0,8 m concrete tank and then every 10 fishes carefully transferred to the 8 L-aquarium filled with filtered water for 7 days acclimation. The fishes were fed with a commercial diet (30% total protein) twice a day at satiation and provided with adequate aeration. The freshwater mussels with average bodyweight 72,27±1,65 g obtained from the ponds of the experimental facility, Fisheries and Marine Science IPB University. The mussels were pooled and reared in separate 3x2x0,8 m concrete tank which filled with naturally enriched- ponds water.

2.2. The pathogen *A. hydrophila*

A local isolate of *A. hydrophila* obtained from the Fish Health Laboratory of Aquaculture Department, IPB University. Prior to use in the experiment, the isolate was axenically cultured overnight in a slant medium of trypticase soy agar (TSA, Himedia, India) at room temperature and subsequently recultured in 8 ml trypticase soy broth (TSB, Himedia, India) for another overnight before harvested and used in a passage trial to recover the virulence. The passage has done to the African catfish juvenile by mean injecting the live cells of pathogen intraperitoneally at dose 10^5 CFU ml^{-1} ind^{-1}. The fish that showed the symptoms then sacrificed and dissected to

reisolated the pathogenic *A. hydrophila* from the kidney to the Rimler-Shotts (RS) medium (Himedia, India). The yellowish colonies in RS medium then purified in the TSA medium and a confirmation test was done by using KIT API 20E (Biomerieux, France). Regular maintenance of the selected and the confirmed colonies of *A. hydrophila* was performed in a slant agar of TSA, while to obtain a mass of pathogen live cells were conducted by harvesting an overnight culture of *A. hydrophila* in 50 ml of TSB medium

2.3. Experimental design

The experiment was performed in a set aquarium within randomized design with three replications. The animals were left for another 48 hours for acclimation. Afterwards, once stable condition achieved (noticeable by no mortalities among animals) the treatment then run for total 14 days duration. The fishes fed with commercial diets containing 30% total protein twice a day at satiation and provided with adequate aeration. The cohabitation of fish and mussels was conducted by adding 10 fishes for each aquarium and the mussels density was set according to **Table 1** below.

Table 1. Detail of experimental design

Code	Description of treatments
M+	Positive control, no mussels added and the fishes were challenged by *A. hydrophila*
M-	Negative control, no mussels added and the fishes were not challenged by *A. hydrophila*
M1	One mussel for a two-liter of water, the fishes were challenged by *A. hydrophila*
M2	Two mussels for a two-liters of water, the fishes were challenged by *A. hydrophila*

2.4. Challenge test

On day 8, all treatments were challenged (except the M-) by mean the immersion method. One liter of an overnight culture of *A. hydrophila* (approximately density 10^9 CFU ml^{-1}) used as a stock culture. The live cells were harvested by precipitation (3000 rpm, 20 min) and resuspended within the sterile water to obtained its initial volume. A direct simple dilution was performed by adding the suspension of *A. hydrophila* live cells into the aquarium to achieve a final density of 10^5 CFU ml^{-1}. This procedure was conducted only once and animals left for another 7 days until day 14. During the course of the challenge test, the fish were fed twice a day with commercial fish diet.

2.5. Water quality maintenance

The quality of water was maintenanced regularly by replacing 20% of the water from total volume daily before the challenge test. To prevent a biased result in the experiment, at the day of challenge test and afterward, the water replacements were halted in order to avoid the decreasing of pathogen population in the water column.

2.6. Parameters

The parameters were observed at random sampling including the survival rate (SR), the blood profile of fishes, total hemocyte count (THC), and total bacterial count. The SR data were observed daily during seven days of challenge test while other parameters were observed three times during the course of the challenge test. The blood profiles were done by taking the fish blood through the vein vessels of the tail. For total erythrocytes, blood is taken as much as 0.5 scales, it then diluted 200 times with a solution of Hayem and counted in a hemocytometer. Total leucocytes will be determined by diluting fish blood 20 times in Turk's solution and then counted in a hemocytometer. For hemocyte count, a freshly 0.10 mL of hemolymph withdrawn from adductor muscle then gently mixed with 0,10 mL of anticoagulant and diluted with formaldehyde to make a 1:1 ratio. Cell concentration then counted with a hemocytometer.

For assessment of the bacterial load in the water column, one mL of water sample withdrawn from each trial and serially diluted in test tubes containing sterile water. An aliquot (50µL) then spread on the surface of the TSA medium and then incubated in 37^0C overnight. The grown colonies were counted, expressed as CFU mL^{-1}.

2.7. Data analysis

All collected data were presented in either graphical or table using Microsoft Excel 2010. Data analysis of survival rate, total

erythrocytes, total leucocytes, total hemocyte count, and water-bacterial load were processed using SPSS 10 and one-way analysis of variance (ANOVA) to determine the difference between treatments and then processed to the Duncan's test.

3. Results

3.1 Tilapia survival rate

Before challenged by the pathogenic *A. hydrophila*, there were no mortalities in fish population among the treatments. However, after a challenge test during 7 days period, the treatment of M+ and M1 showed a significant decreasing in fish survival rate while the treatment M- and M2 were sustaining 100±0.0% of the fish population. The lowest value of the final survival rate was recorded in M+ with a value of 40±14,14% and marked a significant difference (P<0.05) with M2 and M-.

3.2 Total erytrocytes of tilapia

A high value of total erythrocyte noticeable at the beginning of the infection which indicating a level of stress among fish in all treatments. There were significantly different (P <0.05) total erythrocyte values before the challenge test between control and mussel treatments as shown in Figure 2. However, a remarkable decline occurred on the 4th day after the challenge except for the M2 treatment. The M2 treatment was sustaining the highest total erythrocyte reaching 2.53 ± 0.35 × 10^6 cells mm^{-3} and significantly different (P<0.05) from other treatments. On the last day of the challenge test, all treatment showed insignificant differs from others (P>0.05).

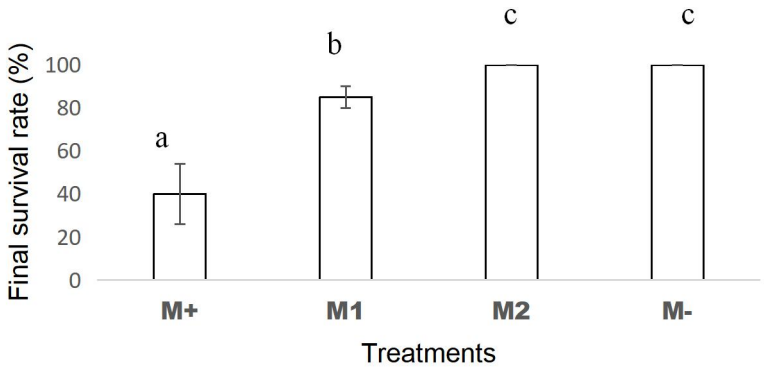

Figure 1. The survival rate of tilapia after challenged by live cells of *A. hydrophila* at seven days post infection. The different letter above the bars showed the significant difference (P<0.05) among the treatments. M+ positive control, M- negative control, M1 and M2 is the treatments with one and two mussel for a two-liter of water respectively.

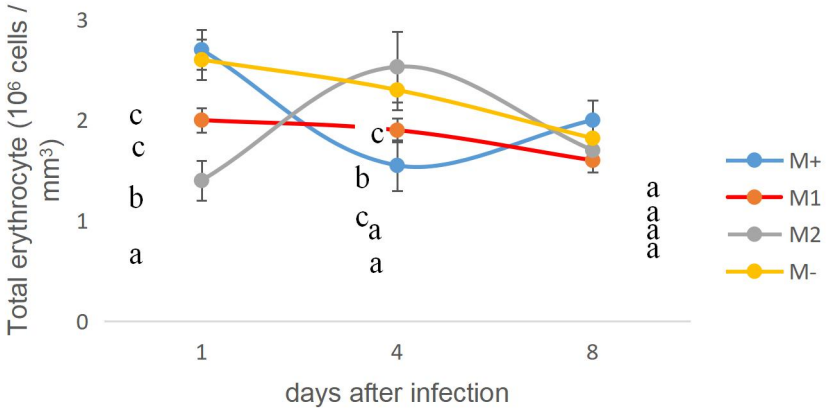

Figure 2. Total fish erythrocytes after challenged by live cells of *A. hydrophila.* The different letter above the lines in the same day showed the significant difference (P<0.05) among the treatments.

3.3 Total leucocytes of tilapia

There was a significant increase in the total leucocytes in the M+ treatment until the end of the challenge test as seen in Figure 3. The peak on the last day of testing, the total leucocytes in the M+ was 7.40 ± 0.77 × 104 cells mm^{-3}, significantly different (P <0.05) with the M2 treatments, whereas in the M1 and M2 treatments the total leucocytes were fluctuating with a tendency to decrease when compared to day 1. The graphic shows the total leucocytes of M2 treatment on the last day of testing was the lowest with 4.30 ± 0.7 × 10^4 cells mm^{-3} significantly different (P <0.05) with M+ and M1 treatments.

3.4 Total hemocytes count (THC) of mussel

Due to no mortalities among the mussel in the course of challenge test, then the impacts of pathogenic bacteria on the immune status of the feeding mussels was assessed by comparing the dynamics of circulating hemocytes among the mussels in the treatments. Since the control treatments were not used the mussels, the comparison of THC fluctuation was done between M1 and M2 only as shown in Figure 4 below. There was no significant fluctuation of THC on the M1 mussel during the challenging test. Different dynamics were found in the M2 with significantly different fluctuation on the mussel's THC mussel (P <0.05). The lowest value was found on M2 mussels on the 7th day with a value of 4.75 ± 0.35 × 10^2 cells mm^{-}

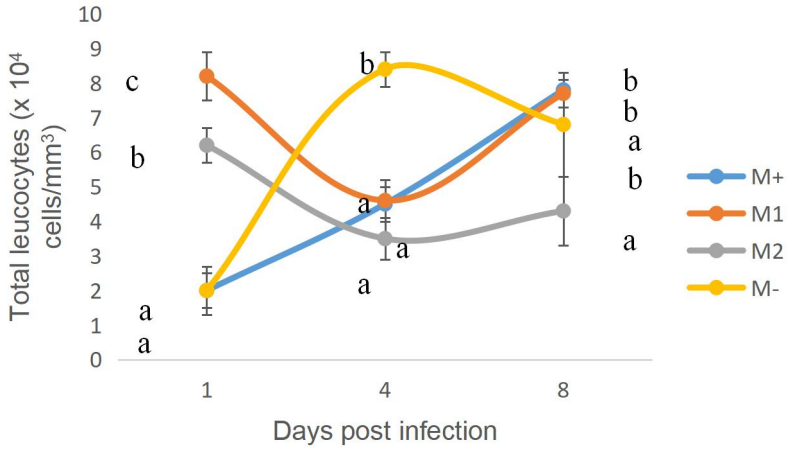

Figure 3. Total fish leucocytes after challenged by live cells of *A. hydrophila.* The different letter above the lines in the same day showed the significant difference (P<0.05) among the treatments.

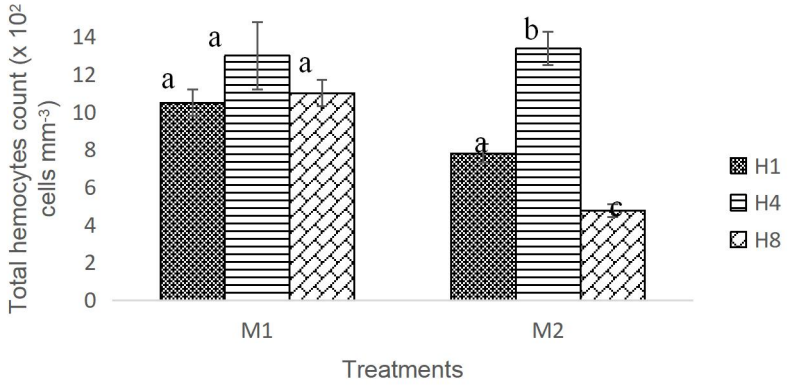

Figure 4. Total hemocytes count (THC) of mussel in the course of challenge test by live cells of *A. hydrophila.* The different letter above the bars in the same treatment showed the significant difference (P<0.05) among the treatments.

3.4 Total bacterial count

Generally, the total bacteria in the water column from all treatments showed a tendency to increase until the last day of observation. However, a significant different results (P <0.05) between treatments always consistent at day 4 post infection onwards. The M+ treatment always showed the highest bacterial abundance in the water column while the lowest one is shown by the M2 treatment as seen in Figure 5 below.

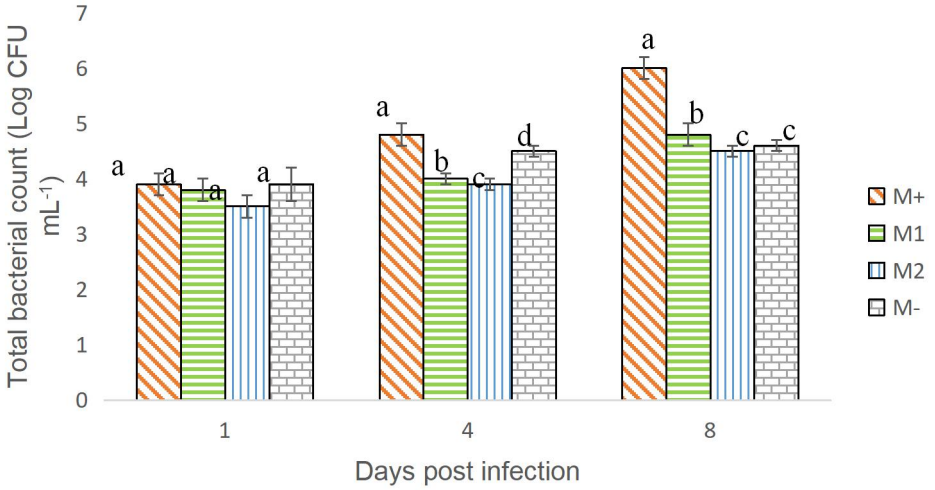

Figure 5. Total bacterial count in the water column in the course of the challenge test. The different letter above the bars in the same day showed the significant difference (P<0.05) among the treatments.

4. Discussion

The ecological services by filter feeder animals in the aquaculture system indeed may have either benefits or adverse impacts to the production i.e. by improving water quality (Brown *et al* 2011, Chopin *et al* 2001, Joyni *et al* 2011) and entrap the swarming pathogen in water column (Molloy *et al* 2011, Rolin *et al* 2016, Webb *et al* 2013) or may also be detriment to the farm biosecurity by accumulating the pathogen and transmitting the pathogen from one species to another (McConnachie *et al* 2013, Ben-Horin *et al* 2015, Desrina *et al* 2013). In this context, the fate of the infection may depend on the particle selectivity of bivalve, the degree of infectivity of pathogens, the transmission mechanism of the pathogen, and the ability of pathogens to resist degradation in the gut of bivalve (Burge *et al* 2016).

In this research, we were able to prove that the presence of *P. exilis* in the tilapia cultivation system was able to suppress the transmission of a waterborne-pathogen *A. hydrophila* to the fish-host. Comparing to the M+ treatment which was experiencing a severe infection and had lost a total 60% of its initial population, the native mussel in M1 and M2 treatment obviously showed a decreasing of the disease severity in the fish population, thus maintaining the survival rate at 85±5% or even reaching 100% as the same level to the M- treatment.

The pathogen *A. hydrophila* has the ability to excrete several compounds such as hemolysin, protease, cholinesterase, enterotoxin, endotoxin, and adhesin which play a role in the development of virulence of pathogenic bacteria (Citarasu et al. 2011). Therefore, observation of the blood profile is somewhat relevant as an indicator of fish health. The highest total erythrocyte was observed in the M- treatment, expecting that the fishes were not infected or affected by the releasing toxins of *A. hydrophila*. However, it did not significantly differ to the outcome of M2 (P>0.05), this findings strengthen the evidence that the feeding activity of *P.exilis* in the M2 treatment may play an eminent role in depleting *A.hydophila* from the water column to a level where the pathogen was not able to infect and then prevent them in poisoning the red blood cells of fish.

Leukocytes in the fish have an important role to destroy the infecting

pathogens through phagocytosis activity as a part of non-specific defense (Sukenda *et al*. 2008). A high pathogen burden in the water column increasing the probability of infection to the fish body. The presence of the pathogen in the body of fish usually provoking the immune system to generate more leucocytes circulating in the fish blood (Austin and Austin 2007). A noticeable increasing leucocytes count in the M+ treatment in the course of challenge test was related to an immune response against infection. On the contrary, decreasing of leucocyte count in the M2 treatment indicating that the fishes were not in suppressive activity against the pathogen due to insignificant infection. However, the increase in leucocytes number is not always related to infection. Stress condition, nutritional factors, and also age factor often tributed to the increasing of leucocytes number in fish (Maftuch *et al* 2011).

Invertebrate animals relied on their innate immunity to respond to an infection. Their circulating hemocytes playing a role to deliver the immune response and their cell abundance have a strong relationship with its immune status. In our research, it was seen that the presence of foreign live particles of *A. hydrophila* inducing the proliferation of hemocytes of *P. exilis* on day 4th either in M1 or M2 treatment. However, the declining of THC occurred on the last day of observation. It seemed that in the final day, the M2 showed insignificant THC fluctuation (Figure 4) and its bacterial load was lesser (Figure 5) compared to the M1. This findings were indicating the pathogen was neither amplified within the mussel body nor exhibiting considerable hostile activities to the immune system of mussel. A non-target host mussel already proposed as a pathogen biocontrol due to its ability to reduce pathogen numbers in the aquatic environment (Burge *et al* 2016) without showing an alteration to their physiological parameters. The high number of mussels in the M2 treatment may have a contribution to the rapid pathogen depletion, lowering the pathogen burden in the mussel, thus recovering the THC to the normal state.

Moreover, even though the details of pathogen reduction by *P. exilis* was not elaborated in this works, we are guessing that the mechanism was related to the feeding activity of the mussel which was, in turn, depleting the pathogen abundance in the water column, similar to what Othman et al (2015) already observed on *Streptococcus agalactiae* in the previous works. He founded that *P. exilis* able to decrease the mortality of tilapia fish and inhibit the outbreak of streptococcosis without accompanied by negative impact from the presence of mussel. Another remarkable mechanism is related to the living space-shifting which was also already observed in *V. anguilarum*. This pathogen previously was in high abundance in the water column and becoming lesser when the blue mussel present. However, the viable of pathogenic cells become accumulated and denser in the pseudofeces of blue mussel and sank on the floor of the codfish tank (Pietrak *et al* 2012). Proper consideration must be taken if the latter mechanism has exactly occurred because a massive spill-back of the pathogen to the susceptible fish host would lead to disease outbreak in the future (Burge *et al* 2016).

5. Conclusion

The native freshwater mussel *P. exilis* able to prevent the transmission of waterborne pathogen *A. hydrophila* when cohabitated with the tilapia. Overall, the best treatment is the M2 based on the survival rate of fish, total leucocytes, and total bacterial loading in the water with value as 100±0,00%, 4,30±0,70x10^4 cell mm^{-3}, and 4,53±0,03 log CFU mL^{-1} respectively.Most of freshwater mussel species have a parasitic stage where their glochidia are the ectoparasite temporarily infesting on the freshwater fish, thus a studies to evaluate the possible trade-off that may occur, and the fate of swallowed pathogen would be a necessity to be addressed in order to obtain the whole picture of this ecological approach before being adopted in the farm level.

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