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Utilization of Tilapia Mucus to Inhibit *Vibrio harveyi* on Vannamei (*Litopenaeus vannamei*)

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ABSTRACT: Shrimp diseases derived from pathogen infection can be caused by bacteria, such as luminous bacteria (*Vibrio*). Toxins produced by *V. harveyi* cells in larvae digestive tract cause luminous *Vibrio*sis disease. One way of preventing or pathogenic *Vibrio sp.* against in shrimp farming is to use shrimp polyculture system with fish. Tilapia fish freshwater or saline has a system in the body that induce antibacterial peptides, its providing an antibacterial effect by inhibiting the growth of *V. harveyi*. The purpose of this study was to determine the ability of antibacterial from Tilapia mucus (*O. niloticus*) in inhibiting growth of *V. harveyi*, knowing the molecular mass of protein Tilapia mucus, knowing the best Tilapia biomass influence on total *V. harveyi* and survival rate of *L. vannamei* through polyculture system. The first stage is a test in vitro, it through antibacterial test based on the test disc, the value of MIC, and MBC Tilapia mucus. Furthermore, the second stage is the characterization of mucus through protein fractions using SDS PAGE. The third stage is a test in vivo, application of antimicrobial Tilapia mucus through polyculture system between *L. vannamei* with Tilapia. Tilapia mucus that has been separated by ethanol has inhibitory diameter against *V. harveyi* by 11 mm (including blank discs with a diameter of 6 mm). MIC of Tilapia mucus are already separated with ethanol was 4.5 ppm protein and MBC of the mucus was 17.99 ppm. Tilapia mucus that has been separated with ethanol has 6 protein fraction contained antibacterial 233, 88, 26, 25, 23, and 17 kDa. *L. vannamei* polyculture with Tilapia was resulting *V. harveyi* amount reduced, but increased survival and THC of *L. Vannamei* reduction was the highest in *L. vannamei* polyculture with Tilapia with biomass of 800 g m^{-3} while the increased survival and THC of *L. vannamei* were observed in Tilapia 600 g m^{-3} and 800 g m^{-3}. The conclusion of this study is polyculture between *L. vannamei* with Tilapia 800 g m^{-3} was proven to increase survival of *L. vannamei*.
Key words: Antibacterial, *L. vannamei*, Polyculture, SDS-PAGE, Tilapia mucus, *V. harveyi*

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INTRODUCTION

Shrimp included in Penaeidae family are species that have high economic value. Panaeid shrimp which is cultivated mainly in Asia and America can be produced as much as 5.5 million tons in 2009 with a value of more than $ 14.6 billion [1]. Although it has a high economic value, the panaeid shrimp industry vulnerable to losses due by disease, causing reduced production.

Panaeid shrimp diseases derived from pathogens infection (viruses, bacteria, fungi and protists) [2]. One of Panaied shrimp diseases caused by bacteria is luminous (*Vibrio*) [3]. *Vibrio*sis, one of which is caused by *V. harveyi*. *V. harveyi* produced exotoxin compounds such as tetrodotoxin and surugatoksin protein that has given the effect of toxins in the digestive tract of Panaeid shrimp which caused the death of Paneaid shrimp in a high number of outbreaks by disease which is defined as luminous *Vibrio*sis [4].

One way that has the potential to prevent or fight the pathogen *Vibrio sp.* is using shrimp polyculture system. System of polyculture between Panaeid shrimp and Tilapia fish can inhibit the spread of *Vibrio* and other pathogenic bacteria in Panaeid shrimp ponds [5]. Fish have a system in the body that induce antimicrobial peptides which lies a layer of mucus [6]. Antimicrobial peptides known to be effective against gram negative and positive. Tilapia (*Oreochromis sp.*) provided an antibacterial effect by inhibiting the growth of *V. harveyi* [7, 8]. On the other hand, Tilapia (*O. niloticus*) is a species of fish that have high economic value and has big market opportunities in local and export markets. The purpose of this study was to determine the ability of antibacterial Tilapia mucus (*O. niloticus*) in inhibiting the growth of *V. harveyi*, knowing the molecular weight of the Tilapia mucus protein, knowing the best Tilapia biomass to effect total of *V. harveyi* and survival of *L. vannamei* through polyculture system.

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MATERIAL AND METHODS

This study conducted in the Parasites and Disease Laboratory Faculty of Fisheries and Marine Science University of Brawijaya, Biomedical Laboratory Faculty of Medicine University of Brawijaya, Biology Molecular University of, and UPT BPBAP Bangil Pasuruan.

Tilapia strain used jatimbulan. Tilapia acclimatized for a week before used for research. During fish acclimatization, fish fed with protein content of 17-19%. Fish put in water with salt as a stressor [9]. Fish were weighed, then mucus taken by fish put into plastic bags and scrubbed to remove mucus from body of Tilapia [10]. Mucus separation method followed by ethanol precipitation [11]. Tilapia mucus concentration which has been separated analyzed protein concentration using the Bradford method [12]. The protein concentration calculated by spectrophotometry with wavelength 595 nm and albumin as standard.

Antibacterial test by *V. harveyi* concentration used 10^9 CFU ml-1. The petri dish was incubated for 28° C. Then observed inhibition zone after 24 hours. Then, if there has a zone of inhibition, inhibition zone diameter formed was measured [7].

MIC test aimed to determine the lowest concentration of mucus to inhibit *V. harveyi*. MIC test used macro dilution [13]. The concentration used for the MIC test was 50%, 40%, 20%, 10%, 5%, 2.5%, 1.25% of Tilapia mucus protein concentration. In addition to the medium used MHB. MIC results determined by the value of the absorbance (OD) of the spectrophotometer (wavelength 570 nm) [14]. Then absorbance squared (Y) plotted obtained by ln of mucus concentration series (Mo) as the x-axis. Values were intersected by the x-axis is the Mt. Mt used for calculations, MIC = 0.25 x Mt [15].

MBC of Tilapia mucus was the minimum concentration (ppm) of Tilapia mucus protein which caused a complete barrier to the growth of *V. harveyi* [16]. MBC value was 4 times that of MIC [15].

SDS-PAGE analysis followed the method of Laemmli. Mucus mixed with 1: 1 loading buffer, incubated for 5 min at 95 ° C, separating gel 12.5%, 3% stacking gel [12]. Proteins were stained with blue coomasie. Tilapia acclimatized in the media with the desired salinity (20 ppt). Every three days, the water used to maintain Tilapia was raised 4 ppt.

Site preparation and polyculture media followed the method of Tendenciana [17] with some changes. Tanks length of 115 cm x width 60 cm filled with water salinity 20 ppt has given aeration system using the blower and installed cage measuring 50 cm x 35 cm x 40 cm at the top of the tank. The tanks filled with sea water bath until the volume to 0.5 m^3. Water was added by 100 ppm chlorine to reduce the total of *Vibrio*, then aerated 24 hours. After 24 hours given sodium thiosulfate 25 ppm. Then the water was left for 24 hours so that the chlorine was gone.

This study used a randomized design group. Four treatments with different biomass used for long maintenance study for 7 days (Table 1).

Table 1. Research Design used in vivo test

Tilapia Biomass ($g\ m^{-3}$)	Treatments			
	1	2	3	4
200	U T V	U V	U T	U
400	U T V	U V	U T	U
600	U T V	U V	U T	U
800	U T V	U V	U T	U

*description: U: *L. vannamei* (biomass 60 shrimp m^{-3}); T: Tilapia 100 g; V: *V. harveyi* (10^7)

Treatment 1 and 3 used Tilapia with biomass 200, 400, 600, and 800 g m^{-3} were included in cage (50 cm x 35 cm x 40 cm). However, treatment 3, *L. vannamei* maintained along Tilapia were included in cage in the pool without the *V. harveyi*. Further treatment 4, *L. vannamei* maintained in the pool without *Vibrio* and without Tilapia. Every day the shrimp and Tilapia fed floating and sinking *L. vannamei* fed as much as 4% of the biomass of the commodity. The parameters were recorded total hemolim (THC), clinical symptoms, survival rate (% SR) *L. vannamei*, and the number of *V. harveyi* (cfu ml^{-1}).

RESULTS AND DISCUSSION

Tilapia with mucus protein was content 11.28 ppm inhibitory diameter 11 mm (including blank discs with a diameter of 6 mm, Figure 1). Research Wei et al. [18] used Snakehead fish mucus for antibacterial against *Aeromonas hydrophila*, produced 8 mm zone of inhibition around the disc. Mucus used in the study extraction was using acid extraction and water that has protein respectively 0.267 and 0.291 mg ml^{-1}. Diameter in Tilapia was lower than expected Snakehead fish because the antibacterial of Tilapia mucus was weaker than the Snakehead fish. Mucus played a role in prevent the attachment of parasites, bacteria, and fungi because the mucus is a source of anti-bacterial products [19].

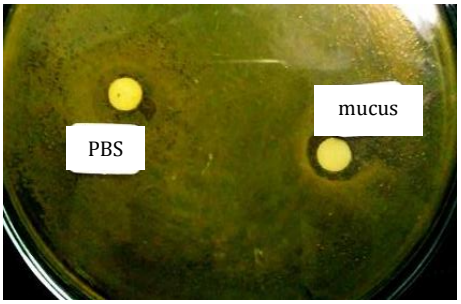

Figure 1. Diameter of inhibition of Tilapia mucus were precipitated with ethanol.

Series protein concentration of 5.64; 2.82; 1.41; 0.71; 0.35; 0.18; and 0.09 ppm used to MIC and MBC test. MIC and MBC test calculation obtained 4.5 ppm as MIC and MBC value of 17.99 ppm as of Tilapia mucus against *V. harveyi*. Antibacterial test research results from Ebran et al. [6], mucus trout, eel, and *Tinca tinca*, against gram-negative bacteria (*E. coli, P. Fluoresaureus*, and *A. hydrophila*) to obtain MIC ranged from 1 to 5 ppm protein with a molecular mass of each was 65 kDa, 45 kDa and 49 kDa. Wei et al. [18] obtained lower the MIC value by using Snakehead Fish mucus, namely mucus with a protein concentration of 0.3 (crude extract) and 0.15 ppm (water extract). Tilapia mucus barrier strength against gram-negative bacteria with power level of the mucus barrier Trout, eels, and fish Tinca tinca, but much weaker than the strength of inhibition of Snakehead fish mucus.

MBC values Tilapia fish against *V. harveyi* was 17.99 ppm. Research Subramanian [16] mentioned that Haddock fish, Hagfish, and Trout mucus had MBC values against gram-negative pathogens with fish protein concentration of 6.1 to 39 ppm. Haddock fish mucus has MBC against gram-negative fish bacteria (*Aeromonas salmonicida*, *Listonella anguilllarum*, *and Ruckeri yersinia*), respectively by 27, 27, 14 ppm protein. Protein found in Haddock fish mucus has a molecular mass of 25 to 6.5 kDa. Hagfish mucus has MBC against gram-negative fish bacteria (*Aeromonas salmonicida*, *Listonella anguilllarum*, and *Ruckeri yersinia*) respectively of 8.3; 16; and 6.1 ppm of protein. Types of proteins in Hagfish has a molecular mass below 20 kDa. Trout mucus had MBC against gram-negative fish bacteria (*Aeromonas salmonicida*, *Listonella anguilllarum*, and *Ruckeri yersinia*), each of which has a protein content of 19, 39, 19 ppm. Various types of proteins found in fish Trout with a molecular mass of 6.5 to 100 kDa. Tilapia mucus MBC results were studied (17.99 ppm) was in a range between Trout and Hagfish. The bacteriostatic and bactericidal in vitro caused by the presence of lysozyme, complement, or immunoglobulin [20].

The molecular weight of the protein contained in the Tilapia mucus range from 10 to 260 Kda (Figure 2). Thickest fraction is 29 kDa. In the mucus lining of many enzymes and antimicrobial proteins, which are known to be involved in the immune system of fish [19].

The molecular weight of the protein obtained Tilapia mucus containing antibacterial is 233, 88, 26, 25, 23, and 17 Kda. The molecular weight of the protein which leads to the antibacterial protein immunoglobulin, complement, lectins, c-reactive protein, protease, and lysozyme. Lysozyme given the defense the form of bacteria and osmotic pressure [21]. Proteases can inhibit pathogen development and attack. In addition, proteases can activate and increase mucus production in the immune component such as complement, immunoglobulins, or antibacterial peptide [22]. C-reactive protein activated complement and defense so as to enable lytic phagocytosis [21]. Immunoglobulin binding microbial surface that interacts with a receptor phagocytosis [23].

Total of *V. harveyi* was lower when given additional Tilapia biomass (Figure 3). Research Tendenciana et al. [8] regarding the antibacterial activity against *V. harveyi* hornorum Tilapia also showed a negative correlation of increased biomass of Tilapia with reduction of *V. Harveyi*. The addition of Tilapia able to suppress the *Vibrio* and many gram-negative pathogens are generally present in shrimp culture depressed [24].

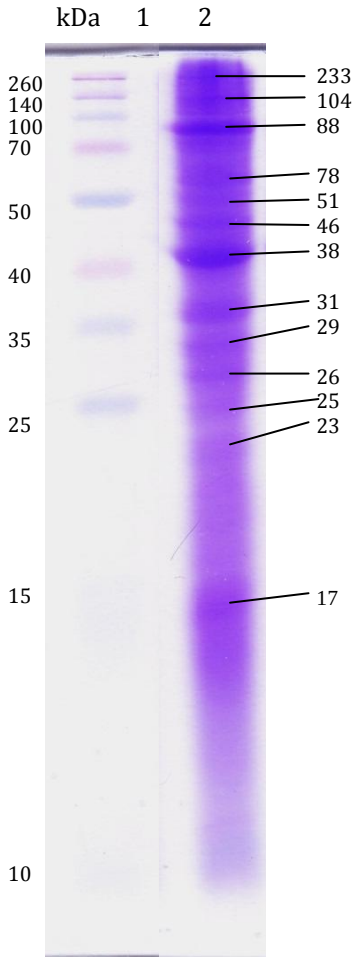

Figure 2. Protein electrophoresis results of some Tilapia mucus.

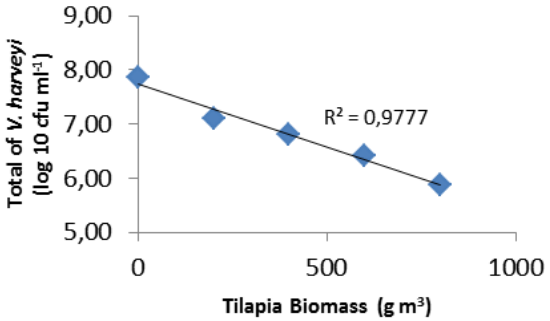

Figure 3. Tilapia granting regression results on the number of *V. harveyi*.

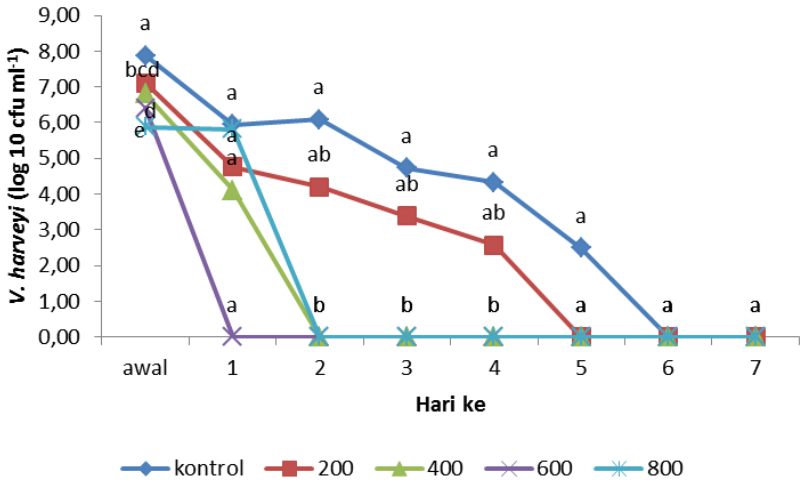

Figure 4. Comparison of the number of *V. harveyi* in each media treatment ($p < 0.05$).

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The number of different *V. harveyi* in control of time of initial treatment, the second, third, and fourth challenge test. Tendenciana and dela Pena [17] research, which examined the components in Green water system to control fluoresce bacteria, showed giving Tilapia hornorum 300 g m^{-3} resulted in *V. harveyi* was not detected after 1 day maintenance. On the other hand, the provision of Tilapia 100 g m^{-3} and control still *V. harveyi* until the sixth day. At the beginning of maintenance, the lowest number of *V. harveyi* in tanks with Tilapia biomassa 800 g m^{-3}. Statistical analysis showed that variations in treatment Tilapia biomass produced the same total of *V. harveyi* (Figure 4).

Anti-bacterial composition of Tilapia mucus in inhibiting the growth of *V. harveyi* directly. Tilapia mucus in vivo test secreted complement, lysozyme, c-reactive protein, immunoglobulins, proteases and lectins. Tilapia mucus hornorum effectively eliminated *Vibrio* luminescence in less than 3 hours after *Vibrio* affected by the suspension of mucus [25].

THC has decreased in the control test when challenged with *V. harveyi*. From research Huang et al. [26] derived the data hemolim shrimp were reduced by 40% when the pathogen infection. THC *L. vannamei* in the treatment Tilapia 600 g m^{-3} and 800 g m^{-3}, was increasing the current challenge test against *V. harveyi* (Figure 5). The effect of the increase in the total of hemocytes will appear when the total is not less or higher than required [27]. The active compound in a material will increase the body's defense system shrimp when entered in the digestion and absorption in the blood [27]. Hemocytes is the first defense in invertebrates, so the number of hemocytes high enough to survive better when there is a pathogen [28].

SR of *L. vannamei* in the media by *V. harveyi* increased after given of Tilapia (Figure 6). The polyculture system reported an increased survival rate higher shrimp [24].

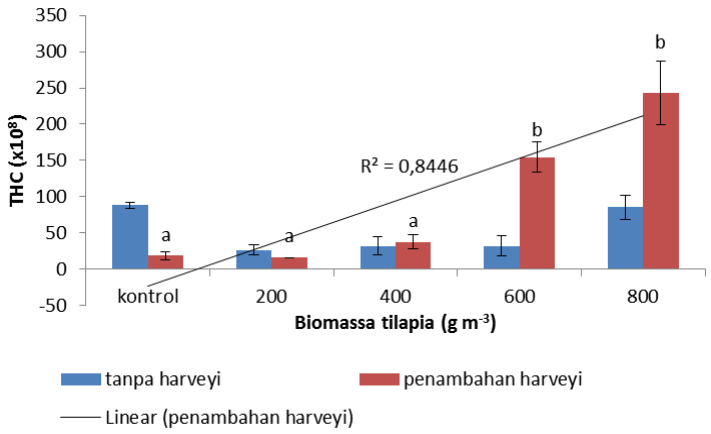

Figure 5. THC *L. vannamei* after administration Tilapia biomass (control: not given Tilapia and p <0.05).

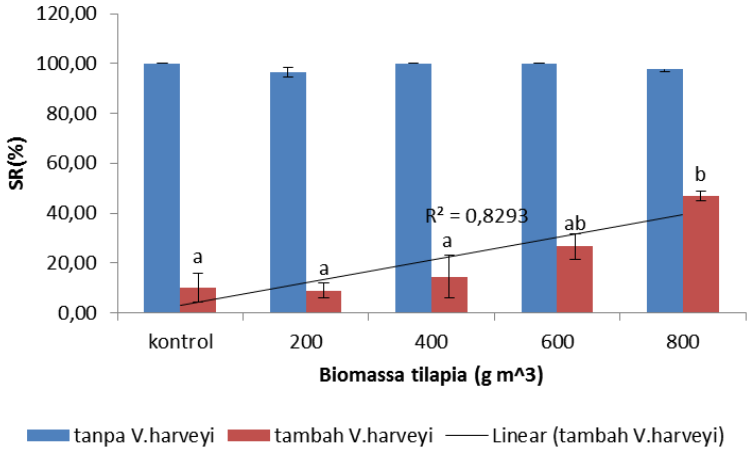

Figure 6. Comparison Survival Rate of *L. vannamei* of each biomass Tilapia (control: not given Tilapia and p <0.05).

Survival rate (SR) of *L.vannamei* results obtained only about 50%. Other studies have shown, giving Tilapia generated SR of *P. Monodon* control 80.58% and 61.54% [29]. Other studies showed no real difference by giving Tilapia hornorum, the SR of *P. Monodon* 54-59% [8]. Both studies using *V. harveyi* inoculation of 10^3 cfu ml^{-1}. SR of *L. vannamei* results obtained on media containing *V. harveyi* was about 50% allegedly because of poor water

quality (Table 2). The concentration of ammonia and nitrite are both below 0.1 ppm [30]. Less optimum environmental cause physiological stress response of the body's defenses and reduce shrimp which causes the shrimp become susceptible to disease [2]. According to Tseng and Chen [31], which examined *L.vannamei* immune response and susceptibility to *V. alginolyticus* to pressure to conclude ammonia nitrite in the water causes pressure on the ability of *L.vannamei* immunity from disease.

Table 2. Ammonia and nitrite concentrations in maintenance medium when testing in vivo

Tilapia (g m^{-3})	without *V. harveyi*		Addition *V. harveyi*	
	Ammonia (ppm)	Nitrite (ppm)	Ammonia (ppm)	Nitrite (ppm)
control	0,31±0,01	7,28±0,1	0,21±0,07	7,28±0,04
200	0,42±0,05	7,12±1,15	0,23±0,01	7,12±0,25
400	0,52±0,05	15,62±0,37	0,25±0,05	13,62±0,98
600	0,25±0,01	15,16±2,82	0,06±0,01	15,16±0,15
800	0,18±0,05	9,262,33	0,34±0,10	9,26±2,17

L. vannamei has infected and dead showed some clinical signs: abdominal of *L. vannamei* was black, hepatopancreas damaged, head and tail was red, and tail damage. According to Rita and Walim [32], The shrimp were infected as a result of *V. harveyi* showed some clinical signs or symptoms, the damage to the outside of the body marked shrimp prawns were visible stress to wound in the body, and the appearance of white spots on the body of shrimp, spotting red on the pleopod and abdominal. According Mikulski et al. [33] and Aguirre-Guzman et al. [34], a sign of *L. vannamei* exposed to *Vibrio*sis was opaque abdominal muscles, chromatophore expanded, abdominal bends with a peak in the third abdominal segment.

CONCLUSION

Tilapia mucus has a value of 4.5 ppm on MIC and MBC of mucus proteins were 17.99 ppm. Molecular mass of mucus Tilapia proteins that have been separated leading to the antibacterial protein, which was 233, 88, 26, 25, 23, and 17 kDa. Type of protein with a molecular mass of the protein, respectively immunoglobulin, complement, lectins, c-reactive protein, protease, and lysozyme. Polyculture systems *L. vannamei* with Tilapia with Tilapia biomass of 800 g m-3 was proven to increase survival of *L. vannamei* through polyculture system *L. vannamei* and Tilapia.

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