

Isolation, identification, and pathogenicity tests of pathogenic bacterial associated with black body syndrome in white barramundi *Lates calcarifer* B.

Isolasi, identifikasi, serta uji patogenisitas bakteri penyebab penyakit black body syndrome pada ikan kakap putih *Lates calcarifer* B.

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ABSTRACT

This study aimed to obtain the pathogenicity of isolated bacteria from maribund barramundi *Lates calcarifer* with black body syndrome (BBS). Moribund barramundi was collected from the Center for Mariculture Development (BBPBL), Lampung Province. Five of pathogenic bacteria were found, i.e. *Pseudomonas stutzeri* L01, *Vibrio harveyi*, *Bacillus cereus*, *Salinococcus roseus*, and *Pseudomonas stutzeri* L02. The bacteria were tested for LD50 to obtain two types of high virulent bacteria to be used for the pathogenicity test. *P. stutzeri* L01 and *V. harveyi* were the most virulent bacteria with a bacterial density of 10^7 CFU/mL. Both bacteria were used for pathogenicity test with three treatments: injection of *P. stutzeri* L01, *V. harveyi*, mix *P. stutzeri* L01 and *V. harveyi*, and phosphate-buffered saline as control. Clinical symptoms showed a blackened body, bleeding under the belly, and inactive. Mortality of fish injected with *P. stutzeri* L01, *V. harveyi* and mix *P. stutzeri* L01 and *V. harveyi* was 53.33%, 55.00%, and 58.33%, respectively. Erythrocyte and hemoglobin in all treatments were not significantly different ($P>0.05$). However, there was significantly different in the total leukocytes of mixed *P. stutzeri* and *V. harveyi* treatment ($P<0.05$). In conclusion, *P. stutzeri* L01, *V. harveyi* and mix of them resulted in black body syndrome (BBS) disease. Coinfection of *P. stutzeri* L01 and *V. harveyi* produce higher mortality than single infection.

Keywords: barramundi, black body syndrome, histopathology, pathogenicity

ABSTRAK

Penelitian ini bertujuan mendapatkan patogenisitas bakteri dari ikan kakap putih *L. calcarifer* yang memiliki gejala black body syndrome (BBS). Ikan diambil dari Balai Besar Pengembangan Budidaya Laut (BBPBL), Lampung. Lima jenis bakteri patogen ditemukan, yaitu *Pseudomonas stutzeri* L01, *Vibrio harveyi*, *Bacillus cereus*, *Salinococcus roseus*, dan *Pseudomonas stutzeri* L02. Bakteri diuji LD50 untuk mendapatkan dua jenis bakteri virulen tinggi yang akan digunakan untuk uji patogenisitas. *P. stutzeri* L01 dan *V. harveyi* adalah bakteri paling virulen dengan kepadatan bakteri 10^7 CFU/mL. Uji patogenisitas dengan tiga perlakuan: injeksi *P. stutzeri* L01, *V. harveyi*, campuran *P. stutzeri* L01 dan *V. harveyi*, serta phosphate-buffered saline sebagai kontrol. Gejala klinis menunjukkan tubuh menghitam, perdarahan di bawah perut, dan pergerakan tidak aktif. Mortalitas ikan yang disuntik dengan *P. stutzeri* L01, *V. harveyi* dan campuran *P. stutzeri* L01 dan *V. harveyi* masing-masing sebesar 53,33%, 55,00%, dan 58,33%. Eritrosit dan hemoglobin pada semua perlakuan tidak berbeda nyata ($P>0,05$). Namun, ada perbedaan yang signifikan dalam total leukosit perlakuan campuran *P. stutzeri* L01 dan *V. harveyi* ($P<0,05$). *P. stutzeri* L01, *V. harveyi* dan campuran *P. stutzeri* L01, *V. harveyi* dapat menyebabkan black body syndrome (BBS). Koinfeksi *P. stutzeri* L01 dan *V. harveyi* menghasilkan mortalitas lebih tinggi daripada infeksi tunggal.

Kata kunci: barramundi, black body syndrome, histopatologi, patogenisitas

INTRODUCTION

Barramundi *Lates calcarifer* is one of the important species of marine culture in Southeast Asia and Australia and this increases the marine culture of barramundi (Venkatachalam *et al.*, 2018). Barramundi intensification provided sufficient rare barramundi juvenile yet caused a new problem. This problem appeared due to the stressful environment and fish density that would cause stress in fish. In this condition, fish exposed to stress will easily expose disease, therefore mass mortality will increase in barramundi culture.

Disease outbreak is one of the common threats against the sustainability of barramundi culture. The common cause of the disease is bacteria, viruses, or fungi. In barramundi culture, the common cause of the disease is bacteria. A previous study (Minaka *et al.*, 2012) stated that bacteria cause disease in fish is a kind of Gram-negative bacteria. Bacteria that cause disease in Barramundi culture commonly comes from *Vibrio harveyi* infection. This bacteria can cause vibriosis in fish. Ransangan dan Saleem (2009) mentioned that *V. harveyi* is the main cause of vibriosis in barramundi cultured in Philipines, caused losses up to ± US 7.4 million dollars. Economic loss in the marine culture industry due to this outbreak was estimated up to US\$ 9 billion per year and resulted in decreasing the total production of cultured fish around the world. Vibriosis disease attacks all cultured marine fish species (Krishnika & Ramasamy, 2014).

Vibrio sp. is primer and secondary pathogen bacteria for marine fish. As primer pathogen, bacteria enter the fish body through direct contact, while as the secondary pathogen, bacteria infect fish body that has infected by other diseases (Novriadi *et al.*, 2014). Pathogenic bacteria from *Vibrio* species is the agent of Vibriosis which may cause high mortality in a short time (Ode, 2012). *Streptococcus* sp. and *Pseudomonas* sp. can also cause mass mortality in cultured barramundi (Suanyuk *et al.*, 2010; Safinska, 2018). This case was similar to mass mortality happen in Australia in 1992, mass mortality was reached 40% in only two days (Kumaran *et al.*, 2010). According to Sukenda *et al.* (2012), some of the species such as *Vibrio alginolyticus*, *V. parahaemolyticus*, *V. anguillarum*, and *Vibrio marinus* was already reported as pathogenic bacteria in fish.

In marine culture, disease infection can cause a common outbreak. Johnny and Roza (2009) reported that disease is one of the obstacles to increasing total production both for freshwater fish and marine water fish. Black body syndrome

(BBS) disease can cause a certain clinical symptom which is the fish body turns black, therefore this disease called black body syndrome (BBS) disease. Other symptoms that may appear during this disease infection are haemorrhagic, lesions, listless behavior, blackened skin, and loss of appetite. This kind of symptoms most likely shows bacterial disease. The cause of this disease is yet unknown, therefore there's an urgent need to find the cause of the disease. This study aimed to identify the cause of BBS disease and test the pathogenicity of this disease agent.

MATERIALS AND METHOD

This study was held from February 2017 to August 2017 in Main Center for Mariculture Development (BBPBL) Lampung and Fish Health Laboratory, Aquaculture Department, Faculty of Fisheries and Marine Science, IPB University.

Bacteria isolation and clinical signs observation

Fish collecting sample referred to Sugiarto *et al.* (2001), which is the sample selected is purposive sampling. The selected sample then was observed according to the characteristics of the tested parameters. This characteristic result was aimed at the research result in a quantitative or qualitative result. The fish sample that was obtained to identify the bacteria is fish with some clinical signs of BBS infection, such as blackened body, lesions, listless swimming behaviour, loss of appetite, and hemorrhage. This sample then was taken to Fish Health and Environment Laboratory, BBPBL Lampung. The pathogen was isolated and identified from the liver, spleen, and kidney. The organ was taken by dissected, cultured in marine agar (MA) media for 24 hours, then cultured in thiosulfate citrate bile salt sucrose (TCBS) agar media and cytophaga agar media to obtain a pure culture of bacteria.

Bacteria characterization

Bacteria characterization was done by separate bacteria colony according to its shape, edge, color, elevation, consistency, and size (Austin & Austin, 2012). Bacteria characterization was used biochemical methods of bacteria identification (API kit) and the molecular method by using PCR of 16S rRNA. The result of 16S rRNA sequencing was then analyzed by BLAST software (NCBI). The 16S rRNA sequencing was aimed to obtain phylogenetic within bacteria species (Drancourt *et al.*, 2000).

Fish health status test

Fish health status test was aimed to ensure fish used in this study was free from bacteria of BBS disease agent. The fish used in this study was a white barramundi size of 6–7 cm obtained from BBPBL Lampung. The external characteristics of fish used for the LD50 test and pathogenicity test should actively swim and the fish body is white. Meanwhile, the internal characteristics of some organs (liver, kidney, and spleen) should be observed in the laboratory to obtain the bacteria of *P. stutzeri* and *V. harveyi* growing within fish body organs or not. White barramundi used for the LD50 test was 12 fish for each replication and 20 fish for each replication for pathogenicity test. Fish were fed with commercial feed twice a day by *at satiation*. The aquarium used in this study was the size of 60 cm × 40 cm × 40 cm with a volume of 80 L. Water used in this study was conducted to treatment by using 400 mg/L of chlorine (Rahardja et al., 2011).

LD50 test

The determination of bacteria virulence level was found by obtaining LD50 value. LD50 test was done by Reed & Muench's method (1938), set with four different dosage, i.e 1×10^9 CFU/mL, 5×10^8 CFU/mL, 2.5×10^8 CFU/mL, 5×10^7 CFU/mL. This bacteria concentration was obtained by the serial dilution method.

Pathogenicity test

LD50 result (fish that tested was showed some clinical symptoms like BBS symptoms) and the highest virulence test result became a reference for pathogenicity test. Healthy white barramundi juvenile was infected by single bacteria, bacteria combination, and control as it is shown in Table 1.

The determination of bacteria dosage for the challenge test was according to the previous study

result. The challenge test was done by injecting 10^7 CFU/mL of *P. stutzeri* L01 isolate, 10^7 CFU/mL of *V. harveyi* isolate, 10^7 CFU/mL of combination isolate of *P. stutzeri* L01 and *V. harveyi*, and 0.1 mL of PBS as control. This injection was done by intraperitoneal. The change of internal organ (liver and kidney) tissue was showed during the infection. The changes during pathogenicity test were observed for 14 days.

Observed parameters

Clinical symptom and bacteria characterization

Bacteria isolate was obtained from white barramundi infected by BBS and then it was identified the biochemical characteristic by using API kit, meanwhile, the molecular characteristic was identified by using PCR 16S rRNA primer.

Mortality and meantime of death

The observation of fish mortality was done every day after pathogen injection. Fish mortality was calculated by using the formula below (Effendie, 1979):

$$\text{Mortality rate (\%)} = \frac{\text{The total dead fish}}{\text{The total fish}} \times 100$$

Fish that was already injected by pathogen was observed the behaviour every day, such as the clinical symptoms, fish mortality, and meantime to death. Meantime to death was calculated by using the formula below (Hubbert, 1980):

$$\text{MTD} = \frac{\sum_{i=1}^n a_i b_i}{\sum_{i=1}^n b_i}$$

Note:

MTD = meantime to death

a = mortality time (hour)

b = number of dead fish

Table 1. The experimental design of pathogenicity test of bacterial disease attacked white barramundi juvenile

Treatment	Note
Control	Fish was injected by PBS
A	Fish was injected by <i>P. stutzeri</i> L01 isolate
B	Fish was injected by <i>V. harveyi</i> isolate
Bacteria combination	Fish was injected by combination isolate of <i>P. stutzeri</i> L01 and <i>V. harveyi</i>

Table 2. Histopathologic scoring and degrees of damage of liver and kidney

Score	Necrosis	Degrees of damage
1	<25 % of total point of view	Normal
2	25–<50% of total point of view	Mild damage
3	necrosis 50–<75% of total point of view	Moderate damage
4	necrosis >75% of total point of view	Severe damage

Histological observation

Histological observation of tissues was observed from day 1, 2, 3, 5, 10, and 14 after the challenge test by using hematoxylin-eosin (HE) staining. The changes of tissues were observed by counting the percentage of damage liver cell and kidney epithelium tubules, replicated three times (Mustaqien et al., 2008), followed by using the formula below:

$$P (\%) = \frac{\sum KS}{\sum TS} \times 100$$

Notes:

- P (%) = The percentage of normal cell and cell degeneration or necrosis cell
- ΣKS = The number of total normal cell and cell degeneration or necrosis cell
- ΣTS = The number of total normal cell (approximately 2000 cells)

Histopathologic scoring of liver and kidney was used previous study method by Wolf et al. (2015), Wolf and Wheeler (2018) as showed below (Table 2).

Hematological observation

Blood samples were obtained at day 0, 1, 7, and 14 after pathogenicity challenge test. The observed parameter of hematology were:

Total erythrocyte

Total erythrocyte was obtained by Blaxhall dan Daisley method (1973). The formula used in total erythrocyte was as follows:

$$\Sigma \text{erythrocyte} = \frac{\Sigma \text{Counted cells}}{\text{Big square volume}} \times \frac{1}{\text{dillution factor}}$$

Total leukocyte

Total leukocyte was obtained by Blaxhall dan Daisley method (1973). The formula used in total leukocyte was as follows:

$$\Sigma \text{Leukocyte} = \frac{\Sigma \text{Counted cells}}{\text{Big square volume}} \times \frac{1}{\text{dillution factor}}$$

Haemoglobin level

Haemoglobin level (Hb) measured by carried out through Sahli method (Wedemeyer & Yasutake, 1977). The scale was read by observing the solution surface and matched with the Sahli tube scale that could be seen on the scale track (yellow scale), signifying that the haemoglobin in g/dL blood was high.

Data analysis

This study used a randomly complete design. The data was tabulated by Microsoft Excel 2013. The mortality rate was analysed by using SPSS ver.24 with an interval of confidence of 95% and was continued by using DMRT (Duncan's multiple range test). Tissue histology was analysed by using the Kruskal-Wallis test and was continued by using the Mann-Whitney test.

Table 3. The results of bacteria biochemical identification found in white barramundi (*L. calcarifer*)

Bacteria	Gram characterization	Biochemical identification	PCR 16S rRNA identification
1	-	<i>Pseudomonas stutzeri</i> 87.7%	<i>Pseudomonas stutzeri</i> L01
2	-	<i>Vibrio harveyi</i> 88.5%	<i>Vibrio harveyi</i>
3	+	<i>Bacillus cereus</i> 99%	<i>Bacillus cereus</i>
4	-	-	<i>Salinococcus roseus</i>
5	-	<i>Pseudomonas stutzeri</i> 99.3%	<i>Pseudomonas stutzeri</i> L02

Table 4. The result of five bacteria density in LD50 of white barramundi juvenile

Bacteria	Mortality (%)	LD50 (CFU/mL)
<i>Pseudomonas stutzeri</i> L01	64.58 ± 0.07^d	8.4×10^7
<i>Vibrio harveyi</i>	81.25 ± 0.35^e	6.2×10^7
<i>Bacillus cereus</i>	54.17 ± 0.24^c	2.9×10^8
<i>Salinococcus roseus</i>	43.75 ± 0.57^b	4.2×10^8
<i>Pseudomonas stutzeri</i> L02	41.67 ± 0.66^a	3.7×10^8

Different superscript in the same column showed significantly different result ($P<0.05$)

RESULTS AND DISCUSSION

Result

Bacteria characterization

Five bacteria isolates were obtained from white barramundi (Table 3). Then these five bacteria isolates were characterised through bacteria biochemical identification using API kit and PCR 16S rRNA primer.

LD50 test

The difference of bacteria type affects bacteria density that causes mass mortality in fish, therefore, it requires LD50 toward the bacteria. LD50 determination was done by calculated total mortality of fish in the first 24 hours after infection through injection (Table 4). However, the mortality will probably occur in the first 24 hours due to the latest infection. Therefore, some previous studies explained that poisoning symptoms should be observed until seven days (Angelina, 2008). The results showed that *P. stutzeri* L01 and *V. harveyi* had highest virulence due to its density reached 8.4×10^7 CFU/mL and 6.2×10^7 CFU/mL and both could cause 50% mortality in fish (Table 4).

Pathogenicity test

Bacteria pathogenicity can be seen when the bacteria caused mortality in fish. Meantime to death of white barramundi juvenile had different result among others (Table 5).

Fish that were infected by bacteria combination (*P. stutzeri* L01 and *V. harveyi*) infection has the longest mean time to death comparing to single infection (A and B). Despite this, it had the longest mean time to death, yet the mortality was higher compared to a single infection. Moreover, bacteria combination infection was also caused worse damage to the macroscopic body and internal organ comparing to other treatments, whereas, the control treatment had no mortality and none mean time to death and it was all normal.

Histopathologic scoring

Total histopathologic score of liver and kidney of each treatment was different (Table 6). The histopathologic score of the liver among the three treatments were different ($P < 0.05$) than the control. *P. stutzeri* L01 treatment was not significantly different ($P > 0.05$) than *V. harveyi* treatment, yet it was significantly different ($P < 0.05$) compared to bacteria combination treatment.

Table 5. Macroscopic condition of fish body, internal organ, and mean time to death

Treatments	Mortality (%)	MTD (hour)	Macroscopic condition of fish body	Macroscopic condition of internal organ
Control	0	0	The body was whitened	Liver: redder Kidney: dusky red
A <i>P. stutzeri</i> L01	53.33 ± 0.33^a	64.76	The body was blackish, lesion in lower abdomen, dropsy	Liver: pale red Kidney: dusky red
B <i>V. harveyi</i>	55.00 ± 0.33^a	67.92	The body was blackish, lesion in lower abdomen, dropsy	Liver: pale red Kidney: dusky red Intestine: yellowish fluids.
Bacteria combination (<i>P. stutzeri</i> L01 and <i>V. harveyi</i>)	58.33 ± 0.67^b	70.69	The body was blackish, lesion in lower abdomen, dropsy	Liver: pale red Kidney: dusky red Intestine: state of decay and had yellowish fluids.

Table 6. Total histopathologic score of liver and kidney of juvenile white barramundi using Kruskal Wallis and Mann Whitney analysis

Treatments	Histopathologic score	
	Liver	Kidney
Control	1.00 ± 0.00^a	1.00 ± 0.00^a
<i>P. stutzeri</i> L01	2.00 ± 0.34^b	1.67 ± 0.34^b
<i>V. harveyi</i>	2.17 ± 0.00^{bc}	2.00 ± 0.17^{bc}
Campuran	2.39 ± 0.10^c	2.39 ± 0.26^c

Different superscript in the same column showed significantly different result ($P < 0.05$)

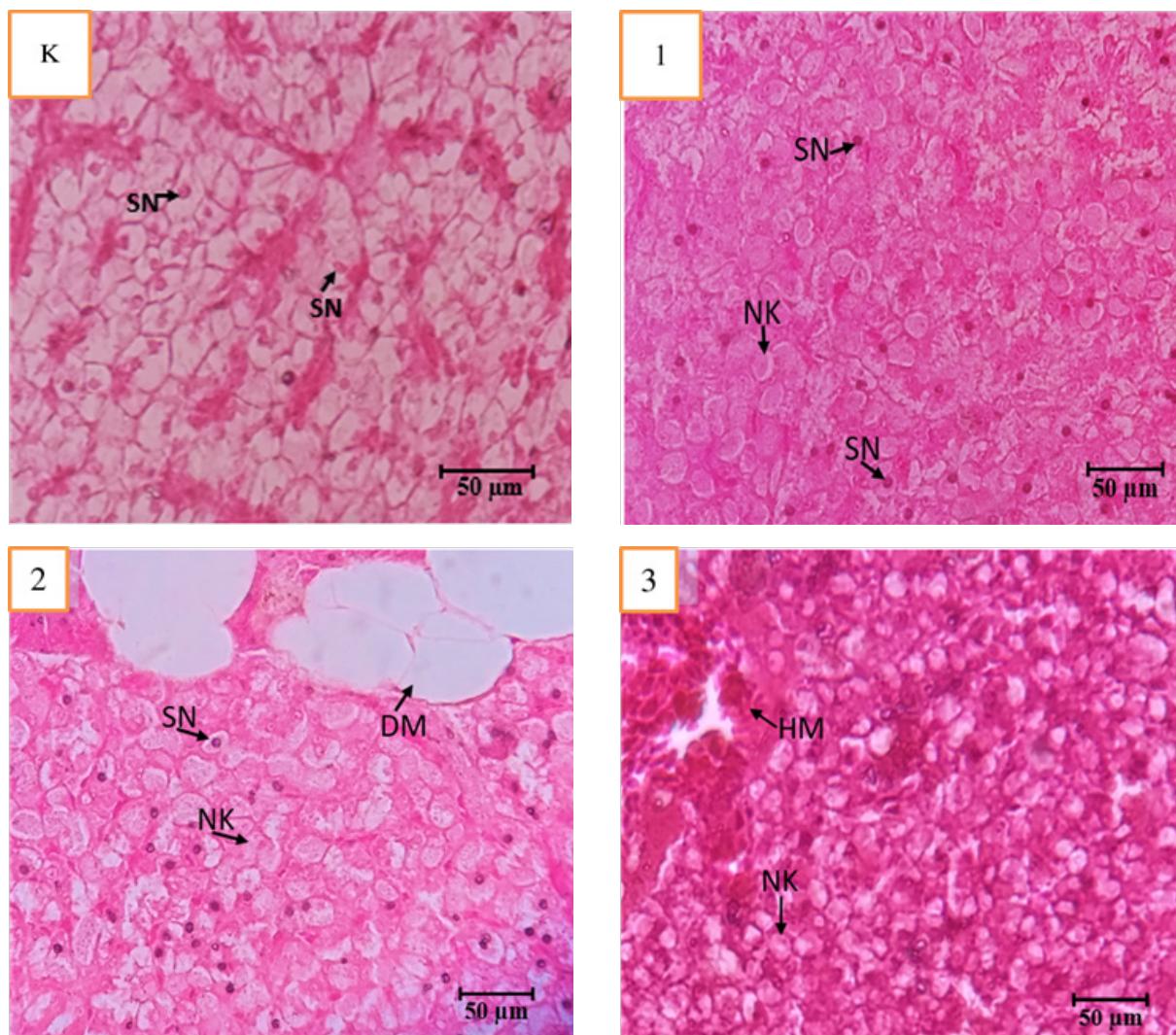


Figure 1. Microscopic condition of liver: control (K), infected with *P. stutzeri* L01 (1), infected with *V. harveyi* (2), and infected with bacteria combination (3). Note: (SN) Normal cell; (NK) necrosis; (MM) melanomacrophage; (DM) lipid degeneration; (HM) hemorrhage. The liver condition was observed by using microscope with 400 times magnification.

Histological observation of liver

The microscopic condition of the liver (Figure 1) between control treatment, *P. stutzeri* L01 treatment, *V. harveyi* treatment, and bacteria combination treatment was different. The worst condition of the liver was showed in bacteria combination treatment, shown some necrosis and haemorrhage.

Hematological observation

The condition of fish hematology in each treatment had a different result for each parameter (Table 7). Total leukocyte among *P. stutzeri* L01, *V. harveyi*, and bacteria combination treatment were higher than control. The highest total leukocyte value was obtained in bacteria combination treatment.

Discussion

Disease in fish is a complex process involving the interaction of a pathogen bacterium, a susceptible fish host and environmental factors. Bacterial fish infection is normally caused by opportunistic bacteria, facultative pathogen bacteria, or obligate pathogen bacteria. Opportunistic bacteria invade immunosuppressed fish, while atypical fish environmental stressors, such as those occurring in intensive aquacultural rearing (hypoxia, abnormal pH, and high population density) generate an optimal setting for such pathogens to thrive (Derome et al., 2016). In general, the survival, multiplication, interaction and pathogenicity of the bacteria require that the pathogen and host exist in the same environment and that the pathogen (1) successfully invades the host, (2) finds a nutritionally compatible niche in the host body, (3) can overcome

Table 7. The hematology of white barramundi at day 0, 1, 7, and 14 after bacteria injection

Observation time	Treatments	Σ Erythrocyte ($\times 10^6$ cell/mm 3)	Σ Leukocyte ($\times 10^4$ cell/mm 3)	Hb (g/dL)
Day 0	Before injection	3.81 ± 0.00 ^a	3.15 ± 0.00 ^a	14.00 ± 0.00 ^a
Day 1	Control	3.78 ± 0.10 ^b	3.20 ± 0.05 ^a	14.00 ± 0.58 ^b
	<i>P. stutzeri</i>	3.73 ± 0.04 ^b	4.45 ± 0.16 ^b	14.00 ± 0.00 ^b
	<i>V. harveyi</i>	3.34 ± 0.09 ^a	4.85 ± 0.24 ^{bc}	13.00 ± 1.00 ^a
	Bacteria combination	3.35 ± 0.07 ^a	5.25 ± 0.13 ^c	13.33 ± 0.58 ^a
Day 7	Control	3.80 ± 0.10 ^b	3.25 ± 0.06 ^a	12.00 ± 0.00 ^b
	<i>P. stutzeri</i>	3.72 ± 0.20 ^b	4.95 ± 0.07 ^b	12.00 ± 0.00 ^b
	<i>V. harveyi</i>	3.45 ± 0.19 ^a	5.80 ± 0.17 ^b	11.67 ± 0.58 ^{ab}
	Bacteria combination	3.32 ± 0.14 ^a	7.45 ± 0.17 ^c	11.00 ± 0.00 ^a
Day 14	Control	3.89 ± 0.18 ^{ab}	3.45 ± 0.15 ^a	12.00 ± 0.58 ^b
	<i>P. stutzeri</i>	3.83 ± 0.21 ^{ab}	4.25 ± 0.05 ^b	12.00 ± 0.00 ^b
	<i>V. harveyi</i>	3.68 ± 0.10 ^a	4.60 ± 0.09 ^{bc}	12.00 ± 0.00 ^b
	Bacteria combination	3.60 ± 0.26 ^a	4.85 ± 0.12 ^c	11.00 ± 0.00 ^a

Different superscript in the same observation time and column showed significantly different result ($P<0.05$).

the host immune response, (4) can use the host cell mechanisms to multiply, (5) can spread to a new host and, finally, (6) possesses a resistance strategy. The virulence and pathogenicity mechanisms developed by bacteria may be associated with the bacterial structure (membrane, fimbriae, pili, etc), to the extracellular products and the host cell defence capacities (Hamed et al., 2018). The result of the LD50 test showed that *P. stutzeri* and *V. harveyi* were the most virulent due to their ability to kill 50% of fish with the lowest bacteria concentration (10^7 CFU/mL). *B. cereus* and *S. roseus* have an LD50 concentration of 10^8 CFU/mL, meanwhile, *P. stutzeri* L02 has the highest LD50 concentration, it was 10^9 CFU/mL. According to the LD50 test result, the bacteria that used for pathogenicity tests in white barramundi juvenile were *P. stutzeri* L01 and *V. harveyi*.

P. stutzeri L01 and *V. harveyi* are common bacteria in tropical seawater and are dominant microflora (Alikunhi et al., 2016; Aguirre et al., 2017). Park et al. (2013) and Deng et al. (2014) stated that *P. stutzeri* was widely distributed to the environment, was occupied in various ecology and was found as an opportunistic pathogen in humans. *P. stutzeri* participates in the denitrification process (biological nitrogen removal from wastewater). *P. stutzeri* that found in human, soil, and seawater is known to have had a different strain and different typical metabolic. *P. stutzeri* is capable to survive under a low oxygen level and has an important role in the denitrification process of water (Diep et al., 2009). *P. stutzeri* is a zoonosis (this type of disease passes from an animal to a human), yet it has lower virulence (Reisler & Blumberg, 1999).

Two bacteria with lowest LD50 then was tested the pathogenicity by checking out the mean time to death, macroscopic, and microscopic fish anatomy (the histology of liver and kidney of fish infected by pathogen bacteria). White barramundi juvenile in bacteria combination treatment has a mortality of 58.33% (highest mortality) and showed significantly different results with *V. harveyi* treatment (55%) and *P. stutzeri* L01 treatment (53.33%), meanwhile there was no mortality in the control treatment ($P<0.05$). This result was directly proportional to the mean time to death of bacteria combination treatment, it was 75.12 hours, it was longer than *V. harveyi* treatment (68.59 hours) and *P. stutzeri* treatment (61.05 hours).

Clinical symptoms in the fish body were characterized by the appearance of hemorrhage in the lower abdomen, dropsy, and ulcer (as found as in chronic disease). This was in line as Ode (2011) stated that fish attacked by vibriosis has some symptoms, such as dropsy and ulcer in some parts of the body. The observation of each macroscopic condition of the liver and kidney after bacteria injection was different. The liver of white barramundi juvenile that has been injected by *P. stutzeri* was turned pale, the kidney was turned dusky red, the intestine was yellowish. The condition of liver and kidney in all treatments of this study were shown in the same condition as mentioned before.

The damage found in three treatments were haemorrhage and necrosis. The total histopathologic score of liver and kidney for 14

days observation was different among treatments than control. The histopathologic score of control was 1.00 (the necrosis was <25%), lower than treatment A that was 1.94 (the necrosis was <25%). The histopathologic score of treatment B was 2.17 (the necrosis was 25–<50%), lower than combination treatment with a histopathologic score of 2.39 17 (the necrosis was 25–<50%). These scores were comparable with histopathologic of the liver in every treatment. The liver and kidney in the control treatment were observed normal (Figure 1), meanwhile, liver and kidney in other treatments were showed some lesions and damage.

Maharajan *et al.* (2016) and Sari *et al.* (2016) stated that the liver became the object that most often examined because the liver is the target organ to obtain an environmental impact and a correlation between environmental factors and structure and function of hepatic cells. Biotoxin, parasite, bacteria, some pollutants such as pesticides and hydrocarbon are sometimes found in the liver. Bacteria that were injected into the fish body is common microflora found in seawater (opportunistic bacteria). This bacteria attacks fish when the water quality is decreased. *P. stutzeri* is a bacteria with a wide geographical area and is known as nitrification bacteria in water (Diep *et al.*, 2009; Hastuti, 2011). The emergence of this bacteria as a pathogen is suspected as a result of water quality reduction in Lampung, therefore the amount of this bacteria is excess.

Hematology parameter has been used as a fish health status indicator in some fish species (Santos *et al.*, 2009; Adedeji, 2010; Akinrotimi *et al.*, 2012; Sayed & Moneeb, 2015). Furthermore, blood is one of the defence components from the attack of the disease. Blood contains antimicrobial that is actively build an innate immune system (Dong *et al.*, 2016). Hematology of white seabass juvenile that was infected by bacteria showed nearly the same. The statistical analysis result showed that the total erythrocyte was not significantly different within treatments on day 1 and day 7, yet the results decreased continuously. On day 14, within treatment A, B, combination treatment, and control were showed a significantly different result. The decrease in these total erythrocyte in treatment B and combination treatment was related to the characteristic of bacteria. *V. harveyi* has hemolysin toxin that can cause lysis of red blood cells by destroying their cell membrane (Suriyani, 2013).

Total leukocyte of white barramundi juvenile that infected by bacteria was increased after 24-hour post-injection. The total leukocyte of *P. stutzeri* L01, *V. harveyi*, and combination were significantly different among treatments with the control. The highest total leukocyte count was found in bacteria combination treatment. Similarly, on day 7 after injection, the total leukocyte count of each treatment was increased continuously and was showed significantly different results among treatments. This increase of total leukocyte since day 1 to day 7 showed that the fish body has responded to antigens. At day 14 after injection, total leukocyte count was decreased, it has shown that there were no mortality found at day 8 to day 14. This result was in line with the statements of Martins *et al.* (2009) and Shahi *et al.* (2014) that the increase of infection intensity caused by pathogen could trigger higher production of leukocyte. Moreover, the increase of leukocyte cells is reflecting on the efficacy of the nonspecific immune system. Mangunwardoyo *et al.* (2010), Priyatna *et al.* (2011) and Kurniawan *et al.* (2014) stated that pathogen in the fish body could activate polymorphonuclear leukocyte cells (melanomacrophage, monocyte, and neutrophils) that plays a role as phagocytic cells. Therefore, the total leukocyte count is often used as an indicator of fish health status

Haemoglobin level of each treatment was increased at day one after injection. This increasing was caused by the response of the body against bacteria. The haemoglobin level of bacteria combination treatment at day seven was significantly different among *P. stutzeri* L01 treatment and control. This was caused by day seven to day 14, there was not found any mortality in both treatment and the fish condition was started recovering, meanwhile, in bacteria combination treatment was still found mortality. Similarly, at day-1, the haemoglobin level of mixed bacteria treatment was lower compared to other treatments ($P<0.05$). Haemoglobin is a nutrient source for bacteria (Pishchany & Skaar, 2012). This showed that the decrease in haemoglobin is caused by bacteria infection. The result of this study was in line with the previous study by Bailone *et al.* (2010) which stated that the decrease in haemoglobin level is caused by bacteria infection inside the host's body.

The bacteria combination treatment showed the highest cell damage in white barramundi juvenile. The clinical symptoms include the body color turned black, haemorrhage, ulcer, and the internal organ

was rotten. These clinical symptoms were concluded as black body syndrome (BBS) disease. Similarly, the histopathologic score of the liver in bacteria combination treatment was 2.39, in which this was the highest score among other treatments. Moreover, the bacteria combination treatment had the highest total leukocyte compared to single bacteria treatment. This was showed that the infection of two bacteria, simultaneously, would cause loss of fish health faster than single bacteria infection. Besides that, high fish mortality showed that the fish immunity could not fight against two bacteria infection simultaneously.

CONCLUSION

The results of bacteria isolation and characterization from white barramundi juvenile showed that black body syndrome (BBS) disease is caused by five different bacteria, such as *P. stutzeri* L01, *V. harveyi*, *B. cereus*, *S. roseus*, and *P. stutzeri* L02. The result of the LD50 test showed that *P. stutzeri* L01 and *V. Harveyi* with the lowest density could cause BBS disease in white barramundi juvenile. Furthermore, the result of the pathogenicity test showed that the infection caused by bacteria combination (*P. stutzeri* L01 and *V. harveyi*) infection had the highest pathogenicity than single bacteria infection.

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