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Kidney and liver histopathology of sea bass *Lates calcarifer* infected with black body syndrome-associated bacteria

Histopatologi ginjal dan hati ikan kakap putih *Lates calcarifer* yang diinfeksi bakteri yang berasosiasi dengan black body syndrome

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ABSTRACT

Bacterial isolation was carried out on sea bass *Lates calcarifer* juvenile with black body syndrome (BBS) and isolates of *Pseudomonas stutzeri* L01, *Vibrio harveyi*, *Bacillus cereus*, *Salinicoccus roseus*, and *Pseudomonas stutzeri* L02 were obtained. Microscopic anatomical pathology of each bacterium needs to be done to determine its pathogenicity. This study aims to compare the degree of damage to sea bass juveniles' liver and kidney organs through histopathological observations due to bacterial infections that cause BBS. The degree of histopathological damage was determined by scoring histopathological changes in the tissue under a microscope with a magnification of 40×10 in five fields of view. The data obtained were analyzed using SPSS 24 software and further tested with Kruskal-Wallis and Post Hoc (Mann Whitney). The results showed that fish liver and kidney organs in all treatments experienced necrosis compared to the control treatment, but not significantly different between bacterial infection treatments. Histopathological observations of kidney and liver organs of sea bass juveniles infected with bacteria that cause BBS show necrosis damage that was classified as normal and mild.

Keywords: bacteria, histopathology, *Lates calcarifer*

ABSTRAK

Isolasi bakteri telah dilakukan pada benih ikan kakap putih yang mengalami *black body syndrome* (BBS) dan diperoleh isolat bakteri *Pseudomonas stutzeri* L01, *Vibrio harveyi*, *Bacillus cereus*, *Salinicoccus roseus*, dan *Pseudomonas stutzeri* L02. Patologi anatomi mikroskopis dari masing-masing bakteri perlu dilakukan untuk mengevaluasi patogenitasnya. Penelitian ini bertujuan membandingkan tingkat kerusakan organ hati dan ginjal benih ikan kakap putih melalui pengamatan histopatologi akibat infeksi bakteri penyebab BBS. Derajat kerusakan histopatologi ditentukan dengan melakukan skoring perubahan histopatologi jaringan di bawah mikroskop dengan perbesaran 40×10 pada lima lapang pandang. Data dianalisis menggunakan *software* SPSS 24 dan diuji lanjut dengan *Kruskal-Wallis dan Post Hoc* (*Mann Whitney*). Hasil pengamatan menunjukkan bahwa organ hati dan ginjal ikan pada semua perlakuan infeksi mengalami nekrosis dibandingkan dengan perlakuan kontrol, tetapi tidak berbeda nyata antar perlakuan infeksi bakteri. Hasil pengamatan histopatologi organ ginjal dan hati benih ikan kakap putih yang diinfeksi dengan bakteri yang menyebabkan BBS menunjukkan kerusakan nekrosis yang tergolong pada derajat normal dan ringan.

Kata kunci: bakteri, histopatologi, kakap putih

INTRODUCTION

Sea bass (*Lates calcarifer*) is one of the most economically fish cultivated in several Asian countries, including Indonesia, due to its high economic potential (Maharajan *et al*., 2016). The target production of sea bass was 30,000 tons in 2017, but the actual production only reached 22,545 tons (KKP, 2018). Technical problems of cultivation and parasitic diseases still disrupt the development of sea bass culture in several coastal areas of Indonesia (Irmawati *et al*., 2020). One of the obstacles to white seabass cultivation is the limited number of white seabass juveniles. Sea bass juveniles measuring 3-4 cm are easily stressed and have body defenses that are vulnerable to disease attacks (Affandi & Tang, 2002).

In addition, disease outbreaks caused by bacteria in aquaculture have increased in recent decades. Bacterial infections dominated the reports for sea bass about 75.0% (Muniesa *et al*., 2020). One disease that attacks sea bass is black body syndrome (BBS). BBS disease is a bacterial disease that infects sea bass juveniles 6-7 cm with characteristics of black body colour, wounds on the body, passive swimming, and decreased appetite. Based on research by Izwar *et al.* (2020), BBS symptoms are associated with five types of pathogenic bacteria including *Pseudomonas stutzeri* L01, *Vibrio harveyi*, *Bacillus cereus*, *Salinicoccus roseus*, and *Pseudomonas stutzeri* L02.

Bacterial infections are primarily the cause of the mass mortality that occasionally occur in marine fish hatcheries (Balachandran *et al*., 2013). Vibrio plays a vital role in marine culture ecosystem as they damage causing diseases and mortality to some aquaculture commodities (Ganesh *et al*., 2010). Vibriosis was the most frequently reported bacterial infection in sea bass in the ongrowing phase (Muniesa *et al*., 2020). Vibrio virulence factors play a crucial role in its pathogenesis, including making the pathogen attach to and enter the host, reproduce in the body, avoid defence mechanisms, and cause damage to the host (Ji *et al*., 2020).

Streptococci typically infect immune-deficient fish that reside in ideal environments (such as a polluted marine environment) and have a weakened immunological response like fish juvenile (Gnanagobal & Santander, 2022). *Pseudomonas* sp. causes damage to internal tissue, such as changing the consistency of the kidneys and heart (Hardi *et al*., 2018). Percentage of fish mortality caused by *Pseudomonas* sp. and vibrio infected were 80% and 90% respectively (Elsayed *et al*., 2018). Histopathological examination of tissues can be used to assess the impact of contaminants on organs and histological biomarkers can be used to infer the presence of various toxins such as carcinogens or certain disease infections (Karim *et al*., 2022).

Liver and kidney organs are suitable for histopathological examination of cell and tissue damage caused by microorganisms in aquatic systems. In the study of Ahmed *et al.* (2013) the presence of disease-causing foreign bodies caused histomorphology deviations in the gills, liver, and kidneys of tilapia. Similar studies revealed that contaminants had little effect on other tissues. Bacteria that enter the blood will infect essential organs in fish, such as the liver, kidneys, and so on.

Bacteria can utilize the kidneys as a place to reproduce themselves and take nutrients that are used for their metabolic processes (Sukenda *et al*., 2008). The liver plays a role in the metabolic process of important substances in the fish body, the formation and secretion of bile, and the body's defense in detoxification. The kidneys are involved in excreting residual nitrogen and regulating water and salt balance. The liver functions in basic metabolic activities (such as glycogen storage, excretion of bilirubin, hormones, etc.) and accumulates, converts, and removes various toxins in fish.

Depending on contamination and concentration, the liver is susceptible to various toxins and changes in the fish liver (Abdel-Moneim *et al*., 2013). Like the kidney, the liver is sensitive to changes indicated by histological alterations due to exposure to pathogens. This study aims to compare the level of damage to sea bass juvenile kidney and liver organs infected with five different bacterial isolates through histopathological observations. Comparison of injection between bacteria determined the level of pathogenicity of each bacterium.

MATERIALS AND METHODS

Preparation of bacteria and rearing site

Stock bacteria were cultured on Tryptic Soy Agar (TSA) media in petri dishes. The media that has been scratched is then incubated in an incubator with a temperature of 37 °C for 24 hours. After incubation, bacteria were observed. Then the bacteria were cultured in Tryptic Soy

Broth (TSB) media at 30 °C for 24 hours. The density of bacteria was made according to the LD50 results.

The LD50 results obtained the density of bacteria injected in healthy sea bass, namely *Pseudomonas stutzeri* L01 with a density of 8.4×10^7 CFU/mL, *Vibrio harveyi* bacteria with a density of 6.2×10^7 CFU/mL, *Bacillus cereus* bacteria with a density of 2.9×10^8 CFU/mL, *Salinicoccus roseus* bacteria with a density of 4.2×10^8 CFU/mL and *Pseudomonas stutzeri* L02 with a density of 3.7×10^8 CFU/mL, as well as a control treatment using PBS solution as much as 0.1 mL. The bacteria stock were obtained from Fish Health Laboratory, Department of Aquaculture, IPB. Fish were injected with one type of bacteria per fish, then reared for 14 days.

Fish were reared in a glass aquarium with a size of $65\times40\times40$ cm^3 with a water height of 20 cm. The aquarium was disinfected with chlorine at a dose of 30 ppm, then filled with ± 15 liters of water, and installed an aerator set for aeration. The test fish were acclimatized in the rearing aquarium for three days. Water quality management was carried out by syphoning in the morning before feeding and changing aquarium water once every three days as much as 50% of the total volume of the aquarium.

Research Design

This study used sea bass juveniles with an average weight of 5.64 ± 0.16 grams. The experiment used a completely randomized design consisting of six treatments and three replications, control (K), *Pseudomonas stutzeri* L01 (A), *Vibrio harveyi* (B), *Bacillus cereus* (C), *Salinicoccus roseus* (D), and *Pseudomonas stutzeri* L02 (E). The number of fish injected was 20 with each treatment.

Preparation of Histopathology

The histology preparation was carried out through several stages instead of fixation, blocking, block cutting, and staining. In the fixation stage, the kidney and liver were put into a 10% BNF fixative solution (buffer normal formalin) for 24-48 hours. Then dehydration was carried out by immersing the tissue in alcohol in stages, including 70% alcohol for 24-48 hours, 80%, 90%, and 95% alcohol for two hours each, and 100% alcohol concentration for 12 hours. Next, the tissue was immersed in alcohol and xylol (1:1) for 30 minutes. After that, the tissue was immersed in xylol three times for 30 minutes.

At the tissue paraffinization stage, the tissue was immersed in a mixed solution of xylol and paraffin (1:1) at a liquid point of 50-60 C. Then, the tissue was immersed using paraffin for 45 minutes on a printing block. Finally, the tissue was cut using a microtome with a thickness of 0.5 micrometres. The colouring process starts from the dewaxing stage, which is carried out by soaking the tissue using xylol twice for three to five minutes.

Furthermore, hydration was carried out using 100% alcohol twice for three minutes. The tissue was soaked using 95%, 90%, 80%, 70% and 50% alcohol each for three minutes, then rinsed with distilled water. Tissue staining used hematoxylin and eosin. The preparations were immersed in hematoxylin for three to five minutes and then rinsed with distilled water.

Tissues were immersed in eosin for three to seven minutes, then rinsed with distilled water. The preparations were soaked gradually in 50%, 70%, 85%, 90%, and 100% alcohol for two minutes. The mounting process was carried out by covering the preparations with cover glass and giving Entellan Rapid Mounting Medium. The preparations were dried for 24 hours and were ready for observation.

Observation of Histopathology

Histopathological tissue observations using an Olympus CX31 microscope with 40×10 magnification and documented using an Optilab camera and Image Raster application. Observations of tissue structure were made on days 1, 2, 3, 5, 10 and 14 post-test. In addition, calculation of the percentage of kidney and liver necrosis was done in each of five fields of view (Izwar *et al*., 2020) using the following formula:

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

P (%) = Percentage of cells with necrosis
Σ KS = Total number of necrotizing cells in five field of view
Σ TS = Total number of cells in 5 field

The scoring method was organized by integers from one to five, with one representing the normal condition and five the maximum level of the evaluated phenomenon. The definition of each score is described in paragraphs to distinguish each level clearly. The scoring of liver and kidney organs of sea bass juveniles refers to Wolf *et al*. (2015) with the division of graded scores Table 1.

Data Analysis

The histopathological condition of the fish organs was calculated using a semiquantitative score protocol. Tissue histopathology data were analyzed using ANOVA through the SPSS program and tested with Kruskal-Wallis. If P<0.05, a Post Hoc test (Mann Whitney) would be analyzed.

RESULTS AND DISCUSSION

Liver histopathology

The results of liver histopathology of sea bass after being injected with bacteria *Pseudomonas stutzeri* L01, *Vibrio harveyi*, *Bacillus cereus*, *Salinicoccus roseus*, and *Pseudomonas stutzeri* L02 showed differences in microscopic anatomical pathology in experimental fish treatment and control. In Figure 1K, the hepatocytes were healthy and normal, while the other Figure showed most of the hepatocytes are necrotizing. The histology profile of the experimental fish was presented in Figure 1.

Kidney histopathology

Histopathology results of the kidney of sea bass after being injected with bacteria *Pseudomonas stutzeri* L01, *Vibrio harveyi*, *Bacillus cereus*, *Salinicoccus roseus*, and *Pseudomonas stutzeri* L02 showed differences between fish injected with

Table 1. Histopathology scoring and degree of liver and kidney tissue damage.

Necrosis	Score	Degree of deterioration
P<25 % total field of view	1	Normal
25≤P< 50% total field of view	2	Mildly damaged
50≤ P <75% total field of view	3	Moderately damaged
P ≥ 75% total field of view	4	Severely damaged

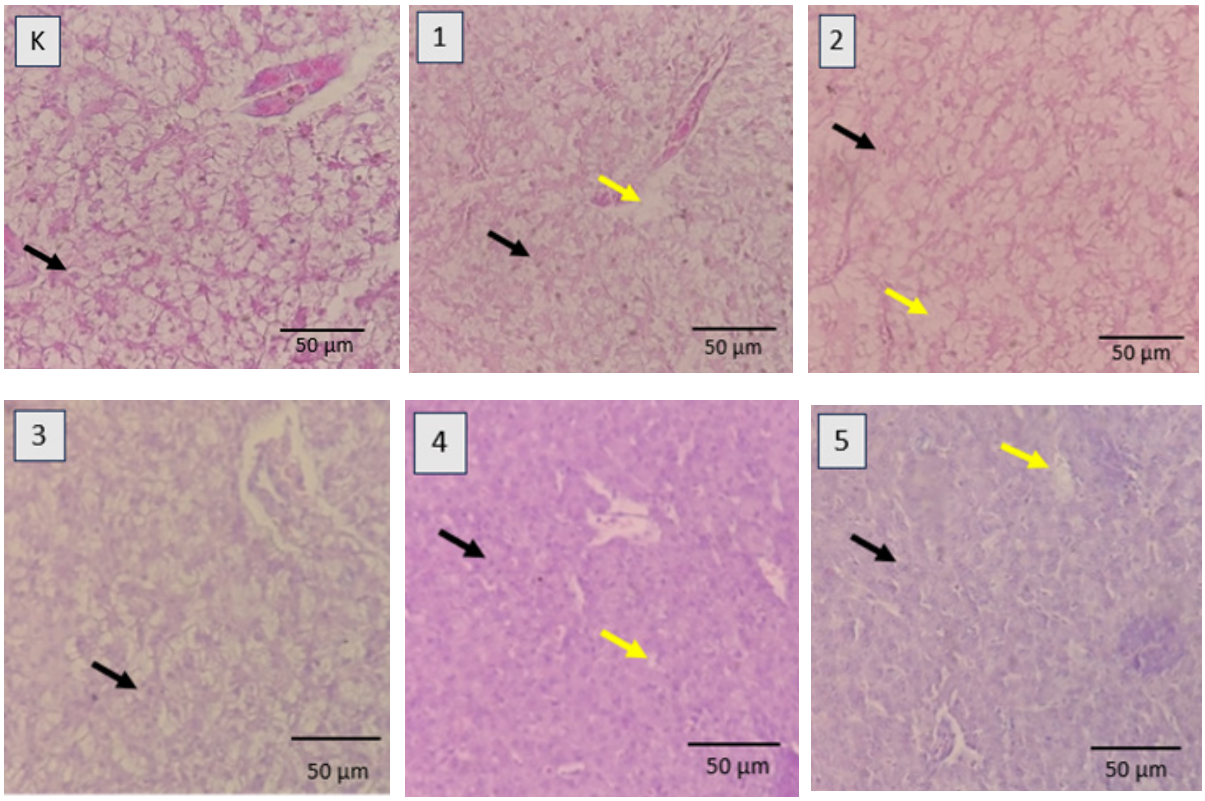

Figure 1. Microscopic anatomical pathology of liver tissue of barramundi on 7th day after bacterial infection. K: Control; 1: *Vibrio harveyi*; 2: *Pseudomonas stutzeri L01*; 3: *Bacillus cereus*; 4: *Salinococcus roseus*; 5: *P. stutzeri* L02. Black: normal cell; yellow: necrosis cell.

bacteria and control (Figure 2K). Hepatocytes in the control group looked healthy and normal, while the other images showed that most of the hepatocytes had necrosis. In addition, the size of the tubes in the kidneys injected with the bacteria looked abnormal compared to the control. The histology profile of the experimental fish was presented in Figure 2.

Histopathology score

Sea bass, after being injected with bacteria associated with Black Body Syndrome, showed the histopathology scores of liver and kidney organs of all treatments were significantly different (P<0.05) with the control treatment. The results of the total score of the histopathology test of the liver and kidneys of white sea bass juveniles can be seen in Table 2.

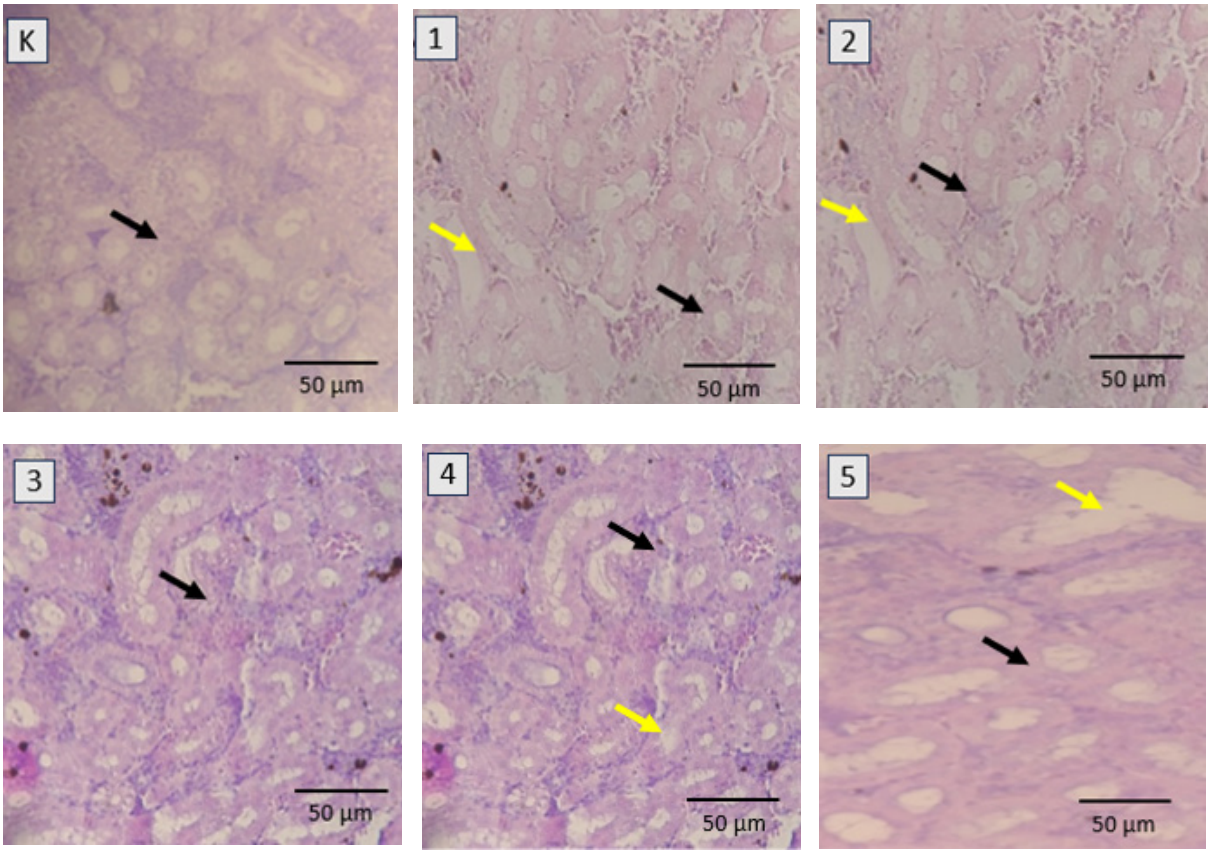

Figure 2. Microscopic anatomical pathology of kidney tissue of barramundi on 7th day after bacterial infection. K: Control, 1: *Vibrio harveyi*, 2: *Pseudomonas stutzeri L01*, 3: *Bacillus cereus*, 4: *Salinococcus roseus*, 5: *P. stutzeri* L02. Black: normal cell; yellow: necrosis.

Table 2. The total score of liver and kidney histopathology test of white sea bass juvenile.

Treatment	Histopathology score			
	Liver	Degree of Deterioration	Kidney	Degree of Deterioration
Control	1.00 ± 0.00^{a}	Normal	1.00 ± 0.00^{a}	Normal
1	2.05 ± 0.22^{bc}	Mild	1.81 ± 0.22^{bc}	Mild
2	2.24 ± 0.09^{c}	Mild	2.05 ± 0.22^{c}	Mild
3	2.00 ± 0.60^{bc}	Mild	1.77 ± 0.25^{bc}	Mild
4	1.83 ± 0.17^{b}	Mild	1.66 ± 0.33^{bc}	Mild
5	1.94 ± 0.38^{bc}	Mild	1.72 ± 0.09^{b}	Mild

Notes: Different superscript letters in the same column indicate significantly different results (P<0.05). 1: *Vibrio harveyi*, 2: *Pseudomonas stutzeri L01*, 3: *Bacillus cereus*, 4: *Salinococcus roseus*, 5: *P. stutzeri* L02

Post hoc Mann-Whitney test determined specific differences between the two treatments. Histopathology results of the kidney and liver of sea bass with the Post hoc Mann-Whitney test can be seen in Table 3.

Discussion

Bacterial diseases in fish generally do not develop simply due to exposure of the infectious agent to the host. Mainly, bacterial diseases occur due to complex interactions between pathogens, fish and environmental stresses that affect the susceptibility of fish to disease. Environmental stresses can affect the homeostatic mechanisms of the fish, thereby reducing its resistance to disease-causing organisms. Fish infected by several bacteria associated with BBS were experimentally observed histologically by H&E staining of kidney and liver organs. BBS is a symptom of bacterial attack on sea bass fish juveniles with characteristics of black body colour, body wounds, passive swimming, and decreased appetite.

Research conducted by Izwar *et al*. (2020) successfully isolated five bacterial isolates suspected of being pathogenic agents that cause BBS disease. The results shown in Figure 1 and Figure 2 indicate that clinically diseased fish collected from fish infected with several bacteria show similar histological changes. Observations of each macroscopic condition of the liver and kidney of sea bass after bacterial injection compared to the control showed differences. Fish injected with bacteria that cause BBS showed blackish red kidneys, paler liver, yellowish intestines, and paler bodies. The condition of the liver and kidneys in all treatments of this study showed the same condition as previously mentioned, except for the control treatment, which did not show any macroscopic changes.

Histopathological examination of abnormal changes in fish tissue is needed to evaluate the damage, especially in the kidney and liver organs. The results of histopathological observations on the liver and kidneys of sea bass fish juveniles experienced necrosis. The kidney is an organ that secretes metabolic products in the form of ammonia. The activity of pathogenic bacteria causes abnormalities in kidney structure and function. Bacteria that enter the kidney, multiply and utilize nutrients for metabolic processes, so the tissue in the kidney undergoes necrosis.

Meanwhile, the liver is an organ that produces bile which acts as an emulsifier in the process of food digestion (Sukenda *et al*., 2008). The challenge test results showed the highest pathogenicity of *Vibrio harveyi* bacteria compared to other bacteria. Kidney and liver damage of sea bass juveniles injected with *Vibrio harveyi* were more severe than fish's kidney and liver in other groups. *Vibrio harveyi* is an opportunistic pathogen that exists in a typical environment and develops pathogenically when the environment and host are in poor condition. Virulence factors in *Vibrio harveyi* are endotoxins that are adhesive to the host cell wall, hemolysins, proteases, lipopolysaccharides (LPS), iron-binding capacity, interaction with bacteriophages,

Table 3. The histopathology of sea bass juveniles kidney and liver were analyzed by Post hoc Mann-Whitney test.

Organ	Variable	*Pseudomonas stutzeri L01*	*Vibrio harveyi*	*B.cereus*	*S.roseus*	*Pseudomonas stutzeri L02*
liver	*Control*	0.037*	0.034*	0.037*	0.037*	0.034*
	Pseudomonas stutzeri L01		0.246	0.827	0.184	0.825
	Vibrio harveyi			0.827	0.046*	0.261
	Bacillus cereus				0.513	0.817
	Salinicoccus roseus					0.507
kidney	*Control*	0.037*	0.037*	0.037*	0.037*	0.034*
	Pseudomonas stutzeri L01		0.261	0.658	0.658	0.507
	Vibrio harveyi			0.184	0.184	0.046*
	Bacillus cereus				0.658	0.653
	Salinicoccus roseus					0.817

*Significant differences are indicated by an asterisk; P≤0.05.

biofilm formation, and quorum sensing, have been identified (Zhang *et al*., 2020).

Histological examination of naturally diseased fish revealed signs of severe necrotic muscles with massive immune-related cell infiltration, severe haemorrhage and blood blockage in the brain, swollen renal tubules, and epithelial cells sloughing into the lumen (Dong *et al*., 2017). In addition, *Vibrio harveyi* bacteria cause the mortality of sea bass juveniles as much as 33.33% (Nugrahawati *et al*., 2019). Pseudomonas is one of the bacteria that can survive in various environmental conditions, including marine habitats, due to its metabolism and genetic versatility. Pseudomonas species have been identified as causing diseases in fish, such as *P. fluorescens*, *P. angulliseptica*, *P. aeruginosa*, and *P. putida* (El-Barbary & Hal, 2016). In this study, the histopathology of sea bass infected with *Pseudomonas stutzeri* L01 and *Pseudomonas stutzeri* L02 showed moderate necrosis.

In Abodayak *et al*. (2016) studied, *Pseudomonas stutzeri* was associated with an opportunistic pathogen that manifested with the disease in European sea bass *Dicentrarchus labrax* with symptoms such as skin ulceration, haemorrhage, and liver congestion. Pseudomonas infects fish mainly through skin and fin trauma and results in a series of characteristic clinical symptoms, such as lethargy, no appetite, disorientation, abdominal swelling with ascites, and abundant white spots on the surface of spleen tissue (Sun *et al*., 2020). During infection, Pseudomonas can spread through the bloodstream and then colonize in the spleen as well as other sampled tissues, including blood, gills, spleen, liver, head kidney, trunk kidney, heart, brain, foregut, hindgut, proximal muscle, distal muscle (Luo *et al*., 2020). The survival rate of Red Sea seabream was only 16.6% due to Pseudomonas infection. In addition, *Pseudomonas stutzeri* showed multi-antibiotic resistance to streptomycin, chloramphenicol, and ciprofloxacin (Emam *et al*., 2022).

Based on the results of histopathological observations of the kidneys and liver of sea bass fish juveniles injected with *Bacillus cereus* bacteria, they showed mild damage. *Bacillus cereus* is a gram-positive facultative anaerobic bacterium that produces endotoxins and can cause disease in aquatic animals. *Bacillus cereus* is ubiquitous because it does not have complex nutritional requirements. The bacteria are widespread in the environment and soil. Thus, processed and non-processed foodstuffs are often contaminated by *Bacillus cereus*, such as vegetables, sauces, rice, cereals, and fish (Nguyen & Tallent, 2019).

Bacillus cereus also causes ulcers in dumbo catfish *Clarias gariepinus* with clinical symptoms of experimentally injected fish showing symptoms of lethargy, skin discolouration, superficial hemorrhagic ulcers, deep ulcers reaching muscle erosion, and underlying tail. Some fish showed haemorrhages in fins and vents, exophthalmia, and congested internal organs (Younes *et al*., 2021). In addition, the mortality of sea bass juveniles injected with *Bacillus cereus* was 54% (Izwar *et al*., 2020). Based on the results of histopathological observations of the kidneys and liver of sea bass fish juveniles injected with *Salinicoccus roseus* bacteria, they showed mild damage. These bacteria were often found in waters with poor environmental quality.

The mortality of sea bass juvenile injected with *Salinicoccus roseus* was 43% (Izwar *et al*., 2020). Bacterial infections isolated from snapper that experienced BBS showed the results of a mild degree of tissue damage, while controls infected with PBS showed no tissue damage (normal). This condition is different from the research of Izwar *et al*. (2020) which showed that co-infection with *Pseudomonas stutzeri* and *Vibrio harveyi* bacteria resulted in a level of damage from the liver and kidneys between mild to moderate degrees with scores of 2.39 ± 0.10 and 2.39 ± 0.26, respectively. Single infection in this study resulted in a score of less than two which indicates the level of damage at a mild degree, except for a single infection with *Vibrio harveyi* bacteria which resulted in scores on the liver and kidneys of 2.24 ± 0.09 and 2.05 ± 0.22, respectively.

CONCLUSION

The research results showed that liver and kidney organs in all treatments exhibited necrosis, but there was no discernible difference between treatments for bacterial infection. According to histopathological observations, the kidney and liver of juvenile sea bass infected with the bacteria that cause BBS exhibit normal and mild necrotic damage.

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