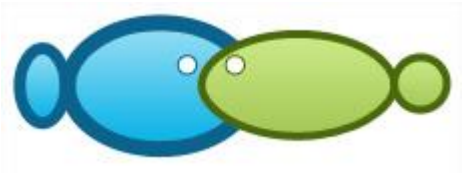

The effects of synbiotics and various herbs during the *Photobacterium damselae* challenge test on the histopathology of seabass (*Lates calcarifer*) internal organs

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Abstract. Seabass (*Lates calcarifer*) is a widely distributed species with high economic potential in fish culture. However, death in the cultivation of seabass is often caused by *Photobacterium damselae* bacteria. Fish can be protected from disease by providing the feed with synbiotics and natural herbal substances. The study examines the internal histological organs of seabass exposed to *P. damselae* to determine the impact of administering synbiotics and various herbs. The study was carried out at the Lampung Marine Aquaculture and Fishery Center with a completely randomized design method, consisting of 5 treatments and 4 replications. The treatments included commercial feed (- control), commercial feed + synbiotics (+ control), synbiotics, commercial feed + some herbs (herbs), and commercial feed + herbs + synbiotics (mixed), which was given to seabass fry with a mean length of 7.7±0.8 cm. The challenge test was conducted by infecting the fish with *P. damselae* bacteria through intra-peritoneal injection. The results indicated that the mixed treatment maintained the lowest mortality rate during the challenge test with *P. damselae*. Meanwhile, internal organ histopathology observations in each treatment showed no differences. According to histological findings, all treatments resulted in organ necrosis, congestion, hemorrhaging, melanomacrophage centers (MMCs), inclusion bodies, and fat degradation. Clinical symptoms appear in fish, such as flaking on the caudal and anal fin, body, and abdomen necrosis with red spots, loose scales, and distended head and abdomen.

Key Words: body resistance, growth, immunity, pasteurellosis, vibriosis.

Introduction. Seabass (*Lates calcarifer*) mostly live in southeast Asian countries, such as Indonesia, Thailand, and Vietnam. This fish has a high economic value and is widely cultivated in Asia and Australia (Eni et al 2017; Sahputra et al 2017; FAO 2020). The consumer interest and high economic value are not balanced with the current production. The cultivation has experienced many obstacles, such as infection with pasteurellosis and vibriosis, which caused mass deaths. *Photobacterium damselae* is one of the causative agents of pasteurellosis and vibriosis that often infects seabass (Korun & Timur 2005; Ransangan et al 2012; Rivas et al 2013; Abu-Elala et al 2015; Miranti 2016; Dong et al 2017; Varvarigos 2020).

The utilization of antibiotics in disease management has impacted the nature of bacterial resistance and the residues may be destructive to human health. Previous studies on *Vibrio harveyi* indicated that this bacteria is resistant to several antibiotics, such as chloramphenicol, erythromycin, and furazolidone (Miranti 2016; Deng et al 2020; Novita et al 2020; Yilmaz et al 2022). One way to securely overcome a fish infection is through the formation of fish body resistance. Many herbs are known to possibly have high immune-

forming and growth-enhancing compounds. Thus, the combined utilization of natural herbal ingredients and synbiotics is an alternative way of avoiding disease in fish.

This study used a mixture of various herbs, including black cumin (*Nigella sativa*), turmeric (*Curcuma longa*), and bitter (*Andrographis paniculata*). These three ingredients are known to increase endurance. The use of black cumin, turmeric, and bitter increases the growth and immunity of fish and shrimp (Fauzy et al 2014; Hidayat et al 2014; Novisa et al 2015). Several previous studies reported that black cumin, turmeric, and bitter independently served as immunostimulants and were effective in increasing growth along with immunity in fish and shrimp (Fauzy et al 2014; Novisa et al 2015; Bektaş et al 2018; Aly et al 2019). Probiotics, prebiotics, and herbs applied independently or separately have been utilized for a long time in aquaculture. In any case, very few are applied in the combined form of synbiotics with various herbs. This becomes the basis for studying the application of plant combinations (black cumin, turmeric, bitter) and synbiotics in feed. This study aimed to determine the body resistance and histopathological structure of seabass internal organs after being treated with a combination of synbiotics and various herbs in the feed and challenged test with *P. damselae.*

Material and Method

Research design. This study was conducted at the Lampung Marine Aquaculture and Fishery Center using a Completely Randomized Design (CRD) method with 5 treatments and 4 replications. The treatments included: P1 - commercial feed + 9 mL commercial synbiotics as a positive control; P2 - commercial feed as negative control; P3 - commercial feed + herbs (37.5 g black cumin + 2 g turmeric + 1 g bitter)/ kg feed; P4 - commercial feed + 9 mL synbiotics; P5 - commercial feed + herbs (37.5 g black cumin + 2 g turmeric + 1 g bitter)/ kg feed + 9 mL synbiotics. *L. calcarifer* with a mean length of 7.7±0.8 cm were reared in fiber tanks put in 50x50x50 cm fishing nets with a density of 25 fish/waring. The water was changed continuously, and the tank was equipped with aeration. The treatment was carried out for 70 days with a 14-day challenge test.

Synbiotic preparation. Bionelaplus probiotics (*Bacillus* IP121, *Bacillus* IBK3, *Lactobacillus sp.*, ammonia-oxidizing bacteria - AOB - A3P, anoxygenic photosynthetic bacteria - BFA) were inoculated into Sea Water Complete (SWC) broth in an Erlenmeyer and incubated for 24 hours at room temperature. The starter was inoculated on an Erlenmeyer with SWC broth media with a composition of 10% starter + 90% SWC media and stored in cold conditions at 4°C to maintain bacterial viability.

The material used in making prebiotics was jicama (*Pachyrhizus erosus*), which refers to the modified method by Park & Han (2015). Yam bean was peeled and washed with distilled water before cutting it into pieces, and the slices were dried at 60°C and ground into flour. The next step was the extraction of oligosaccharides from yam bean flour, which refers to the modified study method of Oktaviana et al (2014). The flour was suspended in 70% ethanol in a ratio of 1:10 and stirred at room temperature. Furthermore, it was precipitated and filtered using filter paper and a sterile funnel, and the filtrate was concentrated using a rotary evaporator at 40°C. The ethanol content in the filtrate was reduced by storing the extract in an oven at 40°C until the paste was formed (Sumardi et al 2021).

Herbal maceration process (black cumin, turmeric, and bitter). This study used black cumin (*Nigella sativa*), turmeric (*Curcuma longa*), and bitter (*Andrographis paniculata*), with an inclusion of 37.5 g, 2 g, and 1 g kg^{-1} feed. Herbal extraction was carried out by the maceration method referring to Miranti (2016). In the initial stage, black cumin, turmeric, and bitter were floured and filtered through a 60 µm mesh sieve. The maceration process followed the stage with a 1:10 ratio of ethanol solution and stirred at room temperature. The herbal flour was precipitated and filtered using filter paper and a sterile funnel. Furthermore, the filtrate was concentrated with a rotary evaporator at 40°C, and the remaining ethanol content was removed until a paste was formed.

Feed formulation. This study used a commercial feed with a protein content of 37%. Prebiotics, probiotics, herbs, and additional feed adhesives were placed in a container with 150 mL of distilled water, then stirred until evenly mixed. The mixture was stirred in the feed evenly and air-dried at room temperature without being exposed to direct sunlight.

Challenge test using P. damselae. The challenge test was conducted by infecting *P. damselae* bacteria in the test fish with the dose of $6.7x10^9$ cfu mL^{-1} through intraperitoneal injection. The number of doses was adjusted to the body weight, and the infected fish were observed based on abnormal symptoms before dying.

Histological preparation. Marwati et al (2016) referred to the histology preparations technique. The test fish from each treatment that showed abnormal symptoms after the challenge test was selected, and the internal organs, such as kidneys, spleen, liver, heart, and gills, were collected. The kidneys, spleen, liver, and heart were fixed in formalin solution, while the gills were fixed in Bouin's solution for 24 hours. The next stage was the dehydration process, starting with putting the sample into an alcohol container with ascending grades of 70%, 80%, 90% alcohol, and absolute alcohol for 1 hour each. The sample was placed into xylol-alcohol (1:1) for 1 hour and pure xylol 1 and 2 for 1 hour each.

The next stage of the paraffin infiltration process was carried out by putting the sample into a mixture of xylol-paraffin (1:1) for 1 hour before putting it into pure paraffin I and II for 1 hour each. The sample was planted in paraffin blocks and allowed to freeze, then attached to the block holder or wood. Before cutting, paraffin blocks were placed on ice pads. They froze quickly, and the samples were cut using a microtome with a thickness of 5-6 μm. To expand the sample or avoid shrinking, the paraffin tape containing the sample was placed in a water bath at a temperature of 45°C. After the expansion, it was taken and affixed to an object glass smeared with new Entellan.

The samples were stained using hematoxylin-eosin by placing them in a solution of xylol I and xylol II for 2 min each. Subsequently, rehydration was carried out in descending graded alcohol from absolute, 90%, 80%, 70%, and 35% alcohol for 2 min before being washed with enough running water. In the next stage, the sample was immersed in a solution of hematoxylin for 5 min and then washed with running water until clean. They were immersed in eosin solution for 2 min, and the excess solution was washed off. The sample was immersed in ascending graded alcohol, from 35%, 70%, 80%, 90% alcohol, and absolute alcohol, for 20 sec, before putting it in xylol I and xylol II. Mounting was performed by covering the sample using a cover slip glued with a new Entellan. The sample was dripped with new Entellan, slowly covered with a slip to avoid bubbles, and was observed under a microscope.

Water quality. Water quality parameters measured during the study included salinity, dissolved oxygen (DO), temperature, pH, nitrite, and ammonia. The measurement was carried out 3 times at the beginning, middle, and end of the study. Water quality measurements were made with: salinity with a refractometer, DO with a DO meter, water temperature with a thermometer kit, and pH with a pH meter.

Data analysis. Data collection included observation of clinical symptoms after the challenge test and the mortality rate of fish. Subsequent observations compared the histopathological structure of fish organs (kidney, liver, heart) with the normal histopathological structure. The data in the form of post-challenge mortality rate, clinical symptoms, the histopathological structure of organs, and water quality were analyzed descriptively.

Results. The lethal dose 50 (LD_{50}) of *P. damselae* was carried out to determine the required dose in the challenge test, and a value of 4.46×107 CFU per head was obtained. The challenge test was carried out using *P. damselae* bacteria being infected intraperitoneally in the fish's body with a syringe. Furthermore, the dose given was adjusted to the body weight, and this test was observed for 14 days. The results showed

that the highest post-challenge mortality rate in the negative control treatment was 60%, and the lowest mortality rate was found in the mixed treatment (0%).

Clinical symptoms of fish after the challenge test with P. damselae. Examination of clinical symptoms was performed after a challenge test with *P. damselae* bacteria for 14 days in all treatments. Clinical symptoms in all treatments were redness (necrosis) on the fish's body, scabs on the caudal fin, and loose scales. Symptoms began to appear on the 3rd day post-injection, with the appearance of redness (necrosis) on several parts of the fish's body (Table 1).

Table 1

Clinical symptoms of fish after being injected with *Photobacterium damselae* for 14 days

Treatments	*External clinical symptoms*	*Clinical symptoms of internal organs*
Control +	Sore and scaly caudal fin, thin body, redness on the head, anus damaged.	Swollen spleen and thymus, pale liver.
Control -	Flaky caudal and anal fins, red spots on the body, sores and loose scales, red spots on the head and around the eyes, yellow discharge during surgery, distended abdomen.	Pale liver, swollen spleen, kidneys, and thymus.
Herbs	Reddish body, blurry eyes, flaky and bleeding anal fin, peeling scales, reddish abdomen, pale and sore upper fin, red mouth.	Pale liver and kidneys.
Synbiotics	Flaky caudal, dorsal, pectoral fins, pale eyes.	Black liver, swollen kidneys.
Mixed	Peeling scales peeling, flaky caudal fin.	Pale liver and kidneys.

Histopathology of seabass internal organs after treatments and challenge test with P. damselae. The results showed that all treatments had melanomacrophage centers (MMC) in the kidneys (Figure 1).

Other tissue damages experienced by the kidney organs are in the form of congestion, hemorrhage, and inclusion bodies (Figure 2).

Liver histopathological structure. The histological structure of the liver is composed of hepatocytes and pancreatic cells. Based on observations, all treatments experienced fat degeneration (Figure 3).

Other tissue damages experienced by the liver are hemorrhage and congestion (Figure 4).

Cardiac histopathological structure. Based on the observation, the heart was subjected to necrosis in the adverse control treatment, and hemorrhage was formed in the positive control, herbal, and mixed treatments. Congestion was also observed in the mixed treatment (Figure 5).

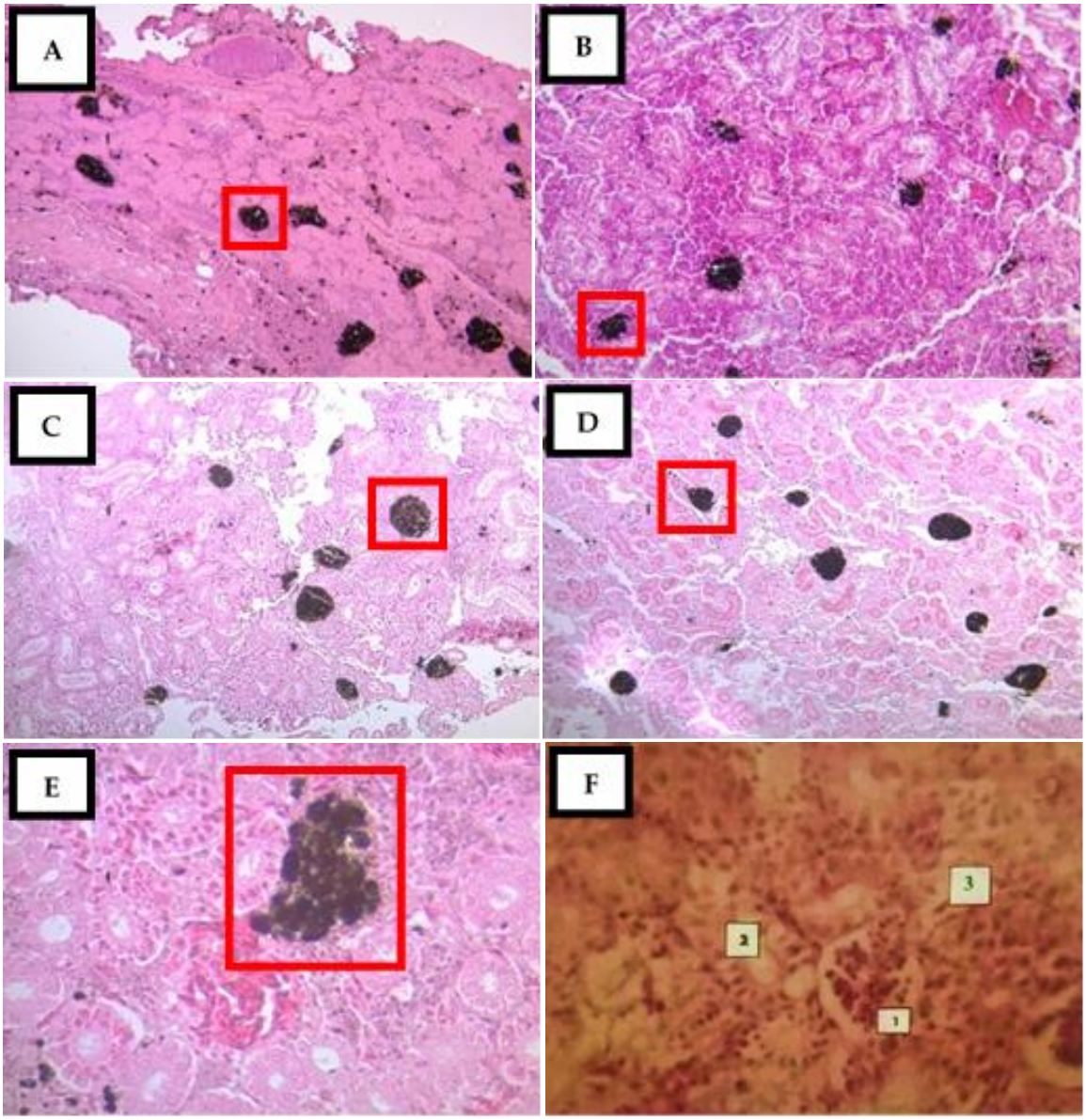

Figure 1. Comparison of kidney histopathological structure of seabass (*Lates calcarifer*) undergoing melanomacrophage centers; A - negative control (10x); B - positive control (10x); C - herbal treatment (10x); D - synbiotic treatment (10x); E - mixed treatment (100x); F - normal fish kidney (100x) (Kurniasih 1999); 1 - glomerulus; 2 - proximal tubules; 3 - blood vessels.

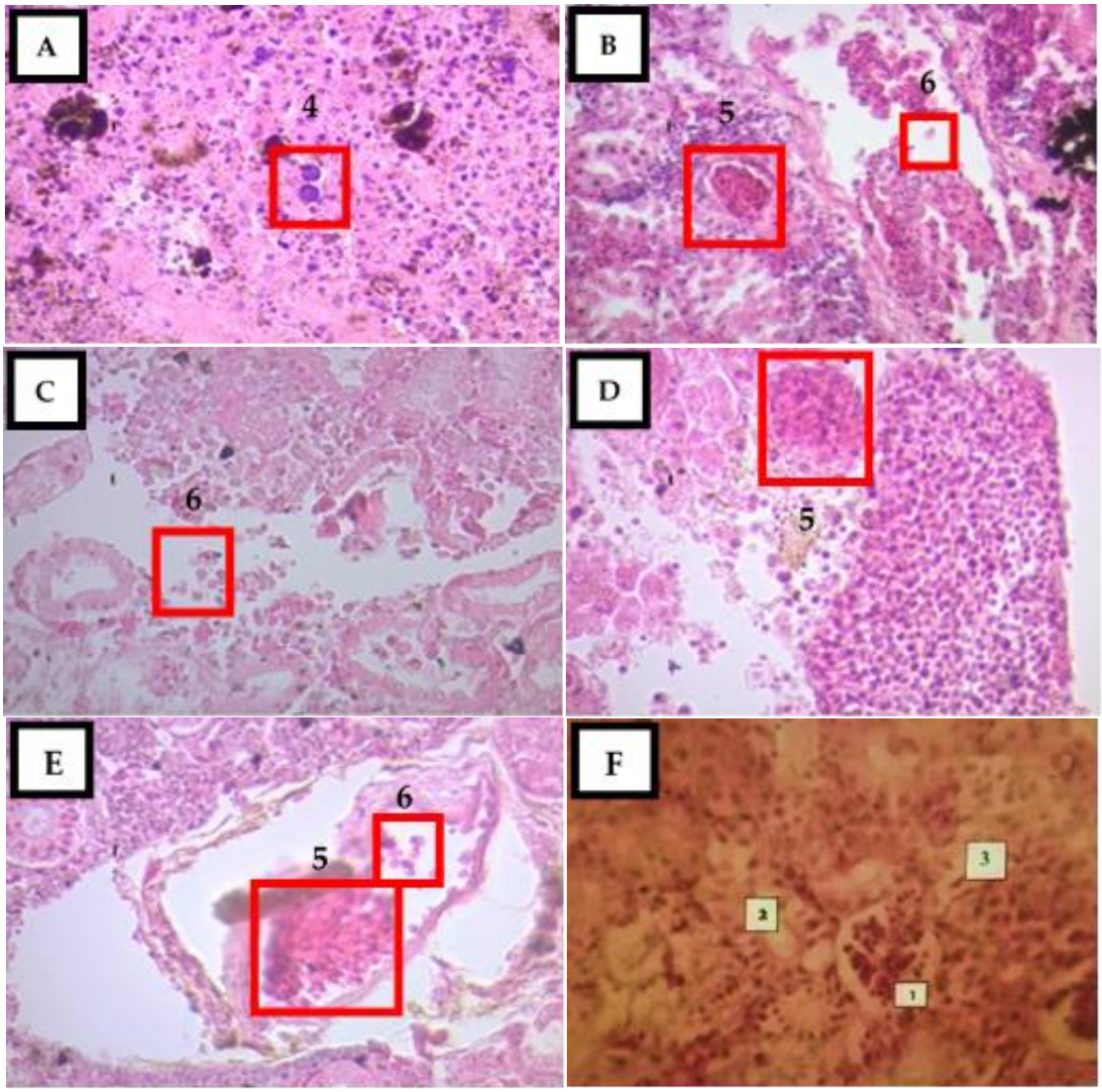

Figure 2. Comparison of the kidney histopathological structure of seabass (*Lates calcarifer*); A - negative control (100x); B - positive control (40x); C - herbal treatment (10x); D - synbiotic treatment (100x); E - mixed treatment (100x); F - normal fish kidney (100x) (Kurniasih 1999); 1 - glomerulus; 2 - proximal tubule; 3 - blood vessels; 4 - inclusion bodies; 5 - congestion; 6 - hemorrhage.

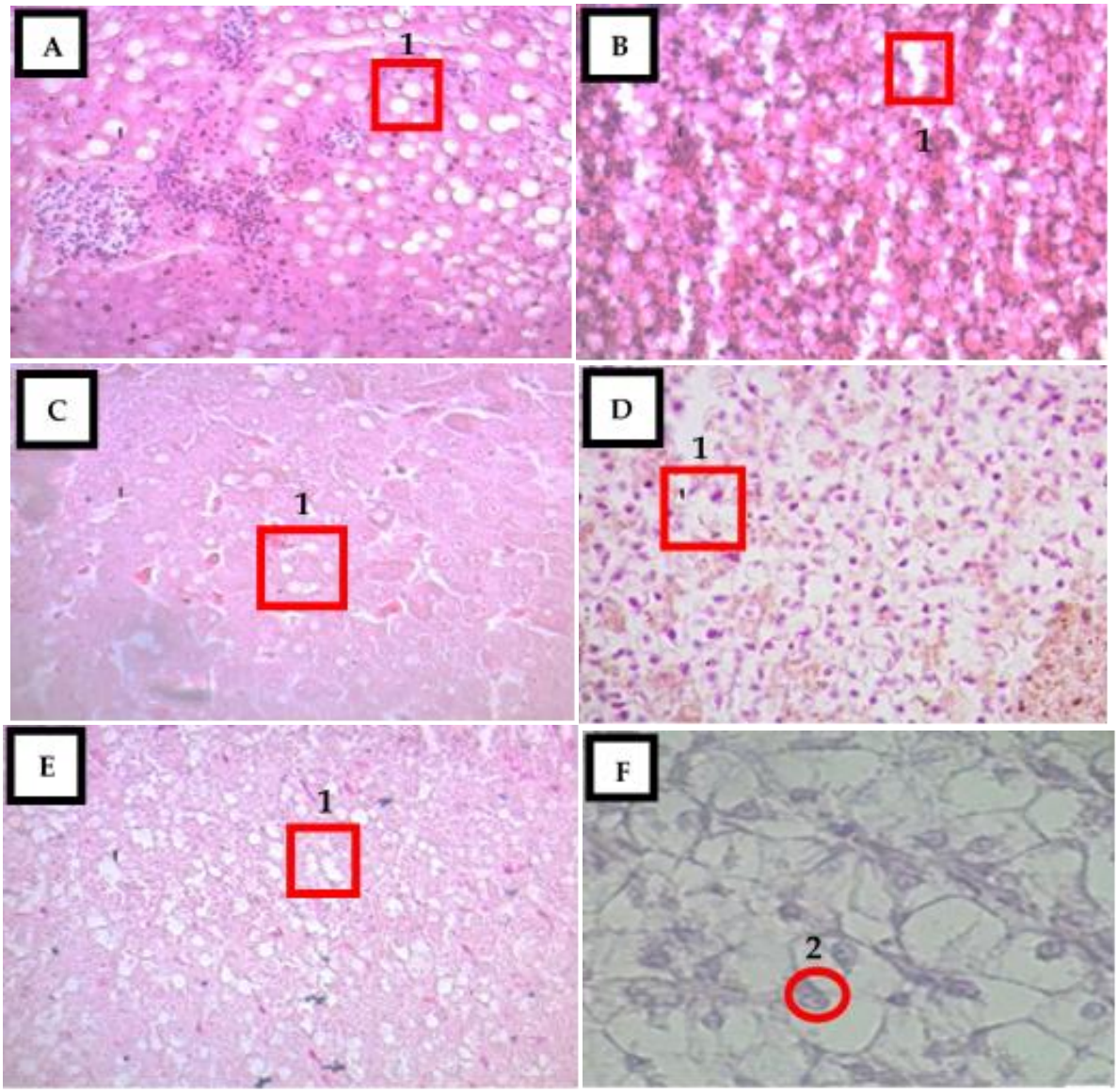

Figure 3. Comparison of the histopathological structure of the liver of seabass (*Lates calcarifer*) with fatty degeneration; A - negative control (40x); B - positive control (40x); C - herbal treatment (40x); D - synbiotics treatment (100x); E - mixed treatment (100x); F - normal fish liver (100x) (Yanti 2018); 1 - cells experiencing fat degeneration; 2 - normal cells.

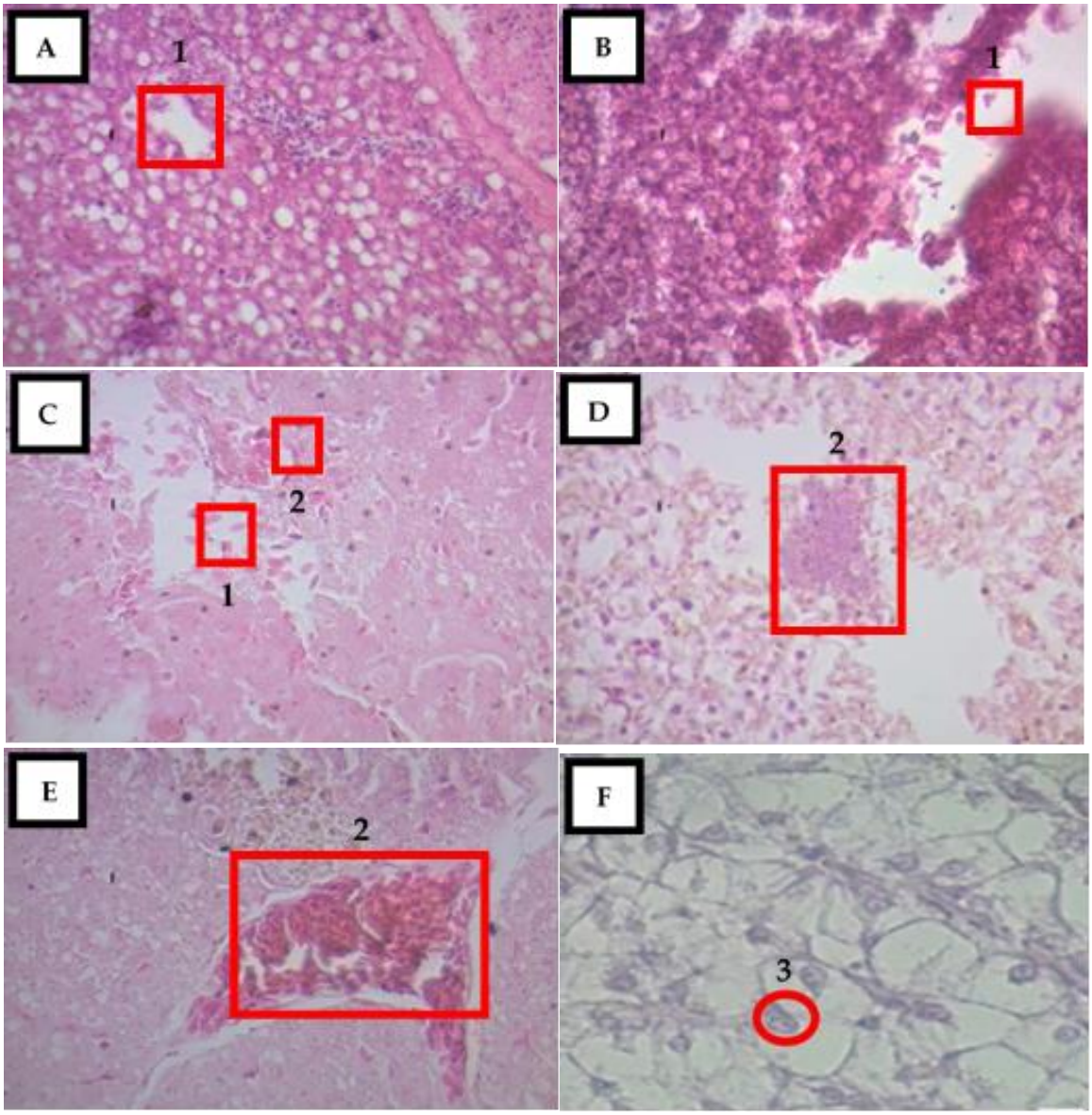

Figure 4. Comparison of the histopathological structure of the liver of seabass (*Lates calcarifer*); A - negative control (40x); B - positive control (40x); C - herbal treatment (40x); D - synbiotics treatment (100x); E - mixed treatment (40x); F - normal fish liver (100x) (Yanti 2018); 1 - hemorrhage; 2 - congestion; 3 - normal cells.

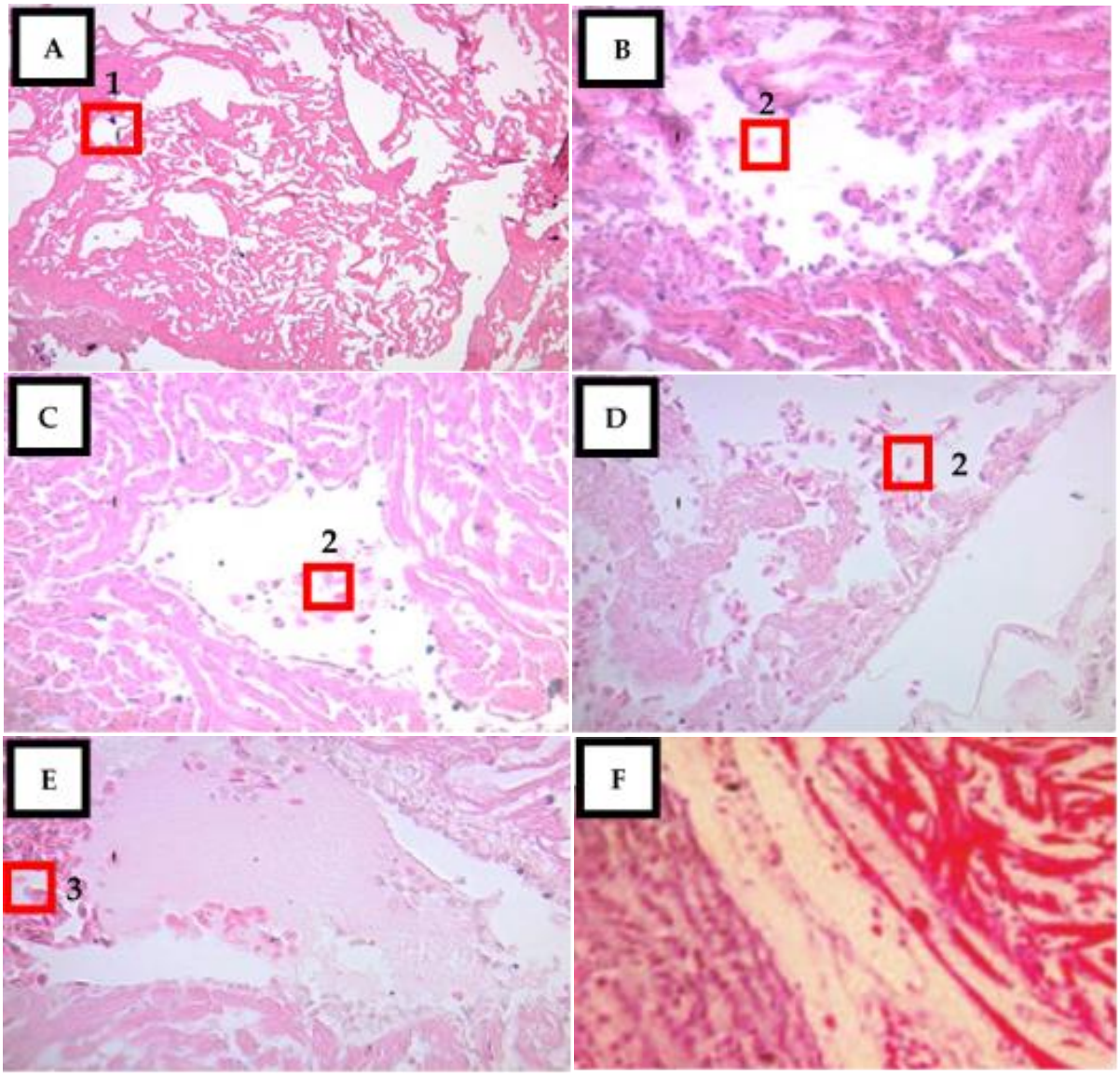

Figure 5. Comparison of the histopathological structure of the seabass heart; A - negative control (10x); B - positive control (40x); C - herbal treatment (40x); D - synbiotic treatment (100x); E - mixed treatment (40x); F - normal fish heart (100x) (Nursyirwani et al 2015); 1 - necrosis; 2 - hemorrhage; 3 - congestion.

Discussion. The mixed treatment maintained the fish alive after the challenge test with *P. damselae*. This was because the active ingredients contained in the three herbs (black cumin, turmeric, and bitter) increased immunity and functioned as antibacterial agents. The addition of synbiotics increased the absorption of nutrients in the treated feed mixed with the active ingredients of the three herbs. The thymoquinone content in black cumin inhibited *P. damselae* infection, hence the bacteria were less infectious. Similarly, Fauzy et al (2014) stated that the main content of black cumin, namely thymoquinone, can inhibit the formation of nucleic acids (RNA) and the protein synthesis process of *P. damselae*. Thymoquinone also causes bacterial proteins to become inactive by forming different complexes with nucleophilic amino acids, hence the protein loses its function.

The active ingredients in turmeric are curcuminoids, and in bitter are saponins, flavonoids, and tannins. Turmeric contains a phenolic group of diarylheptanoids, and the most active ingredients are curcuminoids consisting of curcumin, monodemethyoxycurcumin, and bisdemethoxycurcumin (Pfeiffer et al 2003; Herebian et al 2009). Curcuminoids have anti-inflammatory, antioxidant, anticarcinogenic, antimutagenic, anticoagulant, antidiabetic, antibacterial, antifungal, antiprotozoal,

antiviral, antivenom, and anti-inflammatory properties (Kumar et al 2011). Bitter has three active components of the diterpenoid lactone group in its leaves: andrographolide, neo-andrographolide, and deoxyandrographolide (Chao & Lin 2010). Other active ingredients contained in bitter are saponins, flavonoids, and tannins (Chatterjee et al 2014). Bitter has anti-inflammatory, antioxidant, anticancer, anti-infective, antihepatotoxic, anti-atherosclerosis, and antihyperglycemic properties (Chao & Lin 2010).

Clinical symptoms of fish after challenge test using P. damselae. The observation of the challenge test showed clinical symptoms such as flakes in the caudal and anal fin area, the body and abdomen had sores (necrosis) with red spots, loose scales. Clinical symptoms occurred in all treatments with differences in severity. The negative control showed the most severe symptoms compared to the other treatments.

P. damselae is a gram-negative bacteria. According to Hassanzadeh et al (2015), it is a facultative anaerobic bacteria. This bacterium grows and is found in ocean areas, coral reefs, and the deep sea. Shieh et al (2003) explained that *P. damselae* belongs to the Vibrionaceae family and can infect the host's body through blood circulation. These bacteria will metabolize and multiply in the fish, hence several essential organs such as the kidneys, liver, and spleen will be damaged. Bacteria that can infect and damage fish organs can cause initial clinical symptoms of redness (necrosis), and flakes appear on the caudal fin, dorsal fin, and several other body parts. This follows the findings of Fauzy et al (2014), where bacteria will enter the body through the circulatory system to take up protein. The circulatory system empties into the kidneys, functioning as filters of blood circulated throughout the body. Organs such as the kidneys, liver, and spleen will become the target of bacteria.

Histopathology of seabass internal organs after treatments and challenge test with P. damselae. Histologically, fish kidneys are composed of renal tubules or nephrons. There are two types of tubules, the renal and convoluted corpuscles, which are funnel-shaped and coiled tubes. The renal corpuscle comprises Bowman's capsule and glomerulus (Eroschenko & Pendit 2002). MMC are characterized by inflamed parts and a collection of macrophages (Juanda & Edo 2018). According to Prisoeryanto et al (2010), MMC indicate inflammation, including the collection of macrophages due to a self-protective response against the invasion of parasites in tissues (Hadi & Alwan 2012).

The tissue damage experienced by the kidney is represented by the emergence of congestion in the positive control, synbiotics and mixed treatments. Another tissue damage was the appearance of hemorrhage in the positive control, herbal, and mixed treatments. Meanwhile, the appearance of inclusion bodies only occurred in the negative control treatment. Fauzy et al (2014) found that congestion is characterized by symptoms such as red spots on the tissue, indicating an increase in the amount of blood. Juanda & Edo (2018) added that hemorrhages can be recognized by the presence of blood spots (Figure 2). In contrast, Smith & Jones (1961) explained that hemorrhage may be minor or significant. Minor hemorrhage is bleeding from blood points, and small spots called petechiae. An ecchymosis is called a hemorrhage with large spots on the body's surface or in tissues.

Based on observations, all treatments experienced fat degeneration (Figure 3), which, according to Tavernarakis & Driscoll (2001), is a change in tissue to a less active form. Tissue degeneration causes vacuolation with symptoms of empty spaces and an abnormal size. Kalaiyarasi et al (2017) showed that fat degeneration is characterized by the presence of swollen and vacuolated tissue. The fat accumulation in the cell and loss of the ability to metabolize trigger the degeneration. This is a further response to hydropic degeneration involving advanced cell swelling characterized by empty spaces in the cytoplasm with enlarged vacuoles pushing the nucleus to the edge.

As explained by Loomis (1978), the liver is a vital organ that plays an essential role in the metabolic process and transforming pollutants from the environment. It accumulates the most toxic substances. Some of the toxic substances that enter the body after being absorbed by cells will be carried to the liver by the hepatic portal system, and the liver has the potential to be damaged. Maharjan et al (2016) explained that the liver attacked by

parasites, bacteria, and environmental factors would experience abnormalities in the structure and function of hepatic cells.

According to Sarjito et al (2007), necrosis represents the process of cell or tissue death. Juanda & Edo (2018) explained that tissue damage due to necrosis in fish could be characterized by loss of tissue structure. It can be caused by the presence of pycnosis (Arimbi et al 2017). Blood clots and red tissue characterize inflammation because many erythrocytes come from the blood vessels. This inflammatory response aims to restore tissue and suppress the causative agent of necrosis. It is carried out by regenerating lost cells, forming connective tissue, and emigration of leukocytes to the necrosis areas (Setyowati et al 2010). The disease can be observed in the form of dead cells with faded colors and cells that are not intact (destroyed), hence the tissue becoming fragile (Rahman et al 2018). According to Prisoeryanto et al (2010), necrosis is the death of cells or tissues in every animal's life and is the final stage of irreversible degeneration. Characteristics of necrotic tissue are represented by a paler color, loss of tensile strength, or a poor or pale consistency. This abnormality can be caused by trauma, biological agents, such as bacteria, viruses, fungi, disease, chemical agents, or disruption of the blood supply to a particular area. Thawing necrosis is the most common type in fish.

Water quality. The results showed that salinity, DO, temperature, and pH were within the range of quality standard values. The value of NO_2 before siphoning ranged from 0.056 to 0.077 mg L^{-1} (mean of 0.064 mg L^{-1}) and increased to 0.059-0.073 mg L^{-1} (mean of 0.068 mg L^{-1}). The value of NH_3 before and after siphoning ranged from 0.486 to 0.733 mg L^{-1} (mean of 0.61 mg L^{-1}) and increased to 0.447-0.757 mg L^{-1} (mean of 0.65 mg L^{-1}). The pH ranged from 7-9 (mean of 7.5), the temperature was between 28.6-32°C (mean of 29.4°C), and the salinity was between 31-32‰ (mean of 31.5‰). The DO value was above 5 mg L^{-1}, suitable for aquaculture (Hardianti et al 2016; Shubhi et al 2017). The nitrite and ammonia content range was within normal limits for the cultivation of seabass. This is in line with the provisions of the National Standardization Agency (2014), where the limits of nitrite and ammonia content in water for seabass cultivation are maximum 1 mg L^{-1}.

Conclusions. The mixed treatment with black cumin, turmeric, bitter, and Bionelaplus synbiotics can maintain the lowest mortality rate of seabass, *L. calcarifer* with a mean length of 7.7 cm during the challenge test using *Photobacterium damselae*. The histopathological structures in fish organs (kidneys, liver, and heart) showed damage in all treatments without differences.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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