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HISTOPATHOLOGY OF KIDNEY AND INTESTINE OF TILAPIA (*Oreochromis niloticus*) FED WITH HERB FERMENTED FEED AFTER CHALLENGE OF *Streptococcus agalactiae*

Abdullah Al Furqon[1]*, Morina Riauwaty[1], Iesje Luskityowati[1]
[1]Department of Aquaculture, Faculty of Fisheries and Marine, Universitas Riau
Kampus Bina Widya KM. 12,5, Simpang Baru, Bina Widya, Pekanbaru, Riau 28293
*Abdullah.alfurqon13@gmail.com

ABSTRACT

The natural ingredients' potential as immunostimulants is a plant Curcuma and Aromatic ginger. This research was conducted from March to June 2022 in the Laboratory of Parasites and Fish Diseases, Faculty of Fisheries and Marine, Universitas Riau. Histological preparations were carried out at the Bukittinggi Veterinary Center (BVet). This research aimed to know the structure of the Kidneys and Intestines of tilapia by adding herb-fermented feed to prevent *Streptococcus agalactiae* infection. This research was an experimental method using a Completely Randomized Design (CRD) with 4 treatments and 3 replications. This treatment of the study was; NC (were not immersed and infected), PC (were not immersed in fermented herbs solution but infected of *S.agalactiae*), T1 (Giving 100 mL/kg fermented herbs), T2 (Giving 125 mL/kg fermented herbs), T3 (Giving 150 mL/kg fermented herbs). The fish used were 6 - 7 cm in size and reared with a stocking density of 1 fish/3 L of water and kept for 46 days. Fish were challenged with *S.agalactiae* bacteria with a density of 10^8 CFU/mL at a dose of 0.1 mL/head intramuscularly on day 32, after which the fish were reared again until day 46. Changes that occur in the kidneys of fish are hemorrhage, hypertrophy, and necrosis, while in the intestines there are histological changes in the form of haemorrhage, edema, vacuole degeneration, and necrosis. The results show that the feeding containing the herbal medicine of Curcuma and Aromatic ginger fermented affected the improvement of the histological changes in the kidneys and intestines of tilapia.

Keywords: Tilapia, Fermented Herb, Histopathological.

1. INTRODUCTION

Most fish farmers in Indonesia have used an intensive cultivation system to increase the stocking density of fish. The intensive cultivation system is an effective technology in aquaculture. Even so, intensive aquaculture systems have several negative impacts if not managed properly, namely poor water quality so that fish easily become stressed, can inhibit growth, and can cause the emergence of new elemental bacterial diseases. The type of disease that usually attacks fish in the Asia-Pacific region is disease *Streptococcosis* caused by the bacteria *Streptococcus agalatiae*.

Disease attacks are one of the factors that affect the amount of production from fish farming businesses, which can result in the amount of fish production dropping drastically due to growth disturbances or mass mortality[1]. Efforts to control fish diseases, especially bacterial diseases, can be carried out by using drugs, such as antibiotics, or by vaccination and by applying good cultivation methods[2]. The use of antibiotics can be used to kill harmful microorganisms, but uncontrolled use will negatively affect the body's defense

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system cause resistance to microorganisms, and often give rise to more virulent pathogenic strains[3]. So we need an alternative to prevent fish disease

An alternative that can be used to replace the use of antibiotics is to use natural ingredients as immunostimulants. One of the natural ingredients that have the potential to be used as an immunostimulant are Curcuma and Kampferia plants. The weakness of this herbal ingredient is that it has a pungent aroma and a bitter taste, so it needs to be fermented. According to Syawal et al.[4] giving herbal supplements with a fermentation process in feed can stimulate appetite, increase fish immunity to disease, reduce stress levels against environmental changes, and save feed use so that feed efficiency is high.

Kidneys and intestines are organs that have an important role in the digestive process and are prone to damage. Damage that occurs in the kidneys and intestines of fish can be observed by histological examination methods. Based on information regarding the potential of Curcuma and Kampferia which can be used as natural immunostimulants and the influence of natural ingredients, the authors are interested in researching the histopathology of the kidneys and intestines tilapia who were given post-challenge fermented herbal feed *S.agalactiae*

2. RESEARCH METHOD

Time and Place

This research was carried out from March to June 2022 at the Laboratory for Parasites and Fish Diseases, Faculty of Fisheries and Marine, Universitas Riau, and at the Bukittinggi Veterinary Center (bVet).

Methods

The method used was a one-factor Completely Randomized Design (CRD) method with 5 treatments and 3 replications. The treatment applied is as follows:

NC = Negative control (without fermenting herbs and without being challenged with bacteria *S.agalactiae*
PC = positive control (without fermenting herbs and challenged with bacteria *S. agalactiae*
T1 = Addition of feed containing fermented herbs at a dose of 100 mL/kg of feed and tested by challenge with bacteria *S.agalactiae*
T2 = Addition of feed containing fermented herbs at a dose of 125 mL/kg of feed and tested by challenge with bacteria *S.agalactiae*
T3 = Addition of feed containing fermented herbs at a dose of 150 mL/kg of feed and challenged by bacteria *S.agalactiae*

Procedure

Container Preparation

The preparation of the container starts with the process of cleaning the aquarium measuring 40x30x 30 cm. The maintenance container was first cleaned and then filled with water until it was full and given $KMnO_4$ solution (Potassium Permanganate) as much as 25 ppm for 24 hours. After that, the aquarium is rinsed and dried. The water used comes from a drilled well and has been deposited in a tank that is aerated for 2 x 24 hours. Then each container is filled with water with a volume of 30 L. After that enter the tilapia seeds measuring 6-7 cm with a stocking density of 1 fish/3 L to each aquarium.

Manufacture of herbal supplements

Feed preparation begins with cleaning Curcuma and Aromatic ginger, then peeling, weighing 100 g each. The ingredients are thinly sliced put in a blender and filled with 500 mL of water, then blended until smooth. Then it is filtered and added to clean water (volume 3 L) and boiled until it boils. After cooling, add 175 mg of molasses, 65 ml of probiotic drink, and 50 mg of yeast and stir until homogeneous. Then put them into jerry

cans and close them tightly. Furthermore, it is fermented for 7-10 days until a distinctive aroma changes[4].

Maintenance of Test Fish

The fish used are 6-7 cm in size and are kept from Berkah Farm Pekanbaru. The amount of feed given was 10% of the fish biomass with a frequency of feeding 3 times a day at 08.00 AM, 12.00 PM, and 04.00 PM for 46 days of maintenance.

Preparation of Bacterial Growing Media

The tools used such as petri dishes, test tubes, Erlenmeyer, and measuring cups are sterilized before being used by wrapping each tool using rice paper, then putting it into the autoclave at 121^0C with 1 ATM pressure for 15 minutes. Bacterial inoculant growing media *S.agalactiae* is Blood Agar Base (40 g/L), Brain Heart Agar (BHA) (52 g/L), and Brain Heart Infusion (BHI) (37 g/L)[5].

Histology Preparations

The kidneys and intestines were cleaned of blood and adhering impurities, then fixed using 10% formalin for 24 hours and replaced with 4% formalin. The dehydration process is carried out using graded alcohol starting from 70%, 80%, 90%, and absolute alcohol. Next is the process of clearing using xylol which was then infiltrated with paraffin with a melting point of 56-58^{O}C in an incubator with a temperature of 60^{O}C. Embedding was carried out by immersing the sample in a paraffin block at 62°C and then covering it with a tissue cassette and left to cool/harden. The blocks formed from the process were then cut using a microtome with a thickness of 6 µ. The results of the thin and ribbon-like pieces are placed on the surface of the water inside the water bath (40ºC) until the tissue expands well. The network is then lifted using a glass object and dried using a slide drying bench for 10 minutes. The glass object was arranged in the dye box and then colored using Hematoxylin and Eosin. Observations were made using a microscope.

Parameters Measured

Observation of Clinical Symptoms

Observation of clinical symptoms was carried out for 14 days after infection including body movements, body color, morphology, or physical condition. Observation of clinical symptoms was carried out every day during the maintenance period until after the challenge test.

Damage Scoring

The level of damage and abnormalities that occur in the kidney and intestinal tissue of tilapia is calculated according to Pierce et al. *in* Windarti et al[6].

Survival Rate *(SR)*

Effendi[7] states that to determine the survival rate of fish, the following formula can be used:

$$SR = \frac{Nt}{No} \times 100\ \%$$

Information:

SR : Survival rate of fish during the experiment

Nt : Number of fish at the end of the experiment (fish)

No : Number of fish at the start of the experiment (fish)

Water quality

Water quality parameters measured were temperature, pH, DO, and NH_3. The tools used are a thermometer, pH meter, DO meter, and spectrophotometer. Measurements were made at the beginning and end of the study.

Data Analysis

Observational data on clinical symptoms and survival of the test fish were tabulated in tabular form. While the research data which included histopathological readings of the kidneys and intestines of tilapia were discussed descriptively. The data were then analyzed

for homogeneity and then analyzed using analysis of variance (ANOVA).

3. RESULT AND DISCUSSION

Kidney Histology

The structure of normal fish kidney tissue is marked by the presence of cells that make up the glomerulus which are still visible, not completely round but in the shape of a number six, and Bowmen's capsule looks neatly wrapped around the glomerulus[8].

Necrosis was also found in kidney tissue treated with Pc, T1, and T2. This is presumably due to an inflammatory reaction that causes tissue damage followed by cell death due to infection *S. agalactiae*. Necrosis is indicated by symptoms of loss of tissue structure, and then cells in the kidney tissue experience cell damage. This is according to Hutabarat[8] stating that kidney tissue abnormalities due to necrosis describe a condition in which there is a decrease in tissue activity which in a short time will experience organ structure death. Kidney tissue abnormalities due to necrosis due to decreased tissue activity which begins to disappear in a short time will cause the death of the structure of the kidney organs and also cause abnormalities in the form of congestion.

Table 1. Tilapia kidney damage after being challenged

Treatment	Kidney damage				Damage rate	Category level of damage
	H	K	D	N		
NC	-	-	-	-	0	Normal
PC	+	+	+	+	4	Damaged
T1	+	-	+	+	3	Damaged
T2	+	+	-	+	3	Damaged
T3	-	-	+	-	1	Normal

Description: N= Necrosis; H= Hemorrhage; K= Congestion; D= Fatty Degeneration.

Congestion is also the first pathological symptom of tissue damage and an increase in the amount of blood in the blood vessels so that the blood capillaries appear dilated and the sinusoids in the kidneys are filled with lots of erythrocytes[9]. According to Rafe et al.[10] glomerular congestion is caused by an excessive amount of toxic chemicals which can reduce the ability of glomerular filtration resulting in an increase in blood volume in the blood vessels. These toxic chemicals are thought to be the accumulation of many chemical compounds contained in *Curcuma* sp. herbal medicine and fermented aromatic ginger. These chemical compounds can reduce the ability of glomerular filtration and cause glomerular congestion.

Another damage that occurred in the PC, T1, T2, and T3 treatments was the presence of hemorrhage. According to Jamin & Erlangga[11] states that hemorrhage is bleeding in cells caused by the rupture of blood vessels, causing blood to flow where it shouldn't both outside the body and into body tissues. Hemorrhage on histopathology observed was seen with dark red or black spots. This is by Andayani et al.[12] that hemorrhages are characterized by the presence of blood spots in the blood vessels of the body's tissues which are caused by congestion in the kidneys which is so severe that it causes the blood vessels to become damaged.

Fatty degeneration damage was found in all treatments that were infected with the bacteria *S.agalatiae*. Fatty degeneration occurs due to white vacuoles resulting from the dehydration process which causes accumulation of fat in the cell cytoplasm. The level of damage to kidney tissue that is reduced or experiencing healing is the effect of adding Curcuma and aromatic ginger fermented herbs to tilapia feed during rearing. Curcuminoids provide antioxidant effects on cell membranes,

thereby reducing damage to erythrocyte cell membranes due to oxidation. According to Regar et al.[13] damage to the erythrocyte cell membrane can affect the life span of the erythrocyte cell. Furthermore, curcuminoids can improve the work of blood-producing organs, such as the spleen and kidneys. The addition of probiotic drinks (*Lactobacillus casei shirota strain*) in herbal supplements (fermented herbs) can improve the performance of the immune system; bacteria *Lactobacillus* helps fight pathogens that enter the body of the fish[14].

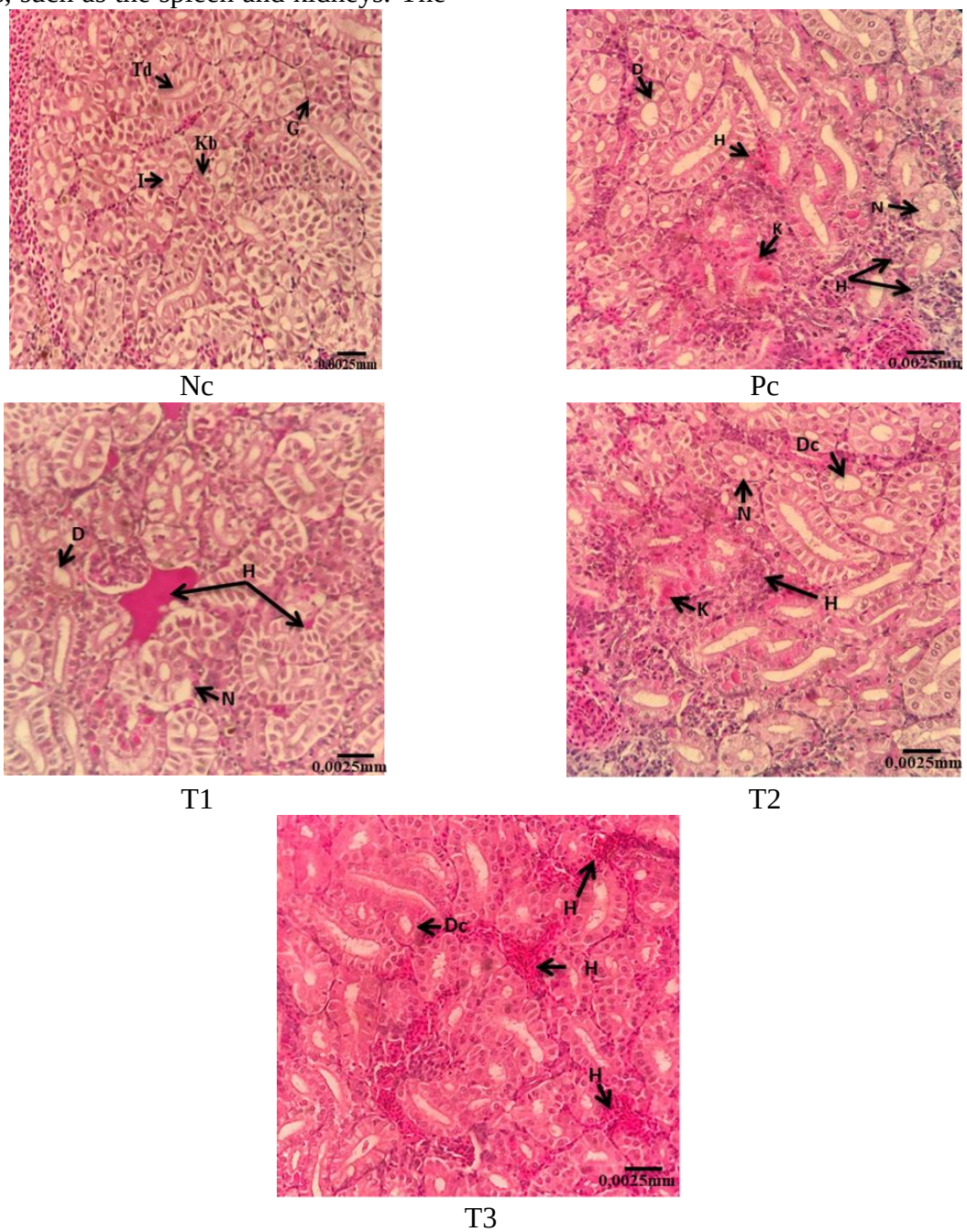

Figure 1. Photomicrograph of *Oreochromis niloticus* kidney structure after being challenged; HE staining (400X Magnification).

Information: I = cell nucleus; Kb = Bownman's capsule; Td = Distal tubule; Dc = Distal convoluted tubule; G = Glomerulus; N = Necrosis; H= Hemorrhage; K= Congestion; D = Fatty Degeneration

Intestinal Histology of *O. niloticus*

The intestinal tissue structure of normal fish is different from the intestinal tissue structure of the fish-challenged *S.agalactiae*. Normal intestinal tissue structure consists of serous, mucous, sub-mucosal, and muscular membranes. In the NC treatment, the intestinal conditions were normal because the intestinal villi, intestinal mucosa, sub-mucosa, muscularis, and serosa layers were still clearly visible (Figure 2). In the PC treatment, intestinal damage occurred in tilapia, namely hemorrhage, edema, necrosis, and vacuolar degeneration. According to Susanto[15], the damage that is often found in the intestines of fish is the occurrence of goblet cell proliferation, hemorrhage, atrophy of intestinal villi, metaplasia, necrosis of epithelial cells, and bleeding.

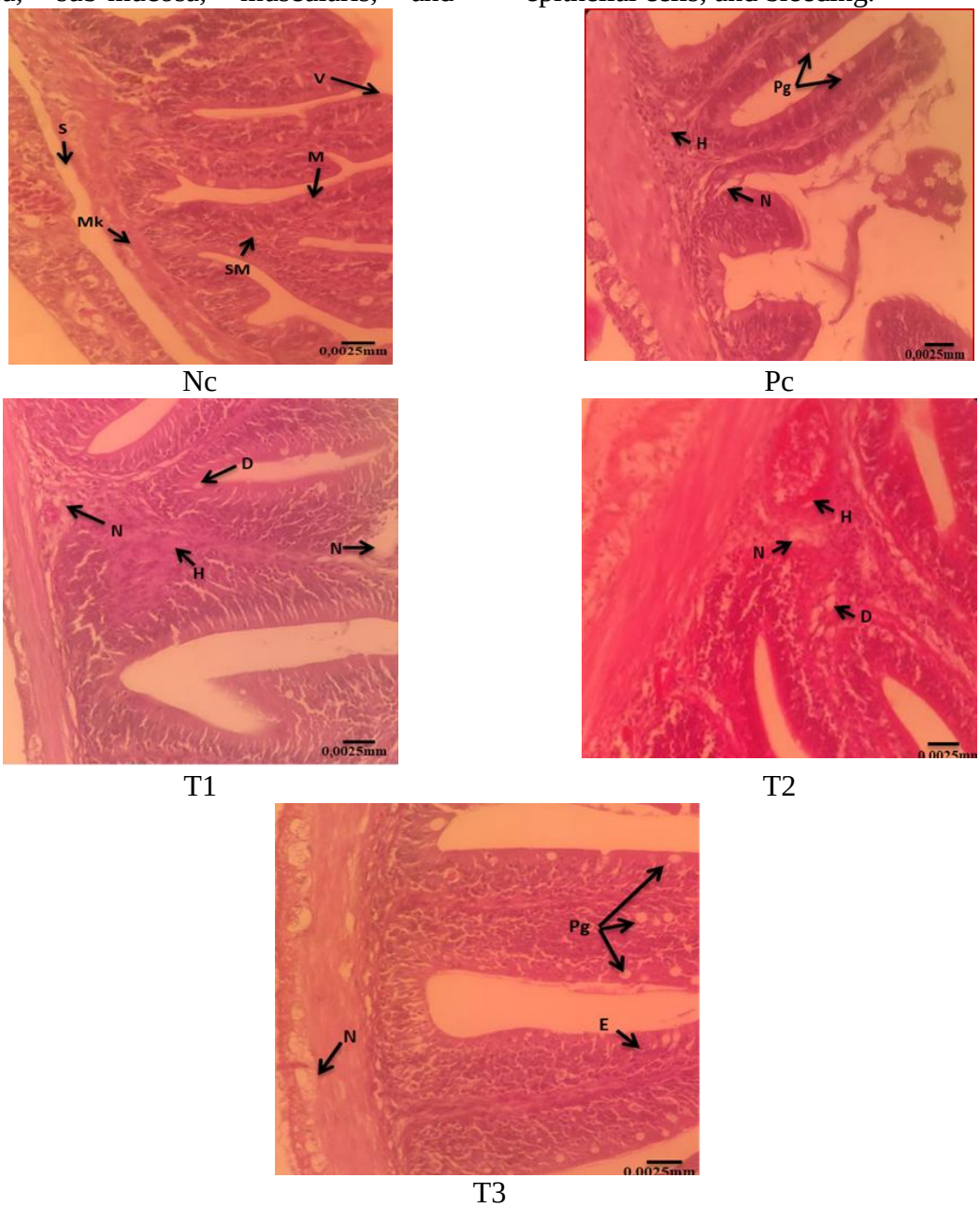

Figure 2. Photomicrograph of the gut structure of tilapia after being challenged; HE staining (400X Magnification).

Information: V (intestinal villi); M (intestinal mucosa); SM (sub mucosa); S (serosal layer); H (hemorrhage); E (edema); N (Necrosis); D (vacuole degeneration). pg; (goblet cell proliferation)

Table 2. Tilapia intestine damage after being challenged

Treatment	Kidney damage				Damage rate	Category level of damage
	H	K	D	N		
NC	-	-	-	-	0	Normal
PC	+	+	-	+	3	Damaged
T1	+	-	+	+	3	Damaged
T2	+	+	-	+	3	Damaged
T3	-	+	-	+	2	Normal

Description: N= Necrosis, H= Hemorrhage, K= Congestion, D= Fatty Degeneration

In the T1 treatment, goblet cell proliferation, necrosis, and vacuole degeneration occurred. In the T2 treatment, there were changes in the form of necrosis, edema, and goblet cell proliferation. At T3 there is damage of the necrotic type, Haemoragi, and vacuolar degeneration. Similar results in histopathological studies on the intestinal organs of catfish also experienced necrosis, edema, and hemorrhage[16]. Likewise, research on the histological structure of the intestines of Asang fish conducted by Sulastri et al.[17] showed damage in the form of villous erosion, lysis, necrosis, hemorrhage, and edema.

Hemorrhage in the intestine is characterized by the presence of dark red parts of the intestinal tissue which are observed and occur mostly in the submucosa of the intestine. According to Juanda and Edo[16] hemorrhage in the intestine indicates the presence of erythrocytes that have come out of the blood vessels and are in the intestinal tissue. This is caused by the entry of foreign objects through the food consumed by fish which causes lesions in the fish's intestines and weakens the vascular walls so that blood vessels are prone to leaks.

Saponin is one of the compounds that can cause damage to the intestinal epithelium resulting in hemorrhage in the cell wall. In certain doses, saponins can also become toxins because they can hemolyze red blood cells. This abnormality is seen in Pc. According to Asih[18] that damage is in physical form which damages the tissue in the leak area, infection with infectious agents especially which results in septicemia, toxins that damage the capillary endothelium, and increased porosity as well as other factors that cause weak vascular walls so that blood vessels are prone to bleeding.

Necrosis in the intestine was found in PC, T1, T2, and T3 treatments. Necrosis is characterized by the presence of intestinal tissue that is damaged and loses its structure. Necrosis can be caused by the content of Curcuma and Aromatic ginger which have bioactive compounds from plants when used in large doses and for a long time can cause necrosis. According to Ersa[19] necrotic damage is irreversible, which means this damage cannot be repaired. However, cells that experience necrosis will be replaced by cells that regenerate. This cell regeneration will work well if the fish get good and sufficient nutrient intake. Giving feed containing ginger and fermented aromatic ginger is expected to be able to prevent this damage to the intestine because curcuma and aromatic ginger have bioactive compounds that are good for metabolism.

In PC, T1, T2, and T3, vacuolar degeneration was marked. Vacuole degeneration is a condition in which a cell loses its normal structure due to influences from within or from outside the cell. This cell degeneration is caused by metabolic disorders that can cause materials to enter intra-extracellular. Degeneration can be caused by a lack of essential materials (e.g., oxygen or nutrients), lack of an energy source that interferes with metabolism, mechanical heat, or can be caused by

injury, accumulation of abnormal substances in the cells caused by viruses, bacteria, or pathogens such as parasites and toxins produced or by toxic chemicals, nutritional imbalances, and mild irritants[19].

Water quality

The results of measuring water quality parameters during the study showed that all parameters measured were still within the normal range for tilapia survival (Table 3).

Table 3. Water quality measurements during the study period

Parameter	Water quality in treatment					Quality standard[20]
	NC	PC	T1	T2	T3	
Temperature (^{o})	27,7 - 28	27,7 - 28	27,7 - 28	27,3 - 27,7	28	25-30 ^{o}C
pH	6,9	6,8 – 6,9	6,8 – 6,9	6,8 - 7	6,8 – 6,9	6-8
DO (mg/L)	4,9 - 5,1	4,8 – 5,2	4,8 – 5,2	4,7 -5,2	4,9 – 5,1	>3 mg/L
NH_3 (mg/L)	0,00062-0,00132	0,00062-0,00335	0,00062-0,00072	0,00062-0,00210	0,00062-0,00218	< 1 mg/L

The parameters of water quality in tilapia during this study were in optimal conditions. The temperature in fish rearing for each treatment ranged from 27.3-28^{O}C. This temperature range is good for tilapia cultivation. According to Arifin[20], it ranges from 25-31^{O}C.

The pH range for tilapia is 6.8-7. This range is still within the tolerable limit of tilapia. DO measurements on tilapia rearing media ranged from 4.8-5.2 mg/L. This DO range is still in the normal range. In the opinion of Arifin[20] DO is good for the growth of tilapia ranging from >3 mg/L mg/L. The range of ammonia in tilapia is 0.001-0.003. This range is still within the tolerable limit of tilapia. According to Arifin[20] a good ammonia content for aquaculture is <1 mg/L.

4. CONCLUSION

Based on the results of observations on the structure of the kidney and intestinal tissue of tilapia, it was observed that there were changes in the kidneys that showed damage such as edema, hemorrhage, necrosis, and fatty degeneration. Whereas in the intestinal tissue of tilapia, damage such as edema, hemorrhage, necrosis, and goblet cell proliferation was seen. The best results from the addition of Curcuma and fermented Aromatic ginger were (P3) at a dose of 150 mL/kg of feed which resulted in a survival rate of 93.34%. The condition of water quality in the maintenance medium is normal conditions, namely the temperature of 27.3 -28OC, pH 6.7-7, content, DO 4.7 - 5.2 mg/L, and ammonia 0.00062–0.00335 mg/L.

Based on the results of the research that has been carried out, it is advisable to carry out further research on the histology of other organs, namely the gills and liver of tilapia fed with the addition of fermented herbs and challenge-tested *S.agalactiae*.

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