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BIOREMEDIATION POTENCY OF PROBIOTICS ON CADMIUM POLLUTION TO IMPROVE FISH REPRODUCTIVE HEALTH

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ABSTRACT

This study aims to evaluate the potential of probiotics to remediate Cadmium (Cd) pollution in order to restore fish reproductive health. Thirty-six mature gonad male tilapia were divided into 12 groups which were determined based on variations in Cd concentration and supplementary feeds (probiotics, vitamin C, and a combination of both). Therefore, in this study, supplementation of probiotics, vitamin C, and combination of both were provided to repair the size of testicular cells that damage due to cadmium exposure. The parameters evaluated were the size of the testicular cells, including the thickness of the testis layer, the length of the seminiferous tubules, the diameter of the cyst, and the diameter of the germ cells. The results showed that giving supplementary feeds significantly improved testicular cell size (testicular layer density, seminiferous tubule length, cyst diameter, and germ cell diameter) contaminated by Cd. Probiotic supplements showed a better effect than other supplements on restoring the reproductive health of Cd-contaminated fish.

KEY WORDS: fish, Germ cells, Testis, Cyst, Cadmium

INTRODUCTION

Industry, agriculture, and human activities are the main sources of heavy metal contamination in aquatic environments (Hayati *et al.,* 2018). Heavy metals are often used industrially and produce the most pollutants. Environmental pollution by heavy metals has become a serious threat to living organisms in an aquatic ecosystem (Siddiquee *et al.,* 2015). Metal toxicity is a major environmental concern because of its bioaccumulation and its non-biodegradability character in nature (Lakherwal, 2014). Small amounts of heavy metals play metabolic and redox functions of body cells, but some others (Hg, Cd, Cu, Pb, etc.) are toxic for living organisms (Siddiquee *et al.,* 2015; Turpeinen *et al.,* 2002). Several studies have shown that heavy metal pollution damages fish testis (Hayati *et al.,* 2017); reduces the quality of fish sperm as well as the ability of fish fertilization (Hayati *et al.,* 2019).

The bioremediation of heavy metals in water is an alternative that can be used to restore polluted environments. It uses microorganisms to reduce pollutants in the aquatic environment. The bioremediation process involves the activity of enzymes produced by microorganisms that modify toxic pollutants by changing the chemical structure of pollutants to non-toxic (Abbas *et al.,* 2014). Many microorganisms have the ability to remove heavy metals, some of them are lactic acid bacteria (LAB), including *Lactobacillus sp*. Lactic acid produced by these bacteria could suppress the growth by making the environment more acidic so effective to remediate heavy metals (Zoghi *et al.,* 2014; Patowary *et al.,* 2016). These bacteria could remove heavy metals through biosorption mechanism or through the formation of bonds between heavy metals and their cells (Jin *et al.,* 2018). The use of microorganisms is a new alternative approach to improve and utilize its bioremediation ability toward heavy metals. Some resistance mechanisms of microorganism on heavy metal include an extracellular barrier, extracellular sequestration, and active transport of metal ions (efflux), intracellular sequestration, and metal ions reduction (Choudhury *et al.*, 2001; Bruins *et al.,* 2000).

Fish culture in reservoirs or ponds whose water is polluted by heavy metal pollution can affect the growth and development of fish. The administration of regular fish food cannot overcome the impact caused by the pollutants. Feeding that contains a probiotic consortium can improve growth performance and affect intestinal microbial populations of fish exposed to heavy metals (Wanguyun *et al.,* 2019). Moreover, probiotics and herbal plant supplements were also proved to improve the quality of sperm damaged by Hg exposure (Hayati *et al.*, 2020). This study aims to analyze the potential of probiotics as cadmium bioremediation agents to improve fish reproductive health, specifically on morphometry changes in fish testicle cells.

MATERIALS AND METHODS

Preparation of the probiotics for fish diet supplementation

The probiotics are the mixed cultures consisting of *Lactobacillus bulgaricus (NBRC13953), Lactobacillus fermentum* (ME3), *Lactobacillus buchneri* (DSM 20057), and *Lactobacillus casei* (DSM 20011) at the ratios of 1:1:1:1 (Giri *et al.,* 2018). The solution of bacteria (1×10^8 CFU/mL) was sprayed at the ratio of 200 mL/kg of the fish diet and then air-dried. The fish diet used in this study was a commercial fish diet (Prima Feed, PF- 500), containing 25.57% crude protein, 6.72% crude fat, crude fiber 3.49%, ash 9.16%, and energy 2974.80 Kcal/Kg. The feed was prepared weekly and stored at 4 ^{o}C.

Experimental design

Tilapia male fish with approximate age around three months and approximate weight around 150 g were obtained from a local fish farm in Pandaan, Pasuruan, East Java, Indonesia. The fish were acclimatized for 2 weeks at 29 ^{o}C and fed with basal diet. The physicochemical parameters of the water were monitored twice a week. Thirty-six tilapia (*Oreochromis niloticus*) weighing 250-300 g were distributed into 12 groups (n=3). Each group was kept in a separate glass tank. The first group was kept in a tank that contained normal water without any treatment, this group was used as a negative control. The fish of test groups were exposed to variation Cd (0, 0.3, and 0.6 ppm) with various type of feed (feed, feed containing vitamin C, feed containing probiotics, and feed containing a combination of both vitamin C and probiotics), and the study was conducted for 15 days. The fish were acclimatized for two weeks.

Histopathological studies on testis

Testis tissues were fixed in Bouins for 48h with a change after 24 hours. Fixed tissues were washed with 50% ethanol and dehydrated further through 70%, 90%, and absolute ethanol and cleared in xylene. The tissues were then embedded in paraffin wax and sections of 5 microns were obtained on a rotary microtome. The sections were then stained with Harrys' hematoxylin and Eosin to observe the architecture of the liver treated and control fish. Stained slides were observed under a compound microscope, photographed and assessed. The thickness of testicle cells and the diameter of germ cells were measured using Software OptiLab Advance Plus.

Statistical analysis

Statistical Package for the Social Sciences (SPSS version 16.0; SPSS, Inc., Chicago, IL) was used for statistical analysis. Data analysis was performed statistically using analysis of variance (ANOVA) to examine differences between treatments. Statistical significance was accepted at the level of $P<0.05$.

RESULTS

The results show that feeding with probiotics can improve the reproductive health of fish exposed to Cd based on the size of testicular cells. It was proved by the change in the size of the testicular layer and the length of the seminiferous tubules. Exposure to variations in Cd concentrations decreased testicular layer thickness and seminiferous tubule length compared to controls. The addition of vitamin C supplements and combination of vitamin C with probiotics on the group exposed with 0.3 ppm Cd show no significant difference in the length of the seminiferous tubules compared to control (P> 0.05), meanwhile it is significant in the group of 0.6 ppm Cd exposure. Likewise, the thickness or density of the testicular layer, supplementation (probiotics, vitamin C, and a combination of probiotics and vitamin C) increases the thickness of the testis layer compared to controls. Based on this data, it can be referred that probiotic supplementation increased the length of seminiferous tubule and the thickness of the testicular layer after exposure to various Cd concentrations rather than vitamin C and their combinations (Table 1).

Cysts are part of the seminiferous tubules where germ cells (spermatogonia and spermatocytes) develop. The results show that the diameter of the spermatogonia was greater than spermatocytes. Cd exposure decreases cyst and germ cell diameter. The cyst diameter among the group of various supplement treatments (probiotics, vitamin C, a combination of probiotics and vitamin C) shows no significant differences (P> 0.05). However, giving supplement has significantly increased (P <0.05) the diameter of fish cysts after Cd exposure. Probiotic supplement feeding significantly increased the diameter of germ cells (P <0.05), but giving vitamin C and a combination of both had no significant effect (P> 0.05). However, supplementation could increase the diameter of germ cells of fish exposed to various Cd concentrations significantly (P <0.05) (Table 2).

DISCUSSION

This study shows the potential of a supplement containing probiotics, vitamin C, and a combination of both on improving the size of the fish testicular cells which was exposed to various Cd concentrations. These results evaluated Cd damage in the testicular layer and seminiferous tubule size, especially in the diameter of cysts and germ cells which can cause testicular atrophy. Although some studies have explained different conditions on other fish species under several contamination conditions, this study proved the damage of Cd exposure at both tissue and cellular levels. Damage caused due to the induction of germ cell death due to Cd oxidants so that it can change the size of the testis.

In this study, a decrease in the thickness of the testis layer and the length of the seminiferous tubules coincided with a decrease in the diameter of

Table 1. The size of fish seminiferous tubules on various Cd exposure after supplement administration

Treatments	Length of Seminiferous tubule (μm)	The density of Testis Layer (μm)
Control	1596.13 ± 8.36[a]	61.16 ± 8.36[a]
Cd-0.3 ppm	1430.13 ± 28.60[c]	40.59 ± 0.59[b]
Cd-0.6 ppm	1223.81 ± 11.30[e]	31.33 ± 11.30[c]
Probiotic (P)	1606.18 ± 12.33[a]	66.52 ± 6.71[d]
PCd-0.3 ppm	1601.57 ± 24.83[a]	54.85 ± 0.03[d]
PCd-0.6 ppm	1442.75 ± 9.30[c]	51.92 ± 6.31[d]
Vitamin C (V)	1522.95 ± 3.54[b]	65.00 ± 4.24[d]
VCd-0.3 ppm	1458.16 ± 6.67[c]	41.42 ± 3.90[e]
VCd-0.6 ppm	1398.33 ± 14.82[d]	34.80 ± 3.42[e]
PV	1531.75 ± 6.20[b]	55.00 ± 7.14[d]
PVCd-0.3 ppm	1480.09 ± 8.14[c]	45.03 ± 1.94[e]
PVCd-0.6 ppm	1454.58 ± 9.59[c]	39.42 ± 0.88[e]

Table 2. The diameter of cysts and testicular germ cells of fish in various Cd exposure after supplement administration

Treatments	The diameter of cysts (μm)	The diameter of Spermatogonia (μm)	The diameter of Spermatocytes (μm)
Control	357.49 ± 12.11[a]	76.54 ± 1.38[b]	69.33 ± 0.38[c]
Cd-0.3 ppm	270.61 ± 16.06[c]	58.06 ± 1.71[e]	53.16 ± 0.79[e]
Cd-0.6 ppm	206.81 ± 15.45[d]	54.54 ± 0.20[f]	43.82 ± 2.60[f]
Probiotic (P)	363.73 ± 16.46[a]	90.28 ± 4.27[a]	78.63 ± 1.42[a]
PCd-0.3 ppm	304.71 ± 4.37[b]	71.98 ± 2.61[b]	64.23 ± 3.33[d]
PCd-0.6 ppm	264.96 ± 24.74[c]	65.57 ± 0.74[c]	63.24 ± 3.75[d]
Vitamin C (V)	364.75 ± 16.42[a]	76.05 ± 1.56[b]	73.50 ± 3.05[b]
VCd-0.3 ppm	292.42 ± 11.76[b]	71.67 ± 1.02[b]	62.71 ± 4.32[d]
VCd-0.6 ppm	290.52 ± 19.91[b]	67.42 ± 0.13[c]	59.95 ± 1.31[d]
PV	356.41 ± 21.20[a]	74.58 ± 1.10[b]	72.35 ± 0.00[b]
PVCd-0.3 ppm	271.77 ± 8.77[b]	71.00 ± 1.41[b]	65.02 ± 1.15[d]
PVCd-0.6 ppm	261.05 ± 30.97[b]	65.20 ± 0.98[d]	61.49 ± 2.11[d]

cysts and germ cells, and both were caused by the Cd toxicity (0.3 and 0.6 ppm). Decreased in cysts and germ cell diameter was associated with cell damage caused by the oxidation of heavy metals Cd, which leads to a decrease in the thickness of the testicular layer and the length of the seminiferous tubules (Hayati *et al.,* 2020). Testicular histology visualized the damage of germ cells, expressing the severity and extent of changes resulting from Cd exposure that indicates pathological conditions. Increased Cd concentrations induce a higher incidence and severity of damage in the testicles of the treated fish. A decrease in germ cell diameter allows an objective assessment of damage in the cell. This pathological condition is an important tool for linking pollution levels with the severity of testicular cell and tissue size changes induced by Cd exposure (Vergilio *et al.,* 2015). The disruption of normal testicular size and the degeneration of cyst have been observed for other fish species exposed to Cd through water or food (El-Ebiary *et al.,* 2013).

Several strategies in utilizing microorganisms have been implemented to prevent poisons production, eliminate and deactivate poisons, or reduce the bioavailability in poisoned food. Between physical, chemical, and biological treatments for toxic degradation, bio removal treatments are beneficial for reducing food toxicity without losing the nutritional quality. The utilization of microorganisms in the removal of toxic heavy metals has been widely carried out. Lactic acid bacteria (LAC) microorganisms are widely known as safe probiotic microorganisms and are best known for reducing toxic metals from heavy metals. When fish eat food that contains probiotics, the microbes will help maintain the balance of the digestive tract and may be useful for the treatment of pathogens or infections (Zoghia *et al.,* 2014). This bioremediation probiotic can adapt to extreme conditions exposed to Cd through resistance mechanisms either by heavy metal ion biosorption mechanisms, compiling complex cell walls, or inducing enzymatic systems to convert toxic heavy metals into non-toxic ones (Abbas *et al.,* 2014; Dwyana *et al.,* 2018).

Supplements containing vitamin C are also good in maintaining optimal health. Vitamins are a heterogeneous group of organic compounds that are important for the growth and maintenance of aquatic animal life. The majority of vitamins are not synthesized by fish bodies and not sufficient to meet the needs of aquatic animals (Santigosa *et al.,* 2015). Vitamin C is important because it is an antioxidant that plays a role in neutralizing heavy metal oxidants, thereby it will reduce the toxicity of heavy metals Cd, and provide protection against oxidative damage from free radicals (Hayati *et al.,* 2020). However, in this study, the use of vitamin C was also good in protecting fish testis against Cd exposure in addition to providing a combination of probiotics and vitamin C.

CONCLUSION

Feeding containing probiotic supplements was better for improving the reproductive health of fish exposed to Cd than the feeding of vitamin C supplements and a combination of both.

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