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Effectiveness of Potassium Diformate on Feed to Improve Immune Performance of Goldfish (*Carassius auratus* L.)

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Authors' contributions

This work was carried out in collaboration among all authors. Author AY designed the study, carried out statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors RTW and AR managed the study analysis. Author IBBS managed the literature search. All authors read and agreed to the final manuscript.

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ABSTRACT

This research aims to determine the optimum potassium diformate (KDF) dosage which was added to feed to increase the immune status of goldfish (*Carassius auratus* L). This study was conducted from September 31 - November 15, 2019 at the Laboratory of Aquaculture and Molecular Biotechnology Laboratory of the Faculty of Fisheries and Marine Sciences, University of Padjadjaran. The research method used is the experimental Complete Random Design (CRD) with 4 treatments and 3 replications. The treatments are A (without KDF/control), B (0.1% KDF), C (0.3% KDF) and D (0.5% KDF). The observed parameters are survival rate, total leukocyte count (white blood cells), total erythrocyte count (red blood cells) and gross clinical sign. Observations were made after 30 days of KDF and post-test challenge by *Aeromonas hydrophila* for 14 days. Data on the number of total leukocyte count and total erythrocyte count were analyzed using F test and Duncan's advanced test at a 95% confidence level, while the gross clinical sign data were analyzed descriptively. The results showed that the KDF 0.3% was the optimum dose and was

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very effective in optimizing the performance of the goldfish immune system optimally, seen from the number of white blood cells and red blood cells which experienced the highest increase of 23.48% and 9.30%. After the challenge test, fish that were given KDF 0.3% had the highest survival rate of 46.67%. In addition, the process of recovering the number of white blood cells and red blood cells can be faster than other treatments, with a value of 12.85×10^4 cells mm^{3-1} and 0.96×10^6 cells mm^{3-1}, marked also by the healing of clinical symptoms in the morphology of goldfish.

Keywords: Aeromonas hydrophila; Carassius auratus; immune system; potassium diformate; red blood cells; survival rate; white blood cells.

1. INTRODUCTION

Goldfish (*Carassius auratus*) is one of the oldest domesticated freshwater ornamental fish species in the world [1]. In Indonesia, the fish is one of the most widely cultivated ornamental fish and almost all exporters include goldfish [2]. However, this fish farming activity has a low level of environmental tolerance that is very susceptible to failure in aquaculture. The cause of the failure was due to a disease problem caused by viruses, bacteria and fungi. The disease can cause losses in various sectors, such as decreasing health, economic quality and reducing the quality of fish production. One of the diseases that attack the goldfish is *Motile Aeromonas Septicemia* (MAS) or also called red spot disease caused by *Aeromonas hydrophila* bacteria.

A. hydrophila is a bacterium that lives and infects freshwater biota. The attack can cause mortality to reach 80 until 100% within 2 weeks. Aeromonas attacks almost all fisheries communities, especially in West Java and even becomes a deadly plague on freshwater fish and causes enormous losses [3].

Prevention of disease in farming goldfish generally still use antibiotics that are proven effective in treating fish, but can cause resistance to pathogenic bacteria [4]. Therefore, one of the safest prevention efforts is done to protect the goldfish from attacking pathogenic organisms, namely by increasing the immune system by providing immunostimulant that can be given to the feed so that the feed becomes of high quality. Quality feed can be made through the addition of other ingredients in a feed that has a specific purpose.

Ingredients commonly used as additional supplements in feed include organic additives. Organic acid and their salts can also be used as organic feed additives which can trigger growth in fish farming, provide energy for metabolism, and increase feed digestibility. These organic acids and their salts are alternatives to antibiotics that have been widely used to enhance the immune system and growth in aquaculture [5]. One of these organic acids and salts is potassium diformate which is often used as a feed additive. However, the current application of potassium diformate as an additional feed ingredient to enhance the immune system of goldfish has not been done in depth. So, the problem faced in this research is how many optimal dosages of potassium diformate in the feed to improve the immune system of the goldfish.

2. MATERIALS AND METHODS

The materials used include goldfish, potassium diformate (KDF), *Aeromonas hydrophila* isolates and commercial feed. This research used experimental Complete Random Design (CRD) method with 4 treatments and 3 replications. The treatments are A (control), B (0.1% KDF), C (0.3% KDF) and D (0.5% KDF). Goldfish were randomly divided into 12 aquariums, each of which was with number 10. Then, it was challenged by *A. hydrophila* by injecting 0.1 mL of *A. hydrophilla* to obtain a density of 10^8 cfu/mL.

2.1 Cbservation Procedure

2.1.1 Sterilization and fish acclimatization

The container used for raising fish is first sterilized by soaking chlorine using a dose of 30 ppm for 24 hours and given aeration. Then, rinsed clean and dried. After that, the fish acclimatized before being used in research for ± 3 days in a fiber bath. Acclimatization is intended so that test fish can adapt to the research environment.

2.1.2 Mixing potassium diformate on feed

Potassium diformate weighed according to each treatment as much as 0.1%, 0.3% and 0.5% of

100 grams of commercial feed given, then mixed with commercial feed in a tray, stirred evenly and sprayed with water as much as 10% as a binder. Then air-dried for about 30 minutes. Feeding in this research was given as much as 3% of fish biomass per aquarium by SNI: 01-6137-1999.

2.1.3 Observation of white blood cells and red blood cells

White blood cells and red blood cells were observed six times, which was carried out before the giving of potassium diformate, after the addition of potassium diformatte in the feed for 30 days and after the challenge tests on the 3rd, 7th, 10th and 14th days. Blood collection is obtained from the base of the fishtail, using a thomma pipette plus Turk solution for white blood cells and hayem solution for red blood cells. Homogeneous blood fluid can be dropped in the hemocytometer room and observed under a microscope.

2.1.4 Cultures of *Aeromonas hydrophila*

Bacterial culture was carried out during the 25th day of maintenance. The isolate from *Aeromonas hydrophila* was inoculated on Sodium Agar to obtain a single colony, then incubated at 30ºC for 24 hours. The results of growing bacteria are taken as much as 1 *ose* and dissolved with NB (Nutrient Broth) media as much as 15 mL. After that, the bacteria were incubated in an incubator shaker for 24 hours at 37ºC at a speed of 150 rpm. Bacteria culture was put into 2 ml cuvette and counted using a spectrophotometer with a wavelength of 540 nm and an absorbance value of 0.235, to obtain a density of 10^8 CFU / ml.

2.1.5 Challenge test with *Aeromonas hydrophila*

A. hydrophila bacterial infection of goldfish was carried out using a 0.1 ml *intraperitoneal* injection method.

2.2 Observation Parameters

2.2.1 White blood cells count

Calculation of white blood cells aims to determine changes in the number of white blood cells in goldfish between treatments. According to Nabib et al. [6], the formula for calculating the number of white blood cells (cells mm^{3-1}) is as follows.

$$\Sigma\text{Leukocyte} = \frac{\text{White blood cells count from all four samples}}{4} \times$$
multiplier factor

2.2.2 Red blood cells count

The formula for calculating the number of red blood cells (cells mm^{3-1}) is as follows.

$$\Sigma\text{Erythrocyte} = \frac{\text{Red blood cells count from all five samples}}{5} \times$$
multiplier factor

2.2.3 Macroscopic clinical symptoms

Observation of macroscopic clinical symptoms include changes in behavior, morphological changes and the response of fish to food. Observations were made after the fish were challenged with aeromonas bacteria for 14 days.

2.2.4 Survival rate

The survival rate of the goldfish was observed by counting the number of seeds that were edited every day after the challenge test, using the following formula [7].

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

Information:

SR = *Survival Rate* (%)
Nt = Number of live fish at the end of rearing
No = The number of fish at the beginning of maintenance

2.2.5 Statistical analysis

Data on the number of white blood cells count and red blood cells count were analyzed using F test and Duncan's advanced test at a 95% confidence level, while the gross clinical sign data were analyzed descriptively.

3. RESULTS AND DISCUSSION

3.1 White Blood Cell (Leukocyte)

The calculation of white blood cells aims to determine changes in the number of white blood cells in goldfish between treatments. The following are the results of the average number of leukocytes of a goldfish during maintenance (Fig. 1).

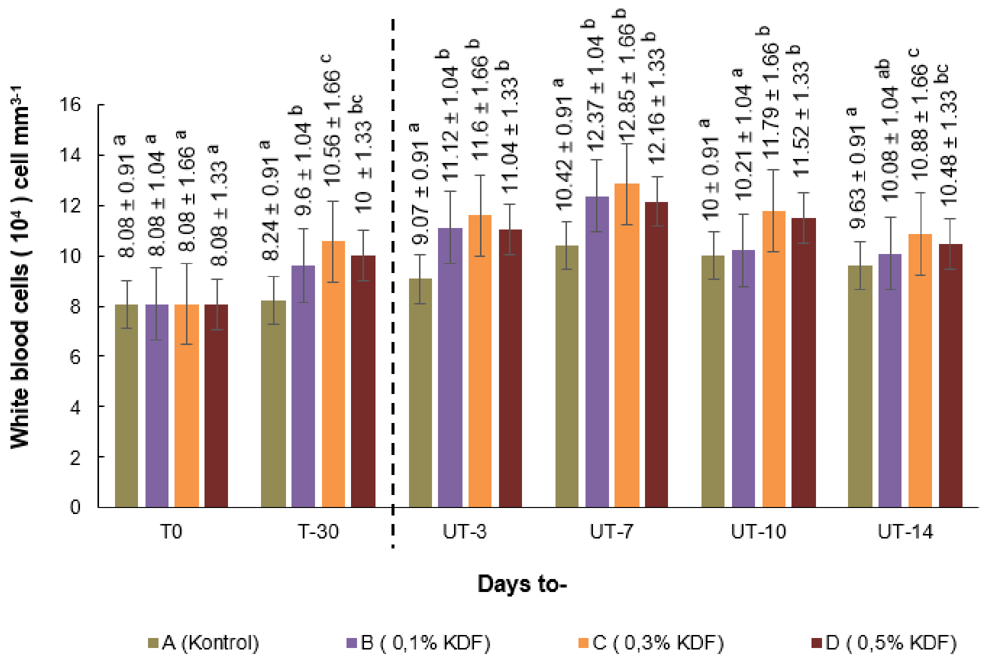


Fig. 1. White blood cells count

Information:

T0: Before giving KDF, UT-7: Day 7 after the challenge test

T-30: After ginving KDF, UT-10: Day 10 after the challenge test

UT-3: Day 3 after the challenge test, UT-14: Day 14 after the challenge test

The graph (Fig. 1) shows that the average leukocytes after giving of potassium diformatte at different doses, the number of white blood cells has increased from each treatment with a range of 8.24-10.56×10^4 cells mm^{3-1}. However, the number of leukocytes counted is still in the normal range. The lowest increase was aimed at treatment A (without KDF) of 1.94% with a value of 8.24×10^4 cells mm^{3-1}. While treatment C (KDF 0.3%) experienced the highest increase in leukocytes by 23.48% with a value of 10.56×10^4 cells mm^{3-1}. This reflects that the goldfish are experiencing the process of induction of the immune system optimally due to the influence of KDF 0.3% that enters the body as an immunostimulant and helps the process of forming the fish's immune system.

The calculation of white blood cells on the 3rd day after the challenge test showed that the number of leukocytes in goldfish from each treatment increased. However, the highest increase in white blood cells was obtained in treatment B (KDF 0.1%) of 13.67% with the amount of 11.12×10^4 cells mm^{3-1}. The high number of white blood cells is thought to be due to phagocytosis in the body which means that the fish is responding to immunity against infection. This is because fish have a severe infection due to the attack of *A. hydrophila* bacteria. According to Dwinanti et al. [8], an increase in the number of leukocytes indicates that the fish has a bacterial infection and the body will anticipate this condition by producing more white blood cells in response to immunity. While treatment C (KDF 0.3%) experienced a low increase of 8.97% with a leukocyte count of 11.60×10^4 cells mm^{3-1}. Low levels of leukocytes indicate that the fish are still in a condition that is not too sick with marked symptoms that are not so severe (Table 1). This is due to the addition of KDF 0.3% to the feed at the appropriate dose to increase the optimal immune system. KDF with a certain dose can increase the immune system in fish [9-11].

The KDF dose of 0.1% is thought to be insufficient to optimally enhance the body of goldfish in attacking *A. hydrophila.* Besides, the higher KDF dose does not always increase the immune system, this is due to interference with the osmoregulation system. Therefore, treatment D (KDF 0.5%) by giving the highest dose is not effective for the body defense system of the goldfish. The increase in the average number of white blood cells lasted until the 7th day, where treatment (A) had the highest increase of 13.64%, and treatment C (0.3%) had a low increase of 9.75%. A decrease in the number of white blood cells in goldfish occurs on the 10th day. This is the initial phase of healing at each treatment. Treatment C (0.3%) experienced a fairly high decrease of 10.41% with several leukocytes of 12.85×10^4 cells mm^{3-1} to 11.79×10^4 cells mm^{3-1}. The lowest decrease is in treatment (A) with a change of 4.27%. The decrease in the number of white blood cells continues on the 14th day. The decrease in all values in all treatments indicates that the attack of Aeromonas bacteria has begun to decline and the fish have begun to be healthy which is characterized by a state of clinical symptoms that are starting to disappear.

3.2 Red Blood Cell (Erythocyte)

The graph in Fig. 2 shows the value of goldfish red blood cells before giving KDF which is 1.17×10^6 cells mm^{3-1}. The amount is still within normal limits. This is consistent with the amount of erythrocytes in Teleostei fish ranging from $1.05\text{-}3.0 \times 10^6$ cells mm^{3-1} [12].

After maintenance with KDF for 30 days, the average value of the number of red blood cells increased between treatments. Treatment C (KDF 0.3%) showed the highest number, namely 1.29×10^6 cells mm^{3-1} with a change of 9.30% and in treatment A (without KDF) the lowest number was obtained 1.20×10^6 cells mm^{3-1} with changes by 2.23%. This is presumably because giving KDF 0.3% is the optimum dose that can increase red blood cells in goldfish.

The results of observations on the 3rd day after the challenge test showed that the average red blood cell of goldfish decreased in each treatment. When viewed from the percentage, treatment A (without KDF) had the highest decrease in the decrease of 22.53% with an average number of erythrocytes 0.98×10^6 cells mm^{3-1}. The lowest percentage was aimed at treatment C (KDF 0.3%) which was 19.51% with an average erythrocyte 1.14×10^6 cell mm^{3-1}. The decrease in the high number of erythrocytes in treatment A indicates that the physiological condition of the goldfish is very disturbed due to Aeromonas bacteria. This is indicated by the presence of bleeding in the kidney organs of fish (Fig. 3b). The process of rupture of red blood cells due to bacteria produces a toxin in the form of the enzyme hemolysin which plays a role in lysis of erythrocytes [13]. According to Anderson et al. [14], the number of red blood cells can provide information related to physiological conditions and indicate the health status of fish. The decrease in red blood cells is considered to be the impact of *A. hydrophila* attacks. This decrease in red blood cells occurs until the 7th day.

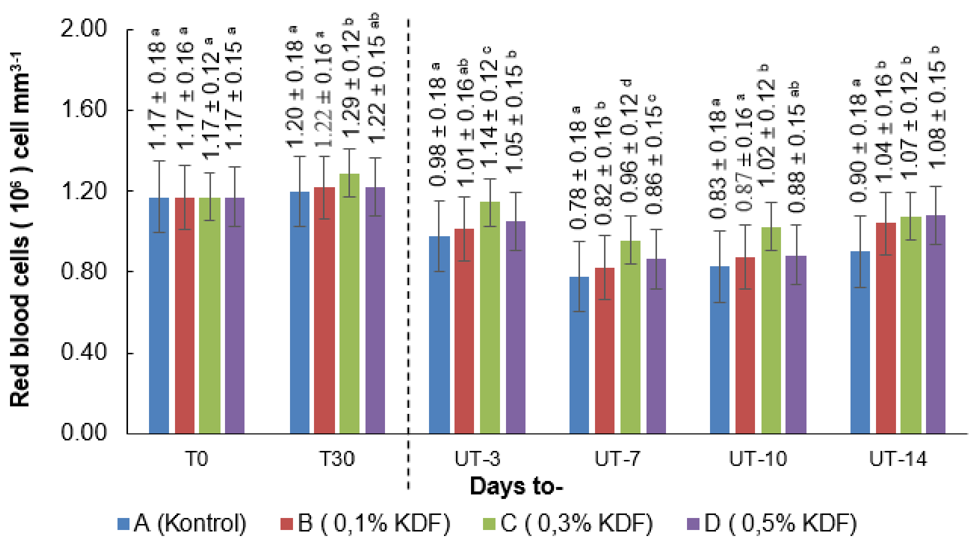


Fig. 2. Red blood cells count

Information:

T0: Before giving KDF, UT-7: Day 7 after the challenge test

T-30: After giving KDF, UT-10: Day 10 after the challenge test

UT-3: Day 3 after the challenge test, UT-14: Day 14 after the challenge test

On the 10th day the number of red blood cells in fish in all treatments increased. This indicates that infected fish have begun to improve and are experiencing healing, marked by the recovery of clinical symptoms in fish that can be seen in Table 1. This healing is thought to be due to homeostasis in the fish's body to produce more red blood cells which are lysed after challenged. The results on the graph show that C (KDF 0.3%) has the highest change of 6.51% with an average number of 1.02×10^6 cells mm^{3-1} and treatment D (KDF 0.5%) has the lowest change of 2.26% with an average number of 0.88×10^6 cells mm^{3-1}. The increase in the number of erythrocytes took place on the 14th day.

3.3 Macroscopic Clinical Symptoms

Macroscopic clinical symptoms observed include morphological changes such as morphological damage, response to feed given and fish swimming motion. Based on observations, the morphological damage that occurred in the test fish was seen on the first day. The symptoms that appear in each treatment are uneven (Table 1), because the test fish have different endurance.

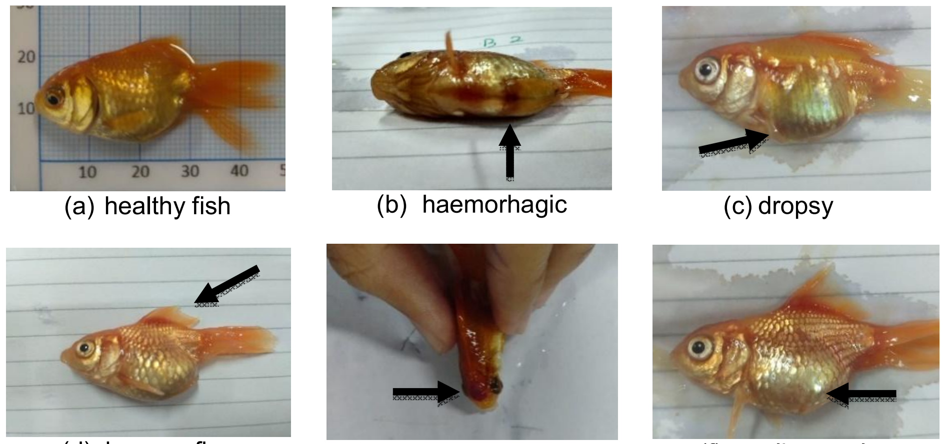
Based on the results (Table 1), the clinical symptoms found on the first day are hemorrhagic bleeding accompanied by swelling in the dorsal part of the fish, where Aeromonas bacteria are injected, causing many fish to die from bodily damage to the fish. This is following the statement of Mulyana et al. [15], fish affected by *A. hydrophila* will experience common symptoms such as bleeding (hemorrhagic), exophthalmia, abdominal swelling (dropsy) and small lesions on the surface of the body (resulting in the release of scales). The bleeding is caused by an enzyme produced by *A. hydrophila* which is haemolysin which enters the blood vessels and can lyse red blood cells causing reddish color on the surface of the injured skin [16]. Where as dropsy of test fish is caused by *A. hydrophila* infection which directly enters the body and rapidly attacks internal organs (Fig. 3c).

Body damage that results in many deaths occur in fish without KDF (treatment A). The addition of potassium formatted can increase the non-specific immune system in fish. This statement was supported by research Yustiati et al. [9-11], that potassium diformate can increase the immune system of pangasius and sangkuriang catfish. Damage to fish fins appearing on observation day 2. This fin damage is caused by a blockage in the blood vessels of the fish near the fin that causes slow decay and fin damage. Damage to fish fins due to the blockage of blood vessels and mucus caused by endotoxins (LPS) [17]. Morphological damage to fish body in treatments A (without KDF), B (KDF 0.1%) and treatment D (KDF 0.5%) lasted until the 7th day. Test fish have experienced healing on days 8 to 14. In contrast to treatments C whose body damage only lasts until the 6th days. After that, the fish begins to recover, marked by the disappearance of symptoms gradually in the test fish.

Table 1. Morphological damage

Days to -	Treatments											
	A			B			C			D		
	1	2	3	1	2	3	1	2	3	1	2	3
1	ab	ab	abc	ab	abc	ab	ab	ab	ab	ab	ab	ab
2	abc	abc	abc	abc	abc	abc	ab	ab	abc	abc	ab	abc
3	abc	abc	abc	abc	abc	abc	ab	abc	abc	abc	abc	abc
4	abcd	abc	abcd	abc	abc	abcd	abc	abc	abc	abc	abcd	abc
5	abcd	abcd	abcd	abc	abc	abcd	abc	abc	abc	abc	abcd	abc
6	abc	abcd	abcd	abc	abcd	abc	abc	ac	abc	abc	abc	abc
7	ac	abc	abc	ac	abc	abc	ac	ac	ac	abc	abc	abc
8	ac	ac		ac	abc	ac	ac	c	ac	ac	abc	ac
9	ac	ac		c	ac	ac	c	c	ac	ac	ac	ac
10	c	c		-	c	c	c	-	c	-	c	-
11	c	c	m	-	c	-	-	-	-	-	c	-
12	c	-		-	-	-	-	-	-	-	-	-
13	-	-		-	-	-	-	-	-	-	-	-
14	-	-		-	-	-	-	-	-	-	-	-

Information:
a: haemorhagic, d: exopthalmia, b: dropsy, -: no clinical symptoms, c: damage fin, m: totally dead



(a) healthy fish (b) haemorhagic (c) dropsy

(d) damage fin (e) exopthalmia (f) peeling scales

Fig. 3. Macroscopic clinical symptoms

Changes in the morphology of these test fish have no difference between treatments. However, based on Table 2, the fish in treatment A had a long recovery, and a total death was found in treatment A3 on the 8th day. While fish that were given KDF 0.3% (treatment C) experienced faster healing compared to other treatments. This is because the fish in treatment C have a better immune system than other treatments due to the addition of the optimal KDF dose. Besides observing the response to feed (Table 2) is also done by looking at the response of fish when given feed and the amount of feed remaining.

Table 2 shows that on day 1 the test fish in treatment C still showed a response to the feed but it was fairly low, indicated by the existence of a small amount of food remaining. In contrast to treatments A, B, and D that the fish have not responded to the feed given. This incident indicates that the pathogenic bacteria injected into the body of the fish have been processed. It is suspected that the bacteria has infected the digestive tract and blood vessels, so that the body's metabolism is disrupted and causes loss of appetite in fish. Following the statement of Yustiati et al. [10], the enterotoxin compound released by *A. hydrophila* will attack the digestive tract in fish which causes disturbed metabolism. So, the fish cannot regulate normal physiological conditions (stress).

On the 3th day, the test fish that received C treatment started responding to feeding properly, visible from the absence of feed left in the aquarium. This shows that the condition of the fish has recovered and the immune system has improved. While treatment A is the longest healing (4th day) in response to the feed given, because it is thought that its energy is widely used as a defense against Aeromonas bacteria.

As for observing the behavior of fish observed from the way the fish swim. The results of observations of fish behavior have been presented in Table 3.

Based on the results, the data obtained that on the 1st day of observation all fish began to experience unstable movements. Test fish experience changes in behavior, i.e. movements of the fish become passive and irregular. Swimming movement stabilizes on the 5th day until the 14th day. Therefore, it can be concluded that increasing the KDF dose of 0.3% can improve the optimal immune system for goldfish, characterized by the recovery of clinical symptoms (morphological damage, response to feed, and fish swimming motion) which is faster than other treatments.

3.4 Survival Rate

Survival rate was observed to determine the percentage of survival of a species of fish kept in a container. After the injection of Aeromonas bacteria, the test fish experienced many deaths from each treatment until the 5th day. Most deaths were obtained in fish that were not given KDF (A). This means giving KDF in feeds gives a good effect on the survival of the goldfish that has been infected by the bacteria. based on the results of the F test (p <0.05), the fish test that was given a dose of 0.3% KDF (C) had a significant influence on the survival rate of goldfish chefs with a percentage of 46.67%. This percentage is the highest value when compared with the D treatment KDF dose 0.5% (26.67%) and treatment B KDF dose 0.1% (20%). Meanwhile, treatment A without KDF administration obtained the lowest survival rate of 6.67%. This shows that giving KDF 0.3% is the optimum dose that can improve the immune system and survival of goldfish because the salt content in KDF is thought to be able to kill bacterial cells and balance the osmotic pressure in the body to improve the osmoregulation process of fish KDF can kill bacterial cells and reduce gastric pH which accelerates the secretion of pepsin and pepsinogen to increase the digestibility of proteins, so the level of health and survival of fish for the better [18].

Table 2. Response to feed

Days to -	Treatments											
	A			B			C			D		
	1	2	3	1	2	3	1	2	3	1	2	3
1	+	-	-	-	+	+	+	+	+	-	-	+
2	-	-	-	-	-	-	+	+	-	-	-	-
3	-	-	-	-	-	-	-	-	+	-	+	-
4	+	+	+	+	-	+	+	+	+	+	+	+
5	+	+	-	+	+	+	+	+	+	+	+	+
6	+	+	-	+	+	+	+	+	++	+	+	+
7	+	+	-	+	++	+	++	++	++	+	++	+
8	+	+		++	++	+	++	++	++	++	++	+
9	++	+		++	++	++	++	++	++	++	++	++
10	++	++		++	++	++	++	++	++	++	++	++
11	++	++	m	++	++	++	++	++	++	++	++	++
12	++	++		++	++	++	++	++	++	++	++	++
13	++	++		++	++	++	++	++	++	++	++	++
14	++	++		++	++	++	++	++	++	++	++	++

Information:
(m): totally died, (-): Response does not exist, (+): Low response (any leftover feed), (++): Response is high (feed is not leftover)

Table 3. Fish swimming movement

Days to -	Treatments											
	A			B			C			D		
	1	2	3	1	2	3	1	2	3	1	2	3
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-
4		-	-	-	+	-	-	-	+	-	-	-
5	+	-	-	+	+	-	+	+	+	+	-	-
6	+	-	-	+	+	-	+	+	+	+	-	+
7	+	+	-	+	+	+	+	+	+	+	+	+
8	+	+		+	+	+	+	+	+	+	+	+
9	+	+		+	+	+	+	+	+	+	+	+
10	+	+		+	+	+	+	+	+	+	+	+
11	+	+	m	+	+	+	+	+	+	+	+	+
12	+	+		+	+	+	+	+	+	+	+	+
13	+	+		+	+	+	+	+	+	+	+	+
14	+	+		+	+	+	+	+	+	+	+	+

Information:
(+) normal swimming movement, (-) passive swimming movement, (m) totally dead

4. CONCLUSION

Based on the results of the study, it can be concluded that the addition of KDF 0.3% is the optimum dose to improve the performance of the goldfish (*Carassius auratus* L.) immune system, which is seen from the number of white blood cells, red blood cell counts, clinical symptoms and survival rate in test fish after the challenge test by *A. hydrophila.*

CONSENT

All authors declare that written informed consent was obtained from the patient (or other approved parties) for publication of this paper.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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