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Effectiveness of potassium diformate addition to feed to improve immune system of Pangasius (*Pangasianodon hypophthalmus*) that challenged by *Aeromonas hydrophila*

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ABSTRACT

This research was conducted from October 2018 until April 2019 in the Faculty of Fisheries and Marine Sciences, Universitas Padjadjaran. This study aims to determine the optimum potassium diformate dosage which was added to feed to increase the immune status of *Pangasianodon hypophthalmus* (Sauvage, 1878). The research method used is the experimental Complete Random Design (CRD) with 4 treatments and 3 replications. The treatments are A (control), B (0.3% KDF), C (0.5% KDF), and D (0.7% KDF). Observed parameters are survival rate, gross clinical signs, intestine pH levels, total leukocyte count (white blood cells), total erythrocyte count (red blood cells), and water quality. The results showed that the 0.5% KDF addition in feed is the most effective to improve immune status. This treatment also is the most effective to improve Pangasius immune status after being challenged with *A. hydrophila* with survival rate of 93.33%, leukocyte count 2.98×10^6 cells/mm^3 and erythrocyte count 1.02×10^6 cells/mm^3. Increased leukocyte count was 65.26% and erythrocyte count -58.23%.

***Keywords*:** *Pangasianodon hypophthalmus*, potassium diformate, survival rate, red blood cells, white blood cells, *Aeromonas hydrophila*, Pangasius

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1. INTRODUCTION

Pangasius (*Pangasianodon hypophthalmus* (Sauvage, 1878) is a popular fish commodity. Its distribution covers: Vietnam, Cambodia, Thailand, Malaysia, Indonesia.

The Ministry of Maritime Affairs and Fisheries states that Pangasius is one of the industrial commodities because it has stability in terms of seeds, enlargement, feed and processing, and the extent of production. Many farmers now cultivate Pangasius. Pangasius is also a fish with high economic value and high export potential. Pangasius cultivation activities have several weaknesses, one of which is the existence of constraints on fish health or a low immune system. The immune system is a mechanism of self-defense against bacteria/pathogens. Disease can attack Pangasius due to bacterial, fungi or virus and cause death. An example of a bacterium that attacks Pangasius is *Aeromonas hydrophila*.

A. hydrophila is the most often cause problems of failure in freshwater aquaculture. There are two solutions to the discussion of diseases, namely prevention and treatment. Therefore prevention is better than treatment. In handling the disease until now there are still those who use antibiotics.

However the antibiotics are dangerous to cause pathogens to become resistant. Antibiotics have now been found in many resistant substances such as Tetracline, Chioramphenicol, and Nitrofuran groups. The use of prohibited antibiotics does not rule out the possibility that there are other safer ingredients and benefits that can be felt.

The material that can be used is organic matter, one of which is organic acid. Organic acids and their salts can also be used as organic additive feeds. The organic acids and their salts can spur growth in aquaculture, provide energy for metabolism, and increase feed digestibility. Potassium diformate can be used as an alternative to growth and health-promoting antibiotics, and it can increase the productivity of *Pangasianodon hypophthalmus*.

However, currently the application of potassium diformate in feeds to increase the immunity of *P. hypophthalamus* has not been done in depth. Then the problem faced in this research is how much the optimal dosage of potassium diformate should be used in feed to increase immune system of Pangasius.

2. MATERIALS AND METHODS

The materials used in this research include pangasius, potassium diformate (KDF), *Aeromonas hydrophila* isolates, and commercial feed. This study used an experimental method with Completely Randomized Design consisting of 4 treatments and 3 replications. The treatment used is A (without the addition of KDF), B (0.3% KDF), C (0.5% KDF) and D (0.7% KDF). Pangasius was randomly divided into 12 aquariums, each of which was with number 20. Then it was challenged by *A. hydrophila*, by injecting 0.1 mL of *A. hydrophilla*, to obtain a density of 10^8 cfu/mL.

The parameters observed were survival rate (SR), macroscopic clinical symptoms, gastrointestinal pH, white blood cell (WBC) count, red blood cell (RBC) count, and water quality. Survival rate, total WBC count, and total RBC count were analyzed using variance analysis and if there were significant differences between treatments they were followed by a double distance, Duncan test with a 5% error rate. Macroscopic clinical symptom data, stomach pH, and water quality were analyzed descriptively.

2.1. Procedure

2.1.1. Mixing of KDF in Feed

Feed and KDF were weighed, then poured into a tray. Next they were mixed until homogeneous and sprayed with water as a binder, then dried.

2.1.2. Counts the number of white blood cells and red blood cells

Blood sampling was done six times, namely before treatment, after being treated, on the third day after the challenge test, on the 7th day after the challenged test, the 10th day after the challenged test, and the 10th day after the challenged test, and after the test challenged. The steps taken are the base of the tail dissected, then the blood coming out is sucked using a Thoma pipette up to a scale of 0.5. Then suction again the Hayem's solution for red blood cells up to a scale of 101 and Turk's solution for white blood cells to 11. The blood is stored above the Haemocytometer, then covered with a glass cover.

2.1.3. Gastrointestinal pH measurement

Gastrointestinal contents were removed, then chopped and measured using pH papers.

2.1.4. Culture of *Aeromonas hydrophila*

The isolates of *Aeromonas hydrophila* were inoculated on NA (*Nutrient Agar*) media using the scratch method to obtain a single colony, then incubated at 30 ºC for 24 hours. The results of the bacteria that were grown were taken by using one needle with as much as 1 ose and dissolved with NB (*Nutrient Broth*) media. After that, the bacteria were incubated in a shaker incubator for 24 hours at 30 ºC at a speed of 150 rpm. Then the density calculation was performed to obtain a density of 10^8 cfu/mL using a spectrophotometer with a wavelength of 540 nm and an absorbent value reaching 0.8-1 for a density of 10^8 cfu/mL.

2.1.5. Challenge Test with *Aeromonas hydrophila*

Infection of *A. hydrophila* against carp was done at intraperitoneally section as much as 0.1 mL/individual with a density of 10^8 cfu/mL. Observation of clinical symptoms, survival rate, WBC count, and RBC count of test fish after A. *hydrophila* infection was carried out every day during the maintenance period of 14 days.

3. RESULT DISCUSSION

3.1. Survival Rate of Pangasius before and after Challenged by *A. hydrophila*

Based on the results of analysis variance F test (ANOVA), the survival rate of fish appeared to be not significantly different. But the results of the survival of Pangasius show different numbers, where the control treatment is the lowest survival rate. However, the treatments C and D are the test fish that have the highest SR (Figure 1).

Based on variance analysis (ANOVA) one may state that survival between treatments is significantly different. Giving KDF to feed has a positive influence on the survival of Pangasius infected with *A. hydrophila*. In treatment C (KDF 0.5%) after being challenged, the highest survival rate was 93.33% compared to the control treatment, which was 66.67%.

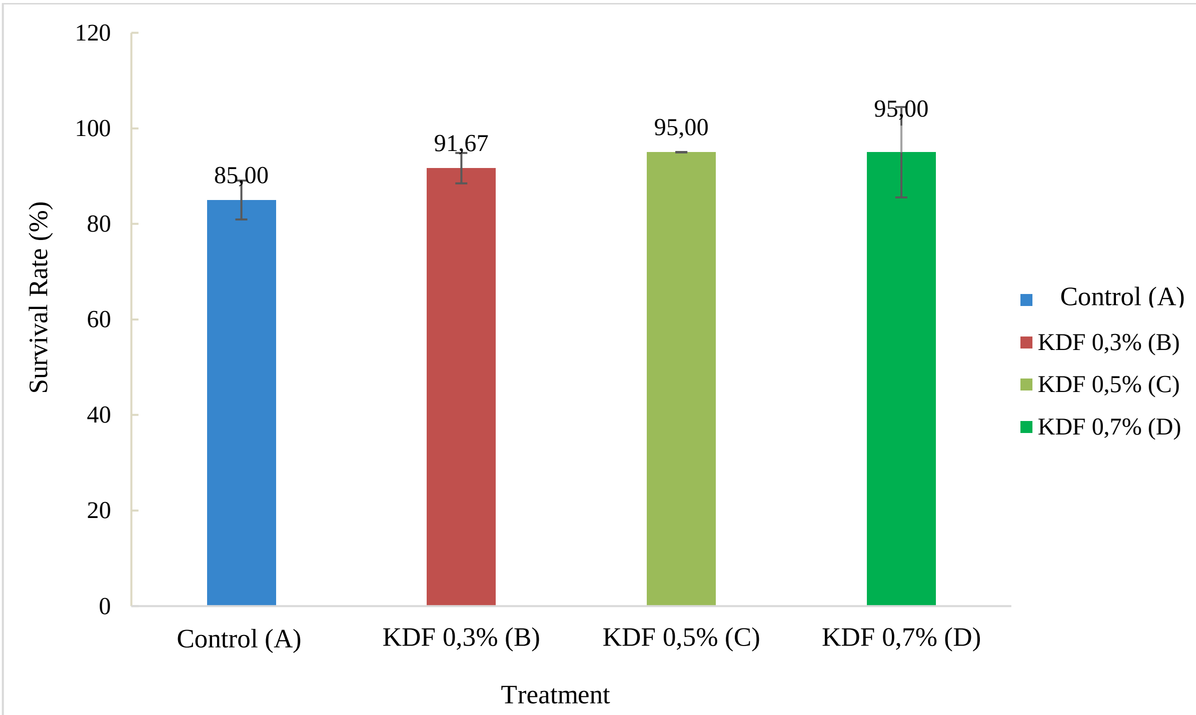

Figure 1. Survival rate before challenge.

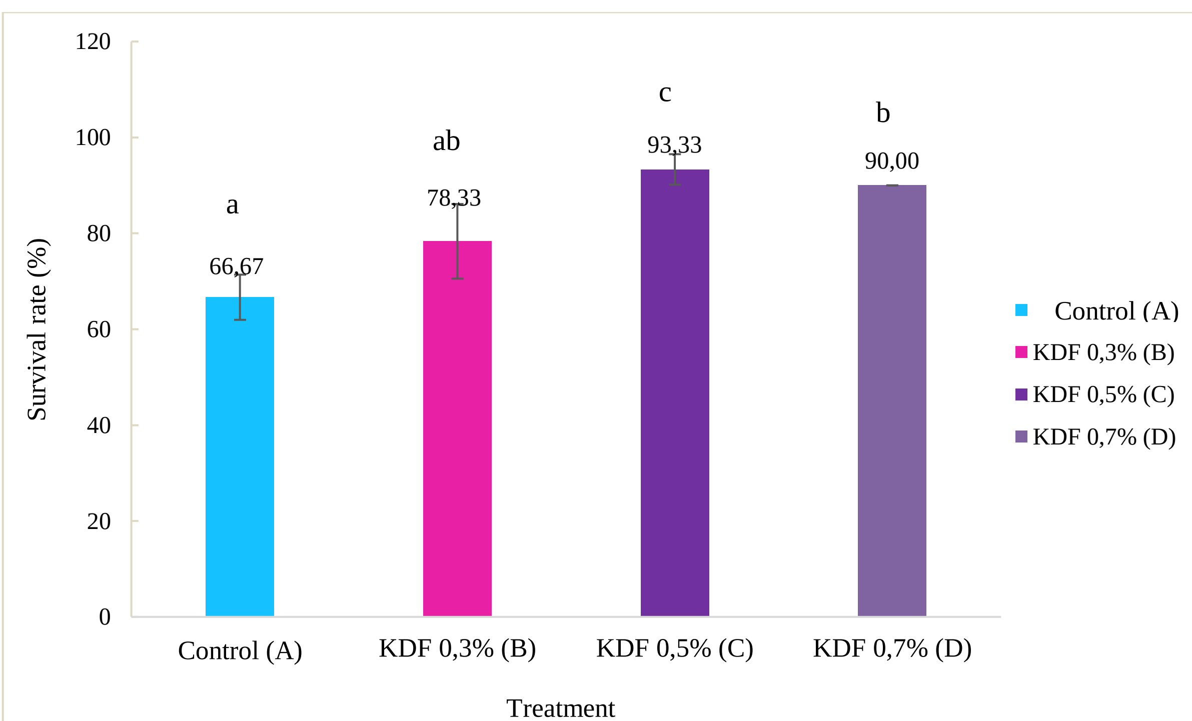

Figure 2. Survival rate after challenge.

Resistance to disease increases with the administration of certain doses of KDF. This shows that administering KDF doses can improve the immune system of Pangasius. The cumulative mortality of fish fed KDF and challenged *A. hydrophila* was lower than the control group. Based on the results of the study treatment C one may notice the group of fish that has the highest survival compared to other KDF dose treatments. It has been noted that in treatment B (0.3% KDF) the dosage of KDF is still not enough to increase the immunity of pangasius to attack *A. hydrophila*.

In treatment D even though the KDF dose has a lower survival than in treatment C (Figure 2), and this is because the dosage given exceeds the proper amount which causes the energy possessed by the fish to be used to balance the metabolism in the body or the osmoregulation system. The content of salt in a medium is closely related to the osmoregulation (mechanism) system in freshwater organisms. Substances that enter the body of the test fish are KDF which is a double salt.

Thus, this salt can interfere with the fish's osmoregulation system if overused. The aquatic organisms have osmotic pressures that vary with their environment. Therefore, fish must prevent an excess of water or lack of water so that the physiological processes in the body are normal.

3.2. Total White Blood Cells Count and Total Red Blood Cells Count

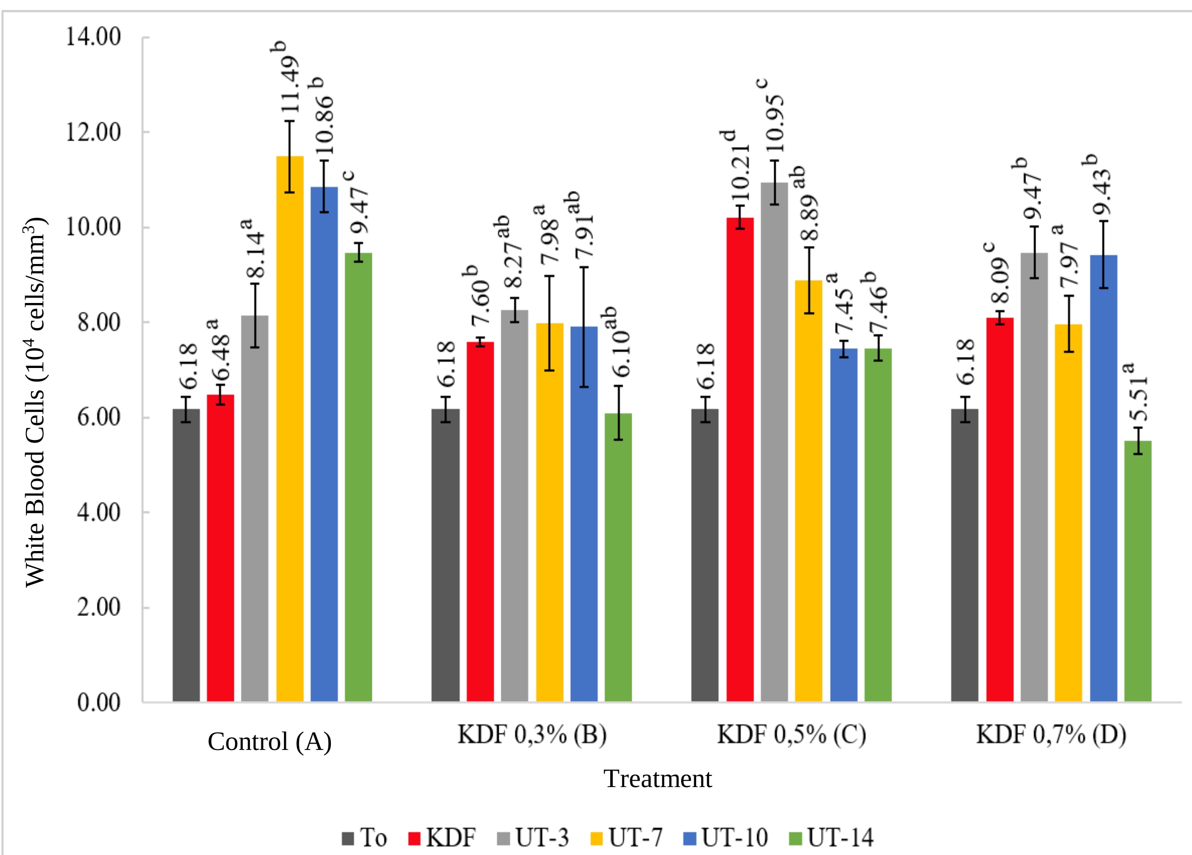

Figure 3. Total white blood cells count.

Information:

T_0:	before treatment	UT-7:	seventh day after challenged
KDF:	after treatment	UT-10:	tenth day after challenged
UT-3:	third day after challenged	UT-14:	fourteenth day after challenged

The total number of leukocytes in teleostei are 2.0×10^4 to 1.5×10^5 cells/mm^3. Control means the lowest increase of RBC 4.85%. Total leukocyte count that added by KDF are higher increase than the control treatment (Figure 3). The highest leukocyte increase after treatment was found in treatment C (0.5% KDF) which increased by 65.26%. Treatment B (KDF 0.3%) increased by 22.97%, and treatment D (0.7% KDF) increased by 30.96%. Pangasius treated with organic acids had an increase in leukocyte count by 10.2%.

This increase in leukocytes shows that KDF can increase leukocytes and optimal at a dose of 0.5% due to the highest increase in leukocytes. Addition of additives to feed can increase Pangasius WBC such as in research by Faith *et al*. (2017) who added curcumin to feed, that Pangasius WBC increased to 7.76-10.55×10^4 cells/mm^3. According to the results of the F test (ANOVA), the number of WBC after this treatment revealed a significant difference, which was further tested using the Duncan test, where the result was the best treatment C because the results were the highest with 0.5% KDF (Figure 3).

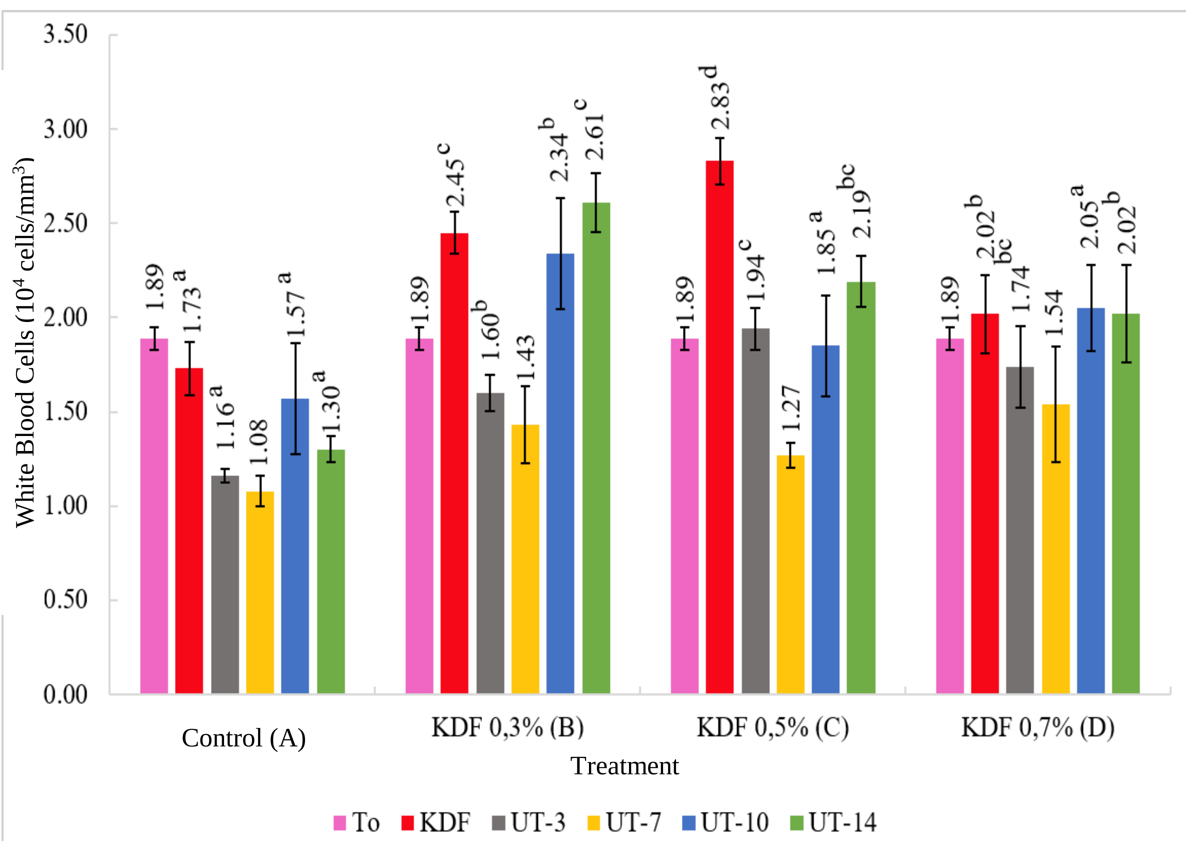

Figure 4. Total Red blood cells count.

Information:

T_0:	before treatment	UT-7:	seventh day after challenged
KDF:	after treatment	UT-10:	tenth day after challenged
UT-3:	third day after challenged	UT-14:	fourteenth day after challenged

The average number of white blood cells increases after infection with *A. hyrophilla*. White blood cells are part of the body's defense system that is non-specific. Changes in total leukocyte values are often used as clues to the state of fish physiology or indicators of the presence of disease in the fish body.

That increase in leukocyte cells is a reflection of the success of the fish immunity system in developing a cellular immunity response as a trigger for an immune response. The active ingredient that functions as an antibacterial and antimicrobial acting in the tissues of the fish body helps leukocyte cells to reduce the amount and pathogenicity of *A. hydrophila*.

Total RBC count given KDF increased more than the control treatment (Figure 4). This is presumably due to the addition of KDF on hematology and the immune system. The amount of total RBC count after challenged has decreased. The decrease in total RBC count indicates the presence of anemia in fish characterized by bleeding in fish kidney organs.

The presence of *A. hydrophila* which produces hemolytic toxins results in the lyse RBC count so that the average of RBC count generally decreases.

The enzyme hemolysin which is one of the exotoxins of *A. hydrophila* has the ability to lyse red blood cells, so that the number of red blood cells in blood vessels decreases. On the tenth day after challenged had entered the healing phase, it was marked by an increase in red blood cells. This total increase in erythrocytes signifies a homeostatic effort in the body of the fish and the body to produce more to replace the RBC that lysis after challenge.

3.3. Macroscopic Clinical Symptoms

Based on observations of the body damage, the most severe damage was the control treatment compared to other treatments because it was not given KDF. However, the group of fish that had the lightest damage and the quickest cure was treatment C (0.5% KDF). Day 1 has dropsy symptoms and fin damage in the control treatment (Table 1).

Dropsy is a disease caused by constriction as a result of inflammation and rupture of blood vessels in the body due to the presence of endotoxin from *A. hydrophila*. Damage to fish fins is due to the blockage of blood vessels and mucus caused by endoctrin of LPS (lipopolysaccharide). Thus the rupture of blood vessels (hemorrhagic) is experienced resulting in a red color on the *A. hydrophila* fin.

Day 2 shows symptoms of exopthalmia. Swelling of the eye is caused by the presence of excess gas produced by the *A. hydrophila*. This gas production is carried out in the body so that there is a buildup of gas in the eye.

A. hydrophila excrete exotoxin or ECP (Extraceluller product) and endotoxin. Exotoxin compounds consist of hemolysin, protease, enteroxin, lecithinase, and leucocidine. Protease determines the occurrence of disease after bacteria pass through the host's first defense. In addition, *A. hydrophila* secretes lesitinase in an effort to enter the bloodstream. The content of hemolysin and lechitinase derived from the *A. hydrophila* is able to lyse red blood cells and destroy various tissue cells.

Therefore the *A. hydrophila* red blood cells in the body of the fish causes fish to become blood deficient while experiencing other symptoms such as red spots due to rupture of blood vessels. Rupture of blood vessels causes red spots on the surface of the body and fins and causes damaged fins/flaky fins, while the endotoxin is a cell wall coated with LPS (Lipopolysaccharide).

This toxin is toxic to the host and can cause fever and inflammation.

Table 1. Symptoms of damage after challenge with *A. hydrophila*.

T	**Days**													
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
A1	abc	abce	abcde	abcde	abc	abc	bc	bc	bc	a	b	a	-	-
A2	abcd	abcde	abcde	abcde	abce	bc	bc	bc	bc	b	b	-	-	-
A3	abce	abcde	abcde	abcde	abce	bce	bc	bc	bc	ab	ab	ab	-	-
B1	ab	abe	abe	abce	bce	bc	b	b	b	a	-	-	-	-
B2	ab	abe	abe	abe	ab	bc	b	b	-	-	-	-	-	-
B3	abc	abce	abce	abce	bce	bc	b	b	-	-	-	-	-	-
C1	ab	abe	abe	bc	bc	b	b	-	-	-	-	-	-	-
C2	ab	abe	abe	abc	bc	b	b	-	-	-	-	-	-	-
C3	ab	ab	ab	abc	abc	bc	b	-	-	-	-	-	-	-
D1	ab	ab	ab	abc	bcd	bc	b	-	b	-	-	-	-	-
D2	ab	abc	abc	abce	abc	bc	bc	b	b	-	-	-	-	-
D3	abc	abc	abc	abce	ab	ab	b	b	b	b	-	-	-	-

Information:

(-): no clinical symptoms
(a): dropsy
(b): haemorrhagic
(c): damaged fin
(d): exophthalmia
(e): necrosis/ulcers

Table 2. Response to feed.

Days	Control (A)			KDF 0.3% (B)			KDF 0.5 % (C)			KDF 0.7% (D)		
	1	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
1	+	+	+	+	+	+	++	++	++	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+
3	-	-	-	-	-	-	+	+	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	+	+	+	-	-	+
6	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	++	++	++	++	++	++
9	+	+	+	+	+	+	++	++	++	++	++	++
10	+	+	+	++	++	++	++	++	++	++	++	++
11	+	+	+	++	++	++	++	++	++	++	++	++
12	++	++	++	++	++	++	++	++	++	++	++	++
13	++	++	++	++	++	++	++	++	++	++	++	++
14	++	++	++	++	++	++	++	++	++	++	++	++

Information:

(-): no response
(+): response to feed decreases
(++): response to feed normal

Pangasius showed a decreased response to feed except in treatment C. The cause of the decrease in response to feed (Table 2) was due to stress after infection with *A. hydrophila*. Treatment A is the longest treatment with no response to feed. This is because the energy used is for bacterial attack resistance. Enteroxin compounds released by *A. hydrophila* attack the gastrointestinal tract in fish. Fish that experience a decrease in appetite are thought to be due to the injured digestive tract and damage to the body. Stress is a condition when an animal is unable to regulate normal physiological conditions due to various adverse factors that affect its health (Table 3). One of the reactions of fish during stress is loss of appetite.

Table 3. Response to shock.

Days	Control (A)			KDF 0.3% (B)			KDF 0.5 % (C)			KDF 0.7% (D)		
	1	2	3	1	2	3	1	2	3	1	2	3
1	++	++	++	++	++	++	++	++	++	++	++	++
2	++	++	++	++	++	++	++	++	++	++	++	++
3	+	+	+	++	++	++	++	++	++	++	++	++
4	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	++	++	++	++
6	+	+	+	++	++	++	++	++	++	++	++	++
7	+	+	+	++	++	++	++	++	++	++	++	++
8	+	+	+	++	++	++	++	++	++	++	++	++
9	+	+	+	++	++	++	++	++	++	++	++	++
10	+	+	+	++	++	++	++	++	++	++	++	++
11	+	+	+	++	++	++	++	++	++	++	++	++
12	+	+	+	++	++	++	++	++	++	++	++	++
13	++	++	++	++	++	++	++	++	++	++	++	++
14	++	++	++	++	++	++	++	++	++	++	++	++

Information:

(-): no response
(+): response to shock decrease
(++): response to shock normal

3.4. Gastrointestinal pH

KDF contains chain of 2 organic acids and contains formic acid to reduce the pH of the digestive tract which can be used to improve the fish's immune system. KDF can be used as an alternative for antibiotic growth and health boosters. Besides, KDF can improve the productivity of *P. hypophthalmus*. KDF also functions as an acidifier, a substance that can reduce pH in the digestive tract. KDF will undergo an ionization process in the digestive tract, which is the release of H^+ ions when it reaches the digestive tract found in a low pH environment.

Another advantage of the low pH of the digestive tract is in absorbing feed nutrients. Bacteria need nutrients for their own metabolism in the fish's body and compete with the host, while the pH becomes low which causes the bacteria to need a lot of energy to balance itself and afterwards it will lose energy and die. Then the energy that is in the body of the fish will only be used to absorb the available feed nutrients. The low pH of the digestive tract will weaken and reduce pathogenic bacteria that are not resistant to acidic pH while more non-pathogenic bacteria will be more than pathogenic bacteria (Figure 5).

Organic acids are antimicrobial, especially for yeast and *E. coli*, whereas for lactic acid bacteria they are not antimicrobial. A similar observation was made by Kirchgessner *et al*. (1992), who reported that formic acid or KDF supplements significantly reduce *E. coli* and increase lactic acid bacteria. Lactic acid bacteria can grow at a relatively low pH, which means that they are more resistant to organic acids/salts than gram negative bacteria. The antimicrobial effects of organic acids have been added to the increase in the density of BAL and antimicrobial products in fish intestines. Colonization of BAL inhibits pathogenic bacterial attachment and invasion.

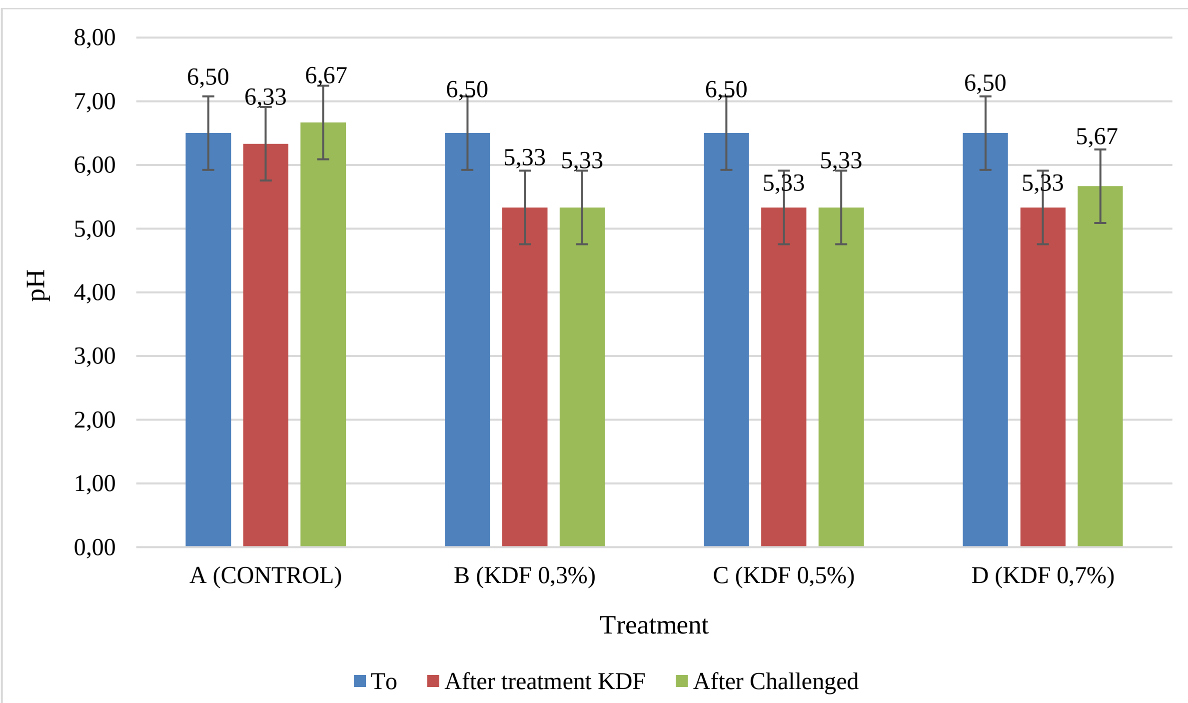

Figure 5. Stomach pH.

In research to rely on adding KDF to tilapia a dose of 0%, 0.3%, 0.6%, 0.9%, and 1.2%, was given where fish that have the highest growth performance and weight are given 0.3% KDF and 0.6% KDF. Potassium diformate is a promoter of tilapia growth that grows in Indonesia. The KDF dose used was 0.1%, 0.2%, 0.3%, and 0.5% which resulted in the highest efficient of feed of 0.5 % KDF. The use of these doses can improve the survival of the tilapia after being challenged with *Vibrio anguillarium*. The research has stated that the addition of 0.5% KDF to snapper juvenile maintained in fresh water resulted in the highest growth, survival rate and productivity index and increased significantly. It was found that 0.5% organic acid can improve fish haematological parameters and improve fish survival after being challenged with *A. sobria*. Moreover, the addition of organic acids to feed produced red blood cells and white blood cells significantly compared to control fish and fish from the 0.5% supplementation group. Much higher total protein and albumin level was obtained than in the control group fish.

3.5. Water Quality

According to the Indonesian National Standards the dissolved oxygen needed for Pangasius is more than 5 mg/L. The results of DO (Dissolved Oxygen) measurements for each treatment did not reach below 5 mg/L and the range was still not much different, likewise with the pH of water which was still within the optimal limits for the life of Pangasius. The pH of the water is not much different between treatments, which is around 6.12-7.85. The last parameter that becomes a benchmark for water quality is temperature. The temperature obtained during the study ranged from 27.0-32.4 °C. It is known that the optimal temperature range for Pangasius life is 25-32 °C. Data shows that the value of water quality obtained shows a normal range and can still be tolerated as catfish habitat. Based on the observations it can be assumed that KDF does not affect water quality. The study shows that all data obtained from the research results are due to differences in the treatment of different doses of KDF and not the effect of water quality (Table 4).

Table 4. Data on water quality measurement results.

Treatment	**Parameters**		
	Temperature (°C)	**pH**	**DO (mg/L)**
A (Control)	27.7-30.6	6.0-7.85	5.8-10.4
B (KDF 0.3%)	27.5-32.4	6.4-7.62	5.1-10.4
C (KDF 0.5%)	27.0-32.1	6.81-7.85	4.7-9.5
D (KDF 0.7%)	28.4-31.1	6.12-7.78	5.2-10.7
Optimal	25-32[2]	6.5-8.5[1]	>5[1]

4. CONCLUSIONS

Addition of 0.5% potassium diformate to feed increased the number of white blood cells from 6.18×10^4 cells/mm^3 to 10.21×10^4 cells/mm^3 (increased by 58.23%) and increased the number of red blood cells by 1.88×10^6 cells/mm^3 to 2.98×10^6 cells/mm^3 (increased by 65.26%) and decreased stomach pH from 6.50 to 5.3. Pangasius that were given a dose of KDF 0.5% had the highest survival rate compared to other treatments after challenge using *A. hydrophila* which was equal to 93.33%.

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