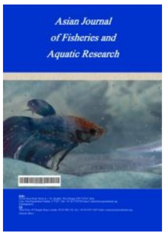


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# The Use of Pottasium Diformate in Feed to Improve Immunity Performance of Common Carp (*Cyprinus carpio*, L)

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***Authors' contributions***

*This work was carried out in collaboration among all authors. Author AY designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft on the manuscript. Authors AR and Sunarto managed the analyses of the study. Author IBBS managed the literature searches. All authors read and approved the final manuscript.*

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## ABSTRACT

This research aims to determine the optimum potassium diformate (KDF) dosage which was added to commercial feed to increase the immune performance of common carp fingerlings (*Cyprinus carpio* L). This study was conducted from October – December, 2021 at the Laboratory of Aquaculture and Molecular Biotechnology of The Faculty of Fisheries and Marine Sciences, Universitas Padjadjaran. The method used in this research was experimental in a Completely Randomized Design (CRD) with 5 treatments and 3 replications. The treatments are A (without KDF as control), B (0.2% KDF), C (0.3% KDF), D (0.4% KDF) and E (0.5% KDF). The observed parameters are total leukocyte count, total erythrocyte count, and gross clinical symptoms. Observations were made for 35 days after KDF administration and then challenged by *Aeromonas hydrophila* for 14 days. Total leukocyte count and total erythrocyte count were analyzed using ANOVA and DMRT at a 95% confidence level, while the gross clinical symptoms was analyzed descriptively. The results showed that the of 0.3% KDF was the most effective dose to increase the immune performance of common carp, the result showed that the total leukocyte count, and total erythrocyte count had the highest increase of 22,95% and 20,55%. In addition, the process of recovering is faster than other treatments it can be shown by the symptoms healing time and number of total leukocyte count and total erythrocyte count are approaching normal condition, with a value of $8{,}38 \times 10^4$ cells/mm$^3$, and $1{,}46 \times 10^6$ cells/mm$^3$.

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*Keywords: Potassium Diformate; Cyprinus carpio; immune system; Aeromonas hydrophila.*

## 1. INTRODUCTION

Common carp is a freshwater fishery favored by the community [1]. The growth of carp is relatively fast but in a less efficient aquaculture system, it can result in unstable water quality which poses various threats to the life and health of fish [2]. One of the diseases that often attack fish is red spot disease caused by *Aeromonas hydrophila* bacteria or commonly called *motile aeromonas septicemia*. This disease often afflicted freshwater fish, causing high mortality outbreaks [3]. Fish disease caused by the bacterium *A. hydrophila* became known in Indonesia around 1980, which caused a disease outbreak in carp in West Java Province, resulting in 125 tons of deaths [4].

One of the alternative preventions carried out by farmers from pathogen infection is by increasing the carp immune system by giving feed additives as immunostimulants. Immunostimulants play a role in activating non-specific defense mechanisms, and specific immune responses [5]. The addition of organic acids to feed is one strategy that can be used to improve the health of cultured fish [6]. Nutrient organic acids can promote growth and control pathogenic bacteria. The active chemicals contained can provide an antimicrobial effect against gram-negative bacteria so that it can increase the host's resistance [7].

The organic acid which is starting to be widely used is potassium diformate (kalium diformate / KDF). Potassium diformate can improve feed efficiency, growth performance, and survival rate. Potassium diformate can lower the pH in the intestine so it can reduce the activity of pathogenic bacteria. The organic acids can also maintain the balance of the bacterial population that is resistant to acidic conditions such as lactic acid bacteria which function as probiotics for immunostimulants. Beneficial bacteria in the digestive tract improved intestinal health resulting in the fish's condition will be more resilient against infection [8].

## 2. MATERIAL AND METHODS

Common carp fingerlings size 3-5 cm bought from the Cibiru Fish Fingerling Center (BBI) in Bandung City, West Java Province, Indonesia. 15 aquariums with a size of 40 cm × 25 cm × 28 cm were used as containers. Carp fingerlings were kept in an aquarium with a density of 10 fish/aquarium. The fish were fed potassium diformate for 35 days. The feeding rate is 3% from biomass, at 08.00 and at 16.00 Western Indonesian Time. The method used is experimental using Completely Randomized Design (CRD) with 5 treatments and 3 replications. The treatments are: Treatment A: control (without addition of potassium diformate), Treatment B: addition of 0.2% potassium diformate, Treatment C: addition of 0.3% potassium diformate, Treatment D: addition of 0.4% potassium diformate, and Treatment E: addition of 0.5% potassium diformate.

### 2.1 Mixing of Potassium Diformate with Feed

The feed used is commercial feed in the form of floating pellets with a crude protein content of 35%. The feed was weighed as much as 100 grams and mixed with potassium diformate according to the treatment, namely 0.2%, 0.3%, 0.4%, and 0.5%. Potassium diformate was added into the commercial feed then stirred for 5 minutes and then mixed with water as binders (10% from total feed used) and aerated until dry.

### 2.2 Culturing of *Aeromonas hydrophila* Bacteria Isolates

*Aeromonas hydrophila* isolate was inoculated on tryptic soy agar media and then incubated at $30^{o}C$ for 24 hours. Bacteria were harvested using an ose needle and dissolved in tryptic soy broth media and then incubated using a shaker incubator at $37^{o}C$ for 24 hours. The cultured bacteria then inserted into the cuvette as much as 2 ml to calculate its density using a spectrophotometer with a wavelength of 540 nm to obtain a density of $10^{8}$ CFU/ml.

### 2.3 Challenge Test

Common carp fingerlings were challenged by *A. hydrophila* using intraperitoneal injection with 0.1 ml/fish with a density of $10^{8}$ CFU/ml. And then observed for 14 days.

### 2.4 Observation of Blood Cells

Blood cells were observed six times, namely before treatment, after administration of potassium diformate, and after day 3, day 7, day 10, and day 14 after challenge. Preparing white blood cells using Turk reagen and red blood cells using Hayem reagen. Both blood were dripped on a hemocytometer to be observed under a microscope.

## 2.5 Data Analysis

Data on total leukocyte count and total erythrocyte count were analyzed using ANOVA and DMRT at a 95% confidence level, while the gross clinical symptoms was analyzed descriptively.

## 3. RESULTS AND DISCUSSION

### 3.1 Leukocytes

Leukocytes are components of blood cells that function as non-specific defenses that play a role in the fish's immune system. Changes in the number of leukocytes can be used as an indicator of disease in fish because the body will produce more leukocytes when antigens enter the body [9]. The following is the average number of leukocytes in common carp during the study (Fig. 1).

The graph (Fig. 1) shows the average number of leukocytes after adding potassium diformate to the feed with different doses, leukocytes increased by 7.72 – 8.52 × $10^4$ cells/$mm^3$, the increase in the number of leukocytes appeared because KDF can increase leukocytes count. Treatment C (0.3% KDF) experienced the highest increase by 22.95% with a value of 8.52 × $10^4$ cells/$mm^3$. The lowest was shown in Treatment A (without KDF) of 1.25% with a value of 6.65 × $10^4$ cells/$mm^3$. This result shows that the KDF addition to feed at a dose of 0.3% can induce the fish immune system.

The leukocytes count on the 3rd day after challenged showed an increase in the number of leukocytes between treatments. The highest increase occurred in treatment A (without KDF) of 22.70% with a value of 8.06 × $10^4$ cells/$mm^3$. The highest changes in leukocyte count indicated that the fish had started infected by

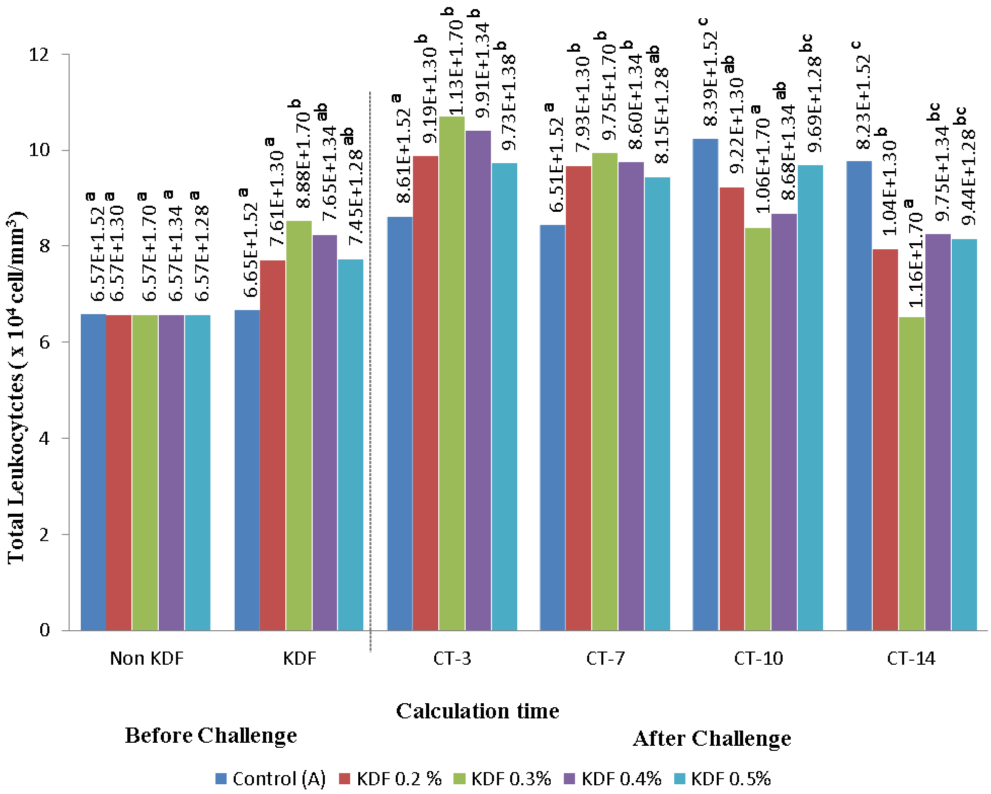


**Fig. 1. Total Leukocytes Count**

*Description:Nonn-KDF: before treatment KDF, KDF: KDF 35 days, CT-3: Third day after challenge, CT-7: Seventh after challenge, CT-10: Tenth days after challenge, CT-14: Fourteenth day after challenge*

*A. hydrophila*. Increases in the number of leukocytes are due to a defense mechanism to infection. These leukocytes performed to phagocytize bacteria to inhibit their growth in the fish's tissue [10]. Infected fish will produce more leukocytes to maintain their immune status. The number of leukocytes with the lowest increase was on treatment C (0.3% KDF) with a value of 10.6 × $10^4$ cells/$mm^3$ (20.21%). This shows that with the addition of 0.3% KDF the fish have a better immune response.

The number of leukocytes on the day 14 after challenge of all treatments decreased. The decrease in the number of leukocytes in the Majalaya carp is the initial phase of healing. The lowest decrease in treatment A (without KDF) was 4.9% with a value of 9.75 × $10^4$ cells/$mm^3$ and the highest decrease in treatment C (0.3% KDF) was 28.8% with a value of 6.51 × $10^4$ cells /$mm^3$. Which means in the 0.3% KDF addition fish are tend to normal condition faster than other treatments.

## 3.2 Red Blood Cells (Erythrocytes)

Erythrocytes function in binding oxygen which will then be used in the catabolism process that produces energy. Graph of the average number of red blood cells (Fig. 2).

The number of erythrocytes after KDF addition for 35 days in treatment C (0.3% KDF) undergone the highest increase of 20.55% with a value of 1.63 × $10^6$ cells/$mm^3$. While treatment A (without KDF) has the lowest increase of 1.89% with a value of 1.32 × $10^6$ cells/$mm^3$.

The number of erythrocytes after 3 day after challenge decreased in each treatment. This happens because the fish erythrocytes are destroyed due to the presence of *A. hydrophila* in the fish's body the presence of these bacteria produces exotoxins and endotoxins that cause a decrease in red blood cells. An increase in the number of erythrocytes indicates an effort from homeostasis in the infected fish body. The body will produce more blood cells to replace the number of lysed erythrocytes. Hemolysin enzymes (exotoxins) from A. hydrophila can lyse erythrocytes so that the number of erythrocytes in blood vessels is reduced [12]. Red blood cells are hemoglobin which works as a carrier of oxygen throughout the body. A low number of erythrocytes will damage the oxygen intake resulted in low metabolism [13]. Low erythrocytes count indicate anemia, which is indicate the fish is in a state of stress [14]. Treatment A (without KDF) experienced the highest decrease in erythrocytes count, namely 50.6% with an average number of erythrocytes 0.88 × $10^6$ cells/$mm^3$, while the lowest percentage decrease was in treatment C (0.3% KDF) of 17% with an average value of 1.39 × $10^6$ cells/$mm^3$. This is due to the presence of hemolysin that cause a decline in erythrocytes.

The number of erythrocytes on day 10 and day 14 after the challenge was in all treatments increased. This happens because of the fish's recovery period to produce cells that have been infected. The highest number of erythrocytes is in C treatment (KDF 0.3%) on the day 10 after challenge with a value of 9.77% and 11.65% on day 14 after challenge. The increased number of erythrocytes indicates a homeostasis attempt in the infected fish.

## 3.3 Macroscopic Clinical Symptoms

Observations of clinical symptoms in the common carp fingerlings were observed after challenge test using *A. hydrophila*. Symptoms observed included morphological damage, response to feed, and fish response to shock.

Symptoms that appeared in each treatment were uneven (Table 1) because each common carp had different immune status. Body surface damage and recovery period for each fish is different because each individual has a different resistance which is determined by age, sex, nutritional status, and stress [15]. Treatment C (0.3% KDF) showed better morphological resistance and a faster recovery period. Treatment A had more morphological damage than other treatments because there was no addition of KDF in the feed so the common carp only got a low immunostimulant effect. *A. hydrophila* is a bacterium that grows in the blood vessels, causing bleeding and swelling symptoms such as ulcers.

Fish that are seriously injured resulted in mortality. Infected fish will experience anemia and necrosis or ulcers on infected organs caused by the hemolysin enzyme in *A. hydrophila* [16].

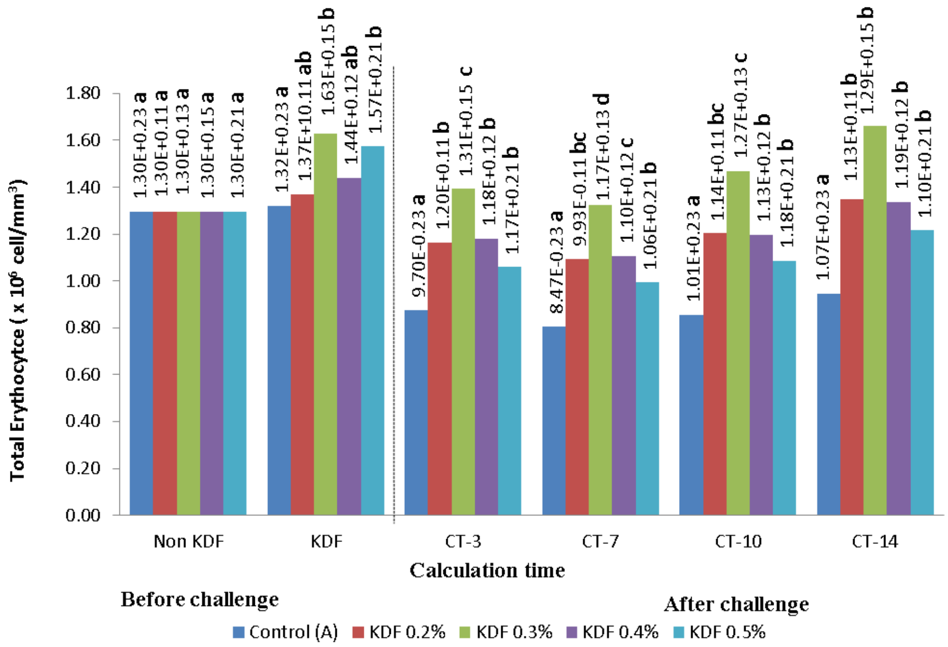


**Fig. 2. Total Erythrocytes Count**

*Description:Nonn-KDF: before treatment KDF, KDF: KDF 35 days, CT-3: Third day after challenge, CT-7: Seventh after challenge, CT-10: Tenth days after challenge, CT-14: Fourteenth day after challenge*

**Table 1. Body Surface Damage for 14 days**

| Treatment | Test | Day to- | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| A | 1 | ab | abc | abcd | abcd | abcd | abc | bc | ac | acd | ac | ad | d | d | c |
| | 2 | ab | abcd | abcd | abcd | abc | abc | cd | c | acd | ac | d | d | d | cd |
| | 3 | ab | abc | abcd | abcd | abcd | abcd | bc | bc | acd | ac | d | d | d | c |
| B | 1 | ab | abc | abcd | acd | abcd | ab | cd | bc | ac | ac | d | d | d | cd |
| | 2 | ab | abc | abcd | abcd | bc | bc | ac | ac | cd | ac | d | d | d | d |
| | 3 | ab | abc | abcd | abcd | bc | ab | bc | ac | ac | ac | d | - | - | - |
| C | 1 | ab | abc | ab | abc | abc | abc | bc | c | cd | cd | d | d | d | d |
| | 2 | ab | abc | ac | bc | bc | bc | c | c | c | ac | d | d | - | - |
| | 3 | ab | abc | abc | ab | ab | ab | cd | cd | cd | d | d | - | - | - |
| D | 1 | ab | abc | abcd | abcd | abc | abc | d | c | cd | d | d | d | d | d |
| | 2 | ab | abc | abcd | acd | bc | bc | bc | cd | cd | ac | d | - | - | - |
| | 3 | ab | abc | abcd | abc | bc | bc | bc | bc | c | ac | d | d | d | - |
| E | 1 | ab | abc | abcd | abc | bc | abc | bc | bc | cd | cd | d | d | d | - |
| | 2 | ab | abc | abcd | abc | abc | ab | bc | bc | cd | ac | ad | d | d | d |
| | 3 | ab | abc | abcd | abc | ab | abc | bc | bc | c | cd | bd | d | d | d |

*Description: (a) scales loss (b) haemorrhage (c) dropsy (d) ulcers*

Treatment E (0.5% KDF) is not effective because the salt contained in KDF can reduce palatability caused by acidifier so that the ability to release H+ is low, so it will inhibit the work of enzymes in the digestive tract [17]. This will cause the energy used mainly to dispose KDF excess in digestive tract than to resist infection.

Observations on the response to feeding that had been challenged by *A. hydrophila* showed a decrease in each treatment (Table 2). Disrupted fish metabolism induces a decrease in feed response. This decrease is caused by internal organ abnormalities such as swelling or inflammation of the liver, kidneys, and bile after infection [18].

Treatments C (0.3% KDF) and D (0.4% KDF) showed the best feed response compared to treatments A (without KDF), B (0.2% KDF), and E (0.5% KDF) which had a lower response to feed. Treatment A had a lower feed response because of the large amount of leftover feed and decreased appetite response. The decreased feed response was caused by infection with *A. hydrophila*. Low erythrocytes will cause the metabolic rate to decrease and resulted in poor energy production. This will cause the fish to become weak and have no appetite and swimming in the bottom or hovering below the water surface.

On the day 7 after injection, the feed response began to improve for treatments C and D, while for treatments A, B, and E on day 9. The addition of KDF in optimum dose gave a better response to feed, this was due to the acidifier contained in KDF helping in boosting the fish's immune system so that the feed response will return to normal faster.

The fish shock response was carried out by knocking on the aquarium for each treatment. The results of post-challenge observations showed various responses to each treatment (Table 3). Shock response on day 1 was low for all treatments. Fish infected with *A. hydrophila* will experience a decrease in a swimming motion and tend to swim on the water surface [19]. This is due to the metabolism and fins damage so that the swimming movement is not stable.

Carp fingerlings shock response in treatment C (0.3% KDF) was normal on day 7, while treatment A (without KDF) on day 11. Stated that *A. hydrophila* infection caused stress in fish, swimming around aeration, and in general, the fish swam sideways due to reduced body balance [20].

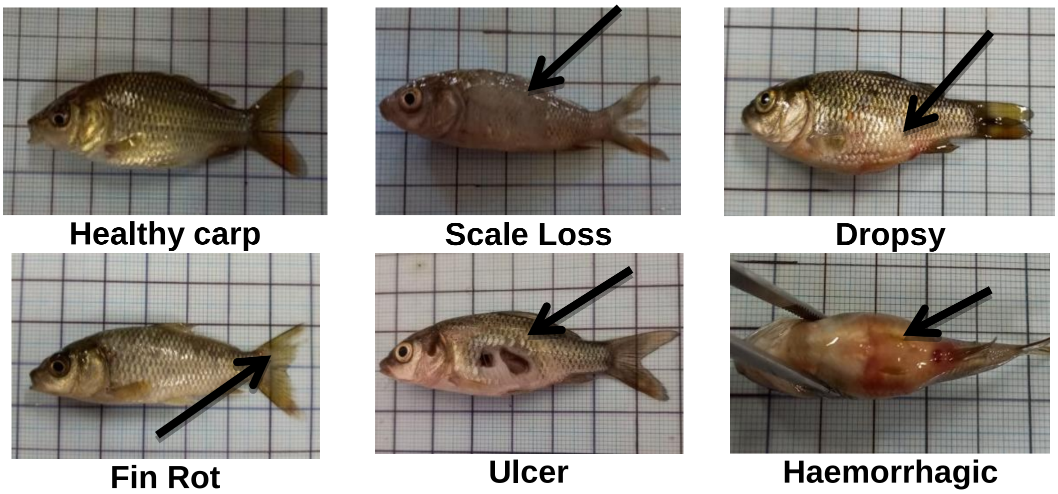


**Fig. 3. Clinical Symptoms in Carp Challenged by *A. hydrophila***

**Table 2. Feed Response**

| Treatment | Test | Day to- | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| A | 1 | ++ | + | + | + | + | + | ++ | + | ++ | ++ | ++ | ++ | ++ | +++ |
| | 2 | + | + | + | + | + | + | + | + | ++ | ++ | ++ | ++ | ++ | +++ |
| | 3 | + | + | ++ | + | + | ++ | ++ | ++ | +++ | ++ | ++ | +++ | +++ | +++ |
| B | 1 | + | + | + | + | ++ | ++ | ++ | ++ | +++ | +++ | +++ | +++ | +++ | +++ |
| | 2 | ++ | + | + | + | + | ++ | ++ | + | +++ | +++ | +++ | +++ | +++ | +++ |
| | 3 | + | + | ++ | + | + | + | ++ | + | ++ | +++ | +++ | +++ | +++ | +++ |
| C | 1 | ++ | + | + | ++ | ++ | ++ | ++ | ++ | +++ | +++ | +++ | +++ | +++ | +++ |
| | 2 | + | + | + | +++ | ++ | ++ | +++ | +++ | ++ | +++ | +++ | +++ | +++ | +++ |
| | 3 | ++ | + | ++ | ++ | ++ | ++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| D | 1 | ++ | ++ | ++ | + | ++ | ++ | ++ | ++ | +++ | +++ | ++ | +++ | +++ | +++ |
| | 2 | ++ | ++ | ++ | ++ | ++ | ++ | +++ | ++ | ++ | +++ | +++ | +++ | +++ | +++ |
| | 3 | + | ++ | + | + | + | + | +++ | + | ++ | ++ | +++ | +++ | +++ | +++ |
| E | 1 | + | + | + | + | + | + | ++ | + | ++ | +++ | +++ | +++ | +++ | ++ |
| | 2 | + | + | + | + | + | ++ | ++ | ++ | ++ | +++ | +++ | +++ | +++ | +++ |
| | 3 | + | + | + | ++ | + | + | ++ | + | ++ | +++ | +++ | +++ | +++ | +++ |

*Description : (+) Low feed response; (++) Moderate feed response; (+++) Normal feed response*

**Table 3. Shock Response**

| Treatment | Test | Day to- | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| A | 1 | + | + | + | + | + | + | + | + | + | + | + + | + + | + + | + + |
| | 2 | + | + | + | + | + | + | + | + + | + + | + + | + + | + + | + + | + + |
| | 3 | + | + | + | + | + | + | + | + + | + + | + + | + + | + + | + + | + + |
| B | 1 | + | + | + | + | + | + | + | + + | + + | + + | + + | + + | + + | + + |
| | 2 | + | + | + | + | + + | + | + | + + | + + | + + | + + | + + | + + | + + |
| | 3 | + | + | + | + | + | + | + | + + | + + | + + | + + | + + | + + | + + |
| C | 1 | + | + | + | + | + + | + + | + + | + + | + + | + + | + + | + + | + + | + + |
| | 2 | + | + | + | + | + | + | + + | + + | + + | + + | + + | + + | + + | + + |
| | 3 | + | + | + | + | + + | + + | + + | + + | + + | + + | + + | + + | + + | + + |
| D | 1 | + | + | + | + | + | + | + | + + | + + | + + | + + | + + | + + | + + |
| | 2 | + | + | + | + | + | + + | + + | + + | + + | + + | + + | + + | + + | + + |
| | 3 | + | + | + | + | + | + | + | + + | + + | + + | + + | + + | + + | + + |
| E | 1 | + | + | + | + | + | + | + + | + + | + + | + + | + + | + + | + + | + + |
| | 2 | + | + | + | + | + + | + | + | + + | + + | + + | + + | + + | + + | + + |
| | 3 | + | + | + | + | + + | + | + | + + | + + | + + | + + | + + | + + | + + |

*Description : (+) Low shock response; (++) Normal shock response*

## 4. CONCLUSION

The optimum dose of potassium diformate addition is 0.3% to increase the immune system of common carp (*Cyprinus carpio* L.) as seen from leukocytes and erythrocytes profiles, lesser clinical symptoms after challenged by *A. hydrophila*.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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