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Supplementation of Trace Mineral in Dry Feed Potential as Immunomodulator Against *Aeromonas hydrophila* Infection of African Catfish

(PENGIMBUHAN *TRACE MINERAL* DALAM PAKAN KERING BERPOTENSI SEBAGAI IMUNOMODULATOR TERHADAP INFEKSI *AEROMONAS HYDROPHILA* PADA IKAN LELE DUMBO)

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ABSTRACT

This study explored the potential trace mineral in increasing the immune response of juvenile African catfish (*Clarias gariepinus*) against the infection caused by *Aeromonas hydrophila*, which is highly prevalent in aquaculture. The experiment involved five groups that were given different treatments, which consisted of A (negative control), B (positive control), C (2 g/kg of trace mineral in feed), D (4 g/kg of trace mineral in feed), and E (6 g/kg of trace mineral in feed), with each treatment repeated thrice. The experimental fish underwent three days of acclimation before the experimental period, followed by two weeks of treatments in each respective group, and a challenge test by *A. hydrophila* injection given post-supplementation accompanied by observation, which lasted for a week. The final observation was made on day 8 post-infection, with significant findings revealed in the results. The results showed the survival rate (%) and total leukocyte counts (TLC) of experimental African catfish treated with trace mineral supplementation (groups C, D and E) were remarkably higher than the positive control (group B) after infected by *A. hydrophila* ($P<0.05$). Furthermore, groups C and D showed increased lymphocyte and monocyte percentages compared to other treatments ($P<0.05$). These results indicate that trace mineral supplementation has the potential to be an immunomodulator through its inclusion within the diet of juvenile catfish and its promising effect in boosting their immunity against infection.

Keywords: *Aeromonas hydrophila*; *Clarias gariepinus*; *fish immune system; trace mineral; dry feed.*

ABSTRAK

Studi ini dilakukan dengan tujuan untuk mengeksplorasi potensi *trace mineral* dalam meningkatkan respons imun benih ikan lele dumbo (*Clarias gariepinus*) terhadap infeksi bakteri *Aeromonas hydrophila* yang diketahui banyak terjadi dalam dunia budidaya perikanan. Percobaan terdiri atas lima kelompok yang diberikan perlakuan berbeda, yaitu: A (kontrol negatif), B (kontrol positif), C (2 g/kg *trace mineral* dalam pakan), D (4 g/kg *trace mineral* dalam pakan), dan E (6 g/kg *trace mineral* dalam pakan) dengan tiga kali ulangan. Ikan lele dumbo percobaan menjalani aklimatisasi selama tiga hari sebelum masa percobaan, dilanjutkan dengan perlakuan selama dua minggu pada masing-masing kelompok dan uji tantang terhadap bakteri *Aeromonas hydrophila* pascasuplementasi disertai observasi yang berlangsung selama seminggu. Pengamatan akhir dilakukan pada hari ke-8 pascainfeksi dengan temuan signifikan terungkap pada hasilnya. Hasil penelitian menunjukkan tingkat kelangsungan hidup (%) dan jumlah leukosit total (TLC) ikan lele dumbo percobaan yang diberi suplementasi *trace mineral* (kelompok C, D dan E) jauh lebih tinggi dibandingkan kontrol positif (kelompok B) pascainfeksi *A. hydrophila* ($P<0.05$). Selanjutnya, kelompok C dan D menunjukkan peningkatan persentase limfosit dan monosit dibandingkan perlakuan lain ($P<0.05$). Hasil ini menunjukkan bahwa suplementasi *trace mineral* berpotensi menjadi imunomodulator melalui aplikasinya ke dalam pakan kering ikan lele dan efeknya yang menjanjikan dalam meningkatkan kekebalan mereka terhadap infeksi.

Kata-kata kunci: *Aeromonas hydrophila; Clarias gariepinus;* pakan kering; sistem imun ikan; *trace mineral.*

INTRODUCTION

African catfish (*Clarias gariepinus*) is one of the most farmed fish in Indonesia. For a while, this fish has been produced many times by people for consumption and was commonly sold across local restaurants. Its meat is consumed because of its beneficial compositions, which contain high nutritional values such as protein, calcium, and phosphor, which strengthens bones accompanied by its fairly low amount of saturated fat contained, allowing it to be consumed in larger amounts while having it accessible in a less costly manner. For such a reason, African catfish has been one of the most important commodities that still needs to be produced more, considering its role as an alternative to other options that may have been more expensive while still being able to fulfill the nutritional needs of people in Indonesia (Nugroho *et al*., 2016).

Research Data of Maritime Affairs and Fisheries in 2022, which was released by the Ministry of Maritime Affairs and Fisheries Republic of Indonesia (KKP RI), has stated that the production of catfish has reached about 347.989 tons within 2021 and increased by around 11.627 tons in the year 2022 which amounts to 359.616 tons of catfish with the increase of the catfish production achieved around 3.34% more than the production during 2021. Intending to increase food production, the stock request for this fish has increased even more over the years. The government has made numerous attempts to increase catfish production in larger amounts while successfully achieving it within shorter periods without unnecessarily large expenses.

These attempts have been made to fulfill people's need for food through a rather intensive farming system, where fish are produced in large amounts quickly

(Adriansyah *et al*., 2020). Farmers have commonly used this system to produce fish in larger amounts while still being able to do it within smaller plots of land, considering that not all of them may have access to larger lands. With the hopes that catfish production will increase, these catfishes have been raised within rather highly populated areas and fed in higher quantities to boost their growth. However, it is still known that this system may hold the risk of causing problems such as organic pollution, oxygen deficiency, and an increase in CO_2 and ammonia-nitrogen, which are more likely to affect the growth of fish because of the lack of control toward maintaining environmental conditions (Sultana *et al.*, 2017).

Furthermore, other factors may affect fish farming externally, such as extreme temperatures, weather changes, and the transportation of fish, which hold the risk of bringing in foreign pathogens from outside of the aquatic environment. These problems may occur from outside or inside of the environment, which can affect the replication and proliferation rate of pathogens, causing them to be distributed within the water and resulting in many infectious problems occurring within the catfish population (Huicab-Pech *et al*., 2016). These problems are more likely to happen when the actions needed to maintain biosecurity are hardly implemented within the farming system, which will cause the catfish to be more prone to infections caused by pathogens in the water. These pathogens may include *Aeromonas sp., Staphylococcus sp., Streptococcus spp., Escherichia coli, Salmonella typhi, Pseudomonas aeruginosa, Vibrio cholera,* and *Shigella dysenteriae* where these pathogens have been known to be causing severe damages in fish (Hardi *et al*., 2018).

The increase in the pathogenicity of infectious microorganisms within aquatic environments may also be caused by the lack of nutrition given to the catfish, followed by higher amounts of feed given. This results in the degradation of water quality within the farm, causing the fish to suffer from stresses that result in declining health, making them more sensitive toward infectious pathogens (Ina-Salwany *et al*., 2019). To prevent such a thing, action needs to be taken to increase the quality of the feed used within the production of catfish without having to increase the quantities of the feed given to maintain the balance of its environment while still being able to efficiently give enough if not more amounts of nutrients to the catfish. In its production, some attempts have been made to increase feed quality by adding things such as leftovers of peas and vegetables and herbal extracts, even to the point of using probiotics in feed fermentation and supplementing fish feed with natural minerals to increase its nutritional values (Subramaniam *et al.*, 2019).

As one of the options, trace minerals are widely known to hold important roles in the growth and development of animals, including those who live in aquatic environments. Supplementing these minerals within animal feeds could potentially improve the natural immune function within the body, causing them to be more resistant to diseases. So far, supplementing trace minerals within feeds has been done for quite a while, especially in animal productions such as livestock, poultry, and even aquatic animals like shrimps and other freshwater fishes, including catfish (Chanda *et al.,* 2015). In an attempt to improve the immune system of the fish raised within this intensive farming system, there needs to be more research done to reveal more of these potentials held by trace minerals as an immunomodulator through its supplementations within fish feeds that are to be given to catfishes tested against infectious pathogens.

Table 1. Average survival rate (SR) (%) of the experimental fish

Experimental groups	No	Ni	Survival Rate (Mean±SD)
A (Negative control)	15	14	93.33±0.12^{c}
B (Positive control)	15	3	20.00±0.20^{a}
C (2 g/kg of trace mineral in feed)	15	9	60.00±0.35^{abc}
D (4 g/kg of trace mineral in feed)	15	10	66.67±0.31^{bc}
E (6 g/kg of trace mineral in feed)	15	4	26.67±0.12^{ab}

Different superscript letters in the same column indicated significant differences ($P<0.05$). No=Number of experimental fish at the beginning of the study period. Ni=Number of experimental fish at the end of the study period. SD=standard deviation.

Table 2. Average total leukocyte count (TLC) of the experimental fish

Experimental groups	Total Leukocyte Count (10^3 cell/µL) (Mean±SD)
A (Negative control)	12.77±0.08^{a}
B (Positive control)	22.38±1.97^{b}
C (2 g/kg of trace mineral in feed)	39.48±2.63^{c}
D (4 g/kg of trace mineral in feed)	56.58±0.78^{e}
E (6 g/kg of trace mineral in feed)	48.45±0.23^{d}

Different superscript letters in the same column indicated significant differences ($P<0.05$). SD=standard deviation.

One such pathogen is *Aeromonas hydrophila,* a rather opportunistic pathogen that spreads widely within aquatic environments and has been known as one of the most common causes of infection in fish. Infections caused by *Aeromonas hydrophila* are commonly known to make fish suffer from acute septicemia that could cause physical damage to fish both externally and internally and potentially result in death, which could happen sooner or later (Musthafa *et al*., 2016). Based on this recorded information, the study was aimed to explore what these trace minerals could do to potentially stave off these dangerous pathogens if mixed with fish feeds.

RESEARCH METHODS

Study Period and Location

Preparation of samples through feeding treatment to each catfish was carried out in a local farmer pond located in Taman Buaran Indah I, Klender, Duren Sawit, Jakarta Timur, DKI Jakarta. The observation and counting of total leukocytes and differential leukocytes were performed in The Microbiology Laboratory, Faculty of Medicine, Universitas Padjadjaran. Overall, this experiment occurred for around a month, which included these phases. The acclimation phase lasts for around three days to allow the experimental fish to adapt to the change in water temperature before being given treatments (Grenchik *et al.*, 2013). The feeding phase lasted for around two weeks to feed the experimental fish according to the respective treatments (Sherif and Mahfouz, 2019). In the inoculation phase, the fish will be inoculated with a strain of *Aeromonas hydrophila* according to each treatment, followed by observation for a full week (Mulia *et al.*, 2022). Data collection was conducted on day 8 post-infection. During

this time, the data regarding the survival rate of the experimental fish and their leukocyte profiles were collected.

Sample

This research was performed quantitatively through an experimental method in five groups, with each group given different treatments and repeated three times. The juvenile catfish belong to strain variant of *Clarias gariepinus,* which originates from Indonesia was used in this study. It is known as Sangkuriang catfish, and its sizes range from 17 to 18 cm. The percentage of survival rate (SR), total leukocyte count (TLC), and differential leukocyte count (DLC) were collected as the data in the current study (Manna *et al.*, 2021).

Experimental Design

The experimental fish were divided into five groups repeated three times, with each having different treatments, which consisted of: A) Negative control, 0 g/kg of trace mineral in feed (AZOMITE®, Nephi, Utah, United States), injected with Brain Heart Infusion Broth (BHIB) sterile as placebo; B) Positive control, 0 g/kg of trace mineral in feed (AZOMITE®), injected with *Aeromonas hydrophila;* C) 2 g/kg of trace mineral in feed (AZOMITE®), injected with *A. hydrophila*; D) 4 g/kg of trace mineral in feed (AZOMITE®), injected with *A. hydrophila;* E) 6 g/kg of trace mineral in feed (AZOMITE®), injected with *A. hydrophila.*

Feed Preparation

According to the treatments for each group, the fish feed was prepared using commercial dry feed with the size of 2 mm, mixed with tapioca starch, which functions as a binding agent and trace mineral (AZOMITE®). For groups A and B, which function as the control treatments, the fish feed was mixed only with 50 g/kg of tapioca to avoid the difference in the result caused by other factors (Azam *et al.*, 2016). Meanwhile, for groups C, D and E, the fish feed was mixed with 50 g/kg tapioca starch added with 2, 4, and 6 g/kg dosages of trace minerals in the feed added within each treatment, respectively.

Maintenance of the Experimental Fish Water Environment

The experimental fish were nurtured within a fish bucket with a water capacity of 80 liters, which also came in sets with an aerator. Within each bucket, around 15 experimental fish with sizes ranging from 17–18 cm were put inside, where these fishes are going to be left to acclimatize for around three days without being given any feed (Grenchik *et al.*, 2013). After going through acclimatization, the experimental fish were given feed according to each treatment that had been decided before, allowing them to grow for a full two weeks (Sherif and Mahfouz, 2019). For each day, the experimental fish were fed twice (Ani *et al.*, 2013). The amount of feed given was equal to 4.5% of the total body weight measured from experimental fish within each bucket (Batool *et al*., 2018).

Water quality parameters such as pH, nitrate (NO_3), ammonia (NH_3), dissolved oxygen (DO), salinity, and temperature were measured periodically before and after water changing (Verma *et al*., 2022). In general, the experimental fish were raised within these conditions: water temperature with the range of 22–33°C, dissolved oxygen with the concentration of 3–6 ppm, and measurement of pH ranging from 6.5–8.5. Water change was performed around 20–30% once every two days within each bucket consistently.

Challenge Test

The experimental fish were injected intracoelomic (IC) with *A. hydrophila* after two weeks of feeding treatments, which was cultured in the brain heart infusion broth

(BHIB) media with a concentration of 9.7 ×10^8 CFU/mL in groups B, C, D and E with each fish was injected by 0.5 mL each fish. Group A was injected with a sterile BHIB with the same amount of 0.5 mL for each fish for a placebo (Dwinanti *et al.*, 2021).

Survival Rate (%)

Survival rates were observed eight days post-infection. The data collected consists of the number of experimental fish left alive on day 8 and its comparison toward its initial numbers before infection. The counting of the survival rate was performed according to this formula: (No) was the number of experimental fish at the beginning of the study period and (Ni) was the number of experimental fish at the end of the study period (Sultana *et al.*, 2018): Survival Rate (SR) Percentage(%)= (Ni x No^{-1}) x 100%.

Blood Sampling

The blood samples were drawn from the experimental fish via caudal venipuncture. The fish were held using a damp towel to restrain the catfish while the needle was drawn to the blood sample through caudal venipuncture (Pollard *et al*., 2022).

Total Leukocyte Count (TLC)

The total leukocytes were performed using the Neubauer hemocytometer chamber using a reagent that consists of 0.65% NaCl mixed with 1% crystal violet to color all the blood cells while avoiding causing lysis to all the blood cells. The total leukocytes are going to be counted using this formula (Rizkiantino, 2023):

Total Leukocyte Count (TLC) (cell/µL of blood) = N × 50

Differential Leukocyte Count (DLC)

The differential leukocyte count was carried out by making blood smear preparations fixed using 98% methanol for 15 minutes, dried, and then colored using Giemsa for around 30 minutes. Under the microscope with an objective lens of 100× , each type of leukocyte found was counted using this formula (Rizkiantino, 2023): Type of Leukocyte (%)= [(Total Leukocyte Type) x (100 Leukocyte Cells)$^{-1}$] x 100%

Data Analysis

The SR, TLC and DLC data were analyzed using an analysis of variance with a 95% confidence interval. If there was data that was significantly different, the data was continued with the Duncan multiple range test (α = 5%).

RESULTS AND DISCUSSION

Group A had the highest percentage of survival rate, with 93.33 ± 0.12% after one week of observation. Meanwhile, group B had the lowest percentage of survival rate, with 20.00 ± 0.20% of the population left after one week. Groups C, D and E had a survival rate of 60.00 ± 0.35%, 66.67 ± 0.31%, and 26.67 ± 0.12%, respectively (Table 1).

Between groups A and B as the negative and positive control, respectively, catfish that were given treatment A had a significantly higher survival rate ($P<0.05$) because it was not being injected with *A. hydrophila;* instead, it was injected with a sterile BHIB. As a result, the experimental fish in group A was supposed to be a healthy fish, which the high survival rate could indicate compared to group B. With this, it could be observed that all the deaths of the juvenile catfish were caused by the *A. hydrophila* injected into the fish while removing other factors that may have been able to cause some biases (Arnold *et al.*, 2016).

Between group B, which was given control feed without trace mineral supplementation and groups C, D and E, which were given trace mineral

supplementation, it could be seen that group D had the highest SR of all those treatments. This could be an indicator that the trace mineral could affect the survival rate of catfish. This occurs because trace minerals can improve the functions of the catfish's immune system. The increase of the activities within the catfish's immune system caused the catfish to be more resilient to an infection caused by *A. hydrophila*. As a result, catfish are less likely to die from infection (Xu *et al.,* 2021).

However, it could be observed that the same case did not happen when the dose of the trace mineral supplement in fish feed increased to 6 g/kg of feed. Of all the groups that were treated with trace mineral supplementation, group E, with the highest dose of trace mineral added, had the lowest SR of them all. This could potentially happen because even if the trace mineral had the potential to stimulate the immune system, within higher doses, the trace mineral seems to cause the opposite instead and inhibits the immune function (Sakai, 1999). As a result, the catfish given treatment E had weaker immune systems and were more likely to die due to *A. hydrophila* infection.

The results in Table 2 indicated that the experimental fish treated in group A as negative control had the lowest TLC with the value of (12.77 ± 0.08) × 10^3 cell/µL, followed by group B as positive control with the value TLC reaching (22.38 ± 1.97) × 10^3 cell/µL. Meanwhile, the group that was treated with trace mineral supplementation had a significantly higher value of total leukocyte count, with group C having a TLC of (39.48 ± 2.63) × 10^3 cell/µL, group D having the highest TLC with a value of (56.58 ± 0.78) × 10^3 cell/µL, and group E with TLC of (48.45 ± 0.23) × 10^3 cell/µL.

Based on those results, it could be seen that both groups that were not treated with trace mineral supplementation (A and B) had significantly lower TLC values. Group A, having a relatively low value of TLC, was an indicator that the fish treated within group A was indeed still in a healthy state compared to group B, which was infected with *A. hydrophila* ($P<0.05$). It was shown that the total leukocyte count within the immune system tends to increase when triggered by an invasion of foreign objects, including pathogens, as a way to start forming antibodies and resisting the invasion (Lestari *et al*., 2021).

Between groups B, C, D and E, it could be seen that the group that was given trace mineral supplementations had significantly higher amounts of TLCs, with group D, which was given 4 g/kg of trace mineral in feed having the highest TLC of them all. This indicated that within a suitable dose, the trace mineral supplementation can affect the production of leukocytes optimally while also increasing the phagocytic activities and antibody production, causing the catfish to be more resistant toward *A. hydrophila* infection (Zhang *et al.*, 2023).

One of the possible ways this could happen is that the trace minerals can stimulate T lymphocyte cells, causing them to produce more colony-stimulating factor (CSF) or cytokine in a shorter time. One such cytokine includes interleukin-3 (IL-3), which holds important roles in regulating the growth and differentiation of hematopoietic progenitor cells, which could differentiate into many types of blood cells while also increasing the activities of neutrophils and macrophages. The increase of IL-3 by this stimulation boosted the production of leukocyte cells within the spleen in fish. As a result, the number of leukocytes recruited to stave off an infection increases, improving the function of the catfish immune system to resist *A. hydrophila* (Weyh *et al.*, 2022).

It should be noted that the increase in the dose of trace minerals given in the feed could cause the reduction of TLC. The group of experimental fish that was given treatment E (6 g/kg of trace minerals in feed) had lower

Table 3. Average percentage of differential leukocyte count (DLC) of the experimental fish

Experimental groups	Lymphocytes (%) (Mean±SD)	Monocytes (%) (Mean±SD)	Neutrophils/ Heterophils (%) (Mean±SD)
A (Negative control)	61.33±1.53^{a}	7.00±1.00^{a}	31.67±1.53^{c}
B (Positive control)	63.67±1.53^{a}	7.67±1.15^{ab}	28.66±2.08^{bc}
C (2 g/kg of trace mineral in feed)	71.67±1.15^{c}	9.67±0.58^{bc}	18.66±1.53^{a}
D (4 g/kg of trace mineral in feed)	73.33±0.58^{c}	11.34±1.15^{c}	15.33±0.58^{a}
E (6 g/kg of trace mineral in feed)	66.33±1.53^{b}	8.00±1.73^{ab}	25.67±3.06^{b}

Different superscript letters in the same column indicated significant differences ($P<0.05$). SD=standard deviation.

TLC compared to the group that was given treatment D (4 g/kg of trace minerals in feed). This could happen because, in a higher dose, trace minerals inhibit the function of the immune system, causing them not to be able to work optimally. This happened because the high dose of trace minerals could change the conformation of the protein in the cell membrane, which resulted in a cytotoxic condition that reduced the immunity of catfish (Mashoof and Criscitiello, 2016).

The results in Table 3 showed that group A percentage of lymphocytes was 61.33 ± 1.53%, followed by the lymphocyte percentage in group B being higher at 63.67 ± 1.53%. Meanwhile, the lymphocyte percentage in groups given trace mineral treatment was higher than the ones that were not, with group C having a lymphocyte percentage of 71.67 ± 1.15%, group D having the highest lymphocyte percentage of around 73.33 ± 0.58%, and group E having lymphocyte percentage of 66.33 ± 1.53%.

The proportion of other types of leukocytes, such as monocytes and neutrophils, suits the lymphocytes percentage, with group A having monocyte and neutrophil percentage at consecutively 7.00 ± 1.00% and 31.67 ± 1.53% while group B having monocyte and neutrophil percentage that was not much different at 7.67 ± 1.15% and 28.66 ± 2.08%. The experimental fish within group C had the average percentage of monocyte and neutrophil at 9.67 ± 0.58% and 18.66 ± 1.53%, while group D had the highest percentage of monocyte of all treatments with the monocyte percentage of 11.34 ± 1.15% and 15.33 ± 0.58% of neutrophil, followed by group E which consisted of 8.00 ± 1.73% monocytes and 25.67 ± 3.06% neutrophil. The results indicated that between the groups given treatments A and B, there were not any significant differences between the compositions of lymphocyte, monocyte, and neutrophil except for the lymphocytes in group B, which were higher than the one in group A.

Meanwhile, there existed some differences between group B, which was not given any trace mineral supplementation, and groups C, D and E, which were given trace minerals. Groups C and D were significantly more composed of lymphocytes compared to group B, where the group that was given treatment D had the highest lymphocyte and monocyte percentages within its composition after being tested against *A. hydrophila* ($P<0.05$). This potentially occurs because the trace mineral has the potential to increase the production of lymphocytes that hold an important role in producing antibodies against infection. In contrast, the group with a lower lymphocyte percentage indicates that there is a decline in the concentration

of the antibody produced, followed by an increase in pathogen invasion (Indriani *et al.*, 2020). The increase in monocyte percentage within the groups given treatments C and D compared to other treatments is caused by the immunostimulant effect given by trace minerals to produce phagocytic cells. It improves its bactericidal activities, thus causing bacterial clearance in the fish body to be more efficiently performed (Yang *et al*., 2021).

CONCLUSION

Based on this study, it can be concluded that supplementing trace minerals within fish feed can improve the survival rate of juvenile African catfish, especially when given the dosage of 4 g/kg of trace minerals in feed, which yields the highest survival rate of catfish infected with *A. hydrophila*.

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