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Immunostimulatory Potential of Dietary Citronella (*Cymbopogon nardus*) Powder Combined with Probiotics in Nile Tilapia Infected with *Aeromonas hydrophila*

Auh Misriani, Indriyani Nur, La Ode Baytul Abidin*

Department of Aquaculture, Faculty of Fisheries and Marine Science, Halu Oleo University
HEA Mokodompit Street, Kendari, Indonesia

Correspondence:
baytul.abidin@uho.ac.id

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ABSTRACT

Motile Aeromonas Septicemia (MAS) caused by *Aeromonas hydrophila*, is a major bacterial disease in Nile tilapia aquaculture and result in substantial production losses. The excessive use of antibiotics for disease control has raised concerns regarding antimicrobial resistance and environmental contamination, thereby necessitating safer and more sustainable alternatives. This study evaluated the immunostimulatory effect of dietary supplementation with citronella (*Cymbopogon nardus*) powder combined with probiotics on Nile tilapia challenged with MAS. A completely randomized design was employed using four dietary treatments: a basal diet without supplementation and three experimental diets supplemented with 15 mL of probiotics and citronella powder at doses of 7.5, 10, or 12.5 g per kg of diet (PC-7.5, PC-10, or PC-12.5). Fish were fed the experimental diets for 14 days prior to challenge with *A. hydrophila* (10^7 CFU/mL), followed by a post-challenge recovery period. Recovery rate, survival rate, and hematological parameters were assessed. The result indicated that dietary supplementation, particularly PC-7.5, tended to improve recovery and survival rates, although these differences were not statistically significant ($P > 0.05$). In contrast, hematological parameters were significantly influenced by the dietary treatments ($P < 0.05$), with PC-7.5 diet producing higher hematocrit, hemoglobin, and leukocyte values, as well as faster hematological recovery following infection. These findings suggest that dietary supplementation with 7.5 g of citronella powder per kg of diet combined with 15 mL probiotics effectively enhances immune response and support disease resistance in Nile tilapia, representing a promising and environmentally friendly strategy for MAS control in aquaculture.

INTRODUCTION

Motile Aeromonas Septicemia (MAS) is a bacterial disease that frequently attacks tilapia and causes losses for farmers. This disease, caused by the *Aeromonas hydrophila*, is acute and characterized by clinical symptoms such as hemorrhages, ulcers on the body surface, fin deterioration, and abnormal swimming behavior (Hasmin *et al*., 2025). In addition, internal organs including the gills, intestine, liver, spleen, and kidney undergo degenerative changes, necrosis, and lesion formation (Abdelhamed et al., 2017), which under certain conditions can lead to mass fish mortality (Rini *et al*., 2024). Therefore, controlling this disease is a priority in efforts to increase of tilapia aquaculture production.

MAS disease control is generally achieved using antibiotics, but uncontrolled use can lead to bacterial resistance (Hossain *et al*., 2022; Suyamud *et al*., 2024), antibiotic contamination in water bodies (Thiang *et al*., 2021), and human health problems (Li *et al*., 2021). Therefore, environmentally friendly and safe alternatives for disease control are needed, one of which is the use of immunostimulants. The application of immunostimulants is an effective method for enhancing the immune response of fish, making them less susceptible to disease (Vijayaram *et al*., 2024). Furthermore, the use of immunostimulants can reduce dependence on antibiotics (Kela, 2022). Immunostimulation in fish can be achieved using natural ingredients known as medicinal plants.

Medicinal plants are a group of plants known to contain various active compounds with antibacterial, antifungal, antiparasitic, and immunostimulant properties. Several studies have shown that medicinal plants can enhance the immune response and resistance of aquaculture organisms to bacterial infections (Elumalai *et al*., 2020; Dev *et al*., 2023). One herbal plant with potential as an immunostimulant is citronella (*Cymbopogon nardus*), which contains active compounds such as saponins, flavonoids, alkaloids, phenolics, triterpenoids, and steroids (Mukhlishin *et al*., 2024). Medicinal plants as immunostimulants can be applied in the form of extracts or powders, administered orally through feed. Using powders has the advantage of being more economical than extracts and easy to apply (Armin *et al*., 2023). The use of medicinal plant powders has been reported to produce an even better immune response than the extracts of the medicinal plants (Munaeni *et al*., 2020).

However, the application of medicinal plants in powder form through feed has limitations because plant cell walls are composed of complex carbohydrates such as cellulose and hemicellulose, which affect bioaccessibility and nutrient digestion (Holland *et al*., 2020). Therefore, to improve the degradation of cell wall structures and optimize the absorption of active compounds in citronella, probiotics were also applied in this study. The use of probiotics has been known to improve feed digestibility and nutrient absorption through the production of digestive enzymes capable of breaking down complex carbohydrates (Yi *et al*., 2020). In addition to improving feed digestibility, probiotics also play a role in improving growth performance, immunity, and disease resistance (Calcagnile *et al*., 2024; Fachri *et al*., 2024). Based on this description, this study aims to evaluate the immunostimulating potential of tilapia by providing feed was supplemented with citronella powder and probiotics for the control of MAS disease.

METHODS

Experimental Design

Citronella was obtained from the Field Laboratory of the Faculty of Agriculture, Halu Oleo University. Citronella powder was prepared by sun-drying the stems and leaves, followed by grinding and sieving of the dried plant material to obtain a fine powder. The citronella powder was incorporated into the experimental diets using a repelleting method, with 3% tapioca flour added as a binder.

The probiotic used in this study was a commercial preparation containing *Lactobacillus casei* and *Saccharomyces cerevisiae*. Probiotics were applied by evenly spraying them onto the feed, following the method described by Hasmin *et al*. (2025).

The experiment was conducted using a completely randomized design with four dietary treatments and three replicates per treatments. The dietary treatments were as follows:

BD : Basal diet without probiotic and citronella powder supplementation
PC-7.5 : Diet supplemented with 15 mL of probiotics and 7.5 g of citronella powder per kg of diet
PC-10 : Diet supplemented with 15 mL of probiotics and 10 g of citronella powder per kg of diet
PC-12.5 : Diet supplemented with 15 mL of probiotics and 12.5 g of citronella powder per kg of diet

The doses of probiotics and citronella powder were determined based on Karel *et al*. (2019) and Hasmin *et al*. (2025).

Rearing Facilities

The rearing facilities consisted of 12 aquarium units measuring 35 cm × 35 cm × 40 cm, each equipped with an aeration system. Prior to use, the aquariums were cleaned using a brush and detergent and then thoroughly rinsed with clean water. The aquariums were subsequently filled with water to a depth of approximately 30 cm. The experimental fish were introduced into the aquaria after 24 hours, with five fish stocked in each aquarium.

Fish Rearing and Bacterial Challenge Test

The experimental fish used in this study were Nile tilapia fingerlings weighing 15–20 g, totaling 60 fish, obtained from the Freshwater Fish Seed Center of Rahandouna, Kendari. The fish were reared for 21 days. During the first 14 days, the fish were fed a diet supplemented with probiotics and citronella powder according to the respective treatment doses. The bacterial challenge test using the pathogenic bacterium *A. hydrophila* was conducted on day 14. The experimental fish were injected with *A. hydrophila* at a dose of 0.1 mL per fish, with a bacterial density of 10^7 CFU/mL. From day 15 to day 21, corresponding to the pot-challenge rearing period, the fish were fed a commercial diet. Post-challenge rearing was carried out to observe the recovery rate, survival, and hematological parameters (Figure 1). The *A. hydrophila* origin from the Fish Parasites and Diseases Laboratory, Hasanuddin University.

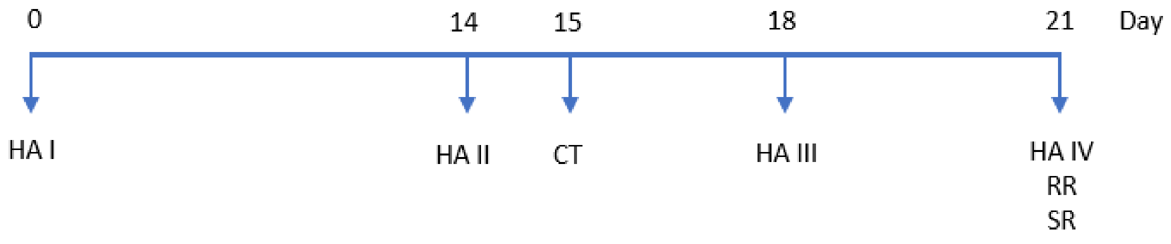

Figure 1. Schedule and Assessment of Experimental Parameters

Where:
HA I : first hematological analysis
HA II : second hematological analysis
HA III : third hematological analysis
HA IV : fourth hematological analysis
CT : challenge test with *A. hydrophila*
RR : recovery rate
SR : survival rate

Observation

Recovery Rate (RR)

RR (%) = (Total recovered fish/Total diseased fish) × 100%

Note:

Recovered fish are defined as individuals showing observable improvement and no longer displaying clinical symptoms (Abidin *et al*., 2022).

Survival Rate (SR)

$$\text{SR (\%)} = \frac{\text{Nt}}{\text{N0}} \times 100\ \%$$

Where:

Nt : Number of fish remaining alive at the end of the rearing period

N0 : Number of fish present at the beginning of the rearing period (Abidin *et al*., 2022)

Hematocrit

Hematocrit (He) was determined using the method described by Anderson *et al*. (1995). Blood samples were introduced into microhematocrit capillary tubes to approximately three-quarters of their length. One end of each tube was sealed with crystoceal to a depth of approximately ±1 mm, after which the tubes were centrifuged at 5,000 rpm for 5 minutes. The length of the settled blood column (a) and the overall length of the blood column in the tube (b) were measured with a ruler, and the hematocrit percentage was calculated using the following formula:

$$\text{He (\%)} = \frac{\text{a}}{\text{b}} \times 100\ \%$$

Hemoglobin

The hemoglobin concentration was determined using the Sahli method (Blaxhall & Daisley, 1973). Initially, blood samples were aspirated using a Sahli pipette up to the 20 mm^3 scale or 0.2 mL, after which the tip of the pipette was wiped clean with tissue paper. The blood was then transferred into an HB meter tube containing 0.1 N HCl up to the 10 marks (red scale). Subsequently, the sample was mixed using a glass stirrer for approximately 3-5 minutes. Distilled water was gradually added until the color of the solution matched the standard reference color in the HB meter. Hemoglobin levels were expressed in g/dL.

Leukocyte Count

The leukocytes or white blood cells (WBC) was performed in accordance with procedure by Blaxhall & Daisley (1973). Blood samples were aspirated into a pipette using a white bead until the 0.5 graduation was attained, followed by the addition of Turk's solution up to the 11 marks. The contents of the pipette were mixed by gently shaking in a figure-eight motion for approximately 3-5 minutes to achieve uniformity. The initial drop was discarded, while the subsequent drop was placed onto a hemocytometer fitted with a cover glass. Leukocyte count was conducted microscopically within four large counting chambers measuring 1 mm × 1 mm. The leukocyte concentration was subsequently calculated using the following formula:

$$\text{WBC} = \frac{\text{n}}{\text{V}} \times \text{P}$$

Where:

WBC : leukocyte concentration per 1 µL of blood (cells/mm^3)

n : Total number of leukocytes counted in four counting chambers

V : volume of the four counting chambers (0.4 mm^3)

P : Dilution factors (20×)

Data Analysis

Data on recovery rate, survival rate, and hematological profiles of the experimental fish were subjected to analysis of variance (ANOVA). When the ANOVA result indicated significant differences among treatments, further comparisons were performed using the Least Significant Difference (LSD) test.

RESULTS

The PC-7.5 treatment tended to result in the highest recovery and survival rates in Nile tilapia affected by MAS diseases. However, variation in dietary citronella powder and probiotic of doses did not have a statistically significant effect on both parameters (P > 0.05) (Figures 2 and 3).

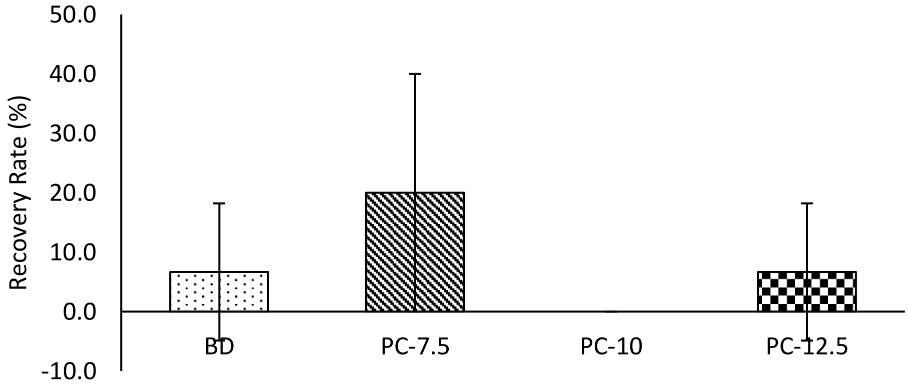

Figure 2. Recovery Rate of Nile Tilapia Following *A. hydrophila* Infection

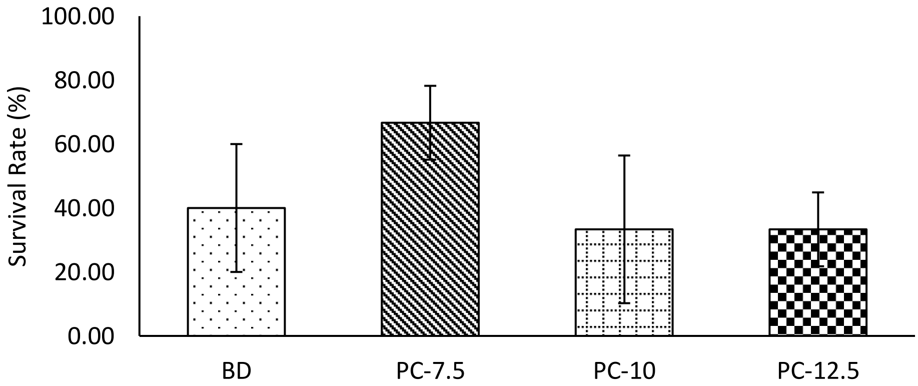

Figure 3. Survival Rate of Nile Tilapia at the End of the Experimental Rearing Period

Dietary supplementation with citronella powder and probiotics significantly affected the hematocrit percentage of fish (P < 0.05). Hematocrit increased on day 14 following supplementation of citronella powder and probiotics, with the highest level observed in the PC-7.5. After the challenge with *A. hydrophila* on day 18, hematocrit decreased in all treatments, but recovery was observed on day 21, especially in the PC-7.5 (Figure 4).

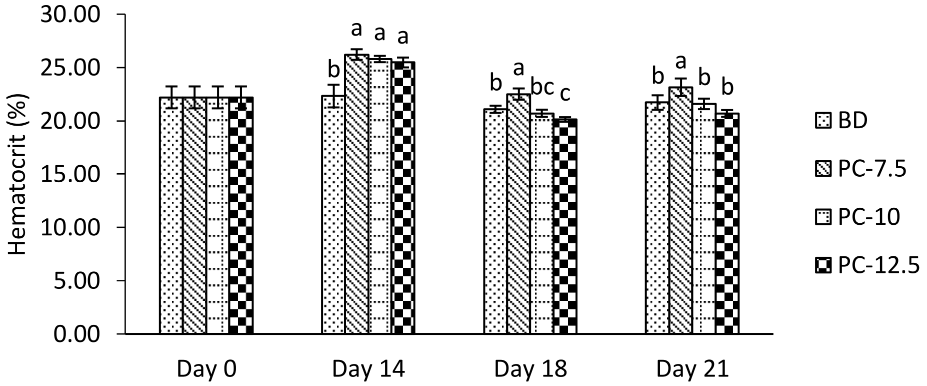

Figure 4. Hematocrit Levels of Nile Tilapia Measured Before and After Challenge with *A. hydrophila*

Dietary supplementation with citronella powder and probiotics increased hemoglobin levels in fish before infection, with the PC-7.5 exhibiting the highest hemoglobin level and a statistically significant difference ($P < 0.05$). Following the challenge with *A. hydrophila*, hemoglobin levels decreased in all treatments and did not differ significantly. However, during the recovery period, hemoglobin levels increased again, particularly in the PC-7.5, which remained significantly higher than the other treatments (Figure 5).

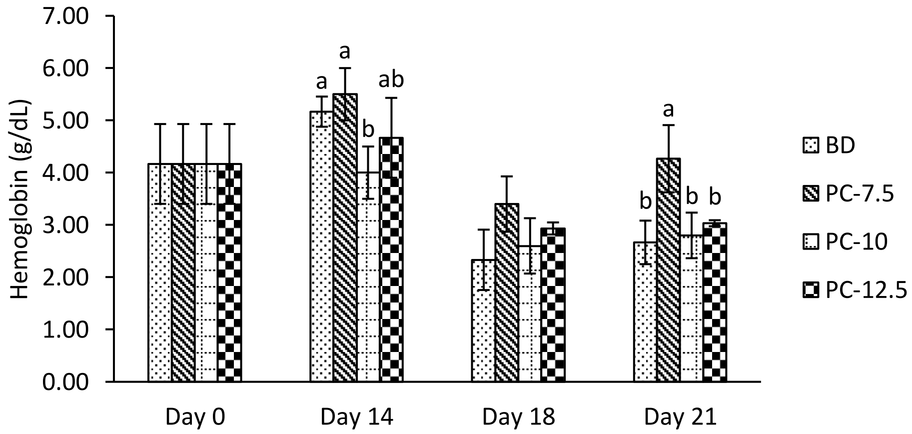

Figure 5. Hemoglobin Levels of Nile Tilapia Measured Before and After Challenge with *A. hydrophila*

Dietary supplementation with citronella powder and probiotics significantly affected the leukocyte count in fish ($P < 0.05$). Leukocyte counts increased before challenge with *A. hydrophila*, with the PC-7.5 exhibiting the highest response, then decreased thereafter. During the recovery phase, leukocyte counts increased again, particularly in the PC-7.5 (Figure 6).

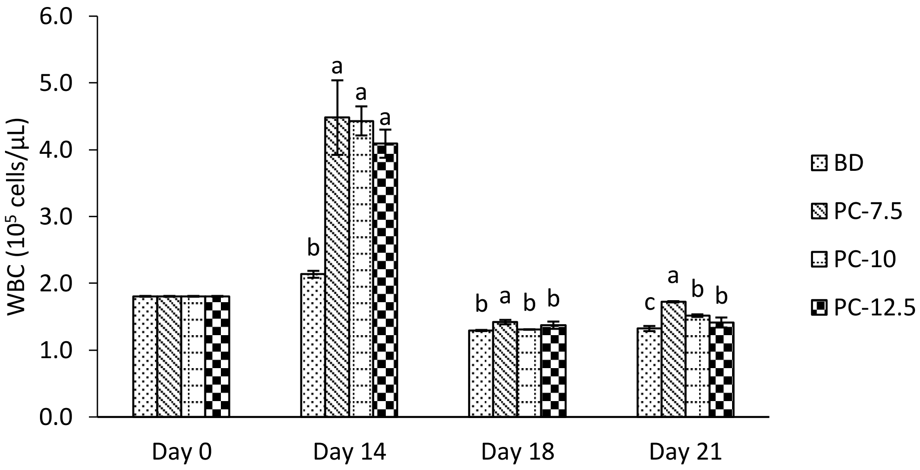

Figure 6. Leukocyte (WBC) Counts of Nile Tilapia Before and After Infection with *A. hydrophila*

DISCUSSION

The recovery rate was defined as the resolution of clinical manifestation following pathogenic infection. In the present study, fish challenged with *A. hydrophila* exhibited typical pathological signs, including abnormal swimming behavior, hemorrhagic lesions, skin ulceration, fin erosion, and mortality. These symptoms are characteristic of motile aeromonad septicemia (MAS) and are consistent with previous descriptions by Hasmin *et al*. (2025). The recovery rate was assessed through visual observation of behavioral recovery and external morphological restoration during the post-challenge period. Initial signs of recovery were observed from day 4 post-challenge, as indicated by the disappearance of hemorrhagic lesions, regeneration of epidermal tissues, and normalization of swimming activity.

The combined supplementation of citronella (*C. nardus*) powder and probiotics at the PC-7.5 dose resulted in the highest recovery rate among treatments, although no statistically significant differences were detected. This response is likely associated with the immunomodulatory activity of bioactive compound present in citronella. Flavonoids and alkaloids have been widely reported to exert antioxidant and anti-inflammatory effects, contributing to the regulation of inflammatory signaling pathways, reduction of tissue damage, and enhancements of host recovery capacity (Jantan *et al*., 2015). In parallel, probiotic supplementation may have supported immune competence through improved nutrient digestibility and absorption, ensuring adequate energy availability for physiological maintenance and immune function (Peng *et al*., 2020). Probiotics have also been shown to modulate both mucosal and systemic immune responses, thereby limiting excessive inflammation and improving resistance to bacterial infections (Shija *et al*., 2023). In contrast, higher inclusion levels of citronella (PC-10 and PC-12.5) resulted in reduced the recovery rate, which may be attributed to elevated concentrations of bioactive and volatile compounds in the diet.

Survival rate showed a clear association with the recovery rate, with the PC-7.5 treatment yielding the highest survival (66.76%), indicative of a more effective host defense response. Conversely, the lower the recovery rates observed in PC-10 and PC-12.5 were accompanied by reduced survival (33.33%). Although citronella possesses immunostimulatory properties, it also contains dominant volatile constituents such as geraniol and citronellol that have been reported to exhibit cytotoxic effects at high concentrations (Fitria *et al*., 2022).

These findings highlight at the importance of dose optimization to achieve beneficial immunomodulation without inducing adverse physiological effects.

Hematocrit is the hematological indicator reflecting oxygen-carrying capacity and overall physiological status in fish subjected to stress or infection (Seibel *et al*., 2021). Dietary supplementation with citronella powder and probiotics effectively maintained hematocrit values within the normal physiological range, particularly at moderate inclusion levels (PC-7.5). Following *A. hydrophila* challenge, a reduction in hematocrit was observed, likely due to hemorrhage and tissue damage. However, fish receiving optimal dietary treatments exhibited faster hematological recovery compared to the control group. This effect may be attributed to the action of flavonoids, which have been reported to support tissue regeneration and hematopoietic activity (Boojar, 2019). Nevertheless, excessive citronella supplementation appeared to impair erythrocyte recovery, resulting in persistently lower hematocrit values.

Hemoglobin concentration, an indicator of blood oxygen transport efficiency, is highly sensitive to bacterial infection and associated pathological stress (Chen & Luo, 2023). Appropriate dietary supplementation with citronella supported hematopoiesis and accelerated hemoglobin recovery following infection. The post-challenge decline in hemoglobin is likely linked to hemolytic activity mediated by aerolysin, a pore-forming toxin produced by *A. hydrophila* that induces erythrocyte lysis (Dong *et al*., 2022; Zhang *et al*., 2025). The more rapid restoration of hemoglobin levels observed in the PC-7.5 treatment suggest a protective role of flavonoids in regulating iron metabolism and mitigating oxidative damage to erythrocytes. These findings are consistent with previous reports describing flavonoids as supportive agents in hematological recovery process (Lesjak & Srai, 2019).

Leukocytes constitute the primary cellular components of the innate immune system in fish, and changes in leukocyte counts reflect the dynamics of host immune responses to pathogenic challenge (Seibel *et al*., 2021). The increase in leukocyte numbers following dietary administration of citronella and probiotics prior to challenge indicates stimulation of innate immune defenses. A subsequent decline in leukocyte counts after bacterial infection likely reflect the intensive recruitment and utilization of immune cells at infection sites, whereas the rebound observed during the recovery phase suggest reactivation and restoration of immune homeostasis. Among treatments, PC-7.5 consistently produced higher leukocyte counts, indicating an optimal balance between immune stimulation and physiological tolerance. The immunological benefits observed may be attributed to citronella-derived bioactive compound, whereby flavonoids and alkaloids modulate inflammatory responses, and terpenoids enhance macrophage phagocytic activity, thereby strengthening both humoral and cellular immune responses (Jantan *et al*., 2015).

CONCLUSION

Dietary supplementation with citronella (*C. nardus*) powder combined with probiotics influences disease recovery, survival rate, and hematological responses in fish infected with MAS diseases. Among the tested treatments, the moderate supplementation level (PC-7.5) consistently produced higher recovery and survival rates, as well as improved hematocrit, hemoglobin level, and leukocyte profiles during the post-challenge period. Supplementation of citronella at levels exceeding the optimal dose was associated with adverse physiological effect, resulting in reduced recovery and survival rate.

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