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Immune Activity of Vannamei Shrimp (*Litopenaeus vannamei*) Under Ozonated and Non-Ozonated Treatments

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Abstract: This study aims to analyze the effect of ozonation treatment on the immune activity of vannamei shrimp, compared to non-ozonization treatment. Parameters measured include Total Haemocyte Count (THC), Differential Haemocyte Count (DHC), and phagocytic activity. The results show that ozonation treatment produces higher THC values, namely an average of 75% more than non-ozonation. The DHC value in the ozonation treatment showed an increase in granular cells by 40% compared to the non-ozonation treatment. In contrast, hyaline cells decreased due to a more active immune response against pathogens. Phagocytic activity in the ozonation treatment reached 15%, higher than non-ozonization which only reached 10.33%. These data indicate that ozonation plays an important role in improving shrimp's immune response by reducing the water's pathogen content and minimizing stress. In conclusion, ozonation was proven effective in increasing the immunity of vannamei shrimp by supporting key immunological parameters.

Keywords: DHC; Immune; Phagocytic activity; THC; Vannamei

Introduction

Vannamei Shrimp Cultivation (*Litopenaeus vannamei*) has rapidly developed in Southeast Asian countries (Lukwambe et al., 2019). The advantage of cultivating name shrimp is that this shrimp is known to be resistant to disease, has a short maintenance period, and has a relatively low level of feed converting ratio (FCR) (Ariadi et al., 2020). The name shrimp cultivation business has great potential for further development with more adaptive production techniques (Wafi et al., 2021). Vannamei shrimp are excellent and the main choice for farmers in East Java. This is because vannamei shrimp have high economic value and are expensive, vannamei shrimp are easy to cultivate because they are resistant to disease can grow quickly, and can be cultivated athigh densities (Dahlan et al., 2017). One system used by several farmers to increase production results is by raising shrimp inan intensive pattern system (Ariadi et al., 2019). Vannamei shrimp cultivation technology in an intensive system can achieve stocking densities ranging from 100-300 individuals/m² (Cahyanurani & Hariri, 2021). However, sometimes cultivation with an intensive system often causes problems. One of the causes of name shrimp cultivation failureis poor water quality management during the production period (Amalo et al., 2023).

Water quality is considered good for cultivation even though there are several parameters that fluctuate (Inayah et al., 2023). Water quality parameters in pond ecosystems play an important role in the level of cultivation productivity. The pond water quality will play a role in the condition and performance of the shrimp being farmed. Fluctuating water quality will make shrimp easily experience stress due to abnormal conditions (Ariadi et al., 2019). White shrimp disease (*Litopenaeus vannamei*) can be caused by viruses,

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bacteria, protozoa, and fungi. Several cases of the disease were found in ponds, especially in the case of a name shrimp, which was caused by vibriosis-type bacteria. The infection process of the pathogenic bacteria Vibrio sp. The pathogenic bacterial infection is one of the problems in the cultivation of vannamei shrimp (*Litopenaeus vannamei*) (Kilawati & Islamy, 2021). This cancause disease if the shrimp are weak and the quality of the cultivation media environment is extreme (Saraswati et al., 2023).

Optimal water quality management is an effort to ensure that water conditions remain in good condition for cultivation so that suitable water is obtained for growth and survival. Ozone will dissolve in water to produce hydroxy radicals (OH-). Hydroxy radicals have the power to oxidize organic compounds and can be used in the process of sterilizing various types of microorganisms, removing odors, and removing color in liquid waste (Badmus et al., 2018). For more than two decades, shrimp farming has suffered viral disease problems like Taura syndrome virus (TSV) and white spot syndrome virus (WSSV), as well as vibriosis due to *Vibrio alginolyticus* and *V. harveyi* (Li & Chen, 2008). The mechanism for disinfecting bacteria by ozone can be carried out in various ways, namely by direct oxidation or usually by destroying the cell wall by removing the contents of the cell. Another way is to react with radiation from ozone decomposition by-products, destroying nucleic acids (purines and pyrimidines), and can also break carbon-nitrogen bonds so that depolymerization occurs (Solomon et al., 1998). The use of ozone (O_3) has been used in both the aquaculture and water treatment industries to improve water quality and reduce pathogens during pre-treatment and waste treatment.

Method

Research Time and Location

This research will be carried out on June 30 – August 14 2024 at PT. KKV Kandang Semangkon Village, Paciran District, Lamongan Regency then for observation of the results of field data collection at the FPIK Laboratory of Brawijaya University and the Aquaculture Laboratory of Trunojoyo University, Madura. A map of the location of this research is presented in Figure 1 following.

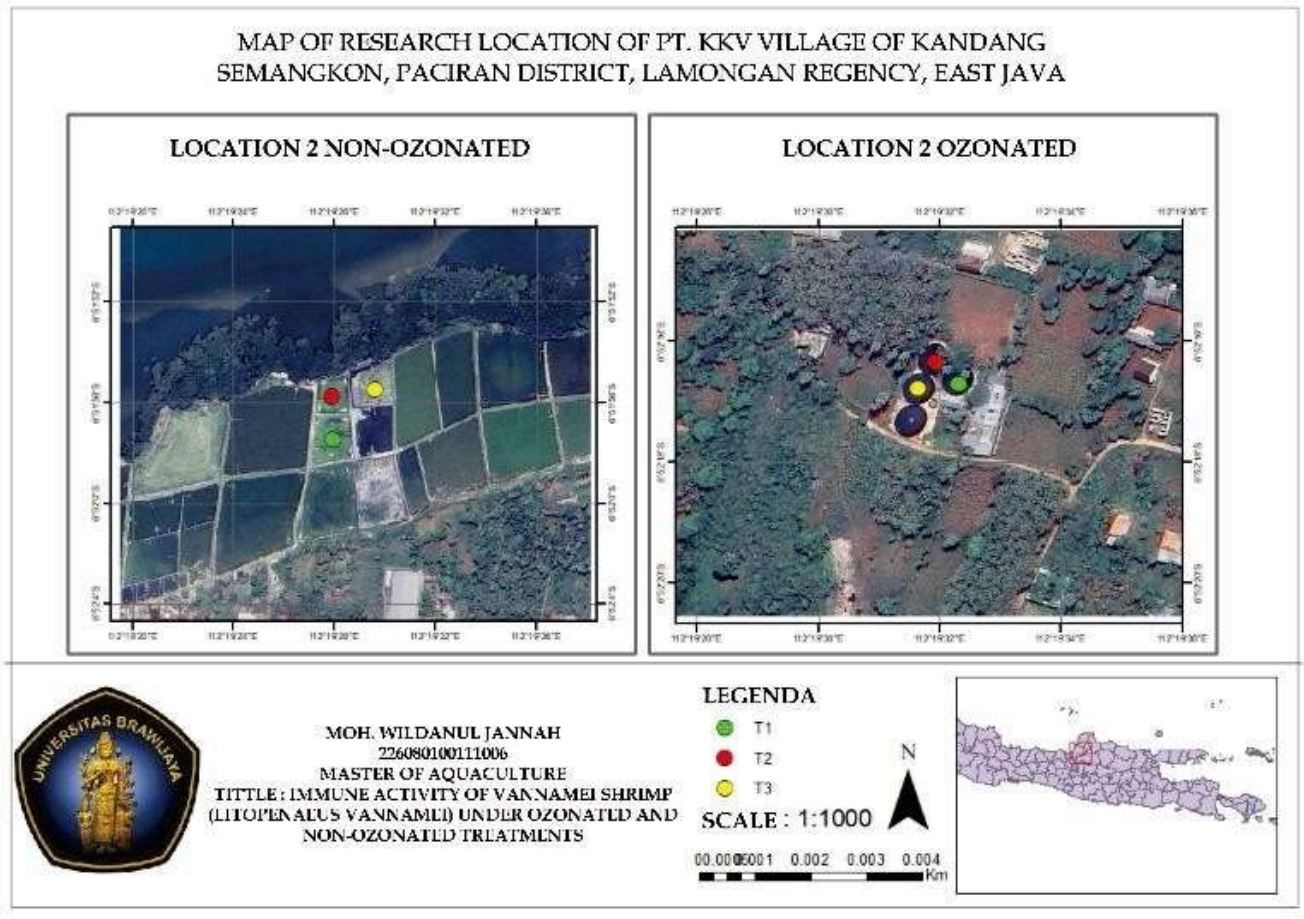


Figure 1. Mapping location (1) ozonated pond; (2) non-ozonated pond

Tools and Material Method

The tools used during this research consisted of sample bottles, writing instruments, sample boxes, refrigerators, Erlenmeyers, volume pipettes, dropper pipettes, measuring flasks, spray bottles, pump pipettes, fume cupboards, scissors, and test tubes. Meanwhile, the materials used during this research consisted of water samples, H_2SO_4, Ammonium molybdate, Ascorbic acid, Potassium antimony tartrate, Kalium nitrate anhydrate (KNO_3), NaCl, Sulfanilic Acid, Magnesium carbonate ($MgCO_3$), Aquades, Filter Paper, Aluminum, Tissue, Latex, and Label Paper.

Sampling Points

Determination of sampling points is carried out using purposive, sampling method carried out based on the policy of the research itself (Muchsin et al., 2017). The sampling point for this research explains the process of collecting data in the field that there were 2 different treatments with 3 ponds each. Meanwhile, field data collection was repeated 3 times every 2 weeks.

Total Haemocyte Count (THC)

10 µL fresh hemolyme was diluted with 20 µL PBS, then the diluted sample was placed on a hemocytometer, then observed under a microscope in the blue box (Permatasari, 2017). The number of hemocytes visible on the microscope is then counted. Total Haemocyte Count (THC) is obtained using the following formula.

$$\text{THC} = \frac{\text{Cell}}{\text{ml}} = \text{Average cell} \times \frac{1}{\text{Blue box volume}} \times \text{FP} \quad (1)$$

FP = Dilution Factor

Differential Haemocyte Count (DHC)

Hemocyte differential or Differential Haemocyte Count (DHC) is calculated based on the method used by Martin & Graves (1985). The slide was soaked in methanol for 5-10 minutes and air-dried. Hemolim is dropped on a glass object and a smear is made and then air-dried. The preparations were fixed with methanol for 5-10 minutes and then air-dried again. The preparations were soaked in 10% Giemsa solution for 15-20 minutes, then washed with running water for 30 seconds and allowed to dry. The hemolymph was observed with a microscope at 400 times magnification and the cell types were identified, namely hyaline, semi-granular, and granular cells. The number of hemocytes was counted to 100 cells and the percentage of each type was determined. Hemocyte differential is calculated using the formula.

$$\text{Hyaline cells} = \frac{\text{Hyaline cells}}{100} \times 100\% \quad (2)$$

$$\text{Semi granular cells} = \frac{\text{Semi granular cells}}{100} \times 100\% \quad (3)$$

$$\text{Granular cells} = \frac{\text{Granular cells}}{100} \times 100\% \quad (4)$$

Phagocytic Activity

0.1 mL of shrimp hemolymph was added to Eppendorf mixed evenly with 25 µL of V. harveyi bacteria and incubated for 20 minutes. Then 5 mL was dropped onto the object glass and a smear preparation was made. Next, it was fixed with 100% methanol for 5 minutes and stained with Giemsa (10%) for 15 minutes. The preparation was then slowly run through water for approximately 5 minutes to remove the remaining Giemsa color. Observations were carried out under a light microscope with a magnification of 400 (Prastiti et al., 2023).

The formula for calculating Vannamei Shrimp Phagocytosis is as follows:

$$\text{Phagocytic activity} = \frac{\text{The numbocytic cells that carry out phagocity}}{\text{Number of phagocitic cells}} \times 100\% \quad (5)$$

Data Analysis

This research was analyzed using the T-test. According to Kainde et al. (2016), an unpaired T-test (independent) is a parametric statistic used to compare two sample mean values that are not paired (independent). Based on the assumption of diversity (variance), in its implementation the t-test is divided into 2, namely: t-test with the assumption that the variance of the two samples is the same (t-Test: Two-Sample Assuming Equal Variances); and t-test assuming the variances of the two samples are different (t-Test: Two-Sample Assuming Unequal Variances). Independent sample t-test is a parametric test used to determine whether there is a difference in the mean between two independent groups or two unpaired groups with the intention that the two groups of data come from different subjects.

Result and Discussion

Total Haemocyte Count (THC)

Calculation of total hemocyte count (THC) aims to determine the health condition of shrimp. Haemocytes are cells that play a central role in the crustacean immune system (Wildarni et al., 2020). Hemocytes are

the front guard of white shrimp defense which plays a role in humoral and cellular defense to fight disease (Suharli et al., 2024). THC and DHC parameters can be used as a reference to see the health condition of vannamei shrimp (Sugiharta et al., 2023). The results of measuring the total hemocytes of vannamei shrimp in the ozonation and non-ozonization treatments are shown in Figure 2.

The vannamei shrimp cultivation system requires preventive efforts to prevent disease attacks, therefore the choice of cultivation system greatly influences the success of vannamei shrimp cultivation (Prastiti et al., 2018). Ozone treatment plays a role in water treatment using ozone to minimize nitrite content and bacterial biomass (Schroeder & DiPersio, 2011). Hemocytes are cells that can release functional particles in the form of macrophages, granulocytes, and NK cells (killer cells) which are responsible for the processes of phagocytosis, encapsulation, and nodular aggregation (Prastiti et al., 2023). The total hemocyte count (THC) is related to many factors, and THC, to a certain extent, reflects the immune response status of the shrimp (HARIS) (Pei-Feng et al., 2009). The higher the total number of hemocytes, the greater the health condition of the vannamei shrimp is in good condition (Oktaviana & Febriani, 2019). The decrease in the total number of hemocytes in replications 2 and 3 of each treatment occurred due to several factors, both internal and external, such as the environment, pathogen infection, and physiological conditions, where in this study there was an increase and decrease in water quality parameters during the cultivation process and the molting process. which occurs periodically so that under certain conditions the total number of hemocytes can decrease according to the conditions (Apún-Molina et al., 2024).

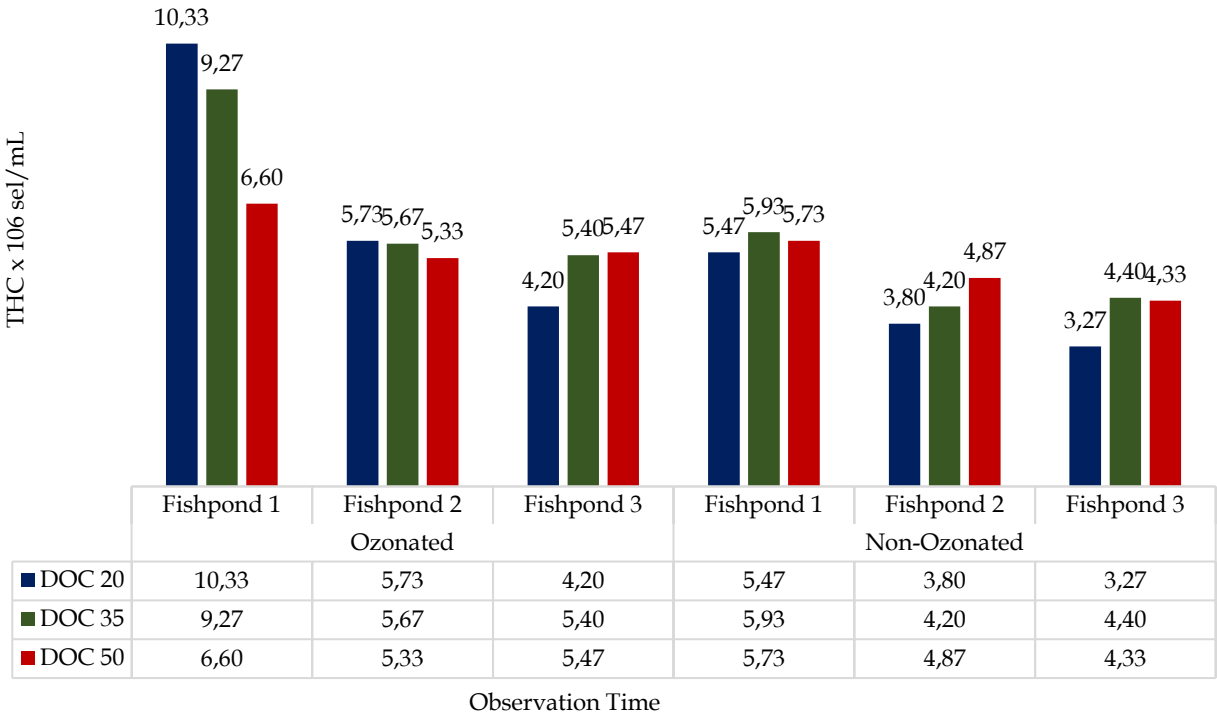


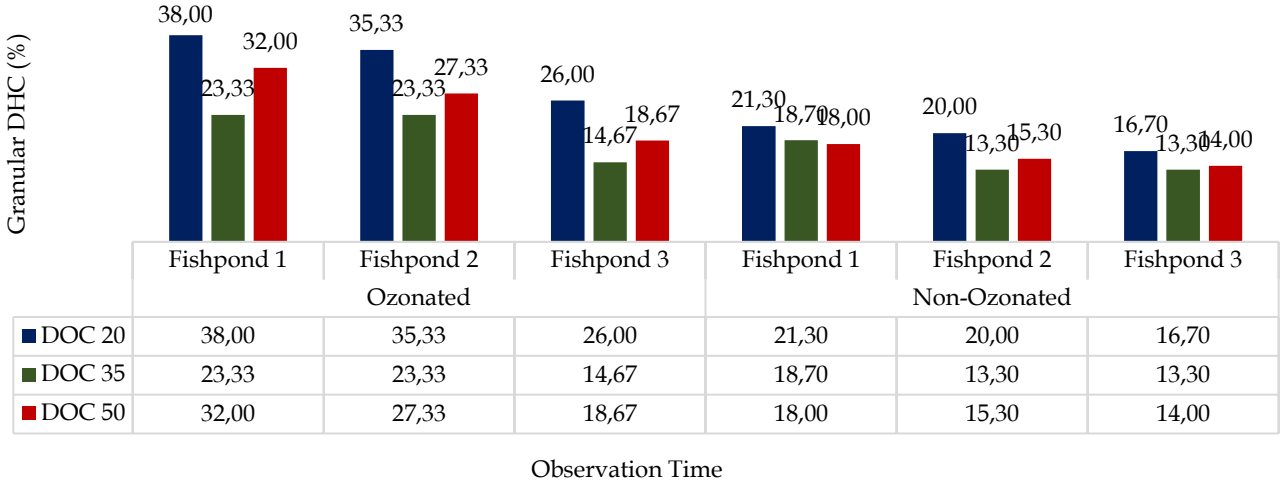
Figure 2. Total Haemocyte Count (THC) of vannamei shrimp

Differential Haemocyte Count (DHC)

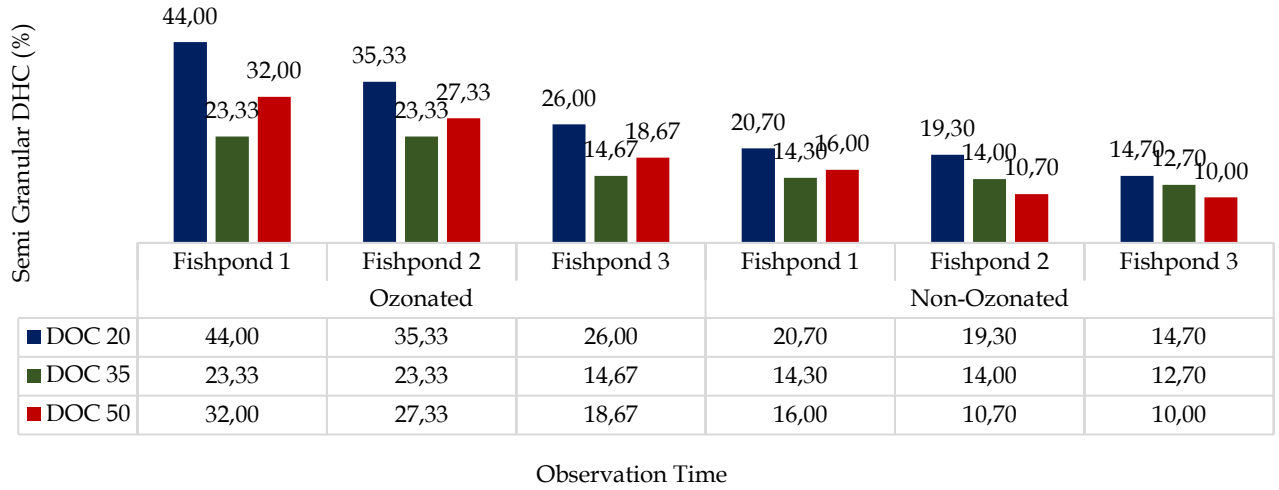
Calculation Differential Haemocyte Count (DHC) aims to determine the number of hemocyte cells in vannamei shrimp. DHC is divided into three types based on the size and granule content, namely granular, semi-granular, and hyaline. The results of measuring the total differential hemocytes of vannamei shrimp in the ozonation and non-ozonization treatments are shown in Figure 3.

The number of granular cells in both treatments showed a decrease in the number of granular cells which correlated with a decrease in the total number of hemocytes. According to Siregar et al. (2024), if foreign material enters the shrimp's body, the granular cells will carry out degranulation, cytotoxicity and lysis of the foreign cells. This process has an impact on reducing the percentage of granular cells. The number of granular cells in shrimp in good condition ranges from 17-40%. Cells that have played a role in fighting pathogens cannot develop into semi-granular cells so that the percentage of semi-granular cells will decrease (Wangi et al., 2019). The number of granular

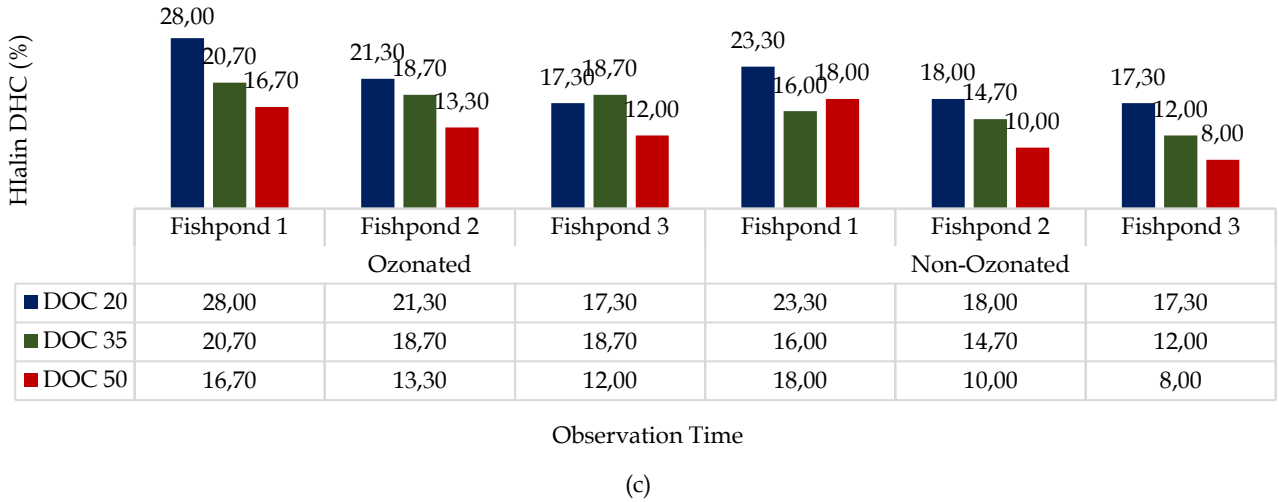
cells in shrimp in good condition ranges from 17-40% (Siregar et al., 2024). An increase in the proportion of granules often indicates infection or immune stimulation, whereas a decrease in hyaline can occur due to environmental damage or deterioration in health (Apún-Molina et al., 2024). The low percentage of hyaline cells and granular cells in the 2nd and 3rd repeat ozonation and non-ozonization treatments indicates that in this phase the cultivated vannamei shrimp were in poor health.



(a)



(b)



(c)

Figure 3. Differential haemocyte count of vannamei shrimp, a) Granular, b) Semi granular, c) Hyaline

Phagocytotic Activity

Phagocytic activity in vannamei shrimp was calculated to determine how the immune response of vannamei shrimp treated with ozonation and non-ozonization was against bacterial infections. vibrio sp. Phagocytic activity can be seen in Figure 4.

Phagocytic activity in the ozonation treatment ranged from 5-15% while in the non-ozonation treatment, it ranged from 4.33-10.33%. Phase through the fusion of phagosomes with lysosomes (Flannagan et al., 2012). Hydrolytic enzymes will degrade and clean up particles or pathogens in phagolysosomes (Underhill & Goodridge, 2012). Phagocytosis has been considered an important defense mechanism of the immune response to pathogens among eukaryotes, which is also involved in various physiological processes, including development, apoptosis, tissue repair, and host defense (Freeman & Grinstein, 2014). The current study has demonstrated heightened phagocytic activity after bacterial challenge that is not present with an unrelated bacterium (Pope et al., 2011). The decrease in phagocytic activity in both treatments could be influenced by several factors such as the environment and shrimp health. Under statement by Apún-Molin et al. (2024), poor water quality conditions can cause stress in shrimp so that phagocytosis decreases significantly. According to Subagiyo et al. (2023), the phagocytic activity of shrimp hemocytes in the control treatment was 30.45%, while in the probiotic treatment, it reached 39.3-44.45%.

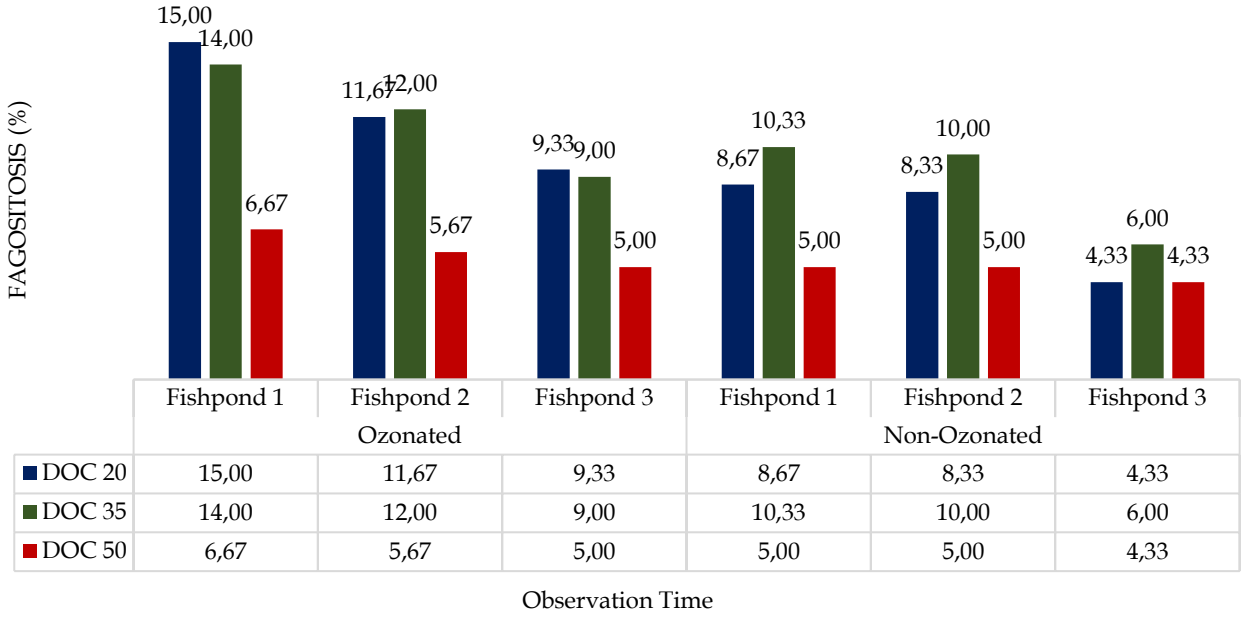


Figure 4. Phagocytotic activity of vannamei shrimp

T-test Analysis

Based on Table 1, it can be concluded that the ozonation treatment in vannamei shrimp ponds at PT. The KKV of Kandang Semangkon Village has a significant effect on total haemocyte count, differential haemocyte count which consists of granular, semi-granular, and hyaline, and phagocytosis of vannamei shrimp. This shows that ozonation treatment can increase the body's defense system of vannamei shrimp against bacteria vibrio spp. by improving water quality which triggers an increase in the total number of hemocytes, hemocyte differential, and phagocytic activity. Ji et al. (2009) stated that granular and semi-granular cells had a significant effect, indicating an increase in cells forming body defenses in shrimp. Both types of cells also play a role in the phagocytosis process. Meanwhile, the hyaline cells in the ozonation treatment were significant, indicating that the shrimp's body system defended itself in responding to foreign objects. Good quality and quantity of hemocyte types can certainly increase the immunity and resistance of vaname shrimp.

Table 1. Unpaired T test analysis of immune profile

Parameters	Significance Value
THC	0.012
Granular	0.002
Semi granular	0.001
Hyaline	0.159
Phagocytotic	0.027

Note: Yellow = Influential

According to Pumkaew et al. (2021), ozonation effectively destroys pathogenic bacteria and viruses, where with proper use of ozone, the pathogen load in

shrimp farming can be reduced, thereby benefiting shrimp survival and growth. Good environmental conditions greatly influence the biota that live in it, this statement is by (Prastiti et al. (2023), water quality has a crucial role in supporting the life, growth and health status of vannamei shrimp, where with good waterquality, pathogens that cause disease in shrimp do not grow easily so that the health of white vannamei shrimp will be maintained optimally.

Conclusion

This research shows that ozonation treatment effectively increases the immune activity of vannamei shrimp (*Litopenaeus vannamei*) compared to non-ozonization treatment. Based on the results obtained, ozonation treatment produces a higher Total Haemocyte Count (THC) value, whichis an average of 75% more than non-ozonization treatment. In addition, in measuring the Differential Haemocyte Count (DHC), ozonation treatment showed an increase in the number of granular cells by 40% compared to non-ozonation treatment. In contrast, hyaline cells decreased due to a more active immune response against pathogens. Phagocytic activity in the ozonation treatment reached 15%, higher than the non-ozonization treatment which only reached 10.33%. These data prove that ozonation plays an important role in enhancing theimmune response of shrimp by reducing the pathogen contentin water and minimizing stress. Thus, ozonation was proven effective in increasing the immunity of vannamei shrimp byimproving key immunological parameters.

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Author Contributions
Conceptualization, methodology, writing—review and editing, software, writing—original draft preparation, M.W.J. and A.M.S.H.; visualization, validation, supervision, project administration, Y.R. and W.I. The authors listed in this article have read and agree to the published version of the manuscript.

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Conflicts of Interest
The authors declare no conflict of interest.

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