

Feed supplementation with calcium from shrimp shells in vannamei shrimp (*Penaeus vannamei*) cultivated in low salinity media

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Abstract. Extensive and unoptimized cultivation land, together with the market demand and large profits, make the vannamei shrimp (*Penaeus vannamei*) commodity very feasible. One of the technical options for developing such a business is cultivation in freshwater media, by exploiting its euryhaline nature. The growth of *P. vannamei* in freshwater media added with various types of minerals shows better value than without the addition of minerals. However, when compared to vannamei shrimp raised in seawater media, its growth is still lower. Therefore, further efforts are still needed, one of which is by providing macro mineral intake in the form of calcium, through feed. This study aims to increase growth through the analysis of the immune response of *P. vannamei* maintained in freshwater media with the addition of calcium sources in the feed and challenged with vibrio parahaemolyticus bacteria. The research method used was experimental, based on a completely randomized design and consisting of 5 treatments, during the maintenance of *P. vannamei* in freshwater media, by providing feed containing different levels of calcium, namely: P1 0%, P2 1%, P3 2%, P4 3%, and P5 4%. The research data were processed using analysis of variance (ANNOVA), at a significance level of 5%. Further analysis (significant difference) was carried out with Duncan's test. The results showed that P5 produced the highest absolute weight value, which was 26 grams. The highest absolute length value was obtained in P5, which was 6.31 cm. The highest SR value occurred in P5, which was 70%. The highest feed utilization efficiency value occurred in P5, which was 61%. The highest body calcium value of shrimp occurred in P5, which was 1.99%. The highest total hemocyte count value occurred in P5, which was 14×10^6 cell mL⁻¹. The highest hyaline value in differential hemocyte count occurred in P5, which was 63. The lowest blood glucose value of shrimp occurred in P5, which was 10.5 mg dL⁻¹. The lowest media calcium content occurred in P5, which was 75 mg L⁻¹. Based on these results, it can be concluded that treatment 5 (P5) is the best treatment that can support *P. vannamei* cultivation in low salinity media.

Key Words: shrimp shell, calcium feed, freshwater media, productivity, vannamei shrimp.

Introduction. Data from Ministry of Maritime Affairs and Fisheries of the Republic of Indonesia, indicates that West Nusa Tenggara Province (NTB) recorded the highest national production volume of white shrimp *Penaeus vannamei* in 2021, totalling 177,427 tons (KKP 2022). The province has a potential aquaculture land area of 27,929 hectares, of which only 4,926 hectares, or approximately 18%, have been utilized. This high production output is driven by a strong market demand and by the significant profitability potential of *P. vannamei* cultivation (Pudyastuti et al 2016). According to Briggs et al (2004), in addition to increasing demand at the international level, *P. vannamei* has also experienced growing domestic market demand. Based on the provisions of the FIM (Federation Internationale de Motocyclisme) and DWO (Dorna WorldSBK Organization), the Mandalika Circuit on Lombok Island in NTB Province serves as the venue for international motorcycle racing events, including the World Superbike Championship (WSBK) and Moto Grand Prix (MotoGP). These events attract significant numbers of visitors, presenting a substantial market opportunity for *P. vannamei* as a global food (Millard et al 2021; Kopot & Taw 2004).

The considerable availability of extensive cultivation land, combined with the potential for substantial market demand and significant profitability, renders vannamei

shrimp a highly viable commodity for widespread development (Elovara 2021). One notable approach is by leveraging the euryhaline characteristic of *P. vannamei*, enabling cultivation in freshwater media (Dugassa & Gaetan 2018). This method warrants serious consideration due to its distinct advantages over conventional cultivation practices that rely on seawater media (Scabra et al 2022).

P. vannamei production in freshwater media has various advantages. First, *P. vannamei* is a commodity that has better economic value compared to several other freshwater fish commodities such as catfish and tilapia (Wickins & Lee 2002). *P. vannamei* cultivation in freshwater media is expected to provide greater production benefits. Second, pathogens that cause disease in *P. vannamei* cultivation in seawater can be avoided. Various sources inform that several types of diseases, such as WSSV and AHPND can be serious obstacles that trigger crop failure in *P. vannamei* cultivation activities (Suryana et al 2022). Vannamei shrimp cultivation in freshwater is expected to suppress pathogens that live and thrive in seawater media. Third, this activity can be carried out on land far from the sea. The water source used for freshwater *P. vannamei* cultivation can come from rivers, lakes, reservoirs, or even groundwater (through wells from inland communities). The development of *P. vannamei* cultivation in freshwater media has begun to be developed since 2013. Scabra et al (2022), based on the results of their research, showed that *P. vannamei* maintained in media with a salinity of 0 ppt (freshwater) has the ability to survive up to 90%. However, it was previously found that *P. vannamei*'s growth in freshwater was not optimal due to the lack of mineral intake (Perry 2001).

The suboptimal growth of *P. vannamei* cultured in fresh water media has been addressed through the addition of various minerals to the cultivation media, including calcium carbonate (CaCO_3) (Scabra et al 2023b), phosphorus (Scabra et al 2021), calcium hydroxide (Ca(OH)_2) (Scabra et al 2023a), calcium carbonate (CaCO_3) + magnesium sulfate (Scabra et al 2023c), calcium hydroxide (Ca(OH)_2) + magnesium sulfate (Scabra et al 2023d), and calcium hydroxide (Ca(OH)_2) and phosphorus (P) (Scabra et al 2023e). The growth performance of *P. vannamei* cultivated in freshwater media with the addition of these minerals demonstrated higher growth rates compared to those in media without mineral supplementation. However, the growth rates remained lower than those achieved in seawater media. Thus, while these studies indicate promising results, the outcomes are not yet optimal. This issue remains a critical problem requiring further solutions.

Mineralization is a limiting factor in the successful cultivation of *P. vannamei* in freshwater media (Davis et al 2005). In addition to mineral supplementation through the media, it is also necessary to add mineral sources through the feed (Scabra et al 2021). This is because the conventional feed for *P. vannamei* cultivated in seawater media contains low mineral content. Information regarding the appropriate dosage of mineral supplementation, such as shrimp shell powder, represents a technological puzzle in the mineralization process for *P. vannamei* cultivation in freshwater media that needs to be resolved. This study aimed to enhance growth by analyzing the immune response of *L. P. vannamei* cultivated in freshwater media with calcium supplementation in the feed.

Material and Method

Description of the study design. This research was conducted for 45 days from August to September 2024, using an experimental method with completely randomized design (CRD). The aspects studied were the effect of adding different shrimp shell flour to the maintenance container with 5 treatments and 3 replications. The treatments consisted of: P1 = control (without adding shrimp shell flour), P2 = adding 1% shrimp shell flour into the feed, P3 = adding 2% shrimp shell flour into the feed, P4 = adding 3% shrimp shell flour into the feed, and P5 = adding 4% shrimp shell flour into the feed. The test parameters in this study were absolute weight, absolute length, total hemocyte count, differential hemocyte count and shrimp blood glucose. Data analysis used ANOVA and, if significantly different, it was further analysed by the Duncan test with a confidence level of 95%.

Preparation of shrimps rearing tank. Plastic containers of a capacity of 45 L were used for shrimp cultivation. Before use, the plastic container is first cleaned and then placed

according to the research design. The amount of water filled into the plastic container is 25 L, equipped with an aerator as an oxygen supply source.

Preparation of shrimps. *P. vannamei* larvae used as experimental specimens were sourced from Sumbawa Island, consisting of 20 days old post-larvae (PL). Prior to the commencement of the research, the shrimp were first acclimatized to their environment. Salinity acclimatization was performed by gradually reducing the salinity levels. A total of 25 shrimp were cultured per container. The number of containers is 15, according to the number of treatments (5 treatments), which are repeated 3 times.

Shrimp shell flour making procedure. The shrimp shells used in this study were obtained from shrimp shell waste collected from ponds. Initially, the shells were washed and sun-dried until fully dried. Subsequently, the shrimp shells were steamed over medium heat until cooked, then sun-dried for 2 to 3 days until the material reached a dry state. Once dried, the material underwent a flouring process. It was then ground using a blender and sieved until ready to be added as a feed mixture according to the treatment (Efianda et al 2020). The addition of shrimp shell flour to the shrimp feed is carried out using the "coating method", which involves egg-white as an adhesive (Martilesa 2018).

Shrimp acclimatization. Prior to cultivation, an acclimatization process was first conducted on the white shrimp larvae. The larvae were placed in a temporary maintenance pond filled with seawater. Acclimatization involved gradually reducing the salinity to 0 ppt over a period of 10 days, with a 10% reduction in salinity each day in order to allow the shrimp larvae to adapt to living at a salinity of 0 ppt and to prevent stress and mortality resulting from a sudden decrease in salinity. Araneda et al (2008) reported that shrimp experience stress when there is a rapid decline in salinity concentration. Salinity reduction was carried out by the dilution method using the formula of Sahir (2022). Salinity reduction is conducted at a rate of 2 ppt per day by adding fresh water to the maintenance container containing seawater until the salinity is reduced by 2 ppt.

Research parameters. During the maintenance of *P. vannamei*, various data were taken and became the main research parameters, including:

The absolute weight growth, calculated by the formula of Effendi (2002):

$$W = W_t - W_0$$

Where:

W - absolute weight growth;

W_t - final shrimp weight;

W₀ - initial shrimp weight.

The absolute length growth, calculated by the formula of Effendi (2002):

$$L = L_t - L_0$$

Where:

L - absolute length growth;

L_t - final shrimp length;

L₀ - initial shrimp length.

The survival rate, calculated by the formula of Effendi (2002):

$$SR = \frac{N_t}{N_0} \times 100$$

Where:

SR - survival rate;

N_t - final amount of shrimp;

N₀ - initial amount of shrimp.

The food efficiency, calculated by the formula of Effendi (2002):

$$FE = \frac{(Wt + D) - W0}{F} \times 100$$

Where:

FE - food efficiency;

Wt - final shrimp weight;

D - dead shrimp weight;

W0 - initial shrimp weight;

F - total food given.

The calcium content on shrimp body was determined by using a spectrophotometric method. A 2 g shrimp sample is first ashed in a furnace at 400–500°C for 4 hours. After ashing, the sample is placed in a ceramic dish, and 25 mL of HCl (37%) is added, followed by heating for 15 minutes. The sample is then filtered and rinsed with distilled water until a final volume of 50 mL is obtained. A 1 mL aliquot is taken, and the pH is neutralized before adding distilled water to make up a total volume of 10 mL. Another 1 mL aliquot is taken and placed in a 25 mL flask, to which 1 mL of murexide, 2 mL of 0.1N NaOH, and distilled water are added, making up the total volume to 25 mL. The absorbance is then measured using a spectrometer at a wavelength of 518 nm (Scabra et al 2023c).

The calcium content on shrimp rearing media was determined by using ammonium purpurate as an indicator by preparing a stronger buffer with a higher pH. To measure calcium hardness, 100 mL of water sample is first pipetted into an Erlenmeyer flask. Then, 4.0 mL of 1N NaOH is added and then homogenized. Next, 0.1–0.2 grams (approximately the size of the stirring rod tip) of murexide is added, stirred, and the sample is titrated slowly with Na-EDTA until the color changes from red-pink to purple (olohydride violet). The endpoint of the titration is indicated when the addition of a drop of titrant no longer changes the purple color (Scabra et al 2023c).

The blood glucose content was tested using a glucometer that employs an enzymatic method. When a drop of blood is placed on the test device, the glucose oxidase enzyme reacts with the glucose in the blood, producing gluconic acid and hydrogen peroxide. These products then react with other substances on the test strip, generating an electron flow or electric current, which is converted into a numerical value.

The total hemocyte count (THC) was determined by taking 0.1 mL of shrimp hemolymph using a syringe and by placing two drops on a hemocytometer covered with a glass cover. The sample was examined under a microscope at 40x magnification (Rahmayanti et al 2018). The results of the observation were counted using the formula:

$$THC = \frac{\text{number of hemolymph cell}}{\text{Amount of hemocytometer squares observed}} \times \text{dilution factor}$$

The differential hemocyte count (DHC) was observed by collecting 0.1 mL of hemolymph using a syringe pre-filled with 0.2 mL of anticoagulant. The hemolymph was then smeared by placing a drop on a object glass and spreading it evenly. The slide was fixed in 100% methanol for 5–10 minutes and allowed to air dry. It was then immersed in Giemsa stain solution for 15–20 minutes and rinsed with water until the stain became lighter. The slide was subsequently observed under a microscope at 40x magnification (Abdi et al 2022). The formula used to calculate hemocyte cells is as follows:

$$DHC = \frac{\text{Amount of every type of hemocyte cell}}{\text{Amount of hemocyte cell observed}} \times 100$$

The phagocytosis activity was observed by taking 0.1 mL of hemolymph and adding 50 µL of *Streptococcus* sp. bacteria with a concentration of 10⁷ CFU mL⁻¹. The mixture was then incubated for 20 minutes. Subsequently, 10 µL of hemolymph was taken, smeared on an object glass, and fixed in methanol for 5–10 minutes, then air-dried. The sample was then immersed in Giemsa stain for 15–20 minutes and rinsed with water until the color

lightened. The sample was then observed under a microscope at 40x magnification (Pujiati 2013). The formula used to calculate phagocytosis activity is as follows:

$$PA = \frac{\text{The number of cells performing phagocytosis}}{\text{The number of cells observed}} \times 100$$

Statistical analysis. All data obtained were analyzed using the analysis of variance (ANOVA). If the test results between treatments were significantly different, a Duncan test was further carried out with a 95% confidence level.

Results

Absolute weight growth. Based on the results of the analysis of variance (ANOVA) test, it was determined that the treatments of the research had significantly different impacts (<0.05) regarding the value of absolute weight growth so further tests with the Duncan method were carried out; P1 was not significantly different from the P2, and P3 but significantly different from P4 and P5. The highest absolute weight growth was found in P5 with the value of 26.63 g, while the lowest absolute weight growth was found in P1, with the value of 11.22 g (Figure 1).

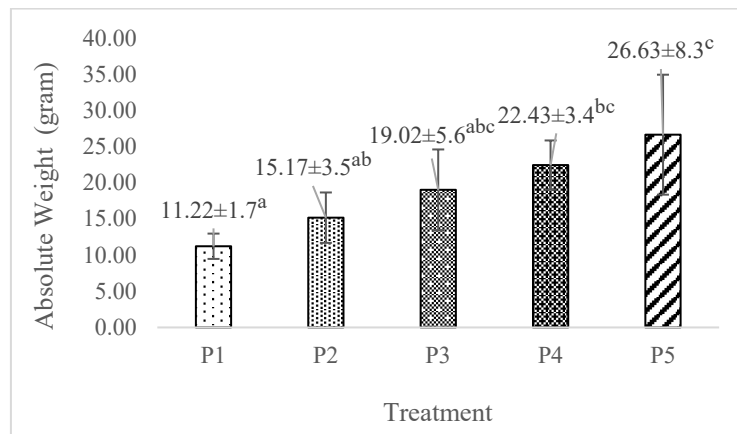


Figure 1. Absolute weight growth (gram).

Absolute length growth. Based on the results of the analysis of variance (ANOVA) test, it was found that the treatments of the research had significantly different impacts (<0.05) regarding the value of absolute length growth so further tests with the Duncan method were carried out; P1 was significantly different from P2, P3, P4 and P5. The highest absolute length growth was found in P5 with the value of 6.31 cm, while the lowest absolute length growth was found in treatment P1 with the value of 5.07 cm (Figure 2).

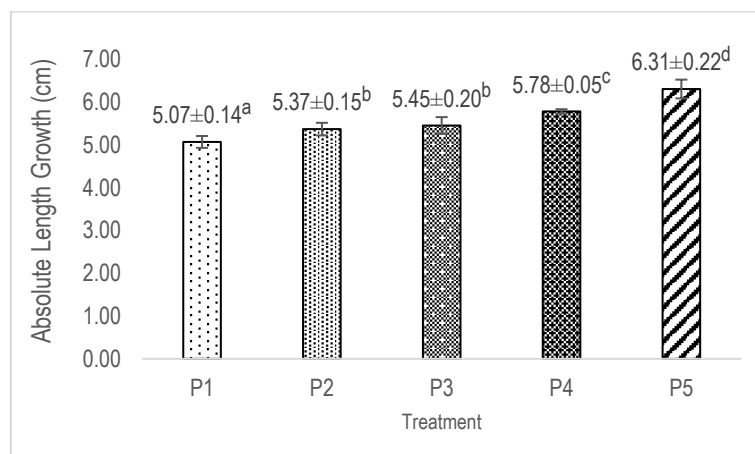


Figure 2. Absolute length growth (cm).

Survival rate (SR). Based on the results of the analysis of variance (ANOVA) test, it was found that the treatments of the research had significantly different impacts (<0.05) regarding the value of survival rate, so further tests with the Duncan method were carried out; P5 was significantly different of P3, P2, and P1 but not of P4. The highest survival rate was found in the P5 with the value of 75%, while the lowest survival rate was found in P1 with the value of 63.3% (Figure 3).

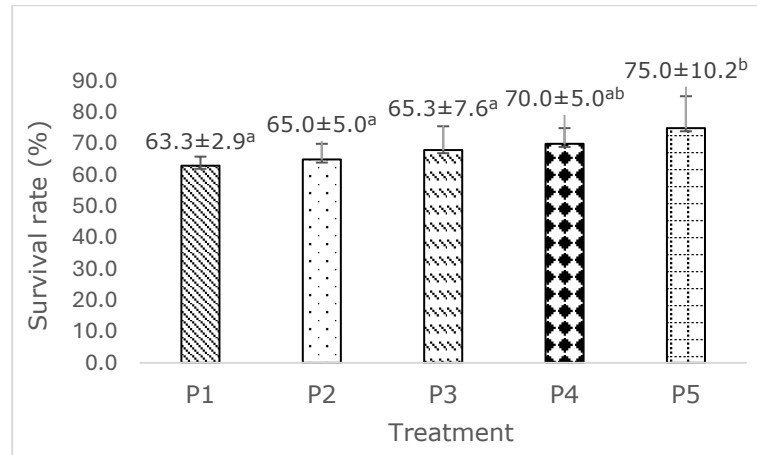


Figure 3. Survival rate (%).

Food efficiency. Based on the results of the analysis of variance (ANOVA) test, it shows that treatment of the research gives significantly different impact (<0.05) regarding the value of food efficiency so further tests with Duncan method were carried out. The Duncan test resulting P1 was not significantly different to P2, but significantly different to P3, P4 and P5. The highest food efficiency was found in the P5 with the value of 62.3% and the lowest food efficiency was found in the P1 with the value of 31.5% (Figure 4).

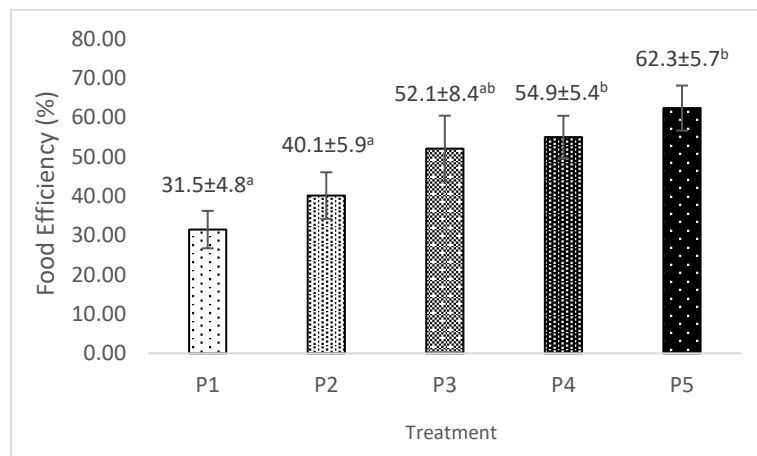


Figure 4. Food efficiency (%).

Calcium content of shrimp body. Based on the results of the analysis of variance (ANOVA) test, it was found that the treatments of the research had significantly different impacts (<0.05) regarding the value of calcium content of the shrimp body, so further tests with Duncan method were carried out; P1 was not significantly different of P2, and P3 but significantly different of P4 and P5. The highest shrimp body calcium was found in P5 with the value of 1.99% and the lowest was found in the P1 with the value of 0.64% (Figure 5).

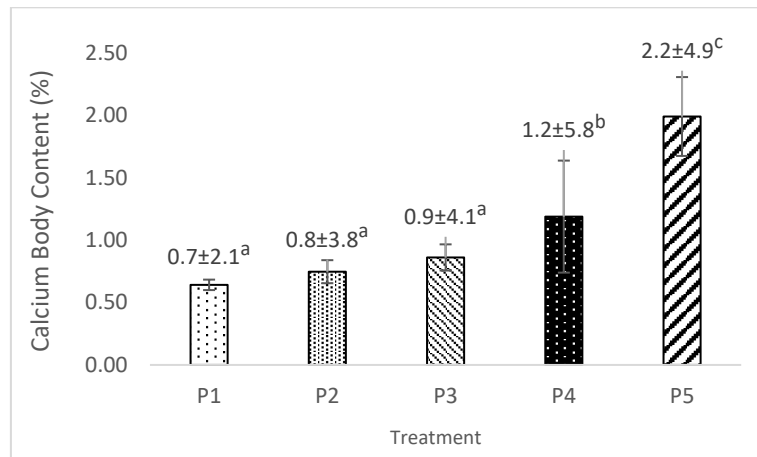


Figure 5. Calcium content of shrimp body (%).

Calcium content on shrimp rearing media. Based on the results of the analysis of variance (ANOVA) test, it was determined that the treatments of the research had significantly different impacts (<0.05) regarding the value of calcium content of rearing the media, so further tests with the Duncan method were carried out; P1 was not significantly different of P2 but significantly different of P3, P4 and P5. The highest media calcium was found in P1 with the value of 85.3 mg L^{-1} and the lowest media calcium was found in P5 with the value of 75.6 mg L^{-1} (Figure 6).

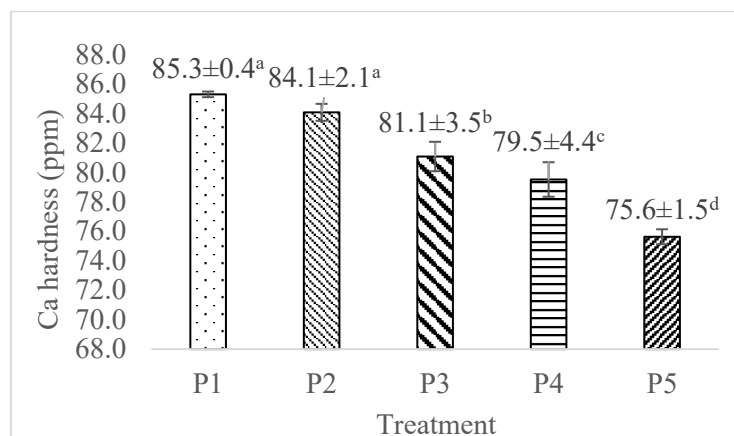


Figure 6. Calcium content on shrimp rearing media (mg L^{-1}).

Shrimp blood glucose. Based on the results of the analysis of variance (ANOVA) test, it was found that the treatments of the research had not significantly different impacts (<0.05) regarding the value of shrimp blood glucose content. Treatment P1 is not significantly different of P2, P3, P4, and P5. The highest blood glucose content was found in the P1 with the value of 14.7 mg dL^{-1} and the lowest glucose value was found in P4 with the value of 11.7 mg dL^{-1} (Figure 7).

Total hemocyte count. Based on the results of the analysis of variance (ANOVA) test, it was found that the treatments were not significantly different (<0.05) regarding the value of total hemocyte count. P1 was not significantly different of P2, P3, P4 and P5. The highest total hemocytes were found in P5 with the value of $14 \times 10^6 \text{ cells mL}^{-1}$ and the lowest total hemocytes were found in P1 with the value of $9.6 \times 10^6 \text{ cells mL}^{-1}$ (Figure 8).

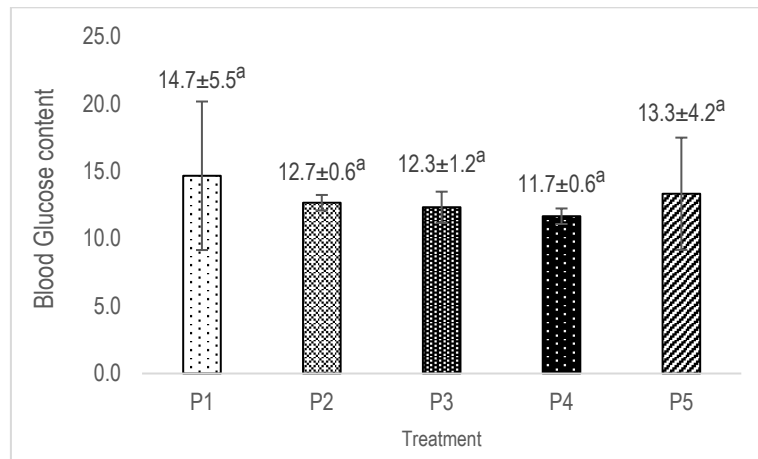


Figure 7. Blood glucose content (mg dL⁻¹).

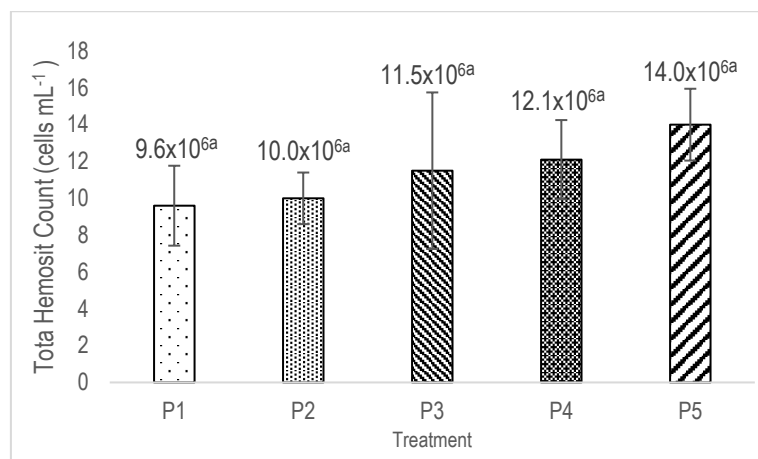


Figure 8. Total hemocyte count (cells mL⁻¹).

Differential hemocyte count. Based on the results of the analysis of variance (ANOVA) test, it was determined that the treatments of the research had significantly different impacts (<0.05) regarding the value of differential hemocyte count so further tests with the Duncan method were carried out; for the granular and semi-granular cells, P1 was not significantly different of P2, P3, P4 and P5; for the hyaline cells, P1 was not significantly different of P2, P3, and P4, but significantly different of P5 (Figure 9).

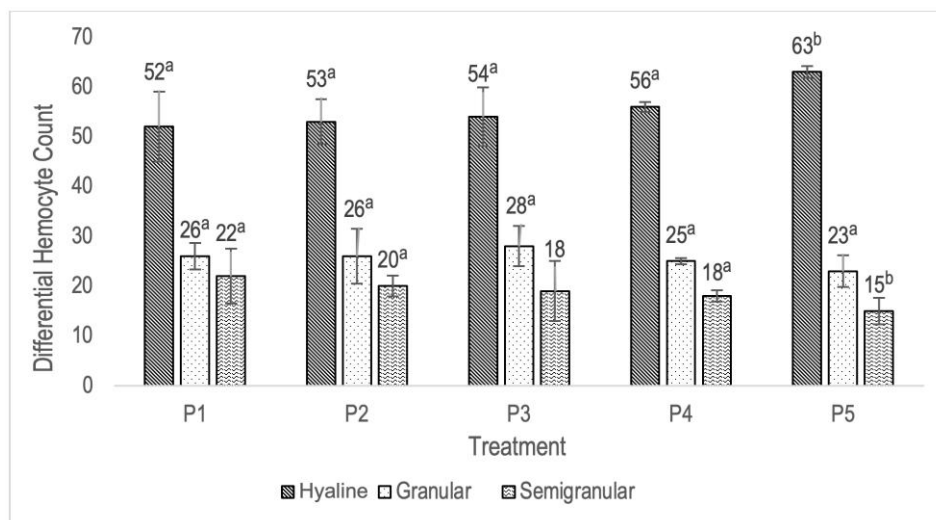


Figure 9. Differential hemocyte count (%).

Phagocytosis activity (PA). Based on the results of the analysis of variance (ANOVA) test, it was determined that the treatments of the research had significantly different impacts (<0.05) regarding the value of phagocytosis activity, so further tests with Duncan method were carried out; P1 was significantly different of P2, P3, P4 and P5. The highest value of phagocytosis activity was found in P5 with the value of 85.1% and the lowest value of phagocytosis activity was found in P1 with the value of 42% (Figure 10).

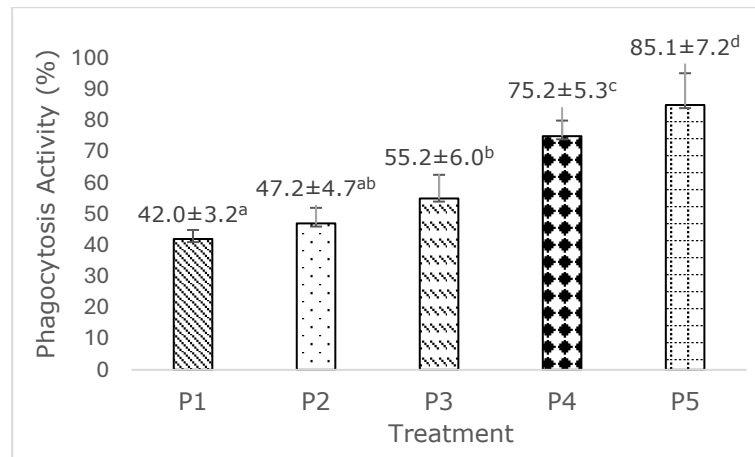


Figure 10. Phagocytosis activity (PA).

Discussion. Absolute growth weight refers to the increase in shrimp weight resulting from the absorption of food, which is chemically processed and stored as energy. When the produced energy exceeds the amount required, it is stored as muscle mass, commonly referred to as growth. As shown in Figure 1, treatment P5 results in the highest shrimp growth. This is hypothesized to be due to the fulfillment of the shrimp's calcium requirements at the provided dosage of 4%. This is further supported by the observed decrease in growth corresponding to a reduction of calcium dosage in the food. Corre-Jr et al (2016) suggests that the addition of calcium at an appropriate dosage in crustacean feed can accelerate carapace formation during molting, thereby facilitating a rapid growth.

The addition of shrimp shell flour in treatment P5 accelerates the molting process and increase muscle mass compared to other treatments. According to Scabra et al (2023b), molting in shrimp occurs more effectively when mineral requirements are adequately met, facilitating metabolic processes such as proper exoskeleton formation. This effect also observed in treatment P1, where a deficiency in mineral intake leads to delayed molting, subsequently impacting exoskeleton formation. Taqwa et al (2021) statement said that during the molting process and exoskeleton hardening, calcium plays a crucial role in various vital functions, including the support of carapace tissue development, which requires minerals, particularly calcium. The addition of calcium also influences the shrimp's physiological homeostasis with its environment. Chong-Robles et al (2014) noted that insufficient calcium levels can disrupt the shrimp's body homeostasis, resulting in an imbalance that triggers heightened osmoregulatory activity, thereby increasing energy expenditure. Consequently, the energy available for molting and growth is diminished.

Length and weight are growth parameters that are intrinsically correlated. As an organism increases in length, there is a concomitant increase in weight. The highest rate of absolute length growth was recorded in the P5 treatment. This result suggests that the incorporation of higher quantities of shrimp shell flour into the feed enhances the absolute length of the shrimp. The addition of shrimp shell flour to the diet provides essential minerals, which are utilized by the shrimp for growth. Variations in absolute length across the treatments highlight the critical role of calcium as a mineral that is indispensable for the physiological processes of shrimp. When calcium requirements are adequately met, optimal growth can be achieved. Conversely, insufficient calcium levels hinder shrimp growth in terms of both length and weight (Boyd 2015). Scabra et al (2021) emphasize that calcium is a vital nutrient for shrimp growth, playing a direct role in the molting

process. Li & Cheng (2012) suggest that optimal calcium concentrations facilitate the separation of the old exoskeleton and the hardening of the new one, thereby accelerating the molting process.

Shrimp cultured in low salinity media require additional minerals during the cultivation process. This is because low salinity media also contains low mineral content. The addition of shrimp shell powder to the feed is an effort to meet the calcium mineral requirements internally. In vannamei shrimp cultivation in freshwater, optimal calcium levels can support the homeostatic process in shrimp, allowing energy that would otherwise be used for metabolism to be redirected towards growth. According to Boyd (2015) suboptimal calcium levels will hinder the homeostatic regulation of calcium ions between the shrimp's body and its environment, resulting in increased energy expenditure and stunted growth. Noviana et al (2018) suggest that when the feed lacks calcium, shrimp attempt to maintain calcium levels in their bodies to meet their needs. Therefore, if the calcium mineral requirement is not adequately met, it will disrupt the shrimp's metabolic processes, ultimately affecting their growth. Boyd (2018) assert that shrimp are organisms capable of maintaining the body fluid balance with their environment. In freshwater-cultured shrimp, the calcium mineral requirement plays a crucial role in osmoregulation, supporting the shrimp's metabolic processes.

The survival rate is a parameter that reflects the success of an aquaculture activity. A high survival rate indicates the success of the cultivation process. The survival rate of shrimp in treatment P1 is notably lower compared to other treatments. This is suspected to be due to the shrimp's inability to maintain the homeostasis (balance) between its body and the surrounding environment, possibly because of insufficient calcium levels. Venkateswarlu et al (2019) suggest that shrimp unable to balance body ions with environmental ions may experience disturbances in metabolic and physiological processes, where the survival of organisms can be influenced by the equilibrium between the ions in their body fluids and those in their environment. This is further supported by the research of Scabra et al (2023b), which indicates that inadequate calcium content disrupts the shrimp's ability to maintain the calcium ion balance between its body and the environment, ultimately leading to mortality. Supono et al (2023) assert that calcium deficiency, as a macro-mineral, can result in mortality during molting.

Feed efficiency defined as the ability of shrimp to absorb and convert feed into growth. A common benchmark used to determine whether the feed is efficient involves observing whether the feed is fully consumed and assessing the growth of the cultured shrimp. The level of feed efficiency influenced by the protein content of the feed (Lee & Lee 2018). There is a direct correlation between feed efficiency and shrimp growth: low feed efficiency typically results in poor growth, whereas high feed efficiency corresponds with enhanced growth. Shrimp growth is indicated by molting, a process in which the shrimp sheds its exoskeleton. This aligns with the findings of Shariff et al (2021), who stated that after molting, the shrimp undergoes a process of exoskeleton hardening facilitated by calcium deposition so it can be efficiently digested and metabolized by the shrimp's body, serving as a readily available reserve for future physiological needs. The appropriate use of feed energy also affects feed efficiency. If the energy requirements of shrimp for various physiological needs are low, the energy can be redirected towards growth (Supono et al 2022). High growth rates under these conditions will naturally lead to an improvement in feed efficiency.

The highest calcium content in the body was observed in Treatment P5. This value gradually decreased as the calcium content added to the feed decreased. A significant difference was observed between Treatment P1 and P5, indicating that providing feed with varying calcium levels affects the calcium content in the shrimp's body. Laining et al (2015) stated that the administration of minerals, either through feed or the rearing medium, at the appropriate dosage, can influence the mineral content in shrimp's bodies. If the mineral content in the feed is provided in insufficient amounts, the shrimp's growth needs will be reduced, which is indicated by the lower calcium content in the body. The calcium content in the shrimp's body in this study was relatively low compared to the study by Laining et al (2015), where the mineral calcium content in shrimp ranged between 2.54-2.74%.

The highest hardness value was found in Treatment 1, at 85.3 mg L⁻¹, while the lowest was observed in Treatment 5, at 75.6 mg L⁻¹. Hardness is caused by the presence of minerals in water, which originate from rocks in the soil, either in ionic form or as part of molecular bonds (references). Calcium is an essential ion for the growth, survival, and osmoregulatory functions of crustaceans. The ionic composition in the rearing medium is an important factor for the survival rate of shrimp. Shrimp's calcium needs can be met through the environment and feed. Excessively high calcium content in aquatic environments can have detrimental effects on the organisms living within, as it forms a scale that attaches to the gills, making it difficult for shrimp to breathe, potentially leading to death (Boyd & Clay 2002). The low hardness value in Treatment P5 is suspected to be due to the shrimp utilized more calcium from the water to support their growth, which led to better growth in Treatment P5 compared to the other treatments.

Glucose in the blood of shrimp serves as an energy source when protein levels decrease. Under certain conditions, glucose levels in the blood reflect the shrimp's overall condition, indicating poor health. Internal homeostasis is maintained by regulating glucose levels through negative feedback in the body. When shrimp require more energy to maintain body homeostasis, glucose levels in the hemolymph increase as energy supply. The hepatopancreas releases crustacean hyperglycemic hormone (CHH) as a result of glycogenolysis, which generates glucose in the hemolymph (Fendjalang et al 2016). CHH functions to regulate glycolysis, a process used by crustaceans to cope with physiologically unstable conditions, leading to metabolic stress. This hormone plays a role in increasing glucose levels in the hemolymph and lactate concentrations during the mobilization of intracellular glycogen stores, carbohydrate and lipid for metabolism, reproduction, osmoregulation, and molting. Boyd (2018) explained that glucose levels in shrimp blood can increase when there are extreme environmental changes that cause stress. In addition to environmental changes, non-ideal conditions such as nutrient deficiencies can also lead to physiological stress in shrimp. Taqwa et al (2021) stated that the addition of calcium at the correct dose can reduce stress levels by minimizing osmoregulatory activity. A body that has adequate mineral supplies is able to better maintain mineral balance with the environment, thereby reducing stress.

Hemocytes are the blood cells of shrimp that function as part of the non-specific defense system in their body. The total number of hemocytes is considered an indicator of shrimp health. According to Oktaviana et al (2020), hemocytes are part of the shrimp's defense system, involved in processes such as phagocytosis, nodulation, and encapsulation. A higher hemocyte count indicates a healthy shrimp, while a low number suggests poor health. The research findings show that the total hemocyte count ranges between 9.6×10⁶ cells mL⁻¹ and 14×10⁶ cells mL⁻¹. This value is relatively high compared to the total hemocyte count in the study by Oktaviana et al (2020), which had an average value of 9.18×10⁶ cells mL⁻¹. The addition of calcium is suspected to affect the number of hemocytes in shrimp, as calcium not only accelerates shrimp growth but also activates enzymes involved in hemocyte formation. This aligns with the statement by Nurfaidah & Agustono (2021), who stated that calcium plays a role in the growth and development of the exoskeleton, regulates blood clotting, heart rate, kidney function, nerves, enzyme activity, and cell function.

Differential hemocyte count is also an indicator that can reflect the health of shrimp, particularly regarding the immune response of hemocyte cells. Hemocytes consist of hyaline cells, granulocytes, and semi-granulocytes, each playing a role in shrimp defense. The percentage of hyaline cells in this study ranged from 52% to 63%, which is considered within the normal range. According to Abdi et al (2022), the percentage of hyaline cells in shrimp hemocytes is between 50% and 80%. The granulocyte percentage observed in this study ranged from 23 to 28%, which is also within the normal range. Chau et al (2011) stated that in healthy shrimp, the granulocyte percentage ranges from 17 to 40%. Meanwhile, the semi-granulocyte percentage in this study ranged from 15 to 22%, which is considered normal, in line with Supono et al (2019) reported that the semi-granulocyte percentage in normal shrimp ranges from 10 to 30%.

The value of phagocytosis activity acts as an indicator of the immune response in shrimp. Phagocytosis is a process experienced by body cells in engulfing and digesting

foreign particles, such as bacteria, viruses, or cellular debris. In blood cells, the phagocytosis mechanism generally occurs in white blood cells, especially neutrophils and macrophages. In shrimp, the blood cells that play a role in phagocytosis are hemocytes. This process is very important in maintaining body health, because it helps clean infections and cellular debris. At high values, phagocytosis activity indicates that the shrimp's immune response is in good condition. According to Kumar et al (2021), shrimp's cellular defense response can be observed through phagocytosis activity. The highest phagocytosis value occurred in Treatment P5, which shows that the immune response of shrimp maintained in this treatment was better than in other treatments. The body's immune response is greatly influenced by adequate nutritional intake. Balanced nutritional needs play an important role in strengthening and maintaining the function of the immune system.

Conclusions. The addition of shrimp shell flour to the feed as an additional mineral in the cultivation of *P. vannamei* in freshwater had significant impacts on the shrimp growth rate, and enhanced the calcium content in both the shrimp's body and media (hardness value). The treatments applied were not significant regarding the shrimp blood parameters, such as the total hemocytes, differential hemocytes and blood glucose. The best results in this study were obtained through Treatment P5 with an absolute weight value of 26.63 g, an absolute length of 6.31 cm, and a body calcium content of 2.2%.

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