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Original Article

The effect of guava leaves (*Psidium guajava* L.) in feed with different extraction methods on the immunity of vaname shrimp (*Litopenaeus vannamei*) challenged by *Vibrio parahaemolyticus*

Andre Rachmat Scabra[*1], Thoy Batun Citra Rahmadani[1], Dela Shinta Maharani[1], Rizka Abdi[2], Muhammad Ridwan[3,4]

[1]Aquaculture Study Program, Mataram University, Indonesia.
[2]Aquaculture and Marine Resource Management Department, Wageningen University and Research, the Netherlands.
[3]Department Ecology and Biogeography, Nicolaus Copernicus University, Toruń, Poland.
[4]Department of Science and Technology, Parthenope University of Naples, Napoli, Italy.

Abstract: Diseases in vaname shrimp can cause economic losses, reduced production, and, in extreme cases, crop failure due to mass mortality. One disease that frequently occurs in intensive ponds of *Litopenaeus vannamei* is vibriosis, caused by *Vibrio parahaemolyticus*. Efforts to enhance shrimp immune responses by administering immunostimulants aim to prevent disease. One natural ingredient that can act as an immunostimulant is guava leaves (*Psidium guajava* L.). Common methods for producing guava leaf extract include maceration, Soxhlet extraction, and ultrasonics, but these methods are expensive. One solution is conventional extraction methods, such as flouring, boiling, and blending. The purpose of this study was to evaluate the effect of adding guava leaf extract to feed, using different extraction methods, on the immune response of whiteleg shrimp and to test it against *V. parahaemolyticus*. The method used was a randomized design with 5 treatments and 3 replications. The measured parameters in this study consisted of SR, FCR, SLGR, SWGR, media TVCs, THC, DHC, PA, and water quality. The results showed that P5 (blender method) with an SR value of 80%, FCR 1.1, SLGR 3 %/day, SWGR 8,2 %/day, media TVCs $20x10^2$ CFU/mL, THC $16x10^6$ cells/mL, hyaline cells 63.3%, and PA 68% showed the highest value compared to other treatments.

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Introduction

Whiteleg shrimp (*Litopenaeus vannamei*) is one of the leading aquaculture commodities in Indonesia. According to the Ministry of Marine Affairs and Fisheries (KKP), Indonesia's shrimp exports in 2022 totaled US$2.16 billion and 241,200 tons, representing 34.5% of the total national fishery export value (KKP, 2023). The high market demand has led intensive-scale farmers to increase stocking densities ranging from 100-300 ind./m² in each pond. This condition degrades water quality and physiological performance in Pacific white shrimp, thereby increasing their susceptibility to stress and disease. Diseases in Pacific white shrimp can cause economic losses and reduced production, and may lead to crop failure due to mass mortality. One of the most commonly encountered diseases in intensive ponds is vibriosis, caused by *Vibrio* spp. (Jannah et al., 2018). *Vibrio parahaemolyticus* can lyse host blood cells (Hatmanti, 2003), causing reddening of the shrimp's body and up to 65% mortality (Khusnah et al., 2023). The production of toxic proteins, Pir-like ToxA (PirA) and ToxB (PirB), encoded by the pVA1 plasmid of *V. parahaemolyticus*, is responsible for Acute Hepatopancreatic Necrosis Disease (AHPND), a highly lethal condition (López-León et al., 2016).

Control of *V. parahaemolyticus* infections can be achieved through antibiotic administration; however, its use has now been restricted or prohibited. This is because excessive and improper dosing can lead to antibiotic-resistant *Vibrio* strains (Kusmarwati et al., 2017). In several cases, antibiotics may leave harmful residues in shrimp consumed by humans. Efforts to enhance shrimp immune responses by administering

[*]Correspondence: Andre Rachmat Scabra
E-mail: andrescabra@unram.ac.id
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immunostimulants offer a means of preventing infectious diseases. Immunostimulants also do not leave residues in aquatic animals and are safe for human consumption (Manoppo and Kolopita, 2016). A drawback of commercial immunostimulants such as β-glucans and lipopolysaccharides is their relatively high cost; a cheaper, more readily produced alternative is required.

The use of natural feed additives in diets has been shown to increase total hemocyte counts and survival rates in Pacific white shrimp (Ismawati et al., 2020). One herbal plant with immunostimulant properties is guava leaf. Guava (*Psidium guajava* L.) leaves possess antibacterial and antimicrobial activities due to their active compounds, including saponins, flavonoids, tannins, and triterpenoids (Handarni et al., 2020). These active compounds can damage and lyse bacterial cell walls and induce protein denaturation, thereby inhibiting bacterial growth. According to Santi et al. (2017), guava leaf extract effectively inhibited the growth of *Vibrio harveyi* in vitro. Hurryah et al. (2015) also reported that guava leaf extract increased the survival rate of humpback grouper (*Cromileptes altivelis*) larvae in water infected with *V. parahaemolyticus*. Based on Dewi et al. (2021), the addition of guava leaf extract at a dose of 5 g/kg feed in Pacific white shrimp diets was proven to enhance immune response, increase survival rate, and reduce *V. parahaemolyticus* population.

Common methods for obtaining guava leaf extract include maceration, Soxhlet extraction, and ultrasonic extraction; however, these methods are relatively expensive. One potential solution is to use conventional extraction methods, such as leaf powdering, boiling, and blending. Studies using conventional extraction methods have identified flavonoids and tannins, which can inhibit bacterial growth (Nihali et al., 2020; Tari et al., 2022). To date, among these three conventional methods, the most effective for incorporating into shrimp feed as an immunostimulant remains unknown. Therefore, this work aimed to examine the effects of guava leaf supplementation in feed using different extraction methods on the immune response of Pacific white shrimp challenged with *V. parahaemolyticus*.

Materials and Methods

The research was conducted for 56 days, in February-March 2025. Shrimp maintenance was carried out in the Fish Production and Reproduction Laboratory, and parameter testing was done in the Fish Health Laboratory, Aquaculture Study Program, Faculty of Agriculture, University of Mataram. This study employed an experimental design by adding guava leaves to whiteleg shrimp feed and extracting them through flouring, boiling, and blending. The extract dosage applied was 5% of the total feed. This study was designed using a completely randomized design (CRD) with 5 treatments and 3 replications, resulting in 15 experimental units.

Preparation of containers and maintenance media: In this study, 15 plastic containers with a capacity of 45L were used to rear whiteleg shrimp. Before use, all containers were cleaned with soap and rinsed with fresh water until no soap residue remained. Next, the rearing containers were placed according to the experimental design and labeled. Each container was filled with sterilized seawater. The final step was installing aeration in each container to supply oxygen to the rearing medium.

Preparation of test animals: The test animals used in this study were PL-20 whiteleg shrimp from PT. Prima Larvae Bali, Jalan Singaraja-Tianyar, Tembok Village, Buleleng, Bali. Before being placed in the rearing tank, the shrimp were acclimatized for 7 days to adapt to their new environment. The purpose of acclimatization was also to reduce stress on the shrimp. In the next stage, the shrimp were placed in tanks at a stocking density of 25 shrimp per treatment.

Test feed preparation

Powdering method: The procedure for producing guava leaf flour is described in Tari et al. (2022). Fresh leaves, weighing 5% of the feed, are gently washed with running water, then dried in an oven at 50°C for 22 hours and 30 minutes, and finally blended to a fine consistency. The resulting powder is sieved to obtain a uniform particle size. Afterward, the flour is mixed into the shrimp feed using a coating method, with 3.5

g of egg white and 10 ml of water per 100 g of feed. The feed is then dried at 40°C until the moisture content reaches 10% (Dewi et al., 2021). The finished feed is placed into sealed plastic bags.

Boiling method: The procedure for preparing boiled guava leaf extract follows Kumar et al. (2021). Fresh leaves, weighing 5% of the feed, are rinsed with running water, finely chopped, and boiled in 75 mL of distilled water at 90°C for 30 minutes. After 30 minutes, the mixture is removed, allowed to stand for 24 hours, and then drained. The solution is filtered and mixed into the feed using a coating method, with 3.5 g of egg white and 10 ml of water per 100 g of feed. The feed is then oven-dried at 40°C until the moisture content reaches 10% (Dewi et al., 2021). The finished feed is placed into sealed plastic bags.

Blendering Method: The procedure for preparing the blender extract of guava leaves is based on the method of Nihali et al. (2020). Fresh leaves weighing 5% of the total feed were rinsed with running water and blended with 100 mL of distilled water until smooth. The resulting slurry is squeezed to separate the pulp from the liquid. The liquid is then filtered using filter paper to remove small leaf particles, producing a clear blender extract. The extract is subsequently mixed into the feed using a coating method, with 3.5 g of egg white and 10 ml of water per 100 g of feed, and then oven-dried at 40°C until the moisture content reaches 10% (Dewi et al., 2021). The finished feed is placed into sealed plastic bags.

Feeding and water exchange: This study employed an *ad libitum satiation* feeding method. The shrimp were fed a 40% protein diet at 07:00, 10:00, 14:00, 18:00, and 21:00 for 55 days. A 10% daily water exchange was performed using the siphoning method prior to the first feeding to maintain optimal water quality.

Preparation of test bacteria: The pure isolate of *V. parahaemolyticus* was obtained from the Fish Health Laboratory, Aquaculture Study Program, University of Mataram. Prior to use, bacterial rejuvenation was performed on TCBS medium to obtain highly virulent bacteria. The rejuvenation procedure began by adding 8.9 g of TCBS powder into an Erlenmeyer flask containing 100 mL of sterile distilled water. The TCBS solution was homogenized and then heated on a hot plate until boiling. The solution was subsequently poured into Petri dishes and allowed to solidify. The bacteria were then cultured using the streak method and incubated at room temperature for 24 hours (Abdi et al., 2022). After the rejuvenation process, the bacteria were cultured in TSB medium. TSB preparation began by adding 4 g of TSB powder to an Erlenmeyer flask containing 100 mL of distilled water, then homogenizing. The TSB solution was heated on a hot plate until it began to boil, then removed from the heat. The Erlenmeyer flask was covered with aluminum foil and sterilized using an autoclave. One loopful of bacterial colonies from the TCBS medium was transferred into the liquid TSB medium. The bacteria were incubated at room temperature for 24 hours.

Challenge test: On day 45, a challenge test was conducted on *L. vannamei* that had been fed guava leaf–supplemented diets to evaluate the enhancement of its immune response. A 0.1 mL aliquot of *V. parahaemolyticus* isolate from TSB medium was taken and diluted with 0.9 mL of NaCl to obtain a final concentration of 10^7 CFU/mL. The bacteria were injected into the dorsal region between the second and third segments at a dose of 100 μL per shrimp.

Research parameters

Survival rate (SR): SR was calculated as the percentage of shrimp that survived to the end of the rearing period. The formula for calculating SR, according to Effendi (2002) and Ratri et al. (2020), is as follows: SR = (Number of shrimp at the end of maintenance)/(Number of shrimps at the start of maintenance)×100%

Feed conversion ratio (FCR): FCR is calculated as the ratio of feed provided to shrimp weight gain. The formula for calculating FCR, according to Zonneveld et al. (1991) and Scabra et al. (2023), is as follows: FCR = F/(Wt-Wo), where Wt = Average final weight (g), Wo = Average initial weight (g), and F = Amount of feed given during maintenance (g).

Specific length growth rate (SLGR): SLGR measures the percentage increase in shrimp length

during rearing. The formula for calculating the specific growth rate, based on Castell and Tiews (1980) and reported in Scabra et al. (2021), is as follows: SLGR (%/day) = ((*Ln Lt-Ln Lo*))/t × 100%, where Lt = Average length of shrimp at the end of maintenance (cm), Lo = Average length of shrimp at the start of maintenance (cm), and T = Maintenance time (day).

Specific weight growth rate (SWGR): SWGR is the percentage of shrimp growth during the rearing process. The formula for calculating SWGR, based on Castell and Tiews (1980) and reported in Scabra et al. (2021), is as follows: SWGR (%/day) = ((Ln Wt-Ln Wo))/t×100%, where Wt = Average shrimp weight at the end of maintenance (g), Wo = Average shrimp weight at the start of maintenance (g), and T = Maintenance time (day).

TVC media: The rearing water was collected in 0.1 mL aliquots, then homogenized with 0.9 mL of NaCl, and serially diluted to a final concentration of 10^{-1}. After that, 100 µL was used to inoculate TCBS medium using the spread plate method, and the inoculated plates were incubated at room temperature for 24 hours. Bacterial colonies were counted using a hand tally counter. According to Alfiyanti and Putri (2020), the TVC calculation uses the following formula: CFU/mL = Number of colonies on a plate × dilution factor.

Total haemocyte count (THC): At the end of the rearing period, on day 56, the total hemolymph of whiteleg shrimp was observed. The syringe was filled with 0.2 mL of anticoagulant, after which 0.1 mL of hemolymph was taken at the base of the fifth pereiopod. The mixture of hemolymph and anticoagulant was homogenized. After 5 minutes, the hemolymph solution was dropped onto a hemocytometer and observed under a microscope at 40X magnification (Jannah et al., 2018). The formula for calculating THC, based on Ekawati et al. (2012) and Jannah et al. (2018), is as follows: THC (cells/mL) = (Number of cells counted)/(Number of fields of view) × 10^4 × diluting factor.

Differential haemocyte count (DHC): Hemolymph was collected using a syringe at 0.1 mL and then dropped onto a glass slide. After that, a smear was made by pulling the cover glass downward. Fixation was then performed in 100% methanol for 5 minutes until dry. Next, the smear was stained with 10% Giemsa solution for 10 minutes, rinsed with distilled water for 30 seconds or until the color faded, and left to dry. The final step was observation under a microscope at 400× magnification. According to Amlacher (1970) and Jannah et al. (2018), the formula for calculating DHC is as follows. DHC (%) = (Number of each hemocyte cell)/(Total hemocytes)×100.

Phagocytosis activity (PA): 100 µL of shrimp hemolymph was collected using a syringe and placed into a microtube. After that, 25 µL of *Staphylococcus* sp. bacterial suspension with a density of 10^7 cells/mL was added and homogenized. It was then incubated for 20 minutes. According to Anderson and Siwicki (1993) and Azuwarita et al. (2021), phagocytic activity is calculated using the formula of PA(%) = (Number of cells that carry out phagocytosis) / (Number of phagocytic cells) × 100.

Water quality: The measured parameters were temperature, pH, DO, salinity, and ammonia. Measurements were conducted four times in each experiment at H0, H22, H45, and H55.

Results

Survival rate: The highest value was observed in P5, which differed significantly from P1, P2, P3, and P4. The lowest value was observed in P1, which differed significantly from P5 but not from P2, P3, or P4 (Fig. 1).

Feed conversion ratio: The highest value was observed in P1, which differed significantly from P2, P3, P4, and P5. The lowest value was observed in P5, which differed significantly from P1, P2, and P4 but not from P3 (Fig. 2).

Specific length growth rate: The highest value was observed in P5, which differed significantly from P1, P2, P3, and P4. The lowest value was observed in P1, which differed significantly from P3, P4, and P5 but not from P2 (Fig. 3).

Specific weight growth rate: The highest value was

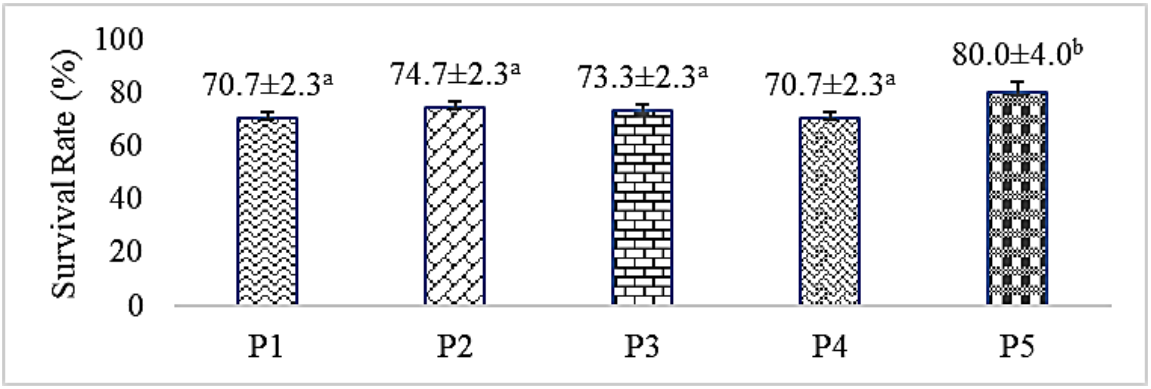

Figure 1. Survival rate of Pacific white shrimp under different guava leaf supplementation treatments challenged with *Vibrio parahaemolyticus*.

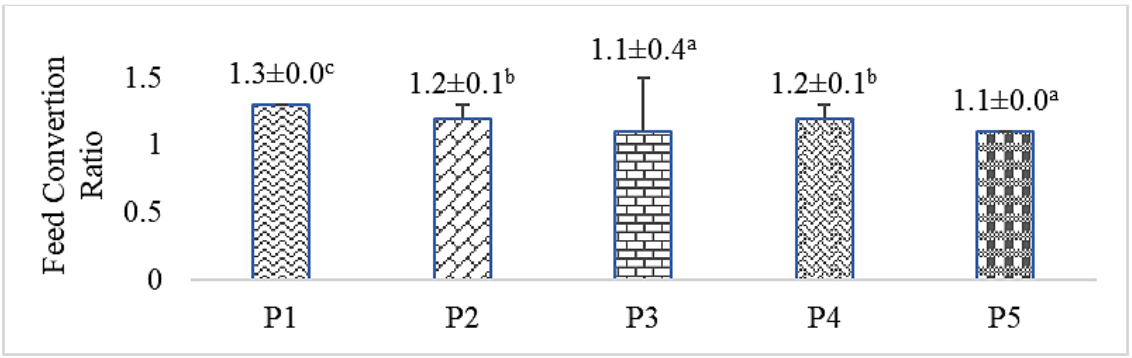

Figure 4. Feed conversion ratio of Pacific white shrimp under different guava leaf supplementation treatments challenged with *Vibrio parahaemolyticus*.

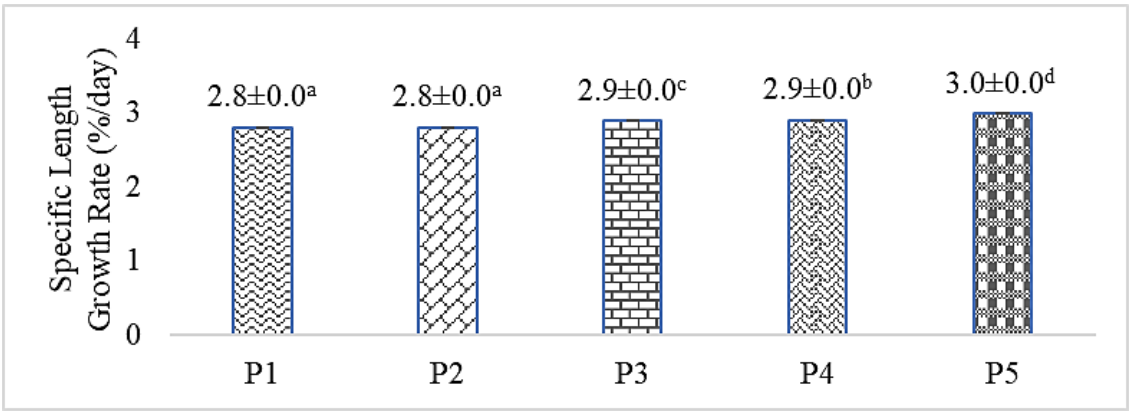

Figure 3. Specific length growth rate of Pacific white shrimp under different guava leaf supplementation treatments challenged with *Vibrio parahaemolyticus*.

observed in P5, which was significantly different from P1 and P2 but not from P3 and P4. The lowest value was observed in P1, which differed significantly from P3, P4, and P5 but not from P2 (Fig. 4).

TVC media: The highest value was observed in P1, which differed significantly from P2, P3, P4, and P5. Meanwhile, the lowest value was observed in P2, which differed significantly from P1 and P4 but not from P3 and P5 (Fig. 5).

Total haemocyte count: The highest value was found in P5, which was significantly different from P1, P2, P3, and P4. The lowest value was observed in P1, which differed significantly from P2, P3, P4, and P5. The results for P2 and P4 were not significantly different, but were significantly different from those of P1, P3, and P5 (Fig. 6).

Differential haemocyte count: Physiologically, hemocytes in whiteleg shrimp consist of three cell types: hyaline, granulocytes, and semigranulocytes, each of which plays a crucial role in the shrimp's

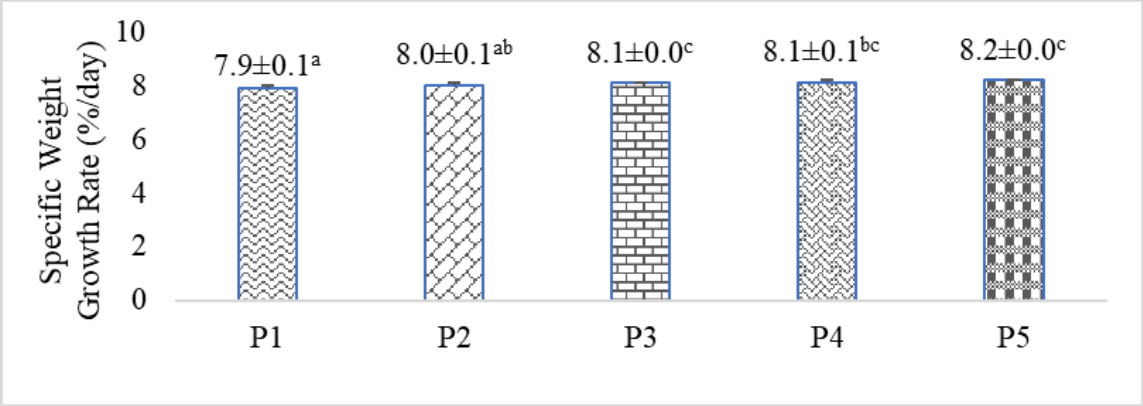

Figure 4. Specific weight growth rate of Pacific white shrimp under different guava leaf supplementation treatments challenged with *Vibrio parahaemolyticus*.

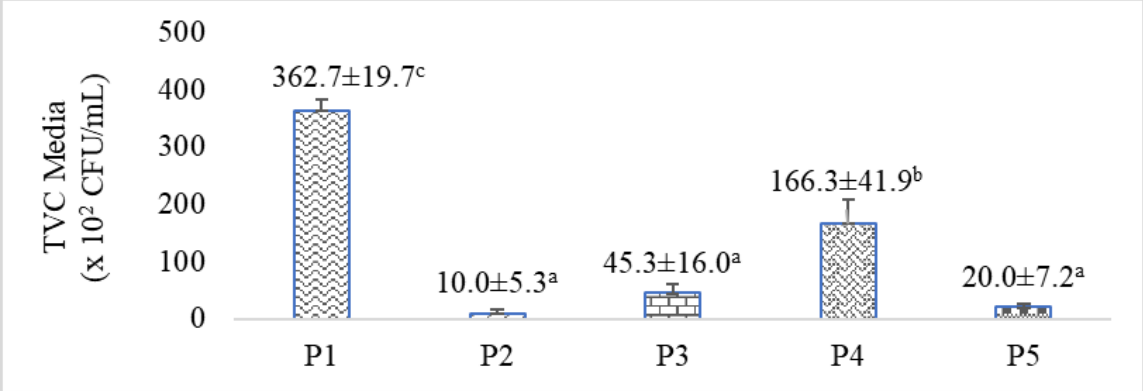

Figure 5. TVC in the media of Pacific white shrimp under different guava leaf supplementation treatments challenged with *Vibrio parahaemolyticus*.

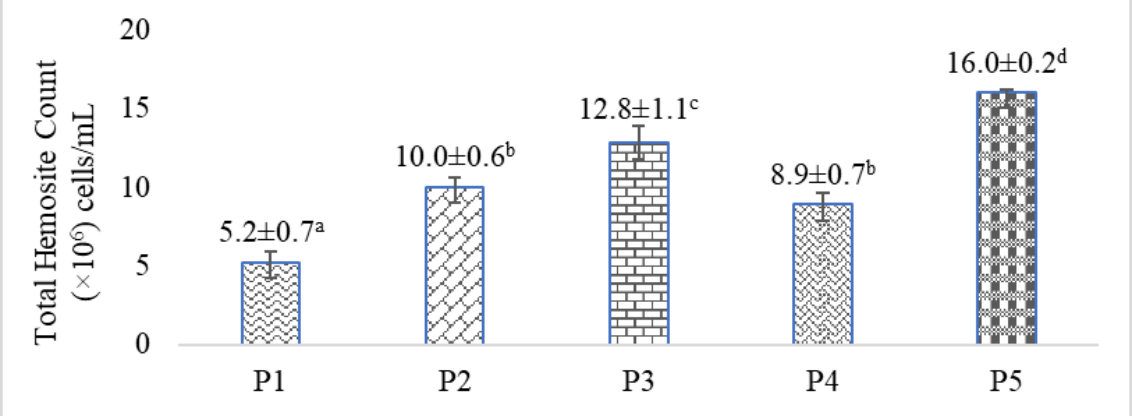

Figure 6. Total haemocyte count in Pacific white shrimp under different guava leaf supplementation treatments challenged with *Vibrio parahaemolyticus*.

defense system. The results indicated that the treatment significantly (P<0.05) affected the DHC value. The results showed that the percentage of hyaline in P1 differed significantly from those in P2, P3, P4, and P5. Meanwhile, P2 was not significantly different from P4. The highest value was found in P5 and the lowest in P1. The percentage of granulocytes in P1 was significantly different from those in P3 and P5, but not significantly different from those in P2 and P4. The highest value was found in P1 and the lowest in P5. Similar results were observed for the percentage of semi-granulocytes, indicating that P1 was significantly different from P5 and P3, but not from P2 and P4. The highest value was found in P1 and the

Table 1. Experimental design for different guava leaf supplementation treatments.

Treatment	Description
P1 (Positive Control)	Feed without guava leaf extract + bacterial infection
P2 (Negative Control)	Feed without guava leaf extract
P3	Feed + 5% guava leaf flour + bacterial infection
P4	Feed + 5% boiled-leaf extract of guava leaves + bacterial infection
P5	Feed + 5% blended guava leaf juice + bacterial infection

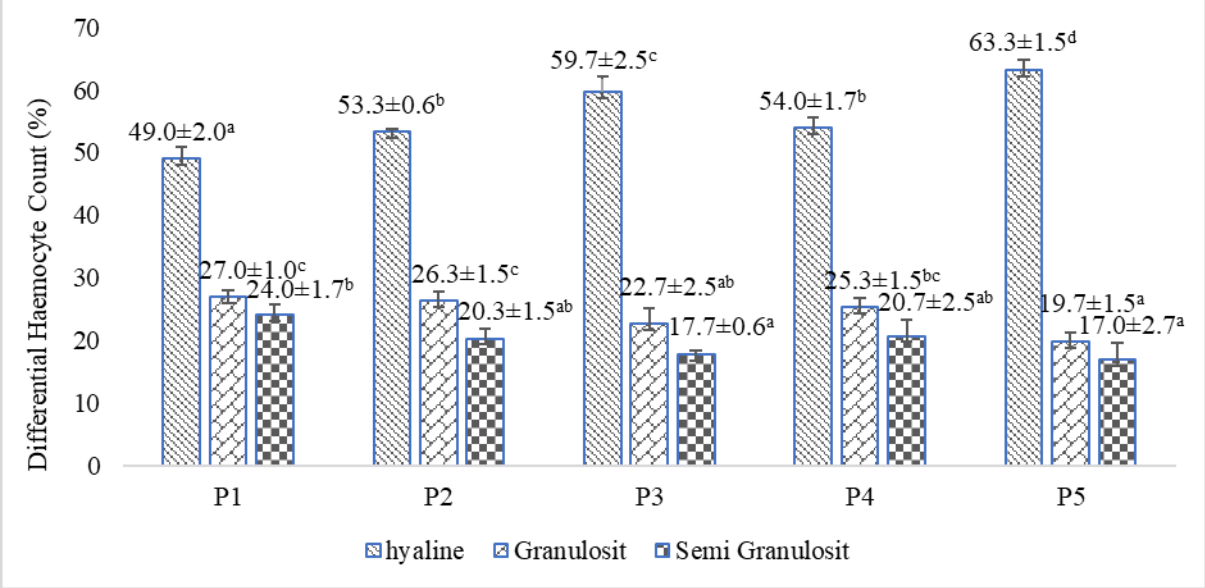

Figure 7. Differential haemocyte count in Pacific white shrimp under different guava leaf supplementation treatments challenged with *Vibrio parahaemolyticus*.

lowest in P5 (Fig. 7).

Phagocytosis activity (PA): The highest value was observed in P5, which differed significantly from P1, P2, P3, and P4. Meanwhile, the lowest value was observed in P1, which differed significantly from those of P2, P3, P4, and P5. The results for P2 and P4 were not significantly different, but were significantly different from those for P1, P3, and P5 (Fig. 8).

Water quality: The results of water quality testing conducted during the research are presented in Table 2.

Discussions

The highest percentage was found in P5 at 80%. Conversely, P1 obtained the lowest percentage, at 70,7%, which was not significantly different from those of P2, P3, and P4. This is presumed to occur because P5 produced the most optimal extract, thereby enhancing the shrimp's immune resistance and metabolic efficiency. The compounds within it act as antioxidants and immunostimulants, increasing immune responses and shrimp growth. This is indicated by the high THC, PA, and DHC values in P5. Thus, the shrimp become more resistant to stress and infection, resulting in a higher SR value in P5 compared to the other treatments. This is supported by Abdi et al. (2022), stating that the higher the THC, DHC, and PA values, the higher the shrimp's survival rate. Likewise, the study conducted by Farman et al. (2025) showed that the treatment with the highest THC concentration (17.67×10^6 cells/mL), hyaline cells at 61%, and PA at 69.98% had the highest survival rate of 77.78%.

A low FCR indicates that the feed has been efficiently converted into biomass. This efficiency can only occur when the shrimp's physiological condition is optimal, maximizing energy from feed for growth (Hasani et al., 2025). Based on the results, the lowest FCR was found in P5 at 1.1. The P5 treatment can be considered to provide the best feed conversion, which directly contributes to increased growth and more efficient production costs. This is presumed to occur

Table 2. Water quality during the rearing period of Pacific white shrimp under different guava leaf supplementation treatments challenged with *Vibrio parahaemolyticus*.

Parameter	Unit	Results (Range)	Optimal Range (75/PERMEN-KP/2016)
Temperature	°C	26.6-28.4	28 - 30
pH	-	7.5-7.8	7.5-8.5
DO	mg/L	5.9-8.5	> 4
Salinity	ppt	32	26-32
Ammonia	mg/L	0.05-0.2	≤ 0.1

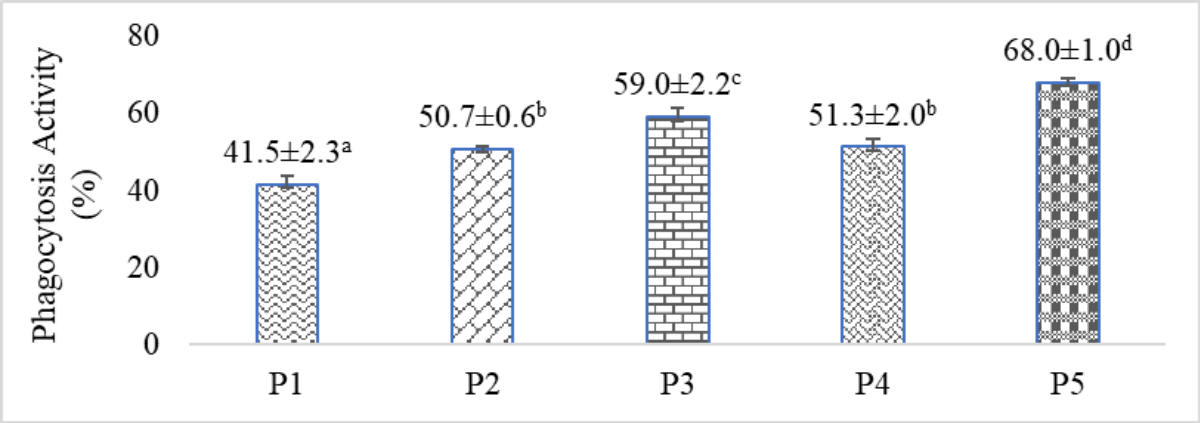

Figure 8. Phagocytosis activity in Pacific white shrimp under different guava leaf supplementation treatments challenged with Vibrio parahaemolyticus.

because administering guava leaf blender extract in the feed increases appetite, allowing more protein and fat to be converted into energy, thereby increasing shrimp growth. This is supported by Amin et al. (2011), who state that as fish metabolism increases, appetite is also stimulated, leading to increased feed consumption. If the feed is used efficiently, more protein can be stored, thereby promoting better growth. As reported in a study by Sadewa (2025) on the administration of turmeric blender extract in whiteleg shrimp feed, the lowest FCR value (1.21) was observed.

Based on the results, the highest SLGR was observed in P5 at 3%/day, significantly higher than in P3 at 2.9%/day. P3 was also significantly different from P4 at 2.9 %/day. All treatments differed significantly from P1 and P2. The SLGR results are consistent with the absolute length growth obtained. This is presumed to occur because the extraction method in P5 did not involve heating, thereby preventing damage to the protein structure and active compounds present in guava leaf extract. Thus, these active compounds can optimally stimulate digestive enzymes and significantly influence the SLGR value in P5. This is supported by the findings of Chairunnisa et al. (2019), who reported that high temperatures can damage active components during extraction. For example, Afandi et al. (2025) reported that excessively high extraction temperatures may degrade bioactive compounds in octopus's ink. Miandare et al. (2019) also added that the addition of immunostimulants in feed can stimulate digestive enzymes. Likewise, the study conducted by Sadewa (2025) showed that feed supplemented with turmeric blender extract at a dose of 5% produced an SLGR value of 4.33%/day, which was significantly higher than the control treatment at 3.73%/day.

Based on SWGR results, the highest percentage was observed in P5 at 8.2 %/day, which was not significantly different from P3 at 8.1%/day and P4 at 8.1%/day. The SWGR results align with the absolute weight growth obtained. Conversely, the lowest percentage was observed in P1 at 7.9%/day, which was not significantly different from that in P2 at 8%/day. This is supported by Darwantin et al. (2016), who reported that shrimp fed immunostimulants in

their feed exhibited better growth performance than those without immunostimulants. Likewise, the study conducted by Dewi et al. (2021) showed that administering immunostimulants in the form of guava leaves to whiteleg shrimp feed resulted in an SGR of 2.13%/day, which was higher than the control treatment at 1.87%/day.

According to Anjasmara et al. (2018), the optimum limit for the presence of *Vibrio* sp. in waters is 10^4 CFU/mL. If the bacterial concentration exceeds this threshold, it can trigger mass mortality in shrimp. The high and uncontrolled population of *Vibrio* can create conditions that threaten shrimp health and survival. Based on the results, the highest TVC media value was observed in P1 at 362.7×10^2 CFU/mL, followed by P4 at 166.3×10^2 CFU/mL. This is presumed to occur because when shrimp are infected by bacteria, their bodies are unable to defend themselves due to a weak immune response. As a result, bacterial growth becomes excessive, potentially causing bacteria to exit the shrimp's body and contaminate the rearing media. Excessively high bacterial density can be fatal and may cause mortality in shrimp. These shrimps will become carriers, causing the rearing media to have a high bacterial density as well. This is in line with Riana (2020), who stated that pathogens, such as bacteria, attack when the shrimp's nonspecific immune system is weakened. Meanwhile, the lowest value was found in P2, which was not significantly different from P5, at 20×10^2 CFU/mL. This is presumed because the high PA value in P5 is able to suppress bacterial growth in the shrimp's intestine, resulting in only a small number of bacteria exiting the shrimp's body and contaminating the rearing media. This is consistent with Rosyida et al. (2022), who stated that high phagocytic activity can suppress bacterial growth in the shrimp's intestine.

THC is closely associated with shrimp immunity, making it an important indicator for assessing immune responses. An increase in THC indicates that the immune response and the shrimp's ability to fight infection are getting better. Yeh et al. (2009) stated that the normal hemocyte count in healthy shrimp ranges from $1.8\text{-}9.28\times10^7$. Based on our findings, P3, P4, and P5 show higher THC values compared to the control treatment. This occurs because guava leaves contain active compounds that can stimulate hemocyte formation, thereby increasing shrimp immune response. According to Díaz-de-Cerio et al. (2017), guava leaf extract contains active compounds such as gallic acid, quercetin, guaijaverin, naringenin, avicularin, hyperin, ellagic acid, flavonoids, saponins, tannins, phenolics, and vitamin C, which possess antioxidant properties and can enhance shrimp immune response. The flavonoid content protects hemocytes from oxidative stress induced by *V. parahaemolyticus* infection.

P3 with a value of 12.8×10^6 cells/mL shows a fairly high THC, but compared to P5, this value is still lower. This occurs because guava leaf flour contains cellulose, which is the main structural component of leaves. This compound cannot be digested by the shrimp's very simple digestive system. In addition, shrimp are carnivorous animals, so they cannot produce the cellulase enzymes used to digest plant fibers. As a result, even though guava leaf flour contains abundant active compounds, the absorption of these compounds is not efficient. According to Lapanda et al. (2025), guava leaf flour contains trans-Caryophyllene (18.43%), 9,12-Octadecadienoic acid (15.3%), Nerolidol isomer (9.65%), and Alloaromadendrene (8.93%). This affects hemocyte stimulation, which is less optimal than in treatments using blender-extracted leaf material, whose compounds are more easily absorbed. This is supported by Lestari (2018), who stated that whiteleg shrimp lack digestive enzymes to break down cellulose, so it is excreted in feces. Agustono et al. (2016) also stated that high cellulose content affects the high crude fiber in the shrimp's digestive tract, resulting in a faster feed passage rate, which decreases the opportunity for the digestive tract to absorb the active compounds contained in the feed. P4 shows a lower value than P3 and P5, i.e., 8.9×10^6 cells/mL. This occurs because during the boiling process, thermolabile compounds such as flavonoids and vitamin C react at high temperatures, thereby decreasing their concentrations. This decrease in

concentration reduces the effectiveness of guava leaf extract in stimulating the immune response, resulting in fewer hemocytes being formed. According to *Syafrida et al. (2018),* the higher the temperature, the lower the antioxidant activity of flavonoids and vitamin C.

P5 shows the highest THC value at 16.03×10^6 cells/mL. This is presumed to occur because the blending method is able to extract more active compounds as it does not involve a heating process that can damage active compounds (Syafrida et al., 2018), and does not contain cellulose that can inhibit the absorption of nutrients and active compounds in the shrimp digestive tract (Lestari, 2018). The study conducted by Dewi et al. (2021) on the addition of guava leaf juice to whiteleg shrimp feed showed that treatments with guava leaf juice produced the highest THC values, reaching 34×10^6 cells/mL, compared to the control treatment, which reached only 27×10^6 cells/mL. P1, with a THC value of 5.2×10^6 cells/mL, shows the lowest value. This occurs because the shrimp were infected with *V. parahaemolyticus* without the administration of extracts, leaving no additional immunostimulant to stimulate hemocyte production. *Vibrio parahaemolyticus* infection can reduce hemocyte count because most hemocytes undergo lysis during phagocytosis, leading to a decrease in total hemocytes in the hemolymph. According to Pratiwi et al. (2016), when pathogens attack, hemocytes engage in phagocytosis and undergo lysis, resulting in decreased hemocyte counts in the hemolymph. Similarly, the study conducted by Febrianti et al. (2025) reported that shrimp not given immunostimulant feed produced the lowest THC of 5.01×10^6 cells/mL, whereas shrimp given kappa-carrageenan extract at a dose of 20 g/kg feed produced a THC of 14.09×10^6 cells/mL.

The hyaline cells, granulocytes, and semigranulocytes play distinct roles in the non-specific immune system of whiteleg shrimp (Rainbow et al., 2025). Hyaline cells are smaller than the other types; they have cytoplasm that lacks granules and play an important role in phagocytosis. Owens and O'Neil (1997) stated that the optimal percentage of hyaline cells for whiteleg shrimp ranges from 60 to 93% of total hemocytes. Based on the results of the current work, the highest hyaline composition, still within the normal range, is found in P5 at 63.3%. This occurs because the blender method optimally extracts guava leaves, as not involve heating and does not contain cellulose, allowing compounds such as flavonoids and saponins, which act as immunostimulants, to be fully absorbed and to stimulate hemocytes to induce hyaline cells, thereby enhancing phagocytic activity. This is consistent with the findings of Febrianti et al. (2025), who reported that an increase in hyaline cells can improve phagocytic activity. Conversely, the lowest hyaline composition is observed in P1, at 49%. When shrimp are attacked by disease, hyaline cells undergo lysis and oxidative stress during phagocytosis, preventing the production of additional hyaline cells. As a result, the composition of hyaline cells in the hemolymph decreases. Similarly, the study by Sadewa (2025) on adding turmeric extract via the blender method to whiteleg shrimp feed produced the highest hyaline cell value of 63.3%.

When pathogens infecting the shrimp body are too large to be phagocytosed by hyaline cells, semi-granulocyte cells will perform encapsulation. This mechanism begins when semigranulocytes attach to the pathogen's cell wall and release the enzyme prophenoloxidase (proPO), which catalyzes the formation of melanin, thereby isolating the infected area. This enzyme will also stimulate the release of phenoloxidase (PO) by granulocyte cells, which plays a role in pathogen destruction. Granulocytes also release the enzyme lysozyme, which can lyse bacterial cell walls. According to Darwantin et al. (2016), the normal percentage of semi-granulocyte cells in shrimp ranges from 13–49%, and the normal percentage of granulocyte cells ranges from 17-40% of total hemocytes. Based on our findings, granulocyte and semigranulocyte counts in each treatment remain within the normal range. This occurs because the *V. parahaemolyticus* bacteria used in the challenge test are small, allowing hyaline cells to still phagocytose them, so no increase in granulocyte and

semigranulocyte cells occurs.

Phagocytic activity is a defense mechanism carried out by phagocytic cells, such as hyaline cells, that engulf and destroy foreign particles, including bacteria, viruses, and cellular debris (Scabra et al., 2025). The mechanism of phagocytosis begins when hyaline cells recognize and attach to the pathogen's cell wall and then engulf it into a phagosome, after which the hyaline cells will produce enzymes that can destroy the pathogen. Based on the results of the current study, the highest phagocytic activity is observed in P5 at 68%, and the lowest in P1 at 41.5%. This occurs because of the high percentage of hyaline cells in the hemolymph of P5, which coincides with the high value of phagocytic activity. P5 is able to extract more active compounds from guava leaves, such as flavonoids, tannins, and saponins, as mentioned before. These compounds cause elevated THC values and hyaline cells in the shrimp hemolymph, which play an important role as phagocytes, thereby increasing phagocytic activity in P5. As a result, shrimp in P5 have an enhanced immune response that is more effective at destroying bacteria. Conversely, P1 had the lowest value, presumably because commercial feed contains no immunostimulants that can increase phagocytic activity; this also coincides with the low THC and hyaline cell values in P1. In addition, the shrimp in P1 were infected with *V. parahaemolyticus* without additional protection, resulting in physiological stress and decreased hemocyte function, including hyaline cells, which play a major role in phagocytosis. Febrianti et al. (2025) stated that an increase in hyaline cells can increase phagocytic activity. Sadewa (2025) showed that the treatment given to turmeric blender extract produced the highest hyaline cell value of 63.3%, which was directly proportional to the high PA value of 69.2%.

Water quality: Water quality plays a crucial role, particularly for health, growth, and survival (Faturrahman et al., 2025). Hutabarat et al. (2025) stated that the success of shrimp aquaculture depends on optimal water quality conditions, such as sufficient oxygen levels, appropriate salinity, pH, and water temperature. Temperature is one of the physical parameters of water quality that can affect shrimp metabolism and appetite. The temperature observed during this study ranged from 26.6 to 28.4°C, which is still within the optimal range. This is supported by Haliman and Adijaya (2005), who stated that temperatures between 26 and 32°C are optimal for whiteleg shrimp growth. Scabra et al. (2023) stated that pH can influence chemical reactions in the water and affect shrimp metabolism. The pH values in this study ranged from 7.5 to 7.8 and were still within the optimal range. According to Permen-KP (2016), the optimal pH for shrimp aquaculture ranges between 7.5 and 8.5. The DO values during this study ranged from 5.9 to 8.5 mg/L, which are within the optimal range. Based on Permen-KP (2016), the optimal DO level for shrimp cultivation is >4 mg/L. Salinity can influence osmoregulation, which is the shrimp's ability to balance the salt concentration inside its body with that of the surrounding environment. The salinity value obtained in this study was 32 ppt. According to Permen-KP (2016), the optimal salinity for shrimp farming ranges between 26 and 32 ppt. According to Permen-KP (2016), the optimal ammonia level in shrimp culture is <0.1 mg/L. However, the ammonia levels during the maintenance period ranged from 0.05 to 0.2 mg/L. This value is above the optimal threshold, although the 0.2 mg/L level did not occur frequently. Based on the data, whiteleg shrimp growth remained within the optimal range. This is presumably because adding guava leaves to the feed can enhance shrimp immunity, making them more resistant to environmental stress.

Conclusion

Based on the results of the study, adding guava leaf extract to the feed, using different extraction methods, enhanced the immune response and growth performance of whiteleg shrimp challenged with *V. parahaemolyticus*. The best treatment was obtained in P5, which showed optimal results for the parameters: SR of 80%, FCR of 1.1, SLGR of 3%/day, SWGR of 8.2%/day, and media TVC of 20×10^2 CFU/mL. The improvement in immune response was indicated by

the THC value of 16×10^6 cells/mL, DHC values (hyaline cells 63.3%, granulocytes 19.7%, semi-granulocytes 17%), and PA of 68%, all of which were higher than those of the other treatments.

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