ORIGINAL ARTICLE

Supplementation of Hairy Eggplant *(Solanum ferox)* and Bitter Ginger *(Zingiber zerumbet)* Extract as Phytobiotic Agents on Whiteleg Shrimp *(Litopenaeus vannamei)*

Statement of novelty: Overall, supplementation of Hairy Eggplant, *S. ferox* and Bitter Ginger, *Z. zerumbet* in feed ingredients was able to increase production performance and immunity parameters in Whiteleg Shrimp *(L. vannamei)*. The two extract ingredients have the potential to be developed and promoted as phytobiotic agents in Whiteleg Shrimp so as to reduce the use of environmentally unfriendly antibiotics.

Ethical approval (if needed): This study does not need ethical approval.

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Supplementation of Hairy Eggplant (Salanum ferox) and Bitter Ginger (Zingiber zerumbet) Extract as Phytobiotic Agents on Whiteleg Shrimp (Litopenaeus vannamei)

ABSTRACT

Objective: This study aimed to evaluate the combination of Hairy Eggplant *(Solanum ferox)* and Bitter Ginger *(Zingiber zerumbet)* on production performance and hematological parameters of Pacific Whiteleg Shrimp *(Litopenaeus vannamei).*

Materials and methods: Four treatments were formulated in the test feed, where P1 (control+commercial vitamin C); P2 (120 ml/L combination of *S. ferox* and *Z. zerumbet*); P3 (100 ml/L); and P4 (80 ml/L) 6,000 post-larvae shrimp with an average initial weight of 0.2 grams were randomly stocked in four groups, with three replications per treatment and 500 were stocked in each pond with a total of 12 ponds.

Results: Based on the results, there were significant differences in production performance (survival, absolute weight growth, specific growth rate (SGR), and feed conversion ratio (FCR)). Biologically, the best performance was found in the P3 treatment (100 ml/L). In this treatment, the total number of hemocytes and the number of hyaline hemocytes were much higher, and this was not the case in the control treatment (P1) where the number of semi-granular and granular cells was significantly higher than the treatment group *($p<0.05$).*

Conclusion: This study confirmed that supplementation of 100 ml/L of *S. ferox* and *Z. zerumbet* could improve production performance and hemato-immunological parameters of Whiteleg Shrimp with functional potential to be developed in phytobiotic-based commercial diets for shrimp.

Keywords: Growth, Haemocytes, Phytobiotics agent, Shrimp *Litopenaeus vannamei*

INTRODUCTION

Crustacean production has increased to 6.09 million tons or equivalent to USD 36.2 million due to increased consumption of shrimp commodities worldwide [1]. Whiteleg shrimp (*Litopenaeus vannamei)* is one of the most popular aquaculture commodities and is relatively easy to cultivate [2]. This can be proven by the high percentage of pacific whiteleg shrimp cultivation worldwide, which is around 90% [3]. This shrimp is able to adapt to a broad salinity (euryhaline) between 5 to 30 ppt, can be cultivated with a high stocking density, and is able to grow well with low protein feeds, making this shrimp a leading commodity in Indonesia.

However, an increase in shrimp production with unmatched implementation of water quality management, quality seeds, or superior broodstock to quality feed can have a negative impact on shrimp productivity by causing various diseases. Several disease outbreaks in shrimp ponds related to viral diseases are white spot syndrome virus (WSSV) and Taura syndrome virus (TSV), while bacterial diseases are *Vibrio harveyi*, *V. alginolyticus*, and *V. parahaemolyticus* which can cause huge losses to shrimp hatcheries worldwide [4].

The Whiteleg Shrimp immune system is also an important focus in the emergence of a disease in the aquaculture environment. Crustaceans, especially shrimp, only have a non-specific and humoral natural immune system [5]. This causes the growth and immunity of shrimp to be increased depending on the quality of the broodstock, the environment, and the application of biocontrol genes [6,7].

Until now, efforts to control bacterial diseases are still through antibiotics and chemotherapy agents [8,9]. However, the use of antibiotics in an unwise manner can lead to the accumulation of residues in the tissue which results in a decrease in product quality [10], the emergence of antibiotic resistance problems in shrimp and other aquatic organisms [11], to pollution of the aquatic environment [12]. Therefore, the use of environmentally friendly materials is needed to control disease in the current aquaculture system. Several approaches such as the use of pro-, pre- and synbiotics [13], immunostimulants [14], vaccination [15], quorum-sensing [16], phage application [17], RNA interference (RNAi) [18], development of molecular-based diagnostic materials [19], to the breeding and spawning of *Specific Pathogen Free* (SPF) shrimp [20] have attracted the attention of researchers and shrimp farmers.

The development of immunostimulant materials is the best effort to improve the shrimp immune system [21]. In addition to being compatible with the shrimp immune system, which is still primitive (non-specific immunity), immunostimulants also have several advantages such as abundant sources of materials, wide target range, the potential for large-scale application, and environmental friendliness.

One of the natural agents that can be used as immunostimulants to increase the immune system of Whiteleg Shrimp are Hairy Eggplant *(Solanum ferox)* and Bitter Ginger *(Zingiber zerumbet)* [22]. Hairy eggplant or *S. ferox* is widely planted in tropical areas such as Indonesia for its fruit. This plant has been listed as a medicinal plant in the ethnobotanical inventory because it is reported to be effective in treating human diseases. Hairy Eggplant contains bioactive compounds such as phenolics, flavonoids, and polyphenols that play an important role in preventing oxidative stress and several biological effects such as antioxidant and anti-inflammatory [23]. Another traditional plant such as *Zingiber zerumbet* (L) which is part of the Zingiberaceae family is commonly found in Southeast Asia such as Indonesia. This plant is used traditionally in various cuisines and drinks because it is reported to have anti-allergic properties found in its rhizomes [24].

Previous studies showed that the combination of Zingiber zerumbet and Curcuma zedoria added to the Orange-spotted grouper (*Epinephelus coioides*) was able to act as an immunostimulant by increasing non-specific immune responses (respiratory burst activity, reactive oxygen species, phagocytic activity, superoxide dismustase, and lysozyme activity [25]. Hardi [26] explained that the combination of *Boesenbergia pandurate, Zingiber zerumbet*, and *Solanum ferox* are effective in preventing infection with *Aeromonas hydrophila* and *Pseudomonas* sp. and modulating the immune system of tilapia *Oreochromis niloticus*.

Based on this report, this study aimed to evaluate the combination of Hairy Eggplant (*Solanum ferox)* and Bitter Ginger *(Zingiber zerumbet)* in increasing the production performance and immune system of Whiteleg Shrimp (*L. vannamei*). This study is expected to provide valuable preliminary information for the application of environmentally friendly immunostimulants in increasing the production of pacific white shrimp.

MATERIALS AND METHODS

Ethical approval: This study does not need ethical approval.

Study area and period

This study was conducted from September 2020 – Desember 2020 at the Loka Pengelolaan Sumber Daya Pesisir & Laut (PSPL) Serang, Directorate General of Marine Space Management, Ministry of Marine and Fisheries, Republic of Indonesia.

Study Design

This study used a completely randomized design (CRD) with four treatments and three replications. The treatments used were a combination of Hairy Eggplant (*S. ferox*) and Bitter Ginger (*Z. zerumbet*) with the following details:

P1 : no combination + vitamin C (control);

P2 : combination of *S. ferox* and *Z. zerumbet* with a dose of 120 ml/L;

P3 : combination of *S. ferox* and *Z. zerumbet* with a dose of 100 ml/L;

P4 : combination of *S. ferox* and *Z. zerumbet* with a dose of 80 ml/L

Preparation of *S. ferox* and *Z. zerumbet*

Preparation of extract refers to the study by Hardi et al. [27]. In short, the plants were collected from traditional markets. The materials were washed and dried in an oven at a temperature of 40-45°C for 48 hours. The dry ingredients were then mashed using a blender to form a fine powder. 100 g of dry samples were mixed with 100 ml of 96% ethanol in an Erlenmeyer flask at room temperature for 72 hours. The mixture was separated using 0.5 m Whatman filter paper to obtain the extract filtrate. The filtrate was re-evaporated using a rotary evaporator to separate the ethanol content for 3-5 hours. The extract was stored in the refrigerator for further testing.

Rearing of whiteleg shrimp

Rearing of Whiteleg Shrimp was carried out in a round tarpaulin container with a pool diameter of 2 meters and a height of 1 meter with a total of 12 pools. Each pond has nine aeration points which are evenly distributed in the rearing pond (Figure 1). The extract was mixed in the feed adjusted to the dose in each treatment. Feeding was carried out four times a day at 07.00 a.m; 11.00 a.m.; 15.00 p.m.; and 19.00 p.m. The amount of feed given was adjusted to the Feeding rate (FR) according to the Indonesian National Standard (SNI) No. 01-7246-2006 namely 15% to 10%.

Figure 1. Rearing pond of *L. vannamei* in this study

Production performance parameters

Survival rate

The survival rate of whiteleg shrimp was calculated at the end of the rearing period. The calculation was carried out according to the equations of Ly et al. [28] and Wiradana et al. [29] namely:

$$SR(\%) = \frac{Nt}{No} \times 100\%$$

Note :

SR : Survival rate (%)

Nt : Number of shrimp at the end of the study (individual)

No : Number of shrimp at the beginning of the study (individual)

Absolute Weight Growth

Weight growth was observed every seven days by weighing the number of shrimp. Absolute weight growth based on Fendjalang et al. [30] is as follows:

$$\Delta W = Wt - Wo$$

Note :

ΔW : Absolute weight growth (gr)

Wt : Final shrimp weight (gr)

Wo : Initial shrimp weight (gr)

Specific Growth Rate (SGR)

The specific growth rate was observed once a week based on Widanarni et al. [31]:

$$SGR(\%/day) = \frac{LnWt - LnWo}{T} \times 100\%$$

Note :

$SGR(\%/day)$: Specific growth rate

Wt : Final shrimp biomass (gr)

Wo : Initial shrimp biomass (gr)

T : Rearing period (days)

***Feed Convertion Ratio* (FCR)**

FCR was calculated at the end of the rearing period based on Zubaidah et al. [32]:

$$FCR = \frac{\Sigma F\ pakan\ yang\ diberikan - \Sigma F\ sisa\ pakan}{(Bt + Bm) - Bo}$$

Note :

FCR : Feed convertion ratio (gr)

ΣF : Feeding amount (gr)

Bt : Final shrimp biomass (gr)

Bm : Dead shrimp biomass (gr)

Bo : Initial shrimp biomass (gr)

Water quality

To maintain the media quality, daily draining of water was carried out [33]. Water quality parameters measured include temperature, dissolved oxygen (DO), pH, Ammonia, Nitrate, and Nitrite. Water quality parameters are used as supporting data in this study.

Immunity parameters

The evaluation of Whiteleg Shrimp immune response was performed by calculating the total hemocyte count (THC) and differential hemocyte counts (DHC). Collection and preparation of shrimp hemolymph were carried out using the procedure by Zahra et al. [34]. Analysis of THC and DHC was carried out by taking 0.1 ml of hemolymph in the fifth pereopod using a 1 ml syringe filled with 0.3 ml of Na-EDTA anticoagulant to prevent blood clots. The sample was homogenized for 5 minutes in a moistened microtube with 10% Na-EDTA. Hemolymph was dripped on the hemocytometer and closed using a cover glass. Calculation of the number and type of cells was carried out under a light microscope with a magnification of 400×. Observation of immune parameters was carried out at the end of the rearing period (56 days). Calculation of THC and DHC was referring to the method by Suleman et al. [35] and Widanarni et al. [36].

$$THC = average\ \Sigma\ counted\ cells \times 250 \times dillution\ factor \times 1000$$

$$DHC\ (\%) = \frac{\Sigma A}{\Sigma B} \times 100$$

Note :

ΣA : The number of each hemocyte cell type

ΣB : Total hemocyte count

Histological analysis

The gill tissue was placed in Davidson's solution until dehydrated, embedded in paraffin, and sliced using a microtome. Tissue was stained using hematoxylin and eosin and analyzed to identify defects such as hyperplasia, vacuolation, and necrosis [37]. The histology was shown in Figures defined by Bonami et al. [38].

Data analysis

Production performance parameters and immunity were tabulated using Ms. Excel 2019 (Microsoft, USA). Statistical analysis was carried out using SPSS Version 25 software (IBM, USA). The data underwent a homogeneity test followed by Single Factor ANOVA with a 95% confidence interval. To find out the differences in each treatment, the data underwent the DUNCAN test. The statistical results were interpreted and presented in the form of tables and figures.

RESULTS AND DISCUSSION

Production performance

Supplementation of Hairy Eggplant and Bitter Ginger in feed can increase absolute weight growth in Whiteleg Shrimp. The highest absolute weight growth was 9.19 g/individual and found in P3, then P4 (6.97 g/individual), and P2 (6.03 g/individual), and P1 (Control) (5.82 g/individual) (Table 1). Weight growth can occur from the energy entering the body. The energy obtained from feed ingredients will first be used for rearing activities, then the remaining energy will be used for the growth process [39]. The absolute weight growth of shrimp with Hairy Eggplant and Bitter Ginger was higher than the control Whiteleg Shrimp. This occurs due to the supplementation of Hairy Eggplant and Bitter Ginger which can stimulate the process of absorption of feed nutrients, especially due to protein and bioactive content. Bioactive content such as flavonoids in traditional plants can act as antibacterial and antioxidant which can minimize the increase in the number of pathogenic microflora in the digestive tract so that it is thought to increase the digestibility of Whiteleg Shrimp [40–42].

Table 1. Production performance parameters of Whiteleg Shrimp

The best specific growth rate (SGR) was found in P3 with a growth of 6.69%/day showing doses of Hairy Eggplant and Bitter Ginger had significant differences ($p<0.05$) on specific growth rate on Whiteleg Shrimp. Increased growth rate can occur due to optimal use of feed ingredients. On the other hand, a low growth rate can occur due to health problems, stress, and suboptimal utilization of feed nutrients used for growth [43].

The lowest FCR value was found in P3, then P2 and P4 respectively. The highest FCR (1.51) was found in the control treatment (Table 1). FCR was inversely proportional to weight growth, so the lower the FCR, the higher the efficiency of the shrimp in utilizing feed for growth [44]. On the other hand, if the shrimp body is in an unstable state, the shrimp may experience a decrease in appetite, and the feed provided is not converted into biomass [45]. Healthy shrimp can be better at converting feed ingredients for weight growth compared to stressed or sick shrimp.

The highest survival rate was found in P3 (90.66%) and of all treatments, the lowest percentage was found in the control treatment (71.92%) (Table 1). This indicated that supplementation of Hairy Eggplant and Bitter Ginger on Whiteleg Shrimp had a significant difference in the survival rate of Whiteleg Shrimp maintained for 56 days. This is presumably due to the content of secondary metabolites in Hairy Eggplant and Bitter Ginger which can increase the immune system against pathogenic bacterial infections to protect the shrimp body from stress. Huang et al.[46] explained that the biological process will increase due to the involvement of phytochemical substances produced by the extract ingredients capable of producing enzymes as detoxification, modulating the immune system, and increasing the survival of shrimp. The increased immune response can have a good impact on increasing body resistance and reducing the number of shrimp mortality. This was confirmed by Jasmanindar et al. [47] which found that the low survival rate in the treatment without the extract had a relationship with a weak immune system compared to the treatment with seaweed extract.

Water quality

All variables observed in the rearing pond remained within the levels recommended for shrimp culture during the test period (Table 2).

Table 2. Water quality parameters during the rearing period

Immunity parameters

The hemato-immunological response is a major physiological mechanism playing a role in protecting animals from disease, environmental stressors, or certain biological agents [48] such as phytobiotics. In this study, total hemocyte count (THC) and differential hemocyte count (DHC) were performed to evaluate the immunity condition of Whiteleg Shrimp with Hairy Eggplant and Bitter Ginger during 56 days of rearing. Based on the results, the total hemocytes of Whiteleg Shrimp in all treatments during the rearing period was around 4.63 – 16.76 ($\times 10^3$ cells/mm^3). The highest THC value was found in P3 and was significantly different when compared to the other three treatments ($P<0.05$). P1 had the lowest THC value of all treatments. P2 and P3 had no significant differences (Table 3). Hemocytes are the main mediators of cellular responses in crustaceans with roles that include self-recognition, phagocytosis, production of reactive oxygen intermediates, wound healing, to the process of melanization by encapsulation of foreign materials [49–51]. The increase in THC of Whiteleg Shrimp with Hairy Eggplant and Bitter Ginger can be suspected by the effect of bioactive compounds that can modulate shrimp immunity. Other studies confirmed that natural hydrolyzed tannin products from sweet chestnut *(Castanea sativa)* are able to act as functional feed additives by promoting growth and hematological parameters of Whiteleg Shrimp [52]. The in vivo effect of Astragalus polysaccharide immunostimulating ingredients, chlorogenic acid, and berberine showed a higher increase in total hemocyte count in Whiteleg Shrimp [53].

The difference in THC values in each treatment may be due to the various concentrations of active ingredients in food due to the treatment that has been determined. The low value of THC at P1 (control) may be influenced by physiological factors such as the slow formation of hemocytes in the shrimp body [54,55]. Interesting discussion confirmed that the low number of shrimp hemocytes is due to infiltration of regenerated tissue and hemocytes cell death due to apoptosis [32].

Table 3. Immunity parameters (THC and DHC) of Whiteleg Shrimp with Hairy Eggplant and Bitter Ginger

Differential hemocyte count (DHC) consists of hyaline as the smallest component of shrimp hemocytes, where granular and semi-granular cells are the other two types of hemocytes. The highest percentage of hyaline was found in P3 (48.67%), followed by P4 (34.67%), P2 (31.33%), and P1 (24.33%) (Table 3). The lowest number of semi-granular cells was found in P3 (23.67%). The decreasing number of semi-granular cells was shown in all treatments except control (P1) with the highest number (Table 3). The same thing happened to the number of granular cells in the treatment group. The highest number of granular cells was found in P1/control (40.33%).

Hyaline cells have a vital role in the shrimp defense system. This cell type has a high ratio of cytoplasmic nuclei and few cytoplasmic granules. An increase in the number of hyaline cells can be associated with phagocytic activity when in contact with antigens or immunostimulating substances will stimulate the body's defense activity so that it will evoke the first defense response

[56]. Semi-granular cells have a relationship with the addition or reduction of hyaline cells so that the decrease in the number of semi-granular cells in the treatment group was due to the process of further development into hyaline cells. As a result, these cells cannot develop into semi-granular cells so that the number of semi-granular cells is low [56]. Semi-granular cells are more involved in the encapsulation mechanism. The encapsulation process is a defense reaction against a large number of foreign particles that are not able to be phagocytized by Hyaline cells. These cells respond more to polysaccharide compounds found in bacterial cell walls [57].

Granular cells are the type of hemocytic cells that have the largest size with an active nucleus in the storage process until the release of prophenoloxidase (proPO) and cytotoxicity. In this study, an increase in granular cells also occurred in the control group (P1). This is due to the low number of hyaline cells involved in the first defense process, thus relying on granular cells for non-specific body defense which is driven by the influence of immunostimulatory components such as vitamin C [58].

Histologi analysis

The condition of gill tissue on Whiteleg Shrimp showed that treatment P1 (control) and P3 experienced vacuolation and hyperplasia (Figure 2), followed by treatment P2 which only experienced hyperplasia, and treatment P4 experienced vacuolation and necrosis.

Figure 2. Gill tissue histology of Whiteleg Shrimp *(L. vannamei)*

The histology can explain that gill tissue damage occurred in all treatments carried out. Physiological differences of each individual Whiteleg Shrimp can cause tissue damage, even though there is an intervention of extract ingredients. High hemocytes can indicate infection or stress factors can cause this, although further studies are still needed to prove this. Clogged blood flow (caused by physical trauma, pollutants, or other physiological disturbances) in the lamellae can cause edema (swelling of cells) between the blood vessels and the epithelial lining of the primary lamellae [59]. Miller and Zachary [60] explained that necrosis is acute cell damage and can be massive, resulting in incomplete tissue formation due to shrinkage or complete shrinkage of the nucleus. Hyperplasia is the formation of excessive tissue due to an increase in the number of cells so that lamellae with hyperplasia will experience thickening of epithelial tissue at the ends of the filaments or the epithelium located near the base of the gill lamellae [61].

Based on the results, 100 ml/L combination of Hairy Eggplant and Bitter Ginger in feed is very promising for Whiteleg Shrimp, related to the production performance and immunity. However, further studies are still needed to emphasize the potential that this combination of extracts can provide against pathogenic bacteria and viruses in Whiteleg Shrimp.

CONCLUSION

Supplementation of Hairy Eggplant and Bitter Ginger into feed was able to affect the production performance of Whiteleg Shrimp as indicated by absolute weight growth, specific growth rate, low feed conversion ratio (FCR), and high survival as shown in P3 (dose 100 ml/L). Similarly, the immunity parameters which include THC and DHC were high in the P3 treatment group, although there was a tendency for higher semi-granular and granular cell values in the control group. All these results indicated that the combination of Hairy Eggplant and Bitter Ginger as phytobiotic ingredients can act as a good modulator of the non-specific immune response of Whiteleg Shrimp which ultimately increases production performance. Further studies are still needed to obtain valuable information regarding the combination of this extract against infection with Whiteleg Shrimp pathogens such as viruses and bacteria so that it can be used as a preventive agent.

LIST OF ABBREVIATIONS

WSSV: White Spot Syndrome Virus; ppt: parts per trillion; RNAi: RNA interference; SPF: Specific Pathogen Free; ml: milliliter; L: Liter; °C: degrees Celcius; gr: gram; µm: micrometer; a.m.; ante meridiem; p.m.: post meridiem; FR: Feeding Rate; SNI: Standard National Indonesia; SR: Survival Rate; SGR: Specific Growth Rate; FCR: Feed Convertion Ratio; DO: Dissolved Oxygen; THC: Total Haemocyte Count; DHC: Differential Haemocytes Counts; proPO: proPhenoloxidase; SPSS: Statistical Package for the Social Sciences; ANOVA: Analysis of Variance.

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CONFLICT OF INTERESTS

The author should declare any conflict of interest.

AUTHORS' CONTRIBUTION

SR designed the study, supervised, and conducted critical checking of this manuscript. DR Interpreted the results and reviewed the manuscript. MATV conducted the study, collected the data, and contributed in manuscript preparation. PAW drafted the manuscript, took part in preparing, and performed critical checking of manuscript. All authors read and approved the final manuscript.

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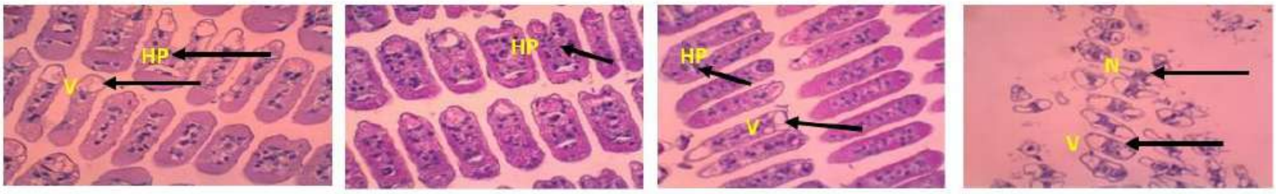
Figure 1. Rearing pond of *L. vannamei* in this study



A. Control (P1) B. 120 ml/L (P2) C. 100 ml/L (P3) D. 80 ml/L (P4)

Figure 2. Histology of gill tissue of whiteleg shrimp (*L. vannamei*). *HP: Hyperplasia; V: Vacuolization; N: Necrosis.

Table 1. The results of the analysis of the production performance parameters of whiteleg shrimp *(L.vannamei)*

Treatments group	Parameter			
	Absolute Growth (g)	SGR (%)	FCR	SR (%)
P1	5.82 ± 0.12[a]	5,49±0,04[a]	1.51±0,05[c]	71.92±1,76[a]
P2	6.03 ± 0.06[a]	5,67±0,07[a]	1.45±0,03[c]	76.95±3,03[a]
P3	9.19 ± 0.52[c]	6,69±0.18[c]	0.90±0,08[a]	90.66±4,61[b]
P4	6.97 ± 0.06[b]	5,93±0,03[b]	1.27±0,01[b]	77.09±1,40[a]

Note: The mean±SD (Standard deviation) values with different superscript letters showed significantly different results ($P<0.05$). *SGR: Specific Growth Rate; FCR: Feed Convertion Ratio; SR: Survival Rate.

Table 2. Water quality parameters during the maintenance period

Parameter	This study	Kisaran Ideal*
pH	6,5-8,5	7,5-8,5
Temperature (°C)	27-33	28 – 30
Dissolved Oxygen (mg/L)	3,8-4,0	≥ 4
Ammonia (mg/L)	0- 0,1	≤ 0,1
Nitrite (mg/L)	0-0,52	≤ 1
Salinity	29,7-33	26-32
Nitrate (mg/L)	0-0,3	0,5

* Ministry of Marine Affairs and Fisheries Regulation (KKP), Republic of Indonesia 2016

Table 3. Immunity parameters (THC and DHC) of Whiteleg Shrimp with Hairy Eggplant and Bitter Ginger

Treatment Groups	THC ($x\ 10^3$ sel/mm^3)	Hialin (%)	Semi Granular (%)	Granular (%)
P1	4.63 ± 2.05^a	24.33 ± 2.08^a	35.33 ± 1.52^c	$40.33 \pm .0.57^d$
P2	8.63 ± 0.87^b	31.33 ± 1.52^b	31.00 ± 1.00^b	$37.67 \pm .0.57^c$
P3	16.76 ± 0.90^c	48.67 ± 4.04^c	23.67 ± 3.51^a	27.67 ± 0.57^a
P4	9.96 ± 1.75^b	34.67 ± 1.52^b	$30.33 \pm .0.57^b$	35.00 ± 1.73^b

Note: The mean values with different superscript letters showed significantly different results ($P<0.05$). *THC : Total Haemocyte Counts

2. NASKAH AKHIR SAAT ACCEPTED

Original Article

Supplementation of hairy eggplant *(Solanum ferox)* and bitter ginger *(Zingiber zerumbet)* extract as phytobiotic agents on whiteleg shrimp *(Litopenaeus vannamei)*

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ABSTRACT

Objective: This study aimed to evaluate the combination of hairy eggplant (*Solanum ferox*) and bitter ginger (*Zingiber zerumbet*) on the production performance and hematological parameters of whiteleg shrimp (*Litopenaeus vannamei*).
Materials and Methods: Four treatments were formulated in the test feed, where P1 (control+commercial vitamin C); P2 (120 ml/l combinations of *S. ferox* and *Z. zerumbet*); P3 (100 ml/l); and P4 (80 ml/l) 6,000 post-larvae shrimp with an average initial weight of 0.2 gm were randomly stocked in four groups, with three replications per treatment and 500 were stocked in each pond with a total of 12 pounds.
Results: Based on the results, there were significant differences in production performance [survival, absolute weight growth, specific growth rate (SGR), and feed conversion ratio (FCR)]. Biologically, the best performance was found in the P3 treatment (100 ml/l). In this treatment, the total number of hemocytes and the number of hyaline hemocytes were much higher, and this was not the case in the control treatment (P1), where the number of semi-granular and granular cells was significantly higher than the treatment group ($p < 0.05$).
Conclusion: This study confirmed that supplementation of 100 ml/l of *S. ferox* and *Z. zerumbet* could improve the production performance and hemato-immunological parameters of whiteleg shrimp, with functional potential to be developed in phytobiotic-based commercial diets for shrimp.

Keywords: Growth; Haemocytes; Phytobiotics agent; Shrimp *Litopenaeus vannamei*

INTRODUCTION

Crustacean production has increased to 6.09 million tons, or equivalent to USD 36.2 million, due to increased consumption of shrimp commodities worldwide [1]. Whiteleg shrimp (*Litopenaeus vannamei)* are one of the most popular aquaculture commodities and are

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relatively easy to cultivate [2]. This can be proven by the high percentage of Pacific whiteleg shrimp cultivation worldwide, at around 90%. This shrimp can adapt to a broad salinity (euryhaline) between 5 to 30 ppt, cultivate with a high stocking density, and grow sufficiently with low protein feeds, making this shrimp a leading commodity in Indonesia.

However, an increase in shrimp production with unmatched water quality management, quality seeds, or superior broodstock to quality feed can harm shrimp productivity by causing various diseases. Several disease outbreaks in shrimp ponds are related to viral diseases like the white spot syndrome virus (WSSV) and Taura syndrome virus (TSV). In contrast, bacterial diseases like *Vibrio harveyi*, *V. alginolyticus*, and *V. parahaemolyticus* can cause considerable losses to shrimp hatcheries worldwide [3].

The whiteleg shrimp's immune systems are also an essential focus in the emergence of a disease in the aquaculture environment. Crustaceans, especially shrimp, only have a non-specific and humoral natural immune system [4]. This causes the growth and immunity of shrimp to be increased depending on the quality of the broodstock, the environment, and the application of biocontrol genes [5,6].

Currently, antibiotics and chemotherapy agents are still used to control bacterial diseases [7, 8]. However, the use of antibiotics unwisely can lead to the accumulation of residues in the tissue, resulting in a decrease in product quality, the emergence of antibiotic resistance problems in shrimp and other aquatic organisms [9], and pollution of the aquatic environment [10]. Therefore, the use of environmentally friendly materials is needed to control disease in the current aquaculture system. Several approaches, such as the use of pro-, pre-, and synbiotics [11], immunostimulants [12], vaccination [13], quorum-sensing, phage application [14], RNA interference (RNAi) [15], development of molecular-based diagnostic materials, and the breeding and spawning of specific pathogen free (SPF) shrimp [16], have attracted the attention of researchers and shrimp farmers.

Phytobiotics are functional additives derived from nature, including food supplement ingredients that have become an alternative to improve health and resistance to disease attacks that are ecologically correct in the modern aquaculture sector [17]. The development of phyobiotics materials is the best effort to improve the shrimp immune system. In addition to being compatible with the shrimp immune system, which is still primitive (non-specific immunity), phytobiotics also have several advantages such as abundant sources of materials, wide target range, the potential for large-scale application, and environmental friendliness. Some of the natural agents that can be used as phytobiotics to increase the immune system of whiteleg shrimp are hairy eggplant *(Solanum ferox)* and Bitter Ginger *(Zingiber zerumbet)* [18]. Hairy eggplant or *S. ferox* is widely planted in tropical areas such as Indonesia for its fruit. This plant has been listed as a medicinal plant in the ethnobotanical inventory because it is reported to be effective in treating human diseases. Hairy eggplant contains bioactive compounds such as phenolics, flavonoids, and polyphenols that play an essential role in preventing oxidative stress and several biological effects such as antioxidant and anti-inflammatory [19]. Another traditional plant such as *Zingiber zerumbet* (L), part of the Zingiberaceae family, is commonly found in Southeast Asia, such as Indonesia. This plant is used traditionally in various cuisines and drinks because it is reported to have anti-allergic properties found in its rhizomes.

Previous studies showed that the combination of *Zingiber zerumbet* and *Curcuma zedoria* added to the orange-spotted grouper (*Epinephelus coioides*) was able to act as an

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immunostimulant by increasing non-specific immune responses (respiratory burst activity, reactive oxygen species, phagocytic activity, superoxide dismustase, and lysozyme activity [20]. Hardi et al [21] explained that the combination of *Boesenbergia pandurate*, *Zingiber zerumbet*, and *Solanum ferox* effectively prevents infection with *Aeromonas hydrophila* and *Pseudomonas* sp., and modulates the immune system of tilapia, *Oreochromis niloticus*. However, studies highlighting the potential of phytobiotics have not been reported in white shrimp, although compound derivatives of phytobiotics have been previously reported [22].

Based on this report, this study aimed to evaluate the combination of hairy eggplant (*Solanum ferox)* and bitter ginger *(Zingiber zerumbet)* in increasing the production performance and immune system of whiteleg shrimp (*L. vannamei*). This study is expected to provide valuable preliminary information for applying environmentally friendly immunostimulants to increase pacific white shrimp production.

MATERIALS AND METHODS

Ethical approval

This research has been licensed by the Fisheries Expert Polytechnic (AUP), Marine and Fisheries Research and Human Resources Agency (BRSDM), Ministry of Marine Affairs and Fisheries (KKP) of the Republic of Indonesia with No: 1832/POLTEK-AUP/TU.210/IX/2020.

Study area and period

This study was conducted from September 2020 to December 2020 at the Loka Pengelolaan Sumber Daya Pesisir & Laut (PSPL) Serang, Directorate General of Marine Space Management, Ministry of Marine and Fisheries, Republic of Indonesia.

Study design

This study used a Completely Randomized Design (CRD) with four treatments and three replications. The treatments used were a combination of hairy eggplant (*S. ferox*) and bitter ginger (*Z. zerumbet*) with the following details:

P1 : no combination + vitamin C (control);
P2: the combination of *S. ferox* and *Z. zerumbet* with a dose of 120 ml/l;
P3: the combination of *S. ferox* and *Z. zerumbet* with a dose of 100 ml/l;
P4: the combination of *S. ferox* and *Z. zerumbet* with a dose of 80 ml/l.

Preparation of *S. ferox* and *Z. zerumbet*

Preparation of the extract refers to the study by Hardi et al. [21]. In short, the plants were collected from traditional markets. The materials were washed and dried in an oven at 40-45°C for 48 h. The dry ingredients were then mashed using a blender to form a fine powder. 100 gm of dry samples were mixed with 100 ml of 96% ethanol in an Erlenmeyer flask at room temperature for 72 h. The mixture was separated using 0.5 m of Whatman filter paper to obtain the extract filtrate. The filtrate was re-evaporated using a rotary evaporator to separate the ethanol content for 3-5 h. The extract was stored in the refrigerator for further testing.

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Rearing of whiteleg shrimp

Rearing of whiteleg shrimp was carried out in a round tarpaulin container with a pool diameter of 2 meters and a height of 1 meter, for a total of 12 pools. Each pond has nine aeration points evenly distributed in the rearing pond (Fig. 1). The extract was mixed into the feed and the dose was adjusted for each treatment. Feeding was carried out four times a day at 07.00 a.m., 11.00 a.m., 3.00 p.m., and 7.00 p.m. The amount of feed given was adjusted to the feeding rate (FR) according to the Indonesian National Standard (SNI) No. 01-7246-2006, namely 15% to 10%.

Figure 1. Rearing pond of *L. vannamei* in this study

Production performance parameters

Survival rate

The survival rate of whiteleg shrimp was calculated at the end of the rearing period. The calculation was carried out according to the equations of Wiradana et al. [23] and Fendjalang et al.[24], namely:

$$SR(\%) = \frac{Nt}{No} \times 100\%$$

Note:
SR: Survival rate (%)
Nt: Number of shrimp at the end of the study (individual)
No: Number of shrimp at the beginning of the study (individual)

Absolute weight growth

Weight growth was observed every seven days by weighing the number of shrimp. Absolute weight growth based on Widanarni et al. [25] is as follows:

$$\Delta W = Wt - Wo$$

Note :
Δ*W*: Absolute weight growth (gm)
Wt: Final shrimp weight (gm)
Wo: Initial shrimp weight (gm)

Specific growth rate (SGR)

The specific growth rate was observed once a week based on Zubaidah et al. [26]:

$$SGR(\%/day) = \frac{LnWt - LnWo}{T} \times 100\%$$

Note:
SGR(%/day): Specific growth rate
Wt: Final shrimp biomass (gm)
Wo: Initial shrimp biomass (gm)
T : Rearing period (days)

***Feed conversion ratio* (FCR)**

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FCR was calculated at the end of the rearing period based on Sarjito et al. [27]:

$$FCR = \frac{\Sigma F\ feed\ given - \Sigma F\ leftover\ feed}{(Bt + Bm) - Bo}$$

Note :
FCR: Feed conversion ratio (gm)
Σ*F*: Feeding amount (gm)
Bt: Final shrimp biomass (gm)
Bm: Dead shrimp biomass (gm)
Bo: Initial shrimp biomass (gm)

Water quality

To maintain the media quality, daily draining of water was carried out [28]. Water quality parameters measured include temperature, dissolved oxygen (DO), pH, ammonia, nitrate, and nitrite. Water quality parameters are used as supporting data in this study.

Immunity parameters

Whiteleg shrimp immune response was evaluated by calculating the total hemocyte count (THC) and differential hemocyte counts (DHC). Collection and preparation of shrimp hemolymph were carried out using Zahra et al. [28]. THC and DHC were analyzed by taking 0.1 ml of hemolymph in the fifth pereopod using a 1 ml syringe filled with 0.3 mL of Na-EDTA anticoagulant to prevent blood clots. The sample was homogenized for 5 minutes in a moistened microtube with 10% Na-EDTA. Hemolymph was dripped on the hemocytometer and closed using a cover glass. Calculation of the number and type of cells was carried out under a light microscope with a magnification of 400×. Observation of immune parameters was carried out at the end of the rearing period (56 days). The calculation of THC and DHC refers to the method by Suleman et al. [29].

$$THC = average\ \Sigma\ counted\ cells\ \times 250\ \times dillution\ factor\ \times 1000$$

$$DHC\ (\%) = \frac{\Sigma A}{\Sigma B} \times 100$$

Note:
Σ*A:* The number of each hemocyte cell type
Σ*B:* Total hemocyte count

Histological analysis

The gill tissue was placed in Davidson's solution until dehydrated, embedded in paraffin, and sliced using a microtome. The tissue was stained using Hematoxylin and Eosin and analyzed to identify defects such as hyperplasia, vacuolation, and necrosis. The histology was shown in figures defined by Mari et al. [30].

Data analysis

Production performance parameters and immunity were tabulated using Ms. Excel 2019 (Microsoft, USA). Statistical analysis was carried out using SPSS Version 25 software (IBM, USA). The data underwent a homogeneity test followed by a single factor ANOVA with a 95% confidence interval. To find out the differences in each treatment, the data underwent the

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Duncan test. The statistical results were interpreted and presented in the form of tables and figures.

RESULTS AND DISCUSSION

Production performance

Supplementation of hairy eggplant and bitter ginger in feed can increase absolute weight growth in whiteleg shrimp. The highest absolute weight growth was 9.19 gm/individual and was found in P3, then P4 (6.97 gm/individual), P2 (6.03 gm/individual), and P1 (Control) (5.82 gm/individual) (Table 1). Weight gain can occur from energy entering the body. The energy obtained from feed ingredients will first be used for rearing activities, then the remaining energy will be used for the growth process [31,32]. The absolute weight growth of shrimp with hairy eggplant and bitter ginger was higher than the control whiteleg shrimp. This occurs due to the supplementation of hairy eggplant and bitter ginger, which can stimulate the absorption of feed nutrients, mainly due to their protein and bioactive content. Bioactive content such as flavonoids in traditional plants can act as antibacterials and antioxidants, minimizing the increase in pathogenic microflora in the digestive tract. It is thought to increase the digestibility of whiteleg shrimp [33–35].

Table 1. The results of the analysis of the production performance parameters of whiteleg shrimp *(L.vannamei)*

The best specific growth rate (SGR) was found in P3 with a growth rate of 6.69%/day, showing that doses of hairy eggplant and bitter ginger had significant differences ($p < 0.05$) in specific growth rate on whiteleg shrimp. Increased growth rates can occur due to optimal use of feed ingredients. On the other hand, a low growth rate can occur due to health problems, stress, and suboptimal utilization of feed nutrients used for growth [36].

The lowest FCR value was found in P3, then P2 and P4, respectively. The highest FCR (1.51) was found in the control treatment (Table 1). The FCR was inversely proportional to weight growth, so the lower the FCR, the higher the efficiency of the shrimp in utilizing feed for growth [37]. On the other hand, if the shrimp body is unstable, the shrimp may experience a decrease in appetite and the feed provided is not converted into biomass. Stressed or unhealthy shrimp can be better at converting feed ingredients for weight growth compared to healthy shrimp [38].

The highest survival rate was found in P3 (90.66%), and of all treatments, the lowest percentage was found in the control treatment (71.92%) (Table 1). This indicated that supplementation of hairy eggplant and bitter ginger on whiteleg shrimp had a significant difference in the survival rate of whiteleg shrimp maintained for 56 days. This is presumably due to the content of secondary metabolites in hairy eggplant and bitter ginger, which can increase the immune system against pathogenic bacterial infections to protect the shrimp body from stress. The biological process will increase due to the involvement of phytochemical substances produced by the extract ingredients capable of producing enzymes for detoxification, modulating the immune system, and increasing shrimp survival. The increased immune response can have a positive impact on increasing body resistance and reducing shrimp mortality. This was confirmed by Jasmanindar et al. [39], which found that the low survival rate in the treatment without the extract had a relationship with a weak immune system compared to the treatment with seaweed extract. The combination of phytobiotics

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(thyme essential oil, red thyme, and rosemary pepper) applied in a supplement for 20 days has significant benefits for improving antioxidant protection, reducing the impact of stressors, and modulating the immunity of tilapia against *Aeromonas hydrophila* infection [17].

Water quality

All variables observed in the rearing pond remained within the levels recommended for shrimp culture during the test period (Table 2).

Table 2. Water quality parameters during the rearing period

Immunity parameters

The hemato-immunological response is a central physiological mechanism playing a role in protecting animals from disease, environmental stressors, or specific biological agents [40,41] such as phytobiotics. In this study, total hemocyte count (THC) and differential hemocyte count (DHC) were performed to evaluate the immunity condition of whiteleg shrimp with hairy eggplant and bitter ginger during 56 days of rearing. Based on the results, the total hemocytes of whiteleg shrimp in all treatments during the rearing period was around 4.63–16.76 $\times 10^6$ cells/mm^3. The highest THC value was found in P3 and was significantly different from the other three treatments ($p < 0.05$). P1 had the lowest THC value of all treatments. P2 and P3 had no significant differences (Table 3).

Hemocytes are the primary mediators of cellular responses in crustaceans, with roles that include self-recognition, phagocytosis, production of reactive oxygen intermediates, wound healing, and the process of melanization by encapsulation of foreign materials [42–44]. The increase in THC of whiteleg shrimp with hairy eggplant and bitter ginger can be suspected by the effect of bioactive compounds that can modulate shrimp immunity. Other studies confirmed that natural hydrolyzed tannin products from sweet chestnut *(Castanea sativa)* could act as functional feed additives by promoting growth and hematological parameters of whiteleg shrimp [45]. The *in vivo* effect of Astragalus polysaccharide immunostimulating ingredients, chlorogenic acid, and berberine showed a higher increase in total hemocyte count in whiteleg shrimp [46].

The difference in THC values in each treatment may be due to the various concentrations of active ingredients in food due to the treatment that has been determined. The low value of THC at P1 (control) may be influenced by physiological factors such as the slow formation of hemocytes in the shrimp body [47,48]. The exciting discussion confirmed that the low number of shrimp hemocytes is due to infiltration of regenerated tissue and hemocyte cell death due to apoptosis [27].

Table 3. Immunity parameters (THC and DHC) of Whiteleg Shrimp with hairy eggplant and bitter ginger

The minor component of shrimp hemocytes in differential hemocyte count (DHC) is hyaline, while the other two types of hemocytes are granular and semi-granular cells.The highest percentage of hyaline was found in P3 (48.67%), followed by P4 (34.67%), P2 (31.33%), and P1 (24.33%) (Table 3). The lowest number of semi-granular cells was found in P3 (23.67%). The decreasing number of semi-granular cells was shown in all treatments except control (P1), with the highest number (Table 3). The same thing happened to the number of granular

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cells in the treatment group. The highest number of granular cells was found in P1/control (40.33%).

Hyaline cells have a vital role in the shrimp's defense system. This cell type has a high ratio of cytoplasmic nuclei and few cytoplasmic granules. An increase in the number of hyaline cells can be associated with phagocytic activity when in contact with antigens or immunostimulating substances that will stimulate the body's defense activity to evoke the first defense response [49]. Semi-granular cells have a relationship with the addition or reduction of hyaline cells, so that the decrease in the number of semi-granular cells in the treatment group was due to the process of further development into hyaline cells. As a result, these cells cannot develop into semi-granular cells, so the number of semi-granular cells is low. Semi-granular cells are more involved in the encapsulation mechanism. The encapsulation process is a defense reaction against a large number of foreign particles that cannot be phagocytized by hyaline cells. These cells respond more to polysaccharide compounds found in bacterial cell walls.

Granular cells are the type of hemocytic cells that have the most significant size with an active nucleus in the storage process until the release of prophenoloxidase (proPO) and cytotoxicity. In this study, an increase in granular cells also occurred in the control group (P1). This is due to the low number of hyaline cells involved in the first defense process, thus relying on granular cells for non-specific body defense, which is driven by the influence of immunostimulatory components such as vitamin C.

Histology analysis

The condition of gill tissue on whiteleg shrimp showed that treatments P1 (control) and P3 experienced vacuolation and hyperplasia (Fig. 2), followed by treatment P2, which only experienced hyperplasia, and treatment P4 which experienced vacuolation and necrosis.

Figure 2. Histology of gill tissue of whiteleg shrimp (*L. vannamei*). *HP: Hyperplasia; V: Vacuolization; N: Necrosis.

The histology can explain that gill tissue damage occurred in all treatments carried out. Physiological differences between each whiteleg shrimp can cause tissue damage even when extracted ingredients are used. More research is needed to prove this results. High hemocytes can indicate infection or stress factors. Clogged blood flow in the lamellae (due to physical trauma, pollutants, or other physiological disturbances) can cause edema (cell swelling) between the blood vessels and the epithelial lining of the primary lamellae. Miller and Zachary [50] explained that necrosis is acute cell damage and can be massive, resulting in incomplete tissue formation due to shrinkage or complete shrinkage of the nucleus. Hyperplasia is the formation of excessive tissue due to an increase in the number of cells so that lamellae with hyperplasia will experience thickening of epithelial tissue at the ends of the filaments or the epithelium located near the base of the gill lamellae [51].

Based on the results, a 100 ml/l combination of hairy eggplant and bitter ginger in the feed is auspicious for whiteleg shrimp, related to the production performance and immunity. However, further studies are still needed to emphasize the potential that this combination of extracts can provide against pathogenic bacteria and viruses in whiteleg shrimp.

CONCLUSION

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Supplementation of hairy eggplant and bitter ginger into feed affected the production performance of whiteleg shrimp as indicated by absolute weight growth, specific growth rate, low feed conversion ratio (FCR), and high survival as shown in P3 (dose 100 ml/l). Similarly, the immunity parameters, including THC and DHC, were high in the P3 treatment group, although there was a tendency for higher semi-granular and granular cell values in the control group. All these results indicate that the combination of hairy eggplant and Bitter Ginger as phytobiotic ingredients could act as an exemplary modulator of the non-specific immune response of whiteleg shrimp, which ultimately increases production performance. Further studies are needed to obtain valuable information regarding the combination of this extract against infection with whiteleg shrimp pathogens such as viruses and bacteria to be used as a preventive agent.

List of abbreviations

WSSV, white spot syndrome Virus; ppt, parts per trillion; RNAi, RNA interference; SPF, Specific Pathogen Free; ml, milliliter; l, Liter; °C, degrees Celcius; gm, gram; µm, micrometer; a.m., ante meridiem; p.m., post meridiem; FR, Feeding Rate; SNI, Standard National Indonesia; SR, Survival Rate; SGR, Specific Growth Rate; FCR, Feed Conversion Ratio; DO, Dissolved Oxygen; THC, Total Haemocyte Count; DHC, Differential Haemocytes Counts; proPO, proPhenoloxidase; SPSS, Statistical Package for the Social Sciences; ANOVA, Analysis of Variance.

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Conflict of interests

The author should declare any conflict of interest.

Authors' contribution

SR designed the study, supervised, and conducted critical checking of this manuscript. DR interpreted the results and reviewed the manuscript. MATV conducted the study, collected the data, and contributed to manuscript preparation. PAW drafted the manuscript, took part in preparing it, and performed critical checking of the manuscript. All authors read and approved the final manuscript.

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Figure 1. Rearing pond of *L. vannamei* in this study

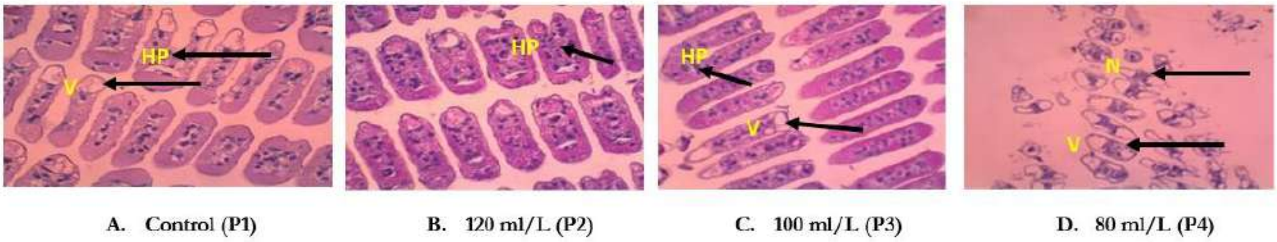

Figure 2. Histology of gill tissue of whiteleg shrimp (*L. vannamei*). *HP: Hyperplasia; V: Vacuolization; N: Necrosis.

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Table 1. The results of the analysis of the production performance parameters of whiteleg shrimp *(L.vannamei)*

Treatments group	**Parameter**			
	Absolute Growth (g)	**SGR (%)**	**FCR**	**SR (%)**
P1	5.82 ± 0.12[a]	5,49±0,04[a]	1.51±0,05[c]	71.92±1,76[a]
P2	6.03 ± 0.06[a]	5,67±0,07[a]	1.45±0,03[c]	76.95±3,03[a]
P3	9.19 ± 0.52[c]	6,69±0.18[c]	0.90±0,08[a]	90.66±4,61[b]
P4	6.97 ± 0.06[b]	5,93±0,03[b]	1.27±0,01[b]	77.09±1,40[a]

Note: The mean±SD (Standard deviation) values with different superscript letters showed significantly different results (P<0.05). *SGR: Specific Growth Rate; FCR: Feed Conversion Ratio; SR: Survival Rate.

Table 2. Water quality parameters during the maintenance period

Parameter	**This study**	**Optimal range***
pH	6,5-8,5	7,5-8,5
Temperature (^{o}C)	27-33	28 – 30
Dissolved Oxygen (mg/l)	3,8-4,0	≥ 4
Ammonia (mg/l)	0- 0,1	≤ 0,1
Nitrite (mg/l)	0-0,52	≤ 1
Salinity	29,7-33	26-32
Nitrate (mg/l)	0-0,3	0,5

* Ministry of Marine Affairs and Fisheries Regulation (KKP), Republic of Indonesia 2016

Table 3. Immunity parameters (THC and DHC) of whiteleg shrimp with hairy eggplant and bitter ginger

Treatment Groups	**THC (x 10^3 cells/mm^3)**	**Hialin (%)**	**Semi Granular (%)**	**Granular (%)**
P1	4.63±2.05[a]	24.33±2.08[a]	35.33±1.52[c]	40.33±.0.57[d]
P2	8.63±0.87[b]	31.33±1.52[b]	31.00±1.00[b]	37.67±.0.57[c]
P3	16.76±0.90[c]	48.67±4.04[c]	23.67±3.51[a]	27.67±0.57[a]
P4	9.96±1.75[b]	34.67±1.52[b]	30.33±.0.57[b]	35.00±1.73[b]

Note: The mean values with different superscript letters showed significantly different results (P<0.05). *THC: Total Haemocyte Counts

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3. PERINTAH PERBAIKAN DARI PARA REVIEWER JURNAL

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ORIGINAL ARTICLE

Supplementation of Hairy Eggplant *(Solanum ferox)* and Bitter Ginger *(Zingiber zerumbet)* Extract as Phytobiotic Agents on Whiteleg Shrimp *(Litopenaeus vannamei)*

Statement of novelty: Overall, supplementation of Hairy Eggplant, *S. ferox* and Bitter Ginger, *Z. zerumbet* in feed ingredients was able to increase production performance and immunity parameters in Whiteleg Shrimp *(L. vannamei)*. The two extract ingredients have the potential to be developed and promoted as phytobiotic agents in Whiteleg Shrimp so as to reduce the use of environmentally unfriendly antibiotics.

Ethical approval (if needed): This study does not need ethical approval.

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Supplementation of Hairy Eggplant (*Salanum ferox*) and Bitter Ginger (*Zingiber zerumbet*) Extract as Phytobiotic Agents on Whiteleg Shrimp (*Litopenaeus vannamei*)

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ABSTRACT

Objective: This study aimed to evaluate the combination of Hairy Eggplant *(Solanum ferox)* and Bitter Ginger *(Zingiber zerumbet)* on production performance and hematological parameters of Pacific Whiteleg Shrimp *(Litopenaeus vannamei).*

Materials and methods: Four treatments were formulated in the test feed, where P1 (control+commercial vitamin C); P2 (120 ml/L combination of *S. ferox* and *Z. zerumbet*); P3 (100 ml/L); and P4 (80 ml/L) 6,000 post-larvae shrimp with an average initial weight of 0.2 grams were randomly stocked in four groups, with three replications per treatment and 500 were stocked in each pond with a total of 12 ponds.

Results: Based on the results, there were significant differences in production performance (survival, absolute weight growth, specific growth rate (SGR), and feed conversion ratio (FCR)). Biologically, the best performance was found in the P3 treatment (100 ml/L). In this treatment, the total number of hemocytes and the number of hyaline hemocytes were much higher, and this was not the case in the control treatment (P1) where the number of semi-granular and granular cells was significantly higher than the treatment group *($p<0.05$).*

Conclusion: This study confirmed that supplementation of 100 ml/L of *S. ferox* and *Z. zerumbet* could improve production performance and hemato-immunological parameters of Whiteleg Shrimp with functional potential to be developed in phytobiotic-based commercial diets for shrimp.

Keywords: Growth, Haemocytes, Phytobiotics agent, Shrimp *Litopenaeus vannamei*

INTRODUCTION

Crustacean production has increased to 6.09 million tons or equivalent to USD 36.2 million due to increased consumption of shrimp commodities worldwide [1]. Whiteleg shrimp (*Litopenaeus vannamei)* is one of the most popular aquaculture commodities and is relatively easy to cultivate [2]. This can be proven by the high percentage of pacific whiteleg shrimp cultivation worldwide, which is around 90% [3]. This shrimp is able to adapt to a broad salinity (euryhaline) between 5 to 30 ppt, can be cultivated with a high stocking density, and is able to grow well with low protein feeds, making this shrimp a leading commodity in Indonesia.

However, an increase in shrimp production with unmatched implementation of water quality management, quality seeds, or superior broodstock to quality feed can have a negative impact on shrimp productivity by causing various diseases. Several disease outbreaks in shrimp ponds related to viral diseases are white spot syndrome virus (WSSV) and Taura syndrome virus (TSV), while bacterial diseases are *Vibrio harveyi*, *V. alginolyticus*, and *V. parahaemolyticus* which can cause huge losses to shrimp hatcheries worldwide [3].

The Whiteleg Shrimp immune system is also an important focus in the emergence of a disease in the aquaculture environment. Crustaceans, especially shrimp, only have a non-specific and humoral natural immune system [4]. This causes the growth and immunity of shrimp to be increased depending on the quality of the broodstock, the environment, and the application of biocontrol genes [6,7].

Until now, efforts to control bacterial diseases are still through antibiotics and chemotherapy agents [7,8]. However, the use of antibiotics in an unwise manner can lead to the accumulation of residues in the tissue which results in a decrease in product quality, the emergence of antibiotic resistance problems in shrimp and other aquatic organisms [9], to pollution of the aquatic environment [10]. Therefore, the use of environmentally friendly materials is needed to control disease in the current aquaculture system. Several approaches such as the use of pro-, pre- and synbiotics [11], immunostimulants [12], vaccination [13], quorum-sensing, phage application [14], RNA interference (RNAi) [15], development of molecular-based diagnostic materials [19], to the breeding and spawning of *Specific Pathogen Free* (SPF) shrimp [16] have attracted the attention of researchers and shrimp farmers.

Phytobiotics is a functional additives derived from nature are included as food supplement ingredients which have become an alternative to improve health and resistance to disease attacks that are ecologically correct in the modern aquaculture sector [17]. The development of phyobiotics materials is the best effort to improve the shrimp immune system [22]. In addition to being compatible with the shrimp immune system, which is still primitive (non-specific immunity), phytobiotics also have several advantages such as abundant sources of materials, wide target range, the potential for large-scale application, and environmental friendliness.

One of the natural agents that can be used as phytobiotics to increase the immune system of Whiteleg Shrimp are Hairy Eggplant *(Solanum ferox)* and Bitter Ginger *(Zingiber zerumbet)* [18]. Hairy eggplant or *S. ferox* is widely planted in tropical areas such as Indonesia for its fruit. This plant has been listed as a medicinal plant in the ethnobotanical inventory because it is reported to be effective in treating human diseases. Hairy Eggplant contains bioactive compounds such as phenolics, flavonoids, and polyphenols that play an important role in preventing oxidative stress and several biological effects such as antioxidant and anti-inflammatory [19]. Another traditional plant such as *Zingiber zerumbet* (L) which is part of the Zingiberaceae family is commonly found in Southeast Asia such as Indonesia. This plant is used traditionally in various cuisines and drinks because it is reported to have anti-allergic properties found in its rhizomes [25].

Previous studies showed that the combination of Zingiber zerumbet and Curcuma zedoria added to the Orange-spotted grouper (*Epinephelus coioides*) was able to act as an immunostimulant by increasing non-specific immune responses (respiratory burst activity, reactive oxygen species, phagocytic activity, superoxide dismustase, and lysozyme activity [20]. Hardi [21] explained that the combination of *Boesenbergia pandurate, Zingiber zerumbet*, and *Solanum ferox* are effective in preventing infection with *Aeromonas hydrophila* and *Pseudomonas* sp. and modulating the immune system of tilapia *Oreochromis niloticus*. However, to date, studies highlighting the potential of phytobiotic have not been reported in Pacific white shrimp.

Based on this report, this study aimed to evaluate the combination of Hairy Eggplant (*Solanum ferox)* and Bitter Ginger *(Zingiber zerumbet)* in increasing the production performance and immune system of Whiteleg Shrimp (*L. vannamei*). This study is expected to provide valuable preliminary information for the application of environmentally friendly immunostimulants in increasing the production of pacific white shrimp.

MATERIALS AND METHODS

Ethical approval: This study does not need ethical approval.

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Study area and period

This study was conducted from September 2020 – Desember 2020 at the Loka Pengelolaan Sumber Daya Pesisir & Laut (PSPL) Serang, Directorate General of Marine Space Management, Ministry of Marine and Fisheries, Republic of Indonesia.

Study Design

This study used a completely randomized design (CRD) with four treatments and three replications. The treatments used were a combination of Hairy Eggplant (*S. ferox*) and Bitter Ginger (*Z. zerumbet*) with the following details:

P1 : no combination + vitamin C (control);

P2 : combination of *S. ferox* and *Z. zerumbet* with a dose of 120 ml/L;

P3 : combination of *S. ferox* and *Z. zerumbet* with a dose of 100 ml/L;

P4 : combination of *S. ferox* and *Z. zerumbet* with a dose of 80 ml/L

Preparation of *S. ferox* and *Z. zerumbet*

Preparation of extract refers to the study by Hardi et al. [22]. In short, the plants were collected from traditional markets. The materials were washed and dried in an oven at a temperature of 40-

45°C for 48 hours. The dry ingredients were then mashed using a blender to form a fine powder. 100 g of dry samples were mixed with 100 ml of 96% ethanol in an Erlenmeyer flask at room temperature for 72 hours. The mixture was separated using 0.5 m Whatman filter paper to obtain the extract filtrate. The filtrate was re-evaporated using a rotary evaporator to separate the ethanol content for 3-5 hours. The extract was stored in the refrigerator for further testing.

Rearing of whiteleg shrimp

Rearing of Whiteleg Shrimp was carried out in a round tarpaulin container with a pool diameter of 2 meters and a height of 1 meter with a total of 12 pools. Each pond has nine aeration points which are evenly distributed in the rearing pond (Figure 1). The extract was mixed in the feed adjusted to the dose in each treatment. Feeding was carried out four times a day at 07.00 a.m; 11.00 a.m.; 15.00 p.m.; and 19.00 p.m. The amount of feed given was adjusted to the Feeding rate (FR) according to the Indonesian National Standard (SNI) No. 01-7246-2006 namely 15% to 10%.

Figure 1. Rearing pond of *L. vannamei* in this study

Production performance parameters

Survival rate

The survival rate of whiteleg shrimp was calculated at the end of the rearing period. The calculation was carried out according to the equations of Ly et al. [23] and Wiradana et al. [24] namely:

$$SR(\%) = \frac{Nt}{No} \times 100\%$$

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Note :

SR : Survival rate (%)

Nt : Number of shrimp at the end of the study (individual)

No : Number of shrimp at the beginning of the study (individual)

Absolute Weight Growth

Weight growth was observed every seven days by weighing the number of shrimp. Absolute weight growth based on Fendjalang et al. [25] is as follows:

$$\Delta W = Wt - Wo$$

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Note :

ΔW : Absolute weight growth (gr)

Wt : Final shrimp weight (gr)

Wo : Initial shrimp weight (gr)

Specific Growth Rate (SGR)

The specific growth rate was observed once a week based on Widanarni et al. [26]:

$$SGR(\%/day) = \frac{LnWt - LnWo}{T} \times 100\%$$

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Note :

$SGR(\%/day)$: Specific growth rate

Wt : Final shrimp biomass (gr)

Wo : Initial shrimp biomass (gr)

T : Rearing period (days)

***Feed Convertion Ratio* (FCR)**

FCR was calculated at the end of the rearing period based on Zubaidah et al. [27]:

$$FCR = \frac{\Sigma F\ feed\ given - \Sigma F\ leftover\ feed}{(Bt + Bm) - Bo}$$

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Note :

FCR : Feed convertion ratio (gr)

ΣF : Feeding amount (gr)

Bt : Final shrimp biomass (gr)

Bm : Dead shrimp biomass (gr)

Bo : Initial shrimp biomass (gr)

Water quality

To maintain the media quality, daily draining of water was carried out [28]. Water quality parameters measured include temperature, dissolved oxygen (DO), pH, Ammonia, Nitrate, and Nitrite. Water quality parameters are used as supporting data in this study.

Immunity parameters

The evaluation of Whiteleg Shrimp immune response was performed by calculating the total hemocyte count (THC) and differential hemocyte counts (DHC). Collection and preparation of shrimp hemolymph were carried out using the procedure by Zahra et al. [29]. Analysis of THC and DHC was carried out by taking 0.1 ml of hemolymph in the fifth pereopod using a 1 ml syringe filled with 0.3 ml of Na-EDTA anticoagulant to prevent blood clots. The sample was homogenized for 5 minutes in a moistened microtube with 10% Na-EDTA. Hemolymph was dripped on the hemocytometer and closed using a cover glass. Calculation of the number and type of cells was carried out under a light microscope with a magnification of 400×. Observation of immune parameters was carried out at the end of the rearing period (56 days). Calculation of THC and DHC was referring to the method by Suleman et al. [30] ~~Suleman et al. [36] and Widanarni et al. [37].~~

$$THC = average\ \Sigma\ counted\ cells\ \times 250\ \times dillution\ factor\ \times 1000$$

$$DHC\ (\%) = \frac{\Sigma A}{\Sigma B} \times 100$$

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Note :

ΣA : The number of each hemocyte cell type

ΣB : Total hemocyte count

Histological analysis

The gill tissue was placed in Davidson's solution until dehydrated, embedded in paraffin, and sliced using a microtome. Tissue was stained using hematoxylin and eosin and analyzed to identify defects such as hyperplasia, vacuolation, and necrosis ~~[38]~~. The histology was shown in Figures defined by Bonami et al. [31].

Data analysis

Production performance parameters and immunity were tabulated using Ms. Excel 2019 (Microsoft, USA). Statistical analysis was carried out using SPSS Version 25 software (IBM, USA). The data underwent a homogeneity test followed by Single Factor ANOVA with a 95% confidence interval. To find out the differences in each treatment, the data underwent the DUNCAN test. The statistical results were interpreted and presented in the form of tables and figures.

RESULTS AND DISCUSSION

Production performance

Supplementation of Hairy Eggplant and Bitter Ginger in feed can increase absolute weight growth in Whiteleg Shrimp. The highest absolute weight growth was 9.19 g/individual and found in P3, then P4 (6.97 g/individual), and P2 (6.03 g/individual), and P1 (Control) (5.82 g/individual) (Table 1). Weight growth can occur from the energy entering the body. The energy obtained from feed ingredients will first be used for rearing activities, then the remaining energy will be used for the growth process [32]. The absolute weight growth of shrimp with Hairy Eggplant and Bitter Ginger was higher than the control Whiteleg Shrimp. This occurs due to the supplementation of Hairy Eggplant and Bitter Ginger which can stimulate the process of absorption of feed nutrients, especially due to protein and bioactive content. Bioactive content such as flavonoids in traditional plants can act as antibacterial and antioxidant which can minimize the increase in the number of pathogenic microflora in the digestive tract so that it is thought to increase the digestibility of Whiteleg Shrimp [33–35].

Table 1. Production performance parameters of Whiteleg Shrimp

The best specific growth rate (SGR) was found in P3 with a growth of 6.69%/day showing doses of Hairy Eggplant and Bitter Ginger had significant differences ($p<0.05$) on specific growth rate on Whiteleg Shrimp. Increased growth rate can occur due to optimal use of feed ingredients. On the other hand, a low growth rate can occur due to health problems, stress, and suboptimal utilization of feed nutrients used for growth [36].

The lowest FCR value was found in P3, then P2 and P4 respectively. The highest FCR (1.51) was found in the control treatment (Table 1). FCR was inversely proportional to weight growth, so the lower the FCR, the higher the efficiency of the shrimp in utilizing feed for growth [37]. On the other hand, if the shrimp body is in an unstable state, the shrimp may experience a decrease in appetite, and the feed provided is not converted into biomass [38]. Healthy shrimp can be better at converting feed ingredients for weight growth compared to stressed or sick shrimp.

The highest survival rate was found in P3 (90.66%) and of all treatments, the lowest percentage was found in the control treatment (71.92%) (Table 1). This indicated that supplementation of Hairy Eggplant and Bitter Ginger on Whiteleg Shrimp had a significant difference in the survival rate of Whiteleg Shrimp maintained for 56 days. This is presumably due to the content of secondary metabolites in Hairy Eggplant and Bitter Ginger which can increase the immune system against pathogenic bacterial infections to protect the shrimp body from stress. Huang et al.[39] explained that the biological process will increase due to the involvement of phytochemical substances produced by the extract ingredients capable of producing enzymes as detoxification, modulating the immune system, and increasing the survival of shrimp. The increased immune response can have a good impact on increasing body resistance and reducing the number of shrimp mortality. This was confirmed by Jasmanindar et al. [40] which found that the low survival rate in the treatment without the extract had a relationship with a weak immune system compared to the treatment with seaweed extract. The combination of phytobiotics (thyme essential oil, red thyme, and rosemary pepper) which is applied in a supplement for 20 days has significant benefits for greater antioxidant protection, reducing the impact of stressors, and modulating the immunity of tilapia against *Aeromonas hydrophila* infection [17].

Water quality

All variables observed in the rearing pond remained within the levels recommended for shrimp culture during the test period (Table 2).

Table 2. Water quality parameters during the rearing period

Immunity parameters

The hemato-immunological response is a major physiological mechanism playing a role in protecting animals from disease, environmental stressors, or certain biological agents [41] such as phytobiotics. In this study, total hemocyte count (THC) and differential hemocyte count (DHC) were performed to evaluate the immunity condition of Whiteleg Shrimp with Hairy Eggplant and Bitter Ginger during 56 days of rearing. Based on the results, the total hemocytes of Whiteleg Shrimp in all treatments during the rearing period was around 4.63 – 16.76 ($\times 10^6$ cells/mm^3). The highest THC value was found in P3 and was significantly different when compared to the other three treatments ($P<0.05$). P1 had the lowest THC value of all treatments. P2 and P3 had no significant differences (Table 3).

Hemocytes are the main mediators of cellular responses in crustaceans with roles that include self-recognition, phagocytosis, production of reactive oxygen intermediates, wound healing, to the process of melanization by encapsulation of foreign materials [42–44]. The increase in THC of Whiteleg Shrimp with Hairy Eggplant and Bitter Ginger can be suspected by the effect of bioactive compounds that can modulate shrimp immunity. Other studies confirmed that natural hydrolyzed tannin products from sweet chestnut *(Castanea sativa)* are able to act as functional feed additives by promoting growth and hematological parameters of Whiteleg Shrimp [45]. The in vivo effect of Astragalus polysaccharide immunostimulating ingredients, chlorogenic acid, and berberine showed a higher increase in total hemocyte count in Whiteleg Shrimp [46].

The difference in THC values in each treatment may be due to the various concentrations of active ingredients in food due to the treatment that has been determined. The low value of THC at P1 (control) may be influenced by physiological factors such as the slow formation of hemocytes in the shrimp body [47,48]. Interesting discussion confirmed that the low number of shrimp hemocytes is due to infiltration of regenerated tissue and hemocytes cell death due to apoptosis [27].

Table 3. Immunity parameters (THC and DHC) of Whiteleg Shrimp with Hairy Eggplant and Bitter Ginger

Differential hemocyte count (DHC) consists of hyaline as the smallest component of shrimp hemocytes, where granular and semi-granular cells are the other two types of hemocytes. The highest percentage of hyaline was found in P3 (48.67%), followed by P4 (34.67%), P2 (31.33%), and P1 (24.33%) (Table 3). The lowest number of semi-granular cells was found in P3 (23.67%). The decreasing number of semi-granular cells was shown in all treatments except control (P1) with the highest number (Table 3). The same thing happened to the number of granular cells in the treatment group. The highest number of granular cells was found in P1/control (40.33%).

Hyaline cells have a vital role in the shrimp defense system. This cell type has a high ratio of cytoplasmic nuclei and few cytoplasmic granules. An increase in the number of hyaline cells can be associated with phagocytic activity when in contact with antigens or immunostimulating substances will stimulate the body's defense activity so that it will evoke the first defense response [49]. Semi-granular cells have a relationship with the addition or reduction of hyaline cells so that the decrease in the number of semi-granular cells in the treatment group was due to the process of further development into hyaline cells. As a result, these cells cannot develop into semi-granular cells so that the number of semi-granular cells is low ~~[49]~~. Semi-granular cells are more involved in the encapsulation mechanism. The encapsulation process is a defense reaction against a large number of foreign particles that are not able to be phagocytized by Hyaline cells. These cells respond more to polysaccharide compounds found in bacterial cell walls ~~[50]~~.

Granular cells are the type of hemocytic cells that have the largest size with an active nucleus in the storage process until the release of prophenoloxidase (proPO) and cytotoxicity. In this study, an increase in granular cells also occurred in the control group (P1). This is due to the low number of hyaline cells involved in the first defense process, thus relying on granular cells for non-specific body defense which is driven by the influence of immunostimulatory components such as vitamin C ~~[51]~~.

Histology analysis

The condition of gill tissue on Whiteleg Shrimp showed that treatment P1 (control) and P3 experienced vacuolation and hyperplasia (Figure 2), followed by treatment P2 which only experienced hyperplasia, and treatment P4 experienced vacuolation and necrosis.

Figure 2. Gill tissue histology of Whiteleg Shrimp *(L. vannamei)*

The histology can explain that gill tissue damage occurred in all treatments carried out. Physiological differences of each individual Whiteleg Shrimp can cause tissue damage, even though there is an intervention of extract ingredients. High hemocytes can indicate infection or stress factors can cause this, although further studies are still needed to prove this. Clogged blood flow (caused by physical trauma, pollutants, or other physiological disturbances) in the lamellae can cause edema (swelling of cells) between the blood vessels and the epithelial lining of the primary lamellae [52]. Miller and Zachary [53] explained that necrosis is acute cell damage and can be massive, resulting in incomplete tissue formation due to shrinkage or complete shrinkage of the nucleus. Hyperplasia is the formation of excessive tissue due to an increase in the number of cells so that lamellae with hyperplasia will experience thickening of epithelial tissue at the ends of the filaments or the epithelium located near the base of the gill lamellae [54].

Based on the results, 100 ml/L combination of Hairy Eggplant and Bitter Ginger in feed is very promising for Whiteleg Shrimp, related to the production performance and immunity. However, further studies are still needed to emphasize the potential that this combination of extracts can provide against pathogenic bacteria and viruses in Whiteleg Shrimp.

CONCLUSION

Supplementation of Hairy Eggplant and Bitter Ginger into feed was able to affect the production performance of Whiteleg Shrimp as indicated by absolute weight growth, specific growth rate, low feed conversion ratio (FCR), and high survival as shown in P3 (dose 100 ml/L). Similarly, the immunity parameters which include THC and DHC were high in the P3 treatment group, although there was a tendency for higher semi-granular and granular cell values in the control group. All these results indicated that the combination of Hairy Eggplant and Bitter Ginger as phytobiotic ingredients can act as a good modulator of the non-specific immune response of Whiteleg Shrimp which ultimately increases production performance. Further studies are still needed to obtain valuable information regarding the combination of this extract against infection with Whiteleg Shrimp pathogens such as viruses and bacteria so that it can be used as a preventive agent.

LIST OF ABBREVIATIONS

WSSV: White Spot Syndrome Virus; ppt: parts per trillion; RNAi: RNA interference; SPF: Specific Pathogen Free; ml: milliliter; L: Liter; °C: degrees Celcius; gr: gram; μm: micrometer; a.m.; ante meridiem; p.m.: post meridiem; FR: Feeding Rate; SNI: Standard National Indonesia; SR: Survival Rate; SGR: Specific Growth Rate; FCR: Feed Convertion Ratio; DO: Dissolved Oxygen; THC: Total Haemocyte Count; DHC: Differential Haemocytes Counts; proPO: proPhenoloxidase; SPSS: Statistical Package for the Social Sciences; ANOVA: Analysis of Variance.

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CONFLICT OF INTERESTS

The author should declare any conflict of interest.

AUTHORS' CONTRIBUTION

SR designed the study, supervised, and conducted critical checking of this manuscript. DR Interpreted the results and reviewed the manuscript. MATV conducted the study, collected the data, and contributed in manuscript preparation. PAW drafted the manuscript, took part in preparing, and performed critical checking of manuscript. All authors read and approved the final manuscript.

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Figure 1. Rearing pond of *L. vannamei* in this study

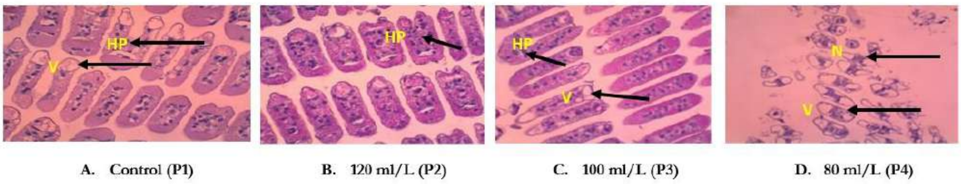

Figure 2. Histology of gill tissue of whiteleg shrimp (*L. vannamei*). *HP: Hyperplasia; V: Vacuolization; N: Necrosis.

Table 1. The results of the analysis of the production performance parameters of whiteleg shrimp *(L.vannamei)*

Treatments group	Parameter			
	Absolute Growth (g)	**SGR (%)**	**FCR**	**SR (%)**
P1	5.82 ± 0.12^{a}	5,49±0,04^{a}	1.51±0,05^{c}	71.92±1,76^{a}
P2	6.03 ± 0.06^{a}	5,67±0,07^{a}	1.45±0,03^{c}	76.95±3,03^{a}
P3	9.19 ± 0.52^{c}	6,69±0.18^{c}	0.90±0,08^{a}	90.66±4,61^{b}
P4	6.97 ± 0.06^{b}	5,93±0,03^{b}	1.27±0,01^{b}	77.09±1,40^{a}

Note: The mean±SD (Standard deviation) values with different superscript letters showed significantly different results ($P<0.05$). *SGR: Specific Growth Rate; FCR: Feed Convertion Ratio; SR: Survival Rate.

Table 2. Water quality parameters during the maintenance period

Parameter	This study	Kisaran Ideal*
pH	6,5-8,5	7,5-8,5
Temperature (^{o}C)	27-33	28 – 30
Dissolved Oxygen (mg/L)	3,8-4,0	≥ 4
Ammonia (mg/L)	0- 0,1	≤ 0,1
Nitrite (mg/L)	0-0,52	≤ 1
Salinity	29,7-33	26-32
Nitrate (mg/L)	0-0,3	0,5

* Ministry of Marine Affairs and Fisheries Regulation (KKP), Republic of Indonesia 2016

Table 3. Immunity parameters (THC and DHC) of Whiteleg Shrimp with Hairy Eggplant and Bitter Ginger

Treatment Groups	**THC (x 10^3 sel/mm^3)**	**Hialin (%)**	**Semi Granular (%)**	**Granular (%)**
P1	4.63 ± 2.05^a	24.33 ± 2.08^a	35.33 ± 1.52^c	$40.33 \pm .0.57^d$
P2	8.63 ± 0.87^b	31.33 ± 1.52^b	31.00 ± 1.00^b	$37.67 \pm .0.57^c$
P3	16.76 ± 0.90^c	48.67 ± 4.04^c	23.67 ± 3.51^a	27.67 ± 0.57^a
P4	9.96 ± 1.75^b	34.67 ± 1.52^b	$30.33 \pm .0.57^b$	35.00 ± 1.73^b

Note: The mean values with different superscript letters showed significantly different results (P<0.05). *THC : Total Haemocyte Counts

Original Article

Supplementation of hairy eggplant *(Solanum ferox)* and bitter ginger *(Zingiber zerumbet)* extract as phytobiotic agents on whiteleg shrimp *(Litopenaeus vannamei)*

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ABSTRACT

Objective: This study aimed to evaluate the combination of hairy eggplant (*Solanum ferox*) and bitter ginger (*Zingiber zerumbet*) on the production performance and hematological parameters of Pacific whiteleg shrimp (*Litopenaeus vannamei*).
Materials and Methods: Four treatments were formulated in the test feed, where P1 (control+commercial vitamin C); P2 (120 mL/L combinations of *S. ferox* and *Z. zerumbet*); P3 (100 mL/L); and P4 (80 ml/L) 6,000 post-larvae shrimp with an average initial weight of 0.2 gm were randomly stocked in four groups, with three replications per treatment and 500 were stocked in each pond with a total of 12 pounds.
Results: Based on the results, there were significant differences in production performance [survival, absolute weight growth, specific growth rate (SGR), and feed conversion ratio (FCR)]. Biologically, the best performance was found in the P3 treatment (100 mL/L). In this treatment, the total number of hemocytes and the number of hyaline hemocytes were much higher, and this was not the case in the control treatment (P1), where the number of semi-granular and granular cells was significantly higher than the treatment group ($p < 0.05$).
Conclusion: This study confirmed that supplementation of 100 mL/L of *S. ferox* and *Z. zerumbet* could improve the production performance and hemato-immunological parameters of whiteleg shrimp, with functional potential to be developed in phytobiotic-based commercial diets for shrimp.

Keywords: Growth; Haemocytes; Phytobiotics agent; Shrimp *Litopenaeus vannamei*

INTRODUCTION

Crustacean production has increased to 6.09 million tons, or equivalent to USD 36.2 million, due to increased consumption of shrimp commodities worldwide [1]. Whiteleg shrimp (*Litopenaeus vannamei)* are one of the most popular aquaculture commodities and are

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relatively easy to cultivate [2]. This can be proven by the high percentage of Pacific whiteleg shrimp cultivation worldwide, at around 90%. This shrimp can adapt to a broad salinity (euryhaline) between 5 to 30 ppt, cultivate with a high stocking density, and grow sufficiently with low protein feeds, making this shrimp a leading commodity in Indonesia.

However, an increase in shrimp production with unmatched water quality management, quality seeds, or superior broodstock to quality feed can harm shrimp productivity by causing various diseases. Several disease outbreaks in shrimp ponds are related to viral diseases like the white spot syndrome virus (WSSV) and Taura syndrome virus (TSV). In contrast, bacterial diseases like *Vibrio harveyi*, *V. alginolyticus*, and *V. parahaemolyticus* can cause considerable losses to shrimp hatcheries worldwide [3].

The whiteleg shrimp's immune systems are also an essential focus in the emergence of a disease in the aquaculture environment. Crustaceans, especially shrimp, only have a non-specific and humoral natural immune system [4]. This causes the growth and immunity of shrimp to be increased depending on the quality of the broodstock, the environment, and the application of biocontrol genes [5,6].

Currently, antibiotics and chemotherapy agents are still used to control bacterial diseases [7, 8]. However, the use of antibiotics unwisely can lead to the accumulation of residues in the tissue, resulting in a decrease in product quality, the emergence of antibiotic resistance problems in shrimp and other aquatic organisms [9], and pollution of the aquatic environment [10]. Therefore, the use of environmentally friendly materials is needed to control disease in the current aquaculture system. Several approaches, such as the use of pro-, pre-, and synbiotics [11], immunostimulants [12], vaccination [13], quorum-sensing, phage application [14], RNA interference (RNAi) [15], development of molecular-based diagnostic materials, and the breeding and spawning of specific pathogen free (SPF) shrimp [16], have attracted the attention of researchers and shrimp farmers.

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Phytobiotics are functional additives derived from nature, including food supplement ingredients that have become an alternative to improve health and resistance to disease attacks that are ecologically correct in the modern aquaculture sector [17]. The development of phyobiotics materials is the best effort to improve the shrimp immune system. In addition to being compatible with the shrimp immune system, which is still primitive (non-specific immunity), phytobiotics also have several advantages such as abundant sources of materials, wide target range, the potential for large-scale application, and environmental friendliness. Some of the natural agents that can be used as phytobiotics to increase the immune system of whiteleg shrimp are hairy eggplant *(Solanum ferox)* and Bitter Ginger *(Zingiber zerumbet)* [18]. Hairy eggplant or *S. ferox* is widely planted in tropical areas such as Indonesia for its fruit. This plant has been listed as a medicinal plant in the ethnobotanical inventory because it is reported to be effective in treating human diseases. Hairy eggplant contains bioactive compounds such as phenolics, flavonoids, and polyphenols that play an essential role in preventing oxidative stress and several biological effects such as antioxidant and anti-inflammatory [19]. Another traditional plant such as *Zingiber zerumbet* (L), part of the Zingiberaceae family, is commonly found in Southeast Asia, such as Indonesia. This plant is used traditionally in various cuisines and drinks because it is reported to have anti-allergic properties found in its rhizomes.

Previous studies showed that the combination of Zingiber zerumbet and *Curcuma zedoria* added to the orange-spotted grouper (*Epinephelus coioides*) was able to act as an immunostimulant by increasing non-specific immune responses (respiratory burst activity, reactive oxygen species, phagocytic activity, superoxide dismustase, and lysozyme activity [20]. Hardi et al [21] explained that the combination of *Boesenbergia pandurate, Zingiber zerumbet,* and *Solanum ferox* effectively prevents infection with *Aeromonas hydrophila* and *Pseudomonas* sp., and modulates the immune system of tilapia, *Oreochromis niloticus*. However, studies highlighting the potential of phytobiotics have not been reported in Pacific white shrimp.

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Based on this report, this study aimed to evaluate the combination of hairy eggplant (*Solanum ferox)* and Bitter Ginger *(Zingiber zerumbet)* in increasing the production performance and immune system of whiteleg shrimp (*L. vannamei*). This study is expected to provide valuable preliminary information for applying environmentally friendly immunostimulants to increase pacific white shrimp production.

MATERIALS AND METHODS

Ethical approval

This research has been licensed by the Fisheries Expert Polytechnic (AUP), Marine and Fisheries Research and Human Resources Agency (BRSDM), Ministry of Marine Affairs and Fisheries (KKP) of the Republic of Indonesia with No: 1832/POLTEK-AUP/TU.210/IX/2020.

Study area and period

This study was conducted from September 2020 to December 2020 at the Loka Pengelolaan Sumber Daya Pesisir & Laut (PSPL) Serang, Directorate General of Marine Space Management, Ministry of Marine and Fisheries, Republic of Indonesia.

Study design

This study used a Completely Randomized Design (CRD) with four treatments and three replications. The treatments used were a combination of hairy eggplant (*S. ferox*) and Bitter Ginger (*Z. zerumbet*) with the following details:

P1 : no combination + vitamin C (control);
P2: the combination of *S. ferox* and *Z. zerumbet* with a dose of 120 mL/L;
P3: the combination of *S. ferox* and *Z. zerumbet* with a dose of 100 mL/L;
P4: the combination of *S. ferox* and *Z. zerumbet* with a dose of 80 mL/L.

Preparation of *S. ferox* and *Z. zerumbet*

Preparation of the extract refers to the study by Hardi et al. [21]. In short, the plants were collected from traditional markets. The materials were washed and dried in an oven at 40-45°C for 48 h. The dry ingredients were then mashed using a blender to form a fine powder. 100 gm of dry samples were mixed with 100 ml of 96% ethanol in an Erlenmeyer flask at room temperature for 72 h. The mixture was separated using 0.5 m of Whatman filter paper to obtain the extract filtrate. The filtrate was re-evaporated using a rotary evaporator to separate the ethanol content for 3-5 h. The extract was stored in the refrigerator for further testing.

Rearing of whiteleg shrimp

Rearing of whiteleg shrimp was carried out in a round tarpaulin container with a pool diameter of 2 meters and a height of 1 meter, for a total of 12 pools. Each pond has nine aeration points evenly distributed in the rearing pond (Fig. 1). The extract was mixed into the feed and the dose was adjusted for each treatment.Feeding was carried out four times a day at 07.00 a.m., 11.00 a.m., 3.00 p.m., and 7.00 p.m. The amount of feed given was adjusted to the feeding rate (FR) according to the Indonesian National Standard (SNI) No. 01-7246-2006, namely 15% to 10%.

Figure 1. Rearing pond of *L. vannamei* in this study

Production performance parameters

Survival rate

The survival rate of whiteleg shrimp was calculated at the end of the rearing period. The calculation was carried out according to the equations of Ly et al. [23] and Wiradana et al. [24], namely:

$$SR(\%) = \frac{Nt}{No} \times 100\%$$

Note:
SR: Survival rate (%)
Nt: Number of shrimp at the end of the study (individual)
No: Number of shrimp at the beginning of the study (individual)

Absolute weight growth

Weight growth was observed every seven days by weighing the number of shrimp. Absolute weight growth based on Widanarni et al. [25] is as follows:

$$\Delta W = Wt - Wo$$

Note :
Δ*W*: Absolute weight growth (gm)
Wt: Final shrimp weight (gm)
Wo: Initial shrimp weight (gm)

Specific growth rate (SGR)

The specific growth rate was observed once a week based on Widanarni et al. [26]:

$$SGR(\%/day) = \frac{LnWt - LnWo}{T} \times 100\%$$

Note:
SGR(%/day): Specific growth rate
Wt: Final shrimp biomass (gm)
Wo: Initial shrimp biomass (gm)
T : Rearing period (days)

***Feed conversion ratio* (FCR)**

FCR was calculated at the end of the rearing period based on Zubaidah et al. [27]:

$$FCR = \frac{\Sigma F\ feed\ given - \Sigma F\ leftover\ feed}{(Bt + Bm) - Bo}$$

Note :
FCR: Feed conversion ratio (gm)
Σ*F*: Feeding amount (gm)
Bt: Final shrimp biomass (gm)
Bm: Dead shrimp biomass (gm)
Bo: Initial shrimp biomass (gm)

Water quality

To maintain the media quality, daily draining of water was carried out [28]. Water quality parameters measured include temperature, dissolved oxygen (DO), pH, ammonia, nitrate, and nitrite. Water quality parameters are used as supporting data in this study.

Immunity parameters

Whiteleg shrimp immune response was evaluated by calculating the total hemocyte count (THC) and differential hemocyte counts (DHC). Collection and preparation of shrimp hemolymph were carried out using Zahra et al. [29]. THC and DHC were analyzed by taking 0.1 ml of hemolymph in the fifth pereopod using a 1 mL syringe filled with 0.3 mL of Na-EDTA anticoagulant to prevent blood clots. The sample was homogenized for 5 minutes in a moistened microtube with 10% Na-EDTA. Hemolymph was dripped on the hemocytometer and closed using a cover glass. Calculation of the number and type of cells was carried out under a light microscope with a magnification of 400×. Observation of immune parameters was carried out at the end of the rearing period (56 days). The calculation of THC and DHC refers to the method by Suleman et al. [30].

$$THC = average\ \Sigma\ counted\ cells\ \times 250\ \times dillution\ factor\ \times 1000$$

$$DHC\ (\%) = \frac{\Sigma A}{\Sigma B} \times 100$$

Note:
Σ*A:* The number of each hemocyte cell type
Σ*B:* Total hemocyte count

Histological analysis

The gill tissue was placed in Davidson's solution until dehydrated, embedded in paraffin, and sliced using a microtome. The tissue was stained using Hematoxylin and Eosin and analyzed to identify defects such as hyperplasia, vacuolation, and necrosis. The histology was shown in figures defined by Bonami et al. [31].

Data analysis

Production performance parameters and immunity were tabulated using Ms. Excel 2019 (Microsoft, USA). Statistical analysis was carried out using SPSS Version 25 software (IBM, USA). The data underwent a homogeneity test followed by a single factor ANOVA with a 95% confidence interval. To find out the differences in each treatment, the data underwent the

Duncan test. The statistical results were interpreted and presented in the form of tables and figures.

RESULTS AND DISCUSSION

Production performance

Supplementation of hairy eggplant and Bitter Ginger in feed can increase absolute weight growth in whiteleg shrimp. The highest absolute weight growth was 9.19 g/individual and was found in P3, then P4 (6.97 gm/individual), P2 (6.03 gm/individual), and P1 (Control) (5.82 gm/individual) (Table 1). Weight gain can occur from energy entering the body. The energy obtained from feed ingredients will first be used for rearing activities, then the remaining energy will be used for the growth process [32]. The absolute weight growth of shrimp with hairy eggplant and Bitter Ginger was higher than the control whiteleg shrimp. This occurs due to the supplementation of hairy eggplant and bitter ginger, which can stimulate the absorption of feed nutrients, mainly due to their protein and bioactive content. Bioactive content such as flavonoids in traditional plants can act as antibacterials and antioxidants, minimizing the increase in pathogenic microflora in the digestive tract. It is thought to increase the digestibility of whiteleg shrimp [33–35].

Table 1. The results of the analysis of the production performance parameters of whiteleg shrimp *(L.vannamei)*

The best specific growth rate (SGR) was found in P3 with a growth rate of 6.69%/day, showing that doses of hairy eggplant and bitter ginger had significant differences ($p < 0.05$) in specific growth rate on whiteleg shrimp. Increased growth rates can occur due to optimal use of feed ingredients. On the other hand, a low growth rate can occur due to health problems, stress, and suboptimal utilization of feed nutrients used for growth [36].

The lowest FCR value was found in P3, then P2 and P4, respectively. The highest FCR (1.51) was found in the control treatment (Table 1). The FCR was inversely proportional to weight growth, so the lower the FCR, the higher the efficiency of the shrimp in utilizing feed for growth [37]. On the other hand, if the shrimp body is unstable, the shrimp may experience a decrease in appetite and the feed provided is not converted into biomass [38]. Stressed or sick shrimp can be better at converting feed ingredients for weight growth compared to healthy shrimp.

The highest survival rate was found in P3 (90.66%), and of all treatments, the lowest percentage was found in the control treatment (71.92%) (Table 1). This indicated that supplementation of hairy eggplant and Bitter Ginger on whiteleg shrimp had a significant difference in the survival rate of whiteleg shrimp maintained for 56 days. This is presumably due to the content of secondary metabolites in hairy eggplant and bitter ginger, which can increase the immune system against pathogenic bacterial infections to protect the shrimp body from stress. Huang et al. [39] explained that the biological process will increase due to the involvement of phytochemical substances produced by the extract ingredients capable of producing enzymes for detoxification, modulating the immune system, and increasing shrimp survival. The increased immune response can have a positive impact on increasing body resistance and reducing shrimp mortality. This was confirmed by Jasmanindar et al. [40], which found that the low survival rate in the treatment without the extract had a relationship with a weak immune system compared to the treatment with seaweed extract. The combination of phytobiotics (thyme essential oil, red thyme, and rosemary pepper) applied in a supplement for 20 days has significant benefits for improving antioxidant protection, reducing the impact of stressors, and modulating the immunity of tilapia against *Aeromonas hydrophila* infection [17].

Water quality

All variables observed in the rearing pond remained within the levels recommended for shrimp culture during the test period (Table 2).

Table 2. Water quality parameters during the rearing period

Immunity parameters

The hemato-immunological response is a central physiological mechanism playing a role in protecting animals from disease, environmental stressors, or specific biological agents [41] such as phytobiotics. In this study, total hemocyte count (THC) and differential hemocyte count (DHC) were performed to evaluate the immunity condition of whiteleg shrimp with hairy eggplant and Bitter Ginger during 56 days of rearing. Based on the results, the total hemocytes of whiteleg shrimp in all treatments during the rearing period was around 4.63–16.76 $\times 10^6$ cells/mm^3. The highest THC value was found in P3 and was significantly different from the other three treatments ($p < 0.05$). P1 had the lowest THC value of all treatments. P2 and P3 had no significant differences (Table 3).

Hemocytes are the primary mediators of cellular responses in crustaceans, with roles that include self-recognition, phagocytosis, production of reactive oxygen intermediates, wound healing, and the process of melanization by encapsulation of foreign materials [42–44]. The increase in THC of whiteleg shrimp with hairy eggplant and Bitter Ginger can be suspected by the effect of bioactive compounds that can modulate shrimp immunity. Other studies confirmed that natural hydrolyzed tannin products from sweet chestnut *(Castanea sativa)* could act as functional feed additives by promoting growth and hematological parameters of whiteleg shrimp [45]. The *in vivo* effect of Astragalus polysaccharide immunostimulating ingredients, chlorogenic acid, and berberine showed a higher increase in total hemocyte count in whiteleg shrimp [46].

The difference in THC values in each treatment may be due to the various concentrations of active ingredients in food due to the treatment that has been determined. The low value of THC at P1 (control) may be influenced by physiological factors such as the slow formation of hemocytes in the shrimp body [47,48]. The exciting discussion confirmed that the low number of shrimp hemocytes is due to infiltration of regenerated tissue and hemocyte cell death due to apoptosis [27].

Table 3. Immunity parameters (THC and DHC) of Whiteleg Shrimp with Hairy Eggplant and Bitter Ginger

The minor component of shrimp hemocytes in differential hemocyte count (DHC) is hyaline, while the other two types of hemocytes are granular and semi-granular cells.The highest percentage of hyaline was found in P3 (48.67%), followed by P4 (34.67%), P2 (31.33%), and P1 (24.33%) (Table 3). The lowest number of semi-granular cells was found in P3 (23.67%). The decreasing number of semi-granular cells was shown in all treatments except control

(P1), with the highest number (Table 3). The same thing happened to the number of granular cells in the treatment group. The highest number of granular cells was found in P1/control (40.33%).

Hyaline cells have a vital role in the shrimp's defense system. This cell type has a high ratio of cytoplasmic nuclei and few cytoplasmic granules. An increase in the number of hyaline cells can be associated with phagocytic activity when in contact with antigens or immunostimulating substances that will stimulate the body's defense activity to evoke the first defense response [49]. Semi-granular cells have a relationship with the addition or reduction of hyaline cells, so that the decrease in the number of semi-granular cells in the treatment group was due to the process of further development into hyaline cells. As a result, these cells cannot develop into semi-granular cells, so the number of semi-granular cells is low. Semi-granular cells are more involved in the encapsulation mechanism. The encapsulation process is a defense reaction against a large number of foreign particles that cannot be phagocytized by hyaline cells. These cells respond more to polysaccharide compounds found in bacterial cell walls.

Granular cells are the type of hemocytic cells that have the most significant size with an active nucleus in the storage process until the release of prophenoloxidase (proPO) and cytotoxicity. In this study, an increase in granular cells also occurred in the control group (P1). This is due to the low number of hyaline cells involved in the first defense process, thus relying on granular cells for non-specific body defense, which is driven by the influence of immunostimulatory components such as vitamin C.

Histology analysis

The condition of gill tissue on whiteleg shrimp showed that treatments P1 (control) and P3 experienced vacuolation and hyperplasia (Fig. 2), followed by treatment P2, which only experienced hyperplasia, and treatment P4 which experienced vacuolation and necrosis.

Figure 2. Histology of gill tissue of whiteleg shrimp (*L. vannamei*). *HP: Hyperplasia; V: Vacuolization; N: Necrosis.

The histology can explain that gill tissue damage occurred in all treatments carried out. Physiological differences between each whiteleg shrimp can cause tissue damage even when extracted ingredients are used. More research is needed to prove this results. High hemocytes can indicate infection or stress factors. Clogged blood flow in the lamellae (due to physical trauma, pollutants, or other physiological disturbances) can cause edema (cell swelling) between the blood vessels and the epithelial lining of the primary lamellae [50]. Miller and Zachary [51] explained that necrosis is acute cell damage and can be massive, resulting in incomplete tissue formation due to shrinkage or complete shrinkage of the nucleus. Hyperplasia is the formation of excessive tissue due to an increase in the number of cells so that lamellae with hyperplasia will experience thickening of epithelial tissue at the ends of the filaments or the epithelium located near the base of the gill lamellae [52].

Based on the results, a 100 mL/L combination of hairy eggplant and Bitter Ginger in the feed is auspicious for whiteleg shrimp, related to the production performance and immunity. However, further studies are still needed to emphasize the potential that this combination of extracts can provide against pathogenic bacteria and viruses in whiteleg shrimp.

CONCLUSION

Supplementation of hairy eggplant and Bitter Ginger into feed affected the production performance of whiteleg shrimp as indicated by absolute weight growth, specific growth rate, low feed conversion ratio (FCR), and high survival as shown in P3 (dose 100 mL/L). Similarly, the immunity parameters, including THC and DHC, were high in the P3 treatment group, although there was a tendency for higher semi-granular and granular cell values in the control group. All these results indicate that the combination of hairy eggplant and Bitter Ginger as phytobiotic ingredients could act as an exemplary modulator of the non-specific immune response of whiteleg shrimp, which ultimately increases production performance. Further studies are needed to obtain valuable information regarding the combination of this extract against infection with whiteleg shrimp pathogens such as viruses and bacteria to be used as a preventive agent.

List of abbreviations

WSSV, white spot syndrome Virus; ppt, parts per trillion; RNAi, RNA interference; SPF, Specific Pathogen Free; ml, milliliter; l, Liter; °C, degrees Celcius; gm, gram; µm, micrometer; a.m., ante meridiem; p.m., post meridiem; FR, Feeding Rate; SNI, Standard National Indonesia; SR, Survival Rate; SGR, Specific Growth Rate; FCR, Feed Conversion Ratio; DO, Dissolved Oxygen; THC, Total Haemocyte Count; DHC, Differential Haemocytes Counts; proPO, proPhenoloxidase; SPSS, Statistical Package for the Social Sciences; ANOVA, Analysis of Variance.

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Conflict of interests

The author should declare any conflict of interest.

Authors' contribution

SR designed the study, supervised, and conducted critical checking of this manuscript. DR interpreted the results and reviewed the manuscript. MATV conducted the study, collected the data, and contributed to manuscript preparation. PAW drafted the manuscript, took part in preparing it, and performed critical checking of the manuscript. All authors read and approved the final manuscript.

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Figure 1. Rearing pond of *L. vannamei* in this study

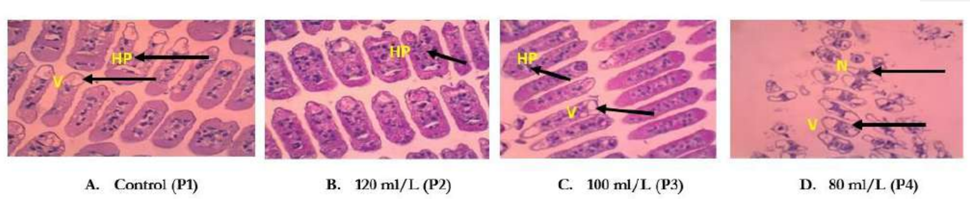

Figure 2. Histology of gill tissue of whiteleg shrimp (*L. vannamei*). *HP: Hyperplasia; V: Vacuolization; N: Necrosis.

Table 1. The results of the analysis of the production performance parameters of whiteleg shrimp *(L.vannamei)*

Treatments group	**Parameter**			
	Absolute Growth (g)	**SGR (%)**	**FCR**	**SR (%)**
P1	5.82 ± 0.12[a]	5,49±0,04[a]	1.51±0,05[c]	71.92±1,76[a]
P2	6.03 ± 0.06[a]	5,67±0,07[a]	1.45±0,03[c]	76.95±3,03[a]
P3	9.19 ± 0.52[c]	6,69±0.18[c]	0.90±0,08[a]	90.66±4,61[b]
P4	6.97 ± 0.06[b]	5,93±0,03[b]	1.27±0,01[b]	77.09±1,40[a]

Note: The mean±SD (Standard deviation) values with different superscript letters showed significantly different results ($P<0.05$). *SGR: Specific Growth Rate; FCR: Feed Conversion Ratio; SR: Survival Rate.

Table 2. Water quality parameters during the maintenance period

Parameter	**This study**	**Optimal range***
pH	6,5-8,5	7,5-8,5
Temperature (^{o}C)	27-33	28 – 30
Dissolved Oxygen (mg/L)	3,8-4,0	≥ 4
Ammonia (mg/L)	0- 0,1	≤ 0,1
Nitrite (mg/L)	0-0,52	≤ 1
Salinity	29,7-33	26-32
Nitrate (mg/L)	0-0,3	0,5

* Ministry of Marine Affairs and Fisheries Regulation (KKP), Republic of Indonesia 2016

Table 3. Immunity parameters (THC and DHC) of Whiteleg Shrimp with Hairy Eggplant and Bitter Ginger

Treatment Groups	**THC (x 10^3 cells/mm^3)**	**Hialin (%)**	**Semi Granular (%)**	**Granular (%)**
P1	4.63±2.05[a]	24.33±2.08[a]	35.33±1.52[c]	40.33±.0.57[d]
P2	8.63±0.87[b]	31.33±1.52[b]	31.00±1.00[b]	37.67±.0.57[c]
P3	16.76±0.90[c]	48.67±4.04[c]	23.67±3.51[a]	27.67±0.57[a]
P4	9.96±1.75[b]	34.67±1.52[b]	30.33±.0.57[b]	35.00±1.73[b]

Note: The mean values with different superscript letters showed significantly different results ($P<0.05$). *THC: Total Haemocyte Counts

Original Article

Supplementation of hairy eggplant *(Solanum ferox)* and bitter ginger *(Zingiber zerumbet)* extract as phytobiotic agents on whiteleg shrimp *(Litopenaeus vannamei)*

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ABSTRACT

Objective: This study aimed to evaluate the combination of hairy eggplant (*Solanum ferox*) and bitter ginger (*Zingiber zerumbet*) on the production performance and hematological parameters of whiteleg shrimp (*Litopenaeus vannamei*).
Materials and Methods: Four treatments were formulated in the test feed, where P1 (control+commercial vitamin C); P2 (120 ml/l combinations of *S. ferox* and *Z. zerumbet*); P3 (100 ml/l); and P4 (80 ml/l) 6,000 post-larvae shrimp with an average initial weight of 0.2 gm were randomly stocked in four groups, with three replications per treatment and 500 were stocked in each pond with a total of 12 pounds.
Results: Based on the results, there were significant differences in production performance [survival, absolute weight growth, specific growth rate (SGR), and feed conversion ratio (FCR)]. Biologically, the best performance was found in the P3 treatment (100 ml/l). In this treatment, the total number of hemocytes and the number of hyaline hemocytes were much higher, and this was not the case in the control treatment (P1), where the number of semi-granular and granular cells was significantly higher than the treatment group ($p < 0.05$).
Conclusion: This study confirmed that supplementation of 100 ml/l of *S. ferox* and *Z. zerumbet* could improve the production performance and hemato-immunological parameters of whiteleg shrimp, with functional potential to be developed in phytobiotic-based commercial diets for shrimp.

Keywords: Growth; Haemocytes; Phytobiotics agent; Shrimp *Litopenaeus vannamei*

INTRODUCTION

Crustacean production has increased to 6.09 million tons, or equivalent to USD 36.2 million, due to increased consumption of shrimp commodities worldwide [1]. Whiteleg shrimp (*Litopenaeus vannamei)* are one of the most popular aquaculture commodities and are

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relatively easy to cultivate [2]. This can be proven by the high percentage of Pacific whiteleg shrimp cultivation worldwide, at around 90%. This shrimp can adapt to a broad salinity (euryhaline) between 5 to 30 ppt, cultivate with a high stocking density, and grow sufficiently with low protein feeds, making this shrimp a leading commodity in Indonesia.

However, an increase in shrimp production with unmatched water quality management, quality seeds, or superior broodstock to quality feed can harm shrimp productivity by causing various diseases. Several disease outbreaks in shrimp ponds are related to viral diseases like the white spot syndrome virus (WSSV) and Taura syndrome virus (TSV). In contrast, bacterial diseases like *Vibrio harveyi*, *V. alginolyticus*, and *V. parahaemolyticus* can cause considerable losses to shrimp hatcheries worldwide [3].

The whiteleg shrimp's immune systems are also an essential focus in the emergence of a disease in the aquaculture environment. Crustaceans, especially shrimp, only have a non-specific and humoral natural immune system [4]. This causes the growth and immunity of shrimp to be increased depending on the quality of the broodstock, the environment, and the application of biocontrol genes [5,6].

Currently, antibiotics and chemotherapy agents are still used to control bacterial diseases [7, 8]. However, the use of antibiotics unwisely can lead to the accumulation of residues in the tissue, resulting in a decrease in product quality, the emergence of antibiotic resistance problems in shrimp and other aquatic organisms [9], and pollution of the aquatic environment [10]. Therefore, the use of environmentally friendly materials is needed to control disease in the current aquaculture system. Several approaches, such as the use of pro-, pre-, and synbiotics [11], immunostimulants [12], vaccination [13], quorum-sensing, phage application [14], RNA interference (RNAi) [15], development of molecular-based diagnostic materials, and the breeding and spawning of specific pathogen free (SPF) shrimp [16], have attracted the attention of researchers and shrimp farmers.

Phytobiotics are functional additives derived from nature, including food supplement ingredients that have become an alternative to improve health and resistance to disease attacks that are ecologically correct in the modern aquaculture sector [17]. The development of phyobiotics materials is the best effort to improve the shrimp immune system. In addition to being compatible with the shrimp immune system, which is still primitive (non-specific immunity), phytobiotics also have several advantages such as abundant sources of materials, wide target range, the potential for large-scale application, and environmental friendliness. Some of the natural agents that can be used as phytobiotics to increase the immune system of whiteleg shrimp are hairy eggplant *(Solanum ferox)* and Bitter Ginger *(Zingiber zerumbet)* [18]. Hairy eggplant or *S. ferox* is widely planted in tropical areas such as Indonesia for its fruit. This plant has been listed as a medicinal plant in the ethnobotanical inventory because it is reported to be effective in treating human diseases. Hairy eggplant contains bioactive compounds such as phenolics, flavonoids, and polyphenols that play an essential role in preventing oxidative stress and several biological effects such as antioxidant and anti-inflammatory [19]. Another traditional plant such as *Zingiber zerumbet* (L), part of the Zingiberaceae family, is commonly found in Southeast Asia, such as Indonesia. This plant is used traditionally in various cuisines and drinks because it is reported to have anti-allergic properties found in its rhizomes.

Previous studies showed that the combination of Zingiber zerumbet and *Curcuma zedoria* added to the orange-spotted grouper (*Epinephelus coioides*) was able to act as an immunostimulant by increasing non-specific immune responses (respiratory burst activity, reactive oxygen species, phagocytic activity, superoxide dismustase, and lysozyme activity [20]. Hardi et al [21] explained that the combination of *Boesenbergia pandurate, Zingiber zerumbet,* and *Solanum ferox* effectively prevents infection with *Aeromonas hydrophila* and *Pseudomonas* sp., and modulates the immune system of tilapia, *Oreochromis niloticus*. However, studies highlighting the potential of phytobiotics have not been reported in white shrimp, although compound derivatives of phytobiotics have been previously reported [22].

Based on this report, this study aimed to evaluate the combination of hairy eggplant (*Solanum ferox)* and Bitter Ginger *(Zingiber zerumbet)* in increasing the production performance and immune system of whiteleg shrimp (*L. vannamei*). This study is expected to provide valuable preliminary information for applying environmentally friendly immunostimulants to increase pacific white shrimp production.

MATERIALS AND METHODS

Ethical approval

This research has been licensed by the Fisheries Expert Polytechnic (AUP), Marine and Fisheries Research and Human Resources Agency (BRSDM), Ministry of Marine Affairs and Fisheries (KKP) of the Republic of Indonesia with No: 1832/POLTEK-AUP/TU.210/IX/2020.

Study area and period

This study was conducted from September 2020 to December 2020 at the Loka Pengelolaan Sumber Daya Pesisir & Laut (PSPL) Serang, Directorate General of Marine Space Management, Ministry of Marine and Fisheries, Republic of Indonesia.

Study design

This study used a Completely Randomized Design (CRD) with four treatments and three replications. The treatments used were a combination of hairy eggplant (*S. ferox*) and Bitter Ginger (*Z. zerumbet*) with the following details:

P1 : no combination + vitamin C (control);
P2: the combination of *S. ferox* and *Z. zerumbet* with a dose of 120 ml/l;
P3: the combination of *S. ferox* and *Z. zerumbet* with a dose of 100 ml/l;
P4: the combination of *S. ferox* and *Z. zerumbet* with a dose of 80 ml/l.

Preparation of *S. ferox* and *Z. zerumbet*

Preparation of the extract refers to the study by Hardi et al. [21]. In short, the plants were collected from traditional markets. The materials were washed and dried in an oven at 40-45°C for 48 h. The dry ingredients were then mashed using a blender to form a fine powder. 100 gm of dry samples were mixed with 100 ml of 96% ethanol in an Erlenmeyer flask at room temperature for 72 h. The mixture was separated using 0.5 m of Whatman filter paper to obtain the extract filtrate. The filtrate was re-evaporated using a rotary evaporator to separate the ethanol content for 3-5 h. The extract was stored in the refrigerator for further testing.

Rearing of whiteleg shrimp

Rearing of whiteleg shrimp was carried out in a round tarpaulin container with a pool diameter of 2 meters and a height of 1 meter, for a total of 12 pools. Each pond has nine aeration points evenly distributed in the rearing pond (Fig. 1). The extract was mixed into the feed and the dose was adjusted for each treatment.Feeding was carried out four times a day at 07.00 a.m., 11.00 a.m., 3.00 p.m., and 7.00 p.m. The amount of feed given was adjusted to the feeding rate (FR) according to the Indonesian National Standard (SNI) No. 01-7246-2006, namely 15% to 10%.

Figure 1. Rearing pond of *L. vannamei* in this study

Production performance parameters

Survival rate

The survival rate of whiteleg shrimp was calculated at the end of the rearing period. The calculation was carried out according to the equations of Wiradana et al. [23] and Fendjalang et al.[24], namely:

$$SR(\%) = \frac{Nt}{No} \times 100\%$$

Note:
SR: Survival rate (%)
Nt: Number of shrimp at the end of the study (individual)
No: Number of shrimp at the beginning of the study (individual)

Absolute weight growth

Weight growth was observed every seven days by weighing the number of shrimp. Absolute weight growth based on Widanarni et al. [25] is as follows:

$$\Delta W = Wt - Wo$$

Note :
Δ*W*: Absolute weight growth (gm)
Wt: Final shrimp weight (gm)
Wo: Initial shrimp weight (gm)

Specific growth rate (SGR)

The specific growth rate was observed once a week based on Zubaidah et al. [26]:

$$SGR(\%/day) = \frac{LnWt - LnWo}{T} \times 100\%$$

Note:
SGR(%/day): Specific growth rate
Wt: Final shrimp biomass (gm)
Wo: Initial shrimp biomass (gm)
T : Rearing period (days)

***Feed conversion ratio* (FCR)**

FCR was calculated at the end of the rearing period based on Sarjito et al. [27]:

$$FCR = \frac{\Sigma F\ feed\ given - \Sigma F\ leftover\ feed}{(Bt + Bm) - Bo}$$

Note :
FCR: Feed conversion ratio (gm)
Σ*F*: Feeding amount (gm)
Bt: Final shrimp biomass (gm)
Bm: Dead shrimp biomass (gm)
Bo: Initial shrimp biomass (gm)

Water quality

To maintain the media quality, daily draining of water was carried out [28]. Water quality parameters measured include temperature, dissolved oxygen (DO), pH, ammonia, nitrate, and nitrite. Water quality parameters are used as supporting data in this study.

Immunity parameters

Whiteleg shrimp immune response was evaluated by calculating the total hemocyte count (THC) and differential hemocyte counts (DHC). Collection and preparation of shrimp hemolymph were carried out using Zahra et al. [28]. THC and DHC were analyzed by taking 0.1 ml of hemolymph in the fifth pereopod using a 1 ml syringe filled with 0.3 mL of Na-EDTA anticoagulant to prevent blood clots. The sample was homogenized for 5 minutes in a moistened microtube with 10% Na-EDTA. Hemolymph was dripped on the hemocytometer and closed using a cover glass. Calculation of the number and type of cells was carried out under a light microscope with a magnification of 400×. Observation of immune parameters was carried out at the end of the rearing period (56 days). The calculation of THC and DHC refers to the method by Suleman et al. [29].

$$THC = average\ \Sigma\ counted\ cells \times 250 \times dillution\ factor \times 1000$$

$$DHC\ (\%) = \frac{\Sigma A}{\Sigma B} \times 100$$

Note:
Σ*A:* The number of each hemocyte cell type
Σ*B:* Total hemocyte count

Histological analysis

The gill tissue was placed in Davidson's solution until dehydrated, embedded in paraffin, and sliced using a microtome. The tissue was stained using Hematoxylin and Eosin and analyzed to identify defects such as hyperplasia, vacuolation, and necrosis. The histology was shown in figures defined by Mari et al. [30].

Data analysis

Production performance parameters and immunity were tabulated using Ms. Excel 2019 (Microsoft, USA). Statistical analysis was carried out using SPSS Version 25 software (IBM, USA). The data underwent a homogeneity test followed by a single factor ANOVA with a 95% confidence interval. To find out the differences in each treatment, the data underwent the

Duncan test. The statistical results were interpreted and presented in the form of tables and figures.

RESULTS AND DISCUSSION

Production performance

Supplementation of hairy eggplant and Bitter Ginger in feed can increase absolute weight growth in whiteleg shrimp. The highest absolute weight growth was 9.19 g/individual and was found in P3, then P4 (6.97 gm/individual), P2 (6.03 gm/individual), and P1 (Control) (5.82 gm/individual) (Table 1). Weight gain can occur from energy entering the body. The energy obtained from feed ingredients will first be used for rearing activities, then the remaining energy will be used for the growth process [31,32]. The absolute weight growth of shrimp with hairy eggplant and Bitter Ginger was higher than the control whiteleg shrimp. This occurs due to the supplementation of hairy eggplant and bitter ginger, which can stimulate the absorption of feed nutrients, mainly due to their protein and bioactive content. Bioactive content such as flavonoids in traditional plants can act as antibacterials and antioxidants, minimizing the increase in pathogenic microflora in the digestive tract. It is thought to increase the digestibility of whiteleg shrimp [33–35].

Table 1. The results of the analysis of the production performance parameters of whiteleg shrimp *(L.vannamei)*

The best specific growth rate (SGR) was found in P3 with a growth rate of 6.69%/day, showing that doses of hairy eggplant and bitter ginger had significant differences ($p < 0.05$) in specific growth rate on whiteleg shrimp. Increased growth rates can occur due to optimal use of feed ingredients. On the other hand, a low growth rate can occur due to health problems, stress, and suboptimal utilization of feed nutrients used for growth [36].

The lowest FCR value was found in P3, then P2 and P4, respectively. The highest FCR (1.51) was found in the control treatment (Table 1). The FCR was inversely proportional to weight growth, so the lower the FCR, the higher the efficiency of the shrimp in utilizing feed for growth [37]. On the other hand, if the shrimp body is unstable, the shrimp may experience a decrease in appetite and the feed provided is not converted into biomass. Stressed or unhealthy shrimp can be better at converting feed ingredients for weight growth compared to healthy shrimp [38].

The highest survival rate was found in P3 (90.66%), and of all treatments, the lowest percentage was found in the control treatment (71.92%) (Table 1). This indicated that supplementation of hairy eggplant and Bitter Ginger on whiteleg shrimp had a significant difference in the survival rate of whiteleg shrimp maintained for 56 days. This is presumably due to the content of secondary metabolites in hairy eggplant and bitter ginger, which can increase the immune system against pathogenic bacterial infections to protect the shrimp body from stress. The biological process will increase due to the involvement of phytochemical substances produced by the extract ingredients capable of producing enzymes for detoxification, modulating the immune system, and increasing shrimp survival. The increased immune response can have a positive impact on increasing body resistance and reducing shrimp mortality. This was confirmed by Jasmanindar et al. [39], which found that the low survival rate in the treatment without the extract had a relationship with a weak immune system compared to the treatment with seaweed extract. The combination of phytobiotics (thyme essential oil, red thyme, and rosemary pepper) applied in a supplement for 20 days has significant benefits for improving antioxidant protection, reducing the impact of stressors, and modulating the immunity of tilapia against *Aeromonas hydrophila* infection [17].

Water quality

All variables observed in the rearing pond remained within the levels recommended for shrimp culture during the test period (Table 2).

Table 2. Water quality parameters during the rearing period

Immunity parameters

The hemato-immunological response is a central physiological mechanism playing a role in protecting animals from disease, environmental stressors, or specific biological agents [40,41] such as phytobiotics. In this study, total hemocyte count (THC) and differential hemocyte count (DHC) were performed to evaluate the immunity condition of whiteleg shrimp with hairy eggplant and Bitter Ginger during 56 days of rearing. Based on the results, the total hemocytes of whiteleg shrimp in all treatments during the rearing period was around 4.63–16.76 $\times 10^6$ cells/mm^3. The highest THC value was found in P3 and was significantly different from the other three treatments ($p < 0.05$). P1 had the lowest THC value of all treatments. P2 and P3 had no significant differences (Table 3).

Hemocytes are the primary mediators of cellular responses in crustaceans, with roles that include self-recognition, phagocytosis, production of reactive oxygen intermediates, wound healing, and the process of melanization by encapsulation of foreign materials [42–44]. The increase in THC of whiteleg shrimp with hairy eggplant and Bitter Ginger can be suspected by the effect of bioactive compounds that can modulate shrimp immunity. Other studies confirmed that natural hydrolyzed tannin products from sweet chestnut *(Castanea sativa)* could act as functional feed additives by promoting growth and hematological parameters of whiteleg shrimp [45]. The *in vivo* effect of Astragalus polysaccharide immunostimulating ingredients, chlorogenic acid, and berberine showed a higher increase in total hemocyte count in whiteleg shrimp [46].

The difference in THC values in each treatment may be due to the various concentrations of active ingredients in food due to the treatment that has been determined. The low value of THC at P1 (control) may be influenced by physiological factors such as the slow formation of hemocytes in the shrimp body [47,48]. The exciting discussion confirmed that the low number of shrimp hemocytes is due to infiltration of regenerated tissue and hemocyte cell death due to apoptosis [27].

Table 3. Immunity parameters (THC and DHC) of Whiteleg Shrimp with Hairy Eggplant and Bitter Ginger

The minor component of shrimp hemocytes in differential hemocyte count (DHC) is hyaline, while the other two types of hemocytes are granular and semi-granular cells.The highest percentage of hyaline was found in P3 (48.67%), followed by P4 (34.67%), P2 (31.33%), and P1 (24.33%) (Table 3). The lowest number of semi-granular cells was found in P3 (23.67%). The decreasing number of semi-granular cells was shown in all treatments except control (P1), with the highest number (Table 3). The same thing happened to the number of granular cells in the treatment group. The highest number of granular cells was found in P1/control (40.33%).

Hyaline cells have a vital role in the shrimp's defense system. This cell type has a high ratio of cytoplasmic nuclei and few cytoplasmic granules. An increase in the number of hyaline cells can be associated with phagocytic activity when in contact with antigens or immunostimulating substances that will stimulate the body's defense activity to evoke the first defense response [49]. Semi-granular cells have a relationship with the addition or reduction of hyaline cells, so that the decrease in the number of semi-granular cells in the treatment group was due to the process of further development into hyaline cells. As a result, these cells cannot develop into semi-granular cells, so the number of semi-granular cells is low. Semi-granular cells are more involved in the encapsulation mechanism. The encapsulation process is a defense reaction against a large number of foreign particles that cannot be phagocytized by hyaline cells. These cells respond more to polysaccharide compounds found in bacterial cell walls.

Granular cells are the type of hemocytic cells that have the most significant size with an active nucleus in the storage process until the release of prophenoloxidase (proPO) and cytotoxicity. In this study, an increase in granular cells also occurred in the control group (P1). This is due to the low number of hyaline cells involved in the first defense process, thus relying on granular cells for non-specific body defense, which is driven by the influence of immunostimulatory components such as vitamin C.

Histology analysis

The condition of gill tissue on whiteleg shrimp showed that treatments P1 (control) and P3 experienced vacuolation and hyperplasia (Fig. 2), followed by treatment P2, which only experienced hyperplasia, and treatment P4 which experienced vacuolation and necrosis.

Figure 2. Histology of gill tissue of whiteleg shrimp (*L. vannamei*). *HP: Hyperplasia; V: Vacuolization; N: Necrosis.

The histology can explain that gill tissue damage occurred in all treatments carried out. Physiological differences between each whiteleg shrimp can cause tissue damage even when extracted ingredients are used. More research is needed to prove this results. High hemocytes can indicate infection or stress factors. Clogged blood flow in the lamellae (due to physical trauma, pollutants, or other physiological disturbances) can cause edema (cell swelling) between the blood vessels and the epithelial lining of the primary lamellae. Miller and Zachary [50] explained that necrosis is acute cell damage and can be massive, resulting in incomplete tissue formation due to shrinkage or complete shrinkage of the nucleus. Hyperplasia is the formation of excessive tissue due to an increase in the number of cells so that lamellae with hyperplasia will experience thickening of epithelial tissue at the ends of the filaments or the epithelium located near the base of the gill lamellae [51].

Based on the results, a 100 ml/l combination of hairy eggplant and Bitter Ginger in the feed is auspicious for whiteleg shrimp, related to the production performance and immunity. However, further studies are still needed to emphasize the potential that this combination of extracts can provide against pathogenic bacteria and viruses in whiteleg shrimp.

CONCLUSION

Supplementation of hairy eggplant and Bitter Ginger into feed affected the production performance of whiteleg shrimp as indicated by absolute weight growth, specific growth rate, low feed conversion ratio (FCR), and high survival as shown in P3 (dose 100 ml/l). Similarly, the immunity parameters, including THC and DHC, were high in the P3 treatment group, although there was a tendency for higher semi-granular and granular cell values in the control group. All these results indicate that the combination of hairy eggplant and Bitter Ginger as phytobiotic ingredients could act as an exemplary modulator of the non-specific immune response of whiteleg shrimp, which ultimately increases production performance. Further studies are needed to obtain valuable information regarding the combination of this extract against infection with whiteleg shrimp pathogens such as viruses and bacteria to be used as a preventive agent.

List of abbreviations

WSSV, white spot syndrome Virus; ppt, parts per trillion; RNAi, RNA interference; SPF, Specific Pathogen Free; ml, milliliter; l, Liter; °C, degrees Celcius; gm, gram; µm, micrometer; a.m., ante meridiem; p.m., post meridiem; FR, Feeding Rate; SNI, Standard National Indonesia; SR, Survival Rate; SGR, Specific Growth Rate; FCR, Feed Conversion Ratio; DO, Dissolved Oxygen; THC, Total Haemocyte Count; DHC, Differential Haemocytes Counts; proPO, proPhenoloxidase; SPSS, Statistical Package for the Social Sciences; ANOVA, Analysis of Variance.

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Conflict of interests

The author should declare any conflict of interest.

Authors' contribution

SR designed the study, supervised, and conducted critical checking of this manuscript. DR interpreted the results and reviewed the manuscript. MATV conducted the study, collected the data, and contributed to manuscript preparation. PAW drafted the manuscript, took part in preparing it, and performed critical checking of the manuscript. All authors read and approved the final manuscript.

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Figure 1. Rearing pond of *L. vannamei* in this study

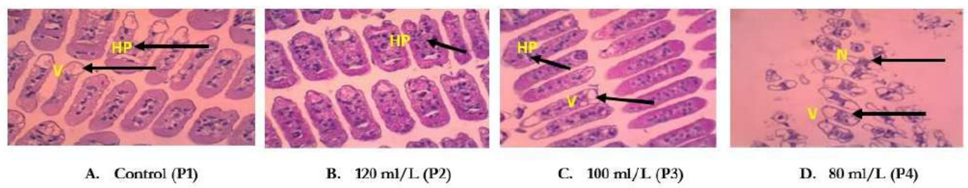

Figure 2. Histology of gill tissue of whiteleg shrimp (*L. vannamei*). *HP: Hyperplasia; V: Vacuolization; N: Necrosis.

Table 1. The results of the analysis of the production performance parameters of whiteleg shrimp *(L.vannamei)*

Treatments group	Parameter			
	Absolute Growth (g)	**SGR (%)**	**FCR**	**SR (%)**
P1	5.82 ± 0.12[a]	5,49±0,04[a]	1.51±0,05[c]	71.92±1,76[a]
P2	6.03 ± 0.06[a]	5,67±0,07[a]	1.45±0,03[c]	76.95±3,03[a]
P3	9.19 ± 0.52[c]	6,69±0.18[c]	0.90±0,08[a]	90.66±4,61[b]
P4	6.97 ± 0.06[b]	5,93±0,03[b]	1.27±0,01[b]	77.09±1,40[a]

Note: The mean±SD (Standard deviation) values with different superscript letters showed significantly different results (P<0.05). *SGR: Specific Growth Rate; FCR: Feed Conversion Ratio; SR: Survival Rate.

Table 2. Water quality parameters during the maintenance period

Parameter	**This study**	**Optimal range***
pH	6,5-8,5	7,5-8,5
Temperature (^oC)	27-33	28 – 30
Dissolved Oxygen (mg/L)	3,8-4,0	≥ 4
Ammonia (mg/L)	0- 0,1	≤ 0,1
Nitrite (mg/L)	0-0,52	≤ 1
Salinity	29,7-33	26-32
Nitrate (mg/L)	0-0,3	0,5

* Ministry of Marine Affairs and Fisheries Regulation (KKP), Republic of Indonesia 2016

Table 3. Immunity parameters (THC and DHC) of Whiteleg Shrimp with Hairy Eggplant and Bitter Ginger

Treatment Groups	**THC (x 10^3 cells/mm^3)**	**Hialin (%)**	**Semi Granular (%)**	**Granular (%)**
P1	4.63±2.05[a]	24.33±2.08[a]	35.33±1.52[c]	40.33±.0.57[d]
P2	8.63±0.87[b]	31.33±1.52[b]	31.00±1.00[b]	37.67±.0.57[c]
P3	16.76±0.90[c]	48.67±4.04[c]	23.67±3.51[a]	27.67±0.57[a]
P4	9.96±1.75[b]	34.67±1.52[b]	30.33±.0.57[b]	35.00±1.73[b]

Note: The mean values with different superscript letters showed significantly different results (P<0.05). *THC: Total Haemocyte Counts