

1 ORIGINAL ARTICLE

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3 **Supplementation of Hairy Eggplant (*Solanum ferox*) and Bitter**

4 **Ginger (*Zingiber zerumbet*) Extract as Phytobiotic Agents on**

5 **Whiteleg Shrimp (*Litopenaeus vannamei*)**

6

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

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

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21 **Supplementation of Hairy Eggplant (*Solanum ferox*) and Bitter**
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23 **Whiteleg Shrimp (*Litopenaeus vannamei*)**

24

25 **ABSTRACT**

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

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

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

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

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

45

46 INTRODUCTION

47 Crustacean production has increased to 6.09 million tons or equivalent to USD 36.2 million due
48 to increased consumption of shrimp commodities worldwide [1]. Whiteleg shrimp (*Litopenaeus*
49 *vannamei*) is one of the most popular aquaculture commodities and is relatively easy to cultivate
50 [2]. This can be proven by the high percentage of pacific whiteleg shrimp cultivation worldwide,
51 which is around 90% [3]. This shrimp is able to adapt to a broad salinity (euryhaline) between 5
52 to 30 ppt, can be cultivated with a high stocking density, and is able to grow well with low protein
53 feeds, making this shrimp a leading commodity in Indonesia.

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

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

65 Until now, efforts to control bacterial diseases are still through antibiotics and chemotherapy
66 agents [8,9]. However, the use of antibiotics in an unwise manner can lead to the accumulation
67 of residues in the tissue which results in a decrease in product quality [10], the emergence of
68 antibiotic resistance problems in shrimp and other aquatic organisms [11], to pollution of the
69 aquatic environment [12]. Therefore, the use of environmentally friendly materials is needed to
70 control disease in the current aquaculture system. Several approaches such as the use of pro-, pre-
71 and synbiotics [13], immunostimulants [14], vaccination [15], quorum-sensing [16], phage

72 application [17], RNA interference (RNAi) [18], development of molecular-based diagnostic
73 materials [19], to the breeding and spawning of *Specific Pathogen Free* (SPF) shrimp [20] have
74 attracted the attention of researchers and shrimp farmers.

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

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

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

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

102

103 **MATERIALS AND METHODS**

104 **Ethical approval:** This study does not need ethical approval.

105

106 **Study area and period**

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

110 **Study Design**

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

114 P1 : no combination + vitamin C (control);

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

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

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

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

119 Preparation of extract refers to the study by Hardi et al. [27]. In short, the plants were collected
120 from traditional markets. The materials were washed and dried in an oven at a temperature of 40-
121 45°C for 48 hours. The dry ingredients were then mashed using a blender to form a fine powder.
122 100 g of dry samples were mixed with 100 ml of 96% ethanol in an Erlenmeyer flask at room

123 temperature for 72 hours. The mixture was separated using 0.5 m Whatman filter paper to obtain
124 the extract filtrate. The filtrate was re-evaporated using a rotary evaporator to separate the
125 ethanol content for 3-5 hours. The extract was stored in the refrigerator for further testing.

126 **Rearing of whiteleg shrimp**

127 Rearing of Whiteleg Shrimp was carried out in a round tarpaulin container with a pool diameter
128 of 2 meters and a height of 1 meter with a total of 12 pools. Each pond has nine aeration points
129 which are evenly distributed in the rearing pond (Figure 1). The extract was mixed in the feed
130 adjusted to the dose in each treatment. Feeding was carried out four times a day at 07.00 a.m;
131 11.00 a.m.; 15.00 p.m.; and 19.00 p.m. The amount of feed given was adjusted to the Feeding rate
132 (FR) according to the Indonesian National Standard (SNI) No. 01-7246-2006 namely 15% to
133 10%.

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

135

136 **Production performance parameters**

137 **Survival rate**

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

$$141 \quad SR(\%) = \frac{N_t}{N_o} \times 100\%$$

142 Note :

143 *SR* : Survival rate (%)

144 *N_t* : Number of shrimp at the end of the study (individual)

145 *N_o* : Number of shrimp at the beginning of the study (individual)

146 **Absolute Weight Growth**

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

$$\Delta W = W_t - W_o$$

149

150 Note :

151 ΔW : Absolute weight growth (gr)152 W_t : Final shrimp weight (gr)153 W_o : Initial shrimp weight (gr)154 **Specific Growth Rate (SGR)**

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

$$SGR(\%/day) = \frac{\ln W_t - \ln W_o}{T} \times 100\%$$

156

157 Note :

158 $SGR(\%/day)$: Specific growth rate159 W_t : Final shrimp biomass (gr)160 W_o : Initial shrimp biomass (gr)161 T : Rearing period (days)162 **Feed Conversion Ratio (FCR)**

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

$$FCR = \frac{\Sigma F \text{ pakan yang diberikan} - \Sigma F \text{ sisa pakan}}{(B_t + B_m) - B_o}$$

164

165 Note :

166 FCR : Feed conversion ratio (gr)167 ΣF : Feeding amount (gr)168 B_t : Final shrimp biomass (gr)169 B_m : Dead shrimp biomass (gr)170 B_o : Initial shrimp biomass (gr)

171

172 **Water quality**

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

176

177 **Immunity parameters**

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

$$188 \quad \text{THC} = \text{average } \Sigma \text{ counted cells} \times 250 \times \text{dillution factor} \times 1000$$

$$189 \quad \text{DHC (\%)} = \frac{\Sigma A}{\Sigma B} \times 100$$

190 Note :

191 ΣA : The number of each hemocyte cell type

192 ΣB : Total hemocyte count

193

194 **Histological analysis**

195 The gill tissue was placed in Davidson's solution until dehydrated, embedded in paraffin, and
 196 sliced using a microtome. Tissue was stained using hematoxylin and eosin and analyzed to

197 identify defects such as hyperplasia, vacuolation, and necrosis [37]. The histology was shown in
198 Figures defined by Bonami et al. [38].

199

200 **Data analysis**

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

207

208 **RESULTS AND DISCUSSION**

209 **Production performance**

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

222

Table 1. Production performance parameters of Whiteleg Shrimp

223
224 The best specific growth rate (SGR) was found in P3 with a growth of 6.69%/day showing doses
225 of Hairy Eggplant and Bitter Ginger had significant differences ($p < 0.05$) on specific growth rate
226 on Whiteleg Shrimp. Increased growth rate can occur due to optimal use of feed ingredients. On
227 the other hand, a low growth rate can occur due to health problems, stress, and suboptimal
228 utilization of feed nutrients used for growth [43].

229
230 The lowest FCR value was found in P3, then P2 and P4 respectively. The highest FCR (1.51) was
231 found in the control treatment (Table 1). FCR was inversely proportional to weight growth, so
232 the lower the FCR, the higher the efficiency of the shrimp in utilizing feed for growth [44]. On
233 the other hand, if the shrimp body is in an unstable state, the shrimp may experience a decrease
234 in appetite, and the feed provided is not converted into biomass [45]. Healthy shrimp can be
235 better at converting feed ingredients for weight growth compared to stressed or sick shrimp.

236
237 The highest survival rate was found in P3 (90.66%) and of all treatments, the lowest percentage
238 was found in the control treatment (71.92%) (Table 1). This indicated that supplementation of
239 Hairy Eggplant and Bitter Ginger on Whiteleg Shrimp had a significant difference in the survival
240 rate of Whiteleg Shrimp maintained for 56 days. This is presumably due to the content of
241 secondary metabolites in Hairy Eggplant and Bitter Ginger which can increase the immune
242 system against pathogenic bacterial infections to protect the shrimp body from stress. Huang et
243 al.[46] explained that the biological process will increase due to the involvement of
244 phytochemical substances produced by the extract ingredients capable of producing enzymes as
245 detoxification, modulating the immune system, and increasing the survival of shrimp. The
246 increased immune response can have a good impact on increasing body resistance and reducing
247 the number of shrimp mortality. This was confirmed by Jasmanindar et al. [47] which found that

248 the low survival rate in the treatment without the extract had a relationship with a weak immune
249 system compared to the treatment with seaweed extract.

250

251 **Water quality**

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

254 **Table 2.** Water quality parameters during the rearing period

255

256 **Immunity parameters**

257 The hemato-immunological response is a major physiological mechanism playing a role in
258 protecting animals from disease, environmental stressors, or certain biological agents [48] such as
259 phytobiotics. In this study, total hemocyte count (THC) and differential hemocyte count (DHC)
260 were performed to evaluate the immunity condition of Whiteleg Shrimp with Hairy Eggplant and
261 Bitter Ginger during 56 days of rearing. Based on the results, the total hemocytes of Whiteleg
262 Shrimp in all treatments during the rearing period was around 4.63 – 16.76 ($\times 10^3$ cells/mm³). The
263 highest THC value was found in P3 and was significantly different when compared to the other
264 three treatments ($P < 0.05$). P1 had the lowest THC value of all treatments. P2 and P3 had no
265 significant differences (Table 3). Hemocytes are the main mediators of cellular responses in
266 crustaceans with roles that include self-recognition, phagocytosis, production of reactive oxygen
267 intermediates, wound healing, to the process of melanization by encapsulation of foreign
268 materials [49–51]. The increase in THC of Whiteleg Shrimp with Hairy Eggplant and Bitter
269 Ginger can be suspected by the effect of bioactive compounds that can modulate shrimp
270 immunity. Other studies confirmed that natural hydrolyzed tannin products from sweet chestnut
271 (*Castanea sativa*) are able to act as functional feed additives by promoting growth and
272 hematological parameters of Whiteleg Shrimp [52]. The in vivo effect of Astragalus

273 polysaccharide immunostimulating ingredients, chlorogenic acid, and berberine showed a higher
274 increase in total hemocyte count in Whiteleg Shrimp [53].

275
276 The difference in THC values in each treatment may be due to the various concentrations of
277 active ingredients in food due to the treatment that has been determined. The low value of THC
278 at P1 (control) may be influenced by physiological factors such as the slow formation of
279 hemocytes in the shrimp body [54,55]. Interesting discussion confirmed that the low number of
280 shrimp hemocytes is due to infiltration of regenerated tissue and hemocytes cell death due to
281 apoptosis [32].

282

283

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

286

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

294

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

299 [56]. Semi-granular cells have a relationship with the addition or reduction of hyaline cells so that
300 the decrease in the number of semi-granular cells in the treatment group was due to the process
301 of further development into hyaline cells. As a result, these cells cannot develop into semi-
302 granular cells so that the number of semi-granular cells is low [56]. Semi-granular cells are more
303 involved in the encapsulation mechanism. The encapsulation process is a defense reaction against
304 a large number of foreign particles that are not able to be phagocytized by Hyaline cells. These
305 cells respond more to polysaccharide compounds found in bacterial cell walls [57].

306

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

313

314 **Histologi analysis**

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

318

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

320

321 The histology can explain that gill tissue damage occurred in all treatments carried out.
322 Physiological differences of each individual Whiteleg Shrimp can cause tissue damage, even
323 though there is an intervention of extract ingredients. High hemocytes can indicate infection or
324 stress factors can cause this, although further studies are still needed to prove this. Clogged blood

325 flow (caused by physical trauma, pollutants, or other physiological disturbances) in the lamellae
326 can cause edema (swelling of cells) between the blood vessels and the epithelial lining of the
327 primary lamellae [59]. Miller and Zachary [60] explained that necrosis is acute cell damage and can
328 be massive, resulting in incomplete tissue formation due to shrinkage or complete shrinkage of
329 the nucleus. Hyperplasia is the formation of excessive tissue due to an increase in the number of
330 cells so that lamellae with hyperplasia will experience thickening of epithelial tissue at the ends of
331 the filaments or the epithelium located near the base of the gill lamellae [61].

332

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

337

338 **CONCLUSION**

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

350

351 LIST OF ABBREVIATIONS

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

359

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361 -

362 CONFLICT OF INTERESTS

363 The author should declare any conflict of interest.

364

365 AUTHORS' CONTRIBUTION

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

371

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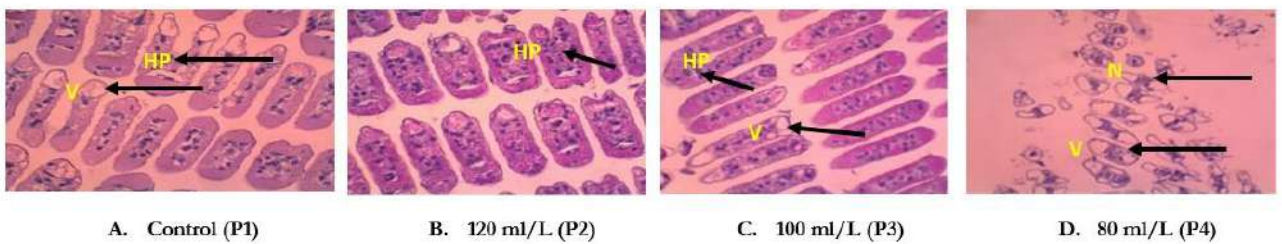
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Figure 1. Rearing pond of *L. vannamei* in this study



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A. Control (P1)

B. 120 ml/L (P2)

C. 100 ml/L (P3)

D. 80 ml/L (P4)

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

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Vacuolization; N: Necrosis.

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590 **Table 1.** The results of the analysis of the production performance parameters of whiteleg
 591 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

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

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595 **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

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

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

Treatment	THC	Hialin	Semi Granular	Granular
Groups	(x 10 ³ sel/mm ³)	(%)	(%)	(%)
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

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

605 Total Haemocyte Counts

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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*)

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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

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 phybiotics 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 zedaria* 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 dismutase, and lysozyme activity [20]. Hardi et al [21] explained that the combination of *Boesenbergia pandurata*, *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:

$$SR(\%) = \frac{N_t}{N_o} \times 100\%$$

Note:

SR: Survival rate (%)

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

N_o: 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:

$$\Delta W = W_t - W_o$$

Note :

ΔW : Absolute weight growth (gm)

W_t: Final shrimp weight (gm)

W_o: Initial shrimp weight (gm)

Specific growth rate (SGR)

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

$$SGR(\%/day) = \frac{\ln W_t - \ln W_o}{T} \times 100\%$$

Note:

SGR(%/day): Specific growth rate

W_t: Final shrimp biomass (gm)

W_o: 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]:

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

Note :

FCR: Feed conversion ratio (gm)

ΣF : 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 \times . 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].

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

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

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. 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 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

(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 $4.63\text{--}16.76 \times 10^6$ cells/mm³. 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

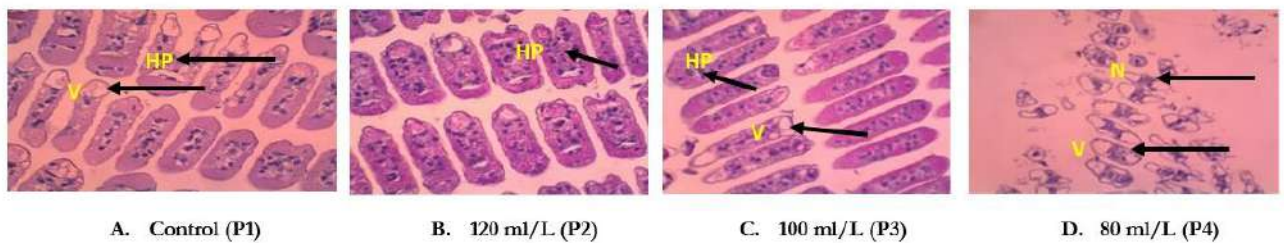


Figure 2. Histology of gill tissue of whiteleg shrimp (*L. vannamei*). *HP: Hyperplasia; V: Vacuolization; N: Necrosis.

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 (°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

3. PERINTAH PERBAIKAN DARI PARA REVIEWER

JURNAL

1 ORIGINAL ARTICLE

2

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

6

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

12

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

14

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21 **Supplementation of Hairy Eggplant (*Solanum ferox*) and Bitter**
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24
25 **ABSTRACT**

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

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

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

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

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

46 INTRODUCTION

47 Crustacean production has increased to 6.09 million tons or equivalent to USD 36.2 million due
48 to increased consumption of shrimp commodities worldwide [1]. Whiteleg shrimp (*Litopenaeus*
49 *vannamei*) is one of the most popular aquaculture commodities and is relatively easy to cultivate
50 [2]. This can be proven by the high percentage of pacific whiteleg shrimp cultivation worldwide,
51 which is around 90% [3]. This shrimp is able to adapt to a broad salinity (euryhaline) between 5
52 to 30 ppt, can be cultivated with a high stocking density, and is able to grow well with low protein
53 feeds, making this shrimp a leading commodity in Indonesia.

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

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

65 Until now, efforts to control bacterial diseases are still through antibiotics and chemotherapy
66 agents [7,8]. However, the use of antibiotics in an unwise manner can lead to the accumulation
67 of residues in the tissue which results in a decrease in product quality, the emergence of
68 antibiotic resistance problems in shrimp and other aquatic organisms [9], to pollution of the
69 aquatic environment [10]. Therefore, the use of environmentally friendly materials is needed to
70 control disease in the current aquaculture system. Several approaches such as the use of pro-, pre-
71 and synbiotics [11], immunostimulants [12], vaccination [13], quorum-sensing, phage

72 application [14], RNA interference (RNAi) [15], development of molecular-based diagnostic
73 materials ~~[19]~~, to the breeding and spawning of *Specific Pathogen Free* (SPF) shrimp [16] have
74 attracted the attention of researchers and shrimp farmers.

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

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

92 Previous studies showed that the combination of *Zingiber zerumbet* and *Curcuma zedoria* added
93 to the Orange-spotted grouper (*Epinephelus coioides*) was able to act as an immunostimulant by
94 increasing non-specific immune responses (respiratory burst activity, reactive oxygen species,
95 phagocytic activity, superoxide dismutase, and lysozyme activity [20]. Hardi [21] explained that
96 the combination of *Boesenbergia pandurata*, *Zingiber zerumbet*, and *Solanum ferox* are effective in
97 preventing infection with *Aeromonas hydrophila* and *Pseudomonas* sp. and modulating the immune

98 system of tilapia *Oreochromis niloticus*. However, to date, studies highlighting the potential of
99 phytobiotic have not been reported in Pacific white shrimp.

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

105

106 MATERIALS AND METHODS

107 **Ethical approval:** This study does not need ethical approval.

Commented [K1]: complete with research ethics

108

109 Study area and period

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

113 Study Design

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

117 P1 : no combination + vitamin C (control);

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

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

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

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

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

124 45°C for 48 hours. The dry ingredients were then mashed using a blender to form a fine powder.
125 100 g of dry samples were mixed with 100 ml of 96% ethanol in an Erlenmeyer flask at room
126 temperature for 72 hours. The mixture was separated using 0.5 m Whatman filter paper to obtain
127 the extract filtrate. The filtrate was re-evaporated using a rotary evaporator to separate the
128 ethanol content for 3-5 hours. The extract was stored in the refrigerator for further testing.

129 Rearing of whiteleg shrimp

130 Rearing of Whiteleg Shrimp was carried out in a round tarpaulin container with a pool diameter
131 of 2 meters and a height of 1 meter with a total of 12 pools. Each pond has nine aeration points
132 which are evenly distributed in the rearing pond (Figure 1). The extract was mixed in the feed
133 adjusted to the dose in each treatment. Feeding was carried out four times a day at 07.00 a.m;
134 11.00 a.m.; 15.00 p.m.; and 19.00 p.m. The amount of feed given was adjusted to the Feeding rate
135 (FR) according to the Indonesian National Standard (SNI) No. 01-7246-2006 namely 15% to
136 10%.

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

138

139 Production performance parameters

140 Survival rate

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

$$144 \quad SR(\%) = \frac{Nt}{No} \times 100\%$$

145 Note :

146 *SR* : Survival rate (%)

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

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

Commented [K2]: Use word equation...

149 **Absolute Weight Growth**

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

$$152 \quad \Delta W = W_t - W_o$$

Commented [K3]: Use word equations

153 Note :

154 ΔW : Absolute weight growth (gr)

155 W_t : Final shrimp weight (gr)

156 W_o : Initial shrimp weight (gr)

157 **Specific Growth Rate (SGR)**

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

$$159 \quad SGR(\%/day) = \frac{\ln W_t - \ln W_o}{T} \times 100\%$$

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160 Note :

161 $SGR(\%/day)$: Specific growth rate

162 W_t : Final shrimp biomass (gr)

163 W_o : Initial shrimp biomass (gr)

164 T : Rearing period (days)

165 **Feed Conversion Ratio (FCR)**

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

$$167 \quad FCR = \frac{\Sigma F \text{ feed given} - \Sigma F \text{ leftover feed}}{(B_t + B_m) - B_o}$$

Commented [K5]: Use word equations

168 Note :

169 FCR : Feed conversion ratio (gr)

170 ΣF : Feeding amount (gr)

171 B_t : Final shrimp biomass (gr)

172 B_m : Dead shrimp biomass (gr)

173 B_o : Initial shrimp biomass (gr)

174

175 **Water quality**

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

179

180 **Immunity parameters**

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

192

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

193

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

194 Note :

195 ΣA : The number of each hemocyte cell type196 ΣB : Total hemocyte count

197

198 **Histological analysis**

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

203

204 **Data analysis**

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

211

212 **RESULTS AND DISCUSSION**

213 **Production performance**

214 Supplementation of Hairy Eggplant and Bitter Ginger in feed can increase absolute weight
215 growth in Whiteleg Shrimp. The highest absolute weight growth was 9.19 g/individual and found
216 in P3, then P4 (6.97 g/individual), and P2 (6.03 g/individual), and P1 (Control) (5.82
217 g/individual) (Table 1). Weight growth can occur from the energy entering the body. The energy
218 obtained from feed ingredients will first be used for rearing activities, then the remaining energy
219 will be used for the growth process [32]. The absolute weight growth of shrimp with Hairy
220 Eggplant and Bitter Ginger was higher than the control Whiteleg Shrimp. This occurs due to the
221 supplementation of Hairy Eggplant and Bitter Ginger which can stimulate the process of
222 absorption of feed nutrients, especially due to protein and bioactive content. Bioactive content
223 such as flavonoids in traditional plants can act as antibacterial and antioxidant which can

224 minimize the increase in the number of pathogenic microflora in the digestive tract so that it is
225 thought to increase the digestibility of Whiteleg Shrimp [33–35].

226 **Table 1.** Production performance parameters of Whiteleg Shrimp

227
228 The best specific growth rate (SGR) was found in P3 with a growth of 6.69%/day showing doses
229 of Hairy Eggplant and Bitter Ginger had significant differences ($p < 0.05$) on specific growth rate
230 on Whiteleg Shrimp. Increased growth rate can occur due to optimal use of feed ingredients. On
231 the other hand, a low growth rate can occur due to health problems, stress, and suboptimal
232 utilization of feed nutrients used for growth [36].

233
234 The lowest FCR value was found in P3, then P2 and P4 respectively. The highest FCR (1.51) was
235 found in the control treatment (Table 1). FCR was inversely proportional to weight growth, so
236 the lower the FCR, the higher the efficiency of the shrimp in utilizing feed for growth [37]. On
237 the other hand, if the shrimp body is in an unstable state, the shrimp may experience a decrease
238 in appetite, and the feed provided is not converted into biomass [38]. Healthy shrimp can be
239 better at converting feed ingredients for weight growth compared to stressed or sick shrimp.

240
241 The highest survival rate was found in P3 (90.66%) and of all treatments, the lowest percentage
242 was found in the control treatment (71.92%) (Table 1). This indicated that supplementation of
243 Hairy Eggplant and Bitter Ginger on Whiteleg Shrimp had a significant difference in the survival
244 rate of Whiteleg Shrimp maintained for 56 days. This is presumably due to the content of
245 secondary metabolites in Hairy Eggplant and Bitter Ginger which can increase the immune
246 system against pathogenic bacterial infections to protect the shrimp body from stress. Huang et
247 al.[39] explained that the biological process will increase due to the involvement of
248 phytochemical substances produced by the extract ingredients capable of producing enzymes as
249 detoxification, modulating the immune system, and increasing the survival of shrimp. The

250 increased immune response can have a good impact on increasing body resistance and reducing
251 the number of shrimp mortality. This was confirmed by Jasmanindar et al. [40] which found that
252 the low survival rate in the treatment without the extract had a relationship with a weak immune
253 system compared to the treatment with seaweed extract. The combination of phytobiotics (thyme
254 essential oil, red thyme, and rosemary pepper) which is applied in a supplement for 20 days has
255 significant benefits for greater antioxidant protection, reducing the impact of stressors, and
256 modulating the immunity of tilapia against *Aeromonas hydrophila* infection [17].

257

258 Water quality

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

261 **Table 2.** Water quality parameters during the rearing period

262

263 Immunity parameters

264 The hemato-immunological response is a major physiological mechanism playing a role in
265 protecting animals from disease, environmental stressors, or certain biological agents [41] such as
266 phytobiotics. In this study, total hemocyte count (THC) and differential hemocyte count (DHC)
267 were performed to evaluate the immunity condition of Whiteleg Shrimp with Hairy Eggplant and
268 Bitter Ginger during 56 days of rearing. Based on the results, the total hemocytes of Whiteleg
269 Shrimp in all treatments during the rearing period was around 4.63 – 16.76 ($\times 10^6$ cells/mm³). The
270 highest THC value was found in P3 and was significantly different when compared to the other
271 three treatments ($P < 0.05$). P1 had the lowest THC value of all treatments. P2 and P3 had no
272 significant differences (Table 3).

273 Hemocytes are the main mediators of cellular responses in crustaceans with roles that include
274 self-recognition, phagocytosis, production of reactive oxygen intermediates, wound healing, to
275 the process of melanization by encapsulation of foreign materials [42–44]. The increase in THC

276 of Whiteleg Shrimp with Hairy Eggplant and Bitter Ginger can be suspected by the effect of
277 bioactive compounds that can modulate shrimp immunity. Other studies confirmed that natural
278 hydrolyzed tannin products from sweet chestnut (*Castanea sativa*) are able to act as functional feed
279 additives by promoting growth and hematological parameters of Whiteleg Shrimp [45]. The in
280 vivo effect of Astragalus polysaccharide immunostimulating ingredients, chlorogenic acid, and
281 berberine showed a higher increase in total hemocyte count in Whiteleg Shrimp [46].

282
283 The difference in THC values in each treatment may be due to the various concentrations of
284 active ingredients in food due to the treatment that has been determined. The low value of THC
285 at P1 (control) may be influenced by physiological factors such as the slow formation of
286 hemocytes in the shrimp body [47,48]. Interesting discussion confirmed that the low number of
287 shrimp hemocytes is due to infiltration of regenerated tissue and hemocytes cell death due to
288 apoptosis [27].

289

290

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

293

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

301

302 Hyaline cells have a vital role in the shrimp defense system. This cell type has a high ratio of
303 cytoplasmic nuclei and few cytoplasmic granules. An increase in the number of hyaline cells can
304 be associated with phagocytic activity when in contact with antigens or immunostimulating
305 substances will stimulate the body's defense activity so that it will evoke the first defense response
306 [49]. Semi-granular cells have a relationship with the addition or reduction of hyaline cells so that
307 the decrease in the number of semi-granular cells in the treatment group was due to the process
308 of further development into hyaline cells. As a result, these cells cannot develop into semi-
309 granular cells so that the number of semi-granular cells is low [49]. Semi-granular cells are more
310 involved in the encapsulation mechanism. The encapsulation process is a defense reaction against
311 a large number of foreign particles that are not able to be phagocytized by Hyaline cells. These
312 cells respond more to polysaccharide compounds found in bacterial cell walls [50].

313
314 Granular cells are the type of hemocytic cells that have the largest size with an active nucleus in
315 the storage process until the release of prophenoloxidase (proPO) and cytotoxicity. In this study,
316 an increase in granular cells also occurred in the control group (P1). This is due to the low
317 number of hyaline cells involved in the first defense process, thus relying on granular cells for
318 non-specific body defense which is driven by the influence of immunostimulatory components
319 such as vitamin C [51].

321 **Histology analysis**

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

325

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

327

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

339
340 Based on the results, 100 ml/L combination of Hairy Eggplant and Bitter Ginger in feed is very
341 promising for Whiteleg Shrimp, related to the production performance and immunity. However,
342 further studies are still needed to emphasize the potential that this combination of extracts can
343 provide against pathogenic bacteria and viruses in Whiteleg Shrimp.

344

345 **CONCLUSION**

346 Supplementation of Hairy Eggplant and Bitter Ginger into feed was able to affect the production
347 performance of Whiteleg Shrimp as indicated by absolute weight growth, specific growth rate,
348 low feed conversion ratio (FCR), and high survival as shown in P3 (dose 100 ml/L). Similarly, the
349 immunity parameters which include THC and DHC were high in the P3 treatment group,
350 although there was a tendency for higher semi-granular and granular cell values in the control
351 group. All these results indicated that the combination of Hairy Eggplant and Bitter Ginger as
352 phytobiotic ingredients can act as a good modulator of the non-specific immune response of
353 Whiteleg Shrimp which ultimately increases production performance. Further studies are still

354 needed to obtain valuable information regarding the combination of this extract against infection
355 with Whiteleg Shrimp pathogens such as viruses and bacteria so that it can be used as a
356 preventive agent.

357

358 **LIST OF ABBREVIATIONS**

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

366

367 **ACKNOWLEDGMENT**

368 -

369 **CONFLICT OF INTERESTS**

370 The author should declare any conflict of interest.

371

372 **AUTHORS' CONTRIBUTION**

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

378

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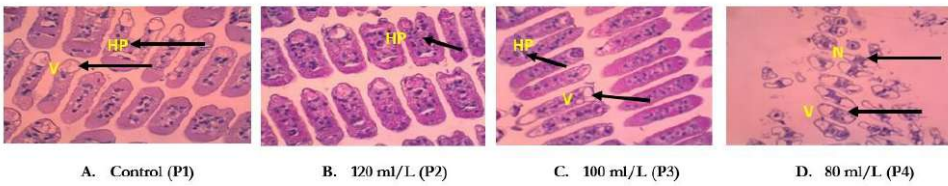
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Figure 1. Rearing pond of *L. vannamei* in this study



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A. Control (P1)

B. 120 ml/L (P2)

C. 100 ml/L (P3)

D. 80 ml/L (P4)

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

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Vacuolization; N: Necrosis.

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551 **Table 1.** The results of the analysis of the production performance parameters of whiteleg
 552 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

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

555

556 **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

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

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

Treatment	THC	Hialin	Semi Granular	Granular
Groups	(x 10 ³ sel/mm ³)	(%)	(%)	(%)
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

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

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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*)**

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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

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 (euhaline) 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 phytobiotics 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 dismutase, and lysozyme activity [20]. [Hardi et al \[21\]](#) explained that the combination of *Boesenbergia pandurata*, *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.

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 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 :

ΔW : 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]:

$$SGR(\%/day) = \frac{\ln Wt - \ln Wo}{T} \times 100\%$$

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]:

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

Note :

FCR: Feed conversion ratio (gm)

ΣF : 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].

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

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

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. 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/mm³. 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

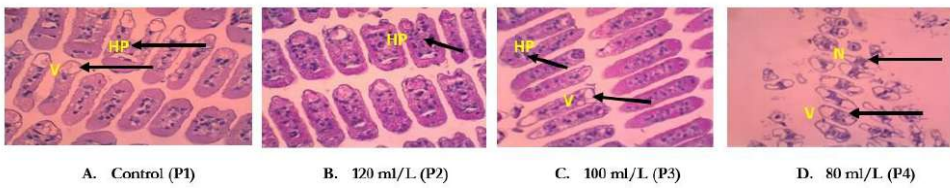


Figure 2. Histology of gill tissue of whiteleg shrimp (*L. vannamei*). *HP: Hyperplasia; V: Vacuolization; N: Necrosis.

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 (°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*)Sinung Rahardjo¹, Merary A. The Vauza², Djumbuh Rukmono¹, Putu Angga Wiradana³¹Department of Aquaculture, Faculty of Utilization Fisheries, Jakarta Fisheries University, Jakarta, 12520, Indonesia.²Master Student of Fisheries Resource Utilization, Postgraduate Program of Jakarta Fisheries University, Jakarta 12520, Indonesia.³Study Program of Biology, Faculty of Health, Science, and Technology, Universitas Dhyana Pura, Provinsi Bali 80351, Indonesia.**Correspondence:** Sinung Rahardjo (snngrahardjo@gmail.com)**ORCIDs**S R (<https://orcid.org/0000-0002-4698-2623>)M A T V (<https://orcid.org/0000-0002-1826-0471>)D R (<https://orcid.org/0000-0001-5667-0669>)P A W (<https://orcid.org/0000-0002-0139-8781>)**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

3rd revision: October 1, 2021

Commented [KNHN1]: Unfortunately, we suggested to work on reference issues (mainly), but you did not make the necessary corrections completely.

Moreover, you have changed our style. It (mL/L) is not our style. ml/l is the style of JAVAR. So, please do the needful along with reference errors.

Also, we requested to mark the modified parts in red fonts, but you have ignored that. Our team intensively worked on the article including language correction, but now we are unable to locate where you have changed.

Please do the needful with maximum care so that we can add this article in our December issue. Otherwise it will be moved to the next issue.

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 (euhaline) 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 phytobiotics 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 dismutase, and lysozyme activity [20]. Hardi et al [21] explained that the combination of *Boesenbergia pandurata*, *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 :

ΔW : 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]:

$$SGR(\%/day) = \frac{\ln Wt - \ln Wo}{T} \times 100\%$$

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]:

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

Note :

FCR: Feed conversion ratio (gm)

ΣF : 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].

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

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

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. 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 $4.63\text{--}16.76 \times 10^6$ cells/mm³. 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

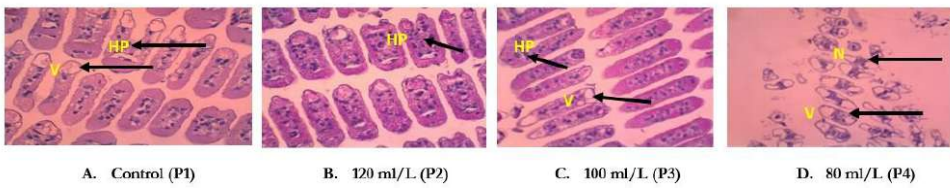


Figure 2. Histology of gill tissue of whiteleg shrimp (*L. vannamei*). *HP: Hyperplasia; V: Vacuolization; N: Necrosis.

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 (°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