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Research Article

Dietary Supplementation with Hydrolyzable Tannin Improves Nonspecific Immune Responses, Intestinal Morphology, and Disease Resistance against *Vibrio alginolyticus* in Whiteleg Shrimp

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This research aimed to evaluate the impact of hydrolyzable tannin on immune responses, intestinal morphology, and resistance to *Vibrio alginolyticus* in whiteleg shrimp (*Penaeus vannamei*). In preliminary *in vitro* experiments, shrimp hemocytes were exposed to different concentrations of hydrolyzable tannin. The findings indicated that hydrolyzable tannin exhibited nontoxic characteristics and could stimulate respiratory burst activity. For the subsequent *in vivo* trial, shrimp (11.83 ± 0.98 g) were subjected to a 28-day oral administration of diets containing diverse hydrolyzable tannin concentrations (0, 1, 2, 4, and 8 g/kg denoted as control, T1, T2, T4, and T8, respectively). Immune parameters, antimicrobial peptide genes, and intestinal morphology were measured. Significant enhancements were observed in total hemocyte count (THC), phagocytic activity, respiratory burst activity, and phenoloxidase activity (PO) across all treatment groups compared to the control group, particularly in T2 and T4 groups ($p < 0.05$). The gene expressions of Penaeidin-2, Penaeidin-3, Penaeidin-4, crustin, and lysozyme were markedly higher in the treatment groups, especially in T2 ($p < 0.05$). Additionally, hydrolyzable tannin contributed to increased intestinal wall thickness after the 28-day feeding trial ($p < 0.05$). In a separate challenge experiment, shrimps (11.36 ± 0.66 g) were exposed to *V. alginolyticus* after 7 days of culture. Notably, T2 and T4 exhibited enhanced resistance, resulting in survival rates of 66.67% and 69.70%, respectively. To conclude, administering 4 g of hydrolyzable tannin per kilogram of feed proved effective in enhancing nonspecific immune responses, improving intestinal morphology, and augmenting resistance to *V. alginolyticus* in whiteleg shrimp.

1. Introduction

Whiteleg shrimp (*Penaeus vannamei*) have many advantages, such as a short growth period and a high tolerance to various salinity ranges and high density farming; thus, it is a major species for aquaculture in Taiwan [1]. However, disease outbreaks due to increased culture density have caused considerable losses in farming ponds. Vibrio is a common pathogen, and *Vibrio alginolyticus* is a lethal pathogen in aquaculture [2, 3]. Whiteleg shrimp infected with *V. alginolyticus* have many symptoms such as poor growth performance, reduced food intake, and decreased activity. To prevent economic losses from disease outbreaks, antibiotics and drugs are used [4, 5]. However, the overuse of antibiotics and drugs not only reduces pathogens but also leads to residue in aquatic animals. Therefore, antibiotics and drugs have become regulated and prohibited [6].

Plants contain compounds with potential pharmacological activity, such as tannins, flavonoids, polyphenols, triterpenes, sterols, and alkaloids [7]. In addition, plants are safe and have minimal side effects on aquatic animals [8]. Many studies have reported that plants, such as *Moringa oleifera* leaves, *Psidium guajava* leaves, *Bidens alba*, and *Plectranthus amboinicus*, can be used to promote growth and enhance

immunity and resistance to pathogens for whiteleg shrimp [9–12]. Plants synthesize aromatic substances, such as terpenoids, tannins, and quinones [13]. Plants use these substances in defense against predators. Tannins are polyphenolic compounds that exist in many plants and fruit [14]. Tannins are classified into two types according to their structure: hydrolyzable tannins and condensed tannins [15]. Hydrolyzable tannins exhibit stronger antioxidant and antibacterial properties compared to condensed tannins [16]. Studies have indicated that hydrolyzable tannins promote biological growth, inhibit bacterial growth, antioxidation, and enhance immunity [17–21]. Therefore, hydrolyzable tannins are widely used as feed additives for pigs, chickens, and bovines [22]. Hydrolyzable tannins can enhance growth performance, total hemocyte count (THC), and lysozyme activity in whiteleg shrimp [23]. According to Zhu et al. [21] dietary supplementation of 0.15% hydrolyzable tannins increased the gene expression level of catalase, glutathione peroxidase, and Cu/Zn superoxide dismutase in whiteleg shrimp [21]. Accordingly, the current study examined the immunostimulant ability of hydrolyzable tannins on whiteleg shrimp and identified the protective effects of these supplements as the prophylactic agents against *V. alginolyticus* infection. This study was conducted to assess the cytotoxicity, phagocytic activity, respiratory burst activity, and phenoloxidase (PO) activity of shrimp hemocytes *in vitro* and to examine the immune parameters, antimicrobial peptide genes, intestinal morphology, and disease resistance to *V. alginolyticus* in whiteleg shrimp fed a diet containing various doses of hydrolyzable tannin.

2. Materials and Methods

2.1. Experimental Animals. Ethical approval was not required for invertebrates according to the guidelines of the Animal Use Protocol of the Institutional Animal Care and Use Committee (IACUC) of National Taiwan Ocean University. Whiteleg shrimp were obtained from the Aquatic Animal Center of National Taiwan Ocean University. Throughout both the acclimatization and experimental periods, the shrimp were fed with commercial shrimp feed (Tairoun Products Co., Ltd., Taiwan) three times a day, with or without the incorporation of hydrolyzable tannin. The commercial feed has a composition of 45% crude protein, 16% ash, 8% crude lipid, and 3% crude fiber. Water conditions were maintained at the following parameters: temperature at 27 ± 1°C, pH at 8.2 ± 0.2, and salinity at $33\% \pm 1\%$.

2.2. Diet Preparation. The commercial diet underwent crushing and was then divided into five distinct groups. Hydrolyzable tannin was introduced into the diets at concentrations of 0 (control), 1 (T1), 2 (T2), 4 (T4), or 8 (T8) g/kg, resulting in a proportional reduction in the quantity of the commercial diet. Subsequently, the feed was sifted to attain a diameter of 2 mm and then allowed to air-dry at 25°C. The feed were securely stored in sealed plastic containers in a dark environment at 4°C. The nutritional composition of diets containing hydrolyzable tannins is detailed in Table 1.

Table 1: Proximate composition of diets containing hydrolyzable tannins.

Ingredients (g/Kg)	Group				
	Control	T1	T2	T4	T8
Commercial feed	1,000	999	998	996	992
Hydrolyzable tannin	0	1	2	4	8
Proximate composition (%)*					
Crude protein	45	44.96	44.91	44.82	44.64
Crude lipid	8	7.99	7.98	7.97	7.94
Ash	16	15.98	15.97	15.94	15.87
Crude fiber	3	3	2.99	2.99	2.98

*Analyzed based on dry weight.

2.3. In Vitro Effect of Hydrolyzable Tannin. Hemolymph was obtained from the ventral sinus of the whiteleg shrimp using a 3 mL syringe equipped with a 23G needle containing an anticoagulant (0.34 M sodium chloride, 0.12 M glucose, 30 mM trisodium citrate, and 10 mM ethylenediaminetetraacetic acid (EDTA); pH 7.4). The THC of the hemolymph was determined using a hemocytometer (Marienfeld™, Marienfeld, Germany). For subsequent utilization in assays evaluating cell viability, phagocytosis, respiratory burst, and PO activity, THC was adjusted to 1×10^6 cell/mL.

2.4. Cell Viability Assay. The assay was measured using a previously described method [24]. In this assay, 100 μL of hemocytes were loaded into 96-well plate with modified complete Hank's balanced salt solution (MCHBSS) alone or with MCHBSS containing different concentrations of hydrolyzable tannin (0, 100, 250, 500, 1,000, 2,000, 4,000, 6,000, 7,000, 8,000, and 9,000 mg/L). The 96 well plates were centrifuged at 800xg for 20 min at 4°C after 1 hr incubation at 25°C. The supernatant was removed, and then 100 μL of (3-(4,5-dimethylthiazol-2-yl)]-2,5-diphenyl tetrazolium bromide; MTT) solution (0.5 mg of MTT/mL in MCHBSS) was introduced to the cells, followed by a 4 hr incubation period in darkness. After incubation, 100 μL of dimethyl sulfoxide (DMSO) were added to the MTT formazan product. Absorbance was immediately recorded at an optical density (OD) of 570 nm on an enzyme-linked immunosorbent assay reader (ELISA reader). The cell viability percentage was calculated using the following formula:

$$\text{Cell viability } (\%) = \frac{\text{OD value of treated cells}}{\text{OD value of control cells}} \times 100. \quad (1)$$

2.5. Phagocytosis Assay. Phagocytic activity was measured using a previously described method [9]. In this assay, 100 μL of hemocytes were loaded onto a glass coverslip. The hemocytes were incubated with MCHBSS alone or with MCHBSS containing different concentrations of hydrolyzable tannin (0, 100, 250, 500, 1,000, 2,000, and 4,000 mg/L) for 1 hr. The fluid containing unattached cells was removed. The attached cells were incubated with 100 μL of latex beads (3×10^7 beads/mL,

0.8 mm; Sigma–Aldrich) in MCHBSS for 1 hr at 25°C. The cells were rinsed with MCHBSS and then treated with 100% methanol for 5 min to fix them. Five percent Giemsa was applied to the stained cells for 20 min and examined under a microscope. Phagocytic activity was characterized as the phagocytic rate (PR), and the phagocytic index (PI). Phagocytic activity was counted using the following formula:

$$\text{PR}\,(\%) = \frac{\text{Numbers of phagocytic cells}}{\text{100 hemocytes}} \times 100, \quad (2)$$

$$\text{PI} = \frac{\text{Total beads in phagocytic cells}}{\text{100 phagocytic cells}}. \quad (3)$$

2.6. Respiratory Burst Activity. The respiratory burst activity of hemocytes was quantified using the method outlined by Abidin et al. [9]. In this assay, 100 μL of hemocytes were loaded into a 96-well plate and cocultured with various concentrations of hydrolyzable tannin, as described in Section 2.3.2, for 30 min at 25°C. The 96-well plate was subjected to centrifugation at 800x *g* for 20 min at 4°C, and the liquid was removed. Cells in each well were then incubated with 100 μL of zymosan (0.1% in MCHBSS; Sigma–Aldrich) for 30 min at 25°C. The liquid above the sedimented cells was aspirated, and the cells subjected to incubation with 100 μL of 0.3% nitro-blue tetrazolium chloride (NBT; Sigma–Aldrich) solution at 25°C. After 30 min, the solution was aspirated. Methanol was added to stop the reaction. Following a 5 min incubation, methanol was removed. The cells were rinsed thrice with 100 μL of 70% methanol, followed by air drying for 30 min. One hundred twenty microlitres 2 M potassium hydroxide and 140 μL dimethyl sulfoxide were added to dissolve the insoluble formazan crystals, and absorbance was measured with an ELISA reader at 630 nm. The quantity of respiratory burst produced was calculated using the following formula:

$$\text{Respiratory burst} = \frac{\text{OD value of sample} - \text{OD value of background}}{\text{OD value of background}}. \quad (4)$$

2.7. Phenoloxidase Activity. The PO activity was measured using a previously described method [9]. In this assay, 1 mL of the hemocyte solution were subjected to centrifugation at 800x *g* for 20 min at 4°C. The liquid above the sedimented cells was removed, and the cells were resuspended in different concentrations of hydrolyzable tannin, as described in Section 2.3.2, for 30 min at 25°C. The mixed solutions were subjected to centrifugation at 800x *g* for 20 min at 4°C. The liquid were removed, and 1 mL of cacodylate citrate buffer solution (0.45 M sodium chloride, 0.01 M sodium cacodylate, and 0.01 M trisodium citrate; pH 7.0) was added. The plate was subjected to centrifugation at 800x *g* for 20 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 200 μL of cacodylate buffer solution (0.45 M sodium chloride, 0.26 M magnesium chloride, 0.01 M sodium cacodylate, and 0.01 M calcium chloride; pH 7.0). Subsequently, 100 μL of each supernatant was subjected to incubation with 50 μL of 1 mg/mL trypsin (Sigma–Aldrich) for 10 min at 25°C, followed by an additional incubation with 50 μL of 3 mg/mL L-dihydroxyphenylalanine (L-DOPA) (Sigma–Aldrich) for 5 min. Finally, 800 μL of cacodylate buffer was added. The absorbance of the samples was measured using a spectrophotometer at 490 nm.

2.8. In Vivo Effects of Hydrolyzable Tannin. A total of 180 shrimp, initially weighing 11.83 ± 0.98 g, were randomly allocated into five groups. In each group, there were 35 shrimp maintained within a 120 L tank. The diet of each group is described in Section 2.2. Each group received the experimental diet, amounting to 5% of body weight, administered three times daily. Immune assays were performed on shrimps at 0, 1, 2, 4, 7, 14, 21, and 28 days. Hemolymph was withdrawn from the ventral sinus and then mixed with an anticoagulant solution at a ratio of 1 : 9. The anticoagulant solution comprised 0.34 M sodium chloride, 0.12 M glucose, 30 mM trisodium citrate, and 10 mM EDTA with a pH of 7.4. Using a mixture of hemolymph and anticoagulant, two parts were used: one was for immune experiments, and the other for gene-related immune tests. Phagocytic activity, respiratory burst, and PO activity were measured using the methods described in Section 2.3.

2.9. Expression of Genes Related to the Immune System. The hemolymph were subjected to centrifugation at 800x *g* for 10 min at 4°C, and the liquid was removed. The cell was lysed with EasyPure Total RNA Reagent (Bioman), followed by chloroform extraction in accordance with the manufacturer's guidelines. The lysed cell solution was subjected to centrifugation at 12,000x *g* for 10 min at 4°C. The upper layer was moved to 1.5 mL microtube, and after the adding 2-propanol, the combination was subjected to centrifugation at 12,000x *g* for 15 min at 4°C. The RNA pellet was rinsed with 75% ethanol and left to air-dry. The RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water. The concentrations of RNA were analyzed using spectrophotometry.

One microgram of RNA was subjected to incubation with 2U of DNase I (Bionovas) for 30 min at 37°C, followed by an additional incubation at 70°C for 5 min to deactivate the DNase. The treated RNA was denatured through heating at 65°C for 5 min in 12 μL of ddH_2O containing Oligo $(dT)_{18}$ primer and 2 × Fast Premix (Bionovas). The synthesis of the first-strand cDNA involved the addition of 1 μL of HiScript I Reverse Transcriptase. The reaction advanced at 42°C for 30 min and then terminated by heating at 85°C for 5 min.

Five immune related genes of shrimp were analyzed. (Table 2). Quantitative polymerase chain reaction (PCR) was performed with SYBR Green on a real-time PCR system (Applied Biosystems). Amplification was carried out in a 96-well plate using a 20 μL reaction mixture comprising 1 μL of 20 ng cDNA, 0.4 μL of 10 μm forward, 0.4 μL of 10 μm

Table 2: Primers of AMP genes.

	Gene	Forward (5'⟶3')	Reverse (5'⟶3')	Reference
Antimictobial peptide	Penaiedin2	TCGTGGTCTGCCTGGTCTT	CAGGTCTGAACGGTGGTCTTC	[25]
	Penaiedin3	CACCCTTCGTGAGACCTTTG	AATATCCCTTTCCCACGTGAC	
	Penaiedin4	GCCCGTTACCCAAACCATC	CCGTATCTGAAGCAGCAAAGTC	
	Crustin	GAGGGTCAAGCCTACTGCTG	ACTTATCGAGGCCAGCACAC	
	Lysozyme	GAAGCGACTACGGCAAGAAC	AACCGTGAGACCAGCACTCT	
Internal control	Elongation factor-α	ATGGTTGTCAACTTTGCCCC	TTGACCTCCTTGATCACACC	[26]

reverse primers, and 10 μL of SYBR Green 2 × Master Mix (Agilent Technologies), with the remaining volume adjusted to 20 μL using ddH_2O. qPCR was performed at 95°C for 30 s, followed by 40 cycles for 15 s at 95°C, and finally for 1 min at 60°C. All the samples were analyzed in triplicate. The normalized expression level of the target gene was determined relative to the expression level of elongation factor 1-α. Data analysis was performed using the $2^{-\Delta\Delta CT}$ method, and the relevant equations are listed below:

(1) CT is the threshold cycle value for the amplified gene.

(2) $\Delta\Delta CT = (C_{t\ \text{target gene of treated sample}} - C_{t\ \text{elongation factor 1-}\alpha\ \text{of treated sample}}) - (C_{t\ \text{target gene of control sample}} - C_{t\ \text{elongation factor 1-}\alpha\ \text{of control sample}})$.

(3) The relative target gene transcript level $= 2^{-\Delta\Delta CT}$.

2.10. Vibrio alginolyticus Challenge Experiment. A total of 132 shrimp (11.36 ± 0.66 g) were divided into four groups. In a 120 L tank, each group consisted of 33 shrimp. Group 1 and Group 2 were fed a commercial diet. Group 3 received the T2 diet, while Group 4 was provided with the T4 diet. Shrimp were fed 5% of their body weight three times a day. After 7 days of the experimental diet, the shrimps were placed in seawater containing 40 mL of *V. alginolyticus* solution (1×10^7 cfu/mL), except for the shrimps in Group 1. The Group 1 shrimp were placed in seawater containing 40 mL of phosphate buffer saline (PBS). At 12, 24, 48, 72, 96, 120, 144, and 168 hr after the challenge, survival rates were recorded. The survival rate was calculated using the formula:

$$\text{Surival rates}\ (\%) = \left(\frac{[\text{surviving shrimp}]}{[\text{number of shrimp injected}]}\right) \times 100\%. \quad (5)$$

2.11. Statistical Data Analysis. Statistical analysis was carried out utilizing SAS (Statistical Analysis System) Software Version 9.4. To assess the normality of the data and homogeneity of variance, Shapiro–Wilk tests and Levene tests were conducted, respectively. The data were subjected to one-way analysis of variance, and differences among treatment means were evaluated using Tukey's multiple range test. Statistical significance was considered at a p value below 0.05. For the *V. alginolyticus* challenge experiment 's survival data, Kaplan–Meier methods in SPSS software version 22.0 were employed, and group differences were evaluated using the Mantel–Cox test. Statistical significance (Sig.) was denoted by p values less than 0.05.

3. Results

3.1. Effect of Hydrolyzable Tannin on Cell Viability In Vitro. Cell viability exceeded 95% at concentrations of 100, 250, 500, 1,000, 2,000, 4,000, 6,000, 7,000, and 8,000 mg/L, with no noteworthy distinctions observed between the treatment groups and the control group (Figure 1(a)). However, at a concentration of 9,000 mg/L, cell viability dropped to 70%, displaying a significant decrease compared to the control group (0 mg/L; $p < 0.05$).

3.2. Effect of Hydrolyzable Tannin on Nonspecific Immune Responses In Vitro. There were no significant differences observed in PR (Figure 1(b)) and PI (Figure 1(c)) between the treatment groups and the control group. Additionally, respiratory burst activity was not significantly affected between 100 and 250 mg/L treatment groups and the control group ($p > 0.05$; Figure 1(d)). Notably, significant increases were detected in the 500, 1,000, 2,000, and 4,000 μg/L treatment groups compared to the control group ($p < 0.05$; Figure 1 (d)), with hydrolyable tannin demonstrating a dose-dependent increase in respiratory burst activity. However, PO activity exhibited no significant differences between the treatment groups and the control group ($p > 0.05$; Figure 1(e)).

3.3. Effect of Hydrolyzable Tannin on Immune Parameters of Whiteleg Shrimp. Figure 2(a) presents the THCs. There were no significant differences in THCs among the groups on days 1, 2, 4, 7, and 28. The THCs significantly increased in all treatment groups in comparison with that in the control group on days 14 and 21. Figure 2(b) presents the PR. No significant increases in the PRs of the experimental groups compared to the control group were observed on days 1, 2, 4, and 28. On day 7, the PR in the T1, T2, and T8 groups showed a significant increase compared to the control group. On day 14, the PR of the T2 group exhibited a significant increase. The PR of the T4 group was notably higher than that of the control group on day 21. Figure 2(c) presents the PI results. No significant differences in the PI were observed between the experimental groups and the control group on days 1, 2, 4, 14, 21, and 28. On day 7, the PI of the T8 group had significantly increased. Figure 2(d) presents respiratory burst activity. On day 1, no significant increases were found in the respiratory burst activity of the

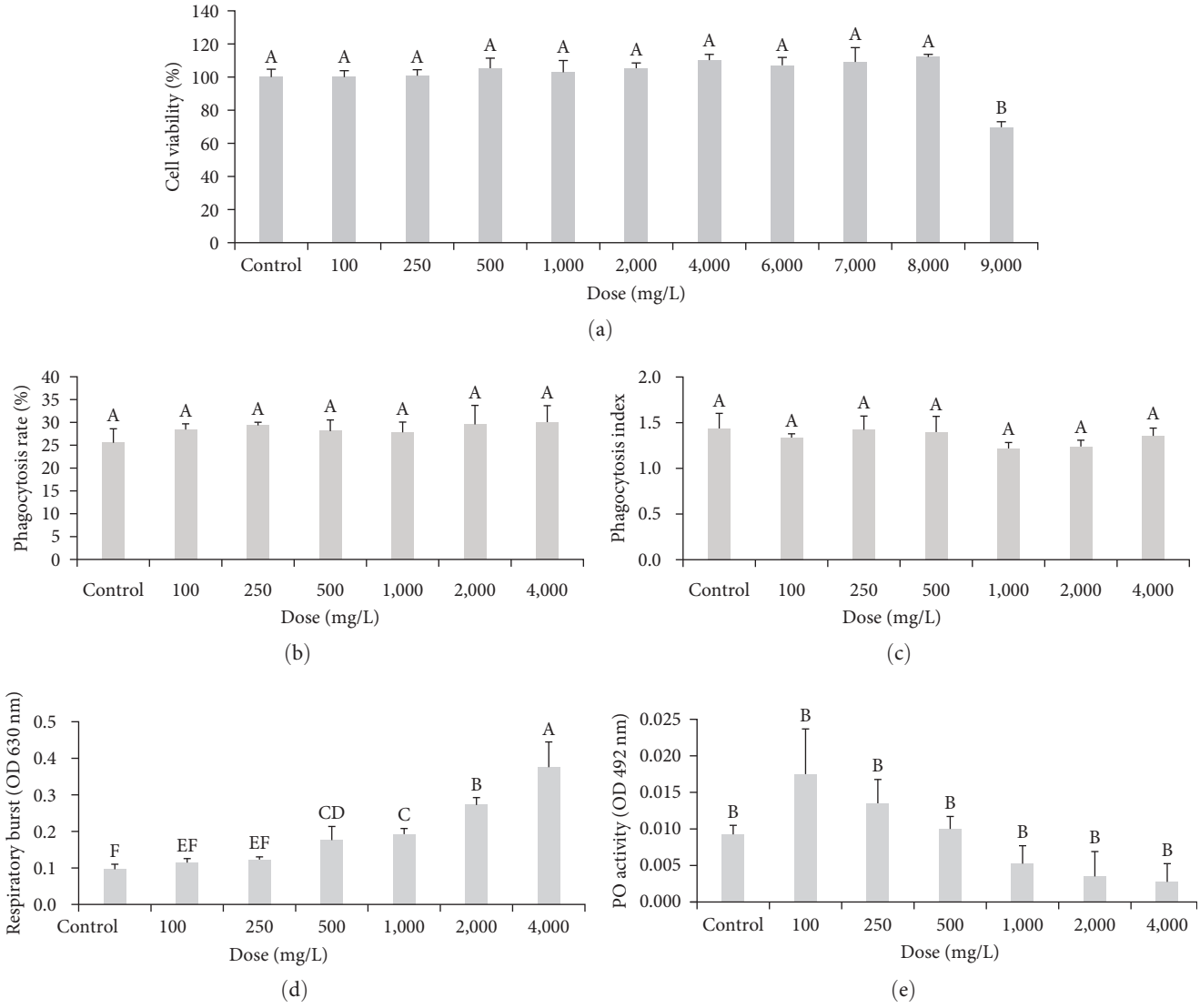
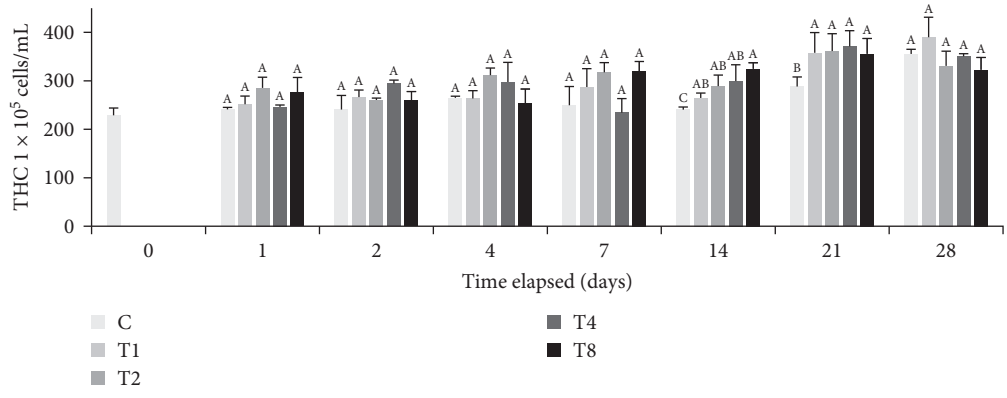


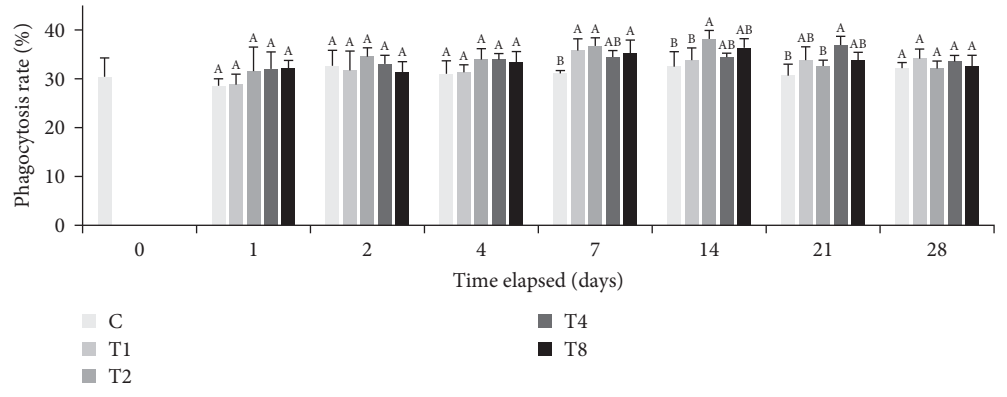
Figure 1: Effects of whiteleg shrimp hemocytes after *in vitro* incubation with different concentrations of hydrolyzable tannins. (a) Cell viability. (b) Phagocytic rate. (c) Phagocytic index. (d) Respiratory burst. (e) PO activity. Control without hydrolysable tannin. The value represent means. Error bars indicate ± standard deviation ($n = 3$). Bars with different letters indicate significantly differences ($p < 0.05$).

experimental groups compared with that of the control group. On days 2, 14, 21, and 28, the respiratory burst activity of all treatment groups had significantly increased. On day 4, the respiratory burst activity in the T1 group showed a significant increase. The respiratory burst activity of the T2 and T4 groups was notably higher than that of the control group on day 7. Figure 2(e) presents PO activity. There was no significant difference in PO activity between the treatment and control groups on days 1 and 2. On day 4, the PO activity in the T2 group showed a significant decrease. On day 7, the PO activity of the T4 and T8 groups showed a significant increase. On day 14, all the treatment groups exhibited significantly increased PO activity. On day 21, PO activity was significantly higher in the T1 group compared with the control group. On day 28, both the T1 and T2 groups showed a significant increase in PO activity compared to the control group.

3.4. Effects of Hydrolyzable Tannin on Antimicrobial Peptide (AMP) Gene Expression of Whiteleg Shrimp. The study also evaluated the expression of five AMP genes, namely Penaeidin-2, Penaeidin-3, Penaeidin-4, crustin, and lysozyme (Figure 3). The T2 group exhibited upregulated Penaeidin-2 expression from day 4 to 21, and T4 group exhibited upregulated Penaeidin-2 expression from day 14 to 21 (Figure 3(a)). Penaeidin-3 expression was significantly increased in all treatment groups compared with the control group on days 4 and 7 (Figure 3(b)). The T2 group exhibited upregulated Penaeidin-4 expression from day 2 to 14 (Figure 3(c)). On days 4 and 7, crustin was significantly upregulated in the T2 group than in the control group, whereas all treatment groups exhibited upregulated crustin expression on days 21–28 (Figure 3(d)). Lysozyme expression was significantly higher in the T2 group than in the control group on days 1, 4, 7, 21, and 28, and the T4 group exhibited upregulated lysozyme expression on days 2, 14, 21, and 28 (Figure 3(e)).



(a)



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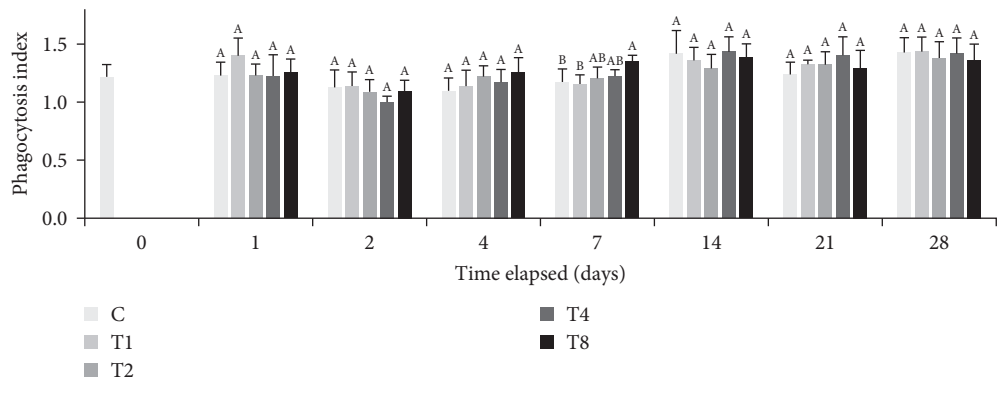

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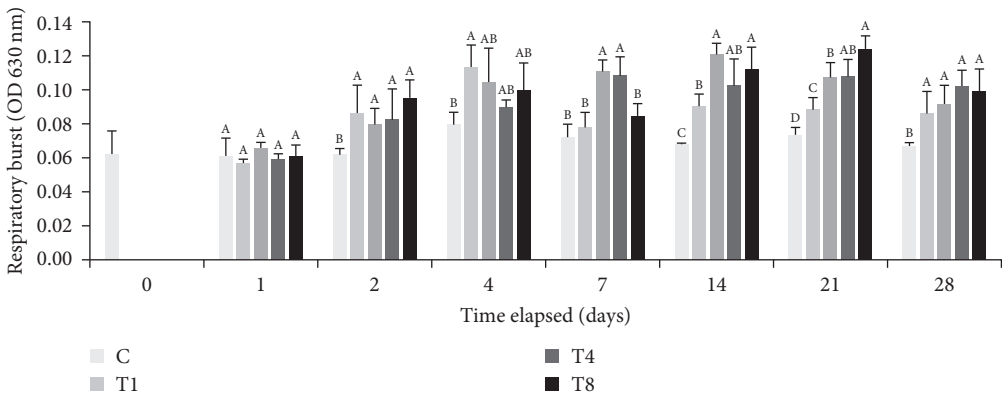

(d)

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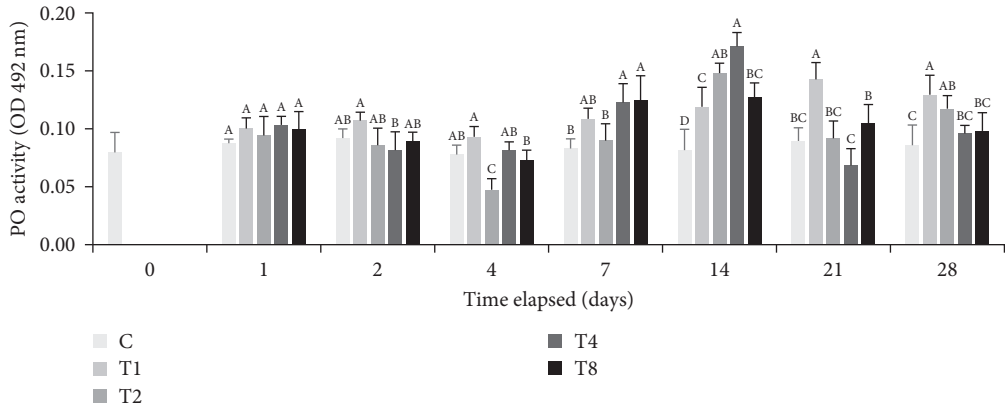
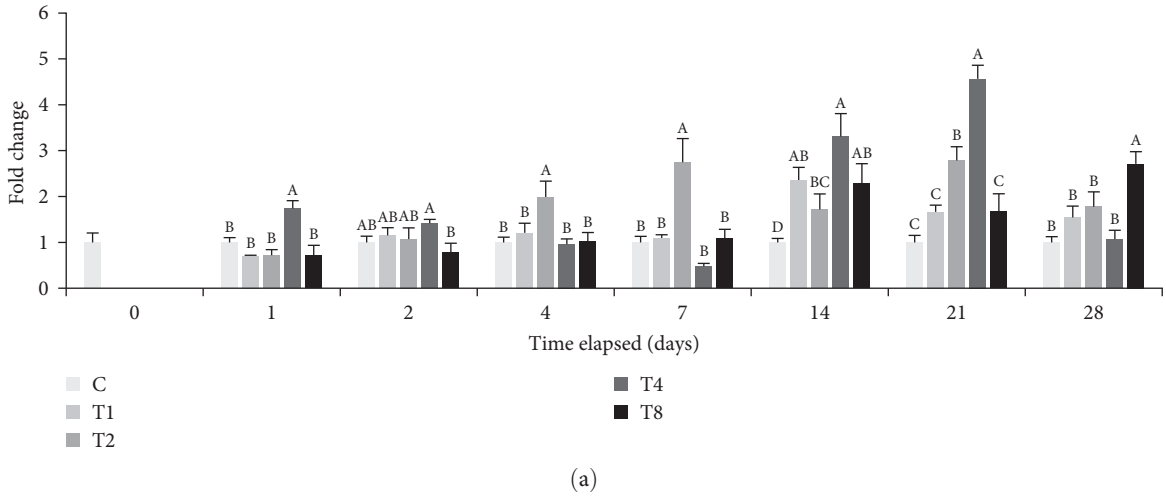
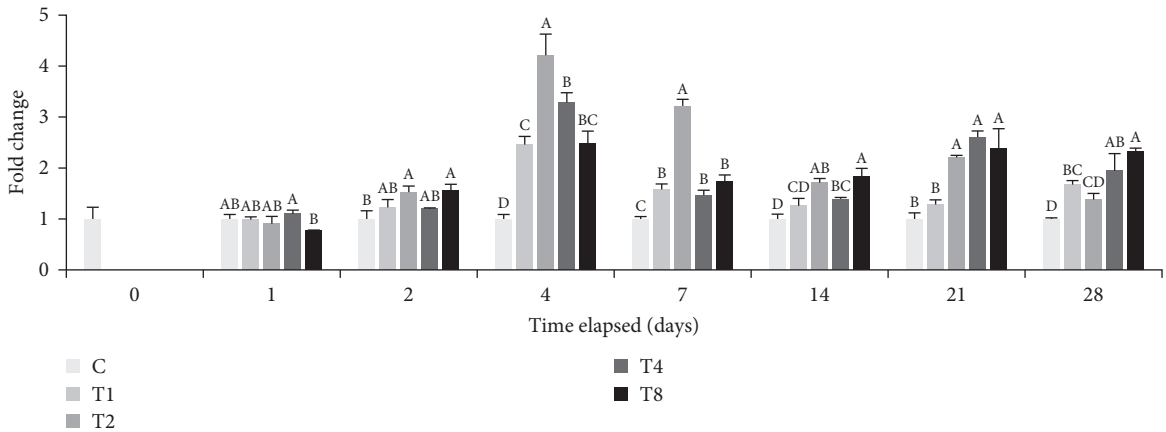


(e)

Figure 2: Nonspecific immune responses of whiteleg shrimp fed with different concentrations of hydrolyzable tannin over 28 days. Control without hydrolyzable tannin (C), 1 g/kg hydrolyzable tannins (T1), 2 g/kg hydrolyzable tannins (T2), 4 g/kg hydrolyzable tannins (T4), or 8 g/kg hydrolyzable tannins (T8) during 28-day feeding: (a) total hemocyte count; (b) phagocytic rate; (c) phagocytic index; (d) respiratory burst; and (e) PO activity; values are presented as mean ± standard deviation ($n = 5$). Significant differences ($p < 0.05$) between treatments are indicated by the different letters above the bars.



(a)



(b)

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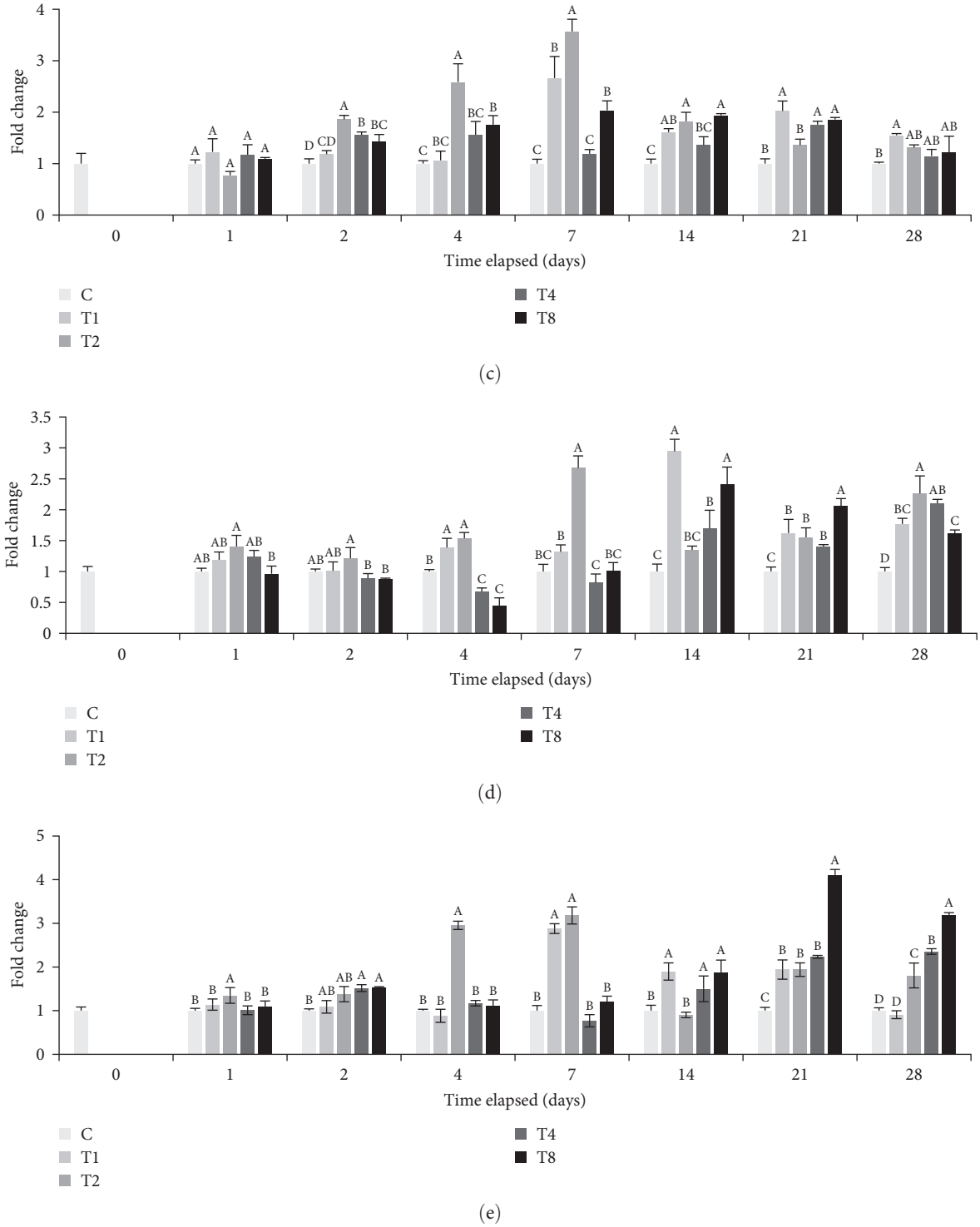


FIGURE 3: Gene expressions of antimicrobial peptides in the hemocytes of whiteleg shrimp fed with different concentrations of hydrolyzable tannin over 28 days: (a) Penaeidin-2, (b) Penaeidin-3, (c) Penaeidin-4, (d) Crustin, and (e) Lysozyme. Results are presented as mean ± standard error of the mean ($n = 5$). Significant differences ($p < 0.05$) between the gene expression of groups are indicated by the different letters above the bars.

3.5. Effects of Hydrolyzable Tannin on Intestinal Histology of Whiteleg Shrimp. Figure 4 presents the intestinal histology of the shrimp. The wall thickness was significantly ($p < 0.05$) increased in all treatment group compared with the control group (Figure 4(a)–4(e), and the T2 group had the highest wall thickness (Figure 4(f)). No significant difference was observed in the height of the intestine between the treatment and control groups (Figure 4(g)).

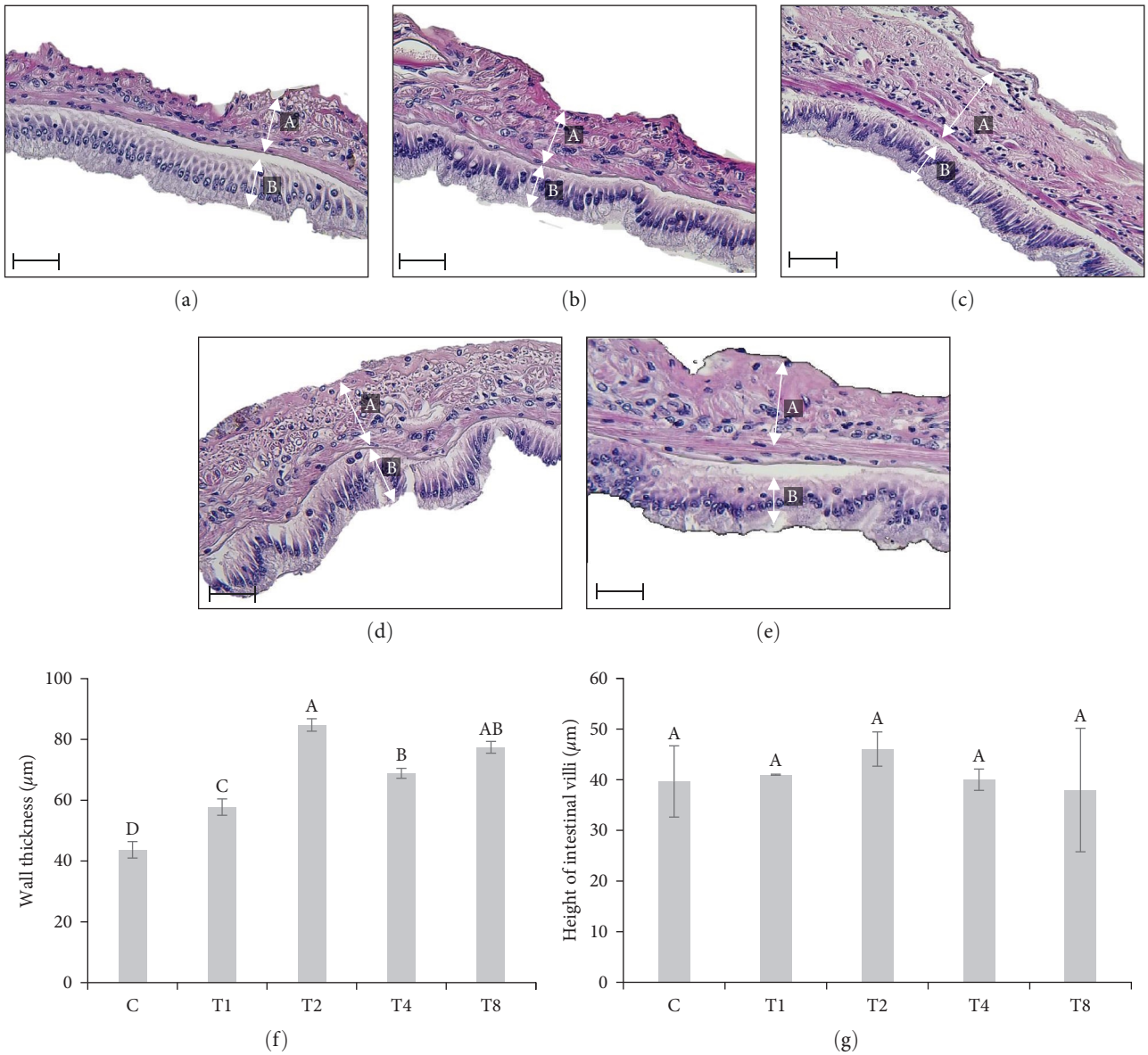
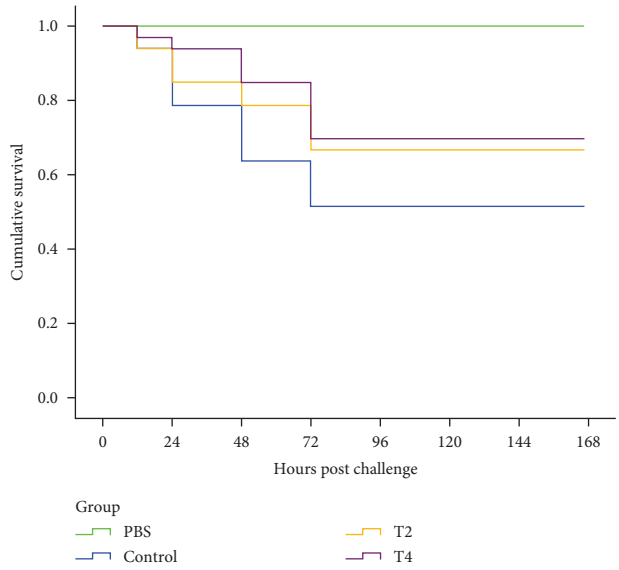


Figure 4: Intestinal tissue of whiteleg shrimp fed with different concentrations of hydrolyzable tannin over 28 days. Transverse sections of intestinal tissue of the whiteleg shrimp with (a) 0 g/kg, (b) 1 g/kg, (c) 2 g/kg, (d) 4 g/kg, and (e) 8 g/kg hydrolyzable tannins. (A) Intestinal wall thickness and (B) intestinal villi height. Scale bar = 40 μm. Original magnification: ×200. The effect of hydrolyzable tannins in the diet (C, T1, T2, T4, and T8) on (f) wall thickness and (g) intestinal villi length of whiteleg shrimp. Data are expressed as mean ± standard error of the mean ($n = 5$). Significant differences ($p < 0.05$) between treatments are indicated by the different letters above the bars.

3.6. Effects of Hydrolyzable Tannin on the Resistance of Whiteleg Shrimp to Vibrio alginolyticus. Figure 5(a) show the survival rate (SR) of shrimp subjected to the experimental diet for 7 days and subsequently challenged with *V. alginolyticus*. The PBS group (negative control) showed no mortality during the 168-hr period, while mortality was observed 24 hr after immersion with *V. alginolyticus* in the control group (positive control). The pairwise comparison analysis of survival among experimental groups is depicted in Figure 5(b). Although there was no significant difference between the infected groups ($p > 0.05$), the T2 and T4 groups exhibited higher SRs compared to the positive control group.

4. Discussion

Many secondary metabolites have pharmacological properties. Among them, hydrolyzable tannins exist in many plants and herbs [14]. Studies have reported the antibacterial and antioxidant effects of hydrolyzable tannin on whiteleg shrimp [21], but these effects on whiteleg shrimp are poorly understood. Therefore, the purpose of this study was to evaluate the potential advantages of using hydrolyzable tannins in aquaculture as an immunostimulant to enhance shrimp immunity. The hydrolyzable tannins are secondary plant metabolites, which can be divided into gallic acid derivatives,



(a)

		Control		PBS		T2		T4	
	Group	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log rank (Mantel– Coz)	Control			20.987	.000	1.653	.198	2.876	.090
	PBS	20.987	.000			13.031	.000	11.602	.001
	T2	1.653	.198	13.031	.000			.133	.715
	T4	2.876	.090	11.602	.001	.133	.715		

(b)

FIGURE 5: (a) Kaplan–Meier survivorship curves of whiteleg shrimp fed with experiment diets for 7 days followed by *Vibrio parahaemolyticus* challenge ($n = 33$ per group). (b) The pairwise comparisons between the groups were analyzed using the Mantel–Cox test.

gallotannins and ellagitannins according to their structure ([27]. Ellagitannins and gallotannins are toxic to brine shrimp (*Artemia salina*), and the LC_{50} is 37–71 and 7–15 μg/mL, respectively [28]. Examining the safety of hydrolyzable tannins for the host cell and their cytotoxicity is crucial. In a previous study, the cytotoxicity of hydrolyzable tannins in whiteleg shrimp hemocyte was evaluated using an MTT assay [10], and the cell survival rate of hemocyte treated with 0–9,000 mg/L hydrolyzable tannins was higher than 70%. This result indicates that hydrolyzable tannins are safe for shrimp hemocyte at concentrations lower than 9,000 mg/L, and that it can be used as an additive for subsequent *in vitro* and *in vivo* experiments.

Dewi et al. [10] reported that a guava (*P. guajava*) leaf extract enhanced the PO activity, phagocytic activity, and O_2^- production of whiteleg shrimp hemocytes in *in vitro* experiments. The phagocytic activity, O_2^- production, and PO activity of whiteleg shrimp hemocytes increased after an *in vitro* culture with a hot-water extract of *Bidens alba* and *P. amboinicus* [11]. Therefore, this *in vitro* hemocyte experiment was performed to determine whether hydrolyzable tannins affect the nonspecific immunity of shrimp. Hydrolyzable tannins increased respiratory burst activity, but no significant difference in phagocytosis and PO activity ($p > 0.05$) was observed. Furthermore, hydrolyzable tannins inhibited PO activity in a dose-dependent manner. Hydrolyzable tannins are polyphenolic compounds that can be oxidized by PO. The amount of PO in the PO cascade decreased after treatment with hydrolyzable tannins. Therefore, the downstream products of the PO promotion cascade decreased, and data for promoting PO activity decreased.

The hemocytes of crustaceans are involved in many immune responses, including phagocytosis, reactive oxygen species (ROS) generation, wound healing, melanization, and encapsulation [29]. Therefore, the THC is considered an indicator of crustacean health. Whiteleg shrimp administered 0.3% hydrolyzable tannins for 60 days exhibited increased THC [23]. In Figure 2(a), the THC increased with 1–8 g/kg hydrolyzable tannins, and on day 21, the treatment groups displayed significantly higher THCs than the control group. This result was consistent with previous results. Moreover, the THCs of whiteleg shrimp fed hydrolyzable tannins increased in a concentration- and time-dependent manner. Phagocytosis is essentially a defensive reaction against infection and invasion of the body by foreign substances [30]. ROS, such as superoxide anion (O_2^-), are released rapidly during phagocytosis, and this process is called respiratory burst [31]. Moderate quantities of ROS are beneficial for several physiological processes,

such as killing pathogens, promoting wound healing, and repairing tissue. The proPO cascade is a key immune defense mechanism in crustaceans and produces melanin to kill pathogens [32]. Many plants, such *Phyllanthus amarus* and *Gynura bicolor*, contain phenolic compounds. The *P. amarus* extract was demonstrated to increase the THC, phagocytosis, respiratory burst activity, and proPO activity of whiteleg shrimp [33]. *G. bicolor*-supplemented feed could enhance the THCs, respiratory burst activity, and proPO activity of hemocytes in whiteleg shrimp [34]. The present study reported that shrimp, when fed diets enriched with hydrolyzable tannins, displayed improved phagocytic activity, respiratory burst activity, and proPO activity. Therefore, it is speculated that hydrolyzable tannins of phenolic compounds can enhance the nonspecific immune response of whiteleg shrimp. In addition, some time points in the treatment periods have resulted in a promotion of immunoparameters, but not all. This phenomenon may be attributed to the process of "immunomodulation" [35]. A continuous immune stimulation could result in inflammation. Thus, immunomodulation would be used to maintain homeostasis [36].

The health of animals rely on the digestion and nutrient absorption of the gut [37]. Gut morphology is crucial for maintaining gut function [38]. When the height of the intestinal villi increases, the surface area for absorbing nutrients increases. Therefore, the ability of the intestinal tract to absorb nutrients increases [39]. Well-functioning peristalsis ability of the intestinal wall can enhance the mixing of digestive enzymes and chyme in the gut and improve digestion efficiency [40, 41]. Hydrolyzable tannins are highly bioavailable in the intestine [42], and hydrolyzable tannins were could effectively increase the thickness of the intestinal wall and improve nutritional absorption. More nutrition was beneficial to growth performance. Therefore, hydrolyzable tannins could improve the growth performance of whiteleg shrimp by increasing the thickness of the intestinal wall [21, 23].

When whiteleg shrimp are infected by pathogens, hemocytes undergo degranulation to release antimicrobial peptides against pathogens. Whiteleg shrimp has many antimicrobial peptides, including penaedin, crustin, anti-LPS factor, and lysozyme [43]. Whiteleg shrimp have three types of penaedin: Penaedin-2, Penaedin-3, and Penaedin-4, which can inhibit the activity of gram-positive bacteria and fungi [44]. Crustin is a cationic cysteine-rich antimicrobial peptide that mainly serves as a defense against gram-positive bacteria. Lysozyme can inhibit the activity of bacteria and viruses [15, 45]. Guava leaf extract added to the diet enhanced the gene expression of antimicrobial peptides in whiteleg shrimp, leading to a high survival rate when the shrimp were challenged with *V. parahaemolyticus*. [10]. The *M. oleifera*-containing diet increased the gene expression of AMPs and improved the survival rate after challenge with *V. alginolyticus* in whiteleg shrimp [9]. The extracts of guava leaf and *M. oleifera* contain hydrolyzable tannins. This result indicates that the diet containing hydrolyzable tannins could increase the gene expression of AMPs. Therefore, it can be speculated that hydrolyzable tannins will increase the expression of AMPs in whiteleg shrimp.

When a respiratory burst occurs, many pathogens will be killed by oxygen free radicals [31]. The *in vivo* experiment showed that respiratory burst was improved in T2 and T4 groups than T1 and T4 at day 7. Moreover, all treatments increased the expression of AMPs. Therefore, the *V. alginolyticus* challenge experiment was performed to determine the optimal hydrolyzable tannin dosage. Figure 5 show that whiteleg shrimp fed 2 and 4 g/kg hydrolyzable tannins for 7 days exhibited an increased survival rate after the *V. alginolyticus* challenge, especially in the T4 group. Therefore, 4 g/kg hydrolyzable tannin is the optimal concentration for resistance to *V. alginolyticus*.

5. Conclusions

This study demonstrated that hydrolyzable tannins, particularly a diet with 2 and 4 g/kg hydrolyzable tannins, have the potential to boost the nonspecific immunity of whiteleg shrimp. This is evidenced by increased levels of PR, respiratory burst activity, PO activity, AMP gene expression, and enhanced resistance to *V. alginolyticus* infection. These findings propose the feasibility of incorporating hydrolyzable tannins as a dietary supplement in shrimp aquaculture.

Data Availability

The authors confirm that the data supporting the findings of this study are available within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Huai-Ting Huang contributed in the data curation and writing–original draft. Yeh-Fang Hu contributed in the writing–review and editing. Zhen-Hao Liao contributed in the formal analysis. Yu-Ru Lin contributed in the data curation. Yin-Yu Chen contributed in the formal analysis. Yung-Chih Wang contributed in the data curation and methodology. Jui-Jen Chang contributed in the formal analysis. Fan-Hua Nan contributed in the supervision.

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References

[1] W. Fang, G. Li, S. Zhou, X. Li, L. Hu, and J. Zhou, "Pharmacokinetics and tissue distribution of thiamphenicol and florfenicol in Pacific white shrimp *Litopenaeus vannamei* in freshwater following oral administration," *Journal of Aquatic Animal Health*, vol. 25, no. 2, pp. 83–89, 2013.

[2] M. D. A. Hannan, M. D. M. Rahman, M. D. N. Mondal, D. E. B. S. Chandra, G. A. Z. L. I. M. A. Chowdhury, and M. D. T. Islam, "Molecular identification of *Vibrio alginolyticus* causing vibriosis in shrimp and its herbal remedy," *Polish Journal of Microbiology*, vol. 68, no. 4, pp. 429–438, 2019.

[3] Y. R. Alfiansah, S. Peters, J. Harder, C. Hassenrück, and A. Gärdes, "Structure and co-occurrence patterns of bacterial communities associated with white faeces disease outbreaks in Pacific white-leg shrimp *Penaeus vannamei* aquaculture," *Scientific Reports*, vol. 10, no. 1, Article ID 11980, 2020.

[4] J. E. Burgents, K. G. Burnett, and L. E. Burnett, "Disease resistance of Pacific white shrimp, *Litopenaeus vannamei*, following the dietary administration of a yeast culture food supplement," *Aquaculture*, vol. 231, no. 1–4, pp. 1–8, 2004.

[5] H. T. T. Thuy, L. P. Nga, and T. T. C. Loan, "Antibiotic contaminants in coastal wetlands from Vietnamese shrimp farming," *Environmental Science and Pollution Research*, vol. 18, no. 6, pp. 835–841, 2011.

[6] R. C. Okocha, I. O. Olatoye, and O. B. Adedeji, "Food safety impacts of antimicrobial use and their residues in aquaculture," *Public Health Reviews*, vol. 39, no. 1, Article ID 21, 2018.

[7] H. O. Edeoga, D. E. Okwu, and B. O. Mbaebie, "Phytochemical constituents of some Nigerian medicinal plants," *African Journal of Biotechnology*, vol. 4, no. 7, pp. 685–688, 2005.

[8] G. Yin, G. Jeney, T. Racz, P. Xu, X. Jun, and Z. Jeney, "Effect of two Chinese herbs (*Astragalus radix* and *Scutellaria radix*) on non-specific immune response of tilapia, *Oreochromis niloticus*," *Aquaculture*, vol. 253, no. 1–4, pp. 39–47, 2006.

[9] Z. Abidin, H.-T. Huang, Z.-H. Liao et al., "Moringa oleifera leaves' extract enhances nonspecific immune responses, resistance against *Vibrio alginolyticus*, and growth in whiteleg shrimp (*Penaeus vannamei*)," *Animals*, vol. 12, no. 1, Article ID 42, 2022.

[10] N. R. Dewi, H.-T. Huang, Y.-S. Wu et al., "Guava (*Psidium guajava*) leaf extract enhances immunity, growth, and resistance against *Vibrio parahaemolyticus* in white shrimp *Penaeus vannamei*," *Fish & Shellfish Immunology*, vol. 118, pp. 1–10, 2021.

[11] H.-T. Huang, Y.-F. Hu, B.-H. Lee et al., "Dietary of *Lactobacillus paracasei* and *Bifidobacterium longum* improve nonspecific immune responses, growth performance, and resistance against Vibrio parahaemolyticus in *Penaeus vannamei*," *Fish & Shellfish Immunology*, vol. 128, pp. 307–315, 2022.

[12] H.-T. Huang, Z.-H. Liao, Y.-S. Wu, Y.-J. Lin, Y.-S. Kang, and F.-H. Nan, "Effects of *Bidens alba* and *Plectranthus amboinicus* dietary supplements on nonspecific immune responses, growth, and resistance to *Vibrio alginolyticus* in white leg shrimp (*Penaeus vannamei*)," *Aquaculture*, vol. 546, Article ID 737306, 2022.

[13] M. R. Kant, W. Jonckheere, B. Knegt et al., "Mechanisms and ecological consequences of plant defence induction and suppression in herbivore communities," *Annals of Botany*, vol. 115, no. 7, pp. 1015–1051, 2015.

[14] M. Brus, L. Gradišnik, M. Trapečar, D. Škorjanc, and R. Frangež, "Beneficial effects of water-soluble chestnut (*Castanea sativa* Mill.) tannin extract on chicken small intestinal epithelial cell culture," *Poultry Science*, vol. 97, no. 4, pp. 1271–1282, 2018.

[15] A. Kaizu, F. F. Fagutao, H. Kondo, T. Aoki, and I. Hirono, "Functional analysis of C-type lysozyme in *Penaeid Shrimp*," *Journal of Biological Chemistry*, vol. 286, no. 52, pp. 44344–44349, 2011.

[16] R. V. Barbehenn, C. P. Jones, A. E. Hagerman, M. Karonen, and J.-P. Salminen, "Ellagitannins have greater oxidative activities than condensed tannins and galloyl glucoses at high pH: potential impact on caterpillars," *Journal of Chemical Ecology*, vol. 32, no. 10, pp. 2253–2267, 2006.

[17] A. Fernandes, I. Fernandes, L. Cruz, N. Mateus, M. Cabral, and V. de Freitas, "Antioxidant and biological properties of bioactive phenolic compounds from *Quercus suber* L," *Journal of Agricultural and Food Chemistry*, vol. 57, no. 23, pp. 11154–11160, 2009.

[18] V. Koleckar, K. Kubikova, Z. Rehakova et al., "Condensed and hydrolysable tannins as antioxidants influencing the health," *Mini-Reviews in Medicinal Chemistry*, vol. 8, no. 5, pp. 436–447, 2008.

[19] X. Ma, H. Wu, L. Liu et al., "Polyphenolic compounds and antioxidant properties in mango fruits," *Scientia Horticulturae*, vol. 129, no. 1, pp. 102–107, 2011.

[20] A. K. Prusty, N. P. Sahu, A. K. Pal, A. K. Reddy, and S. Kumar, "Effect of dietary tannin on growth and haemato-immunological parameters of *Labeo rohita* (Hamilton) fingerlings," *Animal Feed Science and Technology*, vol. 136, no. 1-2, pp. 96–108, 2007.

[21] X.-F. Zhu, H. Guo, G.-L. Li, and C.-H. Zhu, "Effects of dietary hydrolyzable tannins on growth performance, antioxidant capacity, intestinal microflora and resistance against *Vibrio parahaemolyticus* of juvenile Pacific white shrimp, *Litopenaeus vannamei* (Boone, 1931)," *Aquaculture Reports*, vol. 19, Article ID 100601, 2021.

[22] R. Morales and E. M. Ungerfeld, "Use of tannins to improve fatty acids profile of meat and milk quality in ruminants: a review," *Chilean Journal of Agricultural Research*, vol. 75, no. 2, pp. 239–248, 2015.

[23] R. Novriadi, R. Fadhilah, A. E. Wahyudi, and C. Trullàs, "Effects of hydrolysable tannins on the growth performance, total haemocyte counts and lysozyme activity of Pacific white leg shrimp *Litopenaeus vannamei*," *Aquaculture Reports*, vol. 21, Article ID 100796, 2021.

[24] C. Domínguez-Borbor, B. D. Chalén-Alvarado, and J. A. Rodríguez, "A simple *in vitro* method to evaluate the toxicity of functional additives used in shrimp aquaculture," *MethodsX*, vol. 5, pp. 90–95, 2018.

[25] K. C. Han-Ching Wang, C.-W. Tseng, H.-Y. Lin et al., "RNAi knock-down of the *Litopenaeus vannamei* toll gene (LvToll) significantly increases mortality and reduces bacterial clearance after challenge with *Vibrio harveyi*," *Developmental and Comparative Immunology*, vol. 34, no. 1, pp. 49–58, 2010.

[26] H.-T. Huang, H.-J. Lin, H.-J. Huang, C.-C. Huang, J. H.-Y. Lin, and L.-L. Chen, "Synthesis and evaluation of polyamine carbon quantum dots (CQDs) in *Litopenaeus vannamei* as a therapeutic agent against WSSV," *Scientific Reports*, vol. 10, no. 1, Article ID 7343, 2020.

[27] V. Ossipov, J.-P. Salminen, S. Ossipova, E. Haukioja, and K. Pihlaja, "Gallic acid and hydrolysable tannins are formed in birch leaves from an intermediate compound of the shikimate pathway," *Biochemical Systematics and Ecology*, vol. 31, no. 1, pp. 3–16, 2003.

[28] W. L. Braguini, B. B. Alves, and N. V. Pires, "Toxicity assessment of *Lavandula officinalis* extracts in brine shrimp (*Artemia salina*)," *Toxicology Mechanisms and Methods*, vol. 29, no. 6, pp. 411–420, 2019.

[29] E. J. Burge, D. J. Madigan, L. E. Burnett, and K. G. Burnett, "Lysozyme gene expression by hemocytes of Pacific white shrimp, *Litopenaeus vannamei*, after injection with Vibrio,"

Fish & Shellfish Immunology, vol. 22, no. 4, pp. 327–339, 2007.

[30] C. J. Bayne, "Phagocytosis and non-self recognition in invertebrates," *BioScience*, vol. 40, no. 10, pp. 723–731, 1990.

[31] Y.-L. Song and Y.-T. Hsieh, "Immunostimulation of tiger shrimp (*Penaeus monodon*) hemocytes for generation of microbicidal substances: analysis of reactive oxygen species," *Developmental & Comparative Immunology*, vol. 18, no. 3, pp. 201–209, 1994.

[32] J. Rodriguez and G. Le Moullac, "State of the art of immunological tools and health control of penaeid shrimp," *Aquaculture*, vol. 191, no. 1–3, pp. 109–119, 2000.

[33] H.-V.-T. Ngo, H.-T. Huang, P.-T. Lee, Z.-H. Liao, H.-Y. Chen, and F.-H. Nan, "Effects of *Phyllanthus amarus* extract on nonspecific immune responses, growth, and resistance to *Vibrio alginolyticus* in white shrimp *Litopenaeus vannamei*," *Fish & Shellfish Immunology*, vol. 107, pp. 1–8, 2020.

[34] S.-L. Hsieh, C.-C. Wu, C.-H. Liu, and J.-L. Lian, "Effects of the water extract of *Gynura bicolor* (Roxb. & Willd.) DC on physiological and immune responses to *Vibrio alginolyticus* infection in white shrimp (*Litopenaeus vannamei*)," *Fish & Shellfish Immunology*, vol. 35, no. 1, pp. 18–25, 2013.

[35] A. Gopalakannan and V. Arul, "Immunomodulatory effects of dietary intake of chitin, chitosan and levamisole on the immune system of *Cyprinus carpio* and control of *Aeromonas hydrophila* infection in ponds," *Aquaculture*, vol. 255, no. 1–4, pp. 179–187, 2006.

[36] S. Hachimura, M. Totsuka, and A. Hosono, "Immunomodulation by food: impact on gut immunity and immune cell function," *Bioscience, Biotechnology, and Biochemistry*, vol. 82, no. 4, pp. 584–599, 2018.

[37] R. Jha, J. M. Fouhse, U. P. Tiwari, L. Li, and B. P. Willing, "Dietary fiber and intestinal health of monogastric animals," *Frontiers in Veterinary Science*, vol. 6, Article ID 48, 2019.

[38] I. Prakatur, M. Miskulin, M. Pavic et al., "Intestinal morphology in broiler chickens supplemented with propolis and bee pollen," *Animals*, vol. 9, no. 6, Article ID 301, 2019.

[39] K. Amoah, Q.-C. Huang, B.-P. Tan et al., "Dietary supplementation of probiotic *Bacillus coagulans* ATCC 7050, improves the growth performance, intestinal morphology, microflora, immune response, and disease confrontation of Pacific white shrimp, *Litopenaeus vannamei*," *Fish & Shellfish Immunology*, vol. 87, pp. 796–808, 2019.

[40] P.-C. Chai, X.-L. Song, G.-F. Chen, H. Xu, and J. Huang, "Dietary supplementation of probiotic Bacillus PC465 isolated from the gut of *Fenneropenaeus chinensis* improves the health status and resistance of *Litopenaeus vannamei* against white spot syndrome virus," *Fish & Shellfish Immunology*, vol. 54, pp. 602–611, 2016.

[41] Y. Duan, J. Zhang, J. Huang, and S. Jiang, "Effects of dietary *Clostridium butyricum* on the growth, digestive enzyme activity, antioxidant capacity, and resistance to nitrite stress of *Penaeus monodon*," *Probiotics and Antimicrobial Proteins*, vol. 11, no. 3, pp. 938–945, 2019.

[42] J. Serrano, R. Puupponen-Pimia, A. Dauer, A. M. Aura, and F. Saura-Calixto, "Tannins: current knowledge of food sources, intake, bioavailability and biological effects," *Molecular Nutrition and Food Research*, vol. 53, no. S2, pp. S310–329, 2009.

[43] X. Zhao and J. Wang, "The antimicrobial peptides of the immune response of shrimp," *ISJ-Invertebrate Survival Journal*, vol. 5, no. 2, pp. 162–179, 2008.

[44] D. Destoumieux, P. Bulet, J. M. Strub, A. van Dorsselaer, and E. Bachere, "Recombinant expression and range of activity of penaeidins, antimicrobial peptides from penaeid shrimp," *European Journal of Biochemistry*, vol. 266, no. 2, pp. 335–346, 1999.

[45] P. F. Ji, C. L. Yao, and Z. Y. Wang, "Reactive oxygen system plays an important role in shrimp *Litopenaeus vannamei* defense against *Vibrio parahaemolyticus* and WSSV infection," *Diseases of Aquatic Organisms*, vol. 96, no. 1, pp. 9–20, 2011.