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Research Article

Functional Effects of Hydrolyzable Tannins on the Growth, Health Status, and Hepatopancreas Histology of Pacific White Shrimp *Penaeus vannamei* Reared under Commercial Pond Conditions

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In the present study, the functional effects of hydrolyzable tannins (HT) extracted from the sweet chestnut tree *Castanea sativa* were evaluated either by directly incorporating them into the diet or by using a top-dressing application on the growth, body composition, total hemocyte counts, lysozyme activity, and histomorphological condition of the hepatopancreas of Pacific white shrimp *Penaeus vannamei*. Shrimp was confined in hapa nets installed within commercial outdoor ponds for 90 days. Eight experimental diets included a basal formulated diet (BD) with varying inclusion levels of HT (BD-0%, BD-0.1%, BD-0.2%, and BD-0.3%), a low fish meal (FM) diet with reduction on the inclusion level of FM from 10% to 7.5% and labeled as LFM 0.3% HT, a top-dressing HT application (TC) on basal diet (BDTD-0.4% HT), and a commercial diet (CDTD-0% HT and CDTD-0.4% HT). The final body weight (FBW), percentage weight gain (PWG), thermal growth coefficient (TGC), average daily growth (ADG), and feed conversion ratio (FCR) were significantly better in the group of shrimp fed with BD-0.3% HT compared to BD-0% HT. The administration of CD-0.4% HT was able to enhance the growth performance of shrimp compared to BD-0% HT and CD without HT. Higher protein and fat contents were found in the body of shrimp fed with graded levels of HT compared to shrimp fed with BD or CD without HT. Numerically, the direct inclusion and top-dressing process of HT increased the total hemocyte count and lysozyme activity in shrimp. Both BDTD-0.4% HT and CDTD-0.4% HT were also able to support a better hepatopancreatic condition with reference to histomorphology and integrity. These results indicated that BD-0.3% HT inclusion could significantly improve the growth performance and prevent the alteration in health and histomorphological condition of the hepatopancreas of shrimp *P. vannamei* cultured in hapa nets under commercial farm conditions, but also 0.4% HT could be used as a supplementation dosage for the top-dressing process in diets.

1. Introduction

The global production of Pacific white shrimp *Penaeus vannamei* showed a significant increase from 2.64 Mt in 2010 to 4.96 Mt in 2018 [1]. Other than a high consumer demand, the increasing trend was also driven by the expansion of the *P. vannamei* production systems into the Asian countries and the adoption of technology to increase the productivity [1–3]. Clearly, feed technology plays an important role to sustain the production by providing nutritional requirements

in order to optimize the growth and health conditions of shrimp [4, 5], while minimizing the environmental impact to the culture conditions [6, 7]. Since feed accounts for the largest share of the total farming expenses [8], the selection of ingredients, additives, and supplements needs to be carefully done prior to the production of the formulated feeds.

In recent years, the development of formulated shrimp feed with a low inclusion level of fish meal (FM) as a primary and costly protein source has been evaluated [9–11]. Several alternative ingredients, including soybean meal (SBM), dried distillers grains with solubles (DDGS), and concentrated dephenolization cottonseed protein (CDCP), have been used to replace FM, but these ingredients could also induce stress conditions in shrimp if widely used in the diets formulation [12–14]. Therefore, proper replacement strategies with appropriate blends of plant–protein sources supplemented with functional supplements or additives are needed to prevent the nutritional deficiencies and stress condition of shrimp to manifest. To fulfill the nutrient gap, feed supplementation with phytogenic natural supplements has been suggested and aimed to improve the growth performance, nutrient retention, and protect the aquatic organisms from pathogen infections [15–17].

Phytogenic feed additives can be classified into several group, including sensory, technological, zootechnical, and nutritional additives [16]. Among these, hydrolyzable tannins (HT) have been used in animal feeds as nutritional additives [18–20]. An earlier study in shrimp revealed that HT supplementation at an inclusion level of 0.15% in diets could enhance the growth performance, antioxidant capacity, intestinal microflora, and resistance against *Vibrio parahaemolyticus* [21]. Our previous studies indicated that the use of commercially available HT obtained from the sweet chestnut *Castanea sativa* and consisting of esters of ellagic acid with core molecules composed of polyols such as carbohydrates and phenolics could modulate the hematoimmunological parameters and growth of *P. vannamei* [22]. However, limited studies have demonstrated the utilization of HT in *P. vannamei* reared under outdoor or commercial pond conditions. In addition, there has been no published research on the growth and health condition of *P. vannamei* fed HT as a top-coated additive as a common practice applied in intensive shrimp farms.

Therefore, to confirm the functionality of HT in Pacific white shrimp, the objective of the present study was to investigate the influence of HT supplementation both in diet and top-dressing method on the growth, body composition, total hemocyte count (THC), lysozyme activity, and intestinal morphology of shrimp *P. vannamei* reared in hapa nets or cages installed within outdoor commercial ponds. In addition, the effect of the top-dressing process by using a specific binder on the integrity of HT in the basal and formulated feed was also investigated.

2. Materials and Methods

2.1. Experimental Diets. HTs (purity >65%) were provided by Tanin Sevnica (Slovenia). Eight experimental diets were prepared, with the basal diet (BD) formulated to contain a mixture of 10% FM, 8% corn gluten meal (CGM), 44.9% SBM, and 15% wheat products (WP) as binder and filler, without any supplementation of HT (BD-0% HT). The next three experimental diets had similar levels of FM, CGM, SBM, and WP and were supplemented with increasing inclusion levels of HT: 0.1%, 0.2%, and 0.3% labeled as BD-0.1% HT, BD-0.2% HT, and BD-0.3% HT, respectively. The fifth diet was prepared by decreasing the amount of FM from 10% to 7.5% and supplementing with 0.3% HT (named LFM 0.3% HT). The sixth diet was the basal diet top-dressing with 0.4% HT (BDTD-0.4% HT). The seventh diet was a commercial diet commonly used in Indonesian shrimp production systems (named CD, Code SA, CJ Feed, Serang, Banten, Indonesia). Finally, the last diet was the commercial diet top-dressed with 0.4% HT (named CDTD-0.4% HT). The detailed formulations of the experimental diets are shown in Table 1. Regarding the diet manufacturing process, all ingredients were crushed and passed through <200 mesh sieve (Jinan Shengrun, China) prior to the production and analytically weighted and mixed in a paddle mixer (Marion Mixers, Inc., Marion, IA, USA) for homogenization purposes. The cooking extrusion temperature was kept at 110°C for approximately14 s in five-barrel sections, and the last section was maintained at 62°. Portion of feeds were extruded into 1 and 2 mm in diameter and 2–4 mm in length. Diets were air-dried in a pulse bed dryer (Jinan Shengrun, China) and stored at 4°C in sealed containers until further use. The proximate composition and amino acid profile of the experimental diets were analyzed in Saraswanti Indo Genetech Laboratory (Bogor, West Java, Indonesia) and are shown in Table 2.

2.2. Feeding Trial. Pacific white shrimp *P. vannamei* juveniles (0.87 ± 0.02 g initial mean weight) were obtained from Salira Teknik Benur hatchery located in Serang, Banten, Indonesia, and transported to the pond site at the Karawang Aquaculture Production Business Service Center, West Java, Indonesia, in accordance with the ethical committee guidelines of the Jakarta Technical University of Fisheries, using oxygenated plastic bags. The shrimp juveniles were tested free from pathogens prior to the transport to the research location and acclimated in a nursery tank using similar water conditions used for the feeding trial. Then, 8,000 shrimps were randomly distributed into 40 hapa nets (50 shrimp m^{-2} or 200 shrimps per hapa net) with size of $2 \times 2 \times 1$ m per hapa net installed within the outdoor ponds. Five replicate groups of shrimp per dietary treatment were fed by hand with the previously described experimental diets, four times daily (07 : 00, 11 : 00, 15 : 00, and 20 : 00 hr) for 90 days. Daily feeding amount was preprogrammed assuming a normal growth of the shrimp and an expected feed conversion ratio of 1.5. The daily allowances of feed were adjusted based on the daily observed feed consumption and mortality. The top-dressing procedures were performed following the standard protocol for the on-farm application by preparing 10 g xanthan gum as the binder, 100 mL hot water, 1 kg of feed, and 4 g of HT. The protocol was initiated by mixing 100 L

Table 1: Composition (% *as is*) of the experimental diets.

Ingredients (% *as is*)	Experimental diets code							
	BD	BD-0.1% HT	BD-0.2% HT	BD-0.3% HT	LFM 0.3% HT	BDTD-0.4% HT	CD	CDTD-0.4% HT
Menhaden fish meal[1]	10.00	10.00	10.00	10.00	7.50	Basal diet top-coated with 0.4% HT	Commercial diet	Commercial diet and top-coated with 0.4% HT
Soybean meal[1]	44.90	44.90	44.90	44.90	44.90			
Corn gluten meal[1]	8.00	8.00	8.00	8.00	8.00			
Menhaden fish oil[1]	5.66	5.66	5.66	5.66	5.66			
Corn starch[1]	10.34	10.24	10.14	10.04	12.54			
Hydrolyzable tannins[2]	–	0.10	0.20	0.30	0.30			
Soy lecithin[4]	1.00	1.00	1.00	1.00	1.00			
Wheat products[3]	15.00	15.00	15.00	15.00	15.00			
Mineral premix[5]	0.70	0.70	0.70	0.70	0.70			
Vitamin premix[6]	1.90	1.90	1.90	1.90	1.90			
L-Lysine HCl[4]	0.30	0.30	0.30	0.30	0.30			
DL-Methionine[4]	0.50	0.50	0.50	0.50	0.50			
L-Threonine[4]	0.20	0.20	0.20	0.20	0.20			
KP dibasic[4]	1.50	1.50	1.50	1.50	1.50			

Note: [1]PT FKS Multi Agro. Tbk, Jakarta, Indonesia. [2]Farmatan Aqua™ (Tanin Sevnica d.d., Slovenia). [3]PT. Pundi Kencana, Cilegon, Banten, Indonesia. [4]PT. Fenanza Putra Perkasa, Jakarta Selatan, Indonesia. [5]Trace mineral premix (g/100 g premix): cobalt chloride: 0.004; cupric sulfate pentahydrate: 0.550; ferrous sulfate: 2.000; magnesium sulfate anhydrous: 13.862; manganese sulfate monohydrate: 0.650; potassium iodide: 0.067; sodium selenite: 0.010; zinc sulfate heptahydrate: 13.193; alpha-cellulose: 69.664. [6]Vitamin premix (g/kg premix): thiamine·HCl: 4.95; riboflavin: 3.83; pyridoxine·HCl: 4.00; Ca-pantothenate: 10.00; nicotinic acid: 10.00; biotin: 0.50; folic acid: 4.00; cyanocobalamin: 0.05; inositol: 25.00; vitamin A acetate (500,000 IU/g): 0.32; vitamin D3 (1,000,000 IU/g): 80.00; menadione: 0.50; alpha-cellulose: 856.81.

Table 2: Proximate and amino acid (AA) compositions (% as is wet basis) of the experimental diets.

Parameter*	Unit	Experimental diets code					
		BD	BD-0.1% HT	BD-0.2% HT	BD-0.3% HT	LFM 0.3% HT	CD
Proximate analysis (*as is* %)							
Ash content	%	11.47	10.41	10.60	10.47	9.92	10.36
Total fat	%	6.63	6.59	7.13	7.20	6.84	6.76
Moisture content	%	10.46	11.15	9.28	10.19	8.44	10.46
Carbohydrate	%	34.50	35.49	34.30	35.62	37.45	34.54
Protein content	%	36.96	36.37	38.71	36.53	37.37	35.89
Amino acid profile (*as is* %)							
L-Serine	%	1.85	1.75	2.08	2.00	1.88	1.79
L-Glutamic acid	%	5.31	5.97	5.80	5.26	6.03	5.68
L-Phenylalanine	%	1.99	2.09	2.82	2.05	2.13	1.79
L-Isoleucine	%	1.48	1.40	1.50	1.48	1.44	1.37
L-Valine	%	1.65	1.55	1.67	1.69	1.60	1.38
L-Alanine	%	1.70	1.79	1.77	1.73	1.76	1.51
L-Arginine	%	2.49	2.42	3.07	2.62	2.56	2.31
Glycine	%	2.19	2.03	2.34	2.34	2.09	1.81
L-Lysine	%	1.83	1.85	1.70	1.58	1.78	1.64
L-Aspartic acid	%	2.81	3.32	3.15	2.83	3.15	2.33
L-Leucine	%	2.77	2.65	2.84	2.75	2.75	2.52
L-Tyrosine	%	0.65	0.68	0.90	0.67	0.67	0.96
L-Proline	%	1.98	1.94	2.04	2.03	2.06	1.81
L-Threonine	%	1.55	1.49	1.79	1.66	1.58	1.59
L-Histidine	%	0.85	0.86	1.15	0.93	0.92	0.84
L-Tryptophan	%	0.24	0.30	0.32	0.31	0.28	0.22
L-Cystine	%	2.72	2.84	3.09	3.13	3.25	1.93
L-Methionine	%	0.63	0.67	0.67	0.67	0.68	0.59

Note: *Analysis conducted by the Saraswanti Indo Genetech Laboratory (Bogor, West Java, Indonesia. Website: https://www.siglaboratory.com).

hot water with xanthan gum until reaching a slurry condition. Then, 4 g of HT were added to the slurry solution and mixed properly. The slurry solution containing HT was spread homogeneously into the formulated feed until it covered the surface of the feed evenly. The feed was then left 10–15 min under room temperature prior to use.

2.3. Water Quality Analysis and Sample Collection. The physical parameters, including pH, dissolved oxygen (DO), water temperature (T), salinity, total dissolved solid (TDS), and oxidation–reduction potentials (ORP) of the water, were measured four times daily using a real-time measurement system (Aqua TROLL 500 Multiparameter Sonde instrument) during the 90 days of feeding trial, and the data were stored to an application (AquaEasy apps, Bosch, Singapore) for data traceability and recording system purposes. Meanwhile, the ammonia–nitrogen (NH_3–N), nitrite–nitrogen (NO_2–N), and nitrate–nitrogen (NO_3–N) were measured once a week by using absorption spectrophotometry (DR890, HACH, USA). After 90 days, feeding was halted for 24 hr, and then shrimps in each hapa nets were group counted and bulk weighed to calculate the final body weight, percentage weight gain (PWG), feed conversion ratio (FCR), survival rate (SR), average daily growth (ADG), and thermal growth coefficient (TGC) as follows:

$$\mathrm{PWG} = \frac{(\text{Average individual final weight} - \text{Average individual initial weight})}{(\text{Average individual initial weight})} \times 100, \quad (1)$$

$$\mathrm{ADG} = \frac{\text{Total weight gained by the shrimp (g)}}{\text{Total days of culture}}, \quad (2)$$

$$\mathrm{FCR} = \frac{\text{Feed given (g)}}{\text{Alive weight gain of shrimp(g)}}, \quad (3)$$

$$\mathrm{SR} = \frac{\text{Final number of shrimp}}{\text{Initial number of shrimp}} \times 100, \quad (4)$$

$$\mathrm{TGC} = \frac{\mathrm{FBW}^{1/3} - \mathrm{IBW}^{1/3}}{\sum \mathrm{TD}} \times 100, \quad (5)$$

where FBW is the final body weight, IBW is the initial body weight, T is the temperature (°C), and D is the number of feeding days.

2.4. Biochemical Analysis for the Whole Body of Shrimp. Three shrimps from each hapa nets or 15 shrimps per dietary treatment were collected randomly and pooled at the termination of the hapa net trial to evaluate the proximate and amino acid composition in the whole body of shrimp. Prior to proximate, energy, and amino acid analyses, dried whole shrimps were rigorously blended and chopped in a mixer according to methods described by Helrich [23]. All parameters were analyzed at the Saraswanti Indo Genetech Laboratory (Bogor, West Java, Indonesia).

2.5. Total Hemocyte Count. At the termination of the feeding trial, hemolymph was sampled from three individual shrimps per hapa net, or 15 shrimps per dietary treatment, to determine the THC. Hemolymph (100 μL) of individual shrimp was withdrawn from the pleopod base of the second abdominal segment with a sterile 1-mL syringe (25 G × 13 mm needle). Before the hemolymph extraction, the syringe was loaded with a precooled (4°C) solution (10% EDTA, Na_2) used as an anticoagulant. The hemolymph with anticoagulant solution was diluted in 150 μL of formaldehyde (4%) and then 20 μL were placed on a hemocytometer (Neubauer) for the THC measurement using an optical microscope (Olympus, DP72).

2.6. Lysozyme Activity Analysis. The lysozyme activity of shrimp was measured by using a commercial kit (Sigma-Aldrich, Cat. No. LY0100). During the termination of the growth trial, five shrimps per dietary treatment were collected randomly and used for the lysozyme activity analysis. The results were defined by the lysis of *Micrococcus lysodeikticus* cells. The reactions were conducted at room temperature and the absorbance of the samples was measured at 450 nm using an ultraviolet/visible spectrophotometer (PerkinElmer, Lambda XLS, USA):

$$\text{Lysozyme activity}\left(\frac{\text{Units}}{\text{mL}}\right) = \frac{(\Delta\text{A450/min test} - \Delta\text{A450/minblank})(\text{df})}{(0.001)(0.03)}, \quad (6)$$

where df is dilution factor, 0.001 is ΔA450 as per the unit definition, and 0.03 is volume (in mL) of enzyme solution.

2.7. Histomorphology of the Hepatopancreas. Samples of hepatopancreas were taken from two shrimps per hapa net, or 10 shrimps per dietary treatment, immediately preserved in Davidson's fixative solution at room temperature for 48 hr [24], and then transferred to a 50% ethanol solution (VWR, Radnor, PA, USA) until processed by standard histological analysis procedures. Samples were dehydrated through a standard ethanol series to 100%, embedded in paraffin wax, and sectioned at 4 μm intervals for staining with hematoxylin and eosin (H&E) (Merck, Darmstadt, Germany). For estimations, a double-blinded evaluation with a grading scale of 1–5 was used. Score 1 was considered as the normal condition and subsequent scores accounted for increasing levels of histopathological alterations compared to the normal condition. Images were acquired by using a digital imaging microscope (Olympus BX41, Olympus Optical Co., Ltd., Tokyo, Japan).

Table 3: Overall water quality measurements during the grow-out phase of the experiment.

Parameters	Unit	Results
Dissolved oxygen	mg L^{-1}	7.96 ± 3.77
Temperature	°C	29.86 ± 1.85
pH		8.25 ± 0.24
Salinity	‰	29.72 ± 1.44
Total dissolved solid	mg L^{-1}	29.29 ± 2.15
Oxidation–reduction potential	mg L^{-1}	192.59 ± 105.73
Ammonia–nitrogen (NH_3–N)	mg L^{-1}	0.11 ± 0.05
Nitrite–nitrogen (NO_2–N)	mg L^{-1}	0.08 ± 0.04
Nitrate–nitrogen (NO_3–N)	mg L^{-1}	22.79 ± 6.44

Note: Data are presented as mean ± standard deviation (range).

2.8. Statistical Analysis. Data on the growth parameters, THCs, and lysozyme activity were tested for the normality of distribution (Lilliefors test) and homogeneity of variance (Bartlett's test). Normal and homoscedastic data were analyzed with using one-way analysis of variance (ANOVA) to determine significant differences among treatments, followed by Tukey's multiple comparison tests to determine the difference between the means among the treatments. Score data of the histomorphology of the hepatopancreas of shrimp were treated as categorical data, tested for normality and homoscedasticity, and subsequently analyzed using a nonparametric test. All statistical analyses were conducted using SAS system (version 9.4, SAS Institute, Cary, NC, USA).

3. Results

3.1. Water Quality Parameters. Table 3 summarizes the water quality conditions measured during the 90-day of feeding trial in the outdoor ponds used for the research. The parameters measured with real-time measurement devices, this is the DO, T, pH, salinity, TDS, and ORP, were within the range of 7.96 ± 3.77 mg L^{-1}, 29.86 ± 1.85°C, 8.25 ± 0.24, 29.72 ± 1.44‰, 29.29 ± 2.15 mg L^{-1}, and 192.59 ± 105.73 mg L^{-1}, respectively. Ammonia–nitrogen (NH_3–N), nitrite–nitrogen (NO_2–N), and nitrate–nitrogen (NO_3–N) were within the range of 0.11 ± 0.05 mg L^{-1}, 0.08 ± 0.04 mg L^{-1}, and 22.79 ± 6.44 mg L^{-1}, respectively. In general, the water quality levels were within the acceptable range to support the normal growth and survival of shrimp.

3.2. Growth Performance. Table 4 shows the effects of the different inclusion levels of HT on the growth performance parameters of shrimp *P. vannamei* after 90 days of feeding trial. Diet BD-0.3% HT provided a significant increase in the FBW, PWG, TGC, and ADG compared to the other dietary treatments. Statistically, diets BD-0.1% HT and BD-0.2% HT did not cause any significant increase in FBW, PWG, TGC, and ADG compared to the BD. Top-dressing of basal diet

Table 4: Growth performance of Pacific white shrimp (*Litopenaeus vannamei*) (mean initial weight 0.87 ± 0.02 g) fed the experimental diets for 90 days.

Diet codes	Final body weight (g)	Survival (%)	PWG[1] (%)	FCR[2]	TGC[3]	ADG[4]
BD	13.89^{bc}	82.44	$1{,}496.55^{bc}$	1.39^{bc}	0.5404^{bc}	0.1543^{bc}
BD-0.1% HT	14.04^{b}	84.73	$1{,}514.02^{b}$	1.38^{c}	0.5434^{bc}	0.1560^{b}
BD-0.2% HT	14.13^{b}	85.88	$1{,}524.83^{b}$	1.37^{c}	0.5456^{b}	0.1571^{b}
BD-0.3% HT	14.47^{a}	90.99	$1{,}563.68^{a}$	1.34^{d}	0.5528^{a}	0.1608^{a}
LFM 0.3% HT	13.60^{d}	88.03	$1{,}462.76^{d}$	1.43^{a}	0.5340^{d}	0.1511^{d}
BDTD-0.4% HT	14.09^{bc}	87.50	$1{,}519.77^{b}$	1.37^{c}	0.5446^{b}	0.1566^{b}
CD	13.77^{cd}	82.81	$1{,}482.99^{cd}$	1.41^{ab}	0.5378^{cd}	0.1530^{cd}
CDTD-0.4% HT	13.88^{bc}	81.31	$1{,}495.17^{bc}$	1.39^{bc}	0.5402^{bc}	0.1542^{bc}
p-value	<0.0001	0.7955	<0.0001	<0.0001	<0.0001	<0.0001
PSE[5]	0.0479	3.7664	5.5043	0.0049	0.0001	0.0054

Note: [1]PWG, percentage weight gain; [2]FCR, feed conversion ratio; [3]TGC, thermal growth coefficient; [4]ADG, average daily growth; [5]PSE, pooled standard error. Results in the same row with different superscript letter are significantly different ($P<0.05$) based on analysis of variance followed by the Tukey's multiple comparison test.

with 0.4% HT (BDTD-0.4% HT) did not cause any significant impact in the growth parameters compared to the BD. The growth performance of shrimp fed the commercial diet (CD) was similar to that observed with the BD, but resulted in significantly lower FBW, PWG, TGC, and ADG than the basal diet supplemented with increasing inclusion levels of HT. Top-dressing of the commercial diet with 0.4% HT (CDTD-0.4% HT) increased the growth performance of shrimp slightly, but was not significantly different compared to that of shrimp-fed diets BD-0.1% HT and BD-0.2% HT. Regarding the FCR, diet BD-0.3% HT had significantly lower values compared to the other dietary treatments. However, the supplementation of diet with 0.1% HT and 0.2% HT (BD-0.1% HT and BD-0.2% HT) showed no significant differences in FCR compared to the BD and BDTD-0.4% HT. In general, there were no significant differences ($p = 0.7955$) in the survival rate of shrimp fed with different dietary treatments.

3.3. Whole Body Composition. Table 5 shows the proximate and amino acid compositions of the whole body of shrimp *P. vannamei* (expressed as percentage of wet weight) after being fed with the experimental diets for 90 days. Numerically, the highest protein and fat levels were found in shrimp-fed diets BD-0.1% HT, BD-0.2% HT, and BD-0.3% HT compared to shrimp-fed diets BD and CD. The level of ash content was higher in shrimp-fed diets LFM 0.3% HT and CD compared to other dietary treatment. The top-dressing process to the CD was able to reduce the level of ash and slightly increase the protein level of shrimp compared to the group of shrimp fed only with CD.

3.4. Total Hemocyte Counts (THC) and Lysozyme Activity. The experimental diets did not significantly affect the THC and lysozyme activity of shrimp after 90 days of feeding trial (Figure 1). However, numerically, the THC in the group of shrimp-fed diets BD-0.1% HT, BD-0.2% HT, and BD-0.3% HT was higher compared to those of shrimp fed the other dietary treatments. Numerically, there was a gradual increase in the lysozyme activity with the increasing supplementation level of HT (Figure 2). Interestingly, top-dressing process to the basal and commercial diets with 0.4% HT (BDTD-0.4% HT and CDTD-0.4% HT) was able to numerically increase the THC and lysozyme activity compared to the group of shrimp-fed diets the BD and CD.

3.5. Histomorphological Condition of the Hepatopancreas of Shrimp. The inclusion of HT affected the histomorphological condition of the hepatopancreas of shrimp (Figure 3). In the group-fed BD, the disappearance of the cell nuclei, a disorganized organelle structure, expansion of tubular lumen, and many necrotic and lytic cells were observed. In the group of shrimp-fed diets supplemented with graded levels of HT, there were fewer lipid vacuoles and incomplete cells than in shrimp-fed BD. With the increasing dietary level of HT, the formation of R cells was observed. However, the organelle and cell nucleus became visible with the appearance of star-shaped tubular lumen. In the group of shrimp fed with the top-dressing diets (0.4% HT), the number of R cells and B cells was evident. The top-coating of HT on the commercial diet (CDTD-0.4% HT) also improved the integrity of the hepatopancreas of the shrimp with higher numbers of R and B cells and well-organized tubular structure and star-shaped lumen compared to the hepatopancreas condition of shrimp fed only with the commercial diet.

4. Discussion

In the present study, the functional effects of diets containing graded levels of HT, included in the feeds and top-dressing (TD) on the basal and commercial diets, were evaluated on the growth, body composition, THC, lysozyme activity, and histomorphological condition of the hepatopancreas of shrimp. From a previous controlled study using aquaria tanks, Novriadi et al. [22] reported that the use of BD-0.2% or BD-0.3% of HT in diets with 10% inclusion levels of FM could promote the growth and hematoimmunological parameters in shrimp *P. vannamei*. In the present study, the long-term trial using hapa nets installed within the ponds affirmed the prior results and showed significant increases in terms of FBW, PWG, TGC, and ADG using levels of HT from 0.1% to 0.3% compared to the BD. A study by Zhu et al. [21] demonstrated that after a 60-day feeding trial, the FBW of shrimp *P. vannamei* fed with 0.05%–0.15% HT was

Table 5: Proximate and amino acid (AA) compositions (% as is dry matter basis) of the whole body of Pacific white shrimp (*Penaeus vannamei*) fed the experimental diets for 90 days.

Parameters	Unit	Experimental diets							
		BD	BD-0.1% HT	BD-0.2% HT	BD-0.3% HT	LFM 0.3% HT	BDTD- 0.4% HT	CD	CDTD-0.4% HT
Proximate composition									
Protein content	%	21.99	22.29	22.76	22.47	21.88	21.63	21.79	21.82
Ash content	%	1.70	1.75	1.77	1.77	2.05	1.83	2.04	1.78
Total fat	%	0.25	0.27	0.28	0.28	0.20	0.22	0.19	0.19
Calorie from fat	Kcal $100\,g^{-1}$	2.41	2.42	2.44	2.48	2.14	2.31	2.18	2.23
Crude fiber	%	0.26	0.23	0.23	0.23	0.36	0.41	0.44	0.39
Moisture content	%	73.66	74.25	74.47	74.65	73.88	74.37	73.72	73.82
Total calories	Kcal $100\,g^{-1}$	96.54	96.00	96.89	96.72	95.33	96.15	97.00	96.62
Amino acid composition									
L-Serine	%	0.81	0.80	0.83	0.75	0.75	0.74	0.72	0.89
L-Glutamic acid	%	3.71	3.59	3.54	3.43	3.54	3.52	3.58	3.34
L-Phenylalanine	%	1.12	1.03	1.22	0.95	0.84	0.84	0.86	1.37
L-Isoleucine	%	0.92	0.89	0.92	0.86	0.83	0.87	0.87	0.87
L-Valine	%	0.96	0.94	0.94	0.89	0.94	0.94	0.97	0.96
L-Alanine	%	1.27	1.27	1.23	1.29	1.23	1.24	1.27	1.19
L-Arginine	%	2.18	2.13	2.29	1.97	1.87	1.87	1.91	2.87
Glycine	%	1.72	1.64	1.81	1.80	1.69	1.69	1.50	1.99
L-Lysine	%	1.58	1.59	1.50	1.51	1.49	1.49	1.49	1.42
L-Aspartic acid	%	2.15	2.17	1.99	2.06	1.99	2.00	2.07	1.92
L-Leucine	%	1.54	1.51	1.53	1.59	1.62	1.51	1.53	1.75
L-Tyrosine	%	0.74	0.68	0.77	0.68	0.61	0.60	0.61	0.89
L-Proline	%	1.27	1.22	1.05	1.15	1.13	1.13	1.15	1.18
L-Threonine	%	0.95	0.90	0.97	0.96	0.84	0.85	0.87	0.84
L-Histidine	%	0.57	0.55	0.64	0.62	0.47	0.48	0.55	0.67
L-Tryptophan	%	0.17	0.17	0.17	0.17	0.18	0.18	0.16	0.17
L-Cystine	%	0.66	0.46	0.48	0.45	0.43	0.42	0.46	0.41
L-Methionine	%	0.37	0.36	0.36	0.40	0.38	0.37	0.36	0.36

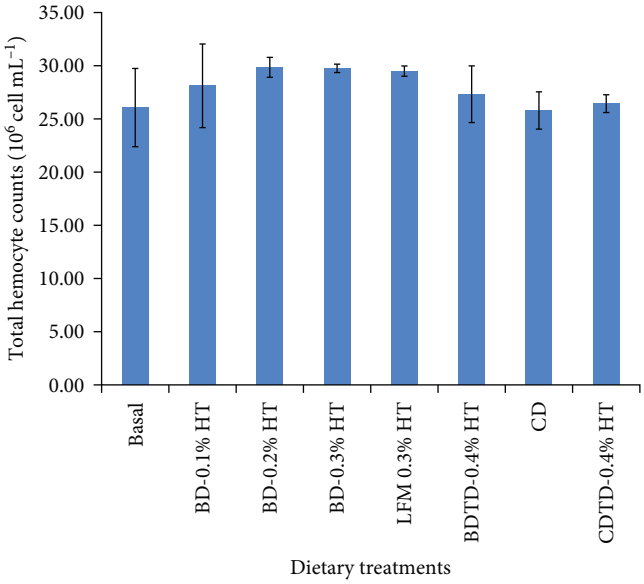

FIGURE 1: Total hemocyte count of Pacific white shrimp (*Penaeus vannamei*) (10^6 cell mL^{-1}) at the end of the 90-day feeding trial. Values represent the mean of 15 replicates (p-value = 0.8989).

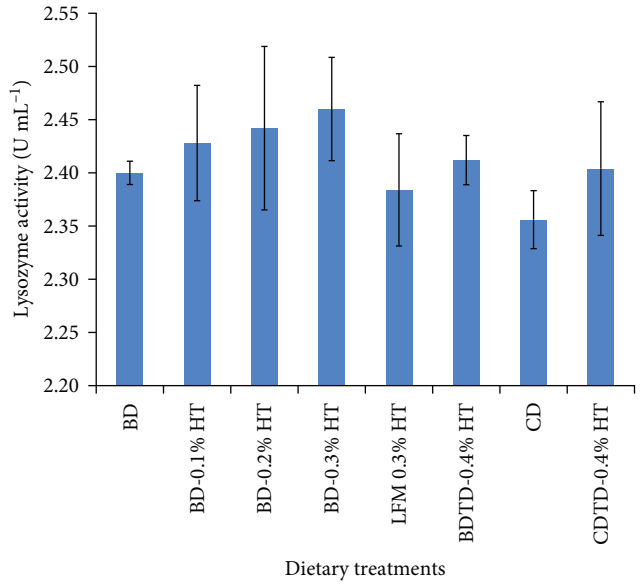

FIGURE 2: Lysozyme activity of Pacific white shrimp (*Penaeus vannamei*) (U mL^{-1}) at the end of the 90-day feeding trial. Values represent the mean of five replicates (p-value = 0.4954).

significantly higher compared to the diet without any inclusion of HT. The ability of HT to induce the activity of trypsin (TRY), a digestive enzyme that provides protein availability and facilitates a better nutrient absorption, could explain this superior growth in shrimp fed with HT [21]. To date, there is little or no information concerning the efficacy of HT for white shrimp *P. vannamei* cultured under pond conditions during long-term feeding periods. Thus, the present results provide essential information on the use of HT, and the significant effects on the growth performance of the shrimp could be due to the longer duration of feeding trials used in this study (90 days) compared to the 60 days from the previous work published by Novriadi et al. [22], so that shrimp can optimally utilized the functional benefits of HT.

Commercial studies on shrimp feed has shown that shrimp feeds containing 30%–35% crude protein can include levels of FM as 7.5%–12.5% without compromising the growth performance of the shrimp [25]. In this study, the reduction of the inclusion of dietary FM from 10% to 7.5% and supplemented with 0.3% HT did not significantly support the optimum growth of shrimp compared to the BD and BD-0.3% HT. This is also in agreement with the previous work [22], where using the same diet formulation under controlled conditions, the shrimp did not show better FBW and biomass compared to the BD. Many studies have been conducted to replace FM with alternative ingredients or supplements in the development of practical diets for Pacific shrimp *P. vannamei* [25–29]. These authors concluded that the reduction of FM in diets will face two major issues: the reduction of the feed intake and the lower apparent digestibility of the alternative nutrients [30]. Therefore, further studies are needed in order to determine the proper inclusion levels of HT to compensate for the gradual reduction of FM in the diet formulation.

Nowadays, the use of a top-dressing technique has become an option in farm-feeding practices to deliver the functional nutrients of ingredients, additives, or supplements to aquatic organisms, in order to enhance the immune system activity and the growth performance [31, 32]. A recent study from Klongklaew et al. [33] showed that the use of top-dressing methods using various inclusion levels of crude extract from *Ulva intestinalis* were able to induce better growth, immune system status, and protect the shrimp against yellow head virus (YHV). In the present study, the top-dressing method conducted by using xanthan gum as the binder to the basal and commercial diet led to superior FBW, PWG, TGC, and ADG compared to the group of shrimps that were only fed with the BD or CD. However, the incorporation of 0.3% HT directly into the diet (BD-0.3% HT) had better growth and feed efficiency compared to the top-dressing group. There is a concern related to top-dressing method in general, where the target substances will disperse in the water before fish or shrimp has the chance to eat the feed, which may lead to the reduction of the dose ingested by the animals [34, 35]. In addition, Emerenciano et al. [36] stated that top-dressing method will have some negative impacts, including uncertainties as to whether the desired dosage has been properly delivered to the tested organisms. Since the diet was coated with the binder, one of the possible reasons for the lower growth performance could be addressed by a potential lack of homogeneity of the top-dressing application during the observation period.

Dietary HT seemed to have a positive effect on the protein and lipid deposition in the body of shrimp. For the protein, the possible hypothesis is that the activity of trypsin increased with the inclusion of HT. Thus, the digestibility of protein also increased, resulting in an improved assimilation and retention of protein. This hypothesis is also supported by the study from Yao et al. [37], where the use of HT in grass

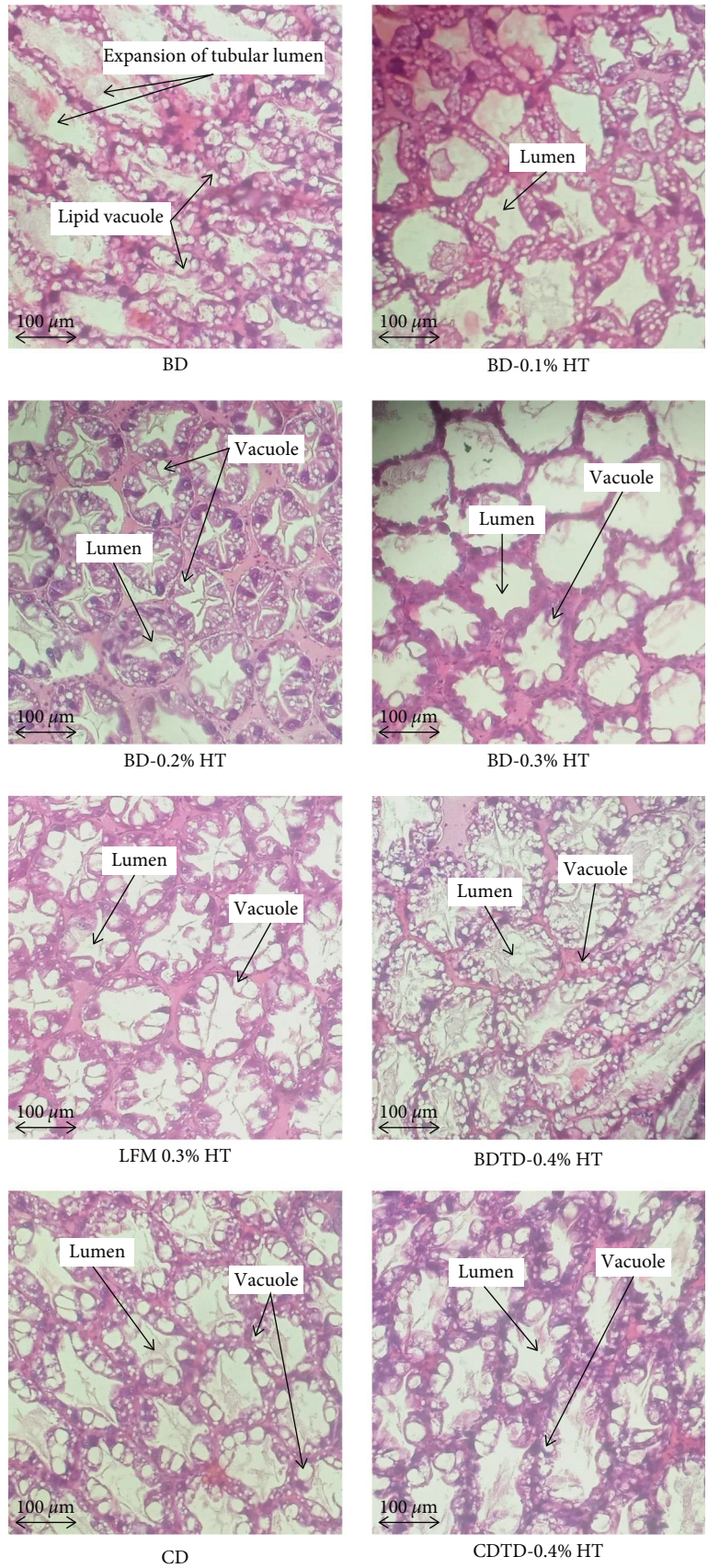

FIGURE 3: Representative histopathological images of hematoxylin and eosin-stained sections of hepatopancreas of Pacific white shrimp (*Penaeus vannamei*) fed the experimental diets for 90 days.

carp *Ctenopharyngodon idellus* increased the expression of the target of rapamycin (TOR) mRNA in the intestine that eventually modulated the protein translation and stimulated the synthesis of protein. Interestingly, for the lipid metabolism, Yao et al. [37] reported that the inclusion of HT ranging from 0.75% to 1.75% in the diet formulation reduced the muscle lipid content of grass carp due to the low activity of lipase. According to Nyman and Bjorck [38] and Bell [39], because of the structure of lipase, the binding strength between tannin and lipases was large and hard to modify *in vivo*, and this partly accounted for the low lipase activity. Since the information on patterns of nutrient deposition in shrimp reared under aquaculture conditions is very limited [40], the better deposition of nutrients in shrimp-fed diets with HT could be due to the positive effects of HT on the digestion and absorption functions of organisms [41].

In shrimp, the hepatopancreas is the major digestive gland within a complex arrangement, and also a sensitive organ to the change in feed ingredients, and consequently is an essential indicator for metabolism [42, 43]. It was reported that a low dosage of tannins had positive effects on the digestion and absorption [44], including shrimp *P. vannamei* [21]. In the present research, the basal diet exhibited a disorganized organelle structure and absence of B and R cells. The addition of HT, either included in the formulated feeds or top-dressing, was able to improve the hepatopancreas condition of the shrimp by increasing the number of R and B cells, improving the tubular structure and lumen star-shaped morphology. The R cells have an essential role to mediate the digestion, absorption, storage, and probably excretion and detoxification [45]. The absence of R cells and the structural changes in the hepatopancreas of shrimp fed with basal diet found in the present study could be due to the high inclusion level of SBM (44.9% *as is* in diet formulation). Several authors reported that the inclusion of a wider level of plant–protein sources could affect the integrity and condition of the hepatopancreas of shrimp [46, 47]. Taking this into account, the supplementation of HT either directly included or using top-dressing process to diet might have improved the nutritional status in Pacific white shrimp.

In shrimp, the nonspecific immunity plays an important role to protect the shrimp from harmful microorganisms and environmental stressors during the critical culture period [48, 49]. To initiate the immune response, some compounds have to be present in the system to recognize the presence of the foreign materials that have gained access to enter the shrimp body [50]. According to Söderhäll and Cerenius [51], the blood cells of invertebrates, termed hemocytes, are the primary effectors of host defense and are involved in numerous immune-related responses, as demonstrated in phagocytosis, melanization, encapsulation, and coagulation. In the present study, despite there were no statistical differences, there was an increasing numerical trend of the THC as the inclusion of HT into the diet increased from 0.1% to 0.3%. In addition, the TD process also resulted in an elevated level of THC in shrimp compared to the group of shrimp fed with either basal or the commercial diets without the inclusion of HT into the formulated diets. Our previous work indicated that, during the 60-day feeding period under a controlled environment, the shrimp fed with 0.2% and 0.3% HT had the significantly highest level of THC compared to the basal diet [22]. These differences may be attributed to the culture condition where shrimps consistently face the environmental stressors during the whole feeding period. According to Guo et al. [52], the apoptotic cell ratio of hemocytes increased as the shrimps were exposed to nitrite-N, one of the major environmental stressors in shrimp farming systems, for 48 hr.

We also examined the lysozyme activity within the tissues of shrimp at the end of the feeding trial. A study from Burge et al. [53] suggested that as pathogens enter the shrimp body, hemocyte will naturally migrate to the intrusion site soon after the body is compromised, and a high concentration of lysozyme will remain in the infected site to combat the disease. Lysozyme in shrimp is well characterized and has been shown to possess lytic activity against a wide range of bacterial infection [54, 55]. In this research, the pattern of the lysozyme activity was similar to that of the THC, being significantly higher in shrimp-fed diets with HT compared to shrimp-fed BD. The reduction of FM influenced the lysozyme activity of the shrimp, as it was lower compared to the group of shrimp-fed BD. An elevated activity of lysozyme was also observed in the commercial diet top-dressing with HT compared to the same commercial diet without HT, suggesting a direct relationship between the HT and the modulation of the nonspecific immune system in shrimp. It will be interesting to evaluate other mechanisms associated with the complex shrimp immune system, such as various antioxidant enzymes, as well as penaeidins, that are important antimicrobial peptides in the defense of shrimp against bacterial pathogens. The potential stimulation of this system would complement the other mechanisms discussed and add to our understanding of how HT acts.

5. Conclusion

The findings in this study clearly showed that the HT supplementation, either directly incorporated or using top-dressing on the feed, enhanced the growth performance and prevent the alteration in the health and histomorphological condition of the Pacific white shrimp *P. vannamei*. This can have positive consequences in real-time practical shrimp production, in order to promote shrimp resilience. The cost–benefit analysis remains to be validated.

Data Availability

The data that support the findings of this study are available from the corresponding author (RN) upon reasonable request.

Ethical Approval

All the procedures and handling process in the present study were approved by the recommendations in the Guide for the Use of Experimental Animals of the Jakarta Technical University of Fisheries.

Disclosure

The mention of trademark or proprietary product does not constitute an endorsement of the product and does not imply its approval to the exclusion of other products that may also be suitable.

Conflicts of Interest

Dr. Giridhar Rahul Gaddipati and Dr. Clara Trullàs are employed by Tanin Sevnica. The remaining authors state no conflicts of interest.

Acknowledgments

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