

Effect of Chestnut Wood Polyphenols on Growth, Survival, and Disease Resistance in Pacific White Shrimp (*Litopenaeus vannamei* Boone, 1931)

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ABSTRACT

Chestnut wood polyphenols (CWP) represent a natural feed additive with potential antibacterial and immunostimulant properties. This study evaluated the *in vitro* antibacterial activity of CWP against *Photobacterium damsela* subsp. *damsela* (PDD) and its effects on the growth performance, immune responses, and disease resistance of Pacific white shrimp (*Litopenaeus vannamei* Boone, 1931). The minimum inhibitory concentration and minimum bactericidal concentration of CWP against PDD were 10 µg·mL⁻¹ and 20 µg·mL⁻¹, respectively. In the feeding trial, shrimp (initial weight, 5.58±0.19 g) were given diets supplemented with CWP at 0 (control), 1, 3, or 5 g·kg feed⁻¹ for 30 days. The results indicate that dietary CWP significantly improved survival rates and feed conversion ratios in a dose-dependent manner, with the highest survival (92.00±4.00%) and lowest FCR (0.99±0.04) observed in the 5 g·kg feed⁻¹ group. In addition, CWP supplementation notably reduced intestinal *Vibrio* spp. counts and enhanced superoxide dismutase activity, while the immune parameters, including total hemocyte count, phagocytic activity, and phenoloxidase activity, remained unaffected. Following experimental challenge with PDD, shrimp fed 5 g·kg feed⁻¹ CWP exhibited higher survival (70.00%) compared to the control group (43.33%). On day 7 post-challenge, attenuation of PDD-associated lesions in the hepatopancreas and muscle by dietary CWP was revealed by histopathological examination. These findings thus demonstrate that dietary supplementation with CWP, particularly at 5 g·kg feed⁻¹, enhances growth performance and immune responses, while reducing pathogenic bacterial loads and improving disease resistance in Pacific white shrimp.

Keywords: Antibiotic alternatives, Disease resistance, Immunostimulant, Natural feed additives, Pale shrimp disease, Tannins

INTRODUCTION

Pacific white shrimp (*Litopenaeus vannamei* Boone, 1931), is one of the most economically important crustacean species in global aquaculture, accounting for approximately 60% of total farmed crustacean production worldwide (FAO, 2024). However, intensive shrimp farming practices have

been associated with disease outbreaks, particularly those involving bacterial infections, which have caused significant economic losses, thereby threatening the sustainability of the industry under threat. Among these bacterial pathogens, *Photobacterium damsela* subsp. *damsela* (PDD) has emerged as a notable threat, causing pale shrimp disease, a condition characterized by lethargy,

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reduced feeding, pale coloration, and high mortality rates (Chuchird *et al.*, 2024).

It should also be considered that the excessive use of antibiotics in aquaculture to control these aforementioned bacterial diseases has raised serious concerns regarding the risk of antibiotic resistance, environmental contamination, and food safety (Cabello *et al.*, 2013). As such, there is an urgent need to develop more sustainable disease management strategies. For instance, natural feed additives derived from plant sources have gained traction as alternatives due to their antimicrobial, immunostimulant, and antioxidant properties, along with their biodegradability and relatively low environmental impact (Reverter *et al.*, 2014).

Polyphenolic compounds, particularly tannins, are secondary plant metabolites that are known for their broad-spectrum antimicrobial activities and immunomodulatory effects (Smeriglio *et al.*, 2016). Chestnut wood (*Castanea sativa*) is a rich source of such hydrolyzable tannins, primarily ellagitannins and gallotannins, which have been shown to possess potent antibacterial activity against various pathogenic bacteria (Prapaiwong *et al.*, 2021). The antimicrobial mechanisms of polyphenols include their ability to disrupt bacterial cell membranes, inhibit bacterial enzymes, chelate metal ions that are essential for microbial growth, and interfere with bacterial quorum sensing systems (Daglia, 2012; Borges *et al.*, 2013).

Moreover, in addition to their previously established antimicrobial properties, polyphenolic compounds have been reported to improve innate immune responses in aquatic animals through pathways such as stimulating hemocyte proliferation, enhancing phagocytic activity, and upregulating antioxidant enzyme activities (Reverter *et al.*, 2014). Recent studies have demonstrated that dietary supplementation with various polyphenol-rich plant extracts, including hydrolyzable tannins, fermented pomegranate peel polyphenols, and sugarcane extracts, significantly augments growth performance

and immune parameters, and improves disease resistance in Pacific white shrimp (Huang *et al.*, 2024; Yu *et al.*, 2024; Wimanhaemin *et al.*, 2025). The innate immune system comprises the primary defense mechanism in crustaceans, as they lack the adaptive immunity present in mammals. Key components of crustacean innate immunity include cellular responses (i.e., hemocyte-mediated phagocytosis, encapsulation, and nodule formation) and humoral factors (i.e., the prophenoloxidase system, antimicrobial peptides, and antioxidant enzymes) (Jiravanichpaisal *et al.*, 2006).

Previous investigations have shown that tannins can modulate immune responses and hence reduce pathogenic bacterial loads in shrimp. Novriadi *et al.* (2023) reported that sweet chestnut-derived hydrolyzable tannins improved growth performance and hepatopancreas histomorphology in Pacific white shrimp under commercial pond conditions. Similarly, Gong *et al.* (2022) demonstrated that dietary tannic acid elevated digestive enzyme activities, antioxidant capacity, and stress resistance while modulating intestinal microbiota composition. Furthermore, several studies have documented the efficacy of plant-derived polyphenols in controlling *Vibrio* infections and improving shrimp survival after challenge with pathogens (Chaweepack *et al.*, 2015; Mantaring *et al.*, 2024; Wimanhaemin *et al.*, 2025).

Nonetheless, despite the growing interest in plant polyphenols as functional feed additives, information regarding the effects of chestnut wood polyphenols (CWP) on shrimp health, immune responses, and disease resistance is quite limited. To the best of the authors' knowledge, the antibacterial effect of CWP against PDD and improvement of disease resistance against PDD infection in shrimp have yet to be investigated. The primary objective of this study was to investigate the effects of dietary CWP on growth performance, survival, immune responses, *Vibrio* spp. counts, and disease resistance against PDD in Pacific white shrimp. It was expected that the CWP-supplemented feed would confer these beneficial effects to the shrimp.

MATERIALS AND METHODS

Experiment 1: In vitro antibacterial activity of chestnut wood polyphenols against Photobacterium damsela subsp. damsela

Chestnut wood polyphenols (CWP)

The commercial CWP product (Actinol®, Cischem Agricultural Co., Ltd, Bangkok, Thailand) used in this study was a crude extract of sweet chestnut (*Castanea sativa*) wood and was characterized by high polyphenolic content. Total phenolic content (TPC) and total tannin content (TTC) were quantified at 826 mg gallic acid equivalents·g sample⁻¹ and 794 mg gallic acid equivalents·g sample⁻¹, respectively. Analyses were conducted by the Kasetsart Agricultural and Agro-Industrial Product Improvement Institute (KAPI), Kasetsart University, Bangkok, Thailand, following the methods described by Wolfe *et al.* (2003) for TPC and Megat Rusydi and Azrina (2012) for TTC.

Bacterial culture preparation

Photobacterium damsela subsp. damsela (PDD) isolate SRT-1-O, originally isolated from Pacific white shrimp that exhibited clinical signs of pale shrimp disease, was used for antibacterial testing. The bacterial isolate was cultured on tryptic soy agar (TSA) supplemented with 1.5% NaCl using the streak plate method and incubated at 37 °C for 18–24 h. A fresh subculture was prepared prior to each experiment to ensure the consistency and viability of bacteria.

Determination of minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration (MIC), which is defined as the lowest concentration that inhibits visible bacterial growth, and the minimum bactericidal concentration (MBC), which is defined as the lowest concentration that kills bacteria, were determined following the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2020). A single PDD colony was suspended

in 0.85% NaCl solution and adjusted to an optical density at 625 nm of 0.08–0.13, corresponding to approximately 1×10^8 CFU·mL⁻¹. This suspension was subsequently diluted 100-fold in 0.85% NaCl to achieve a working concentration of 1×10^6 CFU·mL⁻¹.

MIC determination was performed in 96-well microtiter plates using the broth microdilution method. Serial two-fold dilutions of CWP, ranging from 2,560 to 5 µg·mL⁻¹, were prepared in 50 µL of Mueller-Hinton Broth (MHB). Subsequently, 50 µL of the bacterial inoculum was added to each well; this yielded the final CWP concentrations ranging from 1,280 to 2.5 µg·mL⁻¹ and a bacterial density of 5×10^5 CFU·mL⁻¹. Following incubation at 37 °C for 18–24 h, the MIC was identified as the lowest concentration exhibiting no visible bacterial growth. The experiment was conducted in quadruplicate.

For MBC determination, 10-µL aliquots from all wells showing no visible growth were streaked onto TSA supplemented with 1.5% NaCl and incubated at 37 °C for 18–24 h. The MBC was defined as the lowest concentration that resulted in complete absence of colony formation on the agar plates.

Experiment 2: Effect of chestnut wood polyphenols on the growth performance, survival, immune responses, and Vibrio spp. counts in Pacific white shrimp

Experimental diets

Four experimental diets were formulated to contain CWP at different concentrations: (1) Control (CWP 0 g·kg feed⁻¹), (2) CWP 1 g·kg feed⁻¹, (3) CWP 3 g·kg feed⁻¹, and (4) CWP 5 g·kg feed⁻¹. The required amount of CWP was first dispersed in 150 mL distilled water and then uniformly sprayed onto 1 kg of commercial shrimp pellet feed. Following CWP application, in order to improve palatability and water stability, the pellets of all groups, including the control diet, were top-coated with 15 mL tuna oil (Thai Union Feedmill, Samut Sakhon, Thailand). The prepared feeds were air-dried for 24 h and subsequently stored in sealed plastic containers at -20 °C until use.

Experimental animals and culture conditions

Three hundred healthy Pacific white shrimp with an initial body weight of 5.58 ± 0.19 g were obtained from a commercial farm in Suphanburi Province, Thailand. Following a seven-day acclimation period, shrimp were randomly distributed into 12 experimental tanks (four dietary treatments $\times$ three replicate tanks) at a stocking density of 25 shrimp per tank (100 L capacity). Throughout the 30-day feeding trial, water quality parameters were monitored daily and maintained within the optimal ranges for Pacific white shrimp culture (Jory, 2019; Songsangjinda, 2019). Dissolved oxygen (DO) was measured using a YSI PRO 20 dissolved oxygen meter (YSI Inc./Xylem Inc., Yellow Springs, OH, USA) and maintained above $5 \text{ mg} \cdot \text{L}^{-1}$. Water pH was monitored using an EcoScan pH 5 meter (Thermo Fisher Scientific Inc.) and maintained between 8.1 and 8.3. Total ammonia nitrogen and nitrite-nitrogen concentrations were determined following standard methods (APHA, 2017) and maintained below $0.1 \text{ mg} \cdot \text{L}^{-1}$. Alkalinity was maintained between 120 and 200 $\text{mg} \cdot \text{L}^{-1}$ as CaCO_3 , and salinity was maintained at 10 ppt using a salinometer. All experimental procedures were conducted in accordance with the guidelines approved by the Kasetsart University Institutional Animal Care and Use Committee (IACUC approval number: ACKU66-FIS-016).

Growth performance and survival assessment

Shrimp were fed the experimental diets to apparent satiation four times daily (08:00 a.m., 12:00 a.m., 04:00 p.m., and 08:00 p.m.) for 30 days. At the conclusion of the feeding trial, 10 randomly selected shrimp from each tank were individually weighed to determine final body weight. The number of shrimp in each tank was counted to determine the survival rate. Feed conversion ratio (FCR) was calculated using the following formula:

$$\text{FCR} = \text{Total feed consumed (g)} / \text{Total weight gain (g)}$$

Immune response parameters

At the end of the 30-day feeding trial, three shrimp were randomly sampled from each tank

for immunological analysis. Four innate immune parameters were evaluated, including total hemocyte count (THC), phagocytic activity, phenoloxidase (PO) activity, and superoxide dismutase (SOD) activity.

Hemolymph samples (0.5 mL) were collected from the base of the third walking leg using a 1-mL syringe containing 1.5 mL of modified anticoagulant solution (K-199 medium supplemented with 5% L-cysteine). THC was determined by transferring a 100- μL aliquot of the hemolymph-anticoagulant mixture to a hemocytometer and counting cells under a light microscope at $400\times$ magnification.

Phagocytic activity was assessed following the method of Itami *et al.* (1994). Briefly, a 200- μL aliquot of the hemolymph-anticoagulant mixture was placed on a glass coverslip and incubated for 30 min at room temperature (27°C) to allow hemocyte adherence. Non-adherent cells were removed by gentle washing with phosphate-buffered saline. Heat-killed *Saccharomyces cerevisiae* cells were added and incubated for 2 h at room temperature. Following incubation, coverslips were fixed in methanol and stained with Giemsa solution. Phagocytic activity was expressed as the percentage of hemocytes that demonstrated phagocytosis among 200 counted cells.

PO activity was measured spectrophotometrically according to Söderhäll and Hall (1984). Hemolymph samples were centrifuged, and the supernatant was used for enzyme assay. The oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) was monitored at 490 nm. Enzyme activity was normalized to total protein content, determined by the method of Lowry *et al.* (1951), and expressed as $\Delta A_{490} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$.

SOD activity was determined using a commercial SOD Assay Kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. The results were expressed as percentage inhibition of formazan dye formation. The principle relies on the reduction of WST-1 [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] by superoxide anions to yield

a water-soluble formazan dye. Because SOD competes with WST-1 for superoxide anions, an increased inhibition of formazan formation corresponds to increased SOD activity.

*Total *Vibrio* spp. counts*

The same individual shrimp sampled for immune analysis were also used for determination of total *Vibrio* spp. counts. Hepatopancreas and intestine tissues were aseptically dissected, weighed, and homogenized in sterile 0.85% NaCl solution. Serial ten-fold dilutions of each tissue homogenate were prepared in sterile 0.85% NaCl. From each dilution, 0.1 mL was spread-plated onto Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar and incubated at 37 °C for 24 h. Following incubation, yellow and green colonies characteristic of *Vibrio* spp. were enumerated. Total *Vibrio* spp. counts were expressed as colony-forming units per gram of tissue (CFU·g⁻¹). Note that this technique is not definitive for *Vibrio* spp. identification, and the result should only be viewed as presumptive *Vibrio* spp. counts.

Experiment 3: Effect of chestnut wood polyphenols on disease resistance against Photobacterium damsela subsp. damsela infection in Pacific white shrimp

Experimental animals

Following the completion of the 30-day growth trial (Experiment 2), 30 shrimp from each dietary treatment group were randomly redistributed into three glass tanks containing 50 L of seawater at a stocking density of 10 shrimp per tank.

Bacterial preparation and challenge procedure

PDD isolate SRT-1-O was cultured on TSA supplemented with 1.5% NaCl, then transferred to nutrient broth (NB) supplemented with 1.5% NaCl and incubated at room temperature (27 °C) for 24 h. The bacterial culture was centrifuged at 4,000 × g for 15 min, and the supernatant was discarded. The bacterial pellet was resuspended in 1.5% NaCl solution, and the

optical density was adjusted to 0.08–0.10 at 625 nm, which corresponded to approximately 1×10⁸ CFU·mL⁻¹.

Experimental infection was conducted via oral gavage. Each shrimp received 0.1 mL of bacterial suspension (1×10⁷ CFU·shrimp⁻¹) administered directly into the mouth using a 1-mL syringe fitted with a 25-gauge needle. Following bacterial challenge, shrimp mortality was monitored and recorded daily for 7 days. Shrimp continued to receive their respective experimental diets (Control, CWP 1, 3, or 5 g·kg feed⁻¹) throughout the challenge period.

Histopathological examination

At the conclusion of the challenge experiment (day 7 post-infection), three shrimp from each treatment group were randomly selected for histopathological analysis. Whole shrimp were fixed in Davidson's fixative for 24–48 h. Fixed tissues were processed following standard histological protocols (Bell and Lightner, 1988), including dehydration through a graded ethanol series (70%, 80%, 95%, and 100%), clearing in xylene, infiltration with molten paraffin, and embedding in paraffin blocks. Tissue sections (5 µm thickness) were cut using a rotary microtome, mounted on glass slides, deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E). Stained sections were examined under a light microscope for pathological changes.

Statistical analysis

All data were presented as mean±standard deviation (SD). Statistical analyses were performed using IBM SPSS Statistics version 27 (IBM Corp., Armonk, NY, USA). Differences among treatment groups were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) post hoc test for multiple comparisons. Survival data from the bacterial challenge experiment were analyzed using the Kaplan-Meier survival analysis method, and differences among treatment groups were evaluated using the log-rank test. Statistical significance was set at p<0.05 for all analyses.

RESULTS

Experiment 1: In vitro antibacterial activity of chestnut wood polyphenols against Photobacterium damsela subsp. damsela

In vitro antibacterial susceptibility testing demonstrated that CWP exhibited potent antimicrobial activity against PDD. The MIC was determined to be $10 \mu\text{g}\cdot\text{mL}^{-1}$ (Figure 1), while the MBC was $20 \mu\text{g}\cdot\text{mL}^{-1}$. Although turbidity was observed at CWP concentrations from 10 to $1,280 \mu\text{g}\cdot\text{mL}^{-1}$, this was attributed to particulate residue from the CWP extract rather than bacterial growth. Growth observed in one replicate at $40 \mu\text{g}\cdot\text{mL}^{-1}$ was considered likely due to contamination and did not affect MIC determination.

Experiment 2: Effect of chestnut wood polyphenols on the growth performance, survival, immune responses, and Vibrio spp. counts in Pacific white shrimp

Growth performance and survival

The effects of dietary CWP supplementation on survival rate, final body weight, and FCR after 30 days of feeding are presented in Table 1. Survival

rates showed a dose-dependent increase with CWP supplementation. The highest survival rate ($92.00 \pm 4.00\%$) was observed in shrimp fed $5 \text{ g}\cdot\text{kg feed}^{-1}$ CWP, which was significantly higher ($p < 0.05$) than that in the control group ($80.00 \pm 4.00\%$). Survival rates in the $1 \text{ g}\cdot\text{kg feed}^{-1}$ ($84.00 \pm 4.00\%$) and $3 \text{ g}\cdot\text{kg feed}^{-1}$ ($86.67 \pm 4.62\%$) groups were intermediate and not significantly different from either the control or the highest CWP level.

Final body weights did not differ significantly among treatment groups, ranging from $9.74 \pm 0.29 \text{ g}$ to $10.26 \pm 0.65 \text{ g}$, indicating that CWP supplementation did not adversely affect growth. Feed conversion ratio (FCR) improved significantly with increasing CWP supplementation. Shrimp fed CWP at 1, 3, and $5 \text{ g}\cdot\text{kg feed}^{-1}$ exhibited significantly lower ($p < 0.05$) FCR values (1.14 ± 0.08 , 1.06 ± 0.06 , and 0.99 ± 0.04 , respectively) compared to those in the control group (1.37 ± 0.08), demonstrating enhanced feed utilization efficiency.

Total Vibrio spp. counts

Total *Vibrio* spp. counts in the hepatopancreas and intestine of shrimp after 30 days of feeding are shown in Table 2. In the hepatopancreas, *Vibrio* spp. counts ranged from 1.52 ± 0.09 to $1.70 \pm 0.08 (\times 10^4)$

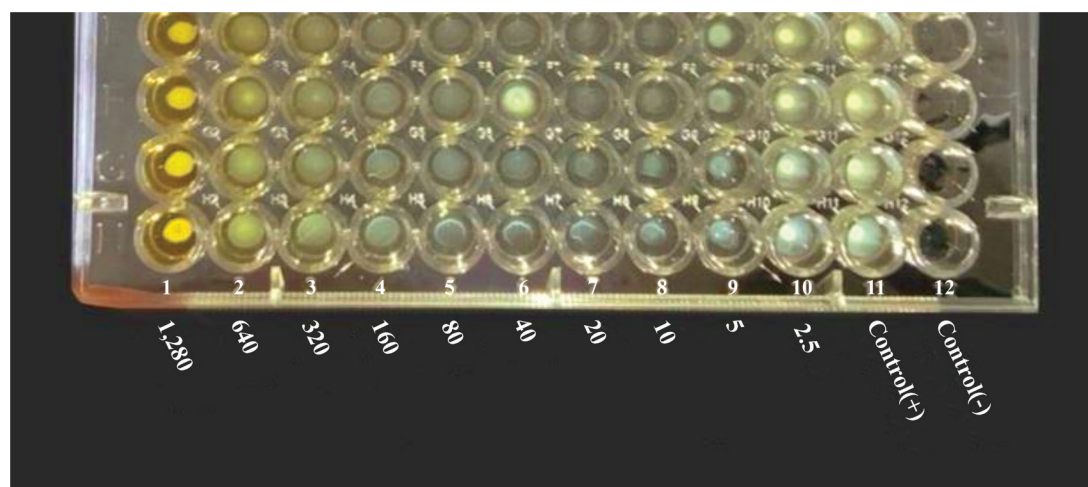


Figure 1. The minimum inhibitory concentration of chestnut wood polyphenols against *Photobacterium damsela* subsp. *damsela*. The MIC was determined as $10 \mu\text{g}\cdot\text{mL}^{-1}$; Turbidity at higher concentrations reflected CWP particulate residue rather than bacterial growth. Growth in one replicate at $40 \mu\text{g}\cdot\text{mL}^{-1}$ was considered likely contamination and did not affect MIC determination.

CFU·g⁻¹) and did not differ significantly among treatment groups. In contrast, intestinal *Vibrio* spp. counts were significantly reduced ($p<0.05$) in all CWP-supplemented groups compared to those in the control group. The control group exhibited intestinal *Vibrio* spp. counts of 2.53 ± 0.09 ($\times 10^3$ CFU·g⁻¹), while shrimp fed CWP at 1, 3, and 5 g·kg feed⁻¹ showed significantly lower counts of 1.90 ± 0.09 , 1.89 ± 0.07 , and 1.78 ± 0.08 ($\times 10^3$ CFU·g⁻¹), respectively, with no significant differences among the CWP-supplemented groups.

Immune response parameters

The effects of dietary CWP supplementation on innate immune parameters are presented in Table 3. THC ranged from 4.97 ± 0.50 to 5.87 ± 0.12 ($\times 10^6$ cells·mL⁻¹) and did not differ significantly among treatment groups. Similarly, phagocytic activity, which ranged from $62.67\pm5.70\%$ to $70.44\pm5.67\%$, showed no significant differences among treatments. PO activity also remained unaffected by dietary CWP, with values ranging from 31.24 ± 2.92 to 43.57 ± 10.12 U·min⁻¹·mg protein⁻¹ across all groups.

In contrast, SOD activity showed significant improvement ($p<0.05$) following by CWP supplementation in a dose-dependent manner. The control group exhibited the lowest SOD activity ($3.60\pm1.41\%$ inhibition), while shrimp fed CWP at 1, 3, and 5 g·kg feed⁻¹ showed progressively higher SOD activities of $12.39\pm1.41\%$, $15.09\pm5.12\%$, and $20.50\pm1.95\%$ inhibition, respectively. The highest CWP level (5 g·kg feed⁻¹) resulted in significantly greater SOD activity compared to all other groups.

Experiment 3: Effect of chestnut wood polyphenols on disease resistance against *Photobacterium damsela* subsp. *damsela* infection in Pacific white shrimp

The protective effects of dietary CWP

supplementation against experimental PDD infection are illustrated in Figure 2. Mortality commenced within 24 h post-challenge in all groups and continued throughout the 7-day observation period. By day 7 post-infection, cumulative survival rates varied significantly among treatment groups.

Table 1. Survival rate, body weight, and feed conversion ratio of Pacific white shrimp fed diets supplemented with chestnut wood polyphenols for 30 days.

Treatment	Survival rate (%)	Body weight (g)	FCR
Control	80.00 ± 4.00^b	10.08 ± 0.36^a	1.37 ± 0.08^b
CWP 1 g·kg feed ⁻¹	84.00 ± 4.00^{ab}	9.74 ± 0.29^a	1.14 ± 0.08^a
CWP 3 g·kg feed ⁻¹	86.67 ± 4.62^{ab}	10.17 ± 0.22^a	1.06 ± 0.06^a
CWP 5 g·kg feed ⁻¹	92.00 ± 4.00^a	10.26 ± 0.65^a	0.99 ± 0.04^a

Note: Data are presented as mean±standard deviation (n = 3); Values in the same column with different superscript letters are significantly different ($p<0.05$).

Table 2. Total *Vibrio* spp. counts in the hepatopancreas and intestine of Pacific white shrimp fed diets supplemented with chestnut wood polyphenols for 30 days.

Treatment	Hepatopancreas ($\times 10^4$ CFU·g ⁻¹)	Intestine ($\times 10^3$ CFU·g ⁻¹)
Control	1.63 ± 0.11^a	2.53 ± 0.09^b
CWP 1 g·kg feed ⁻¹	1.70 ± 0.08^a	1.90 ± 0.09^a
CWP 3 g·kg feed ⁻¹	1.58 ± 0.04^a	1.89 ± 0.07^a
CWP 5 g·kg feed ⁻¹	1.52 ± 0.09^a	1.78 ± 0.08^a

Note: Data are presented as mean±standard deviation (n = 3); Values in the same column with different superscript letters are significantly different ($p<0.05$).

The control group exhibited the lowest survival rate (43.33%), while shrimp fed CWP at 1, 3, and 5 g·kg feed⁻¹ showed survival rates of 46.67%, 56.67%, and 70.00%, respectively. The highest survival rate (70.00%) was observed in shrimp fed

5 g·kg feed⁻¹ CWP, which was significantly higher ($p = 0.043$) than that in the control group. Survival rates in the 1 and 3 g·kg feed⁻¹ groups showed intermediate values that did not differ significantly from those in the control group.

Table 3. Innate immune parameters including total hemocyte count (THC), phagocytic activity, phenoloxidase (PO) activity, and superoxide dismutase (SOD) activity of Pacific white shrimp fed diets supplemented with chestnut wood polyphenols for 30 days.

Treatment	THC ($\times 10^6$ cells·mL ⁻¹)	Phagocytic activity (%)	PO activity (U·min ⁻¹ ·mg protein ⁻¹)	SOD activity (% inhibition)
Control	5.77 $\pm$ 1.12 ^a	65.11 $\pm$ 2.34 ^a	43.57 $\pm$ 10.12 ^a	3.60 $\pm$ 1.41 ^c
CWP 1 g·kg feed ⁻¹	4.97 $\pm$ 0.50 ^a	70.44 $\pm$ 5.67 ^a	39.81 $\pm$ 4.46 ^a	12.39 $\pm$ 1.41 ^b
CWP 3 g·kg feed ⁻¹	5.70 $\pm$ 0.10 ^a	75.11 $\pm$ 8.06 ^a	36.52 $\pm$ 9.44 ^a	15.09 $\pm$ 5.12 ^{ab}
CWP 5 g·kg feed ⁻¹	5.87 $\pm$ 0.12 ^a	62.67 $\pm$ 5.70 ^a	31.24 $\pm$ 2.92 ^a	20.50 $\pm$ 1.95 ^a

Note: Data are presented as mean $\pm$ standard deviation ($n = 3$); Values in the same column with different superscript letters are significantly different ($p < 0.05$).

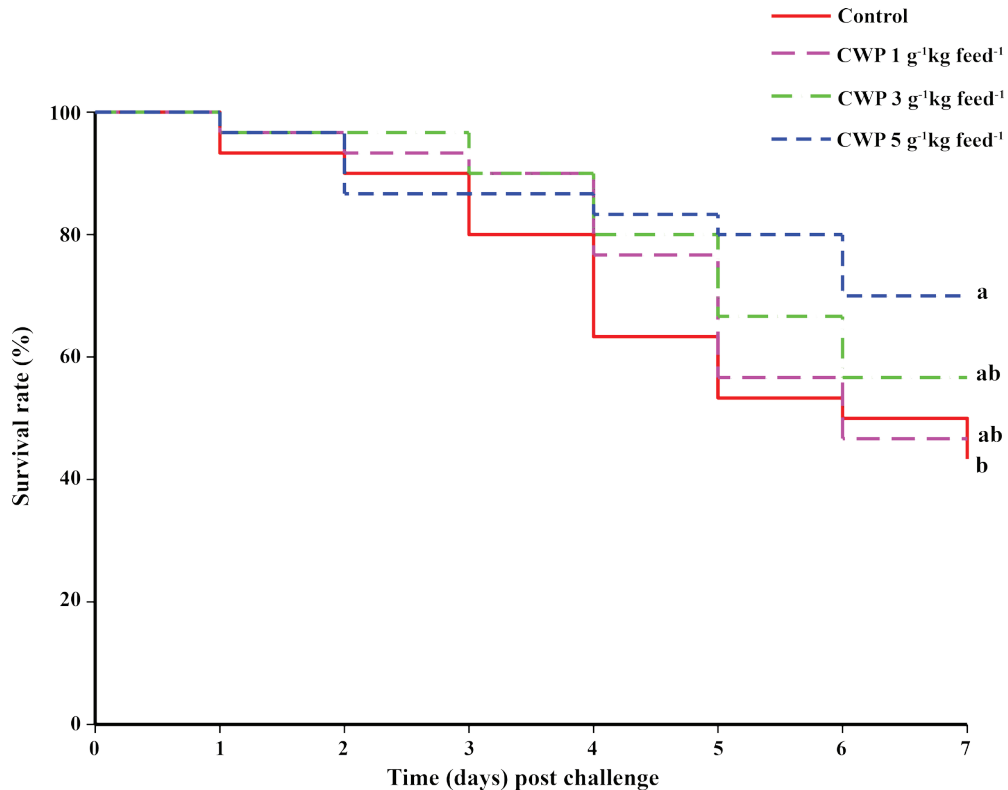


Figure 2. Cumulative survival rates of Pacific white shrimp following experimental challenge with *Photobacterium damselae* subsp. *damselae* (1×10^7 CFU·shrimp⁻¹) after 30 days of feeding diets supplemented with chestnut wood polyphenols (CWP). Different letters indicate significant differences among treatment groups at day 7 post-challenge.

Histopathology of shrimp after the challenge test

At day 7 post-challenge, histopathology demonstrated lesions consistent with PDD-associated pale shrimp disease, with changes observed in the hepatopancreas and muscle. Lesion severity generally followed the survival results, whereby the control group exhibited the most severe pathology, whereas shrimp fed CWP at 5 g·kg feed⁻¹ showed minimal effects. In the control group, hepatopancreatic tubules were severely affected, showing marked

atrophy with degenerative changes. Muscle exhibited the most extensive myopathic changes, with broad areas of muscle necrosis accompanied by hemocytic infiltration. In shrimp fed CWP at 1 and 3 g·kg feed⁻¹, hepatopancreatic and muscle lesions were still evident but were comparatively reduced, showing moderate tubule atrophy and localized muscle degeneration with moderate hemocytic infiltration. Shrimp fed CWP at 5 g·kg feed⁻¹ exhibited the least severe pathological changes. Hepatopancreatic tubules exhibited only minor atrophic changes, while muscle tissues remained unaffected (Figure 3).

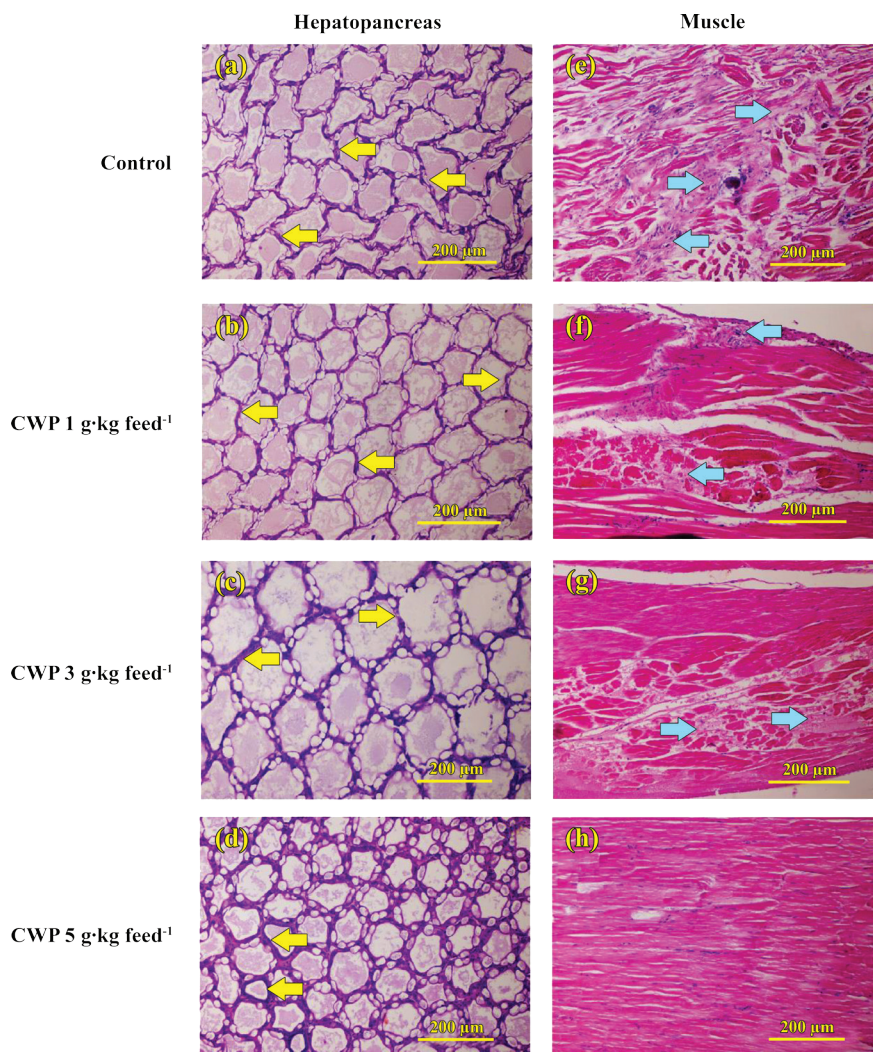


Figure 3. Histopathological alterations in the hepatopancreas (a–d) and muscle (e–h) of Pacific white shrimp challenged with *Photobacterium damsela* subsp. *damsela* and fed chestnut wood polyphenols (CWP) for 7 days. Hematoxylin and eosin (H&E) staining; scale bar = 200 µm. Yellow arrows indicate atrophic hepatopancreatic tubules; blue arrows indicate muscle necrosis and hemocytic infiltration.

DISCUSSION

This study evaluated the antibacterial properties, growth-promoting effects, immunomodulatory activities, and disease resistance enhancement of CWP in Pacific white shrimp. The findings demonstrate that dietary supplementation with CWP, particularly at 5 g·kg feed⁻¹, confers multiple beneficial effects, especially improved survival and FCR, reduced intestinal *Vibrio* spp. counts, and increased resistance against PDD infection.

The *in vitro* antibacterial assay revealed that CWP exhibited potent antimicrobial activity against PDD. These results are consistent with previous studies demonstrating the broad-spectrum antimicrobial properties of plant polyphenols, particularly tannins, against Gram-negative bacteria (Daglia, 2012; Borges *et al.*, 2013). The antibacterial mechanisms of polyphenolic compounds are multifaceted and include disruption of bacterial cell membrane integrity, inhibition of essential bacterial enzymes, chelation of metal ions required for bacterial metabolism, and interference with bacterial quorum sensing systems (Smeriglio *et al.*, 2016; Farha *et al.*, 2020). The relatively low MIC and MBC values observed in this study suggest that CWP could effectively inhibit and eliminate PDD at concentrations achievable through dietary supplementation, thereby contributing to disease prevention in aquaculture systems. Because the CWP used in this study was a crude extract, the specific active ingredients responsible for its antibacterial activity were not identified. Nevertheless, the observed effect against PDD is likely attributable to the synergistic action of multiple phenolic compounds present in the extract, rather than to a single compound.

The growth performance results demonstrated that dietary CWP supplementation significantly improved survival rates and FCR without adversely affecting body weight gain. The dose-dependent improvement in survival, with the highest rate (92%) observed at 5 g·kg feed⁻¹, suggests that CWP enhances shrimp health and stress tolerance. Similar improvements in survival and FCR have been reported for other polyphenol-rich

additives in shrimp. For instance, Wimanhaemin *et al.* (2025) observed that polyphenol-rich sugarcane extract improved body weight and survival in Pacific white shrimp, and Novriadi *et al.* (2023) reported that hydrolyzable tannins from sweet chestnut enhanced growth performance and FCR under commercial pond conditions. The significant reduction in FCR across all CWP-supplemented groups indicates improved nutrient utilization efficiency, which is economically advantageous for commercial shrimp production. Several mechanisms may contribute to these growth-promoting effects, including antimicrobial activity leading to reduced pathogen loads, modulation of gut microbiota composition toward beneficial bacterial communities, enhancement of digestive enzyme activities, and improvement of intestinal morphology and nutrient absorption capacity (Gong *et al.*, 2022; Huang *et al.*, 2024).

The significant reduction in intestinal *Vibrio* spp. counts in CWP-supplemented groups, without corresponding changes in hepatopancreatic counts, suggests that CWP primarily exerts its antimicrobial effects within the gastrointestinal tract. This reduction is particularly relevant for disease prevention, as the intestine serves as a primary entry point for pathogenic bacteria in shrimp. Similar findings have been reported by Vanichkul *et al.* (2010), who observed that turmeric extract reduced total intestinal bacterial and *Vibrio* spp. counts in Pacific white shrimp, and by Chen *et al.* (2023), who demonstrated that *Panax notoginseng* water extract reduced intestinal *Vibrio* spp. counts. The antimicrobial activity of CWP in the gut environment may result from direct bacteriostatic or bactericidal effects, as demonstrated in the *in vitro* assay, or from indirect effects through modulation of gut pH, enhancement of mucus production, and stimulation of beneficial bacterial populations that competitively exclude pathogens (Bolívar-Ramírez *et al.*, 2022).

Regarding the immunomodulatory effect of CWP, it was found that THC, phagocytic activity, and PO activity remained unchanged. Therefore, these findings suggest that CWP did not significantly affect these immune parameters. Nevertheless, SOD activity was significantly enhanced in a dose-dependent manner. SOD is a critical antioxidant

enzyme that catalyzes the dismutation of superoxide radicals into hydrogen peroxide and molecular oxygen, thereby protecting cells from oxidative damage (Holmblad and Söderhäll, 1999). The enhancement of SOD activity suggests that CWP supplementation strengthens the antioxidant defense system in shrimp, which is particularly important under culture conditions where animals are exposed to various environmental stressors. Similar enhancements in SOD activity have been reported for other polyphenol-rich additives. Yu *et al.* (2024) observed that fermented pomegranate peel polyphenols increased SOD, catalase, and glutathione peroxidase activities in Pacific white shrimp, while Hasanthi *et al.* (2024) demonstrated that micelle silymarin enhanced SOD activity. Enhanced antioxidant capacity has been associated with improved immune function, reduced susceptibility to diseases, and better stress tolerance in aquatic animals (Ashour *et al.*, 2024).

However, the absence of significant effects on THC, phagocytic activity, and PO activity in the present study is notable, as it contrasts with some previous reports of immunostimulation by plant polyphenols in crustaceans. Several studies have documented increases in these parameters following dietary supplementation with various plant extracts. Wimanhaemin *et al.* (2025) reported that polyphenol-rich sugarcane extract significantly improved THC, phagocytic activity, PO activity, and SOD activity in Pacific white shrimp. Similarly, Huang *et al.* (2024) demonstrated that hydrolyzable tannin at 4 g·kg feed⁻¹ increased THC, phagocytic activity, respiratory burst, and PO activity. Such discrepancies, nonetheless, may be attributed to several factors, including differences in polyphenol composition, dosage, duration of supplementation, shrimp size and physiological status, and experimental conditions. It is noteworthy that excessive stimulation of immune parameters is not always beneficial and may lead to immunological exhaustion or autoinflammatory responses (Song and Hsieh, 1994).

The bacterial challenge experiment demonstrated that dietary CWP supplementation significantly improved survival following experimental infection with PDD, with the highest

protection observed at 5 g·kg feed⁻¹ (70.00%) compared to 43.33% in the control group. This enhanced disease resistance might be attributed to multiple mechanisms, including the direct antimicrobial action of CWP, reduction of baseline opportunistic bacterial loads in the intestine, and enhancement of antioxidant defenses (Chaweepack *et al.*, 2015; Huang *et al.*, 2024). Similar improvements in disease resistance have been reported for other polyphenol-rich additives. Wimanhaemin *et al.* (2025) observed that shrimp fed polyphenol-rich sugarcane extract had significantly higher survival compared to the control following *Vibrio parahaemolyticus* challenge. Yu *et al.* (2024) reported that fermented pomegranate peel polyphenols significantly increased cumulative survival after *Vibrio alginolyticus* infection. Niyamosatha *et al.* (2015) demonstrated that grape-derived polyphenol additive enhanced survival under recurring *Vibrio parahaemolyticus* exposure. The dose-response relationship observed in disease resistance mirrors the trends in survival rate, FCR, and SOD activity, suggesting that these parameters are interconnected and collectively contribute to improved disease resistance. Furthermore, these results are consistent with the histopathological findings, in which the most pronounced changes were observed in the control group, while shrimp receiving 5 g·kg feed⁻¹ CWP displayed the mildest alterations.

From a practical aquaculture perspective, these results indicate CWP's potential as a functional feed additive for sustainable shrimp production. The supplementation level of 5 g·kg feed⁻¹ can be easily incorporated into commercial feed formulations. Because the CWP-supplemented feed was prepared by top-coating, some leaching of CWP from the pellet surface was unavoidable, making the actual dose lower than intended (Chuchird *et al.*, 2026). Nonetheless, the nominal dose of 5 g·kg feed⁻¹ still benefited shrimp, confirming its effectiveness despite the leaching issue. Furthermore, long-term feeding trials are needed to evaluate the effects of CWP supplementation over complete production cycles and to assess potential cumulative benefits or tolerance development. Detailed microbiome analysis would provide insights into the specific changes in gut bacterial community composition

and diversity induced by CWP supplementation. Transcriptomic and proteomic studies could elucidate the molecular mechanisms underlying the disease resistance-enhancing effects of CWP. Additionally, evaluation of CWP efficacy against other important shrimp pathogens, including viruses and other bacterial species, would broaden the understanding of its protective spectrum. Finally, economic analysis incorporating feed costs, survival improvements, and reduced medication expenses would facilitate decision-making for commercial implementation.

CONCLUSIONS

CWP exhibited strong in vitro antibacterial activity against PDD and improved the performance and health status of Pacific white shrimp when used as a dietary supplement. The CWP at 5 g/kg feed⁻¹ level provided the most consistent benefits, including improved survival and FCR, reduced intestinal *Vibrio* spp., and increased resistance to PDD challenge, but did not significantly affect body weight, immune responses, or hepatopancreatic *Vibrio* spp. counts. Overall, CWP is a promising natural functional feed additive for supporting sustainable health management in shrimp aquaculture. Further validation under long-term and farm-scale conditions is warranted.

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