
**E-ISSN: 2347-5129**
**P-ISSN: 2394-0506**
(ICV-Poland) Impact Value: 76.37
(GIF) Impact Factor: 0.549
IJFAS 2025; 13(4): 01-09

www.fisheriesjournal.com
Received: 03-05-2025
Accepted: 05-06-2025

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# Antioxidant status and Immune parameters in selected tissues and hemolymph of Pacific white shrimp *LitoPenaeus vannamei* during different feeding Trails

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**DOI:** https://www.doi.org/10.22271/fish.2025.v13.i4a.3108

**Abstract**
The present investigation evaluated the immunological and antioxidant responses of *LitoPenaeus vannamei* subjected to dietary supplementation with Probiotics (PB), Bioflocs (BF), and their combination (PB+BF) during shrimp culture operations. The study assessed key hemolymph immune parameters including Total Haemocyte Count (THC), Hyalinocytes (HC), Semi-granulocytes (SGC), and Granulocytes (GC) at different culture stages (0, 40, and 80 Days of Culture-DOC), alongside antioxidant enzyme activities such as Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPx), and Glutathione S-transferase (GST) in hepatopancreas, gill, and haemolymph tissues.
Results indicated a significant enhancement of THC and its sub-populations in shrimp fed with PB, BF, and particularly PB+BF, compared to the control. Phenoloxidase (PO) activity also increased significantly, suggesting an elevated immune readiness and microbial clearance potential. Concurrently, elevated antioxidant enzyme activities across tissues were observed, particularly in PB and BF-fed groups, indicating a strong response to reactive oxygen species (ROS) and oxidative stress, likely induced by microbial presence and hypoxic conditions. Increased malondialdehyde (MDA) levels, especially in hepatopancreas and haemolymph, confirmed oxidative stress occurrence, while enhanced antioxidant responses suggested efficient cellular protection and recovery.
These findings confirm that dietary supplementation with Probiotics and Bioflocs enhances immune competence, antioxidant capacity, and bacterial clearance in *L. vannamei*, ultimately contributing to improved growth performance, feed utilization, and disease resistance. The synergistic application of PB+BF offers a promising approach for sustainable and resilient shrimp aquaculture practices.

**Keywords:** Probiotics, bioflocs, *LitoPenaeus vannamei*, antioxidant status

## 1. Introduction
Aquatic organisms, like all aerobic life forms, rely on oxygen for metabolism but are concurrently exposed to reactive oxygen species (ROS), which are inevitable by-products of aerobic respiration. In crustaceans such as shrimp, ROS-comprising superoxide anions ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals (•OH)-can damage cellular components, including lipids, proteins, and DNA, leading to oxidative stress. To mitigate these harmful effects, shrimp have evolved intricate antioxidant defense systems that include both enzymatic (e.g., superoxide dismutase [SOD], catalase [CAT], glutathione peroxidase [GPx]) and non-enzymatic components (e.g., glutathione, ascorbic acid, vitamin E).
The functionality of these antioxidant systems is known to be influenced by several intrinsic factors, such as the shrimp's phylogenetic lineage, age, sex, and physiological status. Additionally, extrinsic and environmental factors play a significant role in modulating oxidative stress responses. Key contributors include fluctuations in water quality parameters (e.g., temperature, pH, dissolved oxygen, ammonia), seasonal variations, feed composition, stocking density, the presence of phytoplankton and zooplankton, pathogenic microbial loads, and sediment quality [1, 2, 3]. When the balance between ROS production and antioxidant defense is disrupted, shrimp become susceptible to oxidative damage, which can impair growth, immunity, and survival-factors critical to successful aquaculture operations.
In recent years, there has been increasing interest in using functional feeds, particularly those supplemented with probiotics, bioflocs, and synbiotics, to enhance the health and resilience of

*LitoPenaeus vannamei* [4-5]. Probiotics, such as species of *Bacillus* and *Lactobacillus*, are known to improve gut microbiota balance, nutrient assimilation, and immune stimulation [6, 7, 8]. Biofloc technology (BFT), a microbial-based culture approach, contributes additional nutrients (e.g., microbial protein, vitamins, and bioactive compounds), which can bolster physiological functions including antioxidant responses[9-11]. Despite the growing adoption of such strategies, most existing studies have focused on toxicological or pathogenic stressors as triggers of oxidative stress. However, there remains a knowledge gap regarding the modulatory effect of standard culture practices and feed interventions on the baseline antioxidant status of shrimp under non-toxic, production-level conditions.

Therefore, the present study was designed to comprehensively evaluate the effects of probiotics, bioflocs, and their combination (synbiotic approach) on the antioxidant enzyme system and immune parameters of *L. vannamei* during a 80-day feeding trial. By monitoring key antioxidant enzymes (such as SOD, CAT, and GPx) alongside immunological markers, this investigation aims to unravel how functional feeds interact with shrimp physiology to improve oxidative stability and overall health performance under controlled aquaculture conditions.

## 2. Materials and Methods

### 2.1 Experimental Site and Animal Husbandry

The study was conducted at shrimp culture facilities located in Allur, Nellore District, Andhra Pradesh, India (Latitude: 15.00120° N; Longitude: 80.05200° E). Shrimp *LitoPenaeus vannamei* (Pacific white shrimp) were procured from local aquaculture farms and transported to the experimental site in oxygenated, double-layered polythene bags to reduce transportation-induced stress. Shrimp of uniform size were selected following thorough disease screening to ensure a pathogen-free stock. The selected individuals were acclimatized for one week under field conditions in tanks containing seawater adjusted to the desired salinity levels. During the acclimation period, the shrimp were fed twice daily with a commercial feed containing 35% crude protein.

### 2.2 Experimental Setup

The feeding trial was conducted in cement tanks measuring 5 × 10 meters, with earthen bottoms embedded in the submerged earth crust to mimic natural pond conditions. Each tank was maintained at a water depth of 1 meter, holding approximately 3,000 liters of seawater. Water with a salinity of 10 ± 0.5 ppt was sourced from the Buckingham Canal, pre-treated in a sedimentation pond to remove suspended solids, and subsequently distributed to the experimental tanks. To ensure adequate dissolved oxygen levels, continuous aeration was provided using electric blowers and air compressors. Tanks were covered with black netlon plastic sheets to modulate photoperiod and temperature, maintaining a natural 12 h light: 12 h dark cycle. Partial water exchange was carried out biweekly, and chlorinated seawater was periodically added to compensate for losses due to evaporation.

### 2.3 Experimental Design

The study was conducted over an 80-day period at a controlled outdoor aquaculture research facility. A total of 12 rectangular earthen tanks (3,000 L capacity each) were utilized and arranged in a completely randomized design (CRD**)** comprising four treatment groups with three replicates each

- **$T_1$ (Control):** No additives; standard water exchange and traditional management practices applied.
- **$T_2$ (Probiotics only):** Daily supplementation with a commercially available probiotic formulation.
- **$T_3$ (Biofloc only):** Biofloc development was initiated through the addition of an external carbon source (e.g., molasses) to maintain a C: N ratio conducive to microbial floc formation.
- **$T_4$ (Probiotics + Biofloc):** Combined application of probiotics and biofloc-inducing carbon sources to evaluate synergistic effects.

All tanks were continuously aerated using air stones connected to a central air blower system to maintain optimal dissolved oxygen levels (>5 mg/L) and ensure uniform suspension of floc particles. Water quality was monitored regularly, and parameters such as temperature, salinity, pH, and total ammonia nitrogen were maintained within optimal ranges for *LitoPenaeus vannamei* culture.

### 2.4 Preparation of probiotic-enriched feed

Probiotic-supplemented feed was prepared following the protocols outlined by Naresh [12] and Aparna [13], with minor modifications. Pure cultures of *Lactobacillus rhamnosus* and *Bacillus licheniformis* were propagated in nutrient broth under aerobic conditions at 37°C for 24 hours. Following incubation, bacterial cells were harvested by centrifugation at 10,000 rpm for 10 minutes at 4°C. The resulting cell pellets were washed twice with sterile phosphate-buffered saline (PBS; pH 7.4) and re-suspended in the same buffer to maintain cell viability.

The bacterial suspension was uniformly sprayed onto air-dried basal feed pellets using a fine mist sprayer to ensure homogenous distribution. The coated pellets were dried in a hot air oven at 40 °C for 6 hours to reduce moisture and preserve probiotic integrity. Once dried, the probiotic-enriched feed was packed in airtight polythene bags and stored under refrigerated conditions (4°C) until use. The final concentration of viable probiotics in the feed was standardized to $1 \times 10^{10}$ CFU/kg for both *B. licheniformis* and *L. rhamnosus*. Fresh probiotic-coated feed was prepared weekly to ensure microbial potency and stability throughout the feeding trial.

The basal diet was formulated to meet the nutritional requirements of *LitoPenaeus vannamei*, with an approximate composition of 35% protein, 7% lipid, 15% carbohydrate, and 10% fiber. Key ingredients included fishmeal, soybean meal, wheat flour, and a vitamin-mineral premix, providing a nutritionally balanced formulation for optimal shrimp growth [13].

### 2.5 Biofloc Induction

Biofloc development was initiated in the treatment groups $T_3$ (Biofloc only) and $T_4$ (Probiotics + Biofloc) by manipulating the carbon-to-nitrogen (C:N) ratio of the culture water to 15:1. This was achieved by the daily addition of molasses as an exogenous carbon source. The required amount of molasses was calculated based on the nitrogen input from the formulated feed, considering the crude protein content and feed conversion ratio (FCR), following the methodology described by Avnimelech [14].

Carbon supplementation commenced from the first day of culture and was administered consistently after daily water

quality monitoring, ensuring optimal conditions for heterotrophic bacterial proliferation. Water exchange was strictly avoided to maintain the microbial flock structure and promote the natural recycling of nutrients within the system. Siphoning was restricted to the removal of excess sedimented solids to prevent the accumulation of sludge while minimizing disturbance to the established biofloc community.

**Statistical Analysis**

The below mentioned parameters were also estimated by following the respective method in selected tissues and hemolymph.

| Parameter | Method Adopted |
|---|---|
| Protein | Lowry *et al.* [15] |
| Haemocytes | Neubauer Hemocytometer |
| Superoxide desmutase | Kakkar *et al.* [16] |
| Catalase | Takahara *et al.* [17] |
| Glutathione peroxidase | Hafeman *et al.* [18] |
| Prophenol oxidase | Gollas-Galvan *et al.* [19] |
| Reduced glutathione | Tappel [20] |
| Glutathione s-transferase | Habig *et al.* [21] |
| Lipid peroxidation | Ohkawa *et al.* [22] |
| Melondialdehyde | Ohkawa *et al.* [22] |

Data were analyzed using one-way ANOVA with post-hoc Turkey HSD test ($p<0.05$). Software: SPSS v26.

## 3. Results and Discussion

### 3.1 Antioxidant Enzymes

The antioxidant enzyme activity profiles in *LitoPenaeus vannamei* across three major tissues (hepatopancreas, gill, and haemolymph) varied significantly among treatments-Control, Probiotics (PB), Bioflocs (BF), and Probiotics + Bioflocs (PB+BF). The synergistic treatment (PB+BF) consistently exhibited the highest enzyme activity levels, indicating enhanced antioxidative defense (Table 1).

- **Superoxide Dismutase (SOD):** The highest SOD activity was recorded in the hepatopancreas of PB+BF (73.42), followed by BF (63.05) and PB (52.42), with a 35-89% increase over control. Gill and haemolymph tissues followed similar trends, with PB+BF showing the most significant enhancement (+64% and +109%, respectively).
- **Prophenol Oxidase (ProPO):** Enzyme activity significantly increased in all tissues, with the highest values observed in PB+BF (1.71, 1.41, and 2.13 in hepatopancreas, gill, and haemolymph respectively), marking 39-54% elevation over the control.
- **Catalase (CAT):** PB+BF treatment resulted in the highest CAT activity, particularly in hepatopancreas (21.08, +103%) and haemolymph (18.05, +95%). Increases in gill tissues were also substantial (+94%).
- **Glutathione Peroxidase (GPx):** The enzyme showed a substantial increase, particularly in hepatopancreas (+113%) and haemolymph (+92%) under PB+BF, reflecting enhanced oxidative stress defense. **Glutathione S-transferase (GST):** GST activity increased prominently in all tissues, especially in gill (+97%) and hepatopancreas (+94%) with PB+BF.
- **Reduced Glutathione (GSH):** Hepatopancreas GSH levels rose remarkably with PB+BF (88.31; +119%). Conversely, haemolymph showed a decline in GSH levels under treatments, notably in PB+BF (49.88; -54%).
- **Lipid Peroxidation (LPO):** A steady increase in LPO levels was noted across tissues, with the PB+BF group showing the highest values (13.78, 4.91, and 4.31 in hepatopancreas, gill, and haemolymph respectively), suggesting increased oxidative substrate processing.
- **Malondialdehyde (MDA):** MDA levels, an indicator of lipid peroxidation, increased with treatments, especially in hepatopancreas (3.73, +201%) and gill (2.01; +105%) in the PB+BF group.

### 3.2 Haemolymph Immune Parameters

In the present study, haemolymph immune parameters such as Total Haemocyte Count (THC), Hyalinocytes (HC), Semi-granulocytes (SGC), and Granulocytes (GC) were analyzed at 0, 40, and 80 Days of Culture (DOC) and are presented in Table.2. THC typically comprises heterogeneous haemocyte populations, with HC forming the major component (approximately 65-70%), followed by SGC (15-20%) and GC (10-15%).

A significant enhancement ($p<0.05$) in all immune parameters was observed in the experimental groups (Probiotic-fed [PB], Biofloc-fed [BF], and Probiotic + Biofloc-fed [PB+BF]) compared to the control, particularly at 40 and 80 DOC. The THC at 0 DOC was 1823 × $10^4$ cells/ml. In the control group, THC marginally increased to 1940 and 1958 × $10^4$ cells/ml at 40 and 80 DOC, respectively, but this change was statistically non-significant (NS). In contrast, a significant increase in THC was observed in the experimental groups. The PB group showed values of 2674 and 2778 × $10^4$ cells/ml (+41% and +52%), while the BF group recorded 3038 and 3542 × $10^4$ cells/ml (+67% and +94%). The PB+BF group exhibited the highest THC values of 3645 and 4349 × $10^4$ cells/ml at 40 and 80 DOC, with respective increases of +100% and +139%, indicating strong synergistic immune stimulation.

Hyalinocytes (HC), representing 65-77% of the total haemocyte population, ranged from 1185 to 3348 × $10^4$ cells/ml. In the control group, HC counts were 1185, 1319, and 1312 × $10^4$ cells/ml at 0, 40, and 80 DOC, respectively, showing a negligible and NS increase. However, the PB group exhibited HC counts of 1872 and 1890 × $10^4$ cells/ml at 40 and 80 DOC (+58% and +59%). The BF group showed 2308 and 2692 × $10^4$ cells/ml (+95% and +127%), while the PB+BF group demonstrated the highest HC levels at 2697 and 3348 × $10^4$ cells/ml (+128% and +182%).

Semi-granulocytes (SGC) accounted for 15-26% of the THC population, with values ranging from 438 to 658 × $10^4$ cells/ml. In the control group, SGC counts increased modestly from 438 (0 DOC) to 504 and 510 × $10^4$ cells/ml at 40 and 80 DOC, respectively, but the changes were NS. The PB group exhibited values of 539 and 582 × $10^4$ cells/ml (+23% and +33%), and the BF group recorded 556 and 567 × $10^4$ cells/ml (+4% and +30%). The PB+BF group again showed the highest SGC values: 620 and 653 × $10^4$ cells/ml at 40 and 80 DOC, representing significant increases of +42% and +49%.

Granulocytes (GC), which form 8-13% of the total haemocyte population, ranged between 206 and 348 × $10^4$ cells/ml. The control group showed a gradual increase from 206 at 0 DOC to 233 and 253 × $10^4$ cells/ml at 40 and 80 DOC (+13%, +23%, and +30%). In the PB group, GC values reached 268 and 306 × $10^4$ cells/ml (+30% and +49%). The BF group showed 243 and 284 × $10^4$ cells/ml (+18% and +38%). Notably, the PB+BF group recorded the highest GC levels of 328 and 348 × $10^4$ cells/ml, with increments of +59% and +69% at 40 and 80 DOC, respectively.

Overall, the study indicates that the administration of probiotics, bioflocs, and their combination significantly

enhanced haemolymph immune parameters in *LitoPenaeus vannamei*. The synergistic use of PB+BF exhibited the most pronounced immunostimulatory effects, especially by 80 DOC, suggesting their promising potential in boosting shrimp immune defense under aquaculture conditions.

## 4. Discussion

The current study highlights the significant enhancement of antioxidant defense mechanisms in *LitoPenaeus vannamei* fed with probiotics (PB), bioflocs (BF), and their synergistic combination (PB+BF). The most pronounced effects were observed in the PB+BF group across all examined tissues, suggesting a robust systemic response to oxidative stress and improved physiological resilience.

### 4.1 Antioxidant Enzymes and Oxidative Balance

Oxidative stress arises when reactive oxygen species (ROS) production exceeds the neutralizing capacity of antioxidant systems. In aquaculture, factors such as high stocking density, variable water quality, and pathogen exposure often exacerbate oxidative stress in shrimp. To counter this, organisms activate a complex antioxidant network, comprising both enzymatic and non-enzymatic components. The marked increase in superoxide dismutase (SOD) activity in hepatopancreas, gill, and haemolymph in PB+BF-fed shrimp reflects the first line of defense against superoxide radicals. This enzyme catalyzes the dis-mutation of superoxide into hydrogen peroxide, which is subsequently detoxified by catalase (CAT) and glutathione peroxidase (GPx). The elevated SOD activity seen here is in agreement with findings by Zokaeifar *et al*. [23], where synbiotic supplementation led to enhanced oxidative resilience in *L. vannamei*. The simultaneous up-regulation of CAT and GPx under PB+BF feeding further confirms the synergistic effect of these treatments in mitigating oxidative burden. CAT plays a critical role in rapidly converting hydrogen peroxide into water and oxygen, while GPx, utilizing reduced glutathione (GSH) as a cofactor, also detoxifies hydrogen peroxide and lipid hydroperoxides. The increase in GPx suggests a well-activated glutathione redox cycle, particularly in hepatopancreas and haemolymph, essential sites for detoxification and immune modulation.

### 4.2 Prophenoloxidase (ProPO) Activation and Immune Cross-Talk

The prophenoloxidase system is not only a marker of oxidative stress but also a crucial component of the shrimp innate immune defense. Its activation contributes to melanization, encapsulation of pathogens, and production of ROS to kill invading microbes. The up-regulation of ProPO in the PB+BF group suggests that the synbiotic supplementation enhanced pathogen recognition and immune signalling. This aligns with earlier studies by Rengpipat *et al*. [24] and Li *et al*. [25], where probiotics elevated immune enzyme activities, including ProPO, thereby improving resistance to Vibrio infections.

### 4.3 Glutathione S-Transferase (GST) and Reduced Glutathione (GSH)

GST facilitates the detoxification of xenobiotics and peroxidized lipids through conjugation with GSH, providing cellular protection against electrophilic compounds. The significantly higher GST activity in the PB+BF-fed shrimp supports enhanced biotransformation and stress adaptation. Interestingly, the elevated GST was matched by increased GSH levels in hepatopancreas and gill, indicating efficient redox cycling. However, GSH levels in the haemolymph declined, particularly in the PB+BF group, likely due to its rapid utilization under high ROS turnover-an effect similarly observed by Liu *et al*. [26] in stress-challenged crustaceans. The mobilization and consumption of haemolymph GSH might also reflect a shift in resource allocation toward hepatopancreas and gill, where ROS production and detoxification demands are higher due to metabolic activity and direct exposure to environmental stimuli.

### 4.4 Lipid Peroxidation and MDA

Contrary to expectations, levels of lipid peroxidation (LPO) and malondialdehyde (MDA) increased significantly in all tissues, especially under the PB+BF regime. While this might initially suggest cellular damage, it is essential to interpret this within the context of elevated metabolism and immune activation. The increase in MDA and LPO may partly reflect controlled oxidative signaling rather than pathological damage. Moderate ROS levels are known to act as signaling molecules that modulate gene expression, immune responses, and cellular adaptation. The concurrent rise in antioxidant enzymes indicates that the shrimp's defense systems were activated sufficiently to cope with and balance the ROS load, thereby preventing excessive oxidative damage. A similar phenomenon was observed by Moustafa *et al*. [27], who reported increased MDA levels alongside improved antioxidant enzyme profiles in synbiotic-treated shrimp, suggesting a hormetic adaptation to mild oxidative challenges. The prevention and control of diseases are of paramount importance for the sustainable development and stability of the shrimp aquaculture industry. Shrimp immunology has played a pivotal role in formulating strategies aimed at disease regulation, prevention, and control through the modulation of immune responses. The shrimp's resistance to microbial invasion is highly dependent on the integrity and functionality of its immune system. Notably, the immune system in crustaceans is comparatively less developed than in finfish and other vertebrates [28]. Shrimp farming is frequently affected by environmental degradation and outbreaks of infectious diseases, primarily caused by viruses and bacteria. To safeguard shrimp health, it is essential to develop robust strategies for evaluating and monitoring the immune status of cultured shrimp. Internal homeostasis is maintained through two primary systems: the innate immune system and the environmental stress response system, both of which act synergistically to protect cells from biotic and abiotic stressors. During pathogenic infections, the immune system engages microbial clearance and cellular stress-alleviation pathways-processes that must be tightly regulated to avoid pathological consequences [29].

### 4.5 The immune response in crustaceans is orchestrated in three distinct phases [30, 31]

- Recognition phase, involving detection of non-self molecules.
- Effector synthesis phase, where immune-related effectors are produced.
- Humoral and cellular response phase, responsible for organismal recovery.

In decapod crustaceans, the initial defense is provided by physical barriers [32]. Once these barriers are breached,

microbial invasion activates proteolytic pathways, leading to immediate immune reactions [33, 34]. The core effector mechanisms include the coagulation cascade, oxidative burst, and melanization through the pro phenoloxidase (ProPO) system [35, 37]. ProPO activation is critical as it triggers downstream immune functions such as phagocytosis, encapsulation, and nodule formation.

**Table 1:** Defense mechanisms in crustaceans

| Defense Mechanism | Haemocytes Involved | Target |
|---|---|---|
| ProPO System | Semi-granular cells, Granular cells | Bacteria and fungi |
| Antimicrobial Proteins | Granular cells | Bacteria and fungi |
| Phagocytosis | Hyaline cells, Semi-granular cells | Bacteria and microorganisms <10 µm |
| Encapsulation | Semi-granular cells, Granular cells | Fungal spores, yeast, organisms >10 µm |
| Lectins | Hyaline, Granular, and Semi-granular cells | Recognition of non-self; agglutination, phagocytosis |
| Clottable Proteins | Released from haemocytes | Bacteria and fungi |

A close link exists between environmental stress and oxidative stress, as reactive oxygen species (ROS) production is heavily influenced by environmental and physiological factors [38]. Several studies have demonstrated that antioxidant activity and lipid peroxidation levels are effective indicators of oxidative stress in aquatic organisms [39, 40, 41]. The activity of antioxidant enzymes may be modulated by various conditions including hypoxia [42], temperature fluctuations [43], and endogenous factors such as age, diet, sex, seasonal variation, and reproductive stages [44].

Antioxidant enzyme dynamics during embryogenesis and larval development have been well documented in several crustacean species, including *Macrobrachium malcolmsonii*, *M. rosenbergii* [45, 46], and *Palaemonetes pugio* [47]. These enzymes play a vital protective role against oxidative stress during early development and metamorphosis. Dietary supplementation with antioxidants such as vitamins C and E and astaxanthin has been reported to boost antioxidant capacity and resistance to ammonia stress in *L. vannamei* and *P. monodon* [48, 49]. Moreover, the cDNA sequences of critical antioxidant enzymes such as catalase (CAT) and glutathione peroxidase (GPx) have been elucidated in *L. vannamei* [50, 51].

In crustaceans, another multifunctional protein, peroxinectin, with peroxidase activity, plays a key role in preventing oxidative damage caused by hydrogen peroxide ($H_2O_2$) [52, 53]. During pathogenic challenges, ROS generation often exceeds the capacity of the antioxidant defense system, leading to pathological oxidative stress. This overproduction of ROS is a hallmark response to microbial invasion [54, 55]. Among the tissues examined in this study, the hepatopancreas and gill tissues showed greater responsiveness in terms of antioxidant activity compared to haemolymph. The hepatopancreas serves as a central metabolic and detoxification organ in crustaceans, playing a critical role in managing environmental insults such as toxins, heavy metals, pollutants, and pathogenic invasions. Crustaceans possess an open circulatory system, wherein the blue-green haemolymph circulates through a hemocoel and bathes various tissues directly. This haemolymph carries haemocytes along with humoral immune components, facilitating interaction with foreign particles and pathogens [50]. Within the innate immune system, haematopoiesis serves as the primary source of mature effector cells. In crustaceans, haematopoietic tissues form a dense network of lobules primarily located along the dorsal and dorsolateral regions of the stomach, as well as near the base of the maxillipeds and adjacent to the antennal artery. Specifically, in penaeid shrimp, haematopoietic tissue is found in the stomach, maxillipedal region, and antennal glands.

Analogous to vertebrate immune cells such as macrophages, granulocytes, and natural killer cells, shrimp haemocytes perform diverse immune functions including phagocytosis, encapsulation, nodule formation, wound repair, and activation of the prophenoloxidase (ProPO) system. They also contribute to the production of antimicrobial peptides, adhesion molecules, and agglutinins [56, 57, 58, 59]. In many crustaceans, particularly shrimp, the outer wall of haemolymph vessels located in the digestive gland or hepatopancreas plays a significant role in the uptake and processing of foreign particles, resembling the function of lymphoid organs (Oka, 1969; Martin *et al*., 1987; Hose *et al*., 1992). While some researchers have suggested a haematopoietic role for this tissue, the limited presence of mitotic figures does not support this hypothesis. Moreover, phagocytes resident in the hepatopancreas are often destroyed under pathological conditions [63, 64, 65]. Viral and bacterial particles have been frequently detected in the lymphoid organs of penaeid shrimp, indicating that these organs are actively involved in removing foreign materials from the haemolymph. The sudden expansion of the haemal lumen in these organs promotes extensive contact between haemolymph and the epithelial cells of hepatopancreatic tubules, enabling efficient entrapment and immobilization of invasive materials [59].

Haemocytes in penaeid shrimp are generally categorized into three main types: Hyaline cells, Semi-granular cells and Granular cells [66]. Among invertebrates, including crustaceans, phagocytic blood cells serve as the primary line of defense against microbial infections. In shrimp, hyaline and semi-granular haemocytes are recognized as the main phagocytic cells. Phagocytosis in crustaceans is a multiphase process, involving chemotaxis, attachment, ingestion, and pathogen elimination. In the present study, the application of probiotics and bioflocs in *LitoPenaeus vannamei* culture significantly enhanced the Total Haemocyte Count (THC) along with subpopulations of hyalinocytes (HC), semi-granulocytes (SGC), and granulocytes (GC). This immuno-stimulatory response highlights the immune-enhancing potential of probiotics and bioflocs used in the experimental feeding trials. An increase in THC has previously been reported in freshwater prawn *Macrobrachium rosenbergii* [67, 68] and *M. malcolmsonii* [69]. Similarly, an increase in HC was observed in *Penaeus latisulcatus* when supplemented with probiotic strains like *Pseudomonas* [70]. Therefore, THC is considered a reliable indicator of health status, serving as a crucial non-specific immune parameter in shrimp and other crustaceans.

### 4.6 Phenoloxidase (PO) and the ProPO Cascade

Phenoloxidase (PO) plays a central role in melanisation, a key immune response in crustaceans to pathogen invasion. It is activated through a cascade mechanism from its precursor,

prophenoloxidase (ProPO), which is stored in granular haemocytes. This cascade leads to the oxidation of phenolic compounds such as tyrosine and DOPA, ultimately resulting in melanin formation, which encapsulates and neutralizes pathogens. In the present study, PO activity was significantly elevated across all probiotic and biofloc-fed groups, indicating enhanced immune responsiveness. This finding aligns with earlier reports in *P. monodon* [24], *P. vannamei* [71] and *L. vannamei* [72]. PO activity, as a terminal enzyme in the ProPO system, functions in both pathogen recognition and immune effector response by promoting cell-cell communication, phagocytosis, nodule formation, non-self recognition, and pathogen elimination.

## 5. Conclusion

The present study underscores the pivotal role of antioxidant defense mechanisms in mitigating oxidative stress in *LitoPenaeus vannamei* under environmental and immunological challenges. The enhanced activities of key antioxidant enzymes-superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione S-transferase (GST)-in shrimp tissues such as hepatopancreas and gill, reflect a well-coordinated response to elevated reactive oxygen species (ROS) levels induced by environmental stressors, immune challenges, and potential hypoxic conditions. The observed elevation of malondialdehyde (MDA) in hepatopancreas and haemolymph highlights the extent of lipid peroxidation and reinforces the critical need for efficient antioxidant regulation to maintain cellular homeostasis. The study demonstrates that dietary supplementation with Probiotics and Bioflocs, both individually and synergistically, enhances the shrimp's antioxidant enzyme activities, promoting the scavenging of ROS, reducing oxidative damage, and restoring physiological and biochemical balance. This, in turn, contributes to improved feed utilization, immune status, and overall growth performance. Furthermore, the findings affirm that tissue-specific functions and metabolic rates significantly influence oxidative stress susceptibility and antioxidant responses. The hepatopancreas and gill, being metabolically active and functionally critical, are especially vulnerable to oxidative damage and show pronounced antioxidant activity as an adaptive mechanism. The combined application of Probiotics and Bioflocs thus presents a promising strategy to enhance oxidative stress tolerance and health resilience in shrimp aquaculture. In conclusion, strategic dietary interventions using functional feeds enriched with Probiotics and Bioflocs can significantly strengthen the antioxidative and immune defense systems in *L. vannamei*, supporting sustainable and high-performance aquaculture practices.

**Table 2:** Antioxident enzymes in selected tissues of *L. vannamei* during different feeding trails (60 DOC)

| Parameter | Tissue | Control | PB | BF | PB+BF |
|---|---|---|---|---|---|
| Superoxide[a] dismutase | Hepatopancreas | 35.75[a]±1.22 PDC | 52.42[b]±1.34+35 | 63.05[c]±1.67+63 | 73.42[d]±1.89+89 |
| | Gill | 45.74[a]±1.13 PDC | 56.38[b]±1.18+24 | 62.77[c]±1.12+37 | 73.84[d]±1.22+64 |
| | Haemolymph | 13.75[a]±0.78 PDC | 18.41[b]±0.88+34 | 22.94[c]±0.94+67 | 28.79[d]±1.02+109 |
| Prophenol[b] oxidase | Hepatopancreas | 1.23[a]±0.23 PDC | 1.54[b]±0.27+25 | 1.63[c]±0.23+33 | 1.71[d]±0.23+39 |
| | Gill | 0.99[a]±0.09 PDC | 1.22[b]±0.11+23 | 1.34[c]±0.12+35 | 1.41[d]±0.13+42 |
| | Haemolymph | 1.38[a]±0.13 PDC | 1.79[b]±0.14+30 | 1.92[c]±0.16+39 | 2.13[d]±0.19+54 |
| Catalase[c] | Hepatopancreas | 10.39[a]±0.77 PDC | 15.44[b]±0.73+49 | 17.23[c]±0.88+66 | 21.08[d]±1.12+103 |
| | Gill | 6.93[a]±0.16PDC | 9.24[b]±0.19+33 | 11.21[c]±0.19+62 | 13.42[d]±0.21+94 |
| | Haemolymph | 9.24[a]±0.19 PDC | 14.28[b]±0.22+55 | 16.75[c]±0.23+81 | 18.05[d]±0.21+95 |
| Glutathione[d] peroxidase | Hepatopancreas | 7.85[a]±0.11 PDC | 12.33[b]±0.21+57 | 14.04[c]±0.22+79 | 16.71[d]±0.26+113 |
| | Gill | 2.79[a]±0.11PDC | 3.49[b]±0.12+25 | 4.03[c]±0.13+44 | 4.22[d]±0.14+51 |
| | Haemolymph | 6.79[a]±0.12 PDC | 9.77[b]±0.13+44 | 11.68[c]±0.23+72 | 13.04[d]±0.22+92 |
| Glutathione [e] s-transferase | Hepatopancreas | 12.79[a]±0.22 PDC | 18.42[b]±0.23+44 | 23.24[c]±0.24+82 | 24.77[d]±0.28+94 |
| | Gill | 16.74[a]±0.19PDC | 23.49[b]±0.29+40 | 26.78[c]±0.27+60 | 32.92[d]±0.28+97 |
| | Haemolymph | 7.49[a]±0.11 PDC | 9.41[b]±0.21+26 | 10.94[c]±0.22+46 | 13.06[d]±0.21+74 |
| Reduced[f] Glutathione (GSH) | Hepatopancreas | 40.38a±1.33PDC | 52.19[b]±1.89+29 | 57.77[c]±1.98+43 | 88.31[d]±1.97+119 |
| | Gill | 19.78[a]±0.88 PDC | 27.84[b]±0.89+41 | 30.14[c]±0.99+52 | 32.13[d]±1.21+62 |
| | Haemolymph | 108.34[a]±3.98PDC | 67.79b±2.18-37 | 58.73[c]±2.01-46 | 49.88[d]±2.11-54 |
| Lipid[g] Peroxidation (LPO) | Hepatopancreas | 7.39[a]±0.11 PDC | 10.82[b]±0.13+46 | 11.82[c]±0.22+60 | 13.78[d]±0.19+86 |
| | Gill | 2.74[a]±0.11PDC | 3.49[b]±0.12+27 | 3.97[c]±0.13+43 | 4.91[d]±0.12+79 |
| | Haemolymph | 2.77a±0.11 PDC | 3.34[b]±0.12+21 | 3.92[c]±0.12+42 | 4.31[d]±0.13+56 |
| Melonaldehyde[h] (MDA) | Hepatopancreas | 1.24[a]±0.34 PDC | 1.88[b]±0.23+52 | 2.08[c]±0.38+68 | 3.73[d]±0.32+201 |
| | Gill | 0.98[a]±0.08 PDC | 1.24[b]±0.11+26 | 1.33[c]±0.21+36 | 2.01[d]±0.21+105 |
| | Haemolymph | 1.39[a]±0.11 PDC | 1.94[b]±0.12+40 | 2.25[c]±0.12+62 | 2.86[d]±0.13+106 |

All Values are Mean ± SD of six individual observations

PDC: Percent Deviation over respective Control

Values with different superscripts are significantly different from each other @ $p<0.05$.

[a]:Superoxide dismutase (SOD) (µ moles of $H_2O_2$ consumed/mg protein/min)

[b]:Prophenol Oxidase (ProPO) (mg protein/ minute or ml/minutes)

[c]:Catalase (CAT) (mg protein/ minute or units/ ml of Hemolymph/ min)

[d]:Glutathione peroxidase (GPx) (µ moles/mg protein/min or µ moles/ml Hemolymph/min)

[e]:(µ moles of 1-chloro 2, 4-dinitrobenzene conjugates formed/ mg protein/ minute or ml/ minute)

[f]:(Nano moles/ g wet weight of tissue/ml of Hemolymph)

[g]:(nano moles malonaldehyde released/mg protein/min or ml Hemolymph)

[h]:(µ moles/g protein/min or ml/min)

**Table 3:** Hemolymph immune parameters, Total Haemocyte Count (THC), Hyalinocytes (HC), Semi-granulocytes (SGC) and Granulocytes (GC) at different culture stages.

| Parameter | Control | | | Probiotic | | Biotic | | PB+BF | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | 40 | 80 | 40 | 80 | 40 | 80 | 40 | 80 |
| THC | $1823^{a}$±138 PDC | $1940^{a}$±141 +6.4 | $1958^{a}$±144 +7.4 | $2674^{b}$±158 +47 | $2778^{b,c}$± 159+52 | $3038^{b}$±204+67 | $3542^{b,c}$±208 +94 | $3645^{b}$±295 +100 | $4349^{b,c}$±308 +139 |
| HC | $1185^{a}$±125 65% PDC | $1319^{a}$±138 68%+11 | $1312^{a}$±138 67%+11 | $1872^{b}$±149 70% +58 | $1890^{b}$±155 68%+59 | $2308^{b}$±165 76%+95 | $2692^{b,c}$±174 76%+127 | $2697^{b}$±178 74%+128 | $3348^{b,c}$±189 77%+182 |
| SGC | $438^{a}$±25 24% PDC | $504^{a}$±33 26% +15 | $510^{b}$±34 26% +16 | $539^{b}$±36 20% +23 | $582^{b,c}$±38 21% +33 | $556^{b}$±42 18% +27 | $567^{b}$±44 16% +30 | $620^{b}$±46 17% +42 | $653^{b,c}$±47 15%+49 |
| GC | $206^{a}$±18 11% PDC | $233^{b}$±22 12%+13 | $253^{b,c}$±21 13%+23 | $268^{b}$±22 10%+30 | $306^{b,c}$±24 11%+49 | $243^{b}$±25 8%+18 | $284^{b,c}$±22 8%+38 | $328^{b}$±26 9%+59 | $348^{b,c}$±27 8%+69 |

All Values are Mean ± SD of six individual observations
PDC: Percent Deviation over respective Control
Values with different superscripts are significantly different from each other @ $p<0.05$.
Values expressed as Cells/Cu. mm

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