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ANTIOXIDANT STATUS OF PACIFIC WHITE SHRIMP *LITOPENAEUS VANNAMEI* : MODULATION DURING FEEDING WITH PROBIOTIC BLENDED BIOFLOCS

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Abstract: Augmenting Biofloc systems with the addition of Probiotics have been proven to improve not only the water quality and also enhance shrimp performance interms of growth rates and productivity. In the present study, we aimed to evaluate the effects of addition of both Probiotics and Bioflocs on the Antioxidants status and Haemocyte population in *L. vannamei*. All the antioxidant enzymes and haemolymph parameters were found to be significantly ($p<0.05$) enhanced/altered in the Hepatopancreas, Gill and Haemolymph tissues of *L. vannamei* in the Probiotics blended Biofloc treatments than the Control.

These results indicate that microbial protein from recycled waste functions as nutrient, growth promoter and antioxidant stimulator in shrimp reared in BFT systems. Three Experimental diets (ED-1) with combined use of both Probiotics (*Bacillus licheniformis & Lactobacillus rhamnosus* @ 10 billion cfu/kg feed) with Molasses, Tapioca and Maize flour were added as Carbon sources for the production of Bioflocs. ED-2 with Probiotics and Molasses, Maize flour and Sucrose combinations. ED-3 with Probiotics and Molasses, Tapioca, Maize flour and Sucrose combinations and conducted 120 day feeding trail experiments in field.

The data from the Probiotic blended Biofloc fed prawn Experimental trails clearly show that both Probiotics and Bioflocs were able to effectively nullify the ROS production mechanisms by inducing appropriate antioxidant enzyme activities at the tissue and cellular level. This in turn promoted the restoration of normal physiological and biochemical processes, which in turn led to the efficient uptake, utilization, and subsequent absorption of feed, which in turn facilitated the enhancement of growth rate. Both Probiotic and Biofloc distinctly cause the pathogenic forms to be effectively removed from the shrimp's tissues and haemolymph, returning the physiological state to normal allowing for maximum growth. Theerfore, using Probiotics and Bioflocs separately or together will encourage the modification of the antioxidant system to promote favourable production and higher yields.

Keywords: Antioxidant enzymes, Total Haemocyte Count, Hepatopancreas, Gill, Haemolymph

Introduction:

The development of aquatic crops under regulated conditions is known as aquaculture, and the main objective is to create a marketable product as quickly and cheaply as feasible. Aquatic animal production accounted for million tonnes of the predicted 122 million tonnes of global aquaculture production in 2020[1].

The dominating aquaculture industry helps end poverty and malnutrition by producing food that is high in protein. A greater emphasis is being placed on sustainable aquaculture techniques to improve production systems as a result of the expansion of the aquaculture industry and technological advancements[2].

The importance of studying penaeid shrimp immunology has only just grown due to the growing influence of infectious diseases on the long-term viability and sustainability of shrimp aquaculture. Both cellular and humoral components of crustacean immune systems work together to eradicate potentially contagious bacteria. The first immune phase involves the hemocytes and plasma proteins mediating the identification of pathogens[3]. Invertebrates have a well-documented history of using phagocytes, which are a crucial means of getting rid of germs or foreign particles. This microbicidal mechanism in invertebrates was initially shown in gastropods[4]. Later, it was observed in a number of bivalves[5], with particular emphasis on how the oxidative metabolism interacted with particular intracellular parasites. The defence mechanism's agents, the hemocytes, use processes like phagocytosis, encapsulation, nodule formation, clotting, and prophenol oxidase activation to recognise and eliminate foreign chemicals. Hemocytes can also be used to create adhesion molecules, agglutinins, and antimicrobial peptides[6].

Although it is challenging to determine normal values for shrimp, a number of shrimp hemolymph parameters have been used to monitor and assess shrimp stress brought on by unfavourable physiological, environmental, dietary, and illness factors[7]. *Litopenaeus vannamei*, the white prawn, is widely farmed worldwide. Because of its quick growth, strong survival in high-density culture, and resistance to disease, it is a good choice for intensive and bio-secure closed grow-out procedures. In the last ten years, it has become common and sustainable to produce *Litopenaeus vannamei* in intensive systems based on Biofloc that require little to no water exchange[8]. The immunological and antioxidant state of prawns during Biofloc-based culture operations, however, is poorly understood. Thus, the current study's goal was to assess the possible contribution of probiotic-blend Biofloc addition to the enhancement of growth potentials in *L. vannamei* via modifying the immune and antioxidant systems.

Materials & Methods:

Experimental design:

A 120 days Feeding experimental trails were conducted in the field ponds at Allur (Latitude 14.7021 ^{0}N and Longitude 80.0758 ^{0}E) located 20 kms from Nellore, Andhra Pradesh.

Four ponds of 1.0 ha approximately were selected to conduct the present Experimental feeding trails, i.e. one pond was devoted to Control, three ponds were allocated for the conduct of Experimental feeding trails fed with three different Bioflocs, developed by combinations of external carbon sources. The pond preparation procedures were already described[9]. A 120 day feeding trails were conducted and a water depth of 1.5 mts was continuously maintained by taking appropriate measures.

Penaeid shrimp of uniform size 0.67±0.05 g were obtained from local aquaculture farms and were brought in oxygenated polythene bags to the culturing ponds. After acclimatization in the ponds with standard conditions i.e. 15±1 ppt, DO 6.0 mg/lit, Temperature of 22-27 ^{0}C, the shrimp were transferred to the culture ponds. Each pond was stocked with 30,000 nos of individuals. In the present study the Control feed was formulated and its composition was analyzed[10].

Probiotic Feed Preparation:

Probiotic feeds were made by following Naresh's[11] instructions. *Bacillus licheniformis* and *Lactobacillus rhamnosus*, two probiotic bacterial species that are produced at 10 billion CFU/kg every ten days, were added to the control feed to enhance it for use in experimental feeding trails.

Preparation of Bioflocs:

For the purpose of conducting feeding trails with a C/N ratio of 15:1, a fully randomised experimental design was used. Selected external carbon sources were represented by combinations of molasses, tapioca flour, maize flour, and sucrose. The methods previously reported were followed for the manufacture of Bioflocs and the calculation equations utilised[12-16].

Using the proper international standard procedures, water quality parameters, total ammonia nitrogen (TAN) levels, and carbon content Walkley & Black[17] were measured by APHA[18].

Feed Management:

The daily ration of feed was calculated by following total biomass of shrimp i.e. in early stages it was started with 10 % of the total biomass and reduced to 4 % in the later stages of feeding trails. The selected daily ration was split into half each and fed twice a day at 6.00 AM and 6.00 PM, respectively.

Experimental diets:

The following information relates to the preparation of four experimental diets: one Control, three with the inclusion of probiotics, and one with carefully chosen combinations of external carbohydrate sources with 35% crude protein levels, corresponding to a C/N ratio of 15:1.

Control diet	:	Fed with Formulated diet (Table. 1)
Experimental Diet 1 (ED-1)	:	Probiotics blended Control diet along with Bioflocs developed with the combinations of the external Carbon sources including Molasses, Tapioca flour and Maize flour
Experimental Diet 2 (ED-2)	:	Probiotics blended Control diet along with Bioflocs developed with the combinations of the external Carbon sources including Molasses, Maize flour and Sucrose
Experimental Diet 3 (ED-3)	:	Probiotics blended Control diet along with Bioflocs developed with the combinations of the external Carbon sources including Molasses, Maize flour, Tapioca flour and Sucrose

The Experimental feeds were analyzed by following standard methodologies of AOAC[19]. The Proximate composition of Experimental Diets is presented in Table. 2.

Statistical Analysis:

To ascertain the relative importance between the feeding trails, the data from the current study were submitted to One Way Analysis of Variance (ANOVA, SPSS 13.0). To determine the rate of significance between the obtained Mean values, DMRT were performed. The information was presented as Mean ± Standard deviation, with a significance level of $p<0.05$. To determine any differences between the Experimental groups, Tukey's test was employed.

Results and Discussion:

In the present study, an attempt was made to evaluate the Antioxidant status and Hemolymph immune parameters of shrimp during different Experimental feeding trails at selected periods of culture operation and presented in Tables. 3-11.

Haemolymph Immune parameters like Total Haemocyte Count (THC), Hyalinocytes (HC), Semi-granulocytes (SGC) and Granulocytes (GC) were estimated and presented in Table. 3 & Figure. 1. The THC was supposed to contain different proportions of populations i.e. HC constitute 65-70%, followed by SGC @ 23-24% and GC @ 10-11%. All the immune parameters i.e. HC, SGC and GC were found to be significantly ($p<0.05$) increased progressively during all the Experimental feeding trails with increase in the period of Culture operation i.e. 60, 90 DOC.

All the Antioxidant enzymes assayed in selected tissues of shrimp *L. vannamei* i.e. Hepatopancreas, Gill and Haemolymph tissues at different periods of Culture operation i.e. 0, 60 & 90 DOC after subjecting to different feeding trails were presented in Tables. 4-11 & Figures. 2-9.

Superoxide dismutase (SOD), Prophenol oxidase (ProPO), Catalase (CAT), Glutathione Peroxidase (GPx), Glutathione-S-Transferase (GST), Reduced Glutathione (GSH), Lipid Peroxidation (LPO), and malondialdehyde (MDA) are among the antioxidant enzymes assayed. The results are displayed in Tables. 4-11 and were monitored at different DOCs, namely 0, 60, and 90 DOC.

All the Antioxidant enzymes assayed were found to be significantly ($p<0.05$) elevated consequent upon its Experimental feeding trails of Probiotic blended Bioflocs generated with the addition of selected external Carbon sources represented by Molasses, Tapioca flour, Maize flour and Sucrose in different combinations.

The effectiveness of immunostimulants is typically assessed by alterations in parameters like total haemoglobin count (THC)[20], encapsulation, melanization, and blood coagulation[21], phagocytic index, or level of superoxide dismutase (SOD)[22]. In the present study Haemolymph parameters like THC, HC, SGC and GC populations were monitored and found to be significantly increased with progress in the Culture operation period. The changes in the Immune parameters were shown to increase significantly ($p<0.05$) in all the Experimental feeding trails including Control, Probiotic blended Biofloc additions for the development of Bioflocs. Through the addition of external carbohydrate sources in combinations. Among the Feeding trails, the Biofloc added groups i.e. ED-1, ED-2 & ED-3 fed groups recorded maximum increase compared to Control group, ED-3 recorded highest increase followed by ED-2 and a minimum of increase observed with ED-1. The results obtained clearly demonstrate that the changes were more pronounced and significant in all the Experimental feeding trails in the present study.

Over the past 20 years, research on crustacean immunity has been given top emphasis, primarily due to the need to contain disease outbreaks in prawn farms. Adverse immunity does not exist in invertebrates. Rather, they depend on immunological processes that are innate and non-adaptive. Despite this limitation, the majority of microbial threats can be efficiently controlled by prawn defence within hours of their occurrence[23]. The humoral and cellular branches of the innate immune system are composed of haemocytes, which play a vital role in both. These immune effector cells are known to facilitate all of the major immune functions, such as the prophenoloxidase (proPO) system, coagulation, antimicrobial activity, opsonization, phagocytosis, cell agglutination, and nodulation/encapsulation of foreign material [24]. The clotting system and the proPO activation cascade have been researched in decapod crustaceans the most out of all of these processes; in fact, this information is used as a research model. Uncovering the mechanisms underlying other significant immunological responses and the part played by haemoglobin in these responses, however, remains a significant task.

Among animal taxa, the innate immune response is a long-standing and highly conserved system. It is commonly known that robust and efficient innate immune responses are the foundation of crustacean defence against infection. Crustaceans do not appear to have an adaptive immunological defence, in contrast to vertebrates[23, 25]. The two primary branches of their innate immunity are humoral and cellular immunity. The direct fight against invasive pathogens by haemocytes, or immune cells found in invertebrates, is what characterises cellular immunity. A variety of defensive immunological components, mostly created, stored,

and released by hemoglobin-producing cells, make up humoral immunity[23, 24]. Cellular and humoral immunity often coexist because all known immunological responses include haemoglobin, either directly or indirectly. Granular haemoglobin recognises a non-self target, which sets off the immune response. This recognition causes the haemoglobin to degranulate, releasing immune effectors into the haemolymph, or blood of invertebrates[3]. According to Tassanakajon *et al* [26] immune effectors are components of the humoral branch of innate immunity and eventually operate as promoters or triggers for subsequent cellular processes. A wide spectrum of immunological mechanisms occur upon degranulation. According to Theopold *et al* [27] the clotting is the most immediate. Additional components of humoral immunity that show antimicrobial activity include the pro-phenoloxidase-activating system (proPO system), antimicrobial peptides, lectins, reactive oxygen species (ROS), lysosomal enzymes, and agglutinins[28, 29]. Certain constituents additionally instigate and facilitate cellular immune mechanisms, including phagocytosis and pathogen nodulation/ encapsulation.

Prevention and control of diseases are the first priority for the development and stability of shrimp industry. Shrimp immunology played a key role in establishing strategies for the regulation, prevention and control of decreases by manipulation of immune parameters. Due to invading of microorganisms, the resistance power of shrimp was strongly influenced by immune system of shrimp. In crustaceans the "Defence System" was less developed compared to finfish and other vertebrates[30]. The shrimp farming was regularly being affected by problems associated with environmental degradation and also infectious diseases. Viruses and Bacteria were considered to be the main causative agents of shrimp forming activity. To prevent the shrimp from disease, there is a need to develop strategies for the assessment and the monitoring of the immune status. For the maintenance of internal Homeostasis, two major systems are involved i.e. the innate immunity and the environmental stress responding systems, which function together to defend cells from both biotic and abiotic factorial influence. Due to pathogenic infection, the most immune system generally geared up for microbial clearance path ways and cellular stress alleviation pathways that required to be balanced for immune responses leading to lethal consequences[31]. Haemolymph, or invertebrate blood, is made up of two parts: a cellular component made entirely of haemocytes, or shrimp immune cells, and a liquid fraction known as plasma. Since they mediate all known invertebrate immune reactions, either directly or indirectly, haemoglobins play a crucial role in invertebrate immunity. It is customary to separate crustacean haemoglobin into subpopulations or subclasses based on their morphological traits and/or functional properties[32]. Three distinct subpopulations based on their morphology have been identified: (i) hyalinocytes, also known as hyaline cells; (ii) semi-granulocytes, also known as semi-granular cells; and (iii) granulocytes, also known as granular cells.

Generally speaking, phagocytic blood cells seem to be the primary defence mechanism used by invertebrates, especially crustaceans, against germs that cause illnesses. As multiphase, cellular processes that include chemotaxis, attachments, ingestion, and death, hyaline cells and semi-granular haemoglobin were regarded as phagocytes in prawns. Although they include molecules like hemolin and lactone that are part of the immunoglobulin super family, invertebrates do not have particular antibodies, and there is currently no scientific evidence to support the existence of real lymphocyte-like subpopulations in them. The immune defence system of penaeid shrimp is undoubtedly effective, much like that of vertebrates, and is governed by intricate humoral interactions. The most crucial elements in carrying out the immune tasks of penaeid shrimp are haemoglobin and phagocyte cell types.

Subpopulations of crustacean haemocytes and their roles:

Invertebrates have immune cells called haemoglobins. The only cells in the hemolymph are called hemolymphocytes, in contrast to the variety of cells seen in vertebrate blood. In addition to their pivotal involvement in immunity, they take part in a variety of biological processes in crustaceans, including hardening[33, 34] and cuticle and muscle regeneration[35]. There is currently an almost unanimous classification into three subpopulations of haemocytes, despite some disagreement in terminology for naming the various

subpopulations. Hyalinocytes or hyaline cells (HCs), semi-granulocytes or semi-granular cells (SGCs), and granulocytes or granular cells (GCs) are the terms used to describe the subpopulations. On the other hand, uncertainty and dispute regarding haemocyte subtyping are most likely caused by this oversimplified classification method that is based only on the presence and size of cytoplasmic granules. Hose *et al* [36] tried this with decapod crustaceans. Granulocytes and semi-granulocytes are thought to be the cell types in crustaceans that produce and store immune-related molecules in cytoplasmic granules, as well as those that perform cytotoxicity, nodulation, and encapsulation of pathogens[37]. In crayfish, crabs, and prawns, hyalinocytes were discovered to represent the predominant phagocytic cell type. However, several research teams discovered that granular cells were the predominant phagocytic cell type in prawn[38].

Since specific environmental and physiological factors have a significant impact on the rate of ROS formation, there is a close relationship between environmental stress and oxidative stress in an organism[39]. Antioxidants and lipid peroxidation levels have been suggested by a number of writers to be potential markers of oxidative stress in a variety of marine species. Different environmental pro-oxidant conditions, such as increased production of reactive oxygen species (ROS) due to hypoxia[40], temperature[41], age[42], diet, seasons, reproductive cycle stage, and sex, all influence variations in antioxidant enzyme activities[43]. According to a number of authors, cDNA sequencing of antioxidant enzymes like Catalase and GPx in *L. vannamei* and *P. monodon* has been shown to increase or modify the antioxidant capacity of defence mechanism and resistance to ammonia stress. Dietary supplements of antioxidants like vitamin C & E and astaxanthin have also been shown to do the same[30]. Peroxinection, a multifunctional protein with biological activity of peroxidase, is also suggested to play a crucial role in the antioxidant defence of crustacea by reducing oxidative damage from H_2O_2, in addition to GPx and CAT. When the flow of reactive oxygen species (ROS) surpasses the capacity of the antioxidant system, pathogenic circumstances are dramatically exacerbated in the formation of highly hazardous metabolites. When bacteria, fungi, or viruses invade a crustacean, the organisms may produce reactive oxygen species (ROS)[29]. The hepatopancreas and gill tissues are more responsive than the hemolymph among the three tissues used in this study to evaluate the antioxidant system. The hepatopancreas is the main organ that responds to assaults to the body caused by changes in the environment, such as metals, pollutants, poisons, and the invasion of pathogenic microbes.

The crustaceans exhibit an open circulatory system that includes Blue-green haemolymph, Hemocyanin which passes through a hemocoel and reaches the crustacean tissues in different parts of the body. Haemocytes and other humoral constituents are carried by the haemolymph that favours their encounter with foreign bodies[30]. For the innate immune system, the chief source for the mature effector cells is the process of haematopoiesis. In crustaceans, the haematopoietic tissue presents as an extreme network of packed lobules mainly located at the dorsal and dorso lateral sides of the stomach in shrimp, at the base of the maxillipeds and close to the antennal artery. The hematopoietic tissue of penaeid prawns is distributed among the antennal gland, maxillipeds, and stomach. The outer walls of the haemolymph arteries in the digestive gland or hepatopancreas function as lymphoid organs and are involved in the uptake of foreign molecules in the majority of crustaceans, particularly prawns[44, 45]. The lymphoid organs of penaeid prawns are thought to be in charge of eliminating foreign elements from the haemolymph, as evidenced by the detection of viral and bacterial materials in these organs.

proPO system:

The immunological mechanism in prawns that has been investigated the most is the proPO enzymatic cascade. The active form of proPO is phenoloxidase (PO), a tyrosinase that contains copper. Haemocytes manufacture and store the constituents of this enzymatic cascade in their cytoplasmic granules[46, 47]. The elements of the proPO system are released into the bloodstream when haemocytes undergo degranulation, or controlled exocytosis, in response to identification of non-self[47]. Thus, a serine protease cascade initiates the enzymatic cascade by activating the pro-phenoloxidase-activating enzyme. Through a process of restricted proteolysis, this pro-phenoloxidase-activating enzyme catalyses the

transformation of proPO into active phenoloxidase. The synthesis of melanin and hazardous (antimicrobial) intermediate molecules like quinones is the primary role of phenoloxidase. The dark, insoluble pigment known as melanin is responsible for creating the characteristic melanotic capsules that follow wounds and microbial infections.

In the present study, both due to probiotic and Biofloc application to the shrimp farming activity has induced a significant increase in Total Haemocyte Count (THC), along with sub populations of Hyalinocytes (HC), Semi granulocytes (SGC) and Granulocytes (GC) in the shrimp *L. vannamei*, thus indicating increased immune-stimulatory potentials of Probiotics and Bioflocs, used in the present investigation. Increased THC was earlier reported in freshwater prawn *M. rosenbergii*[48], *M. malconsonii*[49] similarly HC count recorded in shrimp *P. latisulcatus* when supplemented with Probiotic strains *Pseudomonas*[50]. So therefore, the THC count can be taken as an index for health indicator since they are important non-specific immunological parameter. The Phenol Oxidase (PO) is an important enzyme in the process of melanisation in crustaceans as a response to entering pathogens. PO is formed from Pro-phenol oxidase (ProPO) activation as cascade phenomenon with numerous steps for organ functioning. The PO cascade constitutes a major element of the shrimp humoral response. Precursors of PO enzyme present in granular haemocytes and catalyzes the oxidation of phenolic compounds as tyrosine and DOPA, which instigate a complex molecular cascade that causes with the formation of a dark pigment, melanin. In the present investigation, TVC was found to be significantly reduced with progress in the days of culture operation (DOC). In the present study Phenol Oxidase activity levels were found to be significantly elevated in all the experimental feeding trails. PO, the final enzyme in the arthropod defence system's ProPO system, functions as an effector and recognition component by fostering cell-to-cell communication, which in turn warms pathogens. Materials created when ProPO systems are activated promote a number of cellular defence mechanisms, such as phagocytosis, nodule formation, haemoglobin localization, non-self identification, and other immune responses. It was discovered that the *L. vannamei* shrimp ProPO system contributed to the removal of Vibrio by lowering the TVC level in the PB and BF feeding paths. Similar observations were made by Yeh *et al.* [51] who also discovered that the hormone ecdyson, which promotes growth, controls moulting, and is involved in the maintenance of multiple physiological and biochemical processes, is in charge. The increased Prophenol Oxidase found in this investigation is consistent with previous findings from studies on *P. monodon* by Rengpipat *et al.*[52], *P. vannamei* by Gullian *et al.*[53], and *L. vannamei* by Tseng *et al.*[54]. According to Martin *et al.*[55], prosport and the storage of protein and amino acids, wound healing, hemolymph coagulation, and circulating haemocytes as the THC of decapods and crustaceans all play significant roles in controlling physiological functions. The number and quality of circulating hemocytic counts of crustaceans are influenced by various factors such as food consumption, life cycle, disease outbreaks, contaminants, and environmental pressures. Haemocytes have been employed as a measure of the immune system's capability in penaeid shrimps, coupled with respiratory burst activity and prophenol oxidase activity. Extrinsic factors influencing numerous species of decapod crustaceans include temperature, salinity, pH and dissolved oxygen. According to Bachere[56], THC is therefore regarded as one of the key factors influencing and disrupting the health condition of crustaceans. In *M. rosenbergii*, a freshwater prawn. After being exposed to low DO, or hypoxic conditions, it was discovered that THC hyaline cells, phenol oxidase activity, and superoxide anions reduced. This was followed by an increase in susceptibility to *Lactococcus garvieae* infection. They also showed a correlation between PO activity and clearance efficiency and prawn resistance to *L. garvieae*. According to Cheng and Chen[57], the freshwater shrimp *M. rosenbergii* exposed to Ammonia-N had a reduced PO activity due to its increased sensitivity. This reduction in PO activity was found to be more significant in determining the prawn's resistance to the infected state than either the haemocyte count or respiratory burst products. The fact that the TVB count in this trial was considerably lower suggests that the pathogenic bacteria were effectively cleared. There have also been reports of increased bacterial clearance in lobsters and shore crabs (*Carcinus maenus*)[58].

One of the most crucial environmental factors for aquatic species is dissolved oxygen (DO); hypoxia will negatively impact *L. vannamei*'s growth, development, and survival. In all experimental feeding trails in the current study, it was discovered that DO levels were somewhat decreased. Thus, research is being done to determine how antioxidant enzyme activity and oxidative stress are affected by reduced DO levels, or hypoxia. Glutathione peroxidase (GPx) can convert peroxide and organic hydroperoxides, while superoxide dismutase (SOD) may convert superoxide radical (O_2^-) to peroxide (H_2O_2). They played a significant part in preventing oxidative damage and preserving the equilibrium of free radicals. The current study's observation of a considerable increase in SOD and GPx activity indicates that employing oxygen loading to produce ROS allowed electrons to accumulate. Oxygen combines with accumulated electrons and breakdown products like xanthine and hypoxanthine when there is enough oxygen present, increasing the generation of reactive oxygen species (ROS) and oxidative stress. Additionally in prawn *L. vannamei*, hypoxia increased oxidative stress and promoted SOD and GPx activities. These outcomes corroborate the findings of the current investigation. PB and BF feeding trail experiments revealed a considerable increase in SOD activity levels in the hepatopancreas, muscle, and hemolymph. The movement and transfer of various foreign molecules or biomolecules into other organs for additional metabolism or biochemical processes was thought to be facilitated by the hemolymph. Because TVC is present in prawn haemolymph, it produces free oxygen radicals and disrupts the body's antioxidant defence mechanisms.

Superoxide anions (O_2^-) are converted into less harmful H_2O_2 by the enzyme superoxide dismutase (SOD), which is specifically designed to scavenge Superoxide radicals.

$$2O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$$

There are three types of SOD, and they vary in terms of the metal ions found in the enzyme's active centre. The predominant form of CuZn SOD found in eukaryotes is mostly described as a cytosolic enzyme (Brand, 2000). MnSOD and FeSOD are the other two types[40].

An oxidoreductase enzyme called catalase (CAT) encourages the breakdown of H_2O_2 into H_2O and oxygen.

$$O_2 + H_2O_2 \rightarrow 2\ H_2O$$

H_2O_2 has the potential to transform into more harmful Hydroxyl radicals if it is not scavenged quickly. The most reactive oxygen species is the hydroxyl radical (OH-), which is commonly suggested to be the cause of DNA damage and lipid peroxidation[59].

The detoxification of H_2O_2 also involves the enzyme glutathione peroxidase (GPx). However, the ideal substrate for GPx is organic hydroxides. When reduced glutathione (GSH) is converted to glutathione disulfide (GSSH; the oxidised form), GPx catalyses the reduction of hydroperioxide[60].

$$GSH + H_2O_2 \rightarrow 2H_2O + GSSG$$

Animal tissues have been found to contain two distinct GPx. Dependent on selenium and not dependent on it.

A class of multifunctional enzymes known as glutathione-S-transferases (GSTs) catalyses the conjugation of reduced glutathione with a wide range of electrophilic substances or cellular elements injured by reactive oxygen species (ROS)[43]. As a result, GSTs have developed to provide defence for cells against various xenobiotics and oxidative metabolic waste products[60].

The flavoenzyme Glutathione reductase (GR) is the enzyme responsible for the reversion of oxidized glutathione (GSSG) to the reduced form (GSH). NADPH is used as electron donor in this process[61].

$$GSSG + NADPH\ + H+ \rightarrow 2\ GSH + NADP\ +$$

Thus GR regenerates GSH, which has been converted to the oxidized form (GSSG) by oxidation and thiol-transfer reactions.

ROS have the ability to destroy and immobilise intruders, however an increased buildup of ROS may harm cells. The initial line of defence is the generation of antioxidant enzymes, which scavenge reactive oxygen species (ROS) brought on by environmental stresses or the presence of specific pathogens. Since crustaceans lack adaptive immunity, phagocytosis together with the generation of ROS serves as a vital line of defence against infections. In this regard, the role of antioxidant enzymes in invertebrates appears to be far more important than in vertebrates[62]. The parent form of intracellular ROS, the superoxide anion, is an active molecule that can be changed by SOD into H_2O_2. H_2O_2 can then be catalysed to create H_2O by GPx and CAT, which lessens its harmful effects[43]. The higher levels of TVC, which cause excessive ROS to be produced in tissues and hemolysis, were the reason for the raised activity levels of the antioxidant enzymes SOD, CAT, and GPx. Following an immunological challenge, Chen *et al* [63] revealed that the haemocytes of the crab *Scylla* sps exhibited a robust antioxidant response. Thus, the shrimp's self-defense mechanism against oxidative stress can account for the increase in antioxidant enzymes seen in this study. Furthermore, phagocytosis produces a number of oxygen species that are controlled by antioxidant molecules and antioxidant enzymes and are crucial to microbial activity. Prior research has verified that immune stimulants like lipo polysaccharides, β-glucon, Peptidoglycon, and Zymosan, which promote phagocytosis, Bactericidal activity, proPO activity, and respiratory bursts to enhance resistance against pathogens, boost immune responses in a number of shrimp species. In order to eliminate pathogens, superoxide anions, hydrogen peroxide, and hydroxyl radicals are typically produced. Since the host may be impacted by these ROS, it is necessary for the host to minimise or rely on antioxidant enzymes like SOD, catalase, and glutathione peroxidase to effectively protect cells from the oxidative damage produced by ROS. Because there was less oxygen available throughout the experimental feeding trials, MDA concentrations in the hepatopancreas and hemolymph were found to be substantially higher. However, when there is enough oxygen available, the concentrations of MDA can revert to normal. This demonstrates unequivocally that "hypoxia" exacerbated oxidative stress in the tissues of HP and HL, and that normal levels of oxygenation will be restored with reoxygenation or the availability of adequate amounts of O_2. MDA was produced oxidatively when free radicals and polyunsaturated fatty acids (PUFA) reacted, and its content may serve as a sign of oxidative stress. MDA concentrations in prawn Gill tissue were comparatively low; this could be due to elevated SOD and GPx activity. In contrast, it was discovered that the MDA concentrations and GPx activity levels were highest in the HP, while the maximum SOD activity was observed in the Gill tissue of the prawn *L. vannamei*. These findings suggested that oxidative stress was relatively high in both the HP and Gill. The primary explanation could be that although HP is engaged in digestion, resume material storage, lipid and carbohydrate metabolism, and the absorption process, Gill is the tissue responsible for swallowing and transferring oxygen. Thus, both tissues have a comparatively high rate of metabolism and are more vulnerable to oxidative damage. The obtained results demonstrate that, under varying oxygen availability conditions, the antioxidant enzyme activities and oxidative stress levels of prawn *L. vannamei* are influenced by the unique functions and metabolism rate of the tissue. According to the theory of the preparative mechanism against oxidative stress, modifications in the activities of important antioxidant enzymes, most likely in conjunction with low molecular weight antioxidants like GSH, were a component of the adaptive mechanism that allowed the body to withstand particular environmental conditions like hypoxia or TVC invasion. According to Oost *et al*. [64] GST is thought to be crucial in preventing membrane damage brought on by LPO. One possible explanation for the shrimp's decreased LPO levels is that their antioxidant defence system may be functioning more actively. Effective ROS elimination depends on the balance between GST, CAT, and GPx . The buildup of uneaten high protein feeds at the bottom of the pond causes several tonnes of organic water to be produced, which can then build up in the prawn pond ecosystem. These organic waste components are highly stable and do not frequently disintegrate into more easily used forms. Additionally, Dierberg & Kiattsimkel[65] noted that the DO in the prawn ponds' deeper levels was reduced due to the oxidation of these organic waste components. By

encouraging the production of harmful metabolites as hydrozen sulphur, methane, ammonia, and nitrite, among others, these pollutants may be able to partially account for variations in the levels of oxidative stress biomarkers. Higher amounts of antioxidants will be required for enhancing immune response if the oxidant and antioxidant balance is a significant factor in determining immune cell activity, including the regulation via signal transduction and gene expression. Furthermore, research design that focuses on the elements that are modulators of the immune response and are controlled by the differentiation and proliferation of circulating haemoglobin following an immunostimulant challenge is crucial. One of the most significant antioxidants believed to be involved in shielding cell membranes from lipid peroxidation is reduced glutathione. Through the action of a complex enzyme system that includes glutathione peroxidase, glutathione s-transferase, and glutathione reductase, the cellular tripeptide glutathione (L-glutamyl cysteinyl glycine) performs beneficial antioxidant actions. Through its interactions with Superoxide, hydroxyl radicals, and singlet oxygen, reduced glutathione also directly contributes to the synthesis of oxidised glutathione and other disulfides, so serving as an antioxidant[60]. The redux balancing phenomenon refers to the general equilibrium between pro-oxidant and antioxidant defence mechanisms seen in normal cells. Oxidative stress happens when pro-oxidants overpower the antioxidant defences[43]. ROS overproduction greatly increases oxidant damage. According to Anchalee *et al*. [43] the lipid mechanism (LPO) is thought to be the main mechanism by which free radicals can damage tissue. This damage can then affect cellular activities and change the physico-chemical characteristics of cell membranes, which can ultimately disrupt essential functions. Because of their conjugated double bond topologies, polyunsaturated fatty acids (PUFA) were thought to be the targets for ROS-driven oxidation. When free radicals attack lipids, conjugated dienes are the first molecules that are produced, and lipid hydroperoxides are the intermediate intermediates. A range of alkanes, alkenes, ketones, and aldehydes are produced by lipid peroxides throughout their breakdown process. Malondialdehyde (MDA) is the most significant byproduct. The most often employed oxidative stress criterion is the assessment of LPO in terms of MDA, the terminal product of LPO.

Overall, immunology data point to the possibility that probiotics differ in their effects on nonspecific immunity. This could be because various probiotic bacteria behave differently in a biofloc environment. A complex of micro and macroorganisms interacting with one another is called the biofloc. The same supply of carbohydrates and methods were used in this study for all treatments in order to produce Biofloc. On the nonspecific immunity, however, the various probiotic strains utilised for various therapies had varying effects. These bacterial strains differ not only in the components of their cell walls but also in their ability to produce enzymes and in their metabolic pathways. Our previous research demonstrated that the colonisation of the microflora and the enzymatic synthesis of cellulase, amylase, and protease facilitate the digestion of feed and external carbon sources for the culturing prawns in the Biofloc system. These bacterial flora that produce enzymes are frequently found in the intestines of prawns and fish, and they are beneficial to the host.

In the BFT system, enzymatic probiotic bacteria play a significant role. The probiotic microorganisms in the bioflc make use of the carbohydrate supply, which improves the reared animals' immunity and performance. Overall, our research showed that adding Bacillus species to Biofloc could enhance *Penaeus indicus* immunity, growth, survival, and yield while lowering the need for additional feed. Nevertheless, it is unclear how Biofloc improves the production and health of prawns. It has been suggested that enhanced growth, survival, and the decrease of commercial food may be caused by the heterotrophic development of diatoms, rotifers, cyanobacteria, and protozoa in the biofloc system. It has already been shown that using *Bacillus* species as probiotics in aquaculture can increase growth, survival, and resistance to physical stress as well as activate digestive enzymes and strengthen immunological responses to thwart pathogens[52]. Additionally, *Bacillus* is well-known for its antagonistic effect against pathogens in aquaculture. According to a report, the peptidoglycan in prawn cell walls may stimulate immunological responses, and Bacillus surface antigens and their metabolites may act as immunogens. Therefore, under Biofloc culture conditions, bioaugmentation of the Biofloc system with Bacillus can increase immunity and

boost prawn output. Commercial probiotics added to the biofloc system may lessen Litopenaeus vannamei infection caused by Vibrio parahaemolyticus[46]. To enhance the humoral immune response, Miao *et al*.[66] employed *Bacillus subtilis* and *Lactobacillus* in *Macrobrachium rosenbergii* in conjunction with the biofloc condition. In *Fenneropenaeus chinensis*, the addition of *Bacillus* sp., *Lactobacillus* sp., and *Rhodobacter* sp. to the biofloc enhanced growth, boosted the immune system, and decreased oxidative stress.

Litopenaeus vannamei and *Penaeus monodon* have improved immunity against infections as a result of bacterial probiotics' ability to change PO enzyme activity. The consumption of the microbial floc in the system as a result of BFT and bio-augmentation of *Bacillus* spp. may be partially responsible for the increased PO activity. Shrimp immunity has enhanced as a result of the improvement of THC and PO activity. It was also due to bio-augmentation in BF that phagocytic activity was elevated. C:N ratio adjustment raises the phagocytosis percentage in shrimps, according to research on the impact of this ratio manipulation on Biofloc-driven immune-stimulation in *Litopenaeus vannamei*.

As of right moment, there are no scientific analyses or causal relationships between these observations. The current study offers some statistically significant and repeated evidence of a series of mechanisms that, when cultured in biofloc systems, boost prawn immunity. Shrimp's immune system is labile, as was said in the introduction (no long term memory). As a result, the prawn population lacks an immediate immune response to infections that enter the systems used to produce prawns. It has been demonstrated, though, that when prawns are grown in a clear water system as opposed to a biofloc system, this is not the case. The dense microbial population linked to the bioflocs is thought to act as a constant trigger for the immune system's development and maintenance, enabling the prawn population to build up a defence mechanism. The use of this technique could be a crucial defence against severe disease outbreaks that frequently cause prawn production systems to collapse and result in enormous losses. It must be underlined that this work is only the beginning and serves as a catalyst for further research.

The data from the Probiotic blended Biofloc fed prawn experimental trails clearly show that both PB and Biofloc were able to effectively nullify the ROS production mechanisms by inducing appropriate antioxidant enzyme activities at the tissue and cellular level. This in turn promoted the restoration of normal physiological and biochemical processes, which in turn led to the efficient uptake, utilisation, and subsequent absorption of feed, which in turn facilitated the enhancement of growth rate Both PB and BF distinctly cause the pathogenic forms to be effectively removed from the shrimp's tissues and hemolymph, returning the physiological state to normal and allowing for maximum growth. Therefore, using Probiotics and Bioflocs separately or together will encourage the modification of the antioxidant system to promote favourable production and higher yields.

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Table. 1: Ingredient Composition of Control Experimental diet (Protein content 35%)

Feed Ingredient	(%)
Shrimp meal	15
Squilla meal	12
Soya bean meal	20
Wheat meal	20
Yeast meal	5
Groundnut oil cake	5
Cod liver oil	5
Vegetable oil	4
Ascorbic acid	2
Choline chloride	1
Vitamin mixture	1
Mineral mixture	1
Chromic oxide	1
Agar Agar	3
Gelatin	5
Total	**100**

Table. 2: Proximate Composition of Experimental Diets (% DM basis).

	Control	Experimental Diet-1	Experimental Diet-2	Experimental Diet-3
Organic Matter (%)	82.49±1.91	82.45±1.93	82.54±1.86	83.42±1.90
Ash (%)	17.51±0.82	17.55±0.80	17.46±0.78	16.58±0.71
Crude Protein (%)	34.73±1.16	36.88±1.21	36.78±1.23	37.43±1.24
Crude Lipid (%)	7.11±0.46	7.17±0.47	7.24±0.47	7.23±0.48
Crude Fiber (%)	4.31±0.35	4.43±0.41	4.43±0.43	4.52±0.45
Nitrogen Free Extract (NFE) (%)	27.80±0.90	25.38±0.87	25.55±0.83	25.62±0.86
Moisture (%)	8.54±0.51	8.59±0.53	8.54±0.53	8.62±0.54
Gross Energy (Kcal/100 g)	394	396	397	401

Organic Matter : 100 – Ash
NFE : 100 – (CP + CL + CF + Ash + Moisture)
Gross Energy : (CP x 5.6) + (CL x 9.44) + (CF x 4.1) + (NFE x 4.1) kcals/100 g
Control Diet : Composition presented in Table. 1

ED-1 Control Feed + Probiotics added + Bioflocs (Molasses +Tapioca+ Maize flour as carbon source)
ED-2 Control Feed + Probiotics added + Bioflocs (Molasses +Maize flour + Sucrose as carbon source)

ED-3 Control Feed + Probiotics added + Bioflocs (Molasses +Tapioca+ Maize flour +Sucrose as carbon source

Table. 3: Haemolymph Immune parameters in *L. vannamei* during Experimental feeding trails at different periods Days of Culture Operation (DOC)

	Days of Culture Operation (DOC)			
	'0' DOC	**'30' DOC**	**'60' DOC**	**'90' DOC**
Control				
Total Haemocytes Count (THC)	2034±117[a] PDC	2138±134[a] +5.11	2225±154[a] +9.39	2328±163[b] +14.45
Hyalinocytes (HC)	1322±89[a] 65% PDC	1411± 93[a] 66% +6.73	1491±98[b] 67% +12.78	1537±101[b] 66% +16.26
Semigranulocytes (SGC)	488±33[a] 24% PDC	513±38[a] 24% +5.12	513±39[a] 23% +5.12	559±43[b] 24% +14.55
Granulocytes (GC)	224±24[a] 11% PDC	214±23[a] 10% -4.46	223±23[a] 10% -0.45	233±24[a] 10% +4.02
Experimental Diet-1				
Total Haemocytes Count (THC)	2034±117[a] PDC	2842±177[b] +39.72	3315±185[b,c] +62.98	3719±220[b,c,d] +82.84
Hyalinocytes (HC)	1322±89[a] 65% PDC	1876±112[b] 66% +41.91	2155±150[b,c] 65% +63.01	2455±170[b,c,d] 66% +85.70
Semigranulocytes (SGC)	488±33[a] 24% PDC	654±47[b] 23% +34.02	796±54[b,c] 24% +63.11	855±62[b,c,d] 23% +75.21
Granulocytes (GC)	224±24[a] 11% PDC	284±25[b] 11% +26.79	365±26[b,c] 11% +62.95	409±30[b,c,d] 11% +82.59
Experimental Diet-2				
Total Haemocytes Count (THC)	2034±117[a] PDC	3135±181[b,c] +54.13	3673±196[b,c] +80.58	3945±233[b,c,d] +93.95
Hyalinocytes (HC)	1322±89[a] 65% PDC	2069±122[b] 66% +56.51	2424±167[b,c] 66% +83.36	2564±177[b,c,d] 65% +93.95
Semigranulocytes (SGC)	488±33[a] 24% PDC	752±52[b] 24% +54.10	845±59[b,c] 23% +73.16	947±63[b,c,d] 24% +94.06
Granulocytes (GC)	224±24[a] 11% PDC	314±28[b] 10% +40.18	404±33[b,c] 11% +80.36	434±33[b,c,d] 11% +93.75
Experimental Diet-3				
Total Haemocytes Count (THC)	2034±117[a] PDC	3618±191[b] +77.88	4042±246[b,c] +98.72	4224±253[b,c,d] +107.67
Hyalinocytes (HC)	1322±89[a] 65% PDC	2388±166[b] 66% +80.64	2668±177[b,c] 66% +101.82	2788±180[b,c,d] 66% +110.89
Semigranulocytes (SGC)	488±33[a] 24% PDC	832±55[b] 23% +70.49	971±66[b,c] 24% +98.98	1014±73[b,c,d] 24% +107.79
Granulocytes (GC)	224±24[a] 11% PDC	398±29[b] 11% +77.68	405±32[b] 10% +80.80	423±33[b,c] 10% +88.84

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 @ $p < 0.05$

Table. 4: Antioxidant enzymes - Superoxide dismutase (SOD) in selected tissues of *L. vannamei* during different Experimental feeding trails at different culture periods of operation

Superoxide dismutase (SOD) (µ moles of H_2O_2 consumed/mg protein/min)									
Parameter	**Control**			**Experimental Diet-1**		**Experimental Diet-2**		**Experimental Diet-3**	
	'0' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC
Hepatopancreas	14.73±0.63[a]	24.78±0.96[b]	38.42±1.18[b]	46.15 ±1.38[b]	59.75 ±1.74[b]	52.18±1.46[b]	64.34 ±1.81[b]	56.77 ±1.64[b]	73.74 ±1.86[b]
	PDC	+68	+161	+207	+306	+254	+337	+285	+401
		PDE[a]	+55[b]	+82[b]	+141[b]	+111[b]	+160[b]	+129[b]	+198[b]
				PDE[a]	+30[b]	PDE[a]	+23[b]	PDE[a]	+30[b]
Gill	22.18±0.84[a]	34.17 ±1.15[b]	45.35 ±1.35[b]	53.11 ±1.46[b]	62.17 ±1.76[b]	57.19 ±1.71[b]	67.45 ±1.85[b]	74.18 ±1.93[b]	91.49 ±2.17[b]
	PDC	+54	+105	+140	+180	+158	+204	+234	+267
		PDE[a]	+33[b]	+55[b]	+82[b]	+67[b]	+97[b]	+117[b]	+139[b]
				PDE[a]	+17[b]	PDE[a]	+18[b]	PDE[a]	+24[b]
Haemolymph	10.24±0.48[a]	15.72±0.70[b]	20.74±0.82[b]	22.17±0.85[b]	30.75±1.14[b]	27.75±1.01[b]	42.93±1.27[b]	35.71±1.18[b]	54.79±1.53[b]
	PDC	+54	+103	+117	+200	+171	+319	+249	+435
		PDE[a]	32[b]	+41[b]	+96[b]	+77[b]	+173[b]	+127[b]	+248[b]
				PDE[a]	+39[b]	PDE[a]	+55[b]	PDE[a]	+53[b]

All Values are Mean ± SD of six individual observations
PDC: Percent Deviation over respective Control
PDE: Percent Deviation over Experimental group
Values with different superscripts are significantly different from each other @ $p < 0.05$.

Table. 5: Antioxidant enzymes - Prophenol Oxidase (ProPO) in selected tissues of *L. vannamei* during different Experimental feeding trails at different culture periods of operation

Prophenol Oxidase (ProPO) (mg protein/ minute or ml/minutes)									
Parameter	**Control**			**Experimental Diet-1**		**Experimental Diet-2**		**Experimental Diet-3**	
	'0' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC
Hepatopancreas	1.38±0.05^{a}	1.79±0.06^{b}	2.95±0.14^{b}	2.25±0.10^{b}	4.12±0.22^{b}	2.79±0.11^{b}	5.22±0.26^{b}	3.34±0.18^{b}	6.93±0.36^{b}
	PDC	+30	+114	+63	+199	+102	+278	+142	+402
		PDE^{a}	+65^{b}	+26^{b}	+130^{b}	+56^{b}	+192^{b}	+87^{b}	+287^{b}
				PDE^{a}	+83^{b}	PDE^{a}	+87^{b}	PDE^{a}	+107^{b}
Gill	0.93±0.04^{a}	1.49±0.05^{b}	2.09±0.09^{b}	1.84±0.06^{b}	3.14±0.16^{b}	2.77±0.11^{b}	4.78±0.27^{b}	3.84±0.22^{b}	6.24±0.32^{b}
	PDC	+60	+125	+98	+238	+198	+414	+313	+571
		PDE^{a}	+40^{b}	+23^{b}	+110^{b}	+86^{b}	+221^{b}	+158^{b}	+319^{b}
				PDE^{a}	+71^{b}	PDE^{a}	+73^{b}	PDE^{a}	+63^{b}
Haemolymph	1.85±0.06^{a}	2.34±0.11^{b}	3.12±0.16^{b}	2.57±0.13^{b}	4.31±0.25^{b}	3.14±0.18^{b}	5.28±0.29^{b}	3.75±0.24^{b}	6.21±0.31^{b}
	PDC	+26	+69	+39	+133	+70	+185	+103	+236
		PDE^{a}	+33^{b}	+10^{b}	+84^{b}	+34^{b}	+126^{b}	+60^{b}	+165^{b}
				PDE^{a}	+68^{b}	PDE^{a}	68^{b}	PDE^{a}	+66^{b}

All Values are Mean ± SD of six individual observations
PDC: Percent Deviation over respective Control
PDE: Percent Deviation over Experimental group
Values with different superscripts are significantly different from each other @ $p < 0.05$.

Table. 6: Antioxidant enzymes - Catalase (CAT) in selected tissues of *L. vannamei* during different Experimental feeding trails at different culture periods of operation

Catalase (CAT) (mg protein/ minute or units/ ml of Hemolymph/ min)									
Parameter	**Control**			**Experimental Diet-1**		**Experimental Diet-2**		**Experimental Diet-3**	
	'0' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC
Hepatopancreas	6.48±0.34[a]	8.79±0.51[b]	12.74±0.56[b]	10.84±0.53[b]	17.38±0.72[b]	13.25±0.63[b]	20.71±0.76[b]	14.34±0.64[b]	23.78±0.84[b]
	PDC	+36	+97	+67	+168	+104	+220	+121	+267
		PDE[a]	+45[b]	+23[b]	+98[b]	+51[b]	+136[b]	+63[b]	+171[b]
				PDE[a]	+60	PDE[a]	+56	PDE[a]	+66
Gill	4.25±0.20[a]	5.70±0.30[b]	7.38±0.39[b]	6.85±0.36[b]	10.12±0.51[b]	7.24±0.37[b]	13.11±0.60[b]	8.74±0.48[b]	15.24±0.68[b]
	PDC	+34	+74	+61	+138	+70	+208	+106	+259
		PDE[a]	+29[b]	+20[b]	+78[b]	+27[b]	+130[b]	+53[b]	+167[b]
				PDE[a]	+48[b]	PDE[a]	+81[b]	PDE[a]	+74[b]
Haemolymph	6.08±0.32[a]	7.41±0.42[b]	8.77±0.53[b]	8.22±0.43[b]	10.47±0.53[b]	10.14±0.36[b]	14.38±0.63[b]	12.18±0.55[b]	17.04±0.64[b]
	PDC	+22	+44	+35	+72	+67	+137	+100	+180
		PDE[a]	+18[b]	+11[b]	+41[b]	+37[b]	+94[b]	+64[b]	+130[b]
				PDE[a]	+27[b]	PDE[a]	+42[b]	PDE[a]	+40[b]

All Values are Mean ± SD of six individual observations
PDC: Percent Deviation over respective Control
PDE: Percent Deviation over Experimental group
Values with different superscripts are significantly different from each other @ $p < 0.05$.

Table. 7: Antioxidant enzymes - Glutathione peroxidase (GPx) in selected tissues of *L. vannamei* during different Experimental feeding trails at different culture periods of operation

Glutathione peroxidase (GPx) (µ moles/mg protein/min or µ moles/ml Hemolymph/min)									
Parameter	**Control**			**Experimental Diet-1**		**Experimental Diet-2**		**Experimental Diet-3**	
	'0' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC
Hepatopancreas	4.93±0.27[a]	6.12±0.33[b]	8.04±0.41[b]	7.18±0.37[b]	9.38±0.48[b]	8.42±0.44[b]	11.14±0.56[b]	10.24±0.52[b]	13.77±0.60[b]
	PDC	+24	+63	+46	+90	+71	+126	+108	+179
		PDE[a]	+31[b]	+17[b]	+53[b]	+38[b]	+82[b]	+67[b]	+125[b]
				PDE[a]	+31[b]	PDE[a]	+32[b]	PDE[a]	+34[b]
Gill	2.17±0.09[a]	3.29±0.18[b]	4.15±0.23[b]	4.25±0.25[b]	5.12±0.29[b]	5.14±0.31[b]	6.73±0.35[b]	6.71±0.34[b]	8.33±0.44[b]
	PDC	+52	+91	+96	+136	+137	+210	+209	+284
		PDE[a]	+26[b]	+29[b]	+56[b]	+56[b]	+105[b]	+104[b]	+153[b]
				PDE[a]	+20[b]	PDE[a]	+31[b]	PDE[a]	+24[b]
Haemolymph	3.99±0.21[a]	5.45±0.33[b]	8.15±0.42[b]	6.95±0.38[b]	8.99±0.47[b]	8.43±0.45[b]	10.32±0.50[b]	10.31±0.50[b]	13.14±0.62[b]
	PDC	+37	+104	+74	+125	+111	+159	+158	+229
		PDE[a]	+50[b]	+28[b]	+65[b]	+55[b]	+89[b]	+89[b]	+141[b]
				PDE[a]	+29[b]	PDE[a]	+22[b]	PDE[a]	+27[b]

All Values are Mean ± SD of six individual observations
PDC: Percent Deviation over respective Control
PDE: Percent Deviation over Experimental group
Values with different superscripts are significantly different from each other @ $p < 0.05$.

Table. 8: Antioxidant enzymes - Glutathione-S-Transferase (GST) in selected tissues of *L. vannamei* during different Experimental feeding trails at different culture periods of operation

Glutathione-S-Transferase (GST) (µ moles of 1- chloro 2,4- dinitrobenzene conjugates formed/ mg protein/ minute or ml/ minute)									
Parameter	**Control**			**Experimental Diet-1**		**Experimental Diet-2**		**Experimental Diet-3**	
	'0' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC
Hepatopancreas	7.18±0.4[a]	10.12±0.48[b]	14.73±0.67[b]	11.14±0.50[b]	16.18±0.75[b]	13.12±0.57[b]	17.75±0.78[b]	14.72±0.67[b]	19.18±0.83[b]
	PDC	+41	+105	+55	+125	+83	+147	+105	+167
		PDE[a]	+46[b]	+10[b]	+60[b]	+30[b]	+75[b]	+45[b]	+90[b]
				PDE[a]	+45[b]	PDE[a]	+35[b]	PDE	+30[b]
Gill	10.17±0.47[a]	14.12±0.65[b]	18.79±0.79[b]	16.77±0.76[b]	21.38±0.88[b]	18.14±0.76[b]	23.12±0.94[b]	20.34±0.85[b]	24.94±0.97[b]
	PDC	+39	+85	+65	+110	+78	+127	+100	+145
		PDE[a]	+33[b]	+19[b]	+51[b]	+28[b]	+64[b]	+44[b]	+77[b]
				PDE[a]	+27[b]	PDE[a]	+27[b]	PDE[a]	+23[b]
Haemolymph	8.77±0.44[a]	9.32±0.45[b]	10.29±0.49[b]	10.79±0.53[b]	12.49±0.54[b]	12.14±0.53[b]	14.31±0.67[b]	13.77±0.87[b]	16.12±0.73[b]
	PDC	+6	+17	+23	+42	+38	+63	+57	+84
		PDE[a]	+10[b]	+16[b]	+34[b]	+30[b]	+54[b]	+48[b]	+73[b]
				PDE[a]	+16[b]	PDE[a]	+18[b]	PDE[a]	+17[b]

All Values are Mean ± SD of six individual observations
PDC: Percent Deviation over respective Control
PDE: Percent Deviation over Experimental group
Values with different superscripts are significantly different from each other @ $p < 0.05$.

Table. 9: Antioxidant enzymes – Reduced Glutathione (GSH) in selected tissues of *L. vannamei* during different Experimental feeding trails at different culture periods of operation

Reduced Glutathione (GSH) (Nano moles/ g wet weight of tissue/ml of Hemolymph)									
Parameter	**Control**			**Experimental Diet-1**		**Experimental Diet-2**		**Experimental Diet-3**	
	'0' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC
Hepatopancreas	24.42±0.92^{a}	30.77±1.13^{b}	41.48±1.25^{b}	38.93±1.22^{b}	63.45±1.62^{b}	49.44±1.33^{b}	70.74±1.94^{b}	58.45±1.45^{b}	89.74±2.11^{b}
	PDC	+26.00	+70	+60	+160	+102	+190	+140	+267
		PDE^{a}	±35^{b}	+27^{b}	+106^{b}	+61^{b}	+130^{b}	+90^{b}	+192^{b}
				PDE^{a}	+63^{b}	PDE^{a}	+43^{b}	PDE^{a}	+54^{b}
Gill	13.93±0.55^{a}	16.93±0.73^{b}	25.93±0.93^{b}	20.34±0.84^{b}	31.77±1.14^{b}	25.13±0.97^{b}	37.13±1.19^{b}	30.75±1.11^{b}	45.77±1.28^{b}
	PDC	+22	+86	+46	+128	+80	+167	+121	+229
		PDE^{a}	+53^{b}	+20^{b}	+47^{b}	+48^{b}	+119^{b}	+82^{b}	+170^{b}
				PDE^{a}	+56	PDE^{a}	+48	PDE^{a}	+49
Haemolymph	65.14±2.21^{a}	80.74±4.40^{b}	105.38±10.25^{b}	65.74±2.19^{b}	60.13±1.83^{b}	60.12±1.85^{b}	54.11±1.85^{b}	52.14±1.36^{b}	41.05±1.31^{b}
	PDC	+24	+62	-1	-7.6	-7.7	-16.9	-19.9	-36.9
		PDE^{a}	+30.5^{b}	-18.5^{b}	-25.5^{b}	-25.5^{b}	-32.9^{b}	-35.4^{b}	-49.1^{b}
				PDE^{a}	-8.5^{a}	PDE^{a}	-9.9^{a}	PDE^{a}	21.2^{b}

All Values are Mean ± SD of six individual observations
PDC: Percent Deviation over respective Control
PDE: Percent Deviation over Experimental group
Values with different superscripts are significantly different from each other @ $p < 0.05$.

Table. 10: Antioxidant enzymes – Lipid peroxidation (LPO) in selected tissues of *L. vannamei* during different Experimental feeding trails at different culture periods of operation

Lipid peroxidation (LPO) (nano moles malonaldehyde released/mg protein/min or ml Hemolymph)									
Parameter	**Control**			**Experimental Diet-1**		**Experimental Diet-2**		**Experimental Diet-3**	
	'0' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC
Hepatopancreas	4.75±0.27^{a}	7.35±0.42^{b}	10.34±0.48^{b}	9.75±0.47^{b}	14.18±0.62^{b}	11.15±0.52^{b}	16.79±0.67^{b}	13.72±0.60^{b}	19.14±0.73^{b}
	PDC	+55	+118	+105	+199	+135	+253	+189	+303
		PDE^{a}	+41^{b}	+33^{b}	+93^{b}	+52^{b}	+128^{b}	+87^{b}	+160^{b}
				PDE^{a}	+45	PDE^{a}	+51	PDE^{a}	+40^{b}
Gill	1.89±0.08^{a}	2.24±0.12^{b}	3.15±0.15^{b}	3.12±0.15^{b}	4.79±0.33^{b}	4.14±0.27^{b}	6.05±0.41^{b}	5.35±0.37^{b}	7.22±0.44^{b}
	PDC	+19	+67	+65	+153	+119	+220	+150	+282
		PDE^{a}	+41	+39	+114	+85	+170	+139	+222
				PDE^{a}	+54	PDE^{a}	+32	PDE^{a}	+35
Haemolymph	1.14±0.04^{a}	1.65±0.08^{b}	2.39±0.15^{b}	2.13±0.10^{b}	3.17±0.15^{b}	3.14±0.15^{b}	4.72±0.32^{b}	3.95±0.27^{b}	6.12±0.43^{b}
	PDC	+45	+110	+87	+178	+214	+314	+246	+437
		PDE^{a}	+45^{b}	+29^{b}	+92^{b}	+90^{b}	+186^{b}	+139^{b}	+271^{b}
				PDE^{a}	+49^{b}	PDE^{a}	+50^{b}	PDE^{a}	+55^{b}

All Values are Mean ± SD of six individual observations
PDC: Percent Deviation over respective Control
PDE: Percent Deviation over Experimental group
Values with different superscripts are significantly different from each other @ $p < 0.05$.

Table. 11: Antioxidant enzymes – Malondialdehyde (MDA) in selected tissues of *L. vannamei* during different Experimental feeding trails at different culture periods of operation

Malondialdehyde (MDA) (µ moles/g protein/min or mi/min)									
Parameter	**Control**			**Experimental Diet-1**		**Experimental Diet-2**		**Experimental Diet-3**	
	'0' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC	'60' DOC	'90' DOC
Hepatopancreas	1.03±0.04^{a}	1.62±0.05^{b}	2.77±0.12^{b}	2.14±0.98^{b}	3.94±0.22^{b}	3.17±0.16^{b}	5.12±0.30^{b}	4.79±0.26^{b}	6.24±0.33^{b}
	PDC	+57	+169	+108	+283	+208	+397	+365	+506
		PDE^{a}	+71^{b}	+32^{b}	+143^{b}	+96^{b}	+216^{b}	+196^{b}	+285^{b}
				PDE^{a}	+84^{b}	PDE^{a}	+62^{b}	PDE^{a}	+30^{b}
Gill	0.93±0.03^{a}	1.12±0.04^{b}	1.23±0.05^{b}	1.38±0.06^{b}	1.49±0.07^{b}	1.54±0.08^{b}	1.72±0.09^{b}	1.71±0.09^{b}	2.15±0.12^{b}
	PDC	+20	+32	+48	+60	+66	+85	+84	+131
		PDE^{a}	+10^{a}	+23^{b}	+33^{b}	+37^{b}	+54^{b}	+53^{b}	+92^{b}
				PDE^{a}	+8^{a}	PDE^{a}	+12^{b}	PDE^{a}	+26^{b}
Haemolymph	1.12±0.04^{a}	1.64±0.08^{b}	2.13±0.12^{b}	1.93±0.10^{b}	2.72±0.18^{b}	2.24±0.16^{b}	3.94±0.25^{b}	3.24±0.20^{b}	5.12±0.29^{b}
	PDC	+46	+90	+72	+143	+100	+252	+189	+357
		PDE^{a}	+30^{b}	+18^{b}	+66^{b}	+37^{b}	+140^{b}	+98^{b}	+212^{b}
				PDE^{a}	+41^{b}	PDE^{a}	+76^{b}	PDE^{a}	+58^{b}

All Values are Mean ± SD of six individual observations
PDC: Percent Deviation over respective Control
PDE: Percent Deviation over Experimental group
Values with different superscripts are significantly different from each other @ $p < 0.05$.

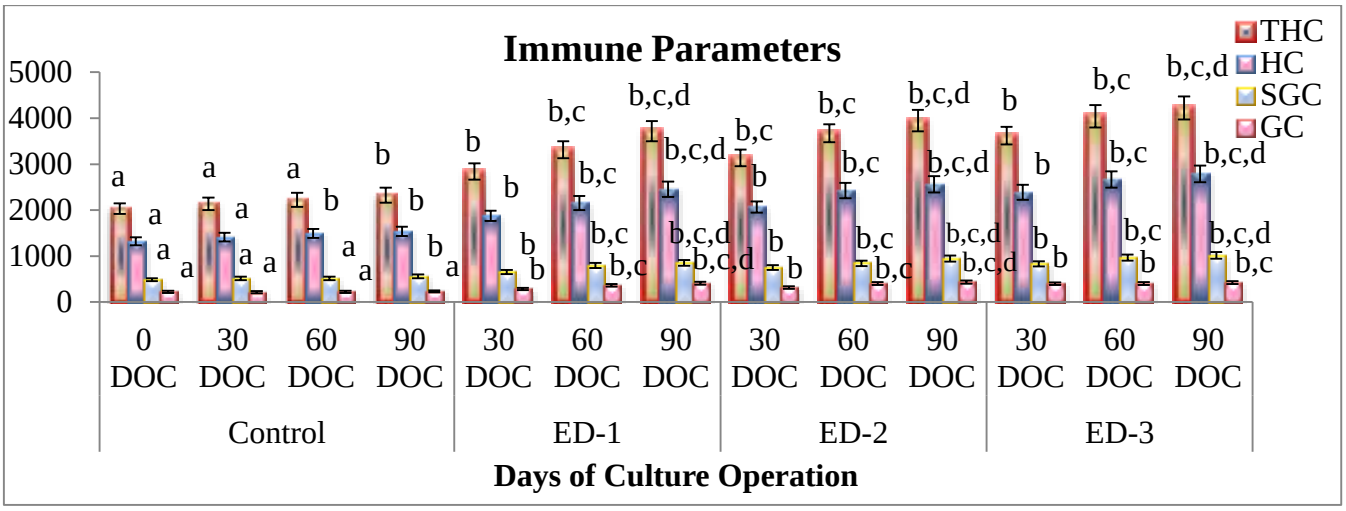

Figure. 1: Immune Parameters (THC, HC, SGC and GC) in shrimp *Litopenaeus vannamei* under different Days of Culture Operation (DOC) – 0, 30, 60 and 90 DOC

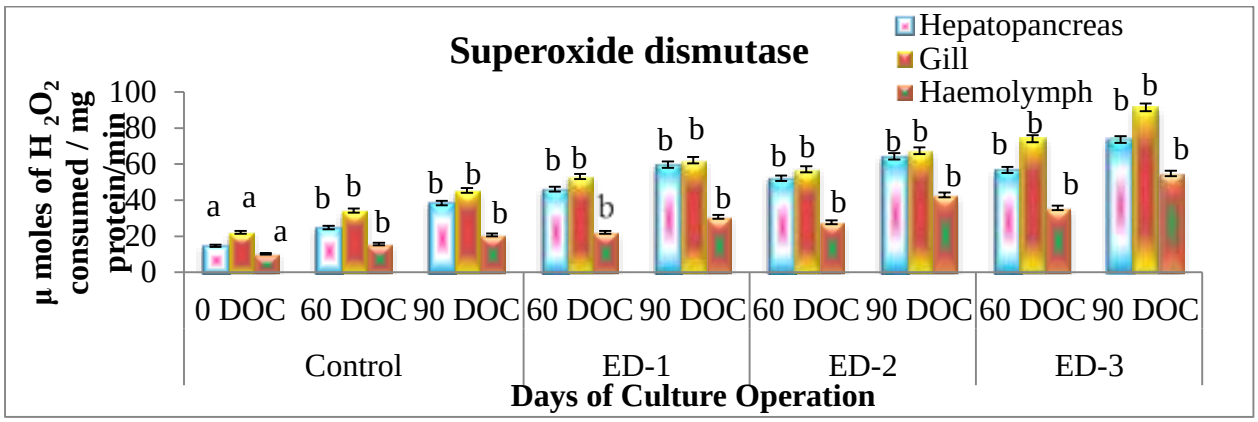

Figure. 2: Antioxidant Parameters (SOD) in shrimp *Litopenaeus vannamei* under different Days of Culture Operation (DOC) – 0, 60 and 90 DOC

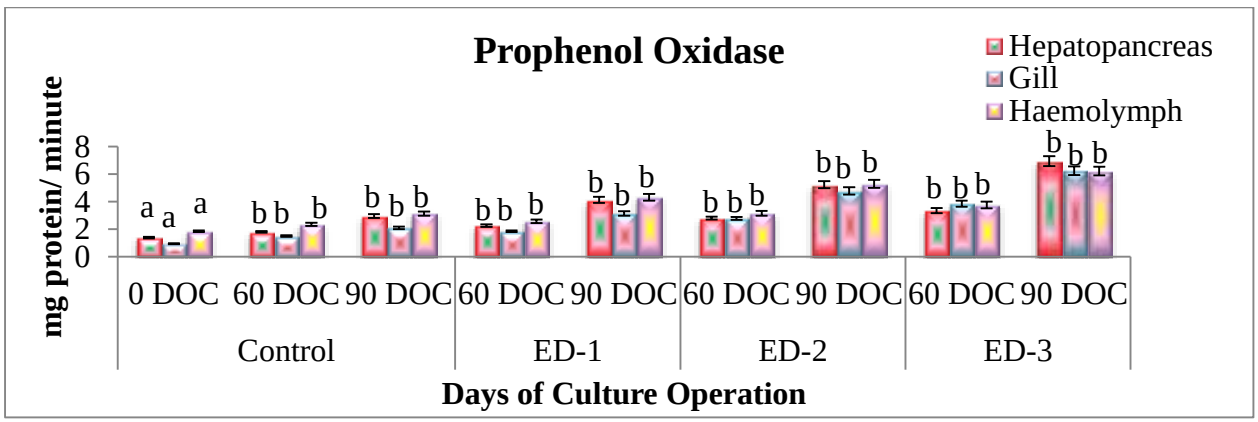

Figure. 3: Antioxidant Parameters (proPO) in shrimp *Litopenaeus vannamei* under different Days of Culture Operation (DOC) – 0, 60 and 90 DOC

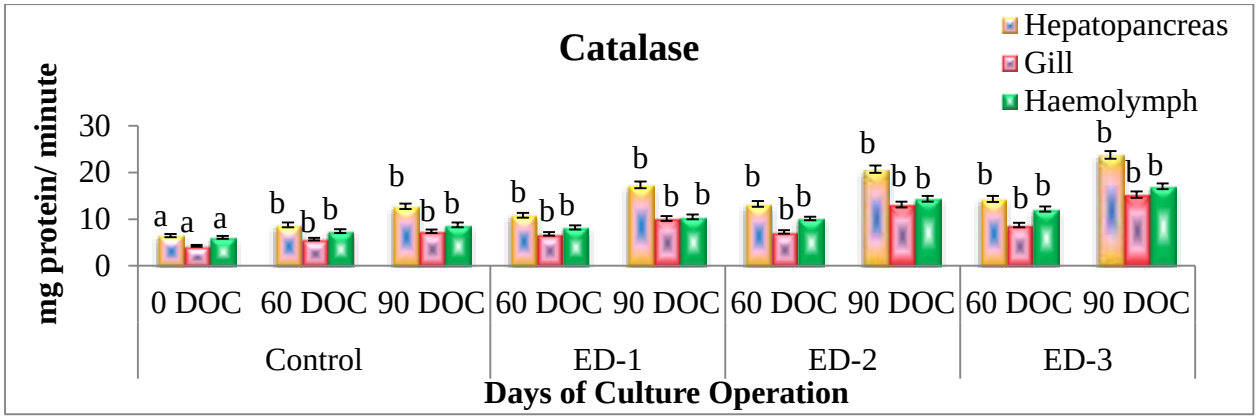

Figure. 4: Antioxidant Parameters (CAT) in shrimp *Litopenaeus vannamei* under different Days of Culture Operation (DOC) – 0, 60 and 90 DOC

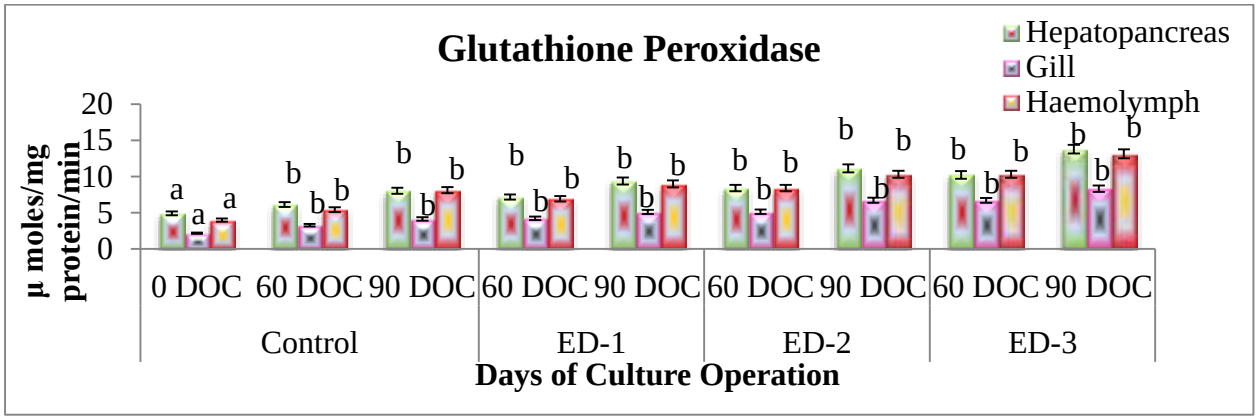

Figure. 5: Antioxidant Parameters (GPx) in shrimp *Litopenaeus vannamei* under different Days of Culture Operation (DOC) – 0, 60 and 90 DOC

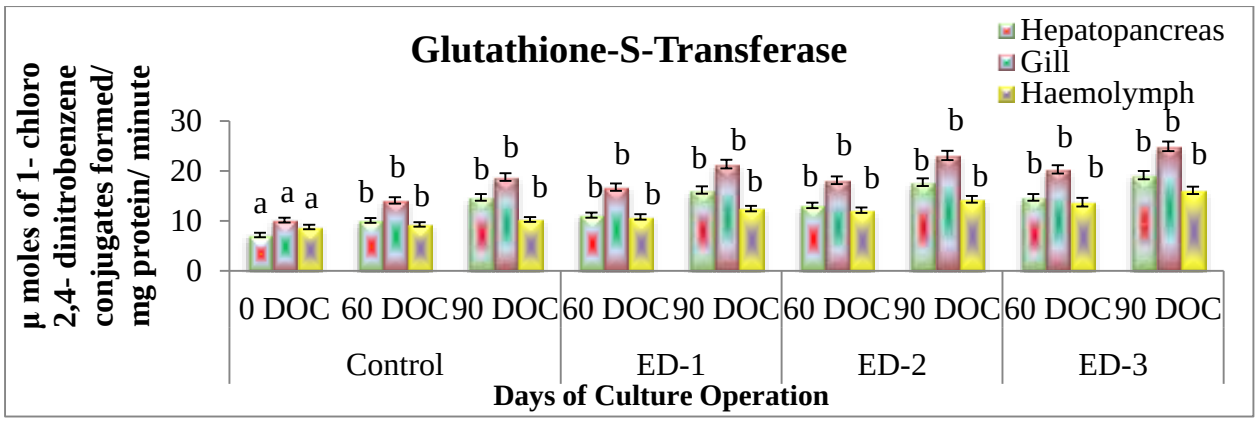

Figure. 6: Antioxidant Parameters (GST) in shrimp *Litopenaeus vannamei* under different Days of Culture Operation (DOC) – 0, 60 and 90 DOC

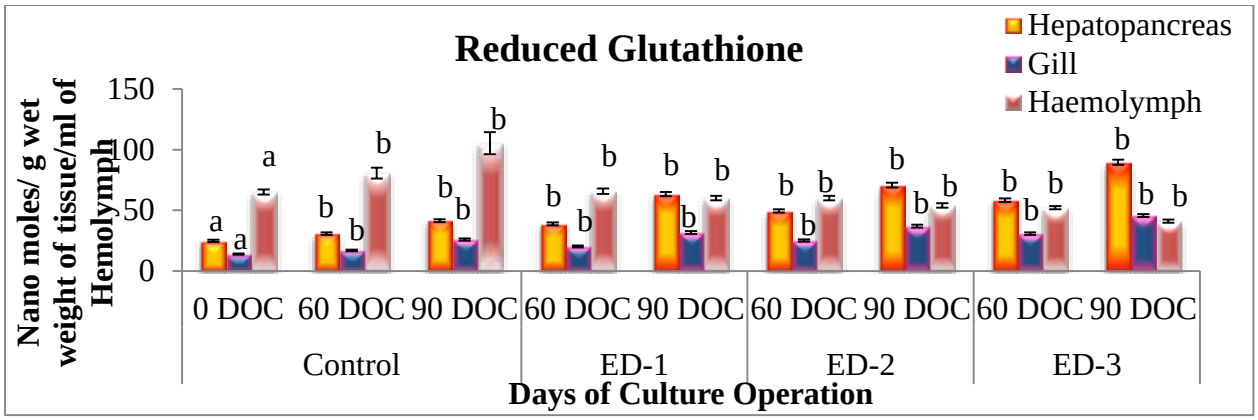

Figure. 7: Antioxidant Parameters (GSH) in shrimp *Litopenaeus vannamei* under different Days of Culture Operation (DOC) – 0, 60 and 90 DOC

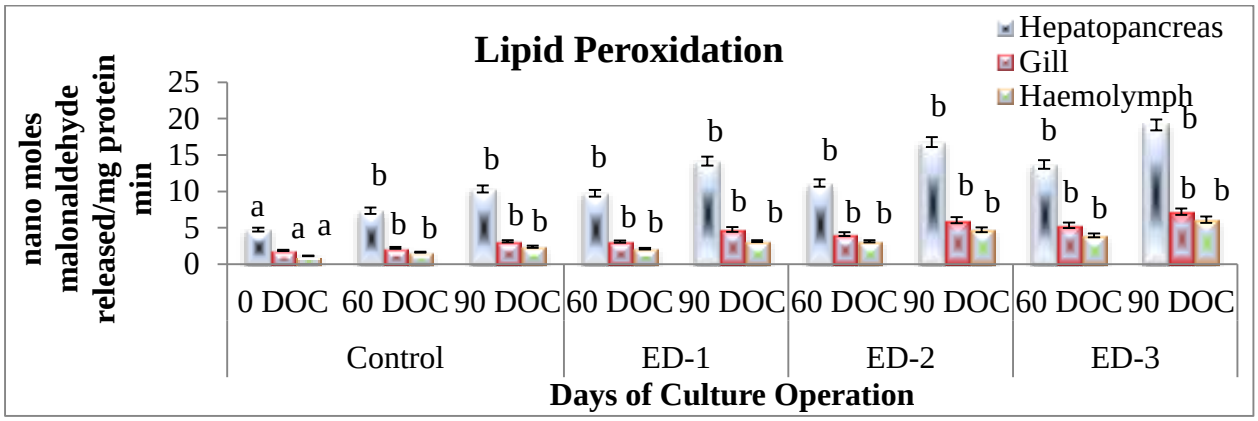

Figure. 8: Antioxidant Parameters (LPO) in shrimp *Litopenaeus vannamei* under different Days of Culture Operation (DOC) – 0, 60 and 90 DOC

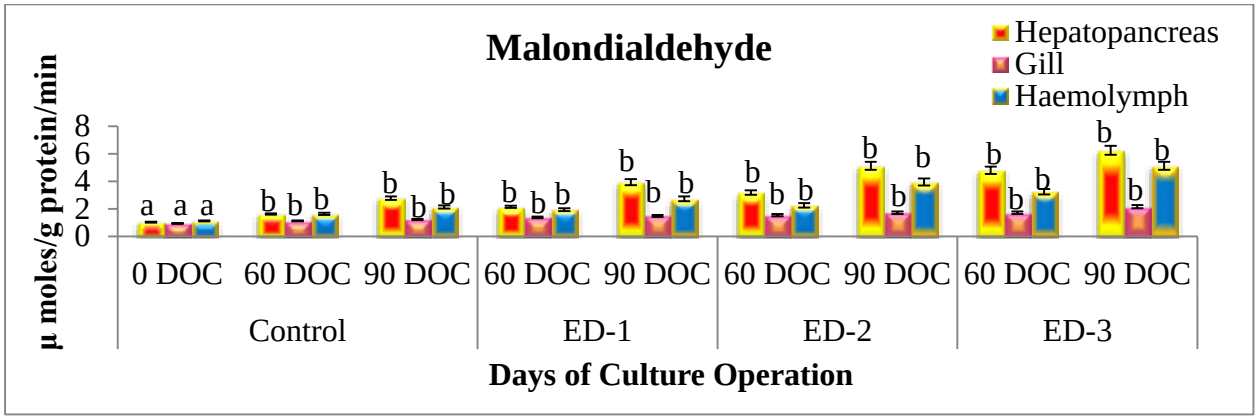

Figure. 9: Antioxidant Parameters (MDA) in shrimp *Litopenaeus vannamei* under different Days of Culture Operation (DOC) – 0, 30, 60 and 90 DOC