Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 28(4): 1259 – 1275 (2024)
www.ejabf.journals.ekb.eg

Physio-Histological Study on the White Leg Shrimp (*Litopenaeus vannamei*) Reared With Different Biofloc Ratios and Densities Levels

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ARTICLE INFO
Article History:
Received: June 27, 2024
Accepted: Aug. 3, 2024
Online: Aug. 15, 2024

Keywords:
Biofloc,
Vannamie,
Antioxidants,
Histology,
Hepatopancreas,
Gills

ABSTRACT

Biofloc systems represent a promising approach for achieving greater sustainability and productivity in aquaculture. This trial aimed to investigate the physio-histological impacts of different concentrations of biofloc on antioxidant capacities and the histology of both hepatopancreas and gills structure and abnormalities of the white leg shrimp (*Litopenaeus vannamei*). The study used three ratios of C:N (1:10, 1:15, 1:20) with three different levels of density (10, 20, 20 individuals/100L) for each ratio with triplicate for 56 days. The results of antioxidants activities showed that antioxidants (SOD, CAT, GPXs, MDH, LYZ) were significantly increased in biofloc treatments and reached its best activity in 1:15 treatment with a stocking density of 20 individuals/100L. The histological records indicated that 1:15 ratio enhanced the structure for both hepatopancreas and gills tissues. Tissues in the control and 1:10 ratio treatment seemed to be normal. In the 1:15 treatment, the hepatopancreas showed all important cells as blaze-Zellen cells (B) and embryonal Zellen cells (E) and normal arrangement of cells and normal interlamellar space for gills. With the increase of C:N ratio and stocking density, hepatopancreas showed a severe cell deformation, vacuoles, while the gills showed epithelial edema and abnormalities, particularly in 1:30 treatment, with 30 individuals/100L.

INTRODUCTION

Biofloc systems were developed in order to meet up the increasing demand for ecologically sustainable aquaculture practices. This technology relies on minimal water exchange, increased biosafety measures, and optimized small-scale regions to achieve high production rates while minimizing the discharge of effluents, containing high concentrations of nutrients, into the aquatic environment **(Krummenauer *et al.*, 2011; Samocha *et al.*, 2020)**. Biofloc systems are employed in shrimp farming to minimize or eliminate water exchange which lead to organic matter accumulation and the later

development of a concentrated microbial community, predominantly found in biofloc aggregates **(Browdy *et al.*, 2012)**. Biofloc is a complex assemblage of bacteria, fungus, microalgae, and other different organisms. These bacteria are associated with providing nutrients for cultivated species and eliminating excess nutrients **(Hargreaves *et al.*, 2013)**.

Systems that build up on no water exchange restrict the selectivity of many cultivated species to those which have ability to multiple stressors, such as high levels of water turbidity, immoderate levels of dissolved oxygen, and suspended practices **(Burford *et al.*, 2004)**. Biofoc (BF) contains a variety of important components including proteins, carbohydrates, lipids, ash, free amino acids, fatty acids, and vitamins that are necessary for aquafeed **(Crab *et al.*, 2010; Ekasari *et al.*, 2014)**. Biofloc technology (BFT) offers numerous advantages for aquaculture by improving the overall well-being of cultivated species, potentially resulting in an increased effectiveness and a sustainability within the aquaculture sector.

Shrimp farming has emerged as a crucial economic activity and the primary export industry in numerous nations where aquaculture is prevalent. This is due to its substantial contribution in meeting the demand for protein and ensuring food security. Shrimp constitutes around 16.4% of the worldwide trade in fish and fishery products in terms of value, positioning it as the second most important commercial food commodity after the tuna.
(FAO, 2022).

The white leg shrimp has recently emerged as one of the most significant crustacean species in global aquaculture sector **(Mansour *et al.*, 2022)**. The white leg shrimp can be genetically chosen as free from viral pathogens strain exhibiting a reduced vulnerability to severe viral infections **(Liao *et al.*, 2011; Castillo-Juárez *et al.*, 2015)**.

Both immunity system and antioxidant activities have a critical physiological role in shrimps through controlling their health and satisfactory growth performance under the environmental stresses **(Xu & Pan, 2014)**. Scientists have examined and utilized many types of microorganisms and its cellular and metabolic components that could be used as probiotics and/or immunostimulants to boost the innate immunity and antioxidant capacity of shrimps. This, in turn, improves their ability to resist pathogens infections **(Vazquez, 2009)**.

Studies showed that incorporating dietary biofloc into cultured shrimp diet will improve their cellular immune response and the antioxidant status of cultured shrimp; this is likely due to the abundance of natural microorganisms and bioactive substances occurring in biofloc **(Xu & Pan, 2013)**. Different authors concluded that biofloc particles are an excellent source of both lipids and fatty acids, which can enhance the nutrition of

the juvenile shrimp **(Crab *et al*., 2010)**. Furthermore, multiple studies demonstrated that, incorporating dietary biofloc into the diet of cultured shrimp can improve their immune cells response and antioxidant status. This is likely due to the plentifulness of natural microorganisms and bioactive substances found in biofloc **(Xu & Pan, 2013)**.

Histopathological procedures offer a more analytical perspective on many causes and their impact on both tissues and cells. The examination of histopathology aids ichthyopathologists in making a clear diagnosis by analyzing a tiny tissue sample from several organs. This special method affirms that the histopathological approach is not limited by laboratory configuration, selection of ecosystem, climate condition or studied aquatic species. This strategy is used on a wide range of species that differ in terms of their evolutionary relationships, age, sex, and physiology. It involves studying different tissues and doing various types of research, both in controlled laboratory settings and in natural environments. Histopathology analysis is commonly employed in trials of fish nutrition to analyze and characterize tiny changes in the tissues and cells of the digestive tract when new components are introduced or fish meal is substituted in fish feeds **(Raškovi *et al*., 2011; Randazzo *et al*., 2021)**.

When fish growth characteristics are unsatisfactory or falling behind in comparison with the control group or previously published data in the field of literature, this technique can give researchers potential explanations for this issue. Frequently, the introduction of novel components in fish feeds does not result in substantial changes in tissue pathology. However, it does affect the morphology of cells responsible for absorbing or converting molecules derived from the feeds, such as hepatocytes in the liver **(Ostaszewska *et al*., 2005)**. Their area or capacity can be measured using different histology programs, and a reduction in cell size serves as an indicator for metabolic and physiological variations in fish organs that are given alternative diets **(Raškovi´c *et al*., 2019)**.

Hence, this investigation was initiated to assess the effects of using varying denisity levels in the different biofloc -based system levels operated on the white leg shrimp, *L. vannamei* on antioxidants activities, *hepatopancreas*, and gills histology.

MATERIALS AND METHODS

Experiment animals

The white leg shrimps (*litopeneaus vannamei)* post larvae (PL12) were obtained from a hatchery located in the Diba -Damietta-Egypt triangle. This strain is considered free of diseases (SFD).

Experiment design

The experiment was conducted at the Suez Canal Aquaculture Company, Qantara Sharq -Ismailia – Egypt. Shrimps were acclimatized to controlled laboratory conditions

for 14 days, and they were received in one plastic tank equipped with suitable ventilation. Four treatments were used: The control (T1) with zero biofloc and three different biofloc C
ratios—T2 (1:10), T3 (1:15), and T4 (1:20)—with three different densities (10, 20, and 30 individuals/100 L) in polyethylene tanks for 56 days. The carbon source used was molasses.

Experiment diet

The shrimp initial weight was 0.6± 0.23g. Shrimps were then fed on a commercial diet containing 38% protein and 9.8% fat and an energy of 3980 calories/kg. The size of the feed granule was 400- 600 microns. Shrimps were hand-fed at 7.5% of their total mass four times/day (9, 11 a.m, 1, and 3 p.m.).

Table 1. Experimental diet formulation and composition

Parameter	Result	Unit
Moisture (%)	8.6	g/100g
Protein (%)	38.4	g/100g
Lipid (%)	9.5	g/100g
Ash (%)	10.7	g/100g
Starch	11.8	g/100g
Cholesterol	972	mg/kg

Water quality

The temperature was maintained at 25- 28ºC inside the tank. The tanks were filled with seawater and diluted with tap water to obtain a salinity of 30mg/ L. In the control tanks (zero biofloc), water was exchanged at a rate of 10% per day. In contrast, for the biofloc treatments, no water exchange was performed, except for adding water to compensate for evaporation.

Antioxidant activities

Hemolymph was collected from approximately 10 shrimps by extracting it from the ventral sinus. In a 1mL sterilized syringe, anticoagulant factors were mixed with about 200μL of hemolymph. The mixture was then centrifuged at 800×g for 10 minutes at 4°C. The resulting supernatant was considered as plasma samples and stored at −80°C for subsequent analysis **(Xu *et al.*, 2013)**. Catalase (CAT) activity was measured using the protocol of **Aebi (1974)**. Superoxide dismutase (SOD) activity was assessed following the method described by **Nishikimi *et al.* (1972)**. Glutathione peroxidase activity (GPXs) was measured using the "turbidimetric method" outlined by **Feng *et al.* (2016)**. Malondialdehyde (MDA) levels were determined according to the procedure of

Uchiyama *et al.* (1987). Lysozyme activity was quantified using the turbidimetric method as described by **Engstad *et al.* (1992)**.

Histological examination

Examination followed the procedure of **Dighiesh *et al.* (2019)**. By the end of the experiment, samples of the hepatopancreas, gut, and gills were collected from three separate individuals/treatments. These samples were then maintained in a fixative solution (10% formalin) for a period of 24 hours. The specimens were rapidly washed with distilled water and thereafter subjected to ethanol alcohol solutions with different concentrations of 70, 80, 90, 95, 100% for dehydration. Following this, they were left in methyl benzoite during the night, and then tissues were embedded in paraffin wax. The specimens were then blocked and sectioned to a thickness of 4µm. With Hematoxylin-Eosin (H×E), sections were stained, and then analyzed using a "Zeiss" microscope. The requested sections of inspected organs were photographed, and scaled at a scale of 40µm.

Statistical analysis

All the columns' data were subjected using the two-way analysis of variance (ANOVA) in SPSS (Version 26). Multiple comparisons and Duncan's test **(Steel & Torrie, 1980)** were employed to assess the disparities between treatments' means and to ascertain their significance at the 95% confidence interval.

Ethical approval

An ethical approval was previously received from the Faculty of Agriculture, Suez Canal University, adhering to both international and national rules for the ethical handling of animals.

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RESULTS

Antioxidants' activities are illustrated in Table (2). Based on the two-way ANOVA, a significant ($P< 0.05$) effect of the stocking densities and C/N ratios on the antioxidant enzymes activities was detected.

Based on the current outcomes, it was noticed that the activities of antioxidant enzyme increased with increasing C/N ratio. Significantly ($P< 0.05$) higher CAT, SOD, GSH and LZY values were observed in the CN15 with all different stocking densities. However, no significant difference ($P> 0.05$) was assessed in the MDA values in the control treatments and the high C/N ratios with all different stocking densities.

Meanwhile, the lower MDA values were recorded in treatments at C: N of 1:10 and 1:15. Whereas there were significantly ($P< 0.05$) increased LZY values in shrimp

samples reared at C: N (1:15) with all different stocking densities in comparison with the control treatments.

The antioxidant enzymes' activity was significantly increased ($P< 0.05$) in different stocking densities and C/N ratios compared to the control treatments, with significantly higher values for C: N, 1:15. There was a positive correlation between the different stocking densities and C/N ratios & antioxidant enzymes (CAT, SOD, GSH, MDA and LZY) ($P < 0.05$).

Table 2. The activities of antioxidants in the hemolymph of *L. vannamei* reared at different stocking densities (10, 20, 30 individuals/100L) and C/N ratios (T1: control; T2, 1:10, T2, 1:10, T3, 1:15 & T4, 1:20) for 56 days

Treatment	**Parameter**				
	CAT(U/mg)	**SOD (U/mg)**	**GSH(U/ml)**	**MDH (Nmol/gm)**	**LZY (U/gm)**
T1/10 individuals/100L	12.8±0.02^{fg}	34.55±0.31^{g}	20.02±0.56^{e}	3.11±0.5^{a}	230±2.00^{h}
T2	21±1.00^{c}	44.82±1.00^{d}	28.87±0.02^{b}	1.83±0.03^{cb}	316±2.00^{c}
T3	24.3±0.1^{a}	62.4±0.1^{b}	33.6±0.2^{a}	1.61±0.01^{c}	389±5.90^{a}
T4	23±1.00^{b}	40.2±0.1^{e}	26.30±1.00^{c}	1.82±0.02^{cb}	302±4.00^{g}
T1/ 20 individuals/100L	13.56±0.02^{fg}	34.16±0.01^{gh}	18.82±0.02^{f}	3.52±0.6^{a}	206±2.00^{i}
T2	17±2.00^{e}	42.44±0.01^{e}	25.44±0.02^{cd}	2.5±0.2^{cb}	341±1.00^{b}
T3	25.3±0.1^{a}	63.03±0.01^{a}	28.1±0.01^{b}	1.68±0.02^{cb}	385±5.00^{a}
T4	18.3±0.1^{d}	36.51±0.1^{f}	27.81±1.00^{b}	2.52±0.02^{ab}	264±5.00^{f}
T1/30 individuals/100L	14.6±0.2^{f}	33.12±0.01^{h}	16.97±0.02^{g}	3.55±0.5^{a}	197±2.45^{j}
T2	16.5±0.1^{e}	34.77±0.01^{g}	20.01±0.01^{e}	2.97±0.42^{b}	290±4.78^{d}
T3	21.5±0.01^{c}	53±0.01^{c}	25.6±1.33^{cd}	2.5±0.2^{cb}	384±4.00^{a}
T4	16.23±0.01^{e}	36.25±0.01^{f}	24.9±1.33^{d}	2.85±0.2^{ab}	275±4.00^{e}

The superscripts in each column indicate statistically significant changes ($P< 0.05$) between the biological replicates.

Histological results are displayed in Figs. (1, 2). Results of hepatopancreas architecture are shown in Fig. (1).

Control treatment (T1) with zero biofloc and three different levels of density showed a normal structure and normal arrangement of the hepatopancreas tissue's cells with normal lumen, nuclei, blaze-Zellen cells and embryonal Zellen cells, slight sloughing with normal arrangement in the hepatopancreas cells appearing in the highest density, 30 individuals/100L group.

In the biofloc treatments with different C:N ratios (1:10, 1:15, and 1:20) and three density levels (10, 20, and 30 individuals/100L for each ratio), the connective tissue exhibited gradual deformation, such as necrosis and sloughing of cells, which varied according to the C:N ratio and density level.

The hepatopancreas in T2 (1:10) showed a normal tissue arrangement in 10 individuals/ 100L density with lumen, blazenzellen cells, and embryonal zellen cells, a good appearance of connective tissue in 20 individuals/100L group.

In 30 individuals/100L group cell deformation, sloughing and some necrosis existed. In T2 (1:15), biofloc treatment tissues showed a better enhancement in lumen, blazenzellen cells, and embryonal zellen cells, a slight deformation in 20 individuals/100L group and some loss of tubules appeared in 30 individuals/100L group.

Treatment 3 of 1:20 biofloc ratio showed a gradual loss of tubules, necrosis, cell deformation according to density level, reaching its highest in 30 individuals/100L group with a total cell deformation and a severe detachment of the hepatopancreas cells.

Fig. (2) shows the histological structure of the white leg shrimp's gills. Gills in the control treatment (T1) illustrated a normal architecture with very little clubbing and melanization and normal interlamellar space in 20 individuals/100L treatment in addition to small vacuoles in 30 individuals/100L treatment.

Gills in T2 (1:15) biofloc treatment was normal at 10 individuals/100L treatment and had clubbing in 20 individuals/100L treatment; the interlamaellar space became bigger in 30 individuals/100L treatment.

Best gills arrangement was observed in T3 (1:15) biofloc treatment in 10 individuals/100L group; slight epithelial edema and deformation in both 20 and 30 individuals/100L groups were detected.

Most gills abnormalities were observed in T4 (1:20) biofloc treatment with severe edema, loss of structure and deformation in 20 and 30 individuals/100L groups.

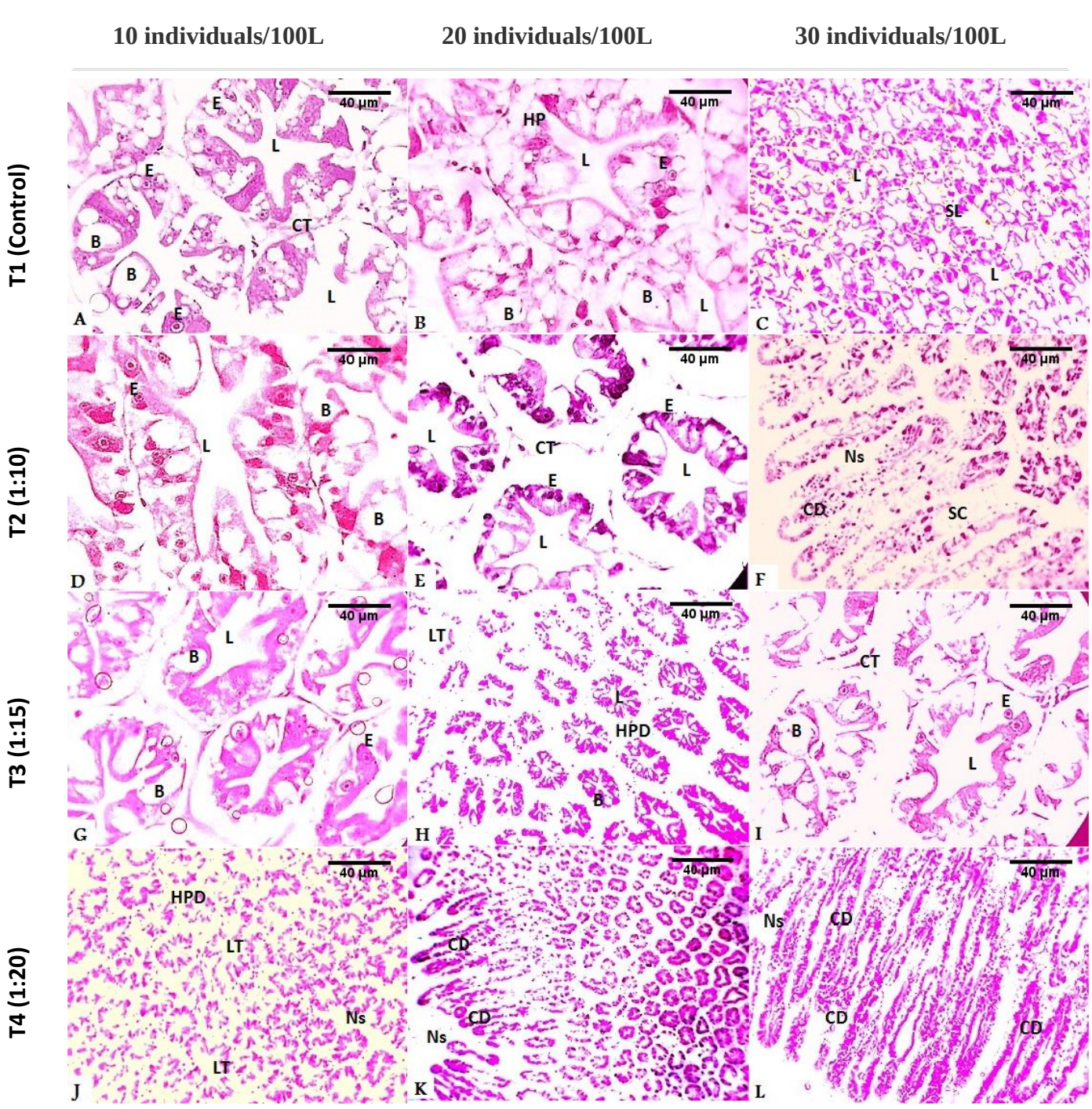

Fig. 1. Histopathological photographs of on the white leg shrimp's (*Litopenaeus vannamei*) hepatopancreas reared in different biofloc C:N ratios and densities. **(A, B, C)** control, T1. **(D, E, F)** T2, 1:10. **(G, H, I)** T3, 1:15. **(J, K, L)** T4, 1:20; with different densities 10, 20, 30 individual/ 100L for each group respectively. (H&E, 40µm). **HP:** hepatocytes; **CT:** connective tissue; **L:** lumen; **B:** blazenzellen cells; **E:** embryonal zellen cells; **SC:** sloughing cells; **N:** nuclei; **HPD:** hepatocytes detachment; **LT:** loss of tubules; **Ns:** necrosis; **CD:** cellular degeneration

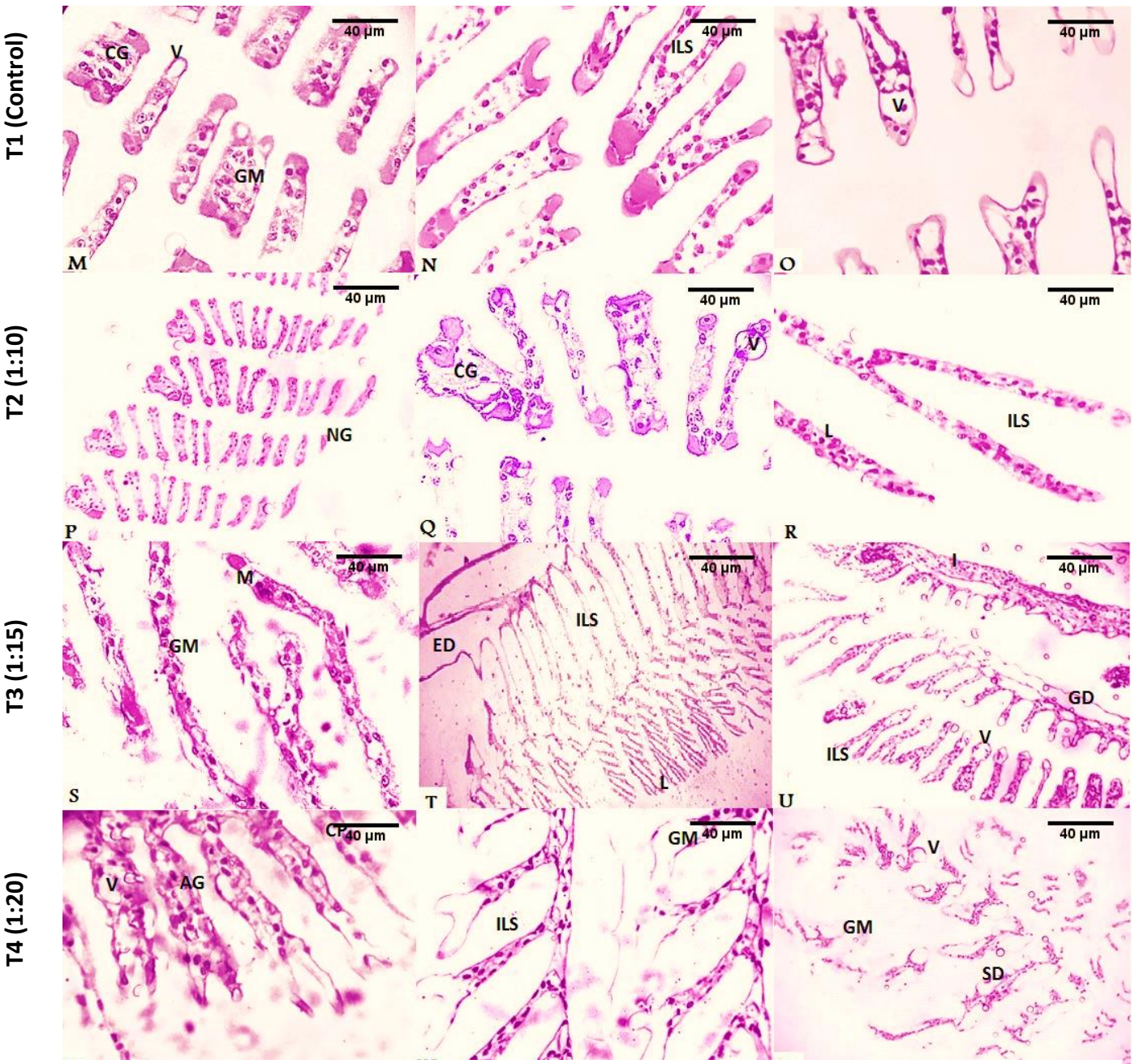

Fig. 2. Histopathological photographs of on White leg shrimp's (*Litopenaeus vannamei*) gills reared in different biofloc C:N ratios and densities. **(M, N, O)** control, T1. **(P, Q, R)** T2, 1:10. **(S, T, U)** T3, 1:15. **(V, W, X)** T4, 1:20; with different densities 10,20&30 individual/ 100L for each group respectively. (H&E, 40µm). **CG:** clubbing gills; **GM:** gill menlaniztion; **V:** Vacuoles; **ILS:** interlamaellar space; **NG:** normal gills; **AG:** abnormal gills; **SP:** cell proliferation; **SD:** sever detachment; **ED:** epithelial edema; **GD:** gill deformation

DISCUSSION

The levels of antioxidants can be a sign that provides information about the health status of shrimp **(Martins *et al.*, 2020; Shourbela *et al.*, 2021)**, and these enzymes serve as a reliable measure of oxidative stress that are caused by pathogen or environmental disruptions, and hence can provide an insight into the overall health of animals. Endogenous antioxidant enzyme such as SOD, CAT, GPX_S, MDA, and LZY, play a crucial role in protecting against oxygen toxicity by effectively combating the oxidative stress through diverse ways **(Wang *et al.*, 2018)**. Superoxide dismutase (SOD) is a radical scavenging factor that is a natural accruing distance by safeguarding active cells from harm, inhibiting the aging process, and enhancing the immune system function **(Campa-Córdova *et al.*, 2002)**. Total antioxidant capacity is an indicator of the strength of the body's antioxidants protecting system. Glutathione (GSH) is the most predominant non-protein thiol within cells that play a crucial role in preserving the equilibrium of the cellular redox state. The enzyme catalase uses hydrogen peroxide (H_2O_2) as a substrate and decomposes it into water (H_2O) and oxygen (O_2), thereby removing free radicals by neutralizing unpaired electrons **(Yin *et al.*, 2018)**. MDA is a by-product of the peroxidation of lipids, which happen when there is an unevenness in the body's antioxidant system. MDA exhibits cytotoxicity and can induce harm to the organism **(Storey, 1996)**. In this current investigation, compared to the control, SOD, CAT, and GPX levels were considerably higher in C/N 15, in accordance with the results of **Xu and Pan (2013)**, **Wang *et al.* (2015)** and **Mansour and Esteban (2017)**. These outcomes indicate that BFT has the potential to decrease the degree of lipid peroxidation in *L. vannamei* and enhance the shrimp's capacity to combat oxygen free radicals. This is a good effect on the shrimp's health, resilience to environmental stress, and survival rate. In addition, BFT has a significant abundance of natural microorganisms and biologically active growth factors, such as phytosterol, carotene, polyphenols, chlorophyll, polysaccharides, taurine, and vitamins. These components contribute as stress responses of shrimp and have a role in antioxidant mechanism **(Liu *et al.*, 2017)**.

Adverse aquaculture systems' circumstances like water quality parameters, both chemical and organic contaminants, nutrition, intensive stocking density, and diseases, always lead to an oxidative stress in shrimp **(Bakhshi *et al.*, 2018; Cavalcanti Nery *et al.*, 2019; Dawood *et al.*, 2019; Chowdhury & Saikia, 2020)**. Catalase (CAT) and superoxide dismutase (SOD) play crucial roles in the elimination of reactive oxygen species (ROS), which operate as factors of oxidative stress in fish **(Kovacik, 2017)**. SOD converts the dismutation of superoxide radicals to oxygen and hydrogen peroxide, which are then eliminated by CAT, avoiding the initiation of lipid peroxidation **(Hoseinifar *et al.*, 2021; Shourbela *et al.*, 2021)**. This investigation found slight notable variations in the CAT and SOD activities among the various stocking densities within the C/N ratio

(15:1) treatments. Shrimp reared in BFT systems showed comparable outcomes in terms of SOD activity **(Bakhshi *et al.*, 2018)**. Nevertheless, additional studies demonstrated that biofloc systems have the ability to improve SOD **(Luo *et al.*, 2014; Liu *et al.*, 2018; Zaki *et al.*, 2020)** and CAT **(Mansour & Esteban, 2017; Shourbela *et al.*, 2021)** activities in shrimp.

Shi *et al.* (2023) findings showed that the antioxidant produced from microbial source in diets enhanced the functional activity of hepatopancreas by boosting the synthesis of enzymes. Biofloc is recognized as a superior and consistent food supply that is linked to enhancing production results, reducing growing duration, ensuring higher survival rates, and promoting well-developed muscle structure **(Cardona *et al.*, 2015; Chan-Vivas *et al.*, 2019)**.

The developmental state of the hepatopancreas might serve as a vital indicator of its functional activity. The hepatopancreas plays an essential physiological function such as storing fat, absorbing nutrients, and producing digesting enzymes **(Suita *at al.*, 2015; Eissa *et al.*, 2024)**. It can be utilized to bolster alternative approaches for assessing the organism's nutritional condition. The primary functions of the hepatopancreas are the synthesis of digestive enzymes, the absorption of nutrients, and the storage of lipids **(Genc *et al.*, 2007)**. B cells are primarily responsible for producing and secreting digestive enzymes **(Felgenhauer, 1992)**. Blasenzellen (B) cells within the hepatopancreas are involved in nutrient accumulation, intracellular digestion, and the transportation of digested materials **(Silva *et al.*, 2018)**. The current findings demonstrated a significant increase in the diameter of the hepatopancreas tubules in shrimp raised in biofloc (BF) systems compared to the control group. Similarly, *L. vannamei* raised in biofloc tanks showed an increase in the thickness of the hepatopancreas tubules, along with a rise in both the quantity and size of enzyme-producing B cells, compared to the control treatment **(Suita *et al.*, 2015)**.

The application of different carbon sources (molasses or biodegradable polymers) in the systems of BFT might affect the structure and function of the hepatopancreas, and this is a result of the transcriptome alterations taking place in the hepatopancreas of *L. vannamie.* It is important to mention that molasses causes greater stress in BFT than biodegradable polymers **(Xue *et al.*, 2019)**. Our study results are corroborated with the outputs of **Moss *et al.* (2001)**, who reported that the usage of natural products in diets can result in the hepatopancreas enhancement through functional activity by promoting the synthesis of enzymes in an optimal level.

The gills of shrimps play a vital role in breathing, osmoregulation, and ionic regulation **(Mantovani & McNamara, 2021)**. The presence of microorganisms obstructing the gills and the accumulation of inorganic nitrogen compounds can have

adverse impacts on the breathing and osmoregulation of shrimp by causing damage to the structure of their organs **(Fregoso-López *et al.*, 2017; Robles-Porchas *et al.*, 2020)**.

The higher-density group had noticeable abnormalities in gill architecture, including vacuole lamellae, hemocytes accumulation, aberrant gill tips, clubbing lamellae and malformation of gill tips. Shrimp mortality in intensive stocking density aquaculture systems, specially without water exchange, is associated with gill blockage and elevated levels of particles suspended in the water column **(Schveitzer *et al.*, 2013; Fregoso-López *et al.*, 2017)**. **Fregoso-López *et al.* (2017)** explained that higher stocking density consistently cause significant damage in shrimp gills, which relates to histological alterations and ultimately contributes to shrimp mortality.

CONCLUSION

The findings of this study demonstrated that the biofloc system possesses a beneficial impact on the health of the while leg shrimp when implemented at appropriate ratios and densities. Physiological and histological indicators are suitable to explain these effects, and it is advisable to conduct a large-scale study to confirm the finding of the current study.

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