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Effect of Agricultural By-Products as a Carbon Source in a Biofloc-Based System on Growth Performance, Digestive Enzyme Activities, Hepatopancreas Histology, and Gut Bacterial Load of *Litopenaeus vannamei* Post Larvae

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Abstract: The present study evaluated the influence of different commercial agricultural by-products as a carbon source in a bifloc-based (BFT) culture system on growth performance, whole-body proximate composition, digestive enzyme activities, gut microbial abundance, and hepatopancreas histology of Pacific whiteleg shrimp, *Litopenaeus vannamei* post larvae (Pls). Three groups were designed, the first group was the control group, where the shrimp was reared in clear water (without carbon source addition and water exchange rate of 100% two times a week) and fed with a commercial diet, in the second and third groups shrimp were reared in BFT systems using two different carbon sources, sugarcane bagasse (SB) and rice bran (RB) without additional feeding or water exchange. The initial stocking density was 16 Pls/liter with an average individual shrimp weight of 0.01 ± 0.002 g and age (PL20). The experiment lasted 90 days. The water quality parameters were maintained at optimum levels during the experiment. The final body weight and specific growth rate of shrimp were significantly ($p \leq 0.01$) higher in the control group than those reared in both SB and RB-based BFT. Meanwhile, the survival rate was significantly ($p < 0.05$) higher in BFT groups than in the control. The protease activity in shrimp stomach did not differ significantly. Meanwhile, protease, lipase, and amylase in the intestine showed a significant increase ($p < 0.01$) in BFT groups than those obtained in the control group. The total heterotrophic bacteria were significantly ($p < 0.05$) higher in BFT groups. Furthermore, the hepatopancreas histological status of shrimp reared in the SB-based BFT group showed an increase in the hepatopancreas tubules in the distal and B-cell zones (blister-like cells) by 16.83 and 34.89%, respectively, compared to the control. This study revealed that BFT could be used as a natural feed without artificial diets, which influenced the gut microbiota of shrimp, increased digestive enzyme activities, as well as improved the histological structure of the hepatopancreas of shrimp. However, the success of this conditions under high stocking density still needs more investigation.

Keywords: Pacific whiteleg shrimp; BFT-based system; agriculture by-product; gut health; zero artificial diet

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1. Introduction

The cultivation of shrimp becomes an important commercial sector of aquaculture, contributing to nutritional security, livelihoods, and national income through export activities in developing countries [1,2]. Global shrimp production has grown substantially over the past decade. Farmed shrimp and prawn output climbed from 3,400,458 Mt in 2008 to 6,004,353 Mt in 2018, accounting for 7.3 percent of worldwide aquaculture production in 2018 [3]. Pacific whiteleg shrimp, *Litopenaeus vannamei*, has recently become one of the world's most significant farmed crustacean species [4]. In 2018, this species accounted for 53 percent of all farmed crustacean output [3]. Farming activities of the marine shrimp have been largely affected by diseases, particularly viral diseases like the white spot syndrome virus [3]. Interestingly, Pacific whiteleg shrimp, *L. vannamei*, could be genetically selected as viral-pathogen-free and has lower susceptibility to serious viral pathogens [5,6].

Biofloc technology (BFT) was created to fulfill the rising need for environmentally friendly aquaculture methods. This technique depends on the little water renewal, high stocking densities, increased biosafety, and the utilization of smaller areas to provide high production rates and fewer nutrient-rich effluents discharged into the aquatic environment [7,8]. Since the early 1970s, the principle of BFT has been recognized. However, since the early 1990s, extensive research on the development and implementation of BFT has been conducted, with an emphasis on shrimp culture [9,10]. Ammonia is eliminated from the culture system that can be recycled into additional food sources for farmed aquatic animals in the BFT [11,12]. The BFT is used in shrimp production with little or no water exchange, leading to the build-up of organic substrates and the subsequent formation of a dense microbial population, which is largely aggregated in biofloc [8,13]. Biofloc is an aggregated organism made up of bacteria, fungus, microalgae, detritus, and other species. These microorganisms have been linked to the nutritional supply of cultivated species as well as the removal of surplus nutrients [14–17].

The beneficial effects of BFT on intensive shrimp culture include a source of macro and micronutrients, water quality control, survival enhancer, promotion of growth, and prevent viral infections [13,18–22]. Microbial aggregates are formed through the manipulation of the carbon/nitrogen ratio of water [23,24], which contains heterotrophic bacteria, protozoa, rotifers, microalgae, dead organisms, and feces [25,26]. This supplement provides a large source of protein for the shrimp and permits the recycling of the nitrogen waste and leached nutrients from the feed [27,28].

One of the main factors affecting biofloc characteristics is the carbon source, which usually differs in carbon content and degradability. For that, some sources were classified as good sources in biofloc production, which promote fast ammonia removal and high biofloc volume [9,29]. The commonly used carbon sources in shrimp culture include dextrose, sugarcane molasses, glucose, sucrose as simple sugars and rice bran, wheat bran, starches wheat flour, gram flour, rice flour, and cornflour as complex sugars [26,30–33]. In addition, glucose and molasses as carbon sources revealed higher performance than starch of *L. vannamei* shrimp during grow-out culture [34]. Moreover, some wastes and by-products were used in BFT systems for shrimp and other aquatic animals, such as tapioca by-products with superior growth and survival of shrimp [35], wheat milling by-products with Nile tilapia [36]. There is a focus on the use of low-cost agriculture by-products as carbon-source wastes in order to establish BFT systems in shrimp farms and reduce the competition with human foodstuffs.

The heterotrophic bacteria (THB) in closed prawn systems have been identified [37], however, the effect of carbon sources on microbial community composition is still not completely revealed. A clear water system and a BFT system were compared in terms of the water bacterial community and gut microbiota, demonstrating that BFT can alter the shrimp intestinal bacteria composition [38]. In addition, the effect of carbohydrate sources (maida flour, wheat flour, gram flour, millet flour, rice flour, cornflour, and multigrain flour) were investigated as alternatives carbon sources to molasses. The bacterial dynamics were significantly affected by the addition of millets and multigrain flour whereas THB increased

and total Vibrio count (TVC) was decreased compared to the control [30]. Furthermore, specific digestive enzyme activities in the hepatopancreas of *L. vannamei* was affected by carbon sources and increased more than in shrimp reared in clearwater [32]. The expression of digestive enzyme-related genes was increased in *Litopenaeus vannamei* reared in BFT-based systems [39]. Meanwhile, using Poly-β-hydroxybutyric acid instead of glucose as a carbon source reduced the activity of intestinal lipase in Nile tilapia, *Oreochromis niloticus* [40].

According to the best of our knowledge, the use of biofloc as a sole food source in shrimp rearing ponds is not completely studied or validated and the only reported study was carried out on pink shrimp, *Farfantepenaeus brasiliensis*, which revealed the possibility of using biofloc as a food source without addition of commercial diet [41]. Therefore, the present study was designed to evaluate the effects of using different carbon sources (sugarcane bagasse and rice bran) in the BFT-based system operated without artificial feed on water quality parameters, gut bacterial abundance, digestive enzymes, hepatopancreas histology, and growth performance of the Pacific white-leg shrimp, *L. vannamei*.

2. Materials and Methods

2.1. Biofloc Setup

The experiment was carried out at the National Institute of Oceanography and Fisheries, Suez Branch (NIOF-Suez) in the outdoor cement tanks (3.5 m width × 7 m length × 1.5 m depth). The experimental design consisted of three groups, the first two groups were fed on biofloc produced using commercial sugarcane bagasse (SB) or rice bran (RB) as organic carbon sources, while the third group had clear water (without carbon addition and water exchange rate of 100% two times a week of sea water) and was fed with a commercial diet as a control. In the current study, the biofloc were ex-situ prepared in two fiberglass tanks (200 L each) filled with a commercial shrimp, *L. vannamei*, wastewater, which was fed on a commercial diet containing 38% protein at a rate of 5%. In each 200 L tank (fermenter) external carbon source of SB and RB, one source for each tank was added to adjust the C:N ratio at 16:1, as described by Avnimelech [42]. Subsequently, the produced biofloc were added into BFT treatments according to the respective treatment to serve as natural shrimp feed, whereas 30% of the fermenter was harvested daily and used to feed the shrimp. In fermenters, continuous aeration was applied to prevent the biofloc sedimentation and biofloc volume was determined weekly using the Imhoff cone, registering the volume of the biofloc in 1000 mL of each water tank after 15–20 min sedimentation according to Avnimelech and Kochba [10]. The experimental design and biofloc volume (ml L^{-1}) for each carbon source was presented in Table 1 and the proximate biochemical analysis of carbon sources was presented in Table 2.

Table 1. Experimental design and rearing conditions of Pacific white-leg shrimp, *L. vannamei*.

Treatments	Culture System	Biofloc Volume (ml L^{-1})	C/N Ratio	Water Exchange Rate	Commercial Feed and Feeding Rate
Control	Clearwater	0	0	100% two times a week	Gradually decreasing level from 10% to 3%
Sugarcane bagasse (SB)	Biofloc (SB)	21.50 ± 1.25	16:1	Zero water exchange	None
Rice bran (RB)	Biofloc (RB)	22.20 ± 0.75	16:1	Zero water exchange	None

Table 2. Proximate biochemical analysis (as % of dry weight basis) of different carbon sources utilized in the current study.

Treatments	Moisture	Protein	Lipid	Carbohydrate	Fiber	Ash
SB	45.34 ± 0.83	1.5 ± 0.01	1.5 ± 0.01	24.4 ± 0.03	65.0 ± 0.01	7.6 ± 0.02
RB	11. 22 ± 0.45	14 ± 0.03	15 ± 0.01	54.8 ± 0.02	8.2 ± 0.02	8 ± 0.01

2.2. Experimental Design

The post larvaes (PLs) of *L. vannamei* were obtained from a commercial shrimp hatchery in the Al-Deba triangle, Damietta, Egypt. Shrimp was acclimated in two indoor fiberglass tanks (2000 L/tank) for two weeks at a constant temperature (28–29 °C), salinity (30–32 ppt) [43], and pH (8–8.2), and continuous aeration. During this period, PLs were fed twice a day with a commercial dry diet obtained from Skretting, EG, containing (crude protein of 38%, fat of 8%, crude fiber of 5.9%, and 3980 Kcal of energy). All experimental tanks were aerated and mixed continuously using a two-horse air blower (IE4 Serial, GOORUI Co., Dongguan, China) to maintain the growth of total heterotrophic bacteria and prevent its accumulation in the tanks, which could cause loss of the biofloc community. The three experimental groups were stocked in triplicates at densities of 16 PL/L with an average weight of 0.01 ± 0.002 g and age (PL20). Shrimp in the control treatment were fed with a commercial diet (crude protein, 38%) at 10% of initial weight and adjusted according to the change of body weight and gradually reduced to 3% at the end of the experiment. In the tanks of the control group, water was exchanged 100% two times/week, while BFT treatments (SB and RB) were maintained for 90 days without any water exchange (zero water exchange), except for the addition of dechlorinated fresh water to compensate for evaporation losses to maintain salinity (32 ppt). Throughout the experimental period, water temperature, salinity, pH, total ammonia nitrogen, nitrite (NO_2-N) and nitrate (NO_3-N) were monitored to maintain water quality at the optimum range for shrimp. These parameters were measured daily between 09:00 and 10:00 am using Lovibond® Water Testing Senso Direct 150 multi-parameters.

2.3. Shrimp Growth Performance

At the end of the feeding trial, shrimp were starved for 1 day to empty the digestive tract [44]. Then, the number and total weight of shrimp in each tank were obtained to calculate weight gain (WG), specific growth rate (SGR), and survival rate (SR) using the given formulas below:

$$\text{WG (g)} = \text{FBW} - \text{IBW} \quad (1)$$

where, FBW and IBW are final and initial body weight (g), respectively.

$$\text{SGR}\ (\%,\ \text{day}) = 100 \times \left(\frac{\ln\ \text{FBW} - \text{In IBW}}{\text{t}} \right) \quad (2)$$

where, ln = natural logarithmic; and t = time in days.

$$\text{SR}\ (\%) = 100 \times \frac{\text{Final number of shrimp}}{\text{Initial number of shrimp}} \quad (3)$$

2.4. Determination of Whole-Body Proximate Composition

To determine the whole-body proximate composition, according to the aforementioned standard methods [45], samples of 5 intact shrimp from each tank (15 shrimp per group) were randomly selected and kept at −20 °C. Briefly, the moisture content was determined by oven drying for 2 h at 120 °C. Crude protein was estimated using an auto Kjeldahl system (Kelplus, DXVA, Pelican Equipments, Chennai, India) and the factor of N × 6.25 [46]. Crude lipid was determined by the ether extraction method using a Soxtec system (Socs

plus, SCS-6, Pelican Equipment, Chennai, India) [47]. In a muffle furnace, ash content was determined by incineration at 600 °C for 2 h.

2.5. Determination of Digestive Enzyme Activities

At the end of the experimental period, three animals were randomly sampled from each replicate (9 shrimp per group) for the measurement of the digestive enzyme activities (Proteases, Lipase, and Amylase) in the stomach and intestine after Euthanasia using clove oil 200 mg/L (El-captain for extracting natural oils, plants and cosmetics directors, Cairo, Egypt). The shrimp was dissected, and samples of the stomach and intestine were placed on sterile tubes, and immediately stored at −80 °C until analysis.

For proteases, the activity was determined by the casein digestion method [48]. Briefly, 1% (*w*/*v*) casein was dissolved in 0.05 M Tris phosphate buffer (pH 7.8) and incubated for 5 min at 37 °C. Tissue homogenate was added to the reaction mixture, and the reaction was halted 10 min later by adding 10% trichloroacetic acid (TCA), followed by filtration of the whole contents. The reagent blank was created by adding tissue homogenate to the reaction right before halting it with TCA and no incubation. The quantity of enzyme required to release acid-soluble fragments equivalent to D 0.001 A 280 per minute at 37 °C and pH 7.8 was defined as one unit of protease activity.

For amylase, the activity was measured with 2% (*w*/*v*) starch solution as a substrate following the method of Rick and Stegbauer [49]. Briefly, in phosphate buffer, a 2% starch solution was prepared (pH 7). The reaction mixture was incubated at 37 °C for 30 min. The process was then stopped by adding dinitrosalicylic acid (DNS) and keeping it in a hot water bath for 5 min. The reaction mixture was diluted with distilled water after cooling, and the absorbance was measured at 540 nm. At 37 °C, the activity was calculated using the maltose standard curve and reported as a mole of maltose released from starch per minute per mg protein.

Meanwhile, lipase activity was determined based on the method of Cherry and Crandall Jr [50]. The amount of NaOH necessary to maintain a steady pH is used to calculate the amount of fatty acid emitted per unit of time. Distilled water, tissue homogenate, phosphate buffer solution (pH 7), and olive oil emulsion made up the reaction mixture. The mixture was thoroughly mixed before being incubated at 4 °C for 24 h. Then 95% alcohol and 2 drops of phenolphthalein indicators were added, and the mixture was titrated against 0.05 N NaOH until a persistent pink color appeared. An enzyme source that had been inactivated before the addition of the buffer and olive oil emulsion was used as a control. The milliequivalent of alkali consumed is used to determine the enzyme's activity.

2.6. Total Bacterial Counts

The total heterotrophic bacteria (THB) and total *Vibrio* bacteria (TVC) count were determined in the gut of shrimp at the 30th, 60th, and the end of the experiment (9 shrimp per treatment). The isolation of bacteria was performed using the serial dilution technique according to Draper and Smith [51]. Briefly, 1 g of gut samples was collected in a sterile polypropylene bottle and subsequently folded to 10-fold serial dilution. Then, 1 mL of appropriate dilutions was applied in duplicates over the plates of trypticase soy agar containing 1.0% *w*/*v* NaCl and thiosulfate citrate bile salts sucrose agar (HiMedia, Mumbai, India) for THB and TVC count [52]. Levels of viable heterotrophic bacteria were determined by counting the colonies that grew on trypticase soy agar plates and thiosulfate citrate bile salts sucrose agar supplemented with (50:50) marine water: distilled water [53]. Each colony in the incubated plates (at 37 and 38 °C for 24 h, respectively) was counted in the range of 30–300 colony-forming unit (cfu). The percentage of TVC/THB was determined using the following formula [54]:

$$\text{TVC/THB } (\%) = 100 \times \frac{\textit{Total vibrio count}}{\textit{Total bacterial counts}} \quad (4)$$

2.7. Histological Status

At the end of the experiment, hepatopancreas of the same nine shrimp that Euthanasia using clove oil and dissected for digestive enzyme determination were used. Hepatopancreas samples were collected/for each group in 10% phosphate-buffered formalin for at least 48 h. The fixed specimens were processed by the conventional paraffin embedding technique, sectioned at 4–5 µm thick sections, and finally stained with hematoxylin and eosin stain (H & E) as previously described Suvarna, et al. [55]. The diameters of hepatopancreas tubules were measured according to [56], using Image-J analysis software, US National Institutes of Health, Bethesda, MD, USA http://rsb.info.nih.gov/ij/ (accessed on 15 October 2021).

2.8. Statistical Analysis

All data were expressed as mean ± SD. The normal distribution was checked before the statistical analysis to assure the parametric test assumption. The percent data were transformed to arcsin before the analysis but expressed in the results as the original value. The one-way analysis of variance (ANOVA) was performed using the SAS v9.0.0 (2004) program. Tukey post hoc tests at a $p < 0.05$ level of significance were used to compare differences among treatments.

3. Results

3.1. Water Quality

During the experimental period, all determined water quality parameters of temperature (28.18 ± 0.5 °C), salinity (32.11 ± 0.4 ppt), pH (7.59 ± 0.4), total ammonia nitrogen (0.11 ± 0.01 mg L^{-1}), NO_2-N (0.16 mg/L), and NO_3-N (0.29 mg/L) were maintained within the range of these optimal conditions for the production requirements of *L. vannamei* (Table 3).

Table 3. Effects of different carbon sources in BFT-based system on water quality of the Pacific whiteleg shrimp, *Litopenaeus vannamei*.

Treatments	Temperature (°C)	Salinity (ppt)	pH	TAN (mg/L)	NO_2-N (mg/L)	NO_3-N (mg/L)
C	28.23 ± 0.81	32.13 ± 0.24	7.59 ± 0.21	0.152±0.05	0.167 ± 0.03	0.298 ± 0.01
SB	28.08 ± 0.73	32.14 ± 0.19	7.54 ± 0.25	0.089 ± 0.05	0.160 ± 0.04	0.288 ± 0.02
RB	28.24 ± 0.76	32.07 ± 0.33	7.65 ± 0.18	0.091 ± 0.04	0.154 ± 0.03	0.291 ± 0.02

C: control treatment of clear water without carbon source, SB: treatment contains sugarcane bagasse as organic carbon sources, RB: treatment contains rice bran as organic carbon sources.

3.2. Growth Performance

The growth performance of *L. vannamei* was illustrated in Table 4. The FBW, WG, and SGR% were significantly ($p < 0.01$) higher in the control group than BFT treatments (SB and RB). On the other hand, SR was significantly ($p < 0.05$) higher in the BFT treatments (SB and RB) than in the control group. In addition, the growth performance and SR (%) of the RB group were higher than the SB group.

Table 4. Effects of different carbon sources in BFT-based system on growth performance and survival of the Pacific whiteleg shrimp, *Litopenaeus vannamei*.

Treatments	Final Body Weight (g)	Weight Gain (g)	Specific Growth Rate (%/day)	Survival (%)
C	11.41 ± 0.09 ^{a}	11.40 ± 0.01 ^{a}	9.61 ± 0.01 ^{a}	51.33 ± 1.35 ^{b}
SB	8.84 ± 0.05 ^{c}	8.83 ± 0.08 ^{c}	9.33 ± 0.01 ^{c}	58.06 ± 5.86 ^{a}
RB	9.57 ± 0.09 ^{b}	9.56 ± 0.04 ^{b}	9.42 ± 0.01 ^{b}	62.22 ± 4.11 ^{a}

C: control treatment of clear water without carbon source, SB: treatment contains sugarcane bagasse as organic carbon sources, RB: treatment contains rice bran as organic carbon sources. Different letters (a > b > c) in the same column are significantly different ($p < 0.05$).

3.3. Whole-Body Proximate Composition

Table 5 shows the whole-body proximate composition of *L. vannamei* reared at different BFT treatments (SB and RB) and the control group. No significant differences were observed in crude protein and moisture contents among shrimp in all groups. The crude lipid (%) of shrimp reared at SB was significantly ($p < 0.05$) higher than shrimp fed on other groups (RB and C). Shrimp reared in BFT groups had higher ash content than the control group.

Table 5. Effects of different carbon sources in the BFT-based system on the whole-body proximate chemical composition of *L. vannamei*, at the end of the experiment (% on a dry weight basis).

Treatments	Moisture (%)	Crude Protein (%)	Crude Lipid (%)	Ash (%)
C	69.18 ± 3.79	64.83 ± 0.42	16.55 ± 0.92 ^{b}	12.05 ± 0.53 ^{b}
SB	75.42 ± 3.17	61.66 ± 3.04	20.07 ± 4.68 ^{a}	14.08 ± 0.39 ^{a}
RB	74.77 ± 3.44	61.92 ± 1.15	17.26 ± 1.36 ^{b}	15.93 ± 1.35 ^{a}

C: control treatment of clear water without carbon source, SB: treatment contains sugarcane bagasse as organic carbon sources, RB: treatment contains rice bran as organic carbon sources. Crude protein, crude lipid, and ash percentages were calculated based on the dry weight content. Different letters (a > b) in the same column are significantly different ($p < 0.05$). The absence of letters in the same column means that there are no significant differences.

3.4. Digestive Enzymes Activities

The activities of different digestive enzymes of proteases in the stomach and proteases, lipase, and amylase were evaluated in the shrimp intestine at the end of the experimental period. In the case of the shrimp stomach, the protease activities did not differ significantly among different treatments. However, the protease activities in shrimp reared in the RB-BFT group tended to increase more than those of SB and C (Figure 1A). In the case of the shrimp intestine, the activities of the digestive enzymes in the BFT groups were significantly ($p < 0.01$) higher than those in the control group. The shrimp reared in the RB group achieved the highest significant digestive enzymes (proteases, lipase, and amylase) activities than those fed SB and C (Figure 1B).

3.5. Microbial Analysis

The microbial abundance over the experimental period among the studied groups was presented in Figure 2A–C. Overall, the addition of carbon sources increased the THB load in the gut of shrimp. Figure 2A showed that the THB count significantly ($p < 0.05$) increased in a time-dependent manner. At all experimentally tested times (30 days, 60 days, and 90 days) the THB count in BFT groups (SB and RB) was significantly higher than in the control group. The TVC count in the RB group was significantly ($p < 0.001$) higher than in the SB group during the different tested times (Figure 2B). The control group had a lower TVC/THB ratio than the BFT groups (Figure 2C) during the experimental period and the lowest TVC/THB ratio was recorded on the 90th day of the experiment.

3.6. Histology of Hepatopancreas

The histological investigation and histometric indices of the hepatopancreas of *L. vannamei* were presented in Figures 3 and 4 as affected by different carbon sources BFT-based system. Hepatopancreas of the control group composed of tubules each is subdivided into the distal zone (DZ), B-cells zone (BZ; blister-like cells), and proximal zone and is lined by a single-layered epithelium and separated by hemolymph sinuses (HS). The tubules in DZ include embryonic cells (E-cells) at the tubular tips. The tubules in BZ are characterized by B-cells with prominent vacuoles. The diameter of the hepatopancreas tubules in the distal zone was 88.04 ± 4.47 µm, while in the B-cell zone is 108.15 ± 6.20 µm.

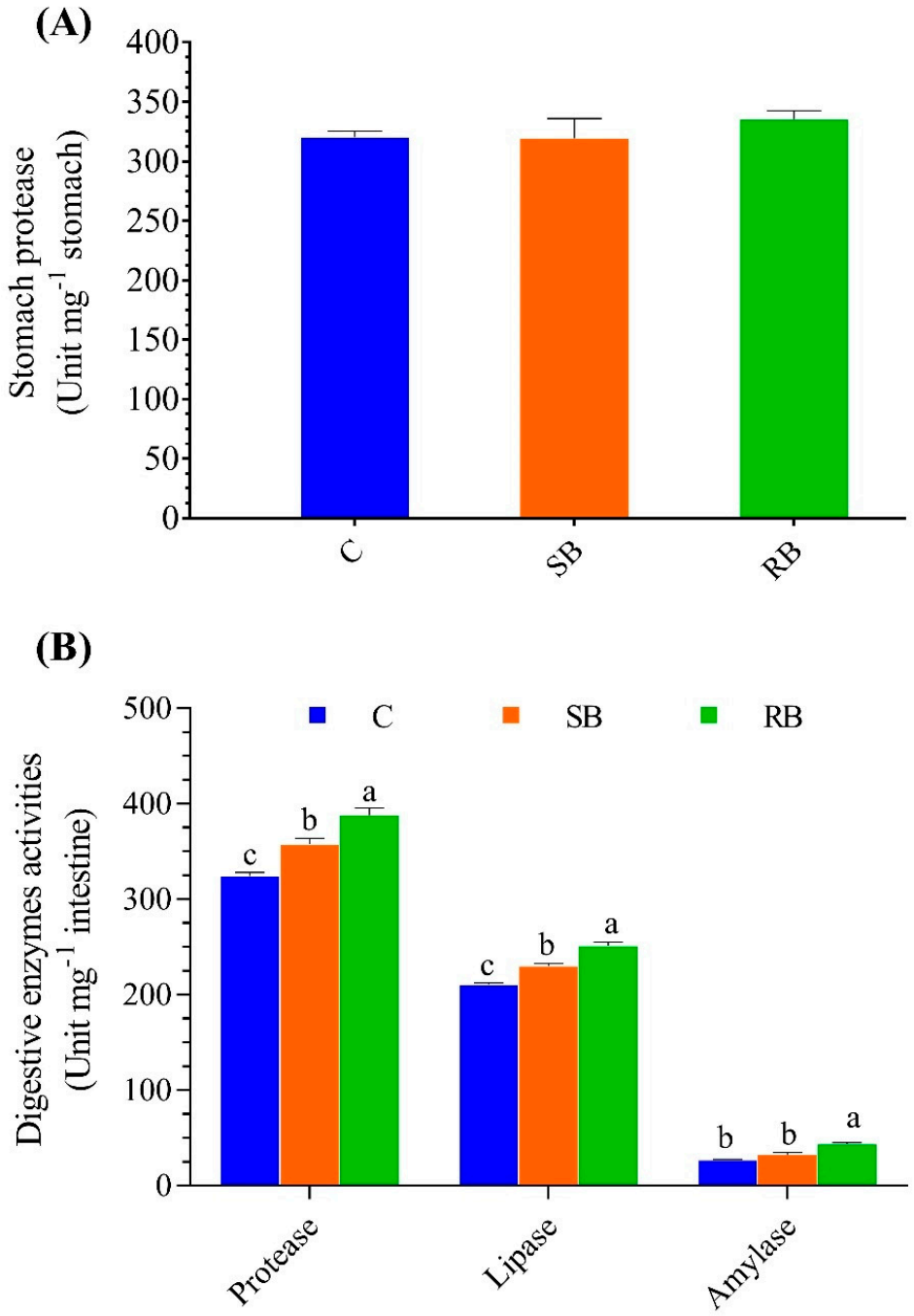

Figure 1. Effects of different carbon sources in BFT-based system on digestive enzymes (proteases, lipase, and amylase) activities of *L. vannamei* stomach (**A**) and intestine (**B**). C: control treatment of clear water without carbon source, SB: treatment contains sugarcane bagasse as organic carbon sources, RB: treatment contains rice bran as organic carbon sources. Different letters (a > b > c) in the same enzyme indicate significantly different ($p < 0.05$).

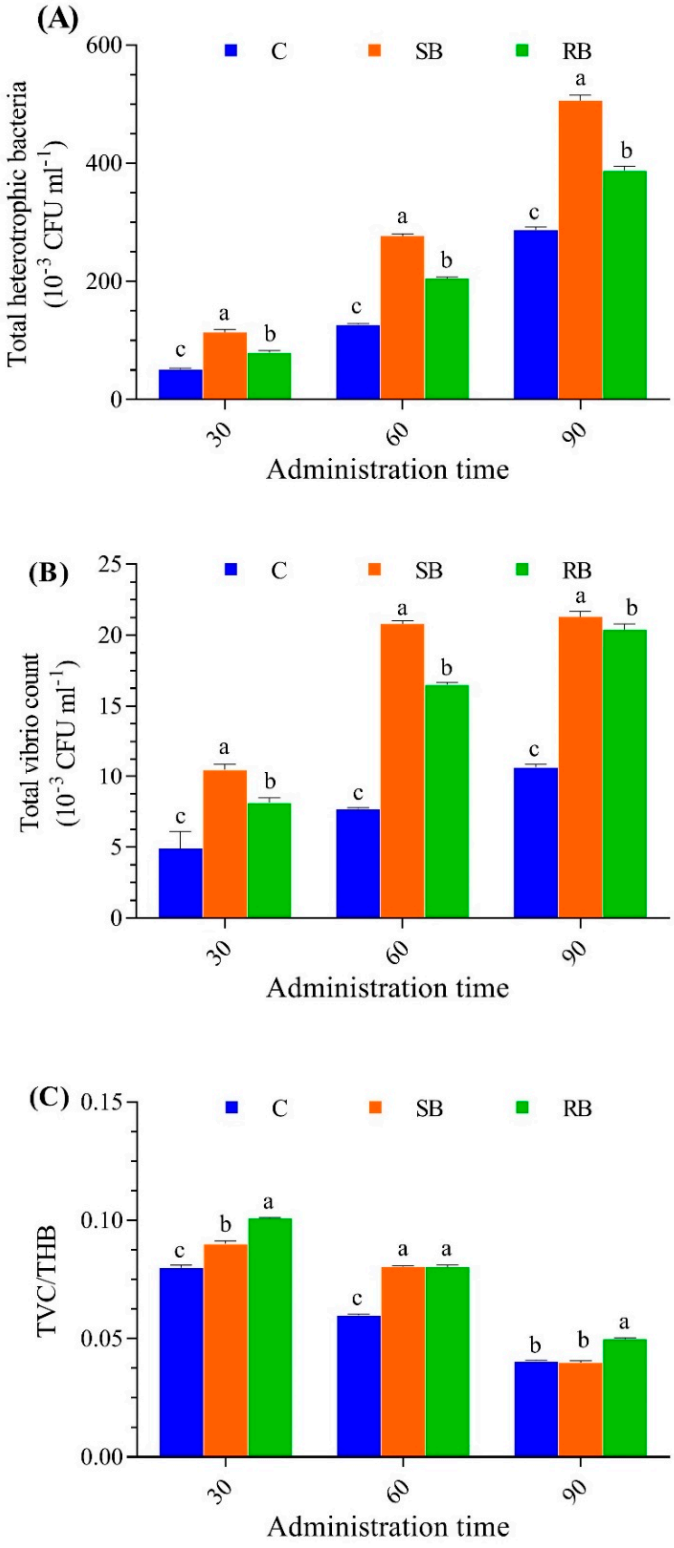

Figure 2. Effects of different carbon sources in BFT-based system on (**A**) total heterotrophic bacteria (10^3 CFU mL^{-1}); (**B**) Total *Vibrio* count (10^3 CFU mL^{-1}); and (**C**) the ratio between TVC and THB in the gut microbial of *L. vannamei.* C: control treatment of clear water without carbon source, SB: treatment contains sugarcane bagasse as organic carbon sources, RB: treatment contains rice bran. Different letters (a > b > c) in the same enzyme indicate significantly different ($p < 0.05$).

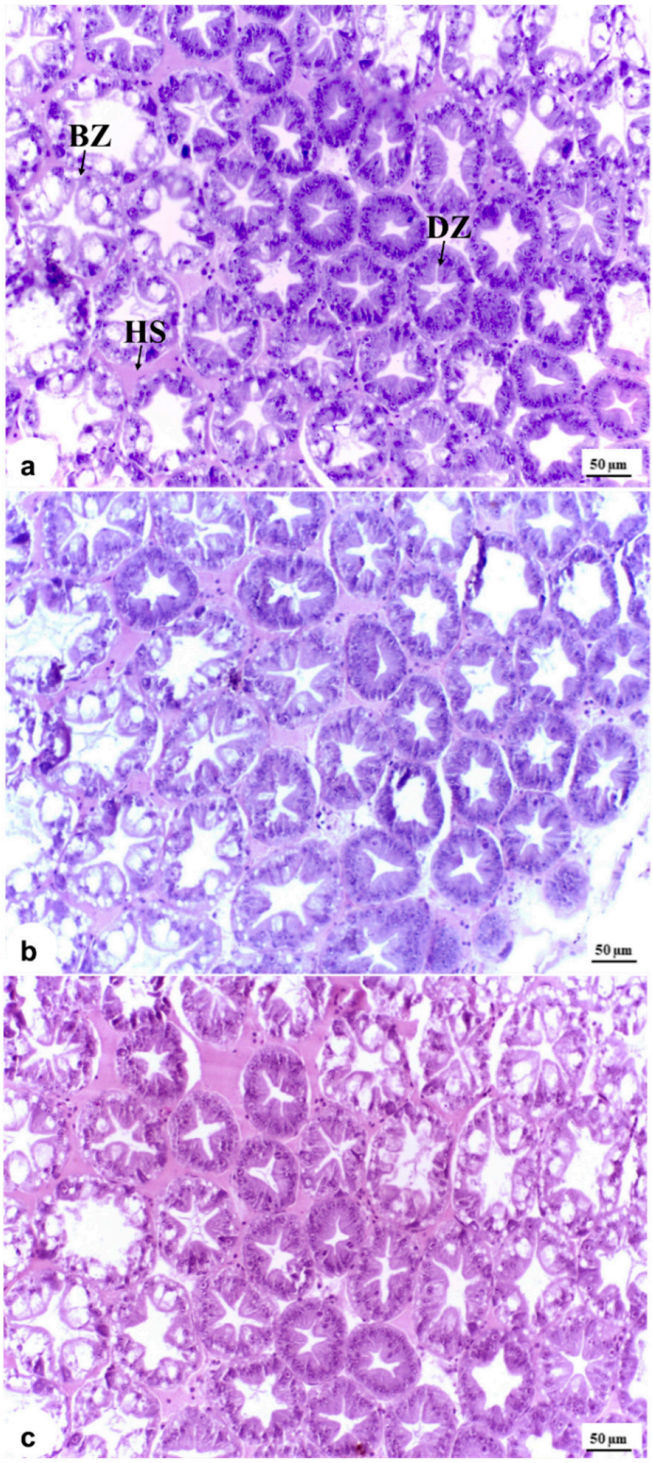

Figure 3. Effects of different carbon sources in BFT-based system on histology of hepatopancreas of *L. vannamei* stained with H&E. The scale par is 50 μm. (**a**): control treatment of clear water without carbon source, (**b**): treatment contains sugarcane bagasse as organic carbon sources, (**c**): treatment contains rice bran asorganic carbon sources, BZ: B-cell zone, DZ: distal zone, HS: hemolymph sinuses.

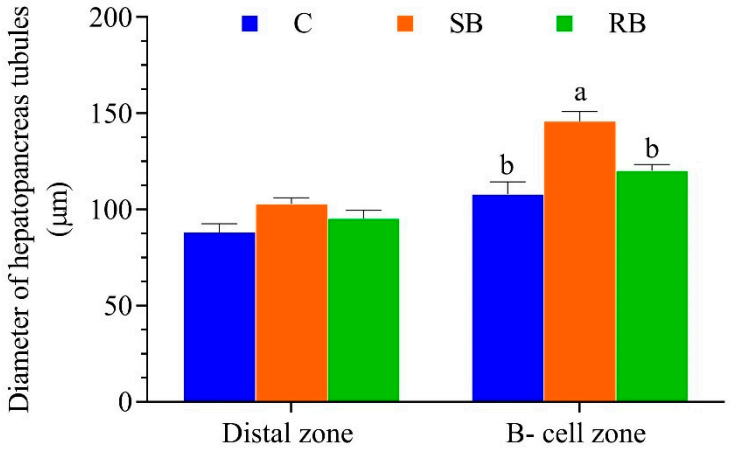

Figure 4. Effect of different carbon sources in BFT-based system on the diameter of hepatopancreas tubules in the distal and B-cells zone (µm) of *L. vannamei*. C: control treatment of clear water without carbon source, SB: treatment contains sugarcane bagasse as organic carbon sources, RB: treatment contains rice bran as organic carbon sources. Different letters (a > b) in the same enzyme indicate significantly different ($p < 0.05$).

Hepatopancreas of the SB-based BFT group showed normal histology (Figure 3) and the diameter of the hepatopancreas tubules in the distal and B-cell zone was increased by 16.83 and 34.89%, respectively, compared to the control group (Figure 4). Also, the hepatopancreas of the RB-based BFT group did not show any pathological signs (Figure 3). The diameter of the hepatopancreas tubules in the distal zone and B-cell zone was increased by 8.27 and 11.26%, respectively, in the RB-based BFT group compared to the control group (Figure 4).

4. Discussion

In the current study, promoted biofloc was used to effectively regulate inorganic chemicals, such as TAN, NO_2-N, and NO_3-N. Although, adding different carbon sources to the BFT system without any diets did not significantly affect these parameters. The current results were in agreement with the results obtained by previous study that used carbon sources without additional diet in the BFT system [54].

The present findings indicated that shrimp fed on the commercial diet (control group) had higher WG and SGR than those fed on BFT alone. However, the survival rates of the groups fed on BFT were higher than those of the control group. Therefore, it could be suggested that *L. vannamei* can be utilized biofloc as a natural feed with higher survival rates compared with the control group. In the same line, *L. vannamei* reared in a BFT-based system had higher survival rates than the control [54]. Meanwhile, pink shrimp *F. brasiliensis* (PL_{25}) that are fed on biofloc only without any supplemental feed have a higher weight gain and survival than shrimp reared in clear water and feed on a commercial diet [41]. The biofloc particles, which is used as a source of nutrients for shrimp, including protein, carbohydrates, vitamins, minerals, and immune stimulants [9]. However, in the present study, the shrimp reared in BFT groups had a lower final weight than the control, whereas the shrimp were forced to eat natural feed that could have lower nutritional quality than the artificial diet. The proximate chemical composition of biofloc produced using SB contained 43.22% protein, 2.30% lipid, 30.35% nitrogen-free extract, and 24.13% ash on dry weight [57]. Meanwhile, the shrimp fed on the biofloc in their fresh or wet form, accordingly its nutritional value not compared to the artificial diets which could cause the growth reduction in BFT treatment with no artificial feed addition compared to the control.

However, the survival rates in the BFT groups were higher than in the control group. Whereas the BFT might be employed as an anti-infective strategy for aquatic animals. The biofloc might provide a more comprehensive supply of cellular immunity bolstered by a

variety of bioactive chemicals [33]. Supplementation of diverse carbon sources improved shrimp development in this study, as evidenced by a considerable rise in survival rates of two BFT groups throughout the 90-day experimental period. Similarly, these results were in agreement with the previous findings of Sharawy, et al. [54], who proposed that using SB to promote biofloc in a high-intensity system with minimal water exchange might boost *L. vannamei* growth and improve immunity and antioxidant capacity. Different combinations or amounts of carbon sources applied to a BFT-based system would alter the biofloc, which might play essential roles in water quality maintenance, food supply, and extracellular enzyme synthesis, as well as contribute to increased growth performance [32,33,58,59].

The results from the whole-body composition of shrimp in this study showed an increase in lipid content with BFT groups higher than the control group. In the same line with the present study, Izquierdo, et al. [60] found that *L. vannamei* raised in mesocosms with biofloc had a higher whole-body lipid content than those raised in clear seawater systems, which could be associated with the successful absorption of various fatty acids from the biofloc. In addition, when comparing BFT groups with the control group in the present study, the crude ash level of the whole-body shrimp was considerably greater in the BFT groups. This might be explained by the BFT constant availability of numerous minerals and trace elements, as evidenced by the biofloc high ash content [61]. Moreover, *L. vannamie* reared in BFT tanks experienced higher lipid and ash contents compared to the shrimp reared in clear water [62]. Using sugarcane molasses as carbon source improved crude protein, lipid and ash contents in the tissues of the *L. vannamie* from the BFT treatments when compared to control [63]. Meanwhile, the protein content in the present study tended to decrease in BFT treatments, this could be due to the low protein level in the BFT, which used alone without additional feed, compared to one's feed commercial diet in the control group. In addition, the reduction in protein content matches the lower growth performance in BFT treatment.

The current results showed that the addition of different organic carbon sources in the BFT system improved the THB count in the gut that could be used as a food source for shrimp without introducing the commercial diet. The BFT rearing systems can alter the water bacterial community and shrimp intestinal bacteria composition [38]. BFT relies on the growth of heterotrophic microbes existing within the culture system, by increasing the C:N ratio [64,65]. Meanwhile, the composition of this microbe is highly associated with carbon sources. The effect of different carbohydrate sources (maida flour, wheat flour, gram flour, millet flour, rice flour, cornflour, and multigrain flour) as an alternative to molasses revealed a significant effect on the bacterial dynamics by increasing THB and decreasing TVC compared to the control [30].

The use of high-throughput amplicon sequencing revealed a significant effects of using different carbon source on gut microbial community in the BFT and the main bacterial phyla were Proteobacteria, Chloroflexi, Actinobacteria, Planctomycetes, Verrucomicrobia and Bacteroidetes [66]. Besides the presence of Vibrionaceae family, its presence in BFT reared animal is much lower than in animal reared in clear water [67,68]. The Vibrio population and TVC/THB percent are used for determining the health of shrimp [69]. When carbohydrates are supplemented, carbon is easily accessible for microbial development, including pathogens. Despite the increased Vibrio load in the current investigation, the TVC/THB percent in the guts of shrimp in SB-based BFT groups was nearly similar to the control. This showed that the development of total heterotrophic bacteria with the used carbon sources affects Vibrio growth in the gut of shrimp. Water and food are two major influences on the gut microbiota of aquatic species, both of which have a significant impact on the host's health and growth. Previous research has suggested that shrimp's gut microbiome is influenced by their cultural milieu [38]. Our findings showed that adding alternative carbon sources (SB and RB) to promote the development of THB had a significant impact on the shrimp gut microbiota. The microbial populations in the shrimp intestine steadily grew throughout the 90-day trial. THB and TVC counts in the shrimp intestine were considerably higher in BFT groups than in control groups. The explanation for this is

that with reducing/zero diets, the shrimp in BFT groups were completely dependent on biofloc as a natural source. This finding showed that the shrimp gut microbiota might be influenced by rearing water or by ingesting floc biomass from the system [70]. In addition, in the present study, the survival of the shrimp reared in BFT treatment was higher than the control group.

In aquatic animals, digestive enzyme activities are crucial for optimizing nutritional processes and boosting digestive capacity [71]. The current results indicated that stomach and intestine proteases, intestinal lipase, and amylase activities significantly increased in the RB group. Debnath, et al. [72] noticed that the type, source, and content of nutrients can alter the digestive enzyme activities in the animal. Due to the direct impact of nutrition on digestive enzyme activity, the same results were found in crustaceans [73].

In agreement with the present findings, the specific digestive enzyme activities in the hepatopancreas of *L. vannamei* were significantly increased in groups reared using BFT-based systems than in shrimp reared in clearwater [32]. Moreover, the expression of digestive enzyme-related genes was increased in *P. vannamei* reared in BFT-based systems [39]. However, the mechanism of increasing digestive enzyme activities in shrimp has not been completely identified, but it could be associated with the nutritional content of the biofloc. The BFT in the tank water, which acted as an additional food supply, was most likely to be responsible for the increased enzyme activity [74,75]. In the RB group, most enzyme activities of proteases in the stomach, amylases, and lipase in the intestine have increased significantly. The presence of the BFT may stimulate endogenous enzymes in the shrimp [76]. In the study of Wang, et al. [32] shrimp in the 50% molasses and 50% wheat bran group had higher extracellular enzyme levels, including protease, amylase, lipase, and cellulose. These enzymes may aid in the breakdown of proteins, carbohydrates, and other essential elements in the feed into smaller units, making it easier for the shrimp to digest and absorb the feed [74].

The observation of the hepatopancreas development may be an indicator of its functional activity [77]. It can be used to support other methods of determining the organism's nutritional status. The hepatopancreas major roles are to produce digestive enzymes, absorb nutrients, and store lipids, [78]. The hepatopancreas "B" cells are primarily responsible for producing and secreting digesting enzymes [79]. The present findings revealed a significant increase in the diameter of hepatopancreas tubules in shrimp reared in SB-based BFT and an insignificant increase in RB-based BFT compared to the control. In the same line, *L. vannamie* reared in BFT tanks showed an increase in the thickness of hepatopancreas tubules and an increase in the number of enzyme-producing cells (B cells) number compared to the control treatment [77]. Also, the use of different carbon sources (molasses or biodegradable polymers) in the BFT systems could affect the hepatopancreas structure and function by inducing transcriptomic changes in the hepatopancreas of *L. vannamie*, whereas molasses induced stress in BFT than biodegradable polymers [80]. The results of the present study are supported by the findings of Moss, et al. [76] who indicated that using natural products as a dietary source can lead to increased shrimp functional activity in the hepatopancreas by increasing the synthesis of particular enzymes. On the other hand, the decrease in feeding rate under the biofloc system did not reveal any histological changes in the hepatopancreas of *Metapenaeus monoceros* [81].

5. Conclusions

The use of sugarcane bagasse and rice bran as carbon sources in a biofloc-based system (BFT) with no additional formulated feed maintained the water quality of different treatments at optimal levels. However, the growth performance of Pacific whiteleg shrimp, *Litopenaeus vannamei* in the BFT groups was lower than that in the control group. Feeding shrimp on biofloc as a natural feed influenced the gut microbiota of shrimp via increasing the total heterotrophic bacteria (THB) and decreasing the ratio of total vibrio count/THB in the gut of shrimp. In addition, shrimp reared in the BFT system experienced higher digestive enzyme activities in the digestive tract, as well as improved the histological structure

of the hepatopancreas of shrimp. All of these findings combined to make the biofloc system a dependable/reliable solution for shrimp farming without artificial feed. However, the success of this conditions under high stocking density still needs more investigation.

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