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The Synergistic Effect of Dietary Protein and Periphyton enhances the Growth, Feed Consumption, and Gene Expression of *Litopenaeus vannamei* in Biofloc systems.

Eman Omran[1], Al-Azab Tahoun[1], Mohamed Said[1], Zaki Sharawy[2,*], Ashraf Suloma[3]

[1]Aquaculture Department, Faculty of Fish Resources, Suez University, Egypt
[2]National Institute of Oceanography and Fisheries (NIOF), Cairo, Egypt
[3]Animal Production Department, Faculty of Agriculture, Cairo University, Giza, Egypt
[*]**Corresponding Author: zaki_sharawy@yahoo.com**

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ABSTRACT

The study examined the impacts of varying protein levels and additional substrate area for periphyton formation on the growth and survival of whiteleg shrimp postlarvae reared in a biofloc system. Shrimp postlarvae were stocked in eighteen 100L tanks each filled with 60L of seawater (28 ‰ salinity). In a 3×2 factorial arrangement, the experimental diets were formulated to contain three varying dietary protein levels (25, 35, and 45% CP). Shrimp diets were prepared and supplied during the subsequent interval of the trial. The dietary protein levels were subjected to study in combinations with two different levels of periphyton surface areas (no periphyton P0% and P100% tank surface area). Different dietary protein and substrate addition levels significantly influenced growth performance, feed utilization, and survival. The dietary protein level in the diets of shrimp postlarvae reared in the Biofloc system can be reduced from 45 to 35% CP without negative effects on growth and feed conversion response. Regardless of the dietary protein level, results indicated much greater benefits for adding periphyton substrate to the shrimp-rearing system. Survival ranged between 76.67 and 91.33% and increased significantly from 84.33 (without substrate) to 91.56% (100% additional substrate area) regardless of the dietary protein level. Irrespective of substrate levels, they were 83.667, 88.167, and 92.0% for 25, 35 and 45% of CP, respectively. Likewise, periphyton enhances the immunity gene expressions of *L. vannamei*. The artificial substrate has economic advantages and is easy to establish. Although the positive impact for white leg shrimp, there is no interaction between the percentages of protein levels and the presence of absent artificial substrate.

INTRODUCTION

Aquaculture represents the fastest-growing food production sector over recent decades, with an average annual growth rate of 6.7% over the past three decades (**FAO, 2022**). In aquaculture, 50–60% of operating costs are related to feed and feeding management. Therefore, feeding management should be done strategically in scientific aquaculture methods to make them profitable. Conversely, fish is the most affordable source of protein to feed the World's rapidly expanding population, which is predicted to

reach 9 billion people by 2050 (**FAO, 2022**). Aquaculture is the only means to satisfy fish demand because the catch fisheries sector's production is static. As a result, feed-based aquaculture will soon play an important part in meeting the targeted level of fish production (**Pailand and Biswas, 2022**). The expensive protein ingredients in commercial diets mostly cause feed expenses. In the context of finding more alternatives of protein sources, many attempts for achieve is application of alternative biofloc technology (BFT). Simultaneously, with more environmentally friendly management techniques, system intensification should be sustainable.

Furthermore, there will be environmental hazards and harmful repercussions from the massive amounts of carbon-based pollutants that will be produced as a result of intensifying cultural systems. Constantly replenishing pond water through exchange can lessen the likelihood of these harmful discharges. The utilization of Bioflocs is a microbial way to produce useful nutrients to be consumed (**Jamal *et al.*, 2020**) by balancing nitrogen and carbon (which add to the system). Wasted nitrogenous metabolites account for about 70–80% of fed proteins. The BFT produces biomass from hazardous waste. **El-Sayed *et al*. (2021) and Nisar *et al.* (2022)** noted that the Biofloc technology was initially created for environmentally friendly, low-cost products in regions with limited water resources.

Affordable, eco-friendly technology is urgently needed for widespread use. Thus, "Biofloc Technology" (BFT) was born as an effective alternative system that is both eco-friendly and cost-effective to operate. BFT provides several benefits, including reduced water usage, recycling of nutrients and organic materials, and reduced pathogen transmission (**Avnimelech, 2007**). Biofloc technology was known to improve shrimp growth performance and water quality in several ways (**Rezende *et al.*, 2018; Ali *et al.*, 2021; El-Sawy *et al*., 2022**). Artificial substrates are used for periphyton formation in many different culture systems in BFT systems (**Schveitzer *et al.*, 2013**). Artificial substrates can be a natural food supplier for cultivated fish and shrimp species (**Arnold *et al.*, 2009**). Expanding the surface area of the raising tank offers greater respite to aquatic animals being raised and acts as an extra location for nitrifying bacteria to attach. Furthermore, it provides an extra area for shrimp, minimizing overcrowding and competition for space, as well as mitigating unfavourable behavioural interactions like cannibalism **(Abdussamad and Thampy, 1994)**.

Crustaceans rely on their innate immunity to defend against infections. This immune response is carried out by cellular and humoral effectors, which detect and kill invading microbes, activating different defence mechanisms (**Söderhäll and Cerenius, 1992**). In invertebrates, a set of conserved host proteins, known as pattern recognition proteins (PRPs), plays a critical role in non-self-recognition (**Anjugam *et al.*, 2016**). β-1, 3-glucan binding protein (*β-GBP*) is one of the most significant PRPs isolated from invertebrates (**Anjugam *et al.*, 2016**). The Prophenoloxidase (*ProPO*)-activating mechanism is initiated when pattern recognition proteins (PRPs) connect to the cell wall components of a microorganism. This mechanism is responsible for activating the immune system of the host (**Lee and Söderhäll, 2002**). Invertebrates may have a high level of immunological surveillance because they contain many hemolymph carotenoids, which control the transcription of several genes associated with the immune system, including the *ProPO* gene (**Babin *et al.*, 2010**). Intending to improve *L. vannamei* immunity, recent studies have concentrated on identifying the specific dietary

components that activate molecular mechanisms. (**Mansour *et al.*, 2022; Sharawy *et al.*, 2022; Abbas *et al.*, 2023**). The expression of various genes in *L. vannamei* reared using the biofloc system was also determined (**Hassan *et al.*, 2022 a,b; Hassan *et al.*, 2023**). **Zhang *et al.*, 20210** found a significant increase in weight gain and survival when more artificial substrates were added. Because the shrimps in all tanks were supplied with suitable water quality and adequate nutritional food, we suggest that the differences in growth and survival were affected mainly by living space added with the addition of artificial substrates.

In this study, the vertical surface of Nylon fabrics was selected as the artificial substrate placed in the experimental tank. We tested the advantage of an artificial substrate and varying dietary protein levels for reducing the density effect by analysis of spatial distribution, survival, and growth in an intensive *L. vannamei* biofloc-based culture system.

MATERIALS AND METHODS

1. Design of experiment and cultivation method

Shrimp postlarvae were acquired from the hatchery (Berket Ghalioun) located in the Kafr AlSheikh Governorate of Egypt. The experiment was conducted over 90 days at the NIOF-Suez laboratory, which is a branch of the National Institute of Oceanography and Fisheries focusing on invertebrates. Shrimp postlarvae were acclimated to laboratory settings for 7 days in two fiberglass tanks, each with a capacity of 2000 L. The tanks were maintained at a temperature of 28–29°C and a salinity of 28‰. A continuous supply of aeration was achieved by utilizing a 0.5 Kilowatt Vortex© ring blower to produce compressed air.

During the acclimation period, the larvae were given a commercial diet (Skretting®) twice a day. 3980 kcal of energy was provided by the meal, which included 5.9% crude fiber, 8% crude fat, and 38% crude protein. In a 3×2 factorial arrangement, three experimental diets were formulated to contain varying crude protein levels (25, 35, and 45%CP) and two different levels of periphyton surface areas (no periphyton P_0% and P_{100} % of tank surface area. The six experimental treatments were subjected to be studied in triplicates in eighteen rectangular plastic tanks each (45×58×40 cm) in width, length, and height, respectively. Each experimental tank was filled with 60 L volume. Under six treatments, all replicate tanks stocked shrimp postlarvae at similar densities. Each tank had the same number of shrimp.

2. Experimental diet and cultured species and feeding

The formulated feed contained different crude protein levels (25, 35, and 45%). All of the experimental tanks were managed under a Biofloc-based system protocol. Feed ingredients were purchased from a local market. The experimental feed was homogenized and screened by 35 μm sieves with a mixing device blender. The biofloc system maintained a carbon-to-nitrogen (CN) ratio of 10:1, as per the methodology outlined by **Panjaitan (2011)**. Each experimental tank was supplemented with molasses, which served as a carbon source. The molasses was fully blended with the water in a glass container and then evenly distributed throughout the tank using vigorous mixing and

aeration. All treatment tanks received 10% water weekly to allow for evaporation and the removal of uneaten feed and feces from tank bottoms.
Shrimp postlarvae of 0.08g (initial weight) were cultured for 90 days and fed with a feeding level of 10% of body weight were applied then reduced to 9% and 8% respectively for the second and the third months. Daily feed amounts were offered twice/day at 9.00 and 15.00h. Molasses addition (as a carbon source) was applied after the second meal introduction by 30 minutes. Shrimp was hand-fed 6 days per week for the overall experiment.

3. Establish the substrate and facilities

The 6 experimental treatments were subjected to be studied as follows: T1: $P0_{25\%CP}$, T2:$P0_{35\%CP}$, T3:$P0_{45\%CP}$, T4:$P100_{25\%CP}$, T5: $P100_{35\%CP}$ and T6: $P100_{45\%CP}$, where the superscripts refer to the dietary protein level, and P refers to the level of periphyton substrate area. This periphyton substrate was made of plastic polyethylene nylon net (0.5-millimeter mesh); it is divided into two boards every one considered 50% of the surface area of the water volume was cultured, installed by using iron wire to be in the middle of all ponds, with (41.5×31.5 cm) length and width in sequence. The Nylon substrate was used to allow attached algae and other periphyton constituents. Experimental tanks were cleaned and disinfected with potassium permanganate ($KMnO_4$) to treat water for impurities or odors; the applied dosage was typically around 1 to 2 milligrams per liter (mg/L) of water. After disinfecting the experimental tanks, the experimental plastic aquaria were filled with Marine water (salinity 28‰) and established the substrate in 9 ponds in every level of crude protein (CP). An air pump (0.50hp) was used to pump the oxygen in the system efficiently. Two air stones were excited in each pond to suspend the floc trapped in the water.

4. Growth parameters and feed utilization

The growth variables were calculated using the formulae provided by **Tacon *et al.* (2002)**:
Weight gain (g) = final weight - initial weight
Average daily Weight gain (ADG) = (W_2-W_1)/days, where W_1 and W_2 are the initial and final weights, respectively.
Specific growth rate (SGR, %/day) = [ln final weight-ln initial weight×100] / time days
Survival (SR, %) = (number of individuals at the end/initial number of individuals stocked)× 100.
Feed conversion ratio (FCR) = dry feed consumed/weight gain (wet weight).
Feed efficiency (FE) = weight gain/ dry feed consumed×100.

5. Laboratory analysis

Analyzed for proximate composition were representative samples of the experimental diets and whole shrimp bodies. The moisture content was measured by subjecting the sample to oven drying at a temperature of 105°C until a consistent weight was achieved. The Kjeldahl method was used to analyze the crude protein (nitrogen ×6.25) after acid digestion. An Auto Kjeldahl System (1030 Auto-analyser, Tecator, Höganös, Sweden) was employed for this purpose. The crude lipid was analyzed through ether extraction using a Soxtec System HT (Soxtec System HT6, Tecator, Höganös,

Sweden). The ash content was determined by combustion at 550 °C for 4 hours. The analysis of moisture, protein, lipid, and ash followed the guidelines provided by **AOAC (2006)**.

Table 1. Composition and proximate analysis of the experimental diets.

Ingredients	**%25**	**%35**	**%45**
Fish meal	10.00	20.00	30.00
Corn gluten	10.00	10.00	10.00
Vegetable Oil	5.00	5.00	5.00
Soybean meal	30.00	30.00	37.50
Corn grain	37.50	27.50	15.00
Rice bran	5.00	5.00	0.00
Di-Calcium Phosphate	2.00	2.00	2.00
Vitamin C	0.10	0.10	0.10
Anti-aflatoxin	0.10	0.10	0.10
Min. / Vit. Mix[1]	0.30	0.30	0.30
	100.00	100.00	100.00
Proximate composition (%)			
Dry matter	90.50	90.50	91.00
Crude protein	25.00	35.00	45.00
Ether extract	7.00	8.25	9.00
Crude Fibre	5.70	5.75	4.95
Ash	11.00	11.00	12.00
NFE[2]	51.40	40.00	29.05

[1]The kilogram of the product contains various vitamins and minerals, including cholecalciferol, retinyl acetate, all-rac-α-tocopheryl acetate, ascorbic acid monophosphate, cyanocobalamin, choline chloride, d-biotin, menadione sodium bisulfite, niacin, folic acid, d-calcium pantothenate, pyridoxine HCl, riboflavin, thiamin, sodium chloride, ferrous sulfate, manganese sulfate, zinc sulfate, copper sulfate, manganese sulfate, potassium iodide, and Celite AW 521 (1000 mg) from Agri-Vet Co., Cairo, Egypt.

[2]The nitrogen-free extract (NFE) is often estimated by subtracting the sum of crude protein (CP), ether extract (EE), crude fiber (CF), and ash from 100.

6. Gene expression analysis

Three shrimp were taken from every replicate using sterile dissection tools under cold circumstances. The samples were stored at a temperature of -80°C until they were analyzed for gene expression. The TRIzol technique (easy-RED, iNtRON Biotechnology) was employed to isolate total RNA using the samples, following the direction provided by the manufacturer. The RNA clarity is assessed by quantifying the OD ratios at 260 and 280 nm with a NanoDrop instrument (BioDrop). Only the isolates with the greatest proportion (A260/A280 1.8) had been chosen for cDNA production at a concentration of 1 ng/µl per reaction. The RNA underwent DNase I treatment (NEB, United States) to eliminate any DNA contamination. The RNA that had undergone treatment was subsequently utilized as a template for the production of first-strand cDNA through the application of reverse transcriptase, Enzynomics, Korea (RT-PCR beads). The manufacturer's instructions were followed for amplification of PCR on an Applied Biosystems Veriti 96-Well Thermal Cycler for cDNA synthesis. The cDNA used in the Real-Time PCR experiment (Bico, Thermo-Fisher) was subjected to an initial

denaturation at 95°C for 15 minutes, followed by 40 cycles with the following parameters: 95°C for 10 seconds, 60°C for 20 seconds, and 72°C for 30 seconds. In 0.5°C increments, temperatures were increased from 60°C to 95°C, resulting in the production of well-defined and accurate products. The study focused on analyzing two genes associated with the immune system (*β-GBP* and *ProPO*). The primers corresponding to these genes are listed in Table 5. The evaluation of gene expression or fold change of the target genes was conducted using the housekeeping gene (*β-actin*) **(Yang et al., 2013)**. The numbers indicate the ratio of fold change relative to the control when employing the $2^{-\Delta\Delta Ct}$ method to standardize the Ct values (cycle threshold) of the targeted genes were normalized using *β-actin* values as described by **Livak and Schmittgen (2001)**.

7. Quantitative data analysis

The average results were obtained using the combined standard error of means (SEM). The data was subjected to a two-way analysis of variance after confirming normal distribution and equal variances. The factors considered in the study were periphyton substrate area levels and dietary protein levels. The analysis was conducted using SPSS software, specifically version 17.0. Utilizing Duncan's multiple comparisons to evaluate disparities among means (Duncan, 1955). The statistical significance was established using a threshold of $P<0.05$.

RESULTS

1. Growth performance and survival rates

Table 2 shows how substrate and dietary protein levels affect *L. vannamei* growth and survival. The highest values for growth parameters regarding weight gain and specific growth rate were recorded for T5 ($P100_{35\%CP}$), while T1 ($P0_{25\%CP}$) recorded the lower values. As shown in Table 2, regardless of different protein levels, the statistical analyses showed significant differences between the experimental substrate areas (0% and 100%, respectively). All assessed growth parameters (AFW, AWG, ADG, and SGR) and survival values (substrate additional area and dietary protein levels) Irrespective of substrate levels, data on the impacts of dietary levels of protein on survivability and growth favored 35 and 45%CP levels compared to the lowest one (25%). Concerning the interaction between the studied factors substrate levels × dietary protein levels, it was found that the highest average final body weight (AFBW) was recorded for $P100_{35\%CP}$ (8.37±0.16). At the same time, $P0_{25\%CP}$ had the lowest value (7.19±0.37). However, the average daily increase, specific growth rate, and weight gain vary greatly across experimental treatments.

2. Feed utilization

Either the interaction (substrate levels x dietary protein levels) or substrate had a significant impact ($P>0.05$) on the feed conversion ratio (Table 3). Significant differences were recorded for the effects of different dietary protein levels. The FCR values were 1.52±0.02, 1.52±0.02, and 1.41±0.02 for (25, 35, and 45% CP levels, respectively).

3. Body proximate composition of L. vannamei

Table 4 shows the chemical composition of shrimp *L. vannamei* reared for 90 days under biofloc-based system conditions. Significant differences among experimental

combinations of substrate area and dietary protein levels. $P100_{45\%CP}$ recorded the highest values for dry matter (31.00±0.60), crude protein (74.00±0.6), ether extract (16.03±0.10), and ash (17.80±0.2) contents. The lowest corresponding values were recorded for $P0_{25\%CP}$, and they were 29.00±0.60, 74.00±0.6, 16.03±0.10 and 17.80±0.2, respectively.

Table 2. Effects of different substrate areas and dietary protein levels on growth performance and survival rates of *L. vannamei* reared in biofloc for 90 days.

Treatments	Periphyton area (%)	CP (%)	AFW (g)	WG (g)	ADG (mg/day)	SGR (%/day)	SR (%)
Substrate area	0		7.65±0.15^{b}	7.56±0.15^{b}	0.08±0.00	5.09±1.68^{b}	84.33±2.34^{b}
	100		8.21±0.16^{a}	8.12±0.15^{a}	0.08±0.00	5.28±1.78^{a}	91.55±0.44^{a}
Protein levels		25	7.52±0.26^{b}	7.44±0.25^{b}	0.08± 0.00	5.68±2.84^{b}	83.66±3.23^{c}
		35	8.10±0.18^{a}	8.02±0.17^{a}	0.08±0.00	5.12±1.96^{a}	88.16±1.94^{b}
		45	8.16±0.13^{a}	8.15±0.12^{a}	0.08±0.00	5.77±1.44^{a}	92.00±0.51^{a}
T1	P_0	25	7.19±0.37	7.19±0.36	0.08±0.00	5.01±0.05^{b}	76.67±1.67^{bc}
T2	P_0	35	7.60±0.00	7.66±0.00	0.08±0.00	5.08±0.01	85.00±2.89^{ab}
T3	P_0	45	7.85±0.14	7.84±0.14	0.08±0.00	5.10±0.02	91.33±0.67^{ab}
T4	P_{100}	25	7.70±0.36	7.69±0.36	0.08±0.00	5.08±0.05	90.67±0.67^{ac}
T5	P_{100}	35	8.37±0.16	8.37±0.15	0.09±0.00	5.18±0.02	91.33±0.67^{ab}
T6	P_{100}	45	8.31±0.10	8.31±0.10	0.09±0.00	5.17±0.01	92.67±0.67^{a}

The Mean±SE values in the same column that are labeled with different letters are statistically significant (P≤0.05).

Table 3. Effects of different substrate areas and dietary protein levels on feed utilization of *L. vannamei* reared in biofloc for 90 days.

Treatments	Periphyton area (%)	CP (%)	FCR	Feed intake
substrate	0		1.47±0.02	11.10±0.15^{b}
	100		1.46±0.03	11.86±0.31^{a}
Protein levels		25	1.52±0.02^{a}	11.28±0.02
		35	1.47±0.03^{ab}	11.82±0.48
		45	1.40±0.02^{c}	11.34±0.22
T1	P_0	25	1.55±0.03	11.15±0.36^{b}
T2	P_0	35	1.44±0.01	11.04±0.25^{b}
T3	P_0	45	1.41±0.01	11.11±0.24^{b}
T4	P_{100}	25	1.49± 0.02	11.41±0.35^{a}
T5	P_{100}	35	1.50±0.06	12.60± 0.69^{a}
T6	P_{100}	45	1.39±0.03	11.58± 0.36^{a}

The Mean±SE in the same column bearing different letters are significantly different (P≤0.05)

Table 4. Effects of different substrate areas and dietary protein levels on body proximate composition of *L. vannamei* reared in biofloc for 90 days.

Treatments	Periphyton area (%)	CP (%)	Dry matter	Crude protein	Ether extract	Ash
substrate	0		29.89±0.30[b]	72.44±0.30[b]	15.22±0.2[a]	16.57±0.10[a]
	100		30.00±0.030[a]	72.89±0.40[a]	14.90±0.10[a]	16.30±0.10[b]
Protein levels		25	29.08±0.30[a]	71.75±0.30[a]	15.15±0.20[a]	16.98±0.20[a]
		35	30.08±0.20[b]	72.75±0.30[ab]	15.62±0.20[a]	16.92±0.30[a]
		45	30.67±0.30[b]	73.50±0.40[b]	15.68±0.20[a]	17.30±0.30[b]
T1	P_0	25	29.00±0.60[a]	71.33±0.03[a]	14.90±0.30[a]	16.57±0.03[a]
T2	P_0	35	30.33±0.30[ab]	73.00±0.06[bc]	15.33±0.30[ab]	16.80±0.03[a]
T3	P_0	45	30.33±0.30[ab]	73.00±0.06[bc]	15.33±0.10[ab]	16.80±0.20[b]
T4	P_{100}	25	29.17±0.40[a]	72.17±0.30[ab]	15.40±0.20[ab]	17.40±0.05[c]
T5	P_{100}	35	29.83±0.20[ab]	72.50±0.30[ab]	15.80±0.20[b]	17.50±0.20[c]
T6	P_{100}	45	31.00±0.60[b]	74.00±0.60[c]	16.03±0.10[b]	17.80±0.20[c]

The Mean±SE values in the same column that are labelled with different letters are statistically significant ($P \leq 0.05$).

4. *Gene expressions*

At the end of the experiment, both *β-GBP* and *proPO* gene expressions were upregulated in all treatments with different protein levels and with or without the periphyton substrate. Out of all the experimental treatments, both T2 and T5 (35% protein with or without substrate) exhibited the maximum expression levels for both genes ($P<0.05$). The groups with different levels of proteins and substrate are significantly higher than those with the protein only. The expression of the *ProPO* gene was greater than that of the *β-GBP* gene. The treatment T5 (35% CP plus the periphyton substrate) had the largest relative fold change of 7.82, whereas the treatment T1 without the substrate had the lowest fold (Figure 1).

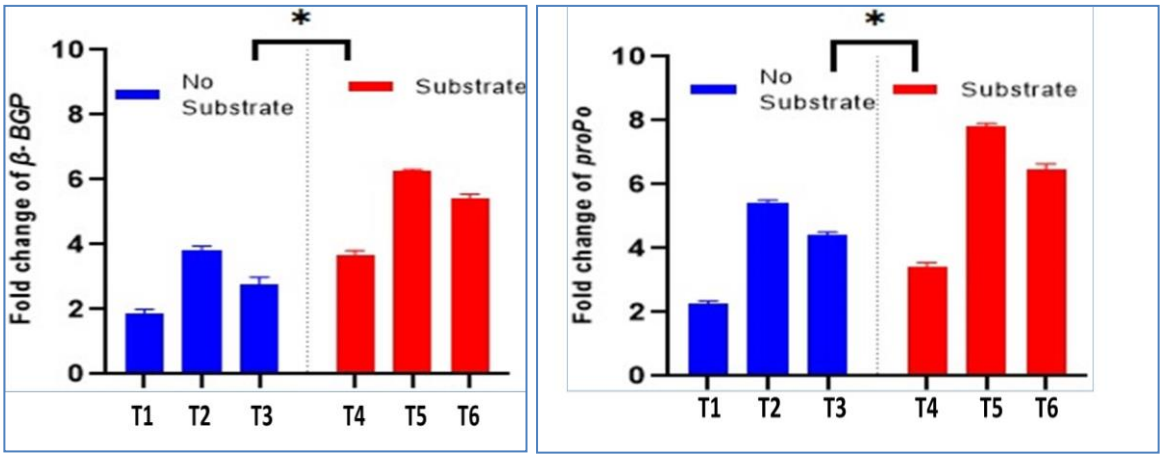

Figure 1. Gene expression analysis of immune-related genes vs. housekeeping gene across various protein levels. The asterisk indicates the presence of significant differences.

Table 5: Primers information used in qRT-PCR for gene expression analysis.

Gene (accession no.)	Sequences	Amplicon length (bp)
β- actin (AF300705)	Forward primer: gcccatctacgagggata Reverse primer: ggtggtcgtgaaggtgtaa	121
β-GBP (AY249858)	Forward primer: acgagaacggacaagaagtg Reverse primer: ttcagcatagaagccatcagg	137
ProPO (AY723296)	Forward primer: cggtgacaaagttcctcttc Reverse primer: gcaggtcgccgtagtaag	122

DISCUSSION

Many reports indicated the superiority of periphyton formation benefits via additional substrate area and, consequently, improved productivity and immunity of shrimp with better water quality (**Chethurajupalli and Tambireddy, 2021)**. *L. vannamei* has increased production and survivability in artificial submerged substrate systems (**Zhang *et al.*, 2010**). They revealed the significance of integrated periphyton substrate-biofloc-based system for shrimp husbandry than systems based only on biofloc protocol. Artificial substrates could disperse the shrimp from the tank bottom onto the artificial substrates and thus alleviate the negative effect of high stocking density on shrimp growth in the tanks. Moreover, the incorporation of the substrate into the biofloc system resulted in improved growth and survival rates in giant river prawn, *Macrobrachium rosenbergii* (**Asaduzzaman *et al.*, 2010**), *L. vannamei* (**Schveitzer *et al.*, 2013; Olier *et al.*, 2020**) than that of only biofloc systems. These findings were confirmed by the positive contribution of periphyton in improving the growth and

survivability of shrimps compared to those reared in tanks without substrates in the present study.

Higher survival rate related to the presence of periphyton, i.e., the advantage of the existing substrate to grow periphyton in bloc tanks as a viable commercial goal. **Kring *et al.* (2023)** demonstrated that adding artificial substrate minimizes the passive impacts of shrimp high stocking rates with significant positive manipulation in growth and yield in shrimp culture. The consequences of adding substrate to high tunnel greenhouses for modifying water quality to support high-density shrimp cultivation were examined. It was shown that this practice allows for greater stocking density without adversely affecting shrimp size. **Arnold *et al.* (2009) and Schveitzer *et al.* (2013)** observed a positive growth rate and survival effect. This can be due to reduced cannibalism with increased surface area (**Schveitzer *et al.*, 2013**).

Additionally, the average weight gain of the shrimp groups in the substrate-added area confirmed the finding of **Audelo-Naranjo *et al.* (2010),** who indicated the importance of existing biota associated with the additional artificial area provided by the addition of the substrate to tanks, which is highly considered as a natural food source for farmed organisms. This intricate food web community evolves, with detritivores and consumers feeding on autotrophic bacteria. To produce new biomass, the autotrophic microalgae-bacteria recycle and use the dissolved organic and inorganic nitrogen that results from their metabolism. Shrimp occupied the highest trophic level in the experiment units, making them the primary consumers and ultimate beneficiaries of the nutrients and energy flow inside these enclosed systems. Adding substrate may enhance water quality manipulation, allowing for increased stocking density of shrimp without affecting animal size. Moreover, **Mani *et al.* (2021)** showed a higher survival rate and better growth for *L. vannamei* in tanks with an artificial substrate, such as a PVC mat and agricultural shed net, than control (tanks without substrate). Artificial substrates may be used due to their convenient accessibility and practical economic benefits. **Fleckenstein *et al.* (2020)** observed enhancing the performance of synthetic substrates under a biofloc system. Furthermore, **David *et al.* (2022)** showed that adding substrates in the production of freshwater prawns (*Macrobrachium rosenbergii*) in ponds and Nile tilapia (*O. niloticus*) is a result of the uptake of nutrients and the build-up of solid particles. Survival rate (>99%) for cultivated fish, whether partial or total feed restriction. The FCR was Final body weight, survival rate, and productivity were higher in treatment with artificial substrates with values.

Garcia *et al.* (2016) showed that periphyton has the potential to substitute at least 50% of the feed used for Nile tilapia in cages without negatively impacting their growth. Currently, there is no available report on the utilization of aquaculture effluent as a nutrient source to enhance the development of natural food to nourish farmed fish. Hence, rather than avoiding utilizing the feed, it is possible to use the nutrient-dense effluent from a conventionally fed monoculture to stimulate the development of periphyton in a shared production system. Furthermore, fish that are only raised on a natural diet, such as periphyton, could be given distinct labeling and potentially marketed to a specialized consumer base seeking more environmentally-friendly items. In a recent study by **Kring *et al.* (2023)**, it was shown that the addition of artificial substrate reduces the negative effects of high stocking rates on shrimp. This led to improvements in feed conversion

ratio (FCR) and yield in shrimp that were cultured in a grow-out phase experiment using *L. vannamei* in BFT housed in basic greenhouses.

Reports showed that the survival rate was higher in treatment with periphyton, which confirmed the advantage of the existing substrate to grow periphyton in bio-floc tanks as a commercial goal. In comparison to traditional systems, including tilapia in ponds (1.7, **Rodrigues *et al.*, 2019**) and cages (1.84, **Garcia *et al.*, 2016**), the FCR in both models (P100-0 and P50-0) is 1.37 and 1.46, which is comparable to or even lower. Although FCR reaches maximum in treatment with only biofloc, still lower than clear water (**Nguyen *et al.*, 2019**); FCR was significantly lower in most Biofloc-RAS systems compared with the same diet protein level (23%, 27%, 31%, 35% CP in clear water–RAS used in rearing Nile tilapia (*Oreochromis niloticus*) values was (1.7, 1.6, 1.4, 1.2) (2.2, 2.0, 1.8, 1.6) respectively.

The result of this study, 35% CP of dietary crude protein helped in shrimp growth performance compared to 25% CP. Comparable results were obtained by (**Pinho and Emerenciano, 2021**). **Nunes *et al.* (2010)** observed the same result in biofloc shrimp *L. vannamei* culture fed 25% crude protein and 37% clear water. We found that the performance of 35% is better than crude protein 25%. Employing a biofloc system for raising vannamei shrimp also enables a decrease in dietary protein levels from 450g to 350g protein kg^{-1}, leading to better growth efficiency and greater feed consumption (**Mansour *et al.*, 2022**). **Biswas *et al.* (2022)** experimented on *Penaeus monodon* subjected to four different periphyton protocols (no periphyton substrate and partial feeding with periphyton grown on nylon net substrate equivalent to 50, 75, and 100) % surface area of polyculture pond stocked with *Mugil cephalus*, *Planiliza parsia* and *Chanos chanos*. The growth of all species exhibited an increasing pattern, suggesting that periphyton had a beneficial impact. *P. monodon* had the greatest specific growth rate, whereas *M. cephalus* displayed the lowest. The combined survival of fish and shrimp and overall production did not exhibit any significant differences among the treatments ($P>0.05$). In addition, reducing the amount of feed by 30% minimizes the feed cost by almost 29%.

One of the advantages of periphyton is its ability to reduce environmental impacts, enhance FCR, and improve water quality (**Tahoun and Abo-State, 2017)**. Also, **Savonitto *et al.* (2022)** demonstrated that substituting 50% of fish meal with waste periphyton resulted in an enhanced FCR (1.2 vs. 1.35 in the control) without adversely affecting fish growth. From an economic standpoint, the feed cost can be reduced by either 11% or 21% by decreasing the amount of fish meal and substituting it with varying levels of periphyton.

The combination of autotrophic and heterotrophic bacteria in biofloc and periphyton enhances the natural defense mechanisms in shrimps. Prior research has shown that shrimps raised with biofloc **(Anand *et al.*, 2013; Panigrahi *et al.*, 2018)** and periphyton **(Kumar *et al.*, 2015)** exhibit increased resistance to infections and improved cellular and humoral responses. The biofloc technique primarily relied on helpful heterotrophic bacteria to effectively combat infections and improve the overall health of shrimp (**Mani *et al.*, 2021**). Disease resistance in treatment with periphyton than conventional because of increased cellular, humoral, and molecular immune parameters when comparing natural and artificial periphyton (**Anand *et al.*, 2013**).

The interactions and reactions between shrimp and pathogens involve the *β-GBP* gene **(Tassanakajon *et al.*, 2013)**. They initiate the prophenoloxidase system, the coagulation cascade, and the creation of antibacterial effectors in reaction to infections **(Tassanakajon *et al.*, 2018)**. The *β-GBP* gene plays a crucial role in activating the *ProPO* system and inducing the synthesis of antimicrobial peptides by detecting microbial components **(Goncalves *et al.*, 2012)**. Regarding the effect of dietary protein levels and the artificial substrate area for periphyton formation during the nursery phase of postlarvae shrimp of *L. vannamei* reared in the biofloc system on immune-related gene expression, the *β-GBP* gene was expressed in all treatments. Its expression was significantly higher with the treatments, which included different protein levels and periphyton in the presence of biofloc. This suggests that the inclusion of a periphyton-based feed additive can enhance the immune system of shrimps by stimulating the shrimp's immune response through the activation of non-specific defense mechanisms. These defense mechanisms are triggered by the microbial cell walls containing peptidoglycans, lipopolysaccharides (LPS), and β-1, 3-glucans **(Panigrahi *et al.*, 2018)**. The results are in contrast with the finding of **Abbas *et al*. (2023)** that the expression of *β-GBP* could be due to the action of the two different stimulations at the same time. The messenger RNA (mRNA) expression of the *ProPO* gene exhibited a significant increase in all treatments using the substrate, particularly as compared to treatments without the substrates. This expression was found to be greater than the expression of the *β-GBP* gene at the same levels of protein and with or without the substrate. The results showed the highest level in the gene expression of *ProPO* at T5; the expression was approximately three-fold higher than the T2 at 35% protein level and without the periphyton with the biofloc. This sentence suggests that a 35% protein concentration and periphyton in the biofloc system work together to have a big effect on starting the shrimp's non-specific immune response. Several studies have shown that the *β-GBP* and *ProPO* messenger RNA transcripts increased when exposed to different biofloc sources **(Hassan *et al.*, 2022a; Hassan *et al.*, 2023; Sharawy *et al.*, 2022)**.

CONCLUSION

Biofloc communities could be used as feed supplements for shrimps. Periphyton communities enhance the growth performance of whiteleg shrimp by adding substrate to higher weight gain and survival rate. Shrimp fed 350 g protein kg^{-1} had reasonable growth performance equal to its analogous at 450 kg^{-1}. A far greater protein-rich diet might be made up for by providing shrimp with biofloc, which has a lower protein content. Likewise, periphyton enhances the immunity gene expressions of *L. vannamei*. The artificial substrate has economic advantages and is easy to establish. Although the positive impact for white leg shrimp, there is no interaction between the percentages of protein levels and the presence of absent artificial substrate.

REFERENCES

Abbas, E.M.; Al-souti, A.S.; Sharawy, Z.Z.; El-Haroun, E. and Ashour, M. (2023). Impact of dietary administration of seaweed polysaccharide on growth, microbial

abundance, and growth and immune-related genes expression of The Pacific White leg Shrimp (*Litopenaeus vannamei*). Life, 13: 1–18.

Abdussamad, E.M. and Thampy, D.M. (1994). Cannibalism in the tiger shrimp *Penaeus monodon* Fabricius in nursery rearing phase. J. Aquacult. Trop., 9(1): 67-75.

Ali, M.A.M.; Sharawy, Z.Z.; Elsayed, N.E.G. and Elfeky, A. (2021). The effect of different stocking densities of marine shrimp larvae *Litopeneaus vannamei* on growth performance and survival rate using biofloc technology. Egypt. J. Aquat. Biol. Fish., 25(3): 551-569.

Anand, P.S.; Kumar, S.; Panigrahi, A.; Ghoshal, T.K.; Dayal, J.S.; Biswas, G.; Sundaray, J.K.; De, D.; Ananda, R.R.; Deo, A.D.; Pillai, S.M. and Ravichandran, P. (2013). Effect of C:N ratio and substrate integration on periphyton biomass, microbial dynamics and growth of *Penaeus monodon* juveniles. Aquac. Int., 21: 511-524.

Anjugam, M.; Iswarya, A. and Vaseeharan, B. (2016). Multifunctional role of β-1, 3 glucan binding protein purified from the haemocytes of blue swimmer crab *Portunus pelagicus* and in vitro antibacterial activity of its reaction product. Fish Shellfish Immunol., 48: 196-205.

AOAC (2006). Official Methods of Analysis. 18th Edition, Association of Official Analytical Chemists, Gaithersburg, MD.

Arnold, S.J.; Coman, F.E.; Jackson, C.J. and Groves, S.A. (2009). High-intensity, zero water exchange production of juvenile tiger shrimp, *Penaeus monodon*: An evaluation of artificial substrates and stocking density. Aquaculture, 293(1): 42-48.

Asaduzzaman, M.; Rahman, M.M.; Azim, M.E.; Islam, A.; Wahab, M.A. and Verdegem, M.C.J. (2010). Effects of C/N ratio and substrate addition on natural food communities in freshwater prawn monoculture ponds. Aquaculture, 306(1-4): 127-136.

Audelo-Naranjo, J.M.; Martínez-Córdova, L.R. and Voltolina, D. (2010). Nitrogen budget in intensive cultures of *Litopenaeus vannamei* in mesocosms, with zero water exchange and artificial substrates. Rev. Biol. Mar. Oceanogr., 45(3): 519-524.

Avnimelech, Y. (2007). Feeding with microbial flocs by tilapia in minimal discharge bioflocs technology ponds. Aquaculture, 264:140-147.

Babin, A.; Biard, C. and Moret, Y. (2010). Dietary supplementation with carotenoids improves immunity without increasing its cost in a crustacean. Am. Nat., 176: 234-241.

Biswas, G.; Kumar, P.; Ghoshal, T.K.; Das, S.; De, D.; Bera, A. and Kailasam, M. (2022). Periphyton: A natural fish food item for replacement of feed at optimized substrate surface area for cost-effective production in brackishwater polyculture. Aquaculture, 561: 738672.

Chethurajupalli, L. and Tambireddy, N. (2021). Rearing of white leg shrimp *Litopenaeus vannamei* (Boone, 1931) in biofloc and substrate systems: microbial community of water, growth and immune response of shrimp. Turk. J. Fish. Aquat. Sci., 22(3).

David, L.H.; Pinho, S.M.; Romera, D.M.; Campos, D.W.; Franchini, A.C. and Garcia, F. (2022). Tilapia farming based on periphyton as a natural food source. Aquaculture, 547, 737544.

Duncan, D.B. (1955). Multiple range and multiple F tests. Biometrics, 11(1): 1-42.

El-Sawy, M.A.; Ashry, O.A.; Abbas, E.M.; Sharawy, Z.Z. and Kelany, M.S. (2022). Potential Application of Agriculture by-Products in Heavy Metals Bioremediation. Egypt. J. Aquat. Biol. Fish., 26(5): 53-67.

El-Sayed, A.F.M. (2021). Use of biofloc technology in shrimp aquaculture: a comprehensive review, with emphasis on the last decade. Rev. Aquac., 13(1): 676-705.

FAO (2022). The State of World Fisheries and Aquaculture 2022, Towards Blue Transformation. Food and Agriculture Organization of the United Nations, Roma.

Fleckenstein, L.J.; Kring, N.A.; Tierney, T.W.; Fisk, J.C.; Lawson, B.C. and Ray, A.J. (2020). The effects of artificial substrate and stocking density on Pacific white shrimp (*Litopenaeus vannamei*) performance and water quality dynamics in high tunnel-based biofloc systems. Aquac. Eng., 90: 102093.

Garcia, F. ; Romera, D.M.; Sousa, N.S. ; Paiva-Ramos, I. and Onaka, E.M. (2016). The potential of periphyton-based cage culture of Nile tilapia in a Brazilian reservoir. Aquaculture, 464: 229-235.

Goncalves, P. ; Vernal, J. ; Rosa, R.D. ; Yepiz-Plascencia, G. ; de Souza, C.R.B.; Barracco, M.A. and Perazzolo, L.M. (2012). Evidence for a novel biological role for the multifunctional β-1, 3-glucan binding protein in shrimp. Mol. Immunol., 51(3-4): 363-367.

Hassan, S.A.; Sharawy, Z.Z.; Hemeda, S.A.; ElNahas, A.F.; Abbas, E.M.; Khalil, H.S. and Verdegem, M. (2023). Sugarcane bagasse improved growth performance, digestive enzyme activity, microbial dynamics, and mRNA transcripts of immune-, growth, and antioxidant-related genes of *Litopenaeus vannamei* in a zero-water exchange system. Aquacult. Rep., 33: 101788.

Hassan, S.A.H.; Sharawy, Z.Z. and El Nahas, A.F. (2022b). Modulatory effects of various carbon sources on growth indices, digestive enzymes activity and expression of related genes in Whiteleg shrimp, *Litopenaeus vannamei* reared under an outdoor zero-exchange system. Aquac. Res., 53: 5594-5605.

Hassan, S.A.H.; Sharawy, Z.Z. and ElNahas, A.F. (2022a). Carbon sources improve water quality, microbial community, immune-related and antioxidant genes expression, and survival of challenged *Litopenaeus vannamei* Postlarvae in biofloc system. Aquac. Res., 53: 5902-5914.

Jamal, M.T.; Broom, M.; Al-mur, B.A.; Al harbi, M.; Ghandourah, M.; Alotaibi, A. and Haque, M.F. (2020). Biofloc Technology: Emerging microbial biotechnology for the improvement of aquaculture productivity. Pol. J. Microbiol., 69(4): 401-409.

Kring, N.A.; Fleckenstein, L.J.; Tierney, T.W.; Fisk, J.C.; Lawson, B.C. and Ray, A.J. (2023). The effects of stocking density and artificial substrate on production of pacific white shrimp *Litopenaeus vannamei* and water quality dynamics in greenhouse-based biofloc systems. Aquac. Eng., 101: 102322.

Kumar, S.; Anand, P.S.; Ravichandran, P.; Panigrahi, A.; Dayal, J.S.; Raja, R.A. and Ponniah, A.G. (2015). Effect of periphyton on microbial dynamics, immune responses and growth performance in black tiger shrimp *Penaeus monodon* Fabricius, 1798. Indian J. Fish., 62: 67-74.

Kumar, S.; Shyne Anand, P.S. ; De, D.; Ghoshal, T.K.; Alavandi, S. V. and Vijayan, K.K. (2019). Integration of substrate in biofloc based system: Effects on growth performance, water quality and immune responses in black tiger shrimp, *Penaeus monodon* culture. Aquac. Res., 50(10s): 2986-2999.

Lee, S.Y. and Söderhäll, K. (2002). Early events in crustacean innate immunity. Fish and Shellfish Immunol., 12: 421–437.

Livak, K.J. and Schmittgen, T.D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods, 25(4): 402-408.

Mani, S.; Mullaivanam Ramasamy, S.; Chakrapani, S.; Krishna, A.; PS, S.A.; Lalramchhani, C. and Panigrahi, A. (2021). The effect of natural and artificial periphytic substrates with biofloc system on shrimp *Penaeus vannamei* (Boone 1931) culture: growth and immune response. Aquac. Int., 29: 651-668.

Mansour, A.T.; Ashry, O.A.; Ashour, M.; Alsaqufi, A.S.; Ramadan, K.M. and Sharawy, Z.Z. (2022). The optimization of dietary protein level and carbon sources on biofloc nutritive values, bacterial abundance, and growth performances of whiteleg shrimp (*Litopenaeus vannamei*) juveniles. Life, 12(6): 888.

Nguyen, T.A.T.; Nguyen, K.A.T. and Jolly, C. (2019). Is super-intensification the solution to shrimp production and export sustainability?. Sustainability, 11(19): 5277.

Nisar, U.; Peng, D.; Mu, Y. and Sun, Y. (2022). A solution for sustainable utilization of aquaculture waste: a comprehensive review of biofloc technology and aquamimicry. Front. Nutr., 8: 1231.

Olier, B.S.; Tubin, J.S.B. and de Mello, G.L. (2020). Does vertical substrate could influence the dietary protein level and zootechnical performance of the Pacific white shrimp Litopenaeus vannamei reared in a biofloc system?. Aquac. Int., 28: 1227-1241.

Pailan, G.H. and Biswas, G. (2022). Dealing the hardship in aquaculture nutrition in a changing climatic condition. In: Sinha, A., Kumar, S., Kumari, K. (eds) Outlook of Climate Change and Fish Nutrition. Springer, Singapore.

Panigrahi, A.; Saranya, C.; Sundaram, M.; Vinoth, K.S.R.; Das, R.R.; Satish, K.R. and Otta, S.K. (2018). Carbon: Nitrogen (C: N) ratio level variation influences the microbial community of the system and growth as well as immunity of shrimp (*Litopenaeus vannamei*) in biofloc based culture system. Fish Shellfish Immunol., 81: 329-337.

Panjaitan, P. (2011). Effect of C: N Ratio levels on water quality and shrimp production parameters in Penaeus monodon shrimp culture with limited water exchange using molasses as a carbon source. Indonesia: The University of HKBP Nommensen.

Pinho, S.M. and Emerenciano, M.G.C. (2021). Sensorial attributes and growth performance of whiteleg shrimp (*Litopenaeus vannamei*) cultured in biofloc technology with varying water salinity and dietary protein content. Aquaculture, 540: 736727.

Rezende, P.C.; Schleder, D.D.; da Silva, H.V.; Henriques, F.M.; de Lorenzo, M.A.; Seiffert, W.Q. and do Nascimento Vieira, F. (2018). Prenursery of the Pacific white shrimp in a biofloc system using different artificial substrates. Aquac. Eng., 82: 25-30.

Rodrigues, C.G.; Garcia, B.F.; Verdegem, M.; Santos, M.R.; Amorim, R.V. and Valenti, W.C. (2019). Integrated culture of Nile tilapia and Amazon river prawn in stagnant ponds, using nutrient-rich water and substrates. Aquaculture, 503: 111-117.

Savonitto, G.; Barkan, R.; Harpaz, S.; Neori, A.; Chernova, H.; Terlizzi, A. and Guttman, L. (2022). Fishmeal replacement by periphyton reduces the fish in fish out ratio and alimentation cost in gilthead sea bream *Sparus aurata*. Sci. Rep., 11(1): 20990.

Schveitzer, R.; Arantes, R.; Costodio, P.F.S.; Santo, C.M.D.; Arana, L.V.; Seiffert, W.Q. and Andreatta, E.R. (2013). Effect of different biofloc levels on microbial activity, water quality and performance of *Litopenaeus vannamei* in a tank system operated with no water exchange. Aquac. Eng., 56: 59-70.

Sharawy, Z.Z.; Abbas, E.M.; Abdelkhalek, N.K.; Ashry, O.A.; Abd El-Fattah, L.S.; El-Sawy, M.A. and El-Haroun, E. (2022). Effect of organic carbon source and stocking densities on growth indices, water microflora, and immune-related genes expression of *Litopenaeus vannamei* Larvae in intensive culture. Aquaculture, 546: 737397.

Söderhäll, K. and Cerenius, L. (1992). Crustacean Immunity. Annu. Rev. Fish Dis., 2: 3–23.

Tacon, A.G.J.; Cody, J.J.; Conquest, L.D.; Divakaran, S.; Forster, I.P. and Decamp, O.E. (2002). Effect of culture system on the nutrition and growth performance of Pacific white shrimp *Litopenaeus vannamei* (Boone) fed different diets. Aquac. Nutr., 8(2): 121-137.

Tahoun, A.M. and Abo-State, H.A. (2017). The positive contributions of probiotic supplementation and artificial substrate to growth performance, feed utilization of nile tilapia. EJNF., 20(1): 125-136.

Tassanakajon, A.; Rimphanitchayakit, V.; Visetnan, S.; Amparyup, P.; Somboonwiwat, K.; Charoensapsri, W. and Tang, S. (2018). Shrimp humoral responses against pathogens: antimicrobial peptides and melanization. Dev. Comp. Immunol., 80: 81–93.

Tassanakajon, A.; Somboonwiwat, K.; Supungul, P. and Tang, S. (2013). Discovery of immune molecules and their crucial functions in shrimp immunity. Fish Shellfish Immunol., 34(4): 954-967.

Yang, S.-P.; Wu, Z.-H. and Jian, J.-C. (2013). Effect of marine red yeast *Rhodosporidium paludigenum* on antioxidant-related gene expression in *Litopenaeus vannamei*. Isr. J. Aquac., 65(817): 1-6.

Zhang, B.; Lin, W.; Huang, J.; Wang, Y. and Xu, R. (2010). Effects of artificial substrates on the growth, survival and spatial distribution of *Litopenaeus vannamei* in the intensive culture condition. Iran. J. Fish. Sci., 9(2): 293-304.