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Performance of Black Tiger Shrimp (*Penaeus monodon*) Postlarvae in *Halamphora coffeaeformis* Enriched Biofloc Systems

Mohd Ihsanuddin Ahmad[1] | Muhammad Farhan Nazarudin[1] | Hazwani Hanim Hasnan[2] | Ninie Diana Baharuddin[3] | Kamil Latif[4] | Murni Karim[5] | Hui Teng Tan[6] | Yam Sim Khaw[1] | Jeffrey K. C. Lee[7] | Ikhsan Natrah[5]

[1]Aquatic Animal Health and Therapeutics Laboratory, Institute of Bioscience, Universiti Putra Malaysia 43400 UPM, Serdang, Selangor, Malaysia | [2]Innoqua Asia Sdn. Bhd. Co Place 10, 2330, Jalan Usahawan, Off Persiaran Multimedia 63000, Cyberjaya, Selangor, Malaysia | [3]Euglena Malaysia Sdn. Bhd, Level 2, Bloc C, Agro-Biotechnology Institute, Malaysia (ABI), Jalan Bioteknologi 43400, Serdang, Selangor, Malaysia | [4]Department of Animal Science and Fisheries, Faculty of Agricultural Science and Forestry, Universiti Putra Malaysia Bintulu Sarawak Campus 97008, Bintulu, Sarawak, Malaysia | [5]Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia 43400 UPM, Serdang, Selangor, Malaysia | [6]Center of Excellence for Research, Value Innovation and Entrepreneurship (CERVIE), UCSI University, Cheras 56000, Kuala Lumpur, Malaysia | [7]Kembang Subur Sdn. Bhd, Lot 948, Tanjong Tengah, Mukim Sungai Karang 26100, Kuantan, Pahang, Malaysia

Correspondence: Ikhsan Natrah (natrah@upm.edu.my)

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ABSTRACT

The intensification of shrimp aquaculture has led to challenges such as declining water quality, reduced growth rates, and increased disease susceptibility. Although biofloc technology improves water quality and supports shrimp growth, it often lacks essential polyunsaturated fatty acids (PUFAs), which are vital for shrimp development and immunity. This study investigates the effects of *Halamphora coffeaeformis* supplementation in a rice bran-based biofloc system on the growth performance, survival, and disease resistance of black tiger shrimp (*Penaeus monodon*) postlarvae (PL). Four rearing conditions were evaluated: control (clear water with water exchange), BFR0 (*H. coffeaeformis* only, 1×10^5 cells mL^{-1}), BFR1 (biofloc only), and BFR2 (biofloc supplemented with *H. coffeaeformis* at 1×10^5 cells mL^{-1}). Shrimp reared in BFR2 exhibited the highest final weight (0.46 ± 0.05 g, $p < 0.05$), specific growth rate and daily weight gain (DWG), along with superior feed conversion efficiency (1.28 ± 0.13). Survival was significantly higher ($95.42 \pm 5.77\%$, $p < 0.05$) compared to all other groups. Additionally, shrimp in BFR2 demonstrated the greatest lipid ($7.08 \pm 0.82\%$) and protein ($49.96 \pm 1.19\%$) contents, along with the highest PUFA levels, particularly eicosapentaenoic acid (EPA; $2.88 \pm 0.59\%$) and docosahexaenoic acid (DHA; $0.49 \pm 0.26\%$). Following *Vibrio parahaemolyticus* exposure, this group exhibited the lowest mortality ($64.58 \pm 3.43\%$) and reduced hepatopancreatic damage. These results suggest that supplementing a rice bran-based biofloc with *H. coffeaeformis* at 1×10^5 cells mL^{-1} enhances shrimp growth, nutritional quality, and disease resistance, reinforcing its potential as a sustainable aquaculture strategy.

Summary

1. *Penaeus monodon* postlarvae (PL) from *Halamphora coffeaeformis*-supplemented rice bran biofloc (BFR2) showed superior growth, daily weight gain (DWG), and lower feed conversion ratio (FCR) than the other groups.
2. *P. monodon* PL from BFR2 exhibited significantly higher lipid and protein content, with elevated eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) levels, indicating improved nutritional quality.
3. *P. monodon* PL from BFR2 showed the highest survival and lowest mortality post-*Vibrio* challenge, with reduced hepatopancreatic damage, suggesting enhanced disease resistance.
4. Supplementation with *H. coffeaeformis* significantly increased floc volume (FV) in BFR2, indicating enhanced biofloc development under zero-exchange conditions.

1 | Introduction

The intensification of shrimp aquaculture has substantially boosted shrimp production to fulfill the growing worldwide demand. In contrast, it has also introduced several challenges such as declining water quality, reduced growth rates, and increased vulnerability to diseases. One of the primary challenges in intensive aquaculture systems is maintaining high survival rates, as shrimp are often vulnerable to environmental stress, poor water quality, and disease outbreaks. Biofloc technology has emerged as a sustainable solution for addressing water quality and shrimp survival issues. Biofloc serves as a natural food source for shrimp, improving growth performance and feed efficiency, indirectly decreasing production costs [1]. Nevertheless, biofloc alone is typically devoid of essential polyunsaturated fatty acids (PUFAs), which are important for shrimp development, immune function, and disease resistance [2].

To overcome this limitation, integrating microalgae into biofloc systems has gained interest due to numerous benefits to aquatic animals [1, 3]. Microalgae, particularly diatoms, are abundant in essential PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These PUFAs have been shown to enhance shrimp growth, overall productivity, and immunity [4]. Moreover, microalgae play a vital role in absorbing and reducing toxic ammonia levels, thereby directly improving water quality [5].

Previous studies have explored the supplementation of various diatom species into biofloc systems to examine their impacts on shrimp culture. For instance, a diatom consortium (*Chaetoceros calcitrans*, *Navicula* sp., and *Phaeodactylum tricornutum*) incorporated into biofloc enhanced the growth performance of *Litopenaeus vannamei* postlarvae (PL), but the findings did not show a significant difference ($p > 0.05$) compared to the control (biofloc only) [6]. Similarly, the integration of *Navicula* spp. into biofloc did not result in significant improvement in the growth performance of *L. vannamei* PL compared with the biofloc-only control ($p > 0.05$) [1]. In contrast, biofloc supplemented with *Navicula* sp. produced significantly higher ($p < 0.05$) final weight and yield of *L. vannamei* PL [7]. In another study, Martins et al. [3] evaluated the contribution of different diatom species separately in biofloc to the performance of *L. vannamei* PL culture. They discovered that these diatoms did not yield significant differences ($p > 0.05$) in growth performance. However, the effects on PL survival and lipid content varied depending on the diatom species. These findings suggest that the influence of biofloc-supplemented diatoms on shrimp performance is highly species-specific.

Halamphora coffeaeformis has emerged as a promising candidate due to its high content of PUFAs such as EPA and DHA. It has shown potential in boosting the growth, nutritional profile and survival rates of *Penaeus monodon* [5]. However, its inclusion in biofloc systems remains largely unexplored. Moreover, previous studies have primarily focused on incorporating diatoms into molasses-based biofloc systems [1, 7], leaving the potential of alternative biofloc substrates (e.g., rice bran) largely unexamined. Given the species-specific nature of diatom interactions in biofloc systems, there is a need to evaluate the combined effects of *H. coffeaeformis* supplementation and alternative biofloc substrates. Therefore, this study aimed to evaluate the effects of *H. coffeaeformis* supplementation in a rice bran-based biofloc system on the growth performance, survival, and disease resistance of black tiger shrimp (*P. monodon*) PL.

2 | Materials and Methods

2.1 | *P. monodon* PL Acclimation and Feeding

A total of 1000 specific pathogen-free (SPF) *P. monodon* PL were acquired from Kembang Subur Sdn. Bhd., Malaysia (3°55′04.3″ N, 103°21′56.7″ E) and acclimated for 7 days in a 1000 L fiberglass tank containing filtered seawater before experimental stocking. Water parameters were maintained as follows: temperature at 28 ± 1°C, dissolved oxygen (DO) at 5 ± 1 mg L^{-1}, salinity at 32 ± 1 ppt, and pH at 8.7 ± 0.1. Starfeed 5001 (Starfeed Mills Sdn. Bhd., Malaysia) was employed as the commercial diet, and the PL were fed three times daily. The feed formulation consisted of 40% protein, 5% fat, and 12% moisture.

2.2 | *H. coffeaeformis* Culture

The benthic diatom *H. coffeaeformis* strain BpSpD2 (Genbank accession number MK005161) was obtained from the Aquatic Bioproduct Laboratory, Aquaculture Department, Faculty of Agriculture, Universiti Putra Malaysia (UPM), Malaysia. The stock culture was maintained in F/2 medium supplemented with silicate (SiO^4; Si) [8] under controlled conditions, including a light intensity of 120 µmol m^{-2} s^{-1} and an 18 h light/6 h dark photoperiod using LED illumination [5]. For the experiment, *H. coffeaeformis* was cultured in 400 L F/2 medium, with water conditions kept the same as those used for shrimp acclimatization. During the culture period, a 12 h light/dark cycle was maintained with fluorescent lamps providing light intensity of 100 µmol m^{-2} s^{-1}. The concentration of *H. coffeaeformis* in each treatment was regulated at 1×10^5 cells mL^{-1}. Before inoculation, the cell density was determined using the hemocytometer cell counting method [9].

2.3 | Experimental Design

The study was carried out indoors over an 8-week period at the hatchery facility of the Institute of Bioscience, UPM. A

randomized experimental design was employed in triplicate, comprising three treatment groups and a control group. The control group consisted of only clear water and underwent regular water exchange (30% every 3 days). The treatment groups were as follows: BFR0 (1×10^5 cells mL^{-1} of *H. coffeaeformis* only), BFR1 (biofloc rice bran only), and BFR2 (biofloc rice bran with 1×10^5 cells mL^{-1} *H. coffeaeformis* addition). No water exchange was performed for the treatment groups, and dechlorinated freshwater was added to compensate for evaporation losses. Similarly, the cell number of *H. coffeaeformis* was assessed weekly, and additional volumes were introduced as needed to maintain the desired concentration. After acclimatization, the PL were size-graded by random sampling, with wet weight and total length measured. Only visually active and intact individuals within a uniform size range (3.0 ± 0.1 mg and 1.2 ± 0.1 cm) were selected. The selected PL were then randomly assigned to experimental tanks (0.68 m × 0.39 m × 0.50 m). Each tank contained 40 PL in 80 L filtered seawater, resulting in a stocking density of 500 PL m^{-3}. The PL were fed a commercial shrimp starter feed (Starfeed 5001, Starfeed Mills Sdn. Bhd., Malaysia; see Section 2.1 for composition) at an initial rate of 12% body weight per day and later adjusted to 8% based on the estimated biomass in each tank [10]. Locally sourced rice bran was incorporated into the feeding regimen daily, approximately 2 h after the initial feeding. The carbon to nitrogen (C:N) ratio was maintained at 20, considering the quantity and protein composition of the feed provided [11]. Feed conversion ratio (FCR) was calculated based on commercial feed input only, as rice bran was applied solely as an external carbon source for biofloc development. The proximate composition of the feed and rice bran was determined according to AOAC [12] standards before the study started (Table 1).

2.4 | Water Quality Parameters

Temperature, DO, salinity, and pH levels in each group were measured daily using the YSI ProQuatro Multiparameter Meter (Yellow Springs, Ohio, USA). Total suspended solids (TSS) were determined weekly using method 2540 D [13], while floc volume (FV; mL L^{-1}) was measured weekly using the Imhoff cone [14]. Total ammonia nitrogen (TAN), nitrite-nitrogen (nitrite-N), nitrate-nitrogen (nitrate-N), and orthophosphate concentrations were analyzed every week using the DR900 Multiparameter Portable Colorimeter (Hach, Colorado, USA). The TAN levels were assessed through the salicylate method, nitrite-N via the USEPA diazotization method, nitrate-N through the cadmium reduction technique and orthophosphate using the PhosVer3 ascorbic acid technique (Hach, Loveland, CO, USA) [15].

TABLE 1 | Proximate composition of commercial feed (Starfeed) and rice bran.

Nutritional composition	Starfeed (%)	Rice bran (%)
Moisture	8.95 ± 0.67^a	8.57 ± 0.15^a
Ash	11.28 ± 0.18^a	7.60 ± 0.42^b
Lipid	4.43 ± 0.35^b	12.93 ± 1.00^a
Protein	34.38 ± 1.95^a	11.60 ± 0.15^b
Fiber	1.51 ± 0.16^b	4.49 ± 0.09^a
Carbohydrate	39.46 ± 0.96^b	54.80 ± 1.25^a

Note: All values are mean ± standard deviation. All values, except moisture, are expressed as percentage on dry weight. Values in the same row with different superscripts differ significantly.

2.5 | Zootechnical Performance of *P. monodon* PL

To assess growth performance and adjust feeding rate, 20 shrimp were randomly sampled weekly from each tank. Shrimp were gently collected using a fine mesh net and immediately transferred to a container with culture water for measurement. Body weight and total length were recorded individually, and the shrimp were immediately returned to their respective tanks. To reduce handling stress and ensure consistency across treatments, sampling was performed by the same personnel, at the same time of day, using a standardized protocol, with minimal air exposure and handling time. Feed amounts were subsequently adjusted according to the estimated biomass. At the end of the culture period, final weight, specific growth rate for weight (SGR_W) and length (SGR_L), daily weight gain (DWG), FCR, and survival rate were calculated using the following equations:

$$\text{Specific growth rates weight } (SGR_w)\left(\frac{\%}{\text{day}}\right) = \frac{\ln\text{final wet weight (mg)} - \ln\text{inititial wet weight (mg)}}{\text{Time (days)}} \times 100,$$

$$\text{Specific growth rates length } (SGR_l)\left(\frac{\%}{\text{day}}\right) = \frac{\ln\text{final total length(cm)} - \ln\text{initial total length (cm)}}{\text{Time (days)}} \times 100,$$

$$\text{Daily weight gains (DWG)}\left(\frac{\text{mg}}{\text{Day}}\right) = \frac{\text{final weight (mg)} - \text{initial weight (mg)}}{\text{Time (days)}},$$

$$\text{Feed conversion ratios (FCR)} = \frac{\text{Dryweight of consumed feed (g)}}{\text{Penaeus monodon wet weight (g)}} \times 100,$$

$$\text{Survival rate (\%)} = \frac{\text{Number of live shrimp}}{\text{Initial number of shrimp}} \times 100.$$

2.6 | Proximate Composition of *P. monodon* PL

At the end of the study, the moisture, ash, lipid, and protein contents of whole-body PL were analyzed in triplicate using standard procedures [12]. Moisture content was assessed by drying shrimp in an oven at 60°C, then further at 105°C until a constant weight was achieved. Total ash was determined by incinerating samples in an oven at 550°C for 5 h, with the weight difference before and after incineration expressed as a percentage. Lipid content was first extracted using the acid hydrolysis method and determined with a Soxhlet extraction unit (SOXTEC 8000, FOSS Ltd., Denmark). Meanwhile, protein content was determined by measuring nitrogen (N × 6.25) using the Kjeldahl method with an automatic analyzer (Kjeltec 8400, FOSS Ltd., Denmark).

2.7 | Fatty Acid Methyl Ester (FAME) Profile of *P. monodon* PL

FAMEs were prepared according to Ichihara and Fukubayashi [16] protocol. Briefly, the lipids from *P. monodon* PL were extracted using a 2:1 chloroform and methanol solution. Hexane was added to a mixture of lipids and a reaction solution of acetyl chloride and methanol (5:100 volume ratio) for methylation purposes. The supernatant containing FAMEs was dried under nitrogen gas. Subsequently, 100 μL of hexane was added, and 10 μL was introduced into a gas chromatograph-mass spectrometer (6890 N GC/5973MS, Agilent Technologies, USA) equipped with a 30 m × 0.25 mm (ID) × 0.25 μm capillary column (Agilent DB-FFAP, USA), and helium was employed at a flow rate of 1.3 mL min^{-1}. The analysis began at 50°C for 1 min, increased to 100°C at 10°C min^{-1}, then continued to 240°C, where it was held for 15 min. Fatty acid concentrations were determined by analyzing peak areas in relation to an internal standard, with compound identification verified via the NIST library.

2.8 | Challenge Test of *P. monodon* PL Against *Vibrio parahaemolyticus*

After the 8-week culture period, 60 PL from each treatment were randomly distributed into three replicate 40 L tanks (20 PL per tank) for the *V. parahaemolyticus* challenge test. An additional negative control group was included, comprising SPF PL of the same age and comparable initial size as those in the challenged treatments but maintained without *V. parahaemolyticus* inoculation [17]. All challenge tanks were provided with continuous aeration throughout the experimental period. *V. parahaemolyticus* strain S12-3 (GenBank accession no. OP198226) was cultured overnight and adjusted to a final concentration of 1×10^7 CFU mL^{-1} for the challenge test. Following inoculation, shrimp survival was monitored at 12 h intervals for 5 days, and cumulative mortality was calculated for each treatment [18]. During the challenge period, shrimp were fed the same commercial diet (Section 2.1) twice daily. Hepatopancreas samples from dead shrimp were aseptically collected and cultured on thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Oxoid, UK) for the isolation of presumptive *V. parahaemolyticus*. Identification was based on selective culture, and no molecular confirmation by PCR was performed.

2.9 | Histological Analysis of Postchallenge *P. monodon* PL Tissues

After the challenge test, both moribund and healthy PL were fixed in Davidson AFA solution for 24 h before being sent to the Veterinary Histopathology Laboratory, Faculty of Veterinary, UPM. These samples underwent tissue processing, embedding, sectioning and hematoxylin and eosin staining. After that, the stained tissues were examined under a light microscope with magnifications ranging from 40 to 100x (Axioskop 2, Zeiss, Jena, Germany) to evaluate histopathological changes.

2.10 | Statistical Analysis

Levene's test was conducted to assess the homogeneity of variances (homoscedasticity), while the Shapiro–Wilk test was employed to check for normality of the data. One-way analysis of variance (ANOVA) was performed to analyze water quality parameters, zootechnical data, nutritional composition and mortality rates. When significant differences were detected among groups, Tukey's post hoc test was applied for multiple comparisons. Data were reported as means ± standard deviations. All statistical analyses were carried out with the aid of IBM SPSS Statistics software version 22 (IBM, Armonk, New York), and significance was determined at $p < 0.05$.

3 | Results and Discussion

3.1 | Zootechnical Performance of *P. monodon* PL

The final weight of PL in BFR1 (0.46 ± 0.12 g) and BFR2 (0.46 ± 0.05 g) was significantly higher ($p < 0.05$) than in the control (0.27 ± 0.04 g) and BFR0 (0.27 ± 0.06 g; Table 2). However, similar final weights were obtained in BFR1 and BFR2 ($p > 0.05$). This implies that either rice bran biofloc or the combination of microalgae and biofloc could serve as additional food sources, contributing to improved shrimp growth. Additionally, BFR1 (SGR_W: 8.18 ± 0.44% day^{-1}; SGR_L: 2.12 ± 0.15 day^{-1}; DWG: 7.42 ± 1.96 mg day^{-1}) and BFR2 (SGR_W: 8.22 ± 0.17% day^{-1}; SGR_L: 2.16 ± 0.08 day^{-1}; DWG: 7.46 ± 0.78 mg day^{-1}) exhibited significantly higher ($p < 0.05$) SGR_W, SGR_L, and DWG compared to the control (SGR_W: 7.37 ± 0.26% day^{-1}; SGR_L: 1.86 ± 0.06 day^{-1}; DWG: 4.43 ± 0.71 mg day^{-1}) and BFR0 (SGR_W: 7.32 ± 0.35% day^{-1}; SGR_L: 1.84 ± 0.09 day^{-1}; DWG: 4.35 ± 0.92 mg day^{-1}). The FCR values in

TABLE 2 | Growth performance of *Penaeus monodon* cultured in control and treatment groups.

	Group			
Parameters	**Control**	**BFR0**	**BFR1**	**BFR2**
Final weight (g)	0.27 ± 0.04^b	0.27 ± 0.06^b	0.46 ± 0.12^a	0.46 ± 0.05^a
SGR_W (% day^{-1})	7.37 ± 0.26^b	7.32 ± 0.35^b	8.18 ± 0.44^a	8.22 ± 0.17^a
SGR_L (% day^{-1})	1.86 ± 0.06^b	1.84 ± 0.09^b	2.12 ± 0.15^a	2.16 ± 0.08^a
DWG (mg day^{-1})	4.43 ± 0.71^b	4.35 ± 0.92^b	7.42 ± 1.96^a	7.46 ± 0.78^a
FCR	2.18 ± 0.35^a	2.25 ± 0.49^a	1.34 ± 0.37^b	1.28 ± 0.13^b
Survival (%)	70.00 ± 3.75^b	78.75 ± 8.75^b	80.00 ± 3.75^b	95.42 ± 5.77^a

Note: All values are mean ± standard deviation. Values in the same row with different superscripts differ significantly. Control (clear water), BFR0 (*Halamphora coffeaeformis* only), BFR1 (biofloc rice bran only) and BFR2 (biofloc rice bran with *H. coffeaeformis* addition).
Abbreviations: DWG, daily weight gain; FCR, feed conversion ratio; SGR_L, specific growth rate in length; SGR_W, specific growth rate in weight.

BFR1 (1.34 ± 0.37) and BFR2 (1.28 ± 0.13) were significantly lower ($p < 0.05$) than those in the control (2.18 ± 0.35) and BFR0 (2.25 ± 0.49). No significant difference ($p > 0.05$) in FCR was observed between BFR1 and BFR2. This observation suggests that the improvement in feed utilization might be associated with the biofloc system itself, whereas the addition of microalgae did not yield further significant improvement in FCR. The relatively lower growth performance observed in this study compared to both pond-based and small-tank studies may be attributed to the controlled indoor cultivation conditions and the utilization of a zero-exchange biofloc system, which may affect energy allocation and growth dynamics in PL. Overall, the current findings are consistent with those of Martins et al. [3] and Brito et al. [1], who reported no significant effects of diatom supplementation in biofloc systems on shrimp final weight, SGR and FCR. For survival rates, BFR2 demonstrated the highest value (95.42 ± 5.77%) among all groups, showing a significant difference ($p < 0.05$) compared to the control (70.00 ± 3.75%), BFR0 (78.75 ± 8.75%), and BFR1 (80.00 ± 3.75%). The superior survival rate in BFR2 underscores the importance of *H. coffeaeformis* addition in rice bran biofloc for improving shrimp survival. This result is consistent with the study by Martins et al. [3], which reported that diatom-supplemented biofloc significantly increased shrimp survival. These findings propose that growth performance in biofloc systems is influenced not only by nutrient availability but also system configuration and microbial dynamics, emphasizing the need to balance between growth optimization and survival under zero-exchange conditions.

3.2 | Proximate Composition of *P. monodon* PL

Proximate composition analysis of whole-body *P. monodon* revealed that all groups displayed dry matter content ranging from 21.82% to 22.91% with no significant differences ($p > 0.05$) among them (Table 3). The ash content of shrimp in BFR1 (10.91 ± 0.29%) and BFR2 (12.08 ± 0.30%) was significantly lower ($p < 0.05$) than that in the control (14.04 ± 0.82%) and BFR0 (13.05 ± 0.61%). Meanwhile, shrimp from BFR1 (lipid: 5.66 ± 0.25%; protein: 48.64 ± 0.37%) and BFR2 (lipid: 7.08 ± 0.82%; protein: 49.96 ± 1.19%) exhibited significantly higher ($p < 0.05$) lipid and protein content compared to those from the control (lipid: 4.32 ± 0.95%; protein: 42.05 ± 0.73%) and BFR0 (lipid: 4.63 ± 1.37%; protein: 40.83 ± 0.16%). These results indicate that rice bran-based biofloc could supply additional lipids and proteins to shrimp, yielding greater nutritional value. This result is supported by a study by Valle et al. [19], who reported that biofloc can provide extra nutrients, minerals and vitamins for shrimp growth. Moreover, microbial protein in biofloc has been proposed to contribute to the increased protein content in shrimp [20].

On the other hand, the lipid content of shrimp in BFR2 was significantly higher ($p < 0.05$) than that in BFR1, whereas no significant difference ($p > 0.05$) in protein content was observed between the two treatments. This may be attributed to the supplementation of microalgae into the biofloc, as this diatom has been shown to contain a substantial amount of lipids [5]. Overall, the integration of *H. coffeaeformis* not only enhances growth performance (Table 2) but also boosts the overall quality of *P. monodon* by improving its nutritional composition, particularly in terms of lipid content. This has considerable implications for sustainable and economically viable shrimp farming practices, offering a promising avenue for improving shrimp aquaculture productivity.

3.3 | FAME Profile of *P. monodon* PL

Saturated fatty acids (SFAs) were the predominant fatty acid component in the control (56.88 ± 0.54% total fatty acids [TFAs]) and BFR0 (50.20 ± 2.66% TFA) groups, while they were the second most abundant component in the BFR1 (49.82 ± 0.73% TFA) and BFR2 (46.43 ± 9.77% TFA) groups (Table 4). BFR2 showed the lowest SFA content among all groups, exhibiting significant difference ($p < 0.05$) compared to the control. Palmitic acid (C16:0) (ranging from 27.12% to 30.41% TFA) and stearic acid (C18:0) (ranging from 14.64% to 21.98% TFA) were the most prevalent fatty acids constituting the majority of SFA and were identified in all groups. This is corroborated by the study by Baharuddin et al. [5], which identified palmitic acid as the dominant SFA in the whole body of *P. monodon* when supplemented with *H. coffeaeformis*. On the other hand, monounsaturated fatty acid (MUFA) content was comparable across all groups ($p > 0.05$), ranging from 24.42% to 27.10% TFA, with a slightly higher level found in BFR1 (27.10 ± 0.74% TFA). Oleic acid (C18:1) constituted the largest portion of MUFA, accounting for 21.79% to 25.48% TFA. This fatty acid was consistently found in all groups.

Although all treatment groups showed numerically higher PUFA values (23.08% to 29.13% TFA) than the control (18.70 ± 0.13% TFA), only BFR2 (29.13 ± 0.17% TFA) was significantly higher ($p < 0.05$). This suggests that the inclusion of microalgae in biofloc systems may improve the PUFA content of shrimp, which is consistent with the findings by de Abreu et al. [21]. Omega-3 and omega-6 fatty acids were the main contributors to total PUFA in

TABLE 3 | Proximate analysis of *Penaeus monodon* cultured in control and treatment groups.

	Group			
Composition (%)	**Control**	**BFR0**	**BFR1**	**BFR2**
Dry matter	21.89 ± 0.55^{a}	21.82 ± 0.56^{a}	21.91 ± 0.35^{a}	22.91 ± 0.23^{a}
Ash	14.04 ± 0.82^{a}	13.05 ± 0.61^{ab}	10.91 ± 0.29^{c}	12.08 ± 0.30^{bc}
Lipid	4.32 ± 0.95^{c}	4.63 ± 1.37^{c}	5.66 ± 0.25^{b}	7.08 ± 0.82^{a}
Protein	42.05 ± 0.73^{b}	40.83 ± 0.16^{b}	48.64 ± 0.37^{a}	49.96 ± 1.19^{a}

Note: All values are mean ± standard deviation. All values, except dry matter, are expressed as percentage on wet weight. Values in the same row with different superscripts differ significantly. Control (clear water), BFR0 (*Halamphora coffeaeformis* only), BFR1 (biofloc rice bran only), and BFR2 (biofloc rice bran with *H. coffeaeformis* addition).

TABLE 4 | Fatty acids methyl ester (FAME) profiles of *Penaeus monodon* cultured in control and treatment groups.

	Fatty acid content (%) of *Penaeus monodon*			
Fatty acid profile	**Control**	**BFR0**	**BFR1**	**BFR2**
C13:0	0.21 ± 0.02^{a}	0.18 ± 0.02^{a}	nd	0.10 ± 0.09^{a}
C15:0	nd	1.23 ± 0.88^{a}	1.49 ± 1.26^{a}	0.25 ± 0.07^{a}
C16:0	30.41 ± 2.90^{a}	30.32 ± 5.74^{a}	27.12 ± 2.83^{a}	28.87 ± 6.94^{a}
C17:0	1.42 ± 0.08^{a}	0.97 ± 0.84^{a}	0.79 ± 0.68^{a}	0.68 ± 0.36^{a}
C18:0	21.98 ± 1.84^{a}	18.83 ± 0.32^{ab}	18.43 ± 0.19^{ab}	14.64 ± 3.06^{b}
C19:0	0.30 ± 0.27^{a}	0.17 ± 0.03^{a}	0.02 ± 0.01^{a}	0.43 ± 0.33^{a}
C20:0	0.92 ± 0.24^{a}	0.68 ± 0.59^{a}	0.95 ± 0.82^{a}	0.75 ± 0.14^{a}
C22:0	0.38 ± 0.06^{a}	0.68 ± 0.59^{a}	0.76 ± 0.66^{a}	0.54 ± 0.09^{a}
C16:1$n-7$	1.36 ± 0.25^{a}	1.94 ± 0.98^{a}	1.31 ± 0.72^{a}	0.70 ± 0.38^{a}
C18:1$n-9$	22.63 ± 0.17^{a}	21.79 ± 0.05^{a}	25.48 ± 3.30^{a}	23.58 ± 1.02^{a}
C20:1$n-9$	0.22 ± 0.08^{a}	nd	nd	0.18 ± 0.03^{a}
C18:2$n-6$	16.20 ± 1.66^{a}	19.94 ± 1.68^{a}	20.61 ± 2.18^{a}	21.60 ± 3.38^{a}
C18:3$n-6$	0.32 ± 0.29^{a}	0.21 ± 0.18^{a}	nd	0.44 ± 0.26^{a}
C20:2$n-9$	0.36 ± 0.34^{a}	0.40 ± 0.35^{a}	0.28 ± 0.09^{a}	1.05 ± 0.40^{a}
C20:4$n-6$	1.60 ± 0.33^{ab}	0.98 ± 0.56^{b}	1.87 ± 0.59^{ab}	2.59 ± 0.18^{a}
C20:5$n-3$	nd	2.85 ± 0.47^{a}	nd	2.88 ± 0.59^{a}
C22:6$n-3$	nd	0.59 ± 0.12^{a}	nd	0.49 ± 0.26^{a}
∑ saturated FA	56.88 ± 0.54^{a}	50.20 ± 2.66^{b}	49.82 ± 0.73^{b}	46.23 ± 3.77^{b}
∑ unsaturated FA	43.12 ± 0.46^{b}	49.80 ± 1.64^{a}	50.18 ± 0.41^{a}	53.77 ± 4.52^{a}
∑ MUFA	24.42 ± 0.11^{a}	24.47 ± 0.22^{a}	27.10 ± 0.74^{a}	24.49 ± 2.14^{a}
∑ PUFA	18.70 ± 0.13^{b}	25.33 ± 0.24^{ab}	23.08 ± 0.16^{ab}	29.13 ± 0.17^{a}
∑ omega 3	nd	3.65 ± 0.83^{a}	nd	3.82 ± 0.14^{a}
∑ omega 6	18.12 ± 0.55^{c}	21.13 ± 0.76^{b}	22.48 ± 0.94^{ab}	24.63 ± 1.21^{a}
∑ EPA	nd	2.85 ± 2.47^{a}	nd	2.88 ± 0.59^{a}
∑ DHA	nd	0.59 ± 0.02^{a}	nd	0.49 ± 0.26^{a}

Note: All values are mean ± standard deviation. Values in the same row with different superscripts differ significantly. Control (clear water), BFR0 (*Halamphora coffeaeformis* only), BFR1 (biofloc rice bran only), and BFR2 (biofloc rice bran with *H. coffeaeformis* addition).
Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; MUFA, monounsaturated fatty acids; nd, not detected; PUFA, polyunsaturated fatty acids.

this study. Higher omega-6 levels were identified in all treatment groups (21.13% to 24.63% TFA) than the control (18.12 ± 0.55% TFA). BFR2 was the only group that demonstrated significantly higher omega-3 content (24.63 ± 1.21% TFA) compared to the control. In contrast, omega-3 fatty acids were identified only in the microalgae-containing groups (BFR0 and BFR2), with BFR2 showing the highest value (3.82 ± 0.14% TFA). This is consistent with previous reports that diatoms are rich sources of omega-3 PUFAs, particularly EPA and DHA [5]. The incorporation of diatoms into shrimp culture may therefore enhance the fatty acid profile of shrimp. These PUFAs have been reported to support shrimp growth and survival [3], and diatoms may provide an alternative PUFA source that enhances resistance to disease and environmental stress [22]. A similar trend was observed in this study, where the highest growth performance and survival rates of shrimp in BFR2 could be related to its elevated PUFA levels derived from microalgae. Therefore, these findings attenuate the potential of integrating diatoms into biofloc as a strategy to improve shrimp growth performance and overall survival rates by enriching their PUFA profile. This highlights the potential of targeted microalgal supplementation to regulate shrimp biochemical composition in biofloc systems, illustrating the relationship between dietary inputs and physiological performance.

3.4 | Water Quality Parameters in *P. monodon* PL Culture Across Control and Treatment Groups

Throughout the experimental period, water quality parameters such as temperature (~27°C), DO (ranging from 5.0 ± 0.2 mg L^{-1} to 5.2 ± 0.2 mg L^{-1}), salinity (31.0 ± 0.0 ppt to 31.4 ± 0.2 ppt), pH (8.35 ± 0.50 to 8.86 ± 0.06), and TSS (485.2 ± 31.7 mg L^{-1} to 511.2 ± 25.7 mg L^{-1}) remained consistent across all the groups with no

TABLE 5 | Water quality parameters of *Penaeus monodon* cultured in control and treatment groups.

	Group			
Water parameters	**Control**	**BFR0**	**BFR1**	**BFR2**
Temperature (°C)	27.2 ± 0.3^{a}	27.1 ± 0.1^{a}	27.1 ± 0.3^{a}	27.2 ± 0.1^{a}
Dissolved oxygen (mg L^{-1})	5.2 ± 0.2^{a}	5.2 ± 0.1^{a}	5.0 ± 0.2^{a}	5.0 ± 0.2^{a}
Salinity (ppt)	31.4 ± 0.2^{a}	31.1 ± 0.2^{a}	31.0 ± 0.0^{a}	31.2 ± 0.3^{a}
pH	8.86 ± 0.06^{a}	8.89 ± 0.05^{a}	8.35 ± 0.50^{a}	8.61 ± 0.35^{a}
TSS (mg L^{-1})	485.2 ± 31.7^{a}	485.8 ± 26.0^{a}	511.2 ± 25.7^{a}	509.8 ± 30.4^{a}
FV (mL L^{-1})	0.2 ± 0.1^{d}	1.6 ± 0.2^{c}	6.4 ± 0.3^{b}	7.9 ± 0.4^{a}
TAN (mg L^{-1})	0.13 ± 0.09^{a}	0.11 ± 0.04^{a}	0.15 ± 0.03^{a}	0.10 ± 0.03^{a}
Nitrite-N (mg L^{-1})	0.29 ± 0.12^{b}	0.79 ± 0.06^{a}	0.77 ± 0.14^{a}	0.78 ± 0.09^{a}
Nitrate-N (mg L^{-1})	1.05 ± 0.18^{b}	1.57 ± 0.27^{a}	1.91 ± 0.27^{a}	1.81 ± 0.29^{a}
Orthophosphate (mg L^{-1})	1.00 ± 0.30^{a}	1.31 ± 0.30^{a}	1.36 ± 0.12^{a}	1.99 ± 0.21^{b}

Note: All values are mean ± standard deviation. Values in the same row with different superscripts differ significantly. Control (clear water), BFR0 (*Halamphora coffeaeformis* only), BFR1 (biofloc rice bran only), and BFR2 (biofloc rice bran with *H. coffeaeformis* addition).
Abbreviations: FV, floc volume; ppt, part per thousand; TAN, total ammonia nitrogen; TSS, total suspended solid.

significant differences ($p > 0.05$; Table 5). These values were within the optimal range for *P. monodon*, as recommended by Chaitanawisuti et al. [23]. FV differed significantly among groups ($p < 0.05$) with BFR2 showing the highest value (7.9 ± 0.4 mL L^{-1}), followed by BFR1 (6.4 ± 0.3 mL L^{-1}), BFR0 (1.6 ± 0.2 mL L^{-1}), and the control (0.2 ± 0.1 mL L^{-1}). FV in BFR1 and BFR2 fell within the suggested range of 5–50 mL L^{-1} for penaeid shrimp [24], whereas values in the control and BFR0 were below this range. This suggests that the incorporation of *H. coffeaeformis* into biofloc may have boosted floc development, potentially enhancing nutrient assimilation and fecal production, which could benefit shrimp growth and health [25].

In terms of chemical water quality, TAN levels were comparable among the control (0.13 ± 0.09 mg L^{-1}), BFR0 (0.11 ± 0.04 mg L^{-1}), BFR1 (0.15 ± 0.03 mg L^{-1}) and BFR2 (0.10 ± 0.03 mg L^{-1}), with no significant differences detected ($p > 0.05$). Meanwhile, all treatments showed significantly higher ($p < 0.05$) nitrite-N (ranging from 0.77 to 0.79 mg L^{-1}) and nitrate-N (ranging from 1.57 to 1.81 mg L^{-1}) compared to the control (nitrite-N: 0.29 mg L^{-1}; nitrate-N: 1.05 mg L^{-1}). This is likely due to the conversion of toxic ammonia into nitrite and subsequently into the less harmful compound, nitrate by nitrifying bacteria and microalgae within the biofloc system [26]. On the other hand, orthophosphate concentrations were significantly higher ($p < 0.05$) in BFR2 (1.99 ± 0.21 mg L^{-1}) compared to the other treatments (ranging from 1.31 ± 0.30 to 1.36 ± 0.12 mg L^{-1}) and the control (1.00 ± 0.30 mg L^{-1}). The decomposition of phosphorus-rich rice bran and the release of phosphorus by microalgae may be the key factors contributing to higher orthophosphate concentrations in BFR2 [27, 28]. Collectively, the nutrient concentrations in all groups remained within the recommended range for penaeid shrimp [29].

3.5 | Challenge Test and Histological Analysis of *P. monodon* PL Against *V. parahaemolyticus*

Shrimp in the positive control group showed a cumulative mortality of $79.24 \pm 3.00\%$ following the *V. parahaemolyticus* challenge (Table 6). This high mortality corresponded with severe histopathological damage observed in the PL hepatopancreas, where extensive necrosis of epithelial cells affected nearly all tubules (Figure 1a). Additionally, hemocyte infiltration was frequently observed in the interstitial spaces, causing space constriction. Moreover, several epithelial cells were detached and present within the tubule lumens, further deteriorating the tissue structure. These tissue damages underscore the detrimental effects of *V. parahaemolyticus* infection on PL survival.

BFR0 ($71.97 \pm 4.51\%$) and BFR1 ($73.58 \pm 3.12\%$) exhibited no significant difference ($p > 0.05$) in mortality compared with the positive control. Histopathological analysis of the hepatopancreas in these groups revealed moderate structural damage, characterized by a reduced number of necrotic hepatopancreatic epithelial cells and fewer sloughed cells within the tubule lumens (Figure 1b,c). Furthermore, hemocyte infiltration was rarely identified.

The inclusion of microalgae into the biofloc (BFR2) yielded the lowest mortality ($64.58 \pm 3.43\%$) in PL, which was significantly

TABLE 6 | The mortality rates of *Penaeus monodon* cultured in control and treatment groups after immersion in 1×10^{7} CFU mL^{-1} of *Vibrio parahaemolyticus*.

Group	**Mortality (%)**
Negative control	0.00 ± 0.00
Positive control	79.24 ± 3.00^{a}
BFR0	71.97 ± 4.51^{ab}
BFR1	73.58 ± 3.12^{a}
BFR2	64.58 ± 3.43^{b}

Note: All values are mean ± standard deviation. Values in the same column with different superscripts differ significantly. Negative control (clear water only), positive control (clear water with *V. parahaemolyticus*), BFR0 (*Halamphora coffeaeformis* only), BFR1 (biofloc rice bran only), and BFR2 (biofloc rice bran with *H. coffeaeformis* addition).

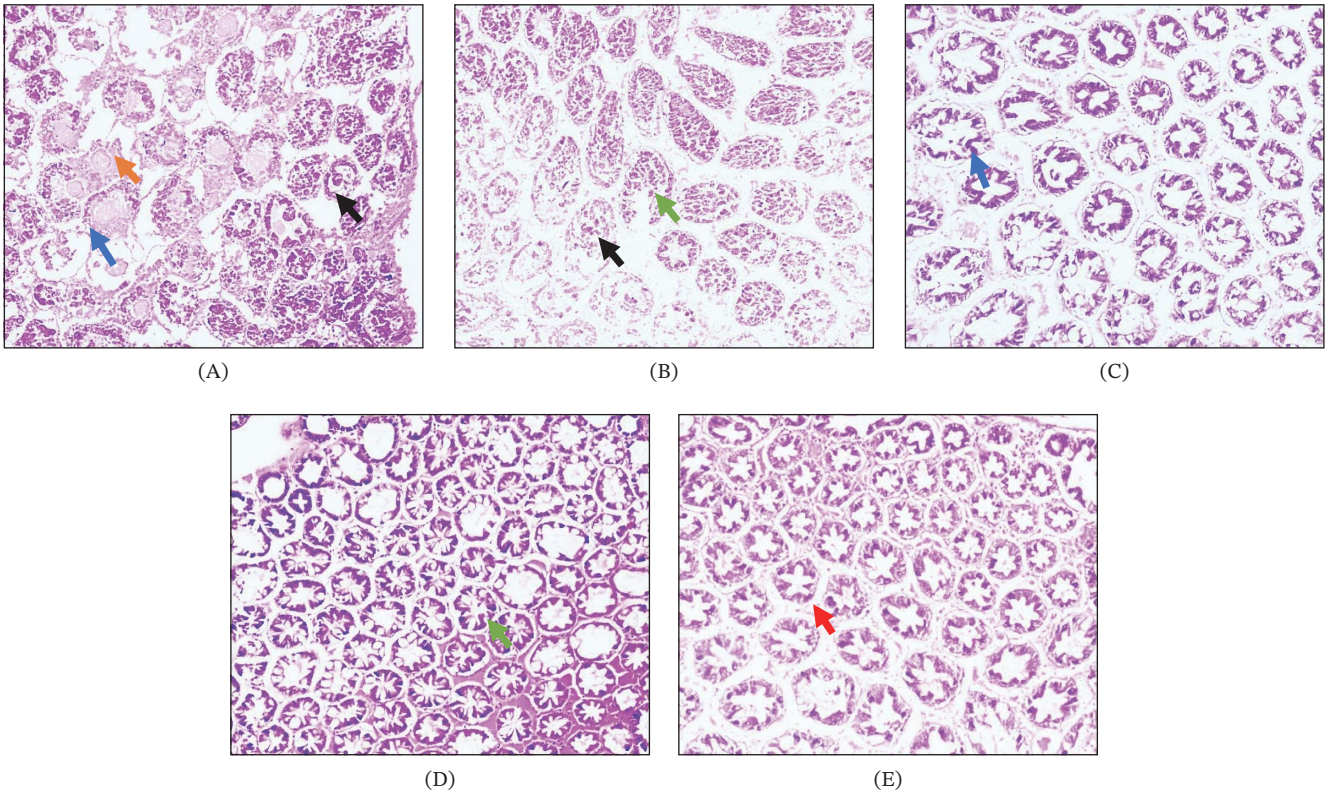

FIGURE 1 | Histological sections of hematoxylin and eosin (H&E)-stained hepatopancreas of *Penaeus monodon* PL following challenge with *Vibrio parahaemolyticus* (1×10^7 CFU mL^{-1}). (A) Positive control (clear water with *V. parahaemolyticus*) showing severe histopathological damage, including extensive epithelial necrosis, marked sloughing of epithelial cells into the tubule lumen and pronounced hemocyte infiltration. (B) BFR0 (*Halamphora coffeaeformis* only) showing moderate tissue damage, with fewer necrotic cells and reduced sloughing compared with the positive control. (C) BFR1 (rice bran biofloc only) showing moderate hepatopancreatic lesions, including necrosis and sloughed epithelial cells, but less severe damage than the positive control. (D) BFR2 (Rice bran biofloc with *H. coffeaeformis* addition) showing the least severe lesions among the challenged groups, with minimal hemocyte infiltration, limited necrosis and fewer sloughed cells in the tubule lumen. (E) Negative control (clear water without *V. parahaemolyticus*) showing normal hepatopancreatic structure without evident pathological lesions. Blue arrows indicate sloughed epithelial cells in the tubule lumen; black arrows indicate necrotic cells; green arrows indicate tubule lumens filled with sloughed cells; orange arrows indicate hemocyte infiltration; red arrows indicate normal hepatopancreatic cells. Magnification: 40x.

lower ($p < 0.05$) compared to the positive control. The least structural distortion in hepatopancreatic cells was found in this group compared to the other groups (Figure 1d). The tubule lumens contained only a limited number of sloughed cells, and necrotic hepatopancreatic epithelial cells were occasionally observed. In addition, hemocyte infiltration was minimal. These findings imply improved hepatopancreatic integrity compared to the other groups. Hepatopancreas samples from dead shrimp yielded presumptive *V. parahaemolyticus* colonies on TCBS agar following the challenge test. These findings provide supportive microbiological evidence of *Vibrio* infection. However, molecular confirmation was not performed, and thus, strain-level identification (S12-3) could not be confirmed.

Shrimp in microalgae-treated groups (BFR0 and BFR2) showed lower mortality than the positive control, suggesting a potential protective effect of microalgae against pathogens such as *V. parahaemolyticus*. This is parallel with the findings of Molina-Cárdenas and Sánchez-Saavedra [30], who demonstrated that six different diatom taxa can inhibit various *Vibrio* strains. This could be attributed to the bioactive compounds produced by microalgae that might serve as antimicrobial agents. For instance, polysaccharide extracts derived from microalgae have been shown to decrease the pathogen transmission [31]. Moreover, microalgal carotenoids can enhance the immune system of aquatic animals, improving resistance to infection [21]. Apart from that, PUFAs such as EPA from the marine diatom *Phaeodactylum tricornutum* have demonstrated antibacterial activity against a wide range of bacteria, including multidrug-resistant *Staphylococcus aureus* [32]. These findings suggest that supplementation of this diatom into rice bran biofloc may enhance disease resistance and improve PL health, leading to more productive aquaculture practices. However, as immune-related biomarkers such as prophenoloxidase (ProPO) activity, antioxidant enzyme responses or immune gene expression were not evaluated in the present study, the underlying mechanism of the improved challenge outcome remains unclear. Thus, future studies incorporating immunological analyses are crucial to validate these mechanisms.

Previous studies have shown that the effects of diatom supplementation in biofloc systems are highly species-dependent, with inconsistent outcomes reported for different taxa. While some studies have demonstrated no significant improvement in PL growth performance with diatom inclusion [1, 6], another study has reported improvements in final weight and yield [7],

suggesting that responses are influenced by the diatom species used. In the present study, BFR2 improved several key performance indicators in *P. monodon* PL, including survival, whole-body lipid enrichment, PUFA profile, and postchallenge performance. One possible explanation is the distinct biochemical composition of *H. coffeaeformis*, which has been reported to contain substantial levels of essential fatty acids, particularly EPA and DHA, as well as favorable biochemical properties under optimized culture conditions [5, 33]. These long-chain PUFAs are important for PL development, health and nutritional quality and may explain the favorable outcomes observed compared with some previously studied diatoms. Thus, the present findings support the view that the benefits of diatom supplementation in biofloc systems are influenced not only by microalgal inclusion, but also by species-specific biochemical characteristics. Taken together, these findings demonstrate incorporating *H. coffeaeformis* into rice bran-based biofloc offers benefits beyond nutrition, potentially strengthening disease resilience through synergistic nutritional and bioactive effects.

4 | Conclusion

This study demonstrates the potential of incorporating *H. coffeaeformis* into a rice bran-based biofloc system to achieve sustainable shrimp aquaculture. The PL cultivated in BFR2 showed improved growth performance, with increased final weight and higher survival rates. Furthermore, the nutritional profile of PL was also enriched, particularly with elevated lipid content and greater concentrations of essential PUFAs such as EPA and DHA, which are important for shrimp health and development. Moreover, supplementation with *H. coffeaeformis* enhanced biofloc development, although TAN was not significantly affected ($p > 0.05$). In addition, the challenge test with *V. parahaemolyticus* further revealed lower mortality rates and reduced hepatopancreatic tissue damage in PL from BFR2, suggesting enhanced disease resilience. These findings highlight the benefits of *H. coffeaeformis* and rice bran in enhancing biofloc development, growth performance, nutritional value, and disease resistance, thereby offering a sustainable and eco-friendly approach to shrimp aquaculture.

Author Contributions

Mohd Ihsanuddin Ahmad: formal analysis, investigation, methodology, writing – original draft, writing – review and editing. **Muhammad Farhan Nazarudin**: investigation, methodology, writing – review and editing. **Hazwani Hanim Hasnan**: formal analysis, writing – review and editing. **Ninie Diana Baharuddin**: formal analysis, writing – review and editing, investigation. **Murni Karim and Jeffrey K. C. Lee**: methodology, supervision, writing – review and editing. **Yam Sim Khaw, Hui Teng Tan, and Kamil Latif**: writing – review and editing. **Ikhsan Natrah**: conceptualization, project administration, supervision, visualization, writing – review and editing, funding acquisition.

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Disclosure

Ikhsan Natrah is affiliated with the Microalgae-Biota Technology and Innovation Research Group (ALBIC), Universiti Putra Malaysia. Further information about the research group is available at albic.my.

Ethics Statement

In the present study, we strictly followed the guideline of ARRIVE (Animal Research: Reporting of In Vivo Experiments). All animals used in the present experiment were treated humanely, according to approved procedures and guidelines of the Universiti Putra Malaysia Animal Ethics Committee Guidelines (IACUCs) (UPM/IAUCAC/AUP-R061/2022). We also followed the relevant guidelines and regulations for caring the animal during the experiment.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data will be made available upon request.

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