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The Impact of *Amphora* sp. Supplementation on Growth, Immunity, and Pathogen Resistance in Post-Larvae Pacific White Shrimp

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Abstract

Shrimp farming plays a critical role in global aquaculture due to increasing demand and economic, and environmental challenges. Sustainable practices, such as microalgae supplementation, are being increasingly adopted. This study evaluates the effects of *Amphora* sp. supplementation on growth, survival, and immune response in Pacific white shrimp (*Litopenaeus vannamei*). Results showed that diets enriched with 15 % *Amphora* sp. was highest significant SDA and EPA content, improved growth, feed efficiency, and resilience against *Vibrio parahaemolyticus*. Total hemocyte count, phagocytotic activity were increased after 3 and 6 weeks of feeding, and LvALF B gene expression was activated after 6 weeks of feeding. These results highlight the potential of using *Amphora* sp. as a sustainable dietary supplement in shrimp aquaculture, particularly for promoting growth performance and resistance to *V. parahaemolyticus* in post-larval Pacific white shrimp.

Keywords: *Litopenaeus vannamei*, *Amphora* sp., *Vibrio parahaemolyticus*, Shrimp growth, Shrimp immunity

Introduction

Sustainable aquaculture practices are being adopted to replace traditional methods that produce substantial wastewater, rely on antibiotics, and pose safety risks while driving up costs. Considerable effort has been invested to address these challenges, with microalgae application emerging as a key approach. Microalgae are not only used for wastewater remediation, biomass production as an ingredient in feed formulation, and water quality control [1,2], but also widely utilized as dietary supplements for aquatic species and aquaculture industry [2]. Moreover, microalgae contain valuable nutrients, including astaxanthin, polyunsaturated fatty acids (PUFAs), and phycocyanin, which enhance immune response, survival rates, and growth in farmed animals [3].

Different microalgae provide unique nutritional profiles that benefit various aspects of aquaculture, from growth and disease resistance to appearance [2]. *Nannochloropsis* spp., with its high lipid (78.88 %) and eicosapentaenoic acid (EPA) content (73.86 %), enhances *Litopenaeus vannamei* resistance to thermal stress when included in their diet (0.5 - 2 %) without affecting midgut microbiota. To enhance shrimp growth and survival rates, improve feed efficiency, and

minimize waste, diet formulations must be optimized for feed management in shrimp culture [3]. There are numbers of microalgae such as *Spirulina platensis* [4], *Chlorella vulgaris* [5,6] *Dunaliella* sp. [7], have been shown to be immunostimulant in shrimp diet against *Vibrio* infection.

Amphora sp. is diatom, a group of microalgae, rich in important nutrition and PUFAs such as EPA. The study by Masour *et al.* [8] demonstrated the effects of *Amphora coffeaeformis* supplementation on growth, survival, and growth-related gene expression in Pacific white shrimp post larvae (*L. vannamei*). Additionally, growth-related genes (Chitinase; CHIT, AMP-activated protein kinase; PRKAG, Cathepsin) were most highly expressed in shrimp fed live *A. coffeaeformis*, indicating enhanced growth performance.

The diatom *Amphora* sp. remains understudied regarding the effects of its PUFAs on the growth and immune response of shrimp when supplemented in their diet. This study aims to evaluate the potential of *Amphora* sp. as a dietary supplement for shrimp, with a focus on survival, growth performance, and response to the exposure to *Vibrio* sp.

Materials and methods

Animal preparation

A total of 1,200 healthy *L. vannamei* shrimp were acclimated for 7 days before the experiment. The shrimp, at the post-larval stage 12 (PL12) were cultured in 200-liter square fiberglass tanks containing seawater at a salinity of 15 parts per thousand (ppt). PL12 shrimps were fed twice daily, with the feed amounting to 3 % of the total biomass, one-fourth of culture water were exchanged daily for water quality monitoring. Aeration and ambient temperature were maintained consistently throughout the acclimatization period.

Diet preparation

Commercial post larvae (PL) shrimp diet (approximately 36 % protein) was powdered and divided into 4 equal portions as 4 treatments of diet. The diet was prepared as described by [9]. A portion without mixing *Amphora* sp. powder was used as control. The other 3 portions were added by 50, 100, and 150 g. *Amphora* sp. powder/kg shrimp diet and used as 5, 10, and 15 % *Amphora* sp. supplement, respectively. All diets were calculated and prepared to be used for 6 weeks feeding trials. Then the mixtures were air-dried at 60 degrees Celsius (°C) in the oven for 30 min and stored in aluminum bag at 4 °C for Fatty acid methyl esters (FAMEs) analysis following the method described by [10].

Feeding trails and sample collection

Feeding trails and sample collection were conducted according to the methods described in the studies by [9,11]. Fifty acclimated PL shrimp, with an average weight ranging from 0.78 ± 0.07 to 0.88 ± 0.04 g/individual, were allocated to each 5 L experimental unit containing 15 ppt seawater. Growth performance was assessed and recorded in the initial weeks, the 3rd week, and the 6th week of the experiment. Shrimp health was evaluated in the study of disease resistance through the challenge test method, conducted in the 3rd and 6th week of culture. Replications 1, 2, and 3 of each treatment were used for shrimp health evaluation in the 3rd week, while the remaining replications 4, 5, and 6 of each treatment were used for shrimp health evaluation in the 6th week. Health parameters including total hemocyte count (THC), phagocytotic activity (PA), and immune related gene expression were investigated after the challenge test.

Growth performance

Growth performance including survival rate, mean body weight, and specific growth rate were recorded and calculated according to the methods described in the studies by [12]. Feed efficiency rate was recorded and calculated according to the methods described in the studies by Lou *et al.* [13] and Hamidoghli *et al.* [14] as follows.

The survival rate (%SR) and Mean body weight (MBW) were determined at weeks 0, 3, and 6 of the experiment by observing the number of shrimp in each experimental unit, using the following formula.

$$SR\ (\%) = 100 \times \frac{Final\ number\ of\ shrimp}{Initial\ number\ of\ shrimp} \quad (1)$$

$$MBW\ (g,) = \frac{Total\ weight\ (g.)}{Total\ number\ of\ shrimp} \quad (2)$$

Specific growth rate (SGR) and feed efficiency rate (FER) were calculated during week 0 - 3, and 3 - 6 of the experiment, using the following formular.

$$SGR\ (\%) = 100 \times (\ln(Final\ weight) - \ln(Initial\ weight)) \quad (3)$$

$$FER\ (\%) = 100 \times \frac{Wet\ weight\ gain\ (g.)}{Dry\ weight\ intake\ (g.)} \quad (4)$$

Water quality analysis Water quality was maintained through approximately 80 % water exchange and continuous daily aeration. The following water quality parameters were weekly monitored, analyzed, and recorded: temperature (Temp.), salinity (Sal.), dissolved oxygen (DO), pH, alkalinity (Alk.), total ammonia nitrogen (TAN), and nitrite (NO_2). Temperature was recorded by Glass Thermometer, Alcohol Filled (SK SATO), Salinity was recorded by hand-held refractometer (RHA-200ATC, JEDTO), DO, pH, Alk., TAN, and NO_2 were analyzed by test kit (SERA).

Disease resistance

Ten PL shrimp from each feeding trail treatment were immersed in bacteria *Vibrio parahemolyticus* solution at final concentration of 10^5 CFU/mL after 3 and 6 weeks of experiment [11]. *V. parahemolyticus* was provided by Songkla Aquatic Animal Health Research and Development Center (SAAHRDC), Department of Fisheries, Ministry of Agriculture and cooperatives, Thailand. The SR of each challenge unit was recorded after 24 h of immersion. Three PL shrimps were collected and preserved in RNAlater® and keep at −20 °C until gene expression analysis. The remaining live PL shrimp were collected for health status assessment.

Health status

Total hemocyte count (THC)

THC was observed from fresh hemolymph collected from the ventral sinus cavity and transferred into a 1.5 mL microtube containing 10 % sodium citrate as an anticoagulant in a 1:1 ratio. A fresh 20 µL sample of hemocytes was then counted using a hemocytometer under a light microscope. The results were presented in units of cells/mL [15] using the following formula.

$$THC\ (cells/mL) = \text{Total number of hemocyte counted in } 0.5\ mm^3 \times 10^4 \times dilution\ number \quad (5)$$

Phagocytic activity (PA)

PA was determined using 20 µL of hemolymph. The hemolymph was pipetted into a 96-well plate, mixed with 20 µL of *Staphylococcus aureus*, and incubated at 30 °C for 20 min. After incubation, 5 µL of each sample was pipetted, dropped, and spread onto a glass slide. The slides were air-dried for 20 min, rinsed with 0.85 % sodium chloride solution, air-dried again for 20 min, and then stained with 10 % Wright stain for 20 min. The stained hemolymph was observed under a light microscope at 400X magnification. The number of phagocytes per 100 hemocytes in each sample was recorded and calculated according to the methods described in the studies by [16]. The results were presented in units of percentage.

$$PA\ (\%) = \frac{Phagocytic\ activity\ cell \times 100}{Total\ number\ of\ counted\ hemocyte} \quad (6)$$

Gene expression analysis

Sample collection

Hepatopancreas samples were collected from preserved 3 shrimp and immediately transferred into 1.5 mL microcentrifuge tubes containing RNAlater®. The samples were kept at −20 °C until further gene expression analysis. All sample collection and processing were conducted using sterile dissecting tools in a cold environment according to the study by [17].

RNA extraction

Total RNA was extracted from hepatopancreases samples using Tri-reagent and chloroform according to the TRIzol methods described in the studies by [9]. The extracted RNA was then stored at –80 °C until further use for cDNA synthesis.

The first stand cDNA synthesis

A total of 1.5 micrograms of DNase-treated total RNA was used for reverse transcription to first-strand cDNA with the ImProm-II™ Reverse Transcription System Kit (Promega, U.S.A.). The reaction was incubated at 25 °C for 5 min, followed by 42 °C for 90 min. To stop the reverse transcriptase activity, the

reaction was heated to 70 °C for 15 min. The concentration and general quality of the synthesized first-strand cDNA were assessed using spectrophotometry (OD260/OD280) and analyzed on 1.0 % agarose gels. The cDNA was diluted tenfold and stored at −20 °C until needed according to the study by [17].

Primer and qRT-PCR amplification

qRT-qPCR amplifications were performed in the CFX Real-time PCR System (BIO-RAD). PCR amplification in a final volume of 10 uL containing 2 uM of each primer, 5 uL of reaction mix (Maxima SYBR Green/Rox qPCR Master Mix 2x; BIO-RAD) and 1 uL of cDNA (diluted 1:10). Primer sequences for elongation factor 1-alpha (LvEF1a) as reference genes are TGG CTG TGA ACA AGA TGG ACA (FW 5'-3') and TTG TAG CCC ACC TTC TTG ACG (Rv 5'-3'). Primer sequences for anti-lipopolysaccharide factors (LvALF B) as target gene are GTG TCT CCG TGT TGA CAA GC (FW 5'-3') and ACA GCC CAA CGA TCT TGC TG (Rv 5'-3'). The thermal profile of PCR consisted of an initial denaturation step of 3 min at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 55 °C for 1 min. Melt curve were 65 - 95 °C at a temperature transition rate of 0.05 °C/s according to the study by. [17].

Expression of Anti-lipopolysaccharide factors: LvALF B gene

The expression of the LvALF B gene was analyzed following the methods outlined by [19]. A 10-fold serial dilution (ranging from 10^3 to 10^8 molecules/µL) was prepared. The copy number of standard DNA molecules was calculated using the formula:

$$Y\ molecules/\mu L = \frac{X\ ng/\mu L\ DNA \times 6.022\times10^{23}}{Plasmid\ length\ in\ bp\times660\times10^{9}} \qquad (7)$$

where X is the plasmid dsDNA concentration (ng/µL), 6.022×10^{23} is Avogadro's number, 660 is the average molecular weight of one base pair, and Y is the copy number of plasmid molecules per microliter. Standard curves with correlation coefficients between 0.995 – 1.000 and efficiencies above 95 % were generated for each run. Standards were analyzed in a 96-well plate, with each point in duplicate. LvEF1a served as the internal control.

Statistical analysis

The results of growth performance and shrimp health were presented as mean ± SD. Statistical analysis was performed by SPSS software. A one-way ANOVA ($p \leq 0.05$) was performed to determine the difference of growth performance and shrimp health in the different *Amphora* sp. supplementation concentration. Pair sample T Test was performed to determine the difference of SR and THC exposure to *V. parahaemolyticus.* The significant differences of the results were indicated at the 0.05 level. Post hoc analysis was done by Duncan.

Results and discussion

Fatty acid methyl esters (FAMEs) analysis in shrimp diet

The analysis of fatty acid concentrations across the different shrimp diets revealed distinct variations among fatty acid groups. The fatty acids were classified into 2 main categories: saturated fatty acids (SFA) and unsaturated fatty acids (UFA). The SFA level was lower than UFA level in all groups of diet. SFA level varied from 0.15 ± 0.26, 2.17 ± 0.54, 2.17 ± 0.47, and 3.25 ± 1.46 mg/100 mg dried algae in control group, 5, 10, and 15 % *Amphora* sp. supplementation diet, respectively. While UFA level varied from 3.11 ± 5.33, 9.33 ± 4.24, 16.53 ± 7.16, and 22.00 ± 16.68 mg/100 mg dried algae in control group, 5, 10, and 15% *Amphora* sp. supplementation diet, respectively. The SFA content in diets supplemented with *Amphora* sp. powder (at 5, 10, and 15 % *Amphora* sp. supplementation) was significantly higher than in the control group (0 % *Amphora* sp. supplementation) as shown in **Figure 1**.

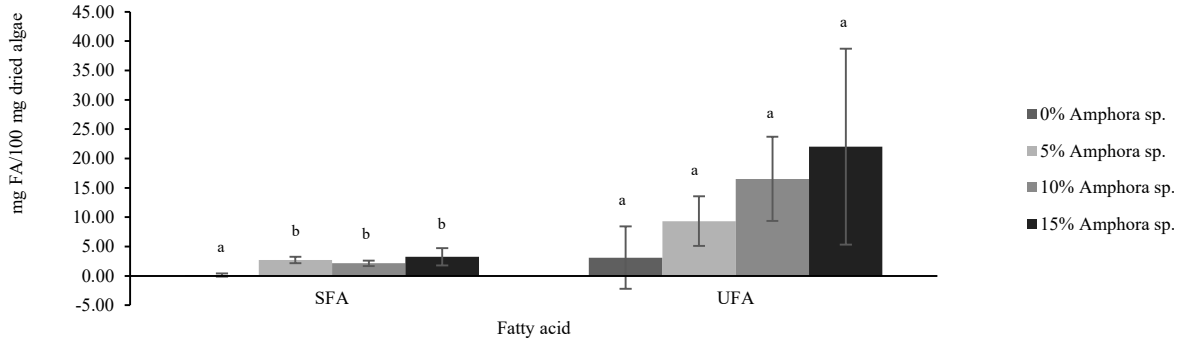

Figure 1 The levels of saturated fatty acids (SFA) and unsaturated fatty acids (UFA) present in various shrimp diets. Mean ± SD values with different lowercase letters are significantly ($p \leq 0.05$) different between each treatment.

UFA group was categorized to be 3 groups including omega 3, 6, and 9. Omega 3 group composed of Stearidonic acid (SDA; C18:4 ω-3), eicosapentaenoic acid (EPA; C20:5 ω-3), docosapentaenoic acid (DPA; C22:5 ω-3), and docosahexaenoic acid (DHA; C22:6 ω-3). Omega 6 composed of Linoleic acid (C18:2 ω6). Omega 9 composed of oleic acid (C18:1 ω9), eicosenoic acid (C20:1 ω9), and erucic acid (C22:1 ω9) (**Figure 2**).

SDA and EPA had significantly higher concentrations compared to other fatty acids. The concentration of SDA was 9.53 ± 8.31 mg/100 mg of dried algae in the diet with 15 % *Amphora* sp. supplementation and 6.49 ± 5.66 mg/100 mg in the diet with 10 % *Amphora* sp. supplementation. While EPA levels were significantly higher in the diet supplemented with 15 % *Amphora* sp. compared to the control group. The EPA concentrations were 9.58 ± 7.98 mg/100 mg of dried algae, 7.66 ± 0.79 and 7.66 ± 7.98 mg/100 mg in the diets supplemented with 15, 10, and 5 % *Amphora* sp., respectively as illustrated in **Figure 2**.

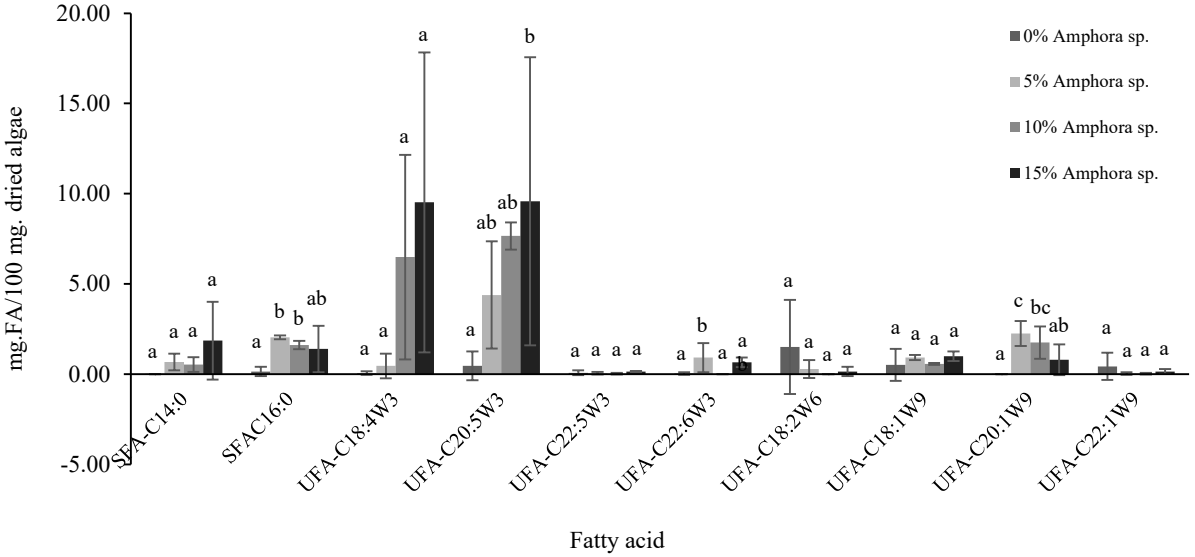

Figure 2 The levels each fatty acids present in various shrimp diets. Mean ± SD values with different lowercase letters are significantly ($p \leq 0.05$) different between each treatment.

The study by Pekkoh *et al.* [18] demonstrated that the diatoms *Anomoeoneis* sp. and *Rhopalodia* sp. contain significant levels of SFAs (41.41 - 41.75 %), monounsaturated fatty acids (MUFAs) (35.53 - 42.71 %), and PUFAs (15.54 - 23.06 %). Notably, the SFA content was lower than the combined UFAs, which ranged between 51.07 and 65.77 %. The major PUFA components were identified within the omega-3 fatty acid group, including EPA at 1.33 to 2.61 % and DHA

at 0.20 to 0.95 %. However, the study did not report levels of SDA.

The *Amphora* sp. powder used in this study was screened, collected, and cultured following the method described in Thongdet *et al.* [10], which produces a substantial proportion of SFAs and UFAs, approximately 7.35 and 24.28 %, respectively. Notably, Bacillariophyceae algae species or diatom group including *Chatoceros* sp., *C. didymus*, *C. affinis*, *T. weissflogii*, *P. tricornutum*, *S. costatum*. can produce SFAs, UFAs, SDA, EPA and DHA at levels of approximately 15 - 60, 5 - 90, 0.2 - 3.7, 9 - 50, and 3 - 50 % as study by [19].

EPA and DHA are recognized as the most essential PUFAs in shrimp diet. They are vital for enhancing growth, feed efficiency, and survival of the shrimp [20]. The optimum requirement of EPA and DHA for penaeid shrimp were reported to be between 0.5 - 1.0 % diet [21]. In shrimp defense system, EPA is considered to play role on the immune system. It is a precursor of eicosanoids which are mediators of the immune response [22].

In this study, DHA in all treatments appeared to be at very low levels (0.04 ± 0.07 - 0.92 ± 0.81 %) while EPA level was significantly higher (0.47 ± 0.79 - 9.58 ± 7.98 %) and increasing in relation to the increasing amount of *Amphora* sp. in tested diets.

Growth performance

Growth performance was assessed using specific growth rate (SGR), mean body weight (MBW), and feed efficiency ratio (FER). These parameters were evaluated in conjunction with water quality data collected during the culture period.

Survival rate (%SR)

As shown in **Figure 3**, shrimp %SR decreased by Week 3, with the control group (0 % *Amphora* sp.) and the treatment group supplemented with 15 % *Amphora* sp. powder exhibiting significantly higher %SR compared to those supplemented with 5 and 10 % *Amphora* sp. By Week 6, all groups experienced a further decline in %SR. The groups supplemented with 5 and 10 % *Amphora* sp. displayed the lowest %SR, while the control group and the 15 % treatment group maintained higher survival rates, recording values of 32.67 ± 10.26 and 36.67 ± 6.12 %, respectively.

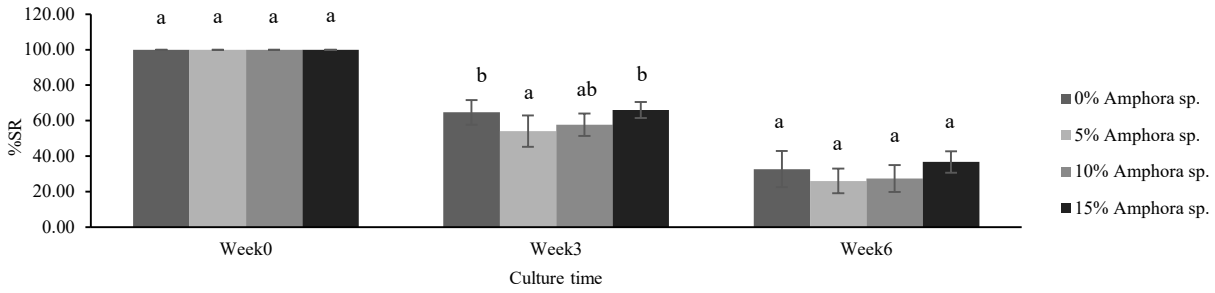

Figure 3 %SR of shrimp determined on week 0, 3, and 6 of culture. Mean ± SD values with different lowercase letters are significantly ($p \leq 0.05$) different between each treatment.

Mean body weight (MBW)

The MBW of shrimp fed with various diets showed a gradual increase over the 6-week culture period, although no statistically significant differences were observed between the weeks of culture. The MBW ranged from 0.78 ± 0.18 to 0.88 ± 0.09 g/individual in the initial week, which increased to 0.97 ± 0.24 to 1.16 ± 0.20 g/individual by week 3. By week 6, the MBW further increased to a range of 1.97 ± 1.02 to 3.21 ± 1.12 g/individual (**Figure 4**). Highlighting the trend of increasing MBW serves as a key indicator of feed performance or cultivation conditions. Tracking MBW trends allows for evaluating the effectiveness of nutritional strategies, feed formulations, or environmental parameters in supporting optimal growth.

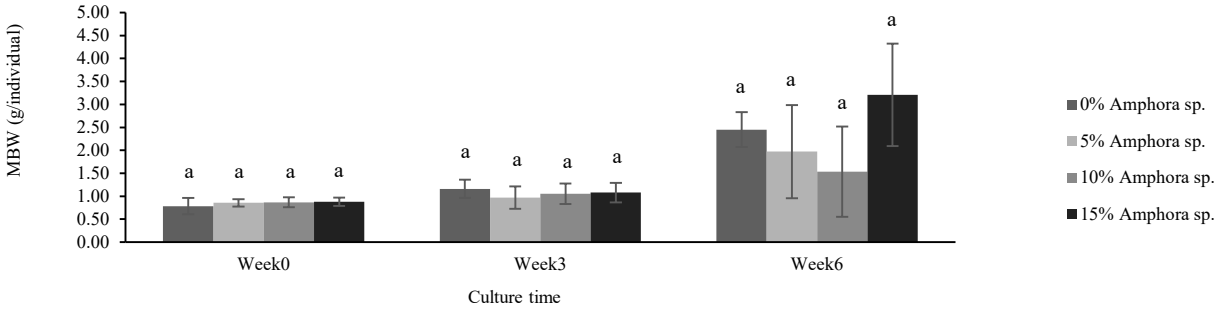

Figure 4 MBW of shrimp determined on week 0, 3, and 6 of culture. Mean ± SD values with different lowercase letters are significantly ($p \leq 0.05$) different between each treatment.

Specific growth rate (SGR)

No significant differences in SGR were detected among treatments between the initial week and week 3. However, a significant difference was observed from week 3 to week 6, as shown in **Figure 5**. During this period, shrimp fed with a diet supplemented with 15 % *Amphora* sp. displayed a significantly higher SGR compared to those fed with a diet supplemented with 5 % *Amphora* sp. However, no significant difference was found between the SGR of shrimp fed with the 15 % *Amphora* sp. diet and those fed with diets either without *Amphora* sp. supplementation or supplemented with 10 % *Amphora* sp. SGR of shrimp cultured during week 3 to week 6 was 25.60 ± 6.12 % ($p < 0.05$) in control group, 18.16 ± 12.56 % ($p < 0.05$), and 31.96 ± 8.25 % ($p < 0.05$) in shrimp fed with diet supplemented with 10 and 15 % *Amphora* sp., respectively.

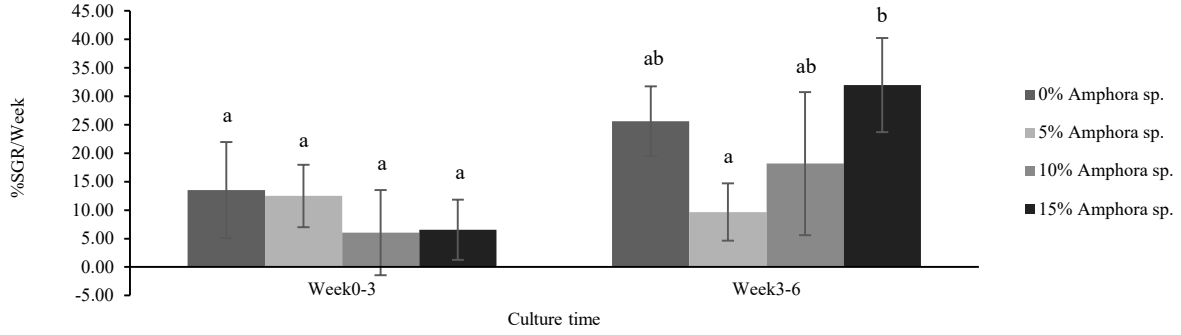

Figure 5 SGR of shrimp determined on week 0 to 3 and 3 to 6 of culture. Mean ± SD values with different lowercase letters are significantly ($p \leq 0.05$) different between each treatment.

Feed efficiency ratio (FER)

No significant differences in FER were observed among the experimental groups across both culture periods. During weeks 0 to 3, The FER ranged from 14.53 ± 12.71 to 47.62 ± 23.14 % in the first period (weeks 0 to 3), with no significant differences between groups ($p \leq 0.05$). During the second period (weeks 3 to 6), significant differences were observed in FER, with shrimp fed 15 % *Amphora* sp. showing the highest FER (316.43 ± 194.59 %), followed by the control (187.17 ± 61.10 %) and 10 % *Amphora* sp. diets (128.98 ± 116.27 %) ($p \leq 0.05$). Notably, shrimp fed with the diet supplemented with 15 % *Amphora* sp. powder achieved the highest FER during both periods of the study (**Figure 6**)

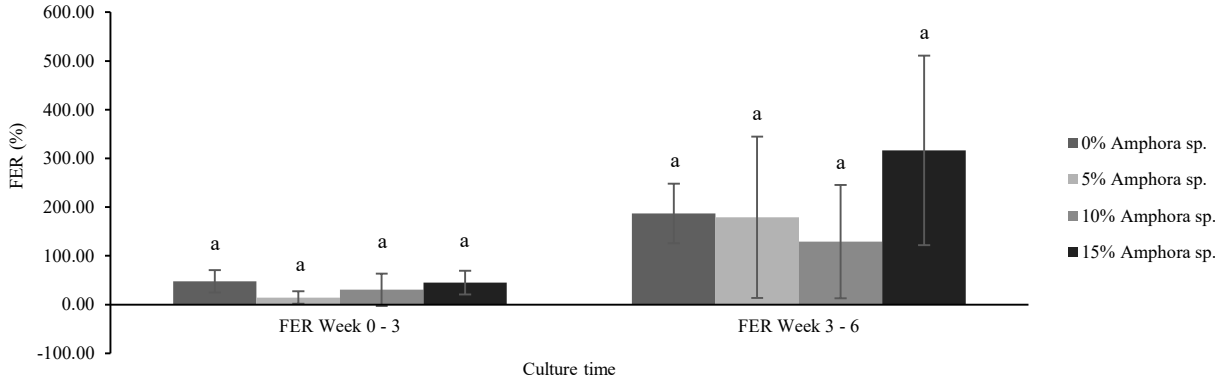

Figure 6 FER of shrimp determined on week 0 to 3 and week 3 to 6 of culture. Mean ± SD values with different lowercase letters are significantly ($p \leq 0.05$) different between each treatment.

Water quality analysis

The water quality parameters were monitored weekly throughout the experiment, including temperature (Temp.), salinity (Sal.), dissolved oxygen (DO), pH, alkalinity (Alk.), total ammonia nitrogen (TAN), and nitrite (NO_2). The ambient temperature ranged from 27.00 ± 0.00 to 29.30 ± 0.00 °C, while salinity remained constant at 15 ppt. DO levels ranged between 4.00 ± 0.00 and 5.00 ± 0.00 ppm. The pH values were recorded within the range of 7.90 ± 0.20 to 8.30 ± 0.20. Alkalinity varied from 138.80 ± 6.90 to 206.80 ± 12.80 ppm. TAN and NO_2 levels were controlled, remaining below 0.5 ± 0.00 and 0.90 ± 0.40 ppm, respectively (**Table 1**).

Table 1 The range of water quality measured weekly, during 6 weeks of culture (Mean ± SD).

Water quality	Treatment			
	0 % *Amphora* sp.	**5 % *Amphora* sp.**	**10 % *Amphora* sp.**	**15 % *Amphora* sp.**
Temp. (°C)	27.0 ± 0.0 - 29.3 ± 0.0	27.0 ± 0.0 - 29.3 ± 0.0	27.0 ± 0.0 - 29.3 ± 0.0	27.0 ± 0.0 - 29.4 ± 0.3
Salinity (ppt)	15.0 ± 0.0	15.0 ± 0.0	15.0 ± 0.0	15.0 ± 0.0
DO (ppm)	4.0 ± 0.0 - 5.0 ± 0.0	4.0 ± 0.0 - 5.0 ± 0.0	4.0 ± 0.0 - 5.0 ± 0.0	4.0 ± 0.0 - 5.0 ± 0.0
pH	7.9 ± 0.2 - 8.2 ± 0.5	8.0 ± 0.2 - 8.2 ± 0.3	7.9 ± 0.2 - 8.3 ± 0.3	8.0 ± 0.1 - 8.3 ± 0.3
Alk. (ppm)	155.8 ± 6.9 - 221.0 ± 21.5	144.5 ± 9.3 - 206.8 ± 12.8	138.8 ± 6.9 - 204.0 ± 17.0	181.3 ± 9.8 - 204.0 ± 15.2
TAN (ppm)	0.0 ± 0.0 - 0.5 ± 0.0	0.0 ± 0.0 - 0.5 ± 0.0	0.0 ± 0.0 - 0.5 ± 0.0	0.0 ± 0.0 - 0.5 ± 0.0
NO_2(ppm)	0.1 ± 0.0 - 0.9 ± 0.4	0.1 ± 0.0 - 0.7 ± 0.4	0.1 ± 0.0 - 0.8 ± 0.4	0.1 ± 0.0 - 0.8 ± 0.4

Growth enhancement through dietary supplement

Comparison between the results of this study and those earlier reported are shown in **Table 2** There were some data adjustments for an appropriate comparison. MBW of shrimp fed with 15 % *Amphora* sp. diet in the present study (1.07 g/week) is clearly higher than that of Ju *et al.* [23] while it is much lower than that of Baharuddin *et al.* [24]. This is possibly due to the higher percentages of DHA and EPA in the *Amphora* sp. supplemented diet. Also, the reason MBW of the shrimp from Baharuddin *et al.* [24] is much higher than the present study and Ju *et al.* [23] is simply because the shrimp were fed with live feed which gains advantage over pellet feed and incorporates with the high levels of EPA and DHA in copepods enriched with *Halamphora* sp. The high level of EPA in *Amphora* sp. used in the present study can significantly improve the growth performance of shrimp.

Table 2 Comparison of growth performance across different studies of dietary supplements.

Ref.	Species	Type of supplement	SR (%)	MBW (g/week)	SGR (%/d)
The present study	*L. vannamei*	15 % *Amphora* sp. 0.92 % DHA 9.58 % EPA	36.67	1.07	1.48
[26]	Greasyback shrimp (*Metapenaeus ensis*)	7.50 % lipid 9.74 % omega-3 0.40 % EPA	Not mentioned	Not mentioned	1.72
[27]	*L. vannamei*	Whole diatoms: *Nannochloropsis* sp. and *T. weissflogii* 0.05 % DHA 0.87 % EPA	93.8 - 100	0.57 - 0.64	Not mentioned
[28]	*P. monodon*	Copepods enriched with *Halamphora* sp. EPA DHA	63.33	4.04	9.47

Comparison of SR according to the study by Hamidoghli *et al.* [14] which reported a %SR of 79.3 ± 2.31 % in indoor biofloc system. While studying by Ju *et al.* [23], shrimp were fed diets containing whole diatoms, specifically *Nannochloropsis* sp. and *T. weissflogii*, demonstrated a SR ranging from 93.8 to 100 % after the eight-week feeding trial. This experimental setup was similar to the culture condition used in the present study, except culture water management. While the present research used water exchanging while the study by Ju *et al.* [23] used a flow-through of seawater (1 L/min). This factor could potentially influence the lower SR observed in the present research. Despite this decline, shrimp fed with a 15 % *Amphora* sp. diet consistently exhibited the highest SR throughout the entire 6 weeks. This highlights the potential positive impact of *Amphora* sp. on shrimp survival, indicating a promising aspect for further investigation and optimization.

Environmental conditions remained stable throughout the experiment, with all water quality parameters maintained within optimal ranges, ensuring water quality did not affect the observed outcomes. These parameters are consistent with those reported in similar studies, which recorded temperature, salinity, DO, pH, Alk., TAN, and NO_2 levels ranging from 25 - 31.5 °C, 4.0 - 6.85, 7.66 - 8.79, 297.55 - 309.59 ppm, below 0.05 - 0.22 ppm, and below 0.05 - 0.14 ppm, respectively according to the study by [12,13,15,26,27].

Health status

Concentration of V. parahemolyticus

The concentration of *V. parahaemolyticus* in the culture water during the challenge test was measured, as presented in **Table 3**. The results showed that the *V. parahaemolyticus* concentration ranged from $5.37\times10^5 \pm 1.56\times10^5$ to $7.57\times10^5 \pm 1.87\times10^5$ CFU/mL in week 3 of challenge test and ranged from $1.87\times10^5 \pm 4.51\times10^4$ to $6.43\times10^5 \pm 6.51\times10^4$ CFU/mL in week 6 of challenge test. A negative control group, which was not exposed to *V. parahaemolyticus*, was used as a control. There were no significant differences in *V. parahaemolyticus* concentration between the challenge test groups in week 3; however, there was a significant difference in *V. parahaemolyticus* concentration between the *Amphora* sp. supplementation group and the control group in week 6 of challenge test experiment. The *V. parahaemolyticus* concentration of *Amphora* sp. supplementation group was significantly higher than control group.

Table 3 Concentration of *V. parahemolyticus* in culture water for challenge test experiment. Mean ± SD values with different lowercase letters are significantly ($p \leq 0.05$) different between each treatment.

Treatment	Week3	Week6
0 % *Amphora* sp. (Negative control)	0.00 ± 0.00	0.00 ± 0.00
0 % *Amphora* sp. (Positive control)	$5.37\times10^5 \pm 1.56\times10^5$ a	$1.87\times10^5 \pm 4.51\times10^4$ a
5 % *Amphora* sp.	$7.57\times10^5 \pm 1.87\times10^5$ a	$4.97\times10^5 \pm 1.22\times10^5$ b
10 % *Amphora* sp.	$6.73\times10^5 \pm 1.53\times10^5$ a	$6.07\times10^5 \pm 1.40\times10^5$ b
15 % *Amphora* sp.	$6.70\times10^5 \pm 1.73\times10^5$ a	$6.43\times10^5 \pm 6.51\times10^4$ b

Survival rate (SR) exposed to *Vibrio parahaemoyticus*

The survival rate (SR) of shrimp was assessed 24 h after immersion in the *V. parahemolyticus* solution. The significant improvement of SR after exposure to *V. parahemolyticus* was observed only in shrimp fed with 10 % *Amphora* sp. supplementation diet, even though the trend of improvement was showed in all *Amphora* sp. supplementation diet. By 6 weeks, the SR of shrimp exposed to *V. parahemolyticus* ranged from 86.67 ± 11.55 to 90.00 ± 0.00 % **Figure 7**.

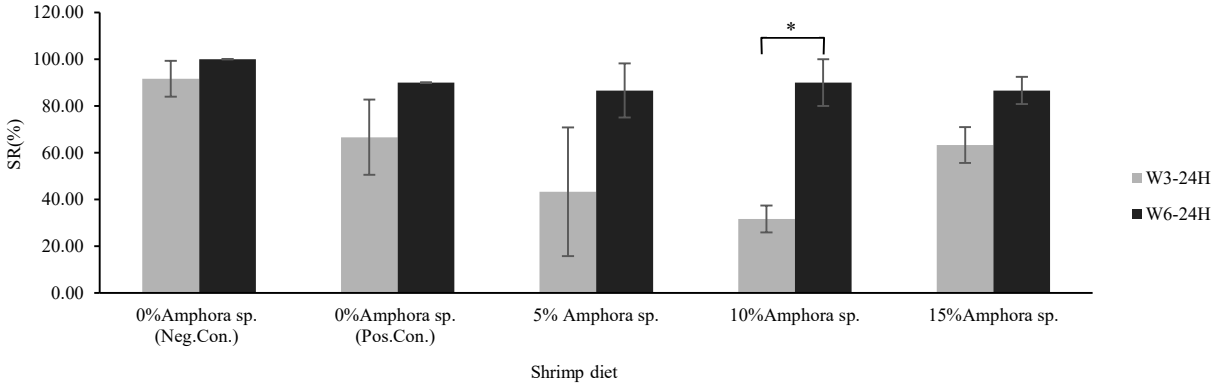

Figure 7 %SR of shrimp determined after 24 h of *V. parahemolyticus* immersion. Mean ± SD values with asterisk are significantly ($p \leq 0.05$) different between each culture day.

In terms of SR after being exposed to *V. parahaemolyticus* for 24 h, the results indicate a positive impact of *Amphora* sp. supplementation, particularly at the 10 and 15 % level. After 3 weeks, shrimp fed with 10 % *Amphora* sp. powder demonstrated the highest SR among all challenge groups, with SR values comparable to the positive control group. By week 6, SR values improved across all groups, reaching near complete survival, suggesting a delayed yet effective immune response associated with *Amphora* sp. supplementation. So, a longer feeding period with *Amphora* sp. supplementation diet positively influences the shrimp's survival rates when exposed to *V. parahaemolyticus*.

Total hemocyte count (THC) as an indicator of cellular immune response

The THC levels in shrimp increased over time in all groups, with the largest increase observed in shrimp fed with diet supplemented with the 15 % *Amphora* sp. powder group, which had a near-zero THC at week 3 but reached nearly $9.07\times10^4 \pm 1.53\times10^4$ cells/mL by week 6, even thought there was not significantly different among each experiment group of each week (**Figure 8(a)**), the THC levels in shrimp from week 6 were significantly higher than that of week 3 ($p \leq 0.05$) but no significant difference was detected between negative control shrimp from each week ($p \leq 0.05$) (**Figure 8(b)**).

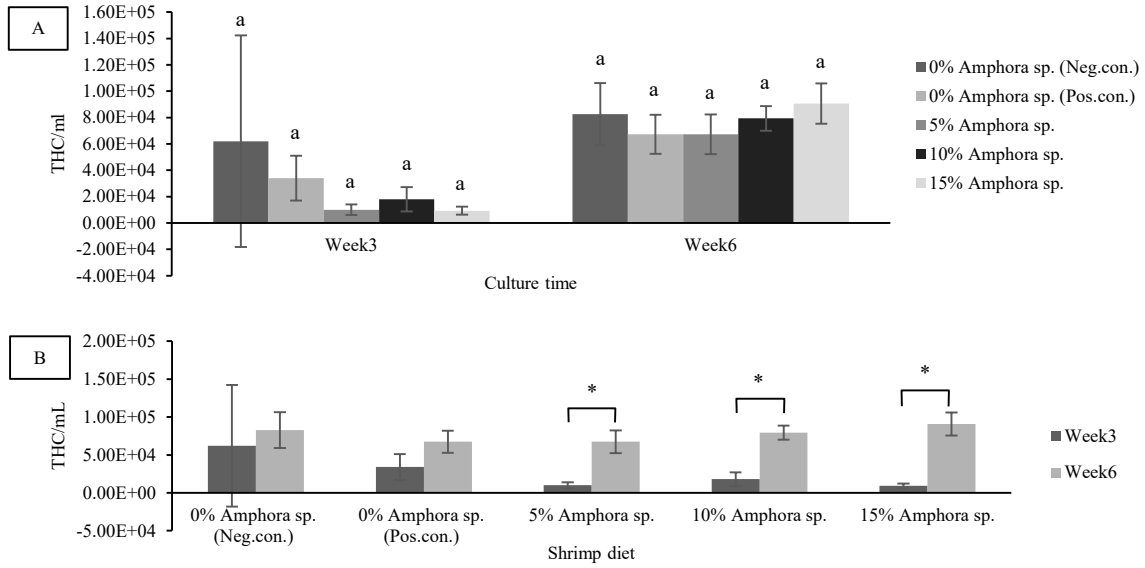

Figure 8 THC (cell/mL) of shrimp determined after 24 h of *V. parahemolyticus* immersion. Mean ± SD values with different lowercase letters are significantly ($p \leq 0.05$) different between each treatment (a) and different between each culture week (b).

The THC results highlight an enhanced immune response in shrimp fed the 15 % *Amphora* sp. supplementation diet, with THC values increasing progressively from week 3 to 6 across all groups. Although no significant differences were noted between groups due to the immune response of the 15 % group is already higher in week 3 and improving in week 6, the higher THC in shrimp receiving the 15 % *Amphora* sp. supplementation diet may indicate a stronger response in cellular immunity, potentially contributing to the observed SR outcomes after 24 h of *V. parahaemolyticus* exposure. The THC is an important indicator of immune response in shrimp, as hemocytes play a critical role in cellular immunity by recognizing and eliminate pathogens. Research by Liang *et al.* [25], highlighted that dietary supplementation with CS-GT increased THC through hemocyte proliferation and enhanced phagocytic activity, aiding pathogen defense of Pacific white shrimp. Similarly, Qin *et al.* [26] found that shrimp fed with dietary supplementation with a multispecies synbiotic increased THC, supporting a heightened immune response following exposure to myonecrosis virus (IMNV) and bacterial *V. parahaemolyticus* pathogens and increase along with supplementation level. Such findings underscore THC's relevance in shrimp health, acting as a frontline defense mechanism against infections. Likewise, the observed increase in THC in this study, following dietary supplementation with *Amphora* sp. powder enriched with PUFAs, indicated the potential of *Amphora* sp. supplementation on cellular defense mechanism of juvenile *L. vannamei*.

Phagocytic activity (PA) and pathogen defense

The PA levels in shrimp increased across all experimental groups from week 3 to week 6, with the exception of the positive control group, where shrimp were fed a non-supplemented diet and exposed to *V. parahaemolyticus*. After 3 weeks of culture, no significant differences in PA were observed among the experimental groups, with PA values ranging from 7.67 ± 2.31 to 23.33 ± 12.34 %. By week 6, the PA levels in the negative control group were significantly higher than that of positive control group ($p \leq 0.05$). Shrimp fed a diet supplemented with 15 % *Amphora* sp. powder and exposed to *V. parahaemolyticus* were significantly higher compared to shrimp fed a diet supplemented with 5 % *Amphora* sp. powder ($p \leq 0.05$). However, there was no significant difference between the 15 and 10 % *Amphora* sp. supplementation groups ($p \leq 0.05$). The PA levels were 32.00 ± 3.61 % in the negative control group and 30.00 ± 12.49 % in shrimp fed a diet supplemented with 15 % *Amphora* sp. powder (**Figure 9**).

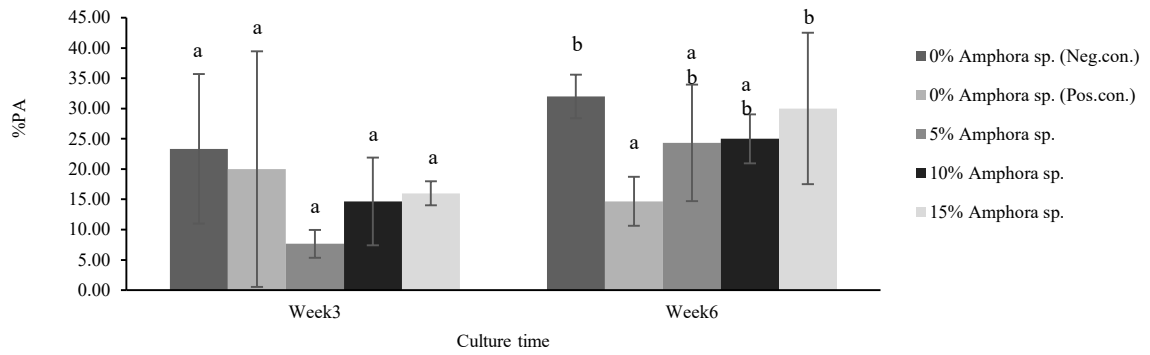

Figure 9 Phagocytic activity of shrimp determined after 24 h of *V. parahemolyticus* immersion. Mean ± SD values with different lowercase letters are significantly ($p \leq 0.05$) different between each treatment.

PA within shrimp hemocytes plays a significant role in eliminating pathogens. When shrimp are exposed to infections, hemocytes are activated, initiating immune responses such as the recognition and elimination of pathogens Liang *et al.* [25] Ambasankar *et al.* [27] demonstrated that juvenile *P. vannamei*, weight 0.55 ± 0.02 g/individual, fed with dietary krill meal and achieved DHA ranged from 0.82 ± 0.02 to 1.14 ± 0.02 % and EPA ranged from 1.14 ± 0.13 to 2.08 ± 0.10 % expressed in THC between 16.48×10^6 to 21.12×10^6 cell/mL. This dietary krill meal supplementation not only increased THC but also enhanced PA.

Similar to other crustaceans, a mean of defense against infection in shrimp rely mainly on phagocytic haemocytes. It is quite clear that many factors such as food, diseases, pollutants, life cycle and environmental stresses can affect the quality and quantity of haemocytes [28]. Therefore, this could probably be the cause of a high variant of THC in some treatments in week 3. However, THC appeared to be more stable after week 6. Similar result was observed in PA value. Both THC and PA are measurements based on the activity of haemocytes. Therefore, agreement of both factors should confirm the similar condition of the sample in the experiment.

Gene expression analysis

The expression of the LvALF B gene was significantly higher ($p \leq 0.05$) in the hepatopancreas of shrimp fed with 10 and 15 % *Amphora* sp. powder supplementation at week 6 compared to those fed with 5 % *Amphora* sp. powder and the control group, as illustrated in **Figure 10**. The LvALF B gene expression levels were investigated at 1.91 ± 0.02 and 1.89 ± 0.11 copyDNA/uL in the hepatopancreas of shrimp supplemented with 10 and 15 % *Amphora* sp. powder, respectively (**Figure 10**).

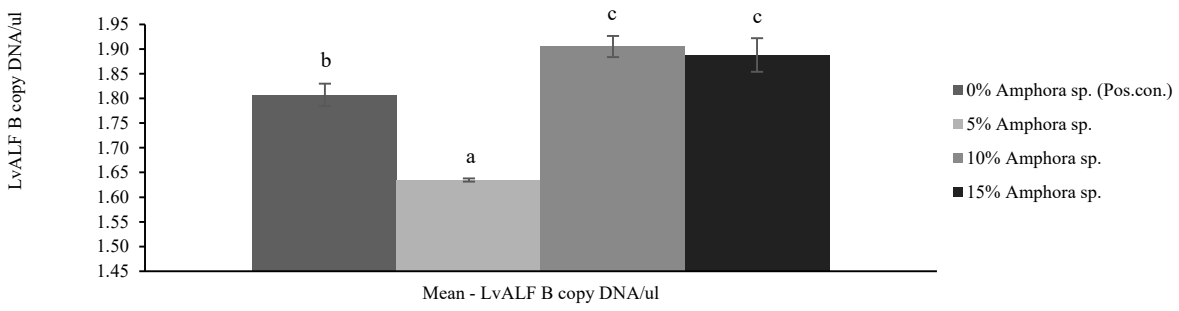

Figure 10 Expression of LvALF B gene determined in hepatopancrease of 6 week feeding trial shrimp, after 24 h of *V. parahemolyticus* immersion. The EF-1α gene was used as an internal control to calibrate the cDNA template for each sample. Mean ± SD values with different lowercase letters are significantly ($p \leq 0.05$) different between each treatment.

Immune-related gene expression analysis revealed significantly elevated LvALF B gene expression levels in the hepatopancreas of shrimp fed with 10 and 15 % *Amphora sp.* powder by week 6. LvALF B, associated with antimicrobial activity, showed higher expression in these groups than in shrimp receiving 5 % *Amphora* sp. powder supplementation or the control diet. This increase in antimicrobial gene expression suggests a dietary influence on genetic responses that bolster pathogen resistance, particularly at higher supplementation levels. In response to pathogen exposure, shrimp upregulate various immune-related genes, contributing to their innate immunity. In penaeid shrimp, ALFs play diverse roles in immunity, participating in antibacterial, antifungal, and antiviral activities. The expression of LvALF AV-R suggested the inducing of the hepatopancreas of shrimp in response to the presence of the Vp_PirABlike toxin, carried by pathogenic *V. parahaemolyticus* with a plasmid encoding Vp PirAB-like toxins. The increasing of this immune-related gene indicated the response of shrimp to against the infection of *V. parahaemolyticus* [29].

LvALF B, monitored in this study, is one of group B isoforms discovered in *L. vannamei* that show antimicrobial activity against natural shrimp bacterial pathogens including V. parahaemolyticus that causes acute hepatopancreatic necrosis disease (AHPND) [29].

Numerous studies performing on the antimicrobial activities of ALFs in various shrimp have confirmed that ALFs play important roles in the innate immunity of the shrimp and their molecular activities can be applied as bioindicator of the shrimp health status [30-32]

Conclusions

The highest level of omega-3 fatty acids, particularly SDA and EPA were detected in the 15 % Amphora sp. diet. This result supports an improvement of nutritional value and suggests potential benefits for shrimp resilience.

Growth performance of the shrimp fed with diets supplemented with Amphora sp. powder (ranging from 0 - 15 % w/w) were determined using various parameters (MBW, SR, SGR, and FER). The results indicate that Amphora sp. supplemented diets considerably improve growth performance in relation to time (within 6 weeks) and doses (between 5 - 15 %). Shrimp fed with the 15 % Amphora sp. supplemented diet exhibited the highest enhanced growth performance.

Health status of the experiment shrimp were monitored through the measurements of THC, PA, SR, and the expression analysis of immune-inducible gene (LvALF B gene) on *V.parahaemolyticus* infected shrimp in comparison with un-infected shrimp. The results demonstrate the potential functions of Amphora sp. as an immunostimulant in shrimp diet. Amphora sp supplemented diet enhances immune responses and potentially improving resilience to pathogenic stress, with optimal effects observed at higher supplementation levels. This confirms by the results of shrimp fed with 15 % Amphora sp. diet that exhibit higher THC, PA level, SR as well as the upregulation of LvALF B gene in experimented shrimp within 6 weeks.

In summary, *Amphora* sp. supplementation in Pacific white shrimp (*L. vannamei*) diets demonstrated notable benefits on shrimp health, growth, and immunity. Shrimp fed with the 15 % *Amphora* sp. supplementation also exhibited enhanced growth performance over time, indicating improved nutrient utilization with higher supplementation. These findings support the inclusion of 15 % *Amphora* sp. in shrimp feed formulations for improved productivity and sustainability in aquaculture.

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