ผลของการเสริมไดอะตอม (*Amphora coffeaeformis*) ในอาหารต่อการเจริญเติบโตและการแสดงออกของยีนที่เกี่ยวข้องกับการเจริญเติบโตในกุ้งขาว (*Litopenaeus vannamei*) ระยะหลังวัยอ่อน

Effects of Diatom (*Amphora coffeaeformis*) Supplementation in Diet on Growth and Growth-Related Genes Expression in Pacific White Shrimp (*Litopenaeus vannamei*) Postlarvae

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บทคัดย่อ

การทดลองนี้มีวัตถุประสงค์เพื่อเปรียบเทียบการเจริญเติบโต อัตรารอดและการแสดงออกของยีนที่เกี่ยวข้องกับการเจริญเติบโตของลูกกุ้งขาว (*Litopenaeus vannamei*) ที่ให้อาหารเสริม *Amphora coffeaeformis* ในระดับที่แตกต่างกัน โดยแบ่งออกเป็น 6 ชุดการทดลอง ได้แก่ 0 4.31 8.26 11.89 15.25 เปอร์เซ็นต์ และ *A. coffeaeformis* สด ตามลำดับ โดยเตรียมไดอะตอมชนิดนี้ด้วยสภาวะปลอดเชื้อในห้องปฏิบัติการ จากนั้นทำการเก็บเกี่ยวและสะสมเซลล์ไว้ เพื่อนำเซลล์ไปทำให้แห้งด้วยเครื่องทำแห้งแบบเยือกแข็ง และนำมาผสมอาหารลูกกุ้งขาวตามชุดการทดลองที่ได้ออกแบบไว้ งานทดลองนี้ใช้ลูกกุ้งขาวระยะโพสต์ลาวา 15 (PL 15) ความยาวเริ่มต้น 1.06±0.10 เซนติเมตร เลี้ยงในน้ำความเค็ม 20 พีเอสยู อุณหภูมิ 30 องศาเซลเซียส เปลี่ยนถ่ายน้ำ 20 เปอร์เซ็นต์ ทุกวัน ให้อาหาร 7 มื้อต่อวัน และวัดคุณภาพน้ำทุก 3 วัน ผลการทดลองจากการเลี้ยงลูกกุ้งเป็นระยะเวลา 14 วัน ได้ชี้ให้เห็นว่าชุดการทดลองที่มีการเสริม *A. coffeaeformis* สด และ 4.31 เปอร์เซ็นต์ มีความยาวสุดท้ายสูงสุด 2.12 และ 1.98 เซนติเมตร เมื่อเทียบกับชุดควบคุมมีความยาวสุดท้ายแตกต่างอย่างมีนัยสำคัญ ($p < 0.05$) ผลการวิเคราะห์การแสดงออกของยีนของลูกกุ้ง ในการศึกษานี้ให้ความสนใจยีนที่เกี่ยวข้องกับการเจริญเติบโต 3 ยีน ได้แก่ Chitinase (CHIT) AMP-activated protein kinase (PRKAG) และ Cathepsin พบว่าลูกกุ้งที่ได้รับอาหารเสริม *A. coffeaeformis* สด มีการแสดงออกของยีนมากที่สุดและมีความแตกต่างกับชุดควบคุมอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) การศึกษาครั้งนี้บ่งชี้ว่าการเสริม *A. coffeaeformis* สด ส่งผลให้กุ้งมีการเจริญเติบโตและการแสดงออกของยีนมากที่สุด และหากเสริม *A. coffeaeformis* ในรูปแบบแห้งควรเสริมที่ไม่เกิน 4.31 เปอร์เซ็นต์

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Abstract

This experiment aimed to compare study the effects of *Amphora coffeaeformis* supplementation on the growth performance, survival rate and growth-related genes expression of Pacific white shrimp postlarvae (*Litopenaeus vannamei*) at different levels. The experiment was designed in 6 treatments using 4 concentrations of *A. coffeaeformis* supplemented into diet as follows: 0%, 4.31%, 8.26%, 11.89%, 15.25% and live *A. coffeaeformis*. This diatom was prepared under laboratory conditions and harvested by nylon mesh filter to amass an amount of *A. coffeaeformis* for drying. The diatom powder was mixed according to the designed treatments. In this study, stage 15 postlarvae (PL15) with the lengths starting from 1.06 ±0.10 cm. were cultured under specific conditions as follows: salinity 20 psu, temperature 30°C, daily water change (20%), feeding shrimps for 7 times/day and water quality analyzed every 3 days. After culturing postlarvae for 14 days, the results showed that the final lengths of white shrimp postlarvae fed by live and 4.31% supplementation of *A. coffeaeformis* were the longest sizes of 2.12 and 1.98 cm. comparing to the controlled groups with significantly different final lengths ($p < 0.05$). Also, the results of growth-related gene expression (Chitinase (CHIT), AMP-activated protein kinase (PRKAG) and protease (Cathepsin) were focused in this study) revealed that white shrimp postlarvae fed with live *A. coffeaeformis* supplemented diet were the highest growth-related gene expression group compared to the controlled groups with statistically significant difference ($p < 0.05$). This study indicated that live *A. coffeaeformis* supplementation affected the highest growth and gene expression of white shrimp postlarvae. It should be noted that no more than 4.31% of dry powder *A. coffeaeformis* supplementation should be added.

Keywords: postlarvae white shrimp, phytoplankton, diatom

Introduction

Microalgae are an essential food source for nurturing in the larval stages of marine animals such as bivalve mollusks, fish, penaeid shrimp, and zooplankton. Microalgae are also a rich source of vitamins, minerals, amino acids, essential fatty acids and pigments for aquaculture (Takeuchi *et al*., 2002). Because of this, they can enhance the health, strength, and disease-resistance of aquatic animals as well as boost the rate of survival (Pimolrat, 2009). The microalgae species most often used for shrimp feed are

Chaetoceros sp., *Thalassiosira* sp., *Tetraselmis* sp., *Isochrysis* sp. and *Nannochloropsis* sp., which can be directly fed to aquatic animals, sometimes for zooplankton and then for food to aquatic animals (Eirik *et al*., 1998). *Amphora coffeaeformis* is a type of diatomic microalgae that has high nutritional value. It is also easy to harvest. Gordon *et al*. (2006) reported that *Amphora luciae* had 32.65% protein, 6.43% lipid and 21.6 fatty acids (% in lipid). Therefore, *Amphora* sp. is an alternative source of nutrients for shrimp culture.

The application of *Amphora* sp. for aquaculture has been used as food for many species of aquatic animals. The *Amphora* sp. has been used to feed abalone and spotted babylons in 1.9×10^4 cells/cm^2 to help increase their growth and survival rate (Gordon *et al*., 2006; Anthasoot, 2007). Phookung (2007) supplemented *Amphora* sp. (3.0×10^6 cell/ml) in the diet for sandworms. The results showed that the pigment (chlorophyll a and carotenoid) and fatty acid composition increased in comparison with control. Besides, *Amphora* sp. can be used for absorption of nitrogen, phosphorus and also improve the water quality (Kumar *et al*., 2016; Sassi *et al*., 2018). Khatoon *et al*. (2009) reported that black tiger shrimp postlarvae (*Penaeus monodon*) were fed with 3 species (*Amphora* sp., *Navicula* sp. and *Cymbella* sp.) diatom supplementation for 19 days and showed a high survival rate as well as growth without the need to exchange water.

Many reports have mentioned the application of *Amphora* sp. for aquacultures, both for nutritional sources and water quality treatment. Therefore, *Amphora* sp. supplementation in shrimp feed could be a feasible option for boosting shrimp quality. As such, this research will be applying *A. coffeaeformis* as supplementation in the diet to compare with different levels of growth and growth related-genes in *Litopenaeus vannamei* postlarvae.

Materials and Methods

1. Cultivation of *A. coffeaeformis*

A. coffeaeformis from the Research Center of Excellence in Shrimp Walailak University was cultivated in batch culture with sterile seawater at salinity of 20 psu, aerated and illuminated continuously with 5,000 lux of fluorescent light. The temperature in the laboratory room was kept at 25±2°C. The culture medium was WU medium consisting of $CO(NH_2)_2$, $Na_2HPO_4.2H_2O$, $Na_2SiO_3.5H_2O$, $NaHCO_3$ and trace metals (Walailak University's phytoplankton medium). The cultures were transferred to the next batch at the exponential phase and harvested using a nylon mesh filter (30 µm) at the early stationary phase of growth. The wet-cell pellets were freeze-dried and stored at -80°C until analysis and diet formulation.

2. Experimental diet formulation and proximate composition analysis

Commercial flakes (Winner®) were used in the experiment. The diets were supplemented with different levels of *A. coffeaeformis* freeze dried at 0%, 4.31%, 8.26%, 11.89% and 15.25%. The level of supplementation was referred from Ju *et al.* (2009) The experimental diets were prepared using 400 ml of sterile water, blended with a mixer and dried using a hot plate at 100˚C, mash and filter the diets with a 600-micron size for feeding white shrimp larvae 15-30 stages (Xie *et al*., 2018). The experimental diets were dried at 105˚C for moisture analysis and at 550˚C for total ash following AOAC (2005). Crude protein was determined by using CN Analyser (LECO®, TruSpecCN, MI, USA) Total lipid content was analyzed according to Bligh and Dyer (1959).

3. Shrimp and feeding experiment

Postlarvae white shrimp (PL10 stage) from a commercial hatchery (in the southern region of Thailand) were transferred into a 200-liter plastic tank with 100 liters of aerated seawater at a salinity of 20 psu. Test white shrimp postlarvae were fed using the control diet for 5 days and then transferred into an 8-liter working volume at PL 15 stage (Initial length at 1.06±0.10 cm). Each experiment tank was stocked with 100 white shrimp postlarvae cultivated to tank 10 liters (8 liters working volume) of aerated seawater at a salinity of 20 psu, 30 controlled temperature and water exchange every 3 days. The ammonia and nitrite were measured using a phenol hypochlorite method and NED method respectively (Jiwyam, 2001) calcium, hardness were measured using EDTA titrimetric method, magnesium by calculations based on the EDTA titrimetric method, alkalinity was measured using indicator method (Tuntoollavest, 2000) and pH with pH pen Smart Sensor (PH818, China). For control, a control diet with live *A. coffeaeformis* and 4 experimental diet treatments were randomly transferred into 5 replicate tanks. The postlarvae were fed 7 times daily at 07.00, 10.00, 13.00, 16.00, 19.00, 21.00 and 01.00 h to satiation (Xie *et al*., 2018) and water was changed daily with 20% sediment removal (Lage *et al*., 2017). After 14 days, white shrimp postlarvae were collected to assess final length, survival rate, SGR based on the length of shrimp (Karim, 2007), and tissue of shrimp (20-50 mg) in 500 microliters TriPure Isolation Reagent (Roche, USA) for gene expression analysis and then bring 20 shrimp (for 3 replicates, n = 60 shrimp/treatment) to the stress test with 100 ppm formalin for 1 hour (Samocha *et al*., 1998; Wangsoontorn *et al*., 2013).

$$\text{Specific growth rate (SGR, \%)} = (\ln L_f - \ln L_0)/t \times 100$$

where "L_0" is the initial body length of shrimp, "L_f" is the final body length of shrimp and "t" is the day of experiment.

Survival rate (%) = $(N_f / N_0) \times 100$

where "N_0" is the initial number of shrimp and "N_f" is the final number of shrimp.

Survival rate stress test (%) = $(N_f / N_0) \times 100$

where "N_0" is the initial number of shrimp before the stress test and "N_f" is the final number of shrimp after the stress test.

4. Gene expression

Gene expression was studied using a real-time PCR technique with the Applied Biosystems 7300 real-time PCR system (Thermo Fisher Scientific, CA, USA). In 10 microliter reaction with white shrimp tissue cDNA as a prototype DNA line and using the forward and reverse primer designed to increase PCR production at 95°C for 15 minutes, 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds, 40 cycles and select dissociation curve to observe the melting curve and beta-actin genes as reference genes, prepared with standard DNA concentrations 1, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} times for comparison of gene expression. This study was interested in the growth-related gene for white shrimp postlarvae. CHIT gene is a gene that is involved in the production of chitin digestion enzymes (Spindler-Barth *et al*., 1990), PRKAG AMP-activated protein kinase (AMPK) is a crucial cellular energy sensor (Hardie *et al*., 2012), Cathepsin gene is enzymes that digestive proteins (McGrath, 1999).

5. Statistical analysis

Statistical significance was considered at $p < 0.05$ by one-way ANOVA following Duncan's Multiple Range Test.

Results

1. The nutritional composition of culture and the experimental diets

The dry weight of *A. coffeaeformis* contained 8.69% moisture, 17.71% crude protein, 23.11% crude lipid and 49.62% ash on average. The nutritional values of diets supplemented with *A. coffeaeformis* at different levels are shown in Table 1. Protein content decreased with the levels of *A. coffeaeformis* supplementation and vice versa for lipid and ash ($p < 0.05$)

Table 1 Protein, lipid and ash (dry weight basis) of diets in the experiment.

Treatment	Protein (%)	Lipid (%)	Ash (%)
Control	49.87±0.11^{e}	11.25±0.21^{a}	17.85±0.46^{a}
A. coffeaeformis 4.31%	48.10±0.10^{d}	11.75±0.26^{b}	19.02±0.44^{b}
A. coffeaeformis 8.26%	47.07±0.40^{c}	13.09±0.29^{c}	20.03±0.06^{b}
A. coffeaeformis 11.89%	46.23±0.22^{b}	14.06±0.27^{d}	21.60±0.55^{c}
A. coffeaeformis 15.25%	44.46±0.25^{a}	15.21±0.15^{e}	21.94±0.09^{c}

Note**:** Values are expressed as means ± SD (n=3) with different superscript (a-e) differ significantly ($p < 0.05$) between treatments

2. Growth performance and survival rate

Based on the final length and SGR length, white shrimp postlarvae fed with live algae exhibited superior performance, followed by 4.31% supplementation and control treatments, respectively ($p < 0.05$). High survival rate was found for white shrimp postlarvae fed with live algae and the treatments containing 4.31% and 8.26% ($p > 0.05$) relative to the remaining treatments. Formalin stress test values did not differ among all treatments (Table 2).

Table 2 Final length, specific growth rate, survival rate, and formalin stress test of white shrimp postlarvae larvae fed with *A. coffeaeformis* at different levels for 14 days

Treatment	Final length (cm)	SGR_{Length} (%/day)	Survival rate (%)	Formalin Stress test (%)
Control	1.92±0.02^{c}	1.83±0.02^{c}	90±1.00^{b}	100
A. coffeaeformis 4.31%	1.98±0.02^{d}	1.92±0.02^{d}	97±2.65^{c}	100
A. coffeaeformis 8.26%	1.82±0.03^{b}	1.67±0.05^{b}	99±1.73^{c}	100
A. coffeaeformis 11.89%	1.75±0.01^{a}	1.54±0.02^{a}	85±1.00^{b}	100
A. coffeaeformis 15.25%	1.74±0.02^{a}	1.52±0.03^{a}	79±3.21^{a}	100
Live *A. coffeaeformis*	2.12±0.02^{e}	2.14±0.02^{e}	97±0.00^{c}	100

Note: Values are expressed as means ± SD (n=3) with different superscript (a-d) differ significantly ($p < 0.05$) between treatments

3. Gene Expression

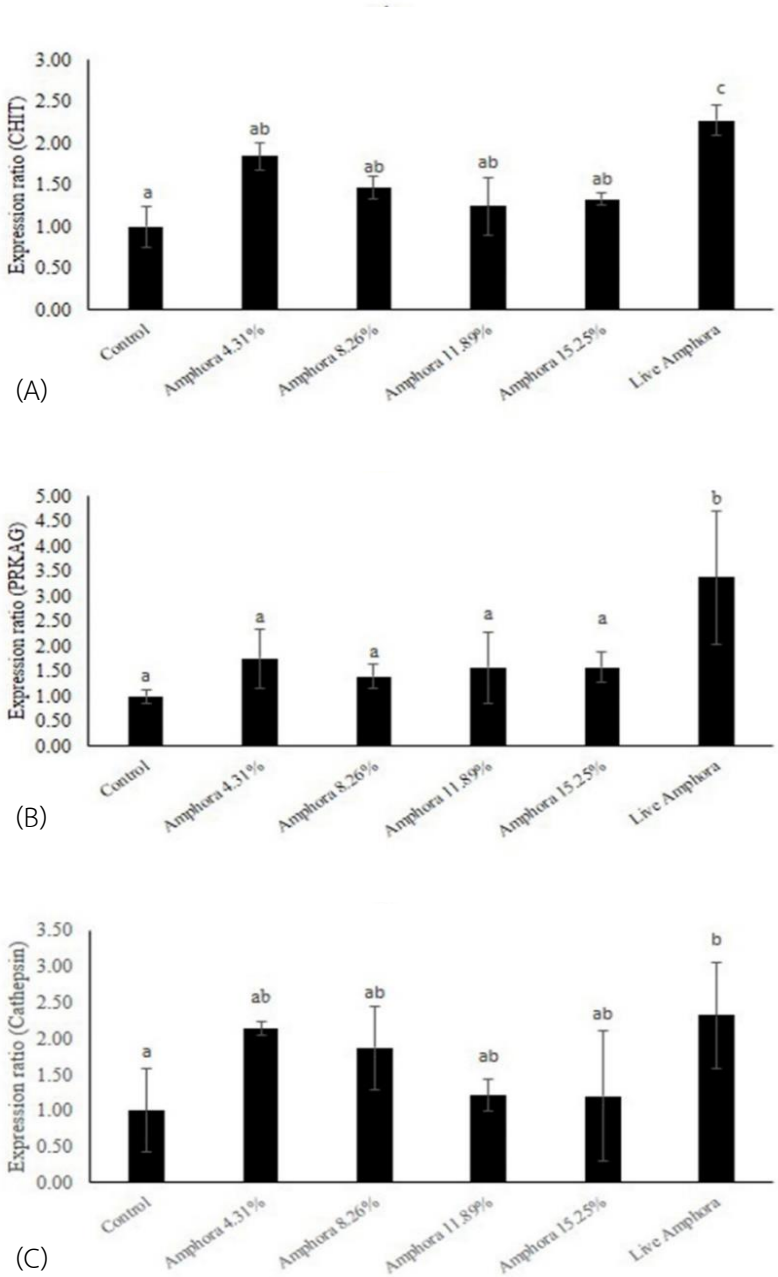

Figure 1 CHIT (A), PRKAG (B) and Cathepsin (C) gene expression from white shrimp postlarvae tissue fed with *A. coffeaeformis* at different levels for 14 days. The values are expressed as means ± SD (n=3) with different superscript (a-c) differ significantly ($p < 0.05$) between treatments.

Significant improvement in CHIT (Fig. 1A), PRKAG (Fig. 1B) and cathepsin (Fig. 1C) expressions were observed in the shrimp fed with live *A. coffeaeformis* supplemented diet. No differences in these three genes were found between the four levels of dried algal supplementation relative to the control treatment.

4. Water quality

The water parameters were assessed during 14 days of white shrimp postlarvae cultivation (every 3 days). The pH value was found to be insignificant for alkaline (7.76-8.11). The values for alkalinity, hardness, calcium, and magnesium were found to vary between 180-220 mg/L, 4134-4800 mg/L, 220-256 mg/L and 970-1134 mg/L, respectively. Ammonia and nitrite measures found that the experiment with live *A. coffeaeformis* had lower ammonia and nitrite values than all other treatments, though there was no statistically significant difference ($p < 0.05$).

Discussion

Cultivation of *A. coffeaeformis* with WU medium in the laboratory, providing 24-hour light and controlled at 24°C for 5 days was used for supplementary diet in the nursery of white shrimp postlarvae. When analyzing nutritional values, *A. coffeaeformis* had high lipid content at 23.11%, though protein content was 17.71%. This is different from a report by Gordon *et al*. (2006), which used cultured *A. luciae* with Guillard F/2 medium. There was high protein at 32.65%, while lipid content was at 6.43% because the nitrogen source in the culture is different. Although nitrite, nitrate, and urea are suitable sources of nitrogen for the growth of algae (Fabregas *et al*., 1989; Yanqun *et al*., 2008), using different nitrogen sources results in the growth and nutritional values of microalgae (Fidalgo *et al*., 1995). This is consistent with the study of Pimolrat (2009), in which *Cheatoceros* sp. was cultured in a laboratory with different nitrogen sources. It was found that the culture content showed high lipid level with urea treatment. There are other factors that can cause the nutritional value of algae to be different. It was reported from Viçose *et al*. (2012), who compared the growth stages of 4 diatoms, that diatom in the exponential phase has higher protein and lipid values in the stationary phase and more carbohydrates accumulate. However, although the nitrogen source of the results in the nutritional value of algae may differ, there are other factors that can affect the nutritional value of algae, such as the type of algae, salinity, and culture time as well as cell harvest.

The results of white shrimp postlarvae supplemented with different *A. coffeaeformis* levels found that a higher level of *A. coffeaeformis* supplementation caused reduced protein values and increased lipid content. The growth of white shrimp postlarvae in

this study showed that white shrimp postlarvae fed with live *A. coffeaeformis* had the longest length. *A. coffeaeformis* at 4.31% showed higher growth than the control because *A. coffeaeformis* supplementation has a high protein value of 48.10 percent. According to Biedenbach *et al*. (1989), optimum protein for growth and survival of white shrimp should be greater than 44 percent. Also, Abdurahman *et al*. (2017) supplemented *Tetraselmis chuii* in diets for white shrimp postlarvae, which showed that *T. chuii* supplementation at 50% had the best survival and growth rate, with protein nutrition at 57.30 percent. However, this study demonstrated that white shrimp postlarvae need high protein content for use in fast growth.

The lipid in this study showed that *A. coffeaeformis* 4.31% had 11.75 percent lipid, which was inconsistent with the report by Perez and Lawrence (2004), who suggested that the lipid for shrimp postlarvae should not exceed 10 percent. However, there are reports that shrimp postlarvae can grow to 30 percent (Banerjee *et al*., 2010). *A. coffeaeformis* supplementation increased lipid and high ash value as well. New (1990) reported that the ash content in diets is suitable for shrimp in the range of 12-18 percent. However, it was found in this study that supplementation with *A. coffeaeformis* is greater than the ash range suitable for white shrimp postlarvae growth. *A. coffeaeformis* at 4.31% enables high growth, but the ash value is slightly higher than the suitable range. In this study, *A. coffeaeformis* supplementation can be performed in diets for white shrimp postlarvae, both live and powder (should not exceed 4.31%). Supplements of this microalgae in live form can be stored by filtering methods. Sedimentation cells can be stored at 4°C for up to 2 weeks. The cells are still alive and can be cultured further (Pankaew *et al.*, 2011).

The formalin stress test was carried out by immersing in water containing a concentration of formalin, 100 ppm for 1 hour (Samocha *et al.*, 1998; Wangsoontorn *et al*., 2013). It was found that all 6 treatments had 100% survival rate and there was no significant difference ($p < 0.05$). The acute stress test for white shrimp postlarvae was able to differentiate between healthy shrimp and weak shrimp larvae (Nietes, 1990). Such a test is an assessment of the quality of shrimp postlarvae that are commonly used on farms (Samocha *et al*., 1993). It is also an easy, convenient and fast method for farmers to check the quality of shrimp postlarvae (Castille *et al*., 1993). Samocha *et al*. (1998) conducted a comparative study between shrimp postlarvae PL2 and PL7 against a formalin stress test. It was found that the older shrimp postlarvae had better survival rates. However, the reports of Anh *et al*. (2018) and Immanuel *et al*. (2007) revealed that stress tests for tiger shrimp postlarvae were used to study tiger shrimp PL40 and showed that even though the shrimp postlarvae

will get older, the stress test using formalin still gave the shrimp mortality rate. The results of formalin stress testing showed no shrimp deaths. Shrimp death is dependent on the environmental conditions as well as the strength of the shrimp.

Gene expression is consistent with the growth for the final length of white shrimp postlarvae. The highest gene expression was found in white shrimp postlarvae that consumed live *A. coffeaeformis.* Although all treatments showed gene expression greater than control, supplementation with dry *A. coffeaeformis* at than 4.31% caused a decrease in the expression of growth related-gene. All of the Chitinase genes (CHIT), PRKAG AMP-activated protein kinase (AMPK) and Cathepsin gene related to the growth of white shrimp postlarvae. Spindler-Barth *et al*. (1990) and Kono *et al*. (1995) reported that CHIT genes were involved in the production of chitin digesting enzymes in crustaceans, whereas AMPK was an important controller in the metabolism of energy that has been stored in the cell and involved the salinity fluctuations (Dan *et al*., 2016). Besides, the Cathepsin gene was also important for shrimp growth. Stephens *et al*. (2012) studied the expression of the Cathepsin B gene from white shrimp with different tissues. The results showed that the amount of food directly affected gene expression and growth. The nutritional value in the treatment showed high nutrition and less shrimp consumption than other treatments, affecting low growth as well as gene expression.

Water quality showed that all treatments had ammonia and nitrite levels in the non-toxic range for water, the salinity of 20 psu. Supplementation of live *A. coffeaeformis* for white shrimp postlarvae can help the shrimp to survive and grow. It also has a better effect on water quality than treatment without adding live *A. coffeaeformis*. While the ammonia and nitrite values for all treatments were not statistically different, the addition of live *A. coffeaeformis* resulted in lower ammonia and nitrite values than other treatments. This is consistent with the reports of Banerjee *et al*. (2010) and Ge *et al*. (2016), who showed that shrimp culture along with supplementing live microalgae without changing water effectively helped to maintain water quality. A report from Ichsan *et al*. (2014) found that microalgae could be used to treat wastewater from agricultural wastewater sources efficiently as well as provide bio-energy. Limheng *et al*. (2005) reported that nitrite content derived from amounts of organic substances accumulated in shrimp ponds, likely due to the large amounts of food leftovers and waste generated in the pond. In this study, the water was exchanged every day, and sucking the sediment helped the nitrite to prevent reaching a level that was toxic to the shrimp postlarvae. In addition, a report from Khatoon *et al*. (2009) studied the feeding of black tiger shrimp postlarvae using 3 diatom species (*Amphora*, *Navicula* and *Cymbella*) without changing

the water for 19 days. They found that, aside from helping to maintain water quality, this diatom group has a high amount of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and amino acids that can increase growth and survival rate.

Conclusion

This study indicated that *A. coffeaeformis* powder supplementation with commercial flake caused reduced protein and increased lipid content. White shrimp postlarvae showed the highest growth rate and growth-related genes expression with live *A. coffeaeformis*. If supplemented with *A. coffeaeformis,* dry powder should be added at no more 4.31%. The water in live *A. coffeaeformis* treatment had lower ammonia and nitrite levels than other treatments

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