DOI: http://dx.doi.org/10.15578/iaj.17.2.2022.139-145 *Indonesian Aquaculture Journal, 17 (2), 2022, 139-145*

Available online at: http://ejournal-balitbang.kkp.go.id/index.php/iaj

EFFECTS OF MICROALGAE SPIRULINA *Arthrospira platensis* SUPPLEMENTATION TO THE PLANT-BASED DIET FOR PACIFIC WHITE SHRIMP *Litopenaeus vannamei*

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(Received: November 10, 2022; Final revision: December 14, 2022; Accepted: December 14, 2022)

ABSTRACT

A sixty-days feeding trial was conducted to evaluate the inclusion effect of spirulina Arthrospira platensis meal (SM) in the diet on growth and health condition of juvenile Pacific white shrimp Litopenaeus vannamei. Four isonitrogenous and iso-lipidic experimental diets were formulated to contain 0%, 0.2%, 0.4%, and 0.8% SM and fed to the shrimp (average initial weight 0.71 ± 0.1 g, 15 shrimp per tank, n = 3). At the end of the growth trial, shrimp were sampled and total haemocyte counts were measured. The growth performances of shrimp were significantly affected by the dietary inclusion of SM, whereas the inclusion of SM provides a better biomass, final body weight (FBW), weight gain (WG) and feed conversion ratio (FCR) compared to the control group. Additionally, the inclusion of SM significantly enhances the total haemocytes count (THC) and lysozyme activity in shrimp compared to control group. Therefore, SM can be considered as the functional ingredients or supplements in diet to improve the growth and health condition of shrimp.

KEYWORDS: Spirulina; *Arthrospira platensis*; growth; health status; *Litopenaeus vannamei*

INTRODUCTION

A growing interest in utilizing supplements, additives and functional ingredients to produce the functional feed has been observed in the last decades in white shrimp *Litopenaeus vannamei* industry, mainly to improve the production efficiency, growth performance and the utilization of nutrients in feed, to overcome the lower survival rate problem as well as to minimize the diseases incidence during the production period (Coutteau, 2016; Encarnação, 2016; Novriadi *et al*., 2022b; Ochoa-Solano & Olmos-Soto, 2006; Olmos *et al*., 2011). The functional ingredients that has been evaluated in shrimp *L. vannamei* including the use of fermented corn protein concentrate (Novriadi *et al*., 2022a), fermented soya bean meal (Yao *et al*., 2020), insect meals (Shin & Lee, 2021) and seaweed (Omont *et al*., 2021). While for additives and supplements, including the use of nucleotides (Novriadi *et al*., 2022b), natural active substances (Novriadi *et al*., 2022c; Orozco Hernández *et al*., 2009), amino acid (Nunes *et al*., 2014) and combination with enzymes (Bulbul *et al*., 2015) until the use of attractants (Nunes *et al*., 2006) to provide positive outcomes in shrimp production system.

Another possibly valuable feed ingredient that can be considered as a functional ingredient in formulated diet is microalgae spirulina *Arthrospira platensis* meal (SM), due to better amino acid profile compared to several alternative ingredients (Hanel *et al*., 2007), high protein level and essential amino acids in dry weight condition (Paoletti *et al*., 1980), and their ability to enhance the immune response of shrimp (Macias-Sancho *et al*., 2014). Spirulina have been reported to have no cell wall, which results in improved digestion and nutrient absorption (Nandeesha *et al*., 2001; 1998). In addition, spirulina could also serve as feed additive or supplement due to the bioactive properties that can stimulate better

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health condition of aquatic organisms (Ragaza *et al*., 2020; Rosas *et al*., 2019; Tayag *et al*., 2010). In shrimp, the dietary inclusion of SM reduces mortality due to white spot syndrome virus (WSSV) (Rahman *et al*., 2006; Rahman, 2007) and bacterial infection (Chen *et al*., 2016). Despite the important role of SM to enhance the growth performance and health condition of shrimp has been studied, but the efficacy of SM used in a very small inclusion level is limited.

Accordingly, the present study investigated the supplementation effect of SM with three inclusion levels, namely: 0.2, 0.4, and 0.8 % on the growth performance of the shrimp *Litopenaeus vannamei*. The total haemocyte count (THC) was also observed to reveal the ability of SM included in small inclusion level to enhance the innate immune system in shrimp.

MATERIALS AND METHODS

Experimental Diets

All experimental diets were formulated to have similar crude protein level (iso-nitrogenous) and crude lipid level (iso-lipidic) to contain 34% protein and 7% lipid as demonstrated in Table 1. In this growth trial, the control diet was designed with 10% fish meal (FM), 41% soybean meal (SBM) and 12% of poultry by-product meal as the primary protein sources. Three experimental diets were formulated to include increasing levels of microalgae *Spirulina platensis* obtained from PT. Bio Cycle Indo (Riau, Indonesia) as a supplement to the diet as much as 0.2, 0.4, and 0.8% and labeled as 0.2, 0.4, and 0.8 SM. All experimental diets were produced at the Aquaculture Production Business Service Center (Karawang, West Java, Indonesia). Prior to production, all ingredients were properly mixed in a paddle mixer (Marion Mixers, Inc., Marion, IA, USA) in a 100 kg batch followed by grinding to a particle size of <200 µm using a disk mill (Jinan Shengrun, China). The cooking-extrusion diets exposed to an average of 110°C for approximately 14 seconds in five-barrel sections and the last section was maintained at 62°C. Pressure at the die head was approximately 50 bars, and screw speed was maintained at 423 rpm. The feeds were extruded through 1- and 2-mm die to produce 1.5- and 2.5-mm particles. Diets were dried in a pulse bed dryer (Jinan Shengrun, China) until moisture readings were below 6%. Pellets were dried at approximately 107°C with an upper limit outflow air temperature of approximately 88°C. Diets were then cooled at ambient air temperatures for final moisture levels of less than 10%. All finished diets were bagged and stored in a temperature-controlled room until further use. Proximate level of the experimental diets were analyzed at Saraswanti Indo Genetech Laboratory, Bogor, West Java, Indonesia and summarized in Table 1.

Growth Trial and Feeding Management

The growth trials were conducted at the Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Diponegoro University (Semarang, Central Java, Indonesia). Pacific white shrimp were obtained from PT. Prima Akuakultur Lestari (Kalianda, Lampung, Indonesia) and randomly distributed into 12 tanks with size of 70 x 35 x 40 cm (98 L) per aquaria tank. The initial mean average weight of the shrimp used in this study was 0.71 ± 0.10 g and 15 shrimps were distributed per tank. Three replicate groups of shrimp were offered experimental diets using nutrition research standard protocol for 60 days and fed by hand four times daily at 07:00, 11:00, 15:00, and 20:00. Feed inputs were pre-programmed assuming the normal growth of shrimp and feed conversion ratio of 1.5. Daily allowances of feed were adjusted based on observed feed consumption, weekly counts of the shrimp and mortality. Uneaten feed, feces, and molts were removed by siphoning the aquaria tank prior to the first feeding.

Water Quality and Growth Sampling

Water quality parameters, consist with dissolved oxygen (DO), pH, water temperature and salinity were measured four times daily using Aqua TROLL 500 Multiparameter Sonde instrument and connected to AquaEasy apps (Bosch, Singapore). Total ammonia nitrogen (TAN), nitrate and nitrite were measured once a week by using absorption spectrophotometry (DR890, HACH, USA). At the end of feeding period, all shrimp were grouped and individually weighed to calculate the final biomass, final weight, percentage weight gain (PWG), feed conversion ratio (FCR), percentage survival (SR) and thermal unit growth coefficient (TGC) as follows:

$$PWG = \frac{(AIFW - AIIW)}{(AIIW)} \times 100$$

where: AIFW = average individual final weight
AIIW = average individual initial weight

$$FCR = \frac{\text{Feed given (g)}}{\text{Total weight gain (g)}}$$

$$SR = \frac{\text{Final number of shrimp}}{\text{Initial number of shrimp}} \times 100$$

$$TGC = \frac{FBW^{1/3} - IBW^{1/3}}{\sum TD} \times 100$$

Table 1. Composition (% *as is*) of diets containing spirulina *Arthrospira platensis* fed to *L. vannamei* for 60 days

Ingredients (% *as is*)	Diet code			
	Control	0.2 SM	0.4 SM	0.8 SM
Soybean meal[1]	41.00	41.00	41.00	41.00
Poultry by-product meal[1]	12.00	12.00	12.00	12.00
Spirulina powder[2]	0.00	0.20	0.40	0.80
Menhaden fish meal[1]	10.00	10.00	10.00	10.00
Soy-lecithin[3]	1.00	1.00	1.00	1.00
Fish Oil[1]	3.20	3.20	3.20	3.20
MCP[1]	0.80	0.80	0.80	0.80
Cholesterol[3]	0.01	0.01	0.01	0.01
Wheat flour[4]	29.79	29.59	29.39	28.99
Mineral premix[5]	0.50	0.50	0.50	0.50
Vitamin premix[6]	0.50	0.50	0.50	0.50
L-lysine[3]	0.25	0.25	0.25	0.25
L-Threonine[3]	0.18	0.18	0.18	0.18
DL- Methionine[3]	0.35	0.35	0.35	0.35
Choline chloride[3]	0.20	0.20	0.20	0.20
Anti-mold[4]	0.12	0.12	0.12	0.12
Stay C – 35[4]	0.10	0.10	0.10	0.10
Proximate analysis (% *as is*):				
Crude protein	34.55	34.63	34.59	34.87
Moisture	8.55	8.67	8.53	8.59
Crude Fat	7.66	7.72	7.72	7.75
Ash	5.88	5.75	5.82	5.84
Amino acids (% as in):				
Lysine	1.72	1.77	1.76	1.75
Methionine	0.81	0.80	0.82	0.88

[1] PT. FKS Multi Agro, Tbk, Jakarta, Indonesia
[2] Bio Cycle Indo, Riau, Indonesia
[3] PT. Rajawali Mitra Pakanindo, Banten, Indonesia
[4] PT. Fenanza Putra Perkasa, Jakarta, Indonesia
[5] Trace mineral premix (g/100 g premix): cobalt chloride, 0.004; cupric sulfate pentahydrate, 0.550; ferrous sulfate, 2.000; magnesium sulfate anhydrous, 13.862; manganese sulfate monohydrate, 0.650; potassium iodide, 0.067; sodium selenite, 0.010; zinc sulfate heptahydrate, 13.193; alpha-cellulose, 69.664.
[6] Vitamin premix (g/kg premix): thiamin·HCL, 4.95; riboflavin, 3.83; pyridoxine·HCL, 4.00; Ca-pantothen ate, 10.00; nicotinic acid, 10.00; biotin, 0.50; folic acid, 4.00; cyanocobalamin, 0.05; inositol, 25.00; vitamin A acetate (500,000 IU/g), 0.32; vitamin D3 (1,000,000 IU/g), 80.00; menadione, 0.50; alpha-cellulose, 856.81

where: FBW = final body weight
IBW = initial body weight
T = water temperature (°C)
D = number of trial days

Total Haemocytes Count

At the end of the growth trial, hemolymph was sampled from two intermolt shrimp per tank or six shrimps per treatment and total hemocytes count was determined. Hemolymph (100 µL) of individual shrimp was withdrawn using sterile 1-mL syringe (25 G × 13 mm needle) at the pleopod base of the second abdominal segment. Before hemolymph extraction, the syringe was loaded with a precooled (4°C) solution (10%-EDTA, Na2) used as an anticoagulant. The haemolymph with anti-coagulant solution was diluted in 150 µL of formaldehyde (4%) and then 20 µL was placed on a hemocytometer (Neubauer) to determine the total haemocytes count (THC) using an optical microscope (Olympus, DP72).

Statistical Analysis

Data for growth parameters and total haemocyte count were analyzed using one-way analysis of variance (ANOVA) to determine the significant difference ($P < 0.05$) among the treatment means followed by the Tukey's multiple comparison test to determine difference between treatments means in each trial. The pooled standard errors (PSE) were used across all the observed parameters as the variance indicator among the treatment. Statistical analyses were conducted using SAS system (V9.4, SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Water Quality

As shown in Table 2, the overall mean and standard deviation of morning and afternoon pH, temperature, dissolved oxygen and salinity are still within the acceptable range for *L. vannamei.*

Growth Performance

The growth performance of Pacific white shrimp *L. vannamei* cultured for 60 days with different dietary treatments are shown in Table 3. The mean final body weight of the cultured shrimp ranged from 7.83–8.73 g. The use of 0.4 and 0.8% of *S. platensis*, produced the highest mean body weight compared to other dietary treatment. Interestingly, the inclusion of 0.2% *S. platensis* also provide better growth compare to the control treatment. The use of *S. platensis* meal in the diet could also lead to the better efficiency compared to the control treatment. The better efficiency was achieved by using 0.8 SM followed by 0.4 and 0.2% SM. Overall, the survival rates were high and similar for all treatments (97.8%).

Total Haemocyte Counts

The effect of adding spirulina meal (SM) on the total haemocyte counts (THC) were displayed in Figure 2. The group of shrimp treated with *S.*

Table 2. Physical water quality data during the grow-out phase of the experiment. Data were presented as mean ± standard deviation (range)

Observed parameters	Time	Dietary Treatments			
		Control	0.2 SM	0.4 SM	0.8 SM
DO (mg L^{-1})	AM	5.61 ± 0.38	5.85 ± 0.40	5.47 ± 0.42	5.73 ± 0.38
	PM	7.92 ± 2.81	5.83 ± 0.07	5.55 ± 0.18	5.81 ± 0.18
pH	AM	7.71 ± 0.27	7.79 ± 0.29	7.64 ± 0.29	7.77 ± 0.41
	PM	7.71 ± 0.09	7.73 ± 0.11	7.59 ± 0.10	7.69 ± 0.10
Temperature (°C)	AM	26.77 ± 0.68	26.72 ± 0.69	26.79 ± 0.65	26.81 ± 0.66
	PM	27.47 ± 0.61	27.44 ± 0.64	27.36 ± 0.65	27.44 ± 0.61
Salinity (‰)	AM	29.5 ± 1.04	29.5 ± 0.86	29.5 ± 0.88	29.4 ± 1.02
	PM	29.1 ± 0.55	29.1 ± 0.54	29.0 ± 0.52	29.0 ± 0.53

Table 3. Growth performance of Pacific white shrimp *Litopenaeus vannamei* (Mean initial weight 0.71 ± 0.10 g) fed experimental diets containing microalgae *Spirulina platensis* for 60 d. Values represent the mean of three replicates. Results in the same columns with different superscript letter are significantly different ($P < 0.05$) based on analysis of variance followed by Tukey's multiple comparison test

Diet code	Biomass	FBW	SR	WG[1]	FCR[2]	TGC[3]
Control	114.70^{c}	7.83^{b}	97.78^{a}	2868.30^{a}	1.75^{b}	0.0819^{a}
0.2 SM	122.57^{b}	8.37^{ab}	97.78^{a}	3192.77^{a}	1.64^{a}	0.0816^{a}
0.4 SM	125.67^{a}	8.57^{ab}	97.78^{a}	3228.29^{a}	1.60^{a}	0.0811^{a}
0.8 SM	127.93^{a}	8.73^{a}	97.78^{a}	3025.57^{a}	1.57^{a}	0.0781^{a}
P-value	< 0.0001	0.0235	10.000	0.3835	0.1056	0.1218
PSE[4]	0.6714	0.1666	22.232	1.540.289	0.0470	0. 0011

Note: [1] WG = Weight gain (%) [3] TGC = Thermal growth coefficient
[2] FCR = Feed conversion ratio [4] PSE = Pooled standard error

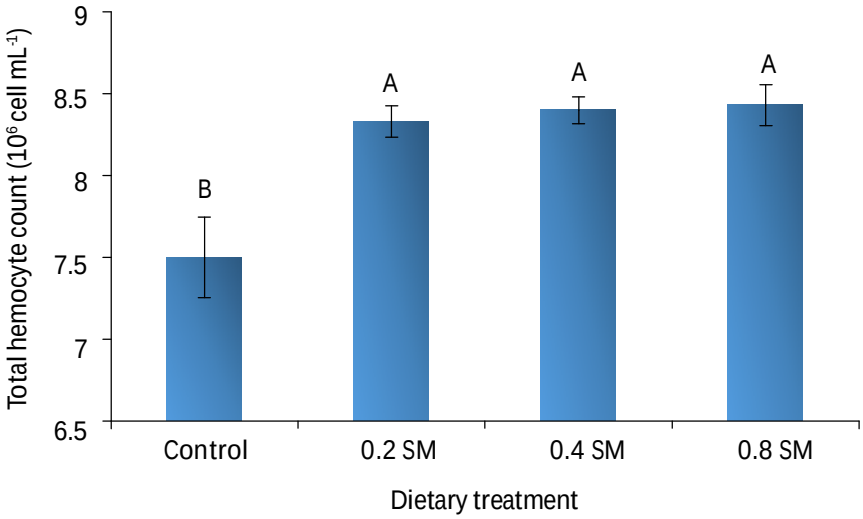

Figure 1. Total hemocyte count of Pacific white shrimp *Litopenaeus vannamei* (10^6 cell mL^{-1}) at the end of growth trial. Values represent the mean of three replicates (P-value: 0.0007).

platensis produced higher number of THC compared to the control treatment ($P < 0.05$)

In this research, Pacific white shrimp *L. vannamei* fed with Spirulina *Arthrospira platensis* (SM) meal showed an increase growth performance and total haemocyte count (THC) compared to the group of shrimp fed with commercial formulation without spirulina. Findings of this study are in agreement with study of Silva Neto *et al*. (2012) where the inclusion of 0.5% SM to the diet with the reduction of fish meal from 18.5% to 13.9% provide better growth compared to the group of shrimp without any addition of SM. The same author indicated that the enhanced growth and performance of the shrimp could be due to the ability of SM to stimulate the feed intake in a very low inclusion levels (Silva Neto *et al*., 2012). In addition, the high protein contents with balanced composition of amino acids and concentration of methionine, tryptophan and other amino acids similarity to the casein (Zidan *et al*., 2021), makes SM has been receiving attention as a potential ingredients in aquafeed formulation (Hanel *et al*., 2007; Macias-Sancho *et al*., 2014). Nowadays, even the nanoparticle size of SM with recorded average size of 183.9 nm or 87.6% smaller than conventional SM has been tested and also able to improve the growth, survival and feed utilization of shrimp *L. vannamei* (Sharawy *et al*., 2022).

Regarding to the sustainability and the efforts to reduce the pressure on the use of marine ingredients, Spirulina *Arthrospira platensis* has been widely studied to replace the use of fish meal (FM) in shrimp feed formulation without compromising the growth of shrimp. Study from Zidan *et al*. (2021) indicated that the inclusion of 4 and 7% of SM to reduce the inclusion of FM from 34 to 30 and 27% within diet formulation still produce significantly better final body weight (FBW), weight gain (WG), specific growth rate (SGR) and survival rate (SR) compared to other treatment. Furthermore, there were no negative effects to the growth performance of shrimp fed with diet up to 75% replacement of FM with SM (Macias-Sancho *et al*., 2014). However, both authors also suggest that the use of excess amount of SM in the diet need to be considered and properly calculated to still provide and meet the essential amino acid and fatty acid requirement of the shrimp.

Besides the ability to enhance the growth, part of the success on the use of functional ingredients is the capacity of that ingredient to enhance the immune function of shrimp against pathogen. It has been found that SM produces higher granular haemocyte and statistically lowers hyalines haemocyte concentration when the level of FM replacement exceeded 25% (Macias-Sancho *et al*., 2014). In addition, incubating shrimp haemocytes in 1 mg L^{-1} spirulina dried powder significantly increase the phenoloxidase (PO) activity, serine proteinase activity and respiratory burst activity of shrimp (Chen *et al*., 2016). Furthermore, the administration of diet containing spirulina dried powder significantly increase the lysozyme activity and resistance of shrimp against *Vibrio alginolyticus* infection (Chen *et al*., 2016). In this research, the inclusion of 0.2, 0.4, and 0.8% SM were also able to increase the number of haemocyte count in shrimp compared to the control group or the group

of shrimp that not receive SM during the culture period. Unlike vertebrates, shrimp do not have separate lymphatic system with hemolymph as the main transport system (Roy *et al*., 2020). As consequence, shrimp have to rely on their innate immune system to protect themselves against wide range of pathogens and haemocytes are the key cells for the shrimp innate immune system (Roy *et al*., 2020). Therefore, the increase in the number of circulating haemocytes after feeding the shrimp with SM are essential for shrimp to enhance their capacity to recognize the pathogen, support the phagocytosis activities, encapsulation, and nodulation of pathogens (Cerenius & Söderhäll, 2004; Roy *et al*., 2020). With all these reported benefits, supplementing the diet with SM could provide better productivity and profitability in shrimp industry.

CONCLUSION

Results in this study confirm the beneficial effects of Spirulina *Arthrospira platensis* (SM) meal as a functional ingredient in shrimp feed formulation to enhance the growth and health condition of Pacific white shrimp *Litopenaeus vannamei*. Under the conditions of the present study, the inclusion of SM up to 0.8% SM produced highest mean final body weight and followed with the inclusion of 0.4 and 0.2% of SM. Inclusion of SM also produces better feed conversion ratio and total haemocyte counts compared to the control treatment. Further research to evaluate the optimum inclusion level of SM that still support the production efficiency and profitability is worth pursuing.

ACKNOWLEDGMENTS

The work was supported by the grants and commercial products from PT. Bio Cycle Indo (Riau, Indonesia) for improving the use of local ingredients in shrimp diet. We would like to thank to the staff and students at the Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Diponegoro University (Semarang, Central Java, Indonesia). The authors would also like to extend the gratitude to those who have taken the time to critically review this manuscript as well as those who helped in supporting this research. Mention of trademark or proprietary product does not constitute an endorsement of the product by the author as a personal and author institution and does not imply its approval to the exclusion of other products that may also be suitable.

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