The impacts of *Spirulina platensis* microalgae supplementation in feed on the growth, immunity, and antioxidant profile of juvenile abalone (*Haliotis squamata* Reeve, 1846)

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Abstract. This research investigated the influence of incorporating *Spirulina platensis* microalgae into feed on the growth performance, immunity, and antioxidant content of juvenile abalone (*Haliotis squamata*). The study aimed to assess the potential benefits of *S. platensis* supplementation as a dietary component for enhancing the cultivation of juvenile abalone. Experimental groups were fed diets with varying levels of *S. platensis*, while a control group received a standard diet without microalgae added. Growth parameters, such as shell length and weight gain, were monitored, and immunity and antioxidant content were assessed. The results indicated no significant improvements in growth, immunity, or antioxidant content in abalone fed with *S. platensis*-supplemented diets. Different results may be observed with various concentrations of *S. platensis* and different animal species.
Key Words: abalone, antioxidant, dietary supplementation, growth, microalgae.

Introduction. Aquaculture is a rapidly expanding method of food production, serving as a dependable means to meet the growing human demand for high-quality animal protein (Lei et al 2024). In aquaculture practices, the sustainable growth and optimal health of farmed organisms, such as abalone, are critical factors for successful commercial production. Abalones, belonging to the genus *Haliotis*, are highly valued marine mollusks with substantial economic importance in the aquaculture industry (Cook 2014; Hernández-Casas et al 2023).

Nutrition plays a pivotal role in promoting growth and enhancing the overall well-being of aquatic species. Optimizing the nutritional composition of abalone feed becomes paramount to enhance growth, immunity, and overall health. Microalgae, particularly *Spirulina platensis*, a nutrient-rich microalga, has gained attention for its potential as a supplementary feed ingredient in aquaculture. Rich in proteins, vitamins, and antioxidants, *S. platensis* has demonstrated positive effects on the growth, immunity, and antioxidant status of various aquatic species (Chen et al 2016; Roohani et al 2019; Sheikhzadeh et al 2019a, b; Jin et al 2020a, b; Alagawany et al 2021; Nagappan et al 2021).

The improvement in the growth of fish by dietary *S. platensis* is also in line with increasing digestive enzyme activity in the intestine (Mohammadiazarm et al 2021; Faheem et al 2022). Moreover, dietary *S. platensis* has improved the intestinal health status of fish by increasing intestinal histomorphology and altering intestinal microbial communities (Ren et al 2022; Zhang et al 2024). The high protein content in *S. platensis*

also has the potential to be used as an alternative protein source to replace fish meal in aquafeed (Cao et al 2018; Jiang et al 2022). In addition, *S. platensis* can be considered a color enhancer due to its rich carotenoid content (Güroy et al 2012; Teimouri et al 2013; Ren et al 2021). A previous study reported that *S. platensis* affected the color of the abalone shell, changing it from yellow-brown to dark brown (Jin et al 2020b). This study aims to investigate the impacts of *S. platensis* supplementation in feed on the growth, immunity, and antioxidant profile of juvenile *Haliotis squamata*.

Material and Method

Experimental design and diet preparation. The entire research was conducted at the Aquaculture Laboratory, Research Center for Marine and Land Bio-Industry, National Research and Innovation Agency (BRIN), situated in Pemenang District, North Lombok Regency, West Nusa Tenggara, from 31st May 2022 to 22nd August 2022. A completely randomized design (CRD) was employed for this study, with four treatments applied in the first study and two treatments in the second study. The focal point of investigation in both studies was the variation in the quantity of *S. platensis* added to the feed for the test organism, specifically juvenile abalone (*H. squamata*).

The control feed used in both studies was devoid of any addition of *S. platensis*. The *S. platensis* utilized in the experiments was procured from PT Algaepark Indonesia Mandiri. In the first study, *S. platensis* was integrated into the abalone feed formulation at different levels: 0%, 2%, 4%, and 8% (Table 1). In the second study, *S. platensis* was added in two different amounts: 0% and 5% (Table 2).

For both studies, other essential raw materials included fish meal, shrimp head meal, soybean meal, and seaweed meal from *Gracilaria* sp., which served as protein sources. Soybean oil and cod liver oil were utilized as sources of fat. Wheat flour was chosen as the carbohydrate source for the juvenile abalone, and sodium alginate and carboxymethyl cellulose (CMC) were included as feed adhesives.

Before the feed manufacturing process, all raw materials in dry form were initially pulverized using a grinding machine. Subsequently, the raw materials were uniformly mixed and supplemented with cod liver and soybean oils. Once mixed, distilled water was added to the dough in a proportion of 40-50%. The homogeneous dough was then shaped into thin sheets with a thickness of approximately 2 mm, which were later molded into pellet flakes measuring 10 x 10 mm. The formed pellets were steamed for approximately 3 minutes to enhance their compactness. Following steaming, the pellets underwent drying in an oven at a temperature of 60ºC until fully dried. The resulting pellets were stored in a refrigerated cabinet at a temperature of 4ºC until ready for use as feed.

The juvenile abalones (*H. squamata*) utilized in this study were obtained from the Lombok Sea Cultivation Center, Ministry of Fisheries and Marine Affairs, located in West Lombok Regency, West Nusa Tenggara. Prior to their involvement in the research activities, the juvenile abalones were acclimatized to facilitate adaptation to the research environment. During the acclimatization process, the juvenile abalones were provided with ad-libitum fresh seaweed (*Gracilaria* sp.) as their feed.

In the first experiment, 240 juvenile abalones with an average weight of 6.46±0.01 grams and an average shell length of 34.90±0.22 mm were distributed across twelve experimental containers. In the second experiment, 120 juvenile abalones with an average weight of 5.81±0.01 grams and an average shell length of 34.51±0.19 mm were distributed across six experimental containers. The stocking density of juvenile abalones in each experimental container was 20 individuals, consistent across both the first and second experiments. The juvenile abalones were fed experimental feed, constituting 1.5% of the total biomass. The feeding regime was conducted over a 12-week maintenance period, with the experimental feed administered once daily in the afternoon for six days per week. Each feeding trial was replicated three times. Excess feed and feces in the experimental containers were removed daily using a siphoning method.

The experimental containers used in this research were plastic baskets with dimensions of 400 x 310 x 220 mm (length x width x height). The containers were lined with mesh at the bottom to prevent feed from falling into the experimental tank. Each

experimental container was equipped with a semi-circular pipe serving as a shelter for the juvenile abalones. All containers were placed within a concrete experimental tank measuring 50 x 55 x 300 cm (width x height x length). The experimental tank featured aeration to ensure oxygen availability for the juvenile abalones and a semi-closed seawater recirculation system with a circulation rate of 60 L per minute. Seawater was added to the experimental tank daily to replace any lost due to cleaning or siphoning, while a complete seawater exchange (100%) was performed once per week. Periodic recordings of seawater temperature and salinity in the experimental tank revealed temperature fluctuations between 26-29°C and salinity levels ranging from 30 to 35 ppm.

To assess the growth of the test organisms, the abalones were weighed every four weeks during the experimental period. The feeding trial was conducted over a period of 12 weeks. The abalones were fed the respective diets twice daily at 08:00 and 17:00, and the feed amount was adjusted based on their biomass and consumption rate.

Table 1

Diet formulation (g $100g^{-1}$) of abalone *H. squamata* in experiment 1

Ingredients	*0%*	*S-2%*	*S-4%*	*S-8%*
Fish meal	10	10	10	10
Shrimp head meal	7.5	7.5	7.5	7.5
Soybean meal	20	20	20	20
Spirulina platensis	0	2	4	8
Seaweed meal	23	23	23	23
Sodium alginate	6	6	6	6
Wheat flour	22	20	18	14
Cod liver oil	1.5	1.5	1.5	1.5
Soybean oil	1.5	1.5	1.5	1.5
Vitamin mixture[1]	3	3	3	3
Mineral mixture[2]	4	4	4	4
Carboxymethyl cellulose	1.5	1.5	1.5	1.5

[1]Vitamin mixture composition (unit kg^{-1}): vitamin A - 60,000,000 IU; vitamin D3 - 12,000,000 IU; vitamin E - 75,000 mg; vitamin K3 - 10,000 mg; thiamine - 10,000 mg; riboflavin - 30,000 mg; pyridoxine - 20,000 mg; cyanocobalamin - 100 mg; biotin - 100 mg; nicotinic acid - 5,000 mg; pantothenic acid - 54,000 mg; folic acid - 5,000 mg; [2]Mineral mixture composition (g $100\ g^{-1}$): NaCl - 1; $MgSO_4.7H_2O$ - 15; $NaH_2PO_4{\cdot}2H_2O$ - 25; KH_2PO_4 - 32; dicalcium phosphate - 20; $FeCl_3$ - 2.5; $ZnSO_4{\cdot}7H_2O$ - 0.4; Ca-lactate - 3.85; CuCl - 0.03; $AlCl_3.6H_2O$ - 0.01; $MnSO_4.H_2O$ - 0.2; $CoCl_2.6H_2O$ - 0.01.

Table 2

Diet formulation (g $100g^{-1}$) of abalone *H. squamata* in experiment 2

Ingredients	*CON*	*S-5%*
Fish meal	10	10
Meat and bone meal	6	6
Soybean meal	20	20
Spirulina platensis	0	5
Seaweed meal	23	23
Sodium alginate	7.5	7.5
Wheat flour	22	17
Cod liver oil	1.5	1.5
Soybean oil	1.5	1.5
Vitamin mixture[1]	3	3
Mineral mixture[2]	4	4
Carboxymethyl cellulose	1.5	1.5

[1]Vitamin mixture composition (unit kg^{-1}): vitamin A - 60,000,000 IU; vitamin D3 - 12,000,000 IU; vitamin E - 75,000 mg; vitamin K3 - 10,000 mg; thiamine - 10,000 mg; riboflavin - 30,000 mg; pyridoxine - 20,000 mg; cyanocobalamin - 100 mg; biotin - 100 mg; nicotinic acid - 5,000 mg; pantothenic acid - 54,000 mg; folic acid - 5,000 mg; [2]Mineral mixture composition (g $100g^{-1}$): NaCl - 1; $MgSO_4.7H_2O$ - 15; $NaH_2PO_4{\cdot}2H_2O$ - 25; KH_2PO_4 - 32; dicalcium phosphate - 20; $FeCl_3$ - 2.5; $ZnSO_4{\cdot}7H_2O$ - 0.4; Ca-lactate - 3.85; CuCl - 0.03; $AlCl_3.6H_2O$, 0.01; $MnSO_4.H_2O$, 0.2; $CoCl_2.6H_2O$ - 0.01.

Growth performance analysis. Throughout the feeding trial, growth parameters such as weight gain, shell length gain and specific growth rate were regularly measured. The growth data observed in this research adheres to the criteria outlined by Ansary et al (2019), encompassing:

a. Weight gain (WG):

$$\text{WG}\ (\%)\ =\ 100\ \text{x}\ \frac{\text{final weight} - \text{initial weight}}{\text{initial weight}}$$

b. Shell length gain (SL):

$$\text{SL}\ (\%)\ =\ 100\ \text{x}\ \frac{\text{final shell length} - \text{initial shell length}}{\text{initial shell length}}$$

c. Specific growth rate SGR):

$$\text{SGR}\ (\%\ \text{day}^{-1})\ =\ 100\ \text{x}\ \frac{\text{Ln final weight}\ -\ \text{Ln initial weight}}{\text{feeding days}}$$

d. Survival rate (SR):

$$\text{SR}\ (\%)\ =\ 100\ \text{x}\ \frac{\text{final number of abalone}}{\text{initial number of abalone}}$$

Antioxidant analysis. At the end of the feeding trial, representative abalone samples from each treatment group were collected for antioxidant content analysis. The whole-body tissues were homogenized, and the total antioxidant capacity (TAC) was determined using standard assays.

At the conclusion of the observations, five abalones from each maintenance container were randomly selected for antioxidant content analysis. The antioxidant content analysis followed the method established by Li et al (2006). The antioxidant content of *S. platensis* was analyzed using the ascorbic acid equivalent antioxidant capacity (AEAC) method. Antioxidant content was measured by determining the capacity of the free radical compound 2,2-diphenyl-1-picrylhydrazyl (DPPH).

To perform the analysis, 10 mg of extract was mixed with methanol p.a solution, and several concentration ratios were made, including 100, 200, 400, and 800 ppm. Then, 160 µL of extract from each sample concentration ratio was added to 40 µL of DPPH. The homogeneous mixture was kept in a dark place for 30 minutes at room temperature, and the absorbance was measured at a wavelength of 517 nm. The blank used was 200 µL of methanol p.a without DPPH, serving as a negative control. The inhibition percentage data obtained were used to determine the inhibition concentration 50 (IC50) value in units of ppm. The IC50 value was determined by probit analysis using Microsoft Excel 2007 software.

Immunity analysis. Following the initial and final weighing of the abalones, a random subset of six individuals from each group underwent testing for total hemocyte count. Hemolymph was extracted from the pedal sinus of the muscular foot of each abalone using a 1 mL sterile syringe equipped with a 23 G × 1 1/4 in. needle containing 250 µL PBS (Phosphate Buffer Saline 0.01 M, pH 7.4). The hemolymph was immediately placed on ice to inhibit cell clumping and agglutination. Total hemocyte counts (THCs) were conducted using a Neubauer hemocytometer (Erma Inc., Tokyo, Japan), with samples appropriately diluted in PBS. A 25 µL aliquot of hemolymph was dispensed onto the hemocytometer and enumerated under a microscope (Olympus CX43, Shinjuku-ku, Tokyo, Japan) with 40x magnification.

Data analysis. Body weight gain, shell width increment, and daily growth rate data were subjected to one-way analysis of variance (ANOVA), with post hoc Tukey's test conducted at a 95% confidence interval and a significance level set at $p < 0.05$. All statistical analyses were performed using SPSS 20 software (IBM® SPSS® Statistics, New York, USA).

Results

Growth performance. The growth of *H. squamata* abalones after being fed with test feed incorporating varying concentrations of *S. platensis* is presented in Table 3 for the first experiment and Table 4 for the second experiment. Throughout the observation period, the survival rate of abalones was exceptionally high (100%), with no abalone mortality observed during the maintenance period in both the first and second experiments.

Moreover, there were no significant differences ($p > 0.05$) in the final weight, weight gain, specific growth rate, final shell length, and shell length gain of abalones fed dietary *S. platensis* in either experiment 1 or experiment 2 after the 12-week feeding period.

Table 3

Growth of abalone *H. squamata* for 12 weeks in experiment 1

Parameters	*0%*	*S-2%*	*S-4%*	*S-8%*
Initial weight (g)	6.46±0.01	6.46±0.01	6.46±0.01	6.47±0.01
Final weight (g)	9.93±0.84	10.04±1.79	8.95±0.46	9.51±0.25
Weight gain (%)	53.78±13.25	55.40±27.56	38.49±6.85	46.98±3.70
SGR (% day^{-1})	0.52±0.10	0.53±0.21	0.40±0.06	0.47±0.03
Initial shell length (mm)	34.81±0.29	34.95±0.04	34.97±0.35	34.90±0.21
Final shell length (mm)	40.50±0.99	40.00±0.45	39.45±0.73	40.11±0.46
Shell length gain (%)	16.36±2.99	14.45±1.44	12.82±1.06	14.94±0.65
Survival rate (%)	100	100	100	100

Values presented as mean±SD (n = 3 tanks/diet). No significant differences were found among dietary treatments ($p > 0.05$). SGR = specific growth rate.

Table 4

Growth of abalone *H. squamata* for 12 weeks in experiment 2

Parameters	*CON*	*S-5%*
Initial weight (g)	5.81±0.01	5.81±0.01
Final weight (g)	9.10±0.56	9.46±0.72
Weight gain (%)	56.56±9.43	62.75±12.18
SGR (% day^{-1})	0.55±0.07	0.59±0.09
Initial shell length (mm)	34.40±0.12	34.63±0.19
Final shell length (mm)	39.41±0.46	40.28±0.56
Shell length gain (%)	14.55±1.67	16.32±1.01
Survival rate (%)	100	100

Values presented as mean±SD (n = 3 tanks/diet). No significant differences were found among dietary treatments ($p > 0.05$). SGR = specific growth rate.

Antioxidant status. In this study, we also investigated the effect of *S. platensis*-supplemented feeds on the percentage of abalone TAC. Abalones were fed with *S. platensis*-supplemented feeds at 0%, 2%, 4%, and 8%. The results were not statistically significant ($p > 0.05$) among all treatments (Table 5).

Table 5

TAC content of abalone *H. squamata* in experiment 1

Parameters	*0%*	*2%*	*4%*	*8%*
TAC (%)	24.22±1.15	23.29±5.31	23.08±4.71	25.59±8.51

Values presented as mean±SD (n = 3 tanks/diet). No significant differences were noticed among dietary treatment ($p > 0.05$).

Similar to the results of experiment 1, TAC values in experiment 2 also indicated no significant differences between both parameters at 0% and 5%, respectively (Table 6).

Table 6

TAC content of abalone *H. squamata* in experiment 2

Parameters	*CON*	*S-5%*
TAC (%)	32.51±4.76	22.70±2.42

Values presented as mean±SD (n = 3 tanks/diet). No significant differences were noticed among dietary treatment ($p > 0.05$).

Immune assay. As shown in Figure 1, the average (±SD) THC values exhibited variability across all experimental groups with distinct concentrations of *S. platensis* in the diet formulation of Experiment 1. No notable differences in THCs were evident at the initial time for all treatments. Nevertheless, when compared with the control group at the conclusion of the experimental period, a significant increase in THC was observed for the 2% concentration of *S. platensis* at approximately 330 $\times 10^4$ cells mL^{-1}). Conversely, a notable decrease was noted in the 4% concentration of *S. platensis* at over 200 $\times$ 10^4 cells mL^{-1}).

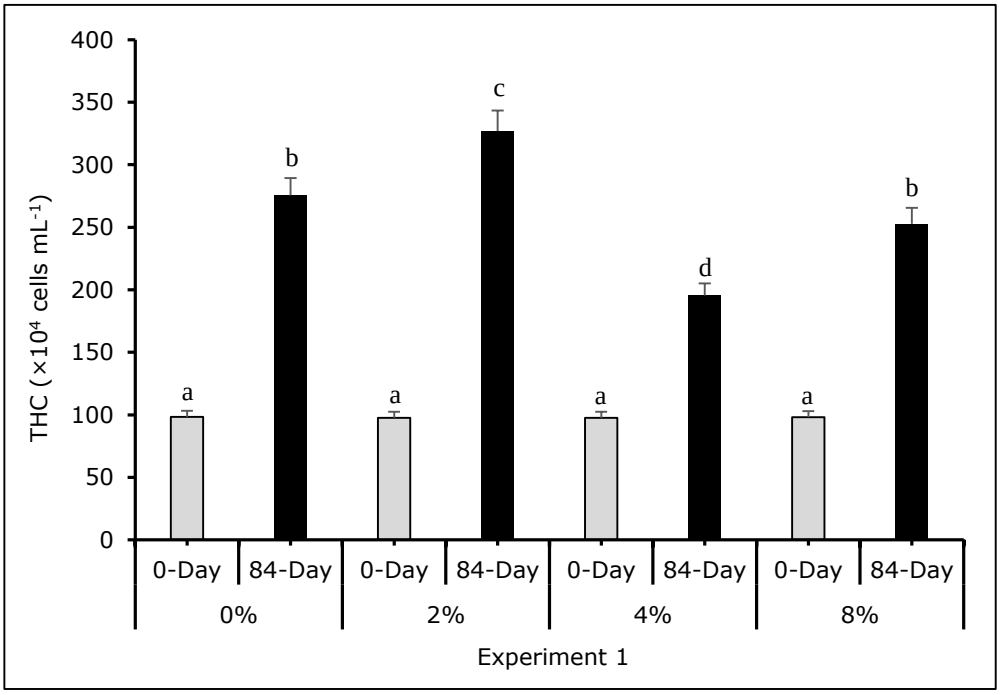

Figure 1. Effect of different concentrations of *S. platensis* in diet formulation of experiment 1 on total haemocytes count (THC) of juvenile *H. squamata* at the beginning and after 84 days experimental period time. Different letters indicate a significant difference ($p < 0.05$) between values.

Meanwhile, Figure 2 showed that there were no significant differences in THC values of juvenile *H. squamata* between the untreated control group and the treatment of diet formulation 2, which contained a 5% concentration of *S. platensis* during the experimental period.

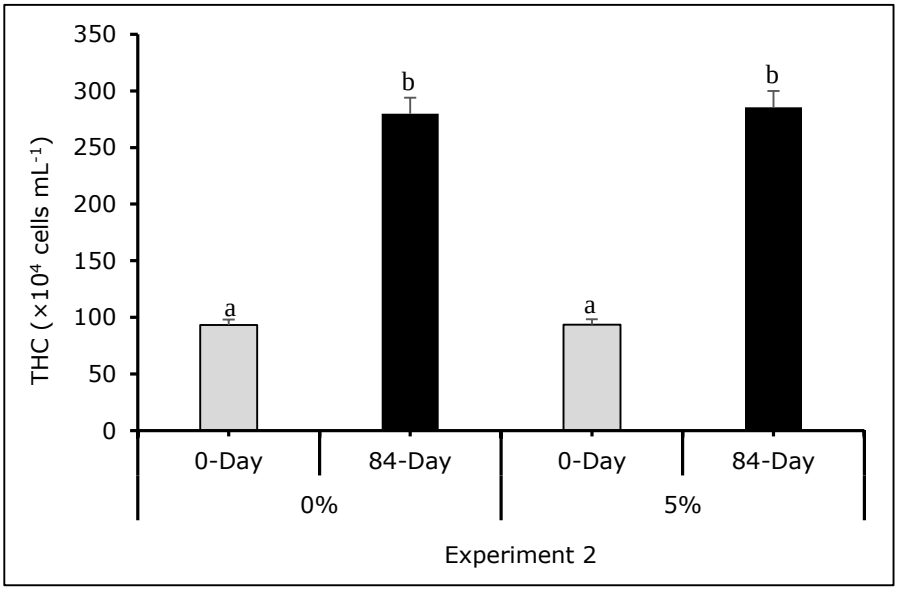

Figure 2. Effect of different concentrations of *S. platensis* in diet formulation of experiment 2 on total haemocytes count (THC) of juvenile *H. squamata* at the beginning and after 84 days experimental period time. Different letters indicate a significant difference ($p < 0.05$) between values.

Discussion. The results of this study showed that supplementation of *S. platensis* up to 8% did not affect the growth and shell performance of abalone. Similar to our study, different inclusion levels of *S. platensis* also did not result in significant differences in growth in gourami (*Trichopodus trichopterus*) (Khanzadeh et al 2016), yellow catfish (*Pelteobagrus fulvidraco*) (Liu et al 2019, 2020), rainbow trout (*Oncorhynchus mykiss*) (Sheikhzadeh et al 2019b), and blunt snout bream (*Megalobrama amblycephala*) (Jiang et al 2022). Although not statistically significant, the inclusion of 2% *S. platensis* showed the highest growth performance in abalone. The improvement in growth performance by dietary *S. platensis* could be attributed to its high protein, fatty acid, vitamin, and mineral content (Ibrahem et al 2013; Li et al 2022). Moreover, the enhancement of growth performance in fish by dietary *S. platensis* is also aligned with increased diet digestibility (Cao et al 2018; Ren et al 2021). In contrast, a high inclusion of dietary *S. platensis* tended to decrease the growth performance of abalone in this study. A decrease in growth performance with increasing dietary *S. platensis* has also been reported by Liu et al (2019) in yellow catfish. The variation in the effect of dietary *S. platensis* on growth is attributed to the different nutrient contents of *S. platensis* used in the study (Mohammadiazarm et al 2021).

The antioxidant activity of abalone feed was determined by analyzing the IC50 values of different concentrations of abalone feeds at 0%, 2%, 4%, 5%, and 8%, respectively. The results showed a dose-dependent antioxidant activity of abalone feed. By increasing the concentration of *S. platensis* in abalone feed, the IC50 values remained lower. When the abalone feed was treated with 0% *S. platensis*, the IC50 result was greater at 203.14 μg mL^{-1}. The strongest antioxidant activity was indicated by abalone feed at 8%, with an IC50 value of 54.69 μg mL^{-1}. These values were followed by abalone feed at 5%, 4%, and 2% with IC50 values of 99.64%, 105.28%, and 187.29%, respectively. All treatments illustrated higher IC50 values than the control, which was ascorbic acid with 7.91 μg mL^{-1} and *S. platensis* powder with 3.26 μg mL^{-1}. Based on the IC50 values of all abalone feed treatments, the higher the concentrations of *S. platensis* in abalone feed, the lower the IC50 values, indicating stronger antioxidant activity of the abalone feed.

Along with the analysis above, antioxidant contents were also analyzed in abalone treated with abalone feed containing different doses of *S. platensis*. The findings showed that after dietary treatment, there were no significant differences in antioxidant contents among all treated abalones.

THC values in abalone after treatment varied in experiment 1 (Figure 1); however, they remained relatively consistent in experiment 2 (Figure 2). These findings showed that different concentrations of microalgae in formulated feeds in both experiments 1 and 2 did not correlate with THC values. These results were not in agreement with previous studies that addressed the use of immunostimulant properties of microalgae as feed ingredients or additives, which have exhibited improved immune responses in THC, phagocytic, and lysozyme activities in fish (Ragap et al 2012; Nagappan et al 2021). While microalgal bioactive compounds are believed to improve immune health, further investigation is needed to determine the ideal dosage and timing, as no beneficial effects on abalone immunity were observed in this study.

Conclusions. This study showed that *Spirulina platensis* powder in supplement animal feeds has improved growth, antioxidant and immunity eventhough it was not significant. In the present study, we conducted abalone culture design to investigate the effects of *Spirulina platensis* powder-supplemented abalone feeds during culture periods of 60 days. Our results can be one of evidence reports that *Spirulina platensis* may be used as an alternative supplement in abalone feed during aquaculture.

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Conflict of interest. The authors declare that there is no conflict of interest.

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