Effect of dietary protein and lipid levels on growth performances and digestive enzymes activities in grouper *(Epinephelus coioides)*

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Abstract. This study investigates the effect of dietary protein and lipid levels on growth and digestive enzymes activities in grouper *(Epinephelus coioides)*. Furthermore, grouper juveniles (8.63±0.47 g) were separated into ten groups and cultivated in a 100-L tank. Five groups were fed in different protein levels of 35, 40, 45, 50, and 55% protein, while the remaining were fed in different lipid levels of 6, 8, 10, 12, and 14% lipid. All experimental diets were mixed with 1.0% *Sauropus androgynus* extract. The fishes were sampled for digestive enzyme activities at 1, 4, and 7 days. The results showed that fish receiving 55% protein affected the growth and increased the activities of protease, lipase, carboxypeptidase A (CPA), and carboxypeptidase B (CPB). Liver tissue included higher levels of lipase, as well as carboxypeptidase A and B, while digestive tract tissue contained measurable levels of protease. In conclusion, the administration of grouper juveniles with *S. androgynus* extract can affect growth performances and increase digestive enzyme activities.

1 Introduction

Fishes constitute the most varied groups of vertebrate animals concerning foods, digestibility, and nutritional contents [1, 2]. Moreover, the diversity of species and habitats caused fishes to consume different types of foods [3, 4], ranging from small to more significant organisms [5, 6]. This means many digestive mechanisms may exist in these aquatic creatures [7]. Fish's digestive processes are recognized to be qualitatively similar to those found in other vertebrates and mammals. However, the process is complex and comprises a scope of sensory, mechanical, and hormonal stimuli [8]. Even though some findings have been conducted on digestive processes, studies on digestive enzyme activities are still limited. The difficulty in studying this activity may be caused by several factors, including no uniformity

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in the tissue used for enzymatic activity analysis, nutritional status, and environmental factors such as substrates, temperature, and pH [9].

A study of fish digestive enzyme activity is vital to build and formulate a new feeding system, as well as answering various issues concerning nutritional physiology [10, 11, 12]. Several studies on digestive enzymes have been investigated in different species such as proteolytic and amylase activities in *Oncohinchus mykiss, Sparus aurata, Anguilla Anguilla, Cyprinus carpio, Carassius auratus*, and *Tinca tinca* [9]; proteolytic enzyme in *Solea senegalensis* [13]; trypsin, amylase, and lipase in prickleback fishes [7]; protease enzymes and free amino acid in *Salmo salar* [14], proteolytic activity and properties of proteins in *S. salar* [15], lipase activity in cichlids fishes [16] and protease and lipase enzyme in yellowfin tuna, skipjack tuna and tonggol tuna[17]. However, there is still rare or limited information about grouper digestive enzymes.

The application of plant-derived materials increases fish appetites [18, 19], and the active compounds such as linolenic acid, palmitic acid, benzoic acid, tocopherol, alkaloids, flavonoids, phenolics, terpenoids, steroids, and essential oils have characteristics to improve the immune system, anti-stress and appetite stimulation [18, 20, 21, 22]. This study used *Sauropus androgynus* extract as a feed additive to investigate the protease, lipase, carboxypeptidase A (CPA) and carboxypeptidase B (CPB) enzymes. This plant is primarily grown in South and Southeast Asia, and recent studies showed that supplementing *S. androgynus* in terrestrial animals' diets can affect growth performance, feeding efficiency, and egg production [23, 24]. Furthermore, *S. androgynus* is a high nutritive-value plant containing several essential materials such as multi-vitamin, metabolic compounds, essential oils, alkaloids and non-alkaloids, and active compounds [23, 25, 26].

Even though some trials proved that dietary *S. androgynus* can affect the growth performances in terrestrial animals due to beneficial substances in this plant, the efficacy in aquaculture is still unknown. Studies on digestive enzyme activities showed the capacity of fish to use protein and lipids. Therefore, they are conducted to investigate the effect of dietary different protein and lipid levels supplemented with *S. androgynus* extract in affecting growth and digestive enzyme activities in grouper (*Epinephelus coioides)*.

2 Materials and methods of research

Experimental Fish Preparation

For experiments on dietary protein and lipid levels, grouper *(E. coioides)* juveniles weighed 8.63±0.47 g. In addition, they weighed 35.73±3.67 g for experiments on digestive enzyme activities obtained from Aquatic Animal Center, National Taiwan Ocean University. They were acclimatized in the hatchery of the Department of Aquaculture for two weeks before experimentation. The fishes were reared and fed twice daily with commercial diets. They were then distributed into 100-L total water volume (60 x 50 x 35 cm), and the storage fiberglass provided well-aerated water. Water quality parameters were maintained at 30±1°C and 33±1 ppt salinity.

Experimental Design

For experiments on dietary different protein and lipid levels, fish were reared in triplicate in 30 tanks (100-L each) at 25 fish/tank and cultured for 56 days. Fish were hand-fed at 3% of total body weight. In different protein level trials, fish were divided into five groups: juveniles were offered diet with 35% (P35), 40% (P40), 45% (P45), 50% (P50) and 55% (P55) protein of total ingredients (Table 1); whereas, in different lipid trials, fish were divided into five groups: juveniles were offered diets with 6% (L6), 8% (L8), 10% (L10), 12% (L12) and 14% (L14) lipid of total ingredients (Table 2).

Table 1. Composition of experimental diets in different protein levels

Treatments	**P 35**	**P 40**	**P 45**	**P 50**	**P 55**
Protein levels (%)	35	40	45	50	55
Ingredients (*g 100^{-1}*) Fish meal	54	62	70	77	83
Fish oil	5	4	3	3	2
α-starch	6	6	6	6	6
Vitamin mix [a]	2	2	2	2	2
Mineral mix [b]	3	3	3	3	3
Vitamin C	0.05	0.05	0.05	0.05	0.05
Choline chloride	0.5	0.5	0.5	0.5	0.5
Carboxymethylcellulose, CMC	2	2	2	2	2
S. androgynus extract [c]	1	1	1	1	1
Cellulose	26.45	19.45	12.45	5.45	0.45

Table 2. Composition of experimental diets in different lipid levels

Treatments	**L 6**	**L 8**	**L 10**	**L 12**	**L 14**
Lipid levels (%)	6	8	10	12	14
Ingredients (*g 100^{-1}*) Fish meal	70	70	70	70	70
Fish oil	0	1	3	5	7
α-starch	6	6	6	6	6
Vitamin mix [a]	2	2	2	2	2
Mineral mix [b]	3	3	3	3	3
Vitamin C	0.05	0.05	0.05	0.05	0.05
Choline chloride	0.5	0.5	0.5	0.5	0.5
Carboxymethylcellulose, CMC	2	2	2	2	2
S. androgynus extract [c]	1	1	1	1	1
Cellulose	15.45	14.45	12.45	10.45	8.45

Data on growth rate was recorded regularly every 2 weeks. The body wet weight was measured using an analytical balance and the total length using a digital caliper. Growth performances were calculated as follows: weight gain (WG, %) = 100 x [(final weight (g) – initial weight (g)] / initial weight (g); specific growth rate (SGR, %/day) = 100 x (ln*W2* – ln*W1*) / *T*; where: *W1* and *W2* are initial weight and final weight, respectively and *T* is the number of days in the feeding periods [27]; and condition factor (K) = [(105 x weight of fish (g) / (length of fish)^3(cm)] [28]. In the experiment on survival rate, all treatments were observed daily and the data was calculated by the following formula: survival rate (SR, %) = (final no. of fish / initial no. of fish) x 100 [29]. This experiment was conducted using a

recirculation water system. Water was provided from storage tanks, filtered, and supplied to the system. The water system was equipped with a mechanical filter (spongy), UV light, and automatic heater, and supplied with compressed air via air- stones from air pumps. Water flows in experimental aquaria were measured and adjusted before the experiment to be proportional to the fish density and to ensure sufficient water circulation.

On the other hand, for the experiment on digestive enzyme activities, the experimental facility was composed of 6 tanks at 10 fish/tank. During the experiment, groupers were randomly distributed into the cultivating system in triplicate. The feeding trial was divided into two treatment groups: Control group (C) were fed the diet without *S. androgynus* extract and treated group (T) was fed the diets mixed with 1.0% of *S. androgynus* extract. Fish were hand fed at ad libitum twice daily (08:00 and 17:00) and all fish received their respective diets for 7 days. Sampling was examined at 1, 4, and 7 days after feeding. Three fish of each treatment were sacrificed for the assay of digestive enzyme activities.

Dietary Preparation

Fresh *S. androgynus* leaves were collected from Bengkulu, Indonesia. The leaves were cleaned and shadow-dried for three days, and after drying, all specimens were ground using an electrical blender. The specimen was filtered using 20 µm mesh to obtain the powder extracted using 70% ethanol in the soxhlet apparatus. This extraction was operated with gently shaking at room temperature for 24 h [30]. The obtained extract was filtered and condensed using a rotary vacuum evaporator at 45°C. *S. androgynus* extract was prepared at a concentration of 1.0% of the total ingredients before mixing into experimental diets. In addition, the ingredients of each diet were mixed for 30 min for pelletization. All experimental diets were administered with 1.0% *S. androgynus* extract from the total ingredients. For the experiment on digestive enzyme activities, the control (C) diet was treated similarly to the supplemented diets, but no extract was added. The diets were then dried in an incubator at 45°C for 24 h. After drying, the pellets were put into plastic bags and stored at 4°C for further use.

Sample Preparation

Protease and lipase activities were determined in the liver and digestive tract of experimental fish. For protease activity, samples were prepared following the method described by [9] with minor modifications. Briefly, 100 mg of liver and digestive tract tissues were isolated from each experimental fish. Then, the tissues were homogenized using 1 ml distilled water. Homogenate was then centrifuged at 30,000 x *g* for 30 min at 4°C. For lipase activity, samples were prepared following the manufacturer's instructions. Briefly, 40 mg of tissues were homogenized using lipase assay buffer and centrifuged at 13,000 x *g* for 10 min to remove insoluble materials and the supernatant was collected for further analysis.

For carboxypeptidase A, 100 mg tissues were isolated from each experimental fish. Then, the tissues were homogenized using 1 ml distilled water and then centrifuged at 30,000 x *g* for 30 min at 4°C. Whereas, for carboxypeptidase B activity, 100 mg samples were rinsed with 1x Phosphate Buffered Saline (PBS) solution, then, homogenized in 1 ml of 1x PBS and stored overnight at -20°C. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 min at 5000 x *g* at 4°C. The supernatant was removed and assayed immediately.

Measurements of the digestive enzyme activities

The protease enzyme assay was conducted using the Protease Fluorescent Detection Kit (Sigma-Aldrich Inc) following the manufacturer's instructions. In brief, 20 µl of incubation buffer, 20 µl of FITC-casein substrate, and 10 µl of the samples were mixed in a microcentrifuge tube. Control samples were prepared by adding 20 µl of incubation buffer, 20 µl of FITC-casein substrate, and 10 µl of the control sample to a tube. For the blank sample, preparation was performed by adding 20 µl of incubation buffer, 20 µl of FITC-casein substrate, and 10 µl of distilled water to the tube. All tubes were mixed gently and

incubated at 37°C in the dark for 60 min. After incubation, 150 µl of the 0.6 N TCA was added to each tube and incubated at 37°C in the dark for 30 min. Then, centrifuge the tubes for 10 min at 10,000 x *g*. Then, the supernatant was used for the fluorescence measurement.

Analysis of lipase activity was assayed using Lipase Activity Assay Kit (Sigma-Aldrich Inc) following the manufacturer's instructions. Briefly, 20 µl of samples were mixed with 100 µl of reaction mix in each of the wells. The solutions were then incubated at 37°C for 3 min and measured the absorbance at 570 nm (A570 initial). Then, continued to incubate the plate at 37°C, and then the absorbance was measured every 5 min. During incubation, the plate was protected from light. The measurement was conducted until the value of the most active sample was greater than the value of the highest standard (10 nmole/well). The final absorbance measurement (A570 final) for calculating the enzyme activity was the penultimate reading or the value before the most active sample is near or exceeds the end of the linear range of the standard curve.

Carboxypeptidase A was assayed using Carboxypeptidase A Assay Kit following the manufacturer's instructions. The analysis was conducted by preparing a blank control by adding 100 µl of ultrapure water (18.2 MΩ.cm) into the wells; and then prepared a test sample reaction by adding 50 µl of sample to the appropriate wells and adjust the volume to 100 µl with ultrapure water. For positive control, it was prepared by adding 2 µl of the carboxypeptidase A control enzyme to the wells, and the volume was adjusted to 100 µl with ultrapure water. Whereas for the control for inhibitor, the reaction was prepared by adding 2 µl of the carboxypeptidase A control enzyme and 1 µl of carboxypeptidase inhibitor to the wells, then adjusting the total volume to 100 µl with ultrapure water. Start the reaction by the addition of 100 µl of the reaction mixture to each well, and incubate the plate for 5 min at 25°C.

For carboxypeptidase B, the assay was conducted using Carboxypeptidase B ELISA Kit following the manufacturer's instructions. Briefly, 100 µl of the sample was added to the wells and incubated for 2 h at 37°C. Then, the liquid was removed. 100 µl of Biotin-antibody (1x) was added to each well and incubated for 1 h at 37°C. Aspirated each well and washed. Each well was washed using Wash Buffer (200 µl) using multichannel pipette and let it stand for 2 min. 100 µ l of HRP-avidin (1x) was added to each well and incubated for 1 h at 37°C. Repeat the wash process for 5 times. After that, 90 µl of TMB Substrate was added to each well and incubated for 15-30 min at 37°C.

3 Results

Fish Growth Performances

Fish growth was significantly affected by different levels of protein and lipid contents in experimental diets. A summary of growth responses, conditional factors, and feeding performances of grouper after feeding experimental diets at different protein levels in each trial is provided in Table 3. Meanwhile, growth responses, conditional factors, and feeding performances of grouper after feeding experimental diets in different lipid levels are provided in Table 4.

Table 3. Growth responses, conditional factor, and feeding performances of grouper after feeding experimental diets in different protein levels

Protein level	Length (cm)		Weight (g)		WG (%)	SGR (%)	K	FI (g)	FCR
(%)	Initial	Final	Initial	Final					
P 35	7.75±0.38	10.74±0.34^{c}	8.63±0.47	17.13±2.08^{d}	98±10	1.22±0.16	1.38±0.04	14.45±0.90	1.84±0.16

P 40	7.75±0.38	11.03±0.26	8.63±0.47	19.42±1.32	125±12	1.45±0.12	1.45±0.03	17.25±0.56	1.64±0.10
P 45	7.75±0.38	11.44±0.30	8.63±0.47	22.17±1.52	157±13	1.68±0.13	1.48±0.03	17.05±0.73	1.28±0.17
P 50	7.75±0.38	11.84±0.64	8.63±0.47	26.15±2.45	203±12	1.97±0.17	1.58±0.04	15.53±0.80	0.90±0.10
P 55	7.75±0.38	12.73±0.54	8.63±0.47	32.08±1.91	272±23	2.34±0.11	1.57±0.03	18.01±1.40	0.77±0.11

Table 4. Growth responses, conditional factors, and feeding performances of grouper after feeding experimental diets in different lipid levels

Lipid level (%)	Length (cm)		Weight (g)		WG (%)	SGR (%)	K	FI (g)	FCR
	Initial	Final	Initial	Final					
L 6	7.75±0.38	11.16±0.38	8.63±0.47	19.52±1.39	126±16	1.46±0.13	1.40±0.04	11.78±1.00	1.72±0.15
L 8	7.75±0.38	11.36±0.46	8.63±0.47	21.31±2.00	147±18	1.61±0.17	1.45±0.03	15.02±0.61	1.21±0.14
L 10	7.75±0.38	11.40±0.56	8.63±0.47	22.03±2.89	155±22	1.66±0.19	1.48±0.02	13.85±1.40	1.13±0.21
L 12	7.75±0.38	11.66±0.62	8.63±0.47	22.93±1.23	166±18	1.74±0.09	1.45±0.10	15.80±1.10	1.15±0.12
L 14	7.75±0.38	12.76±0.73	8.63±0.47	30.19±2.18	250±27	2.23±0.13	1.47±0.10	18.53±1.30	0.88±0.12

The feeding trials on different protein levels (P) showed the highest growth performances in the group with high protein contents (P 55). It showed a significant difference ($P < 0.05$) compared to other groups, with an average final body weight of 32.08±1.91 g and the final length of 12.73±0.54 cm. In the feeding trials on different lipid levels (L), group L 14 showed the best rate, where the juveniles received extract mixed diets containing 14% lipid. The mean final body weight and length were 30.19±2.18 g and 12.76±0.73 cm, respectively. Among treatment groups, the lowest fish growth performances occurred at P 35 with the mean final body weight and length of 17.13±2.08 and 10.74±0.34, where the juveniles received extract mixed diets containing 35% protein.

All groups increased their weight and length steadily every week during the whole experimental period. However, both P-treatments (Fig.1 and Fig.2) and L-treatments (Fig.5 and Fig.6) showed the most significant weight increments, where the highest gain (272%±23%) in P-treatments were attained by group P 55. In L-treatments, the highest weight gain was seen in group L-14 which reached 250%±27% after 8 weeks of rearing. In terms of specific growth rate (SGR), fish that received experimental diets with different protein levels tended to decrease the SGR percentage since week 6 (Fig.3). Meanwhile, those that received experimental diets with different lipid levels increased the SGR percentage until the end of the experiment (Fig.7). No statistical differences were observed on condition factor (K) during the 8-weeks rearing (Table 4).

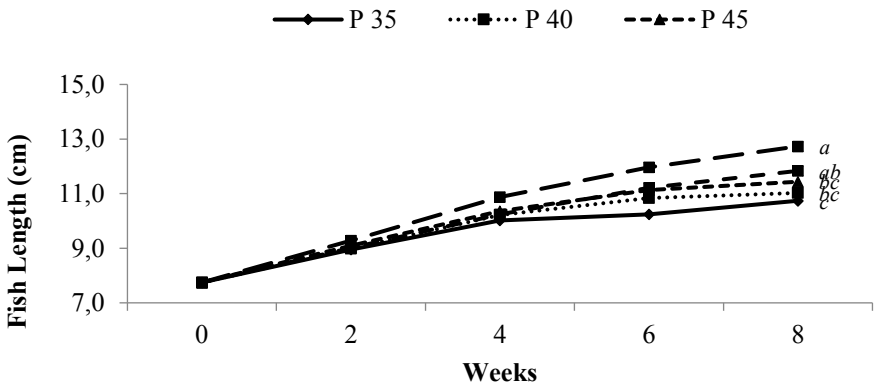

Fig. 1. Fish length after feeding experimental diets in different protein levels.

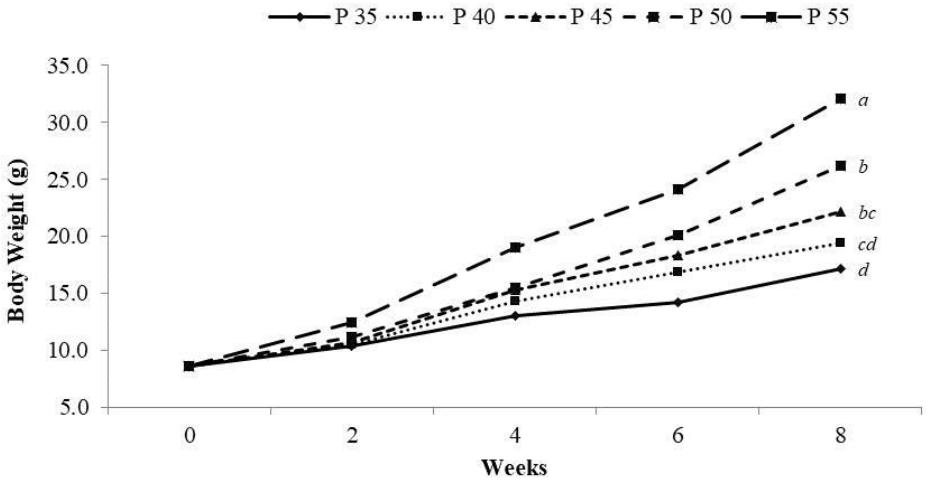

Fig. 2. Fish weight after feeding experimental diets in different protein levels.

The poorest fish growth was obtained in group P 35, with the percentage of weight gain and specific growth rate of 98%±10% and 1.22%±0.16%/day, respectively. In P-treatments, at the second measurement (4 weeks), the mean SGR was 2.05%/day (ranged from 1.47% to 2.81%), then decreased steadily to 1.73%/day (ranged from 1.22 to 2.34%) after 8 weeks. In L-treatments, the mean SGR value increased from 1.24%/day (ranged from 0.64 to 2.01%) to 1.74%/day (ranged from 1.46 to 2.23%) at the end of the experiment. Furthermore, the mean WG increased from 28% (ranged from 20 to 44%) to 171% (ranged from 98 to 272%) in P-treatments; and from 12% (ranged from 9 to 22%) to 169% (ranged from 126 to 250%) in L-treatments, respectively. The results on the WGs and SGRs indicated that fish fed with *S. androgynus* extract mixed diets enhance the overall relative growth rate in grouper *(E. coioides)* juveniles.

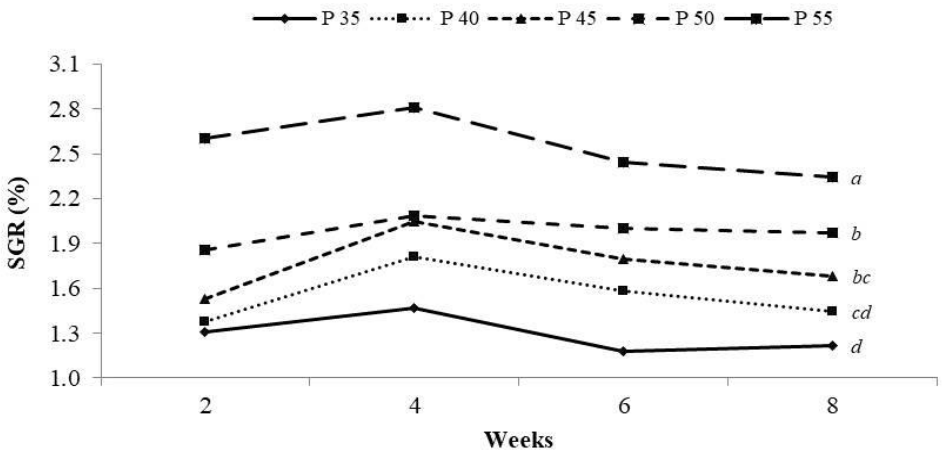

Fig.3. Specific growth rate of grouper after feeding experimental diets in different protein levels.

Feeding Performances

Feeding parameters such as feed intake (FI) and conversion ratio (FCR) were significantly affected by *S. androgynus* extract mixed diets in different protein (P) and lipid (L) levels. Fish cultured in P-treatments consume more feed than those in L-treatments . However, from both treatments (different protein and lipid levels), the highest FI was found at L 14 (18.53±1.30 g), while the lowest was at L 6 (11.78±1.00 g) (Table 4). This study also found that, in P-treatments, the best feed utilization was obtained by P 55, with FCR showing a significantly different ($P < 0.05$) compared to P 35 – P 45 (Fig.4), with the mean FCR being 0.77±0.11. Similarly, group L 14 showed the best feeding activity (Fig.8), with a mean FCR of 0.88±0.12 (Table 4). In both treatments (different protein and lipid levels), the total FCR values were lower in P-treatments than in L-treatments. Data analyses found that the best FCR was obtained by P 55 at 0.77±0.11, while the poorest was seen at P 35 and 1.84±0.16 (Table 3).

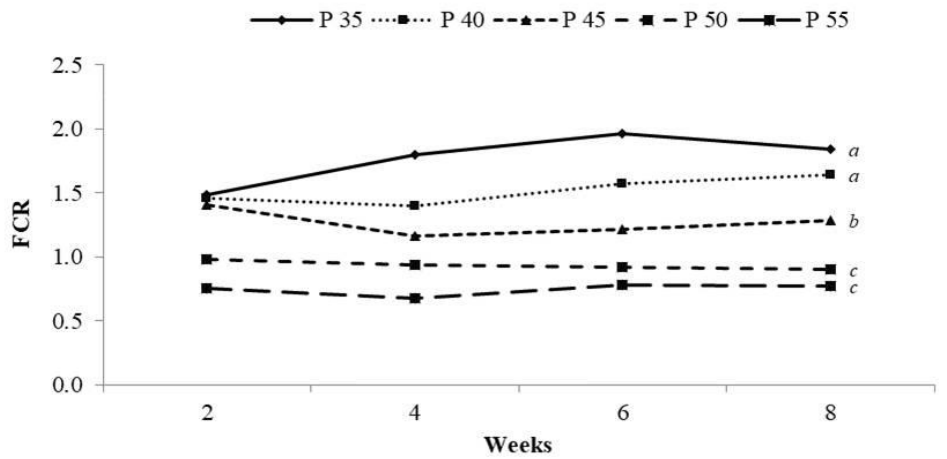

Fig.4. Feed conversion ratio of grouper after feeding experimental diets in different protein levels.

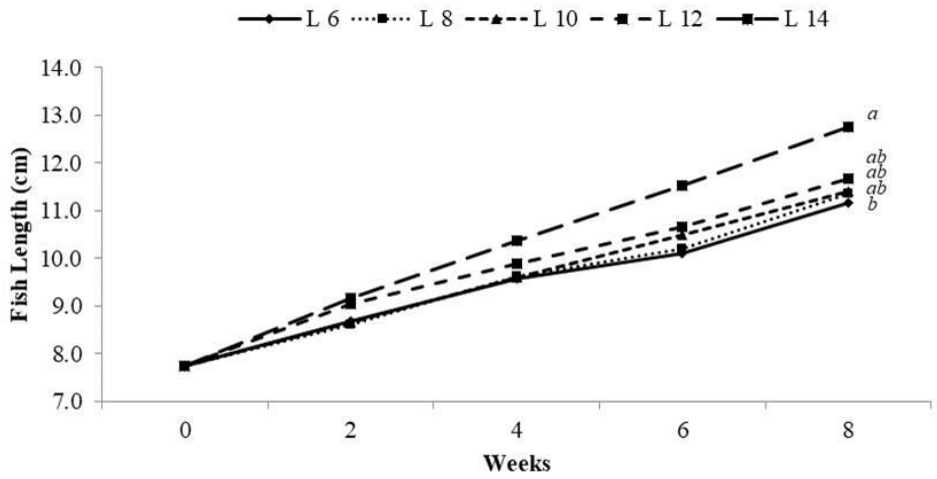

Fig.5. Fish length after feeding experimental diets in different lipid levels. Values are

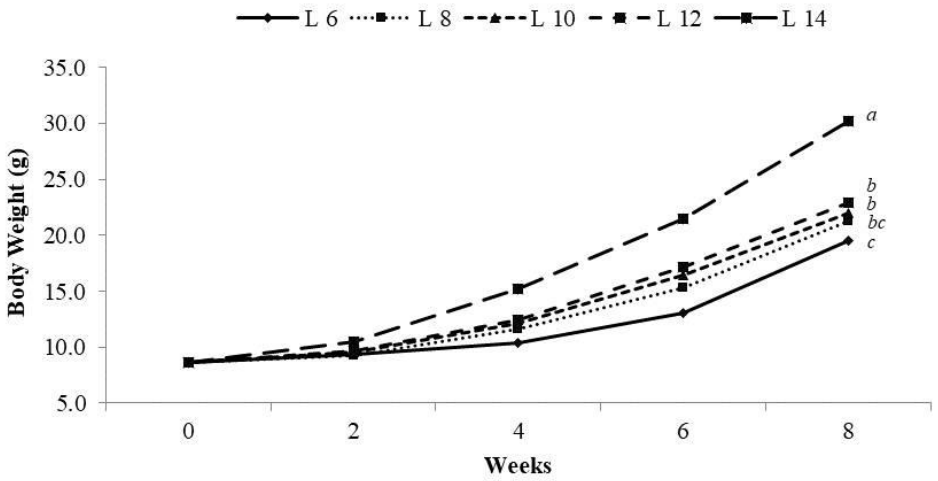

Fig.6. Fish weight after feeding experimental diets in different lipid levels.

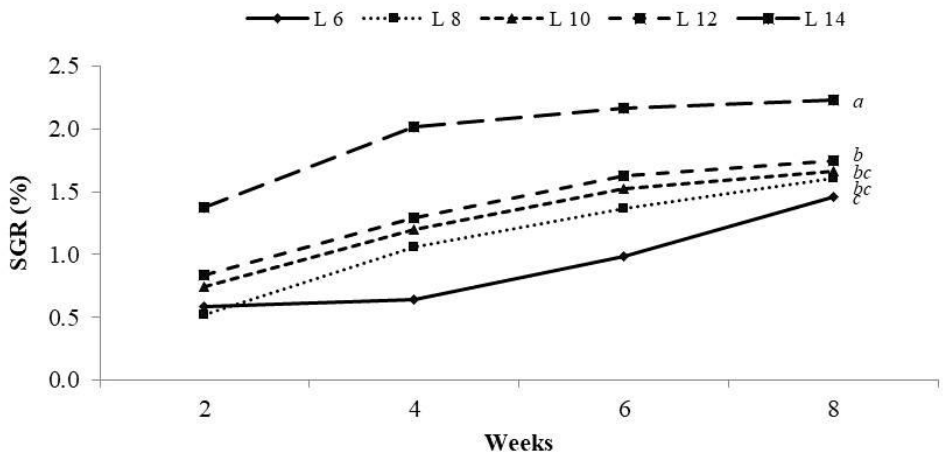

Fig.7. Specific growth rate of grouper after feeding experimental diets in different lipid levels.

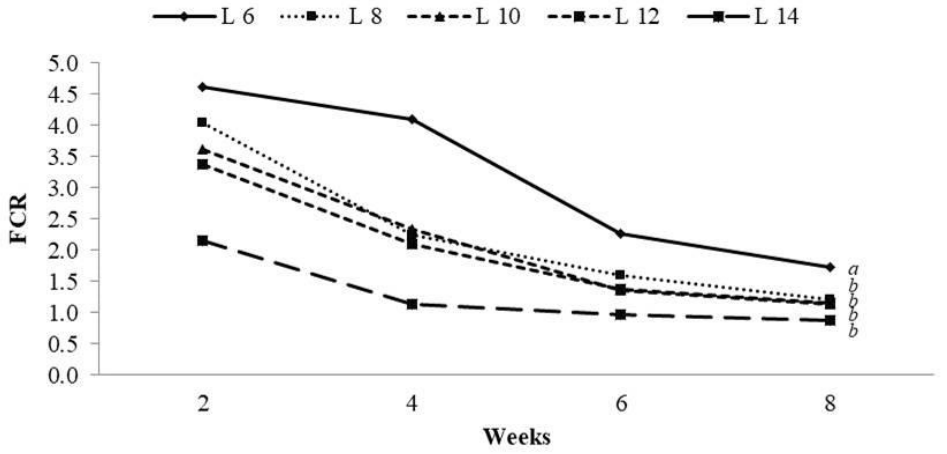

Fig.8. Feed conversion ratio of grouper after feeding experimental diets in different lipid levels.

Enzymatic Activities

In this present study, lipase activity in the liver of treated fish (TLV: groups that received *S. androgynus* extract mixed diets) was higher than in the control group (CLV). In the TLV group, lipase activity increased steadily and reached a peak at day 7, with a mean value of 28.85±0.002 milliunits/ml. On the other hand, lipase activity in the digestive tract of treated fish (TDT) was seen to be lower than in the control group (CDT).

Protease activity in the liver and digestive tract of the treated group (TLV and TDT) was higher than the control. Protease activity in the TLV group peaked (mean value: 9.53±0.02 Units/ml) on the second sampling date, followed by a gradual decline afterward. In contrast, protease activity continuously increased in the digestive tract of the TDT group, with the

highest value at the end of sampling time (13.38±0.03 Units/ml).

CPA analysis shows that only liver tissue exhibited its activity. This trial demonstrated that both treated groups (TLV and TDT) showed lower CPA than the control. Overall, CPA was higher in the control group, with the highest values of 3.15±0.00 Units/ml in CLV, while the lowest was in TDT (0.04±0.00 Units/ml). CPB activity is also expressed in the liver tissues but slightly differs from CPA. The trials found that CPB activity was higher in both treated groups, and even though CPB of CLV was higher than CDT and TDT, the CPB of TLV was always higher than CLV. The same result also can be seen in the digestive tract trial group, while CPB activity in TDT was higher than CDT on days 4 and 7. From this trial, the highest CPB activity was seen in the TLV group at day 7 (1.65±0.00 Units/ml), while the lowest CPB was seen in CDT at day 7 (0.18±0.00 Units/ml).

4 Conclusions

In conclusion, *S. androgynus* extract showed the capability to modulate the growth performances and digestive enzyme activities such as protease, lipase, and carboxypeptidase B in *E. coioides* juvenile.

Therefore, this study establishes that the recommended dosage of this species is 45 - 55% protein source or 10 - 14% supplemented with 1.0% of *S. androgynus* extract in diets. Further investigation is needed to determine the effects of growth performance and digestive enzyme activities of *E. coioides* on the protein or lipid ingredients in a long-term feeding trial.

Thanks.
Authors expresses sincere gratitude to all parties who have aided in the completion of this research.

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