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Effect of Garlic Powder on Enhancing Growth Performance and Health Status of Hybrid Grouper (*Epinephelus fuscoguttatus* × *E. lanceolatus*) Fingerlings During the Nursery Phase

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ABSTRACT

This study evaluated the impact of dietary garlic powder on growth performance and health status in hybrid grouper (*Epinephelus fuscoguttatus* × *E. lanceolatus*) fingerlings during the nursery phase over nine weeks. Five isonitrogenous, isolipidic diets (45% protein, 12% lipid) were prepared: Control (0%), 1.5%, 3%, 4.5%, and 6% garlic. Four hundred fish (initial weight 14.31 ± 0.08 g) were distributed into 20 tanks (1000 L; 20 fish/tank; four replicates). Fish were fed twice daily to satiation, six days a week. No significant differences were observed among the groups for growth performance and the feed efficiency parameters evaluated. Nevertheless, the 3% garlic-supplemented group exhibited numerically better feed conversion ratios (1.31), protein efficiency ratios (1.70), protein productive value (103.92), and specific growth rates (2.32) compared to other treatments. Histological analysis revealed enhanced intestinal morphology at 3% garlic inclusion, whereas higher levels of garlic (4.5% and 6%) reduced villi dimensions and mucosal thickness. The digestive enzyme analysis revealed significantly higher intestinal amylase activity levels in the control group compared to the 3% and 6% garlic-supplemented groups, whereas lipase activity declined with increasing garlic inclusion. In conclusion, the addition of 3% garlic powder improved growth and intestinal structure in hybrid grouper fingerlings without adversely affecting digestive enzyme activity, supporting its moderate use in nursery-stage diets.

INTRODUCTION

Groupers are high-value finfish species in Southeast Asia, including Malaysia. They are currently being grown industrially in Asian countries because they are characterized by a high growth performance, the best feed conversion, and a good fillet quality in addition to a high market price, which has led to high demand in both the local and international markets **(Noor *et al.*, 2019)**. The development of markets for live fish in Hong Kong and China led to an increase in demand for grouper fish species, including hybrid grouper **(Rimmer *et al.*, 2004)**. The production of hybrid grouper was initiated by the Borneo Marine Research Institute, University Malaysia Sabah, as a result of the fertilization of tiger grouper (*Epinephelus fuscoguttatus*) eggs with giant grouper (*E. lanceolatus*) sperm in 2007 **(Ch'ng & Senoo, 2008; Kawamura *et al.*, 2015)**. The objectives of hybridization in aquatic animals are to provide rapid growth leading to a short growth cycle, increase resistance to diseases, improve flesh quality, regulate the sex ratio, and enhance tolerance to environmental influences **(Ch'ng & Senoo, 2008)**. However, hybrid grouper, as well as other groupers, have a high mortality rate that can reach 98% in the larvae-rearing stage due to lack of feed, environmental factors or the presence of diseases, which affects the development of the groupers' farming industry **(Kato *et al.*, 2004; Ch'ng & Senoo, 2008; Ambariyanto *et al.*, 2013; Chu & Sheen, 2016)**.

Considering these challenges, many studies have attempted to enhance survival during the nursery phase, either by using antibiotics or vaccines; however, mortality remains high **(Ching *et al.*, 2012; Ambariyanto *et al.*, 2013; Mabroke *et al.*, 2021)**. As global demand for aquatic animal products continues to grow, the intensification of fish farming has been accompanied by a rise in infectious diseases, threatening the sustainability and resilience of aquaculture systems **(Xu *et al.*, 2020)**. In response, antibiotics have been widely used for disease prevention and treatment in aquaculture **(Cabello, 2006; Laxminarayan *et al.*, 2013; Choi *et al.*, 2014; Rafraf *et al.*, 2016)**. This practice, however, poses significant risks to human health through the development of antimicrobial resistance, the presence of residues in fish products, and negative impacts on aquatic ecosystems **(Xu *et al.*, 2020)**. Therefore, there is a pressing need to explore safe and natural alternatives that not only enhance growth performance and fish health but also contribute to maintaining water quality and ensuring the sustainability of the aquaculture ecosystem **(Xu *et al.*, 2020)**.

Medicinal plants are plants that exert beneficial pharmacological effects due to the presence of bioactive compounds such as alkaloids, flavonoids, terpenoids, glycosides, tannins and essential oils. These plants offer great potential as natural and sustainable feed additives in aquafeed. They are easy to incorporate into diets, supported by a stable market supply, and provide multiple benefits, including enhanced growth, improved immune response, and antibacterial effects, while remaining safe for aquatic animals and human consumers **(Chesti & Chauhan, 2018; Xu *et al.*, 2020)**.

Garlic is one of the most beneficial medicinal plants, widely cultivated around the world and is economically viable. It has been used in alternative medicine and as a functional food to promote human health **(Chesti & Chauhan, 2018)**. Numerous researchers have studied the impact of garlic on aquatic animals, as reported by **Büyükdeveci *et al.* (2018)**, who found that garlic extract has a positive effect on growth performance and intestinal microorganisms of the rainbow trout. Both **Chesti and Chauhan (2018)** and **Xu *et al.* (2020)** demonstrated that garlic powder supplementation enhances fish growth and digestive performance. In Amur carp, the inclusion of 1.5% garlic powder promoted the best growth parameters, while in juvenile Japanese seabass, adding 10g/ kg garlic powder improved digestive enzyme activities and growth performance.

Moreover, garlic has been shown to play a significant role in enhancing fish health across various species. It increases survival rate and antioxidant activity **(Metwally, 2009; Yousefi *et al.*, 2020)**, positively influences hematological and biochemical parameters in sea bass **(Yılmaz & Ergün, 2012)**, and improves immune responses **(Esmaeili *et al.*, 2017; Xu *et al.*, 2020)**. Garlic supplementation also provides protection against a range of pathogens, including *Photobacterium damselae* spp. *Piscicida* & *Streptococcus iniae* in Cobia, *Aeromonas hydrophila* in rainbow trout, *Lernantropus kroyeri* in European sea bass, *Yersinia ruckeri* in brown trout and *Vibrio harveyi* in Asian sea bass **(Nya & Austin, 2009; Talpur & Ikhwanuddin, 2012; Guo *et al.*, 2015; Zaefarian *et al.*, 2017; Yavuzcan Yildiz *et al.*, 2019)**. In addition, garlic has been associated with enhanced gut health and disease resistance in the Nile tilapia **(Abu-Elala *et al.* 2016)**, improved blood parameters in common carp **(Pashaki *et al.*, 2018)** and improved feed utilization and survival rate of red tilapia **(Samson, 2019)**.

This study aims to evaluate the effects of dietary garlic powder on the growth performance, digestive enzyme activities, and intestinal histology of hybrid grouper fingerlings during the nursery phase. To the best of our knowledge, this is the first study to investigate these effects in hybrid grouper within the body weight range of 12– 100g.

MATERIALS AND METHODS

Experimental site

This study was conducted at the Department of Fisheries Bukit Malut Nursery Incubator, Kuah, Langkawi, Kedah, Malaysia.

Hybrid grouper rearing

Hybrid grouper fry at 40 days post-larvae (PL40), weighing 2- 4g and measuring 5-7cm in length, were purchased from Sea Bass Enterprise Nursery, Kota Putera, Besut, Terengganu, Malaysia. The hybrid grouper fry had been reared for 2 months in 15 fiberglass tanks ($3m^3$ capacity each) containing seawater with a salinity of 24– 26ppt, temperature ranging from 26– 29°C, dissolved oxygen levels maintained at 6– 7mg/ L, and pH values ranging from 7.5 to 8.0, all within the optimal range for growth and health. The photoperiod was maintained at 12 hours light and 12 hours dark, throughout the experimental period to simulate natural daylight conditions. Fish were fed a commercial diet (CP company) containing 43% protein and 6% lipid, three to four times daily, until the feeding trial commenced. Before sampling, fish were allowed to acclimatize under experimental conditions for an appropriate period to reduce handling stress. The study was conducted in accordance with approval from the animal ethics committee, USM/IACUC/2021/(129)(1148).

Experimental design and diet preparation

Five experimental diets containing different levels of garlic powder were formulated (Table 1), as follows: the control diet was free of garlic powder, 1.5% garlic diet, 3% garlic diet, 4.5% garlic diet and 6% garlic diet. All feed ingredients used for diet formulation were obtained from the local market through Sri Purta Trading Sdn. Bhd. (Alor Setar, Kedah, Malaysia) and Take It Global Sdn. Bhd. (Pulau Pinang, Malaysia). Five isocaloric, isonitrogenous diets with 45% crude protein and 12% crude lipid were prepared by mixing the feed ingredients in a mixer, followed by pelletization, which produced homogeneous-sized pellets. The pellets were dried using a fan and then stored at -4°C until use.

Table 1. Formulation and proximate composition (g kg^{-1} dry weight basis) of experimental diets

Ingredient (g/kg)	Control	1.5% Garlic	3% Garlic	4.5% Garlic	6% Garlic
Fish meal[a]	450	450	450	450	450
Soybean meal[b]	280	280	280	280	280
Wheat flour[c]	120	120	120	120	120
Fish oil	40	40	40	40	40
Palm oil	30	30	30	30	30
Premix[d]	10	10	10	10	10
Carboxy methyl cellulose	60	45	30	15	0
Mixed vitamin[e]	10	10	10	10	10
Garlic powder[f]	0	15	30	45	60
Total (g)	1000	1000	1000	1000	1000
Proximate composition					
Moisture %	7.57	8.11	7.19	8.16	8.02
Crude protein %	45.90	47.10	46.70	47.20	47.00
lipid %	11.70	11.40	11.80	12.00	12.10
Ash %	11.76	10.25	10.52	10.40	10.90
Total carbohydrate[g]	23.07	23.14	23.79	22.24	21.98
Gross energy kcal/kg[h]	4639.14	4681.08	4723.58	4705.28	4692.56

[a]Danish fishmeal (Crude protein= 71.23% & Crude fat= 12.41%) supplied by Sri Purta Trading Sdn. Bhd. Alor Setar, Kedah, Malaysia.

[b]Soybean meal (Crude protein= 46.17% & Crude fat= 3.14%) supplied by Sri Purta Trading Sdn. Bhd. Alor Setar, Kedah, Malaysia.

[c]Wheat flour (Crude protein= 0.73% & Nitrogen-Free extract= 99.27%) supplied by Sri Purta Trading Sdn. Bhd. Alor Setar, Kedah, Malaysia.

[d]Premix (g/kg)-cobalt carbonate, 100mg; copper sulphate, 780mg; magnesium sulphate, 137mg; manganese oxide, 800mg; potassium chloride, 50g; potassium iodide, 150mg; sodium chloride, 60g; sodium selenite, 200mg and zinc oxide, 1.5g; calcium lactate, 327g; ferrous sulphate, 25g; calcium phosphate (monobasic), 397.5g.

[e]Mixed vitamin (Rovimix 6288; F.Hoffman La-Roche Ltd, Basel, Switzerland), containing (per kg, dry weight): vitamin A, 50 million IU; vitamin D3, 10 million IU; vitamin E, 130g; vitamin B1, 10g; vitamin B2, 25g; vitamin B6, 16g; vitamin B12, 100mg; biotin, 500mg; panthothenic acid, 56g; folic acid, 8g; niacin, 200g; anti-cake20g; antioxidant, 200mg; vitamin K3, 10g; and vitamin C, 35g.

[f]Take It Global Sdn. Bhd. Pulau Pinang, Malaysia.

[g]Total carbohydrate content was determined by the difference: total carbohydrate = 100 − (% crude protein + % crude fat + % total a sh + % Moisture).

[h]Dietary gross energy was calculated using the conversion factors of 5.6, 9.4 and 4.2 kcal/kg for protein, lipids and carbohydrates, respectively.

Experimental fish feeding

A total of 400 hybrid grouper fingerlings with an initial weight of 14.31g ± 0.08 were stocked in 20 circular fiberglass tanks (Fig. 1) at a density of 20 fish per tank (5 treatments and 4 replicates) with a capacity of 1000L of seawater (temperature range was 26- 29°C and dissolved oxygen remained within the optimal range for growth at 6- 7ml/ L). Fish were fed till satiation twice daily at 09:00 am and 4:00 pm, 6 days a week for nine weeks.

Water management system

Seawater was first collected and stored in four storage tanks, each with a capacity of 5 tons. For disinfection, 7–10 chlorine tablets were added to each tank, and the water was left to stand for approximately 3 hours to ensure proper sanitation. To neutralize any residual chlorine, 70– 80g of sodium thiosulfate was added (**Boyd & Tucker, 2012**). The treated seawater was then used for daily water exchange in the experimental tanks, with each tank being refreshed once per day. Prior to each water exchange, tanks were thoroughly cleaned to remove uneaten feed and accumulated waste materials. Water quality parameters were monitored regularly throughout the experimental period. Temperature and dissolved oxygen were measured using a portable dissolved oxygen meter (AIMI DO9100 Digital Dissolved Oxygen Meter, Guangdong, China); salinity was determined using a hand-held refractometer (ATAGO MASTER, Japan), and pH was measured using a digital pH meter (Eutech Digital pH Meter, Expert pH, Singapore)

Growth performance evaluation

Fish were weighed biweekly (every two weeks) to monitor growth performance throughout the experimental period. On each weighing day, fish were fasted for the day of weighing to minimize handling stress and ensure accurate body weight measurements. All fish in each tank were weighed collectively to calculate the mean body weight per tank, which was considered one replicate. The following indices were used to evaluate the growth performance, feed utilization efficiency, and health condition of hybrid grouper fingerlings:

a) Weight gain (WG, g) = final weight (g) – initial weight (g).
b) Specific growth rate (SGR, %/day) = [(ln final weight – ln initial weight) / experimental days] × 100.
c) Feed conversion ratio (FCR) = feed intake (g) / weight gain (g).
d) Protein efficiency ratio (PER) = weight gain (g) / protein intake (g).
e) Protein productive value (PPV, %) = [(final body protein – initial body protein) / protein intake] × 100.
f) Hepatosomatic index (HSI, %) = (liver weight / body weight) × 100.
g) Viscerosomatic index (VSI, %) = (viscera weight / body weight) × 100.
h) Relative gut length (RGL, %) = (gut length / total body length) × 100.

A: Hybrid grouper fry were reared in nursery tanks for a period of two months to reach the fingerling size before being used in the feeding trial.

B: Experimental tanks used in the study had a capacity of 1,000 liters each

C: Hybrid grouper fingerlings

Fig. 1. Phases of hybrid grouper rearing

Fish sampling

Fish were randomly selected from each treatment group for sampling. Prior to sample collection, fish were anesthetized using clove oil at a concentration of 40- 50mg/ L to minimize handling stress and facilitate safe sampling (**Coyle *et al.*, 2004**). From each treatment, 4 to 5 fish were collected for histological examination and enzyme activity analysis, while an additional 3 to 4 fish were taken from each tank for chemical composition analysis.

Proximate analysis

The proximate analysis of fish and diets for all experimental treatments was determined according to the **AOAC (2012)** method. The primary moisture content of the whole body of the fish was specified by drying the fish in an oven at 60°C until the weight was constant, while the secondary moisture content was determined by drying the fish at 120°C for an hour. Crude protein content had been estimated using the Kjeldahl method, where the nitrogen content × 6.25 would be the crude protein. The ash content was measured in a muffle furnace at a temperature of 600°C for 2 hours. The crude fat content was determined by extracting with a mixture of chloroform and methanol.

Preparation of histological samples

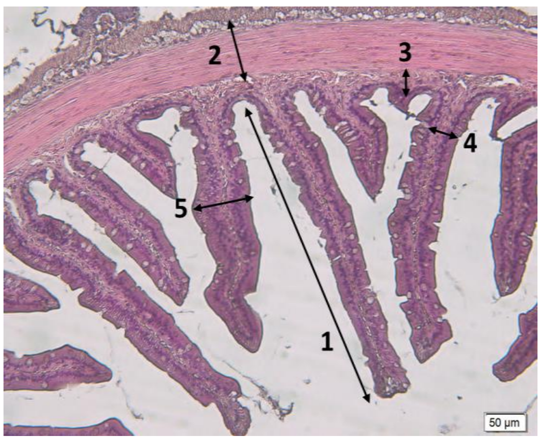
The preparation of tissues for histology followed the method described by **Firdaus-Nawi *et al.* (2013b)**, with modifications. In brief, the intestine, stomach and liver of the fish were dissected from freshly euthanized fish. Three fish of each treatment were collected. The tissues were cut into small pieces, approximately 5mm in size, and immersed quickly in 10% formalin for 2 days to preserve the tissue structure. Following fixation, the tissues were dehydrated using a gradient of ethanol concentrations, progressing from 50 to 100%. Then, the tissues were immersed in xylene to remove any traces of alcohol, after which the tissues were impregnated with paraffin wax. Then, individual tissue was embedded into paraffin blocks using an embedding machine, and slides were prepared through serial sectioning using a microtome at a thickness of 4µm. These glass slides were dried in a 40°C oven overnight before staining with Hematoxylin and Eosin, each for 4-5 minutes. Finally, the stained slides were analyzed using a digital microscope with CellSens Imaging software (Olympus CellSens Standard software, Shinjuku-ku, Tokyo, Japan) at 50×, 100× and 200× magnification, with objectives of 4×, 10×, 20×, and 40× to measure various aspects of the intestine, stomach and liver of hybrid grouper fingerlings (Fig. 2).

Digestive enzymes activity

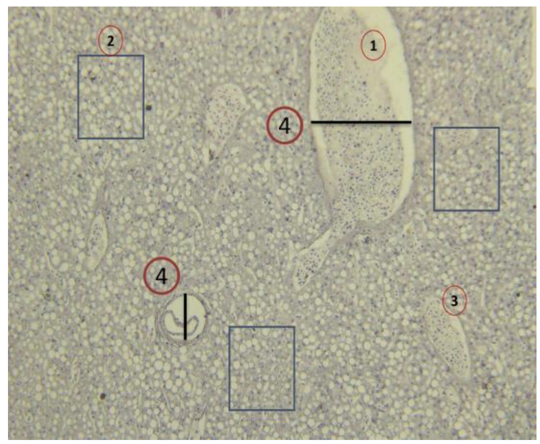
At the end of the feeding trial, the intestines and stomachs from 4 freshly euthanized fish of each treatment were collected and kept in the freezer in preparation for digestive enzyme analysis. Activities of three digestive enzymes were analyzed following the method by **Hamid *et al.* (2022)**. To get the assay for enzyme reaction, the tissue was homogenized together with cold distilled water (3ml/ 1g intestine and 2ml/ 1g stomach) using a hand-held glass homogenizer. Then, the tissue homogenates were centrifuged at

4°C for 15min at 15,000g to separate the supernatant and the tissue debris. The supernatant was aliquoted into new tubes and kept frozen at -20°C until further used. Bradford method was used to determine the total protein content of the homogenates, and bovine serum albumin (BSA) was used as the standard. The absorbance of the reaction was read using spectrophotometer at 595nm. Gastric protease activity was measured using casein hydrolysis method **(Walter, 1984)** using L-tyrosine as the standard. The reaction's absorbance was measured at 280nm. One unit of total proteolytic enzyme activity was defined as the amount of enzyme that releases µg of tyrosine per minute in the reaction mixture.

$$\text{Specific enzyme activity (U)}^{\text{-mg protein}} = \frac{\text{Abs sample (280nm)} - \text{Abs blank (280 nm)}}{60\text{ min} \times \text{mg protein in enzyme extract}}$$



A: Intestine Measurements
1) Villi length, 2) Thickness of muscle and mucous layer, 3) Crypt length, 4) Thickness of lamina propria, 5) Villi width



B: Liver Measurements
1) Protal venule 2) Nuclei concentration 3) Bile ductule 4) Length

C: Stomach Measurements, Muscle thickness

Fig. 2. Phases of histological analysis and measurements

Amylase activity in the intestine was determined using the starch hydrolysis method, as described by **Worthington and Manual (1982)**. Maltose was used as the standard to measure the specific amylase activity of the samples. Amylase specific activity was calculated according to the formula below:

Specific enzyme activity (U$)^{\text{-mg protein}}$ = $\frac{Abs\ sample\ (540\ nm) - Abs\ blank\ (540\ nm)}{60\ min\ \times mg\ protein\ in\ enzyme\ extract}$

Lipase activity in the intestine was determined using the method described by **Bier (1955)**, with slight modifications: 0.5mL of emulsion, 0.25mL of McIlvaine buffer, and 0.25mL of enzyme extract were added in glass test tubes and mixed before being incubated at 37°C for 4 hours. Then, 1.5mL of acetone-ethanol (1:1) solution was added to the test tubes to stop the reaction, followed by10µL of 1% phenolphthalein, as indicator. The reaction mixture was titrated with 0.01 M sodium hydroxide until the color changed.

Statistical analysis

A normality test was performed on the data to assess whether they followed a normal distribution. Regression analysis was used to examine the effect of garlic powder levels on histological measurements, including the internal and external diameters of the intestine as well as the thickness of the mucous and muscle layers. Statistical analyses were conducted using SPSS 17.0 software (SPSS, Chicago, IL, USA), with means ± S.E of four replicates per treatment. Data were analyzed by one-way ANOVA, and Duncan's multiple range test (**Duncan, 1955**) was applied to identify significant differences among treatments, with a $P < 0.05$ considered statistically significant.

RESULTS

1. Growth performance, feed utilization and proximate composition

The effect of garlic powder on growth performance and feed efficiency is summarized in Table (2), while the proximate composition of hybrid grouper fingerlings is presented in Table (3). After nine weeks of feeding, the body weight of all fish increased approximately threefold compared to the initial value, with numerical but not significant differences among treatments. Although the inclusion of garlic powder to the diet did not lead to any statistically significant differences ($P > 0.05$) in final weight, weight gain, or specific growth rate (SGR), it had a notable impact on feed efficiency. In this study, the FCR recorded ranged from 1.31 to 1.55 across all treatments, with the 3% garlic-supplemented diet achieving the most efficient FCR, 1.30. Moreover, garlic supplement influenced the protein efficiency ratio (PER), although an unclear trend was observed. PER values ranged from 1.42 to 1.70, with the highest efficiency again observed in fish fed with the 3% garlic supplement (PER=1.70). For protein productive value (PPV), the highest value was found in fish fed 3% (103.92), and the lowest PPV occurred in the 6% garlic group (81.86).

Table 2. Effect of garlic powder on growth performance, feed utilization and survival rate of hybrid grouper fingerlings (means ± SE)[1]

Parameter	Control	1.5% Garlic	3% Garlic	4.5% Garlic	6% Garlic
Mean initial weight (g/fish)	14.23 ± 0.11	14.28 ± 0.15	14.29 ± 0.08	14.33 ± 0.08	14.42 ± 0.01
Mean final weight (g/fish)	57.11 ± 1.72	55.78 ± 1.19	64.99 ± 1.73	56.63 ± 7.13	57.58 ± 3.29
Weight gain (g/fish)[2]	42.88 ± 1.61	41.50 ± 1.27	50.70 ± 1.64	42.30 ± 7.15	43.16 ± 3.28
Feed intake (g/fish)	60.61 ± 0.48	64.73 ± 2.13	66.42 ± 3.61	61.45 ± 5.10	66.54 ± 4.09
FCR[3]	1.41^{ab} ± 0.04	1.55^{a} ± 0.04	1.31^{b} ± 0.03	1.45^{ab} ± 0.12	1.54^{a} ± 0.06
PER[4]	1.57^{ab} ± 0.04	1.42^{b} ± 0.03	1.70^{a} ± 0.04	1.51^{ab} ± 0.13	1.44^{b} ± 0.05
PPV[5]	94.76^{ab} ± 2.59	88.69^{bc} ± 1.53	103.92^{a} ± 2.12	95.83^{ab} ± 5.76	81.86^{c} ± 4.23
SGR[6]	2.13 ± 0.03	2.09 ± 0.04	2.32 ± 0.03	2.09 ± 0.18	2.12 ± 0.08
SR[7]	93.33 ± 1.66	93.33 ± 1.66	86.66 ± 4.40	88.33 ± 1.66	93.33 ± 1.66
HSI[8]	1.84 ± 0.35	1.39 ± 0.25	1.71 ± 0.07	1.37 ± 0.38	1.39 ± 0.08
VSI[9]	9.44 ± 0.71	8.16 ± 1.24	8.67 ± 1.06	6.76 ± 0.52	6.99 ± 0.62
RGL[10]	1.20 ± 0.08	1.07 ± 0.20	1.21 ± 0.04	1.15 ± 0.13	1.01 ± 0.07

[1] Means in the same row with different superscripts are significantly different (*P*< 0.05) by Duncan's test.
[2] Weight gain (WG) = final body weight (g) - initial body weight (g).
[3] Feed conversion ratio (FCR) = feed intake (g)/body weight gain (g).
[4] Protein efficiency ratio (PER) = wet weight gain / protein fed
[5] Protein productive value (PPV) = (final protein content of the fish/100 × fish final weight) – (initial protein content of the fish/100 × fish initial weight) / gram feed × (%CP in feed/100) * 100
[6] Specific growth rate (SGR) = (ln final body wt − ln initial body wt)/feeding days × 100.
[7] SR = survival rate = survival fish / total fish × 100.
[8] Hepatosomatic index (HSI) =100 × hepatopancreas weight (g)/body weight (g).
[9] Viscerosomatic index (VSI) =100 × viscera weight (g)/body weight (g).
[10] Relative gut length =intestine length / body length

In addition to growth indices, the inclusion of garlic powder had no significant impact on the survival rate, hepatosomatic index (HSI), viscerosomatic index (VSI), or relative gut length (RGL) of the hybrid grouper fingerlings. Additionally, the whole-body chemical composition remained largely unaffected across all dietary treatments. Importantly, even at the highest inclusion level of 6% garlic, there were no adverse effects on the protein or lipid content of the fingerling fish.

Table 3. Proximate composition of hybrid grouper fingerlings fed diets for 9 weeks (means ± SE)[1]

Component	Control	1.5% Garlic	3% Garlic	4.5% Garlic	6% Garlic
Moisture %	74.42 ± 0.13	74.02 ± 0.34	74.62 ± 0.46	74.45 ± 0.65	74.20 ± 0.17
Crude Protein %	59.33^{ab} ± 0.20	60.76^{a} ± 1.52	60.10^{ab} ± 0.85	61.73^{a} ± 1.39	56.70^{b} ± 0.79
Crude Lipid %	16.23^{ab} ± 0.12	16.20^{ab} ± 0.70	17.26^{a} ± 0.63	14.40^{b} ± 0.05	15.56^{ab} ± 0.87
Ash %	13.37 ± 0.51	13.24 ± 0.48	13.23 ± 0.26	13.60 ± 0.14	13.36 ± 0.47

1. **Means in the same row with different superscripts are significantly different (*P* < 0.05) by Duncan's test.**

2. Histological measurements

Table (4) summarizes the effects of dietary garlic powder supplementation on the histological characteristics of the intestines, liver, and stomach in hybrid grouper fingerlings. It was found that the intestinal length was the highest in the control group (21.75 ± 2.49cm) and decreased progressively with increasing garlic powder levels, reaching the lowest value in the 6% group (17.50 ± 1.17cm, Figs. (3, 4)). Both outer and inner diameters of the intestine declined significantly in response to garlic inclusion. The control group showed the highest outer (2204.87 ± 48.67µm) and inner diameters (1919.23 ± 61.45µm), whereas the 6% garlic group recorded the lowest values (797.35 ± 8.60µm and 705.99 ± 11.25µm, respectively). Moreover, muscle and mucosal layer thickness did not differ significantly among most treatments, except for a marked reduction observed at the 6% level. The percentage of muscle layer increased significantly in the 3% garlic group compared to the others.

Table 4. Histological measurements of hybrid grouper fingerlings after 9 weeks of feeding on different levels of garlic powder. (means ± SE)[1]

Measurement	**Control**	**1.5% Garlic**	**3% Garlic**	**4.5% Garlic**	**6% Garlic**
Intestine (µm)					
Length of gut (cm)	21.75 ± 2.49	19.50 ± 4.38	20.25 ± 0.25	19.25 ± 2.66	17.50 ± 1.17
Outer diameter	2204.87^{a} ± 48.67	1499.28^{c} ± 105.5	1669.83^{b} ± 19.48	1729.49^{b} ± 61.77	797.35^{d} ± 8.60
Inner diameter	1919.23^{a} ± 61.45	1263.40^{c} ± 98.33	1329.06^{bc} ± 35.08	1491.37^{b} ± 62.16	705.99^{d} ± 11.25
[1]Thickness of mucous and muscle layers	285.64^{a} ± 42.86	235.87^{a} ± 55.59	340.77^{a} ± 35.38	238.12^{a} ± 41.81	91.36^{b} ± 7.08
[2]Muscle layer %	12.96^{b} ± 1.91	15.46^{ab} ± 3.20	20.35^{a} ± 2.07	13.62^{b} ± 2.33	11.47^{b} ± 0.92
[3]Villi percentage %	87.03^{a} ± 1.91	84.53^{ab} ± 3.20	79.64^{b} ± 2.07	86.37^{a} ± 2.33	88.52^{a} ± 0.92
Villi length	443.71^{a} ± 54.21	255.23^{b} ± 22.06	408.45^{a} ± 32.63	379.56^{a} ± 42.24	175.86^{b} ± 20.44
Villi width	86.61^{a} ± 6.88	69.95^{b} ± 4.20	69.52^{b} ± 4.09	64.59^{b} ± 2.61	45.46^{c} ± 4.25
Number of villi	46.33^{a} ± 2.33	29.00^{c} ± 1.00	36.33^{bc} ± 2.90	40.33^{ab} ± 4.40	42.66^{ab} ± 0.88
Gap between villi	48.23 ± 5.54	38.30 ± 5.06	44.55 ± 4.37	38.83 ± 3.04	49.59 ± 3.68
Thickness of lamina propria	17.92^{a} ± 2.24	19.40^{a} ± 1.69	11.61^{bc} ± 0.64	9.38^{c} ± 0.87	15.83^{ab} ± 1.58
[1]Thickness of mucous and muscle layers = outer diameter - inner diameter [2]Muscle layer % = thickness of mucous and muscle layers / outer diameter × 100 [3]Villi percentage % = inner diameter / outer diameter × 100					
Liver (µm)					
Hepatopancreas weight (g)	2.05 ± 0.51	1.42 ± 0.44	1.53 ± 0.22	1.01 ± 0.24	1.23 ± 0.07
Central vein diameter	470.04^{b} ± 9.65	884.08^{a} ± 1.24	400.42^{b} ± 1.07	326.77^{bc} ± 9.68	100.37^{c} ± 34.26
Central vein length	385.52^{a} ± 1.05	299.0^{a} ± 52.42	118.99^{b} ± 30.63	101.26^{b} ± 11.92	30.32^{b} ± 9.02
Hepatic ductule length	57.48^{cd} ± 6.03	150.01^{ab} ± 23.39	188.66^{a} ± 28.31	106.10^{bc} ± 23.96	39.89^{d} ± 6.87
Hepatic ductule diameter	252.74^{cd} ± 20.85	477.50^{ab} ± 79.11	602.97^{a} ± 85.46	347.57^{bc} ± 82.35	129.48^{d} ± 23.43
Stomach (µm)					
Muscle thickness	316.95^{c} ± 11.12	233.63^{d} ± 8.17	198.38^{d} ± 29.37	618.59^{b} ± 35.95	735.59^{a} ± 12.71

[1] Means in the same row with different superscripts are significantly different (*P*< 0.05) by Duncan's test.

Villus length and width were significantly higher in the control group (443.71 ± 54.21 µm and 86.61 ± 6.88 µm) and declined gradually with increasing garlic levels, with the lowest values reported in the 6% group (175.86 ± 20.44µm and 45.46 ± 4.25µm). The number of villi increased significantly across garlic treatments, ranging from 29 in the 1.5% group to 42.66 in the 6% group; however, the control group exhibited the highest count (46.33). In addition, the lamina propria thickness exhibited a non-linear pattern, with a notable reduction at higher garlic levels. In general, diets containing 4.5 and 6% garlic powder negatively affected intestinal histomorphology, as evidenced by reduced diameters, thinner muscle and mucosal layers, and shorter villi.

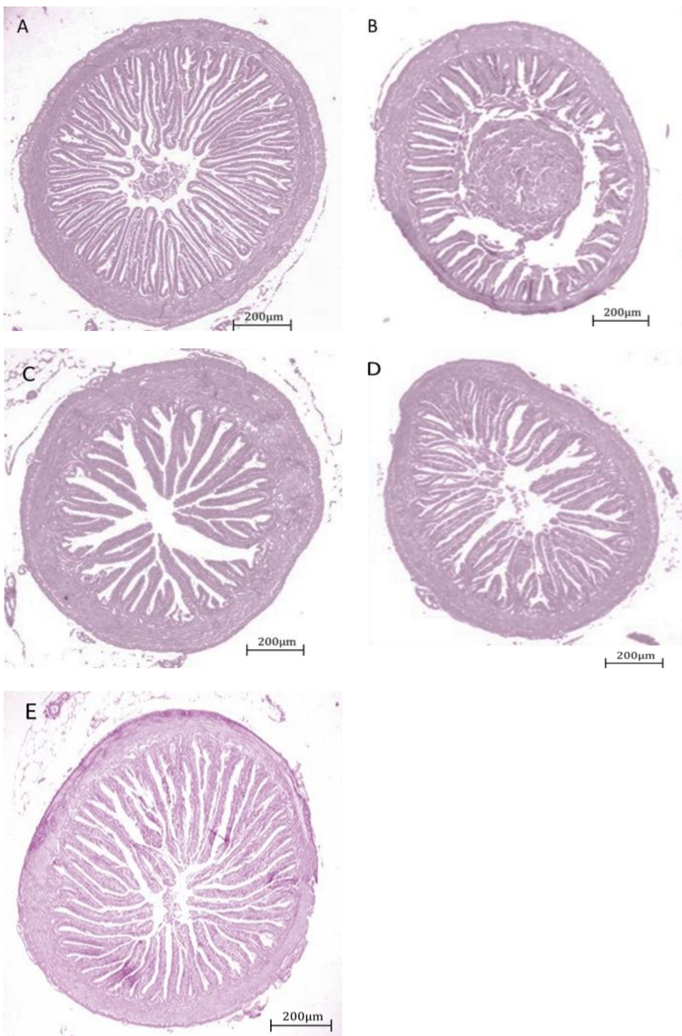


Fig. 3. Cross-sections of hybrid grouper intestine representing the outer diameter, inner diameter, muscle and mucous layer thickness resulting from feeding on various levels of garlic powder. A (control), B (1.5% garlic), C (3% garlic), D (4.5% garlic), E (6% garlic), H&E, bars in 200µm.

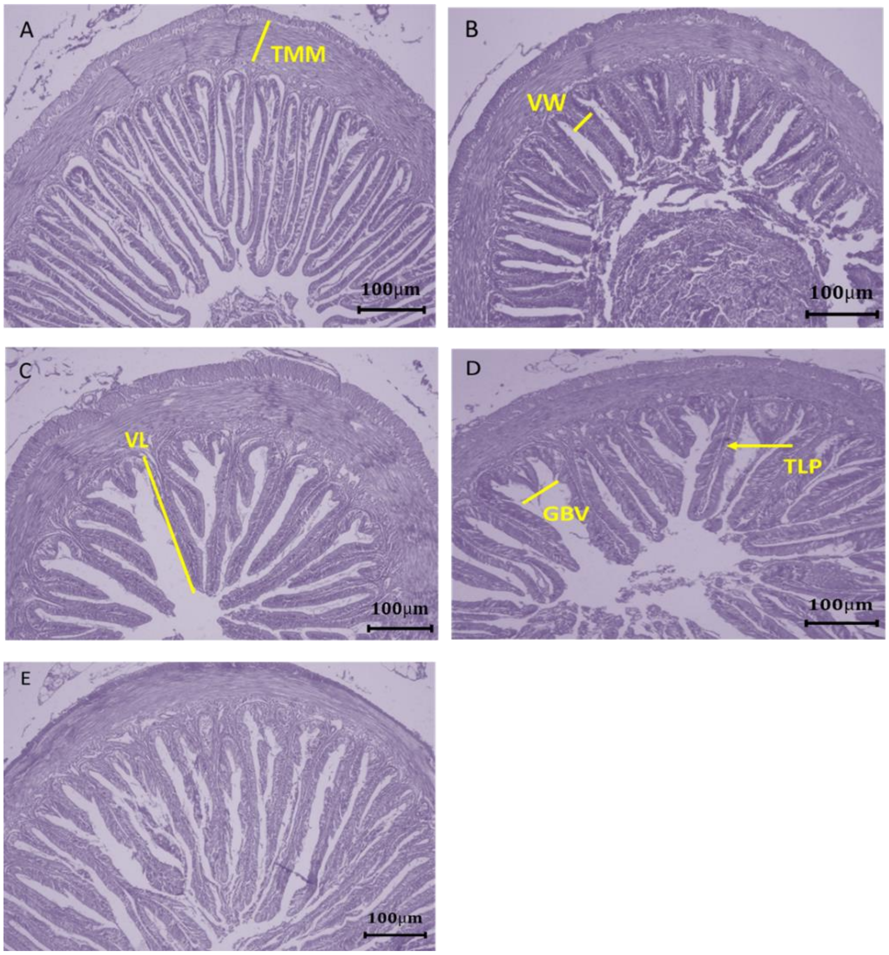


Fig. 4. Cross-sections of hybrid grouper intestine representing (TMM) thickness of mucous and muscle layers, (VW) villi width, (VL) villi length, (GBV) gab between villi, and (TLP) thickness of lamina propria resulting from feeding on various levels of garlic powder compared to control. A (control), B (1.5% garlic), C (3% garlic), D (4.5% garlic), E (6% garlic), H&E, bars in 100µm.

No histopathological abnormalities in the liver, such as inflammation, degeneration, or necrosis, were observed in any treatment (Fig. 5). While liver weight tended to decrease in garlic-fed groups compared to the control, the differences were not statistically significant. A significant increase in central vein diameter was observed in the 1.5% garlic group, followed by a reduction in both diameter and length with higher garlic levels. Hepatic duct length increased significantly in the 1.5 and 3% garlic groups (150.01 and 188.66µm, respectively) compared to the control (57.48µm). Similarly, the hepatic duct diameter peaked in the 3% group and declined in the 6% group.

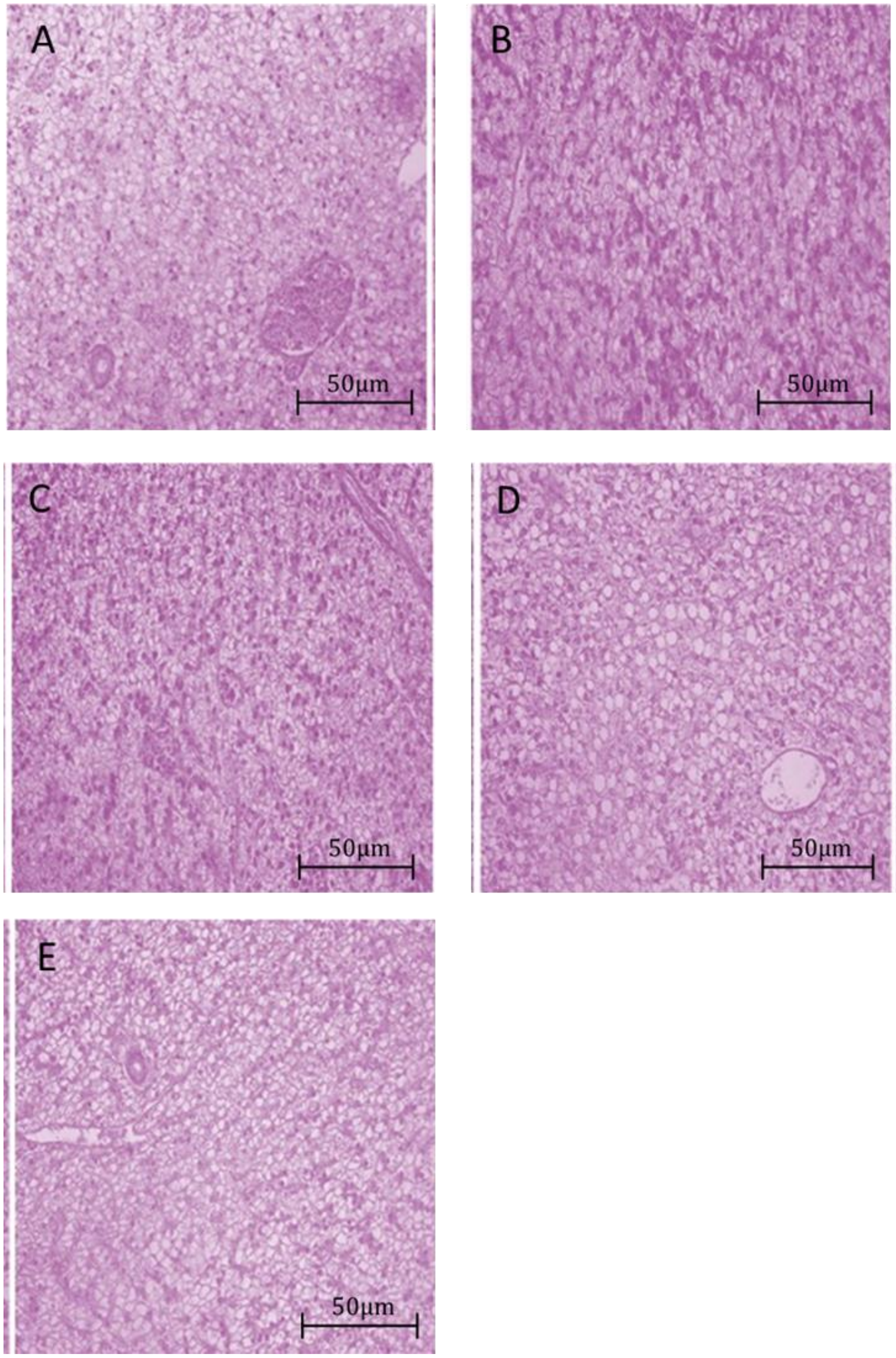


Fig. 5. Cross-section of hybrid grouper liver showing the presence of no pathological effects on the liver, such as deformities, inflammation, or tissue loss. A (control), B (1.5% garlic), C (3% garlic), D (4.5% garlic), E (6% garlic), H&E, bars in 50µm.

Meanwhile, for stomach histology (Fig. 6), significant differences were observed in muscle layer thickness. The 6% garlic group showed the highest value (735.59µm), followed by the 4.5% group (618.59µm). Control fish exhibited a moderate thickness

(316.95µm), while the lowest values were recorded in the 1.5 and 3% garlic groups (233.63 and 198.38µm, respectively).

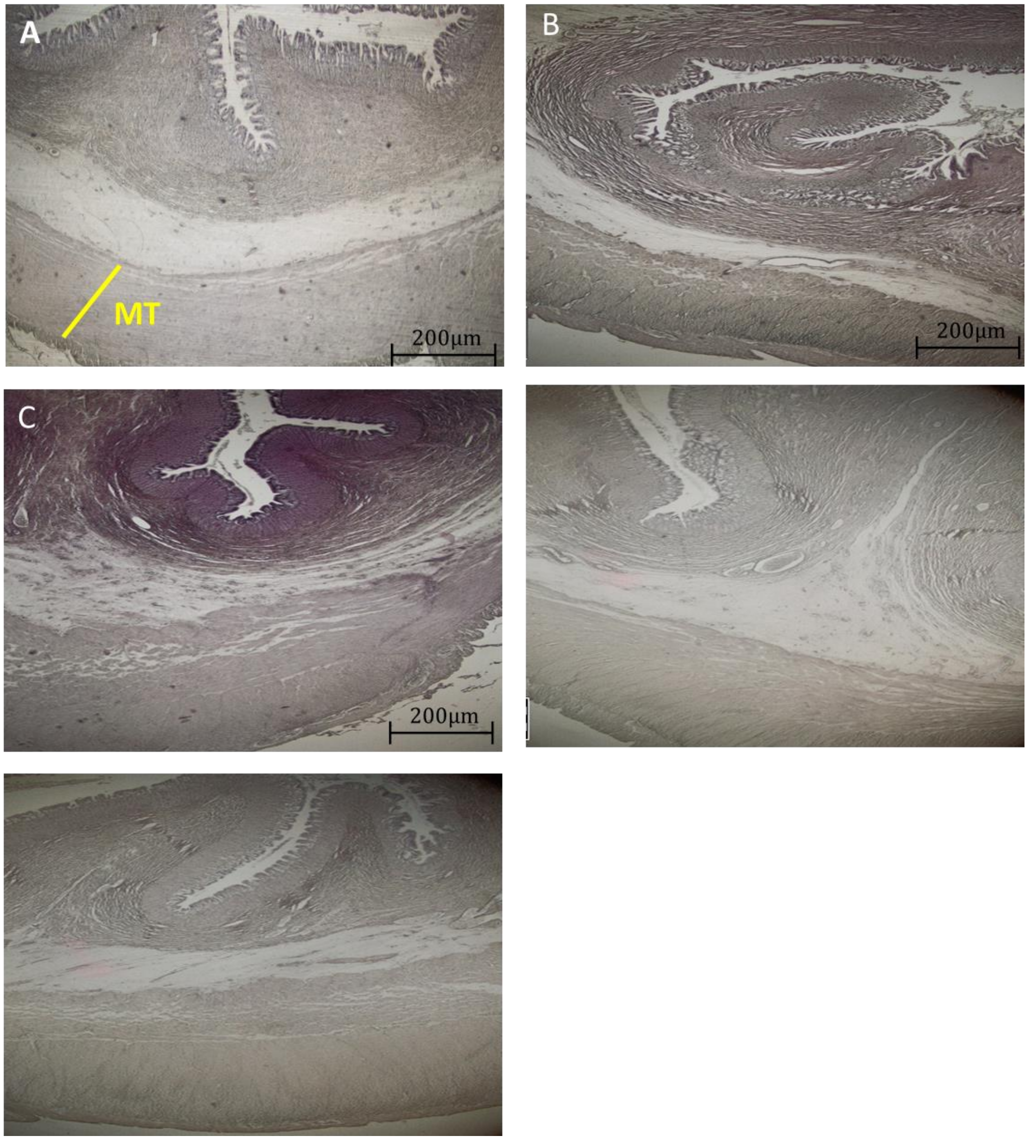


Fig. 6. Cross-section of hybrid grouper representing (MT) muscle thickness of stomach. A (control), B (1.5% garlic), C (3% garlic), D (4.5% garlic), E (6% garlic), H&E, bars in 200µm.

3. Digestive enzymes

Table (5) illustrates the effects of dietary garlic powder on the activity of three digestive enzymes (amylase, protease, and lipase) in the stomach and intestines of hybrid grouper fingerlings. Amylase activity in the intestine was significantly higher in fish fed the control diet (0.18 unit/mg protein) compared to those fed diets containing 3 and 6% garlic powder. The 1.5% garlic group showed moderate activity (0.11 unit/mg protein), followed by the 4.5% group (0.10 unit/mg protein), with no significant difference between them.

Protease activity in the stomach was numerically the highest in fish fed the 3% garlic diet (1.75 unit/mg protein), followed by those fed 4.5% garlic (1.52 unit/mg protein), the control group (1.24 unit/mg protein), and the 6% garlic group (1.20 unit/mg protein). The lowest protease activity was recorded in fish fed the 1.5% garlic diet (0.80 unit/mg protein).

Intestinal lipase activity showed an increasing trend at lower garlic inclusion levels, followed by a gradual decline as garlic concentration increased. The highest activity was observed in fish fed the 1.5% garlic diet (28.40 unit (U) of 0.01 N NaOH), followed by those fed 3% (24.07U), 4.5% (22.57U), and 6% (19.32U), while the control group recorded 21.20U. Fish fed the 1.5% garlic diet exhibited significantly higher lipase activity compared to the control, 4.5%, and the 6% garlic groups ($P < 0.05$).

Table 5. Effect of adding garlic powder on the activity of digestive enzymes of hybrid grouper fingerlings (means ± SE)[1]

Enzymes activity	Control	1.5% Garlic	3% Garlic	4.5% Garlic	6% Garlic
Amylase (U/mg prot)	$0.18^{a} \pm 0.05$	$0.11^{ab} \pm 0.01$	$0.06^{b} \pm 0.02$	$0.10^{ab} \pm 0.02$	$0.06^{b} \pm 0.03$
Protease (U/g prot)	1.24 ± 0.11	0.80 ± 0.27	1.75 ± 0.23	1.52 ± 0.48	1.20 ± 0.23
Lipase (U of 0.01 N NaOH)	$21.20^{b} \pm 0.47$	$28.40^{a} \pm 3.27$	$24.07^{ab} \pm 0.68$	$22.57^{b} \pm 1.70$	$19.32^{b} \pm 0.90$

[1] Means in the same row with different superscripts are significantly different (*P*< 0.05) by Duncan's test.

DISCUSSION

Reducing high mortality rates and enhancing the health status of grouper species, including hybrid grouper, has garnered considerable research attention, particularly in Asia **(Rahimnejad *et al.*, 2015; Luo *et al.*, 2016; Arrokhman *et al.*, 2017; Anthonius *et al.*, 2018; Mohd Faudzi *et al.*, 2018; Tan *et al.*, 2018, 2019)**. Among the various strategies explored, the incorporation of feed additives into fish diets, especially natural ones such as garlic, has shown promising results in improving fish health and reducing mortality. Garlic supplementation has been previously studied in various aquaculture species, including orange-spotted grouper **(Guo *et al.*, 2012)**, Asian sea bass (**Talpur & Ikhwanuddin, 2012)**, kuruma shrimp **(Tanekhy & Fall, 2015)**, rainbow trout **(Esmaeili *et al.*, 2017)**, brown trout **(Zaefarian *et al.*, 2017)**, red tilapia **(Samson, 2019)**, European sea bass **(Yildiz *et al.*, 2019)**, and Japanese seabass **(Xu *et al.*, 2020)**.

In the present study, no adverse effects were observed from incorporating garlic powder into the diets of hybrid grouper fingerlings. On the contrary, the best growth performance, feed conversion ratio (FCR), protein efficiency ratio (PER), and specific growth rate (SGR) were recorded at a 3% garlic inclusion level. Similar findings have been reported in other species. For instance, **Guo *et al.* (2012)** observed an enhanced growth in orange-spotted grouper (initial weight: 19g) fed a diet containing 1.3% garlic powder for 14 days. Likewise, **Samson (2019)** reported an improved performance in red tilapia fed 1-1.5% garlic powder for six weeks. **Zaefarian *et al.* (2017)** found that juvenile brown trout (average weight: 19.94g) exhibited optimal growth when fed 20g/ kg garlic powder for six weeks. **Esmaeili *et al.* (2017)** postulated that supplementing juvenile rainbow trout diets with 30g/ kg garlic in combination with meat and bone meal as a partial replacement for fish meal positively influenced growth and physiological responses. In Japanese seabass, **Xu *et al.* (2020)** reported that dietary supplementation with 10g/ kg garlic powder improved growth and digestive capacity. Furthermore, **İrkin and Yiğit, (2015)** found that feeding European seabass diets containing 4% garlic meal for two months resulted in better FCR and PER compared to diets containing 0, 2, or 6% garlic meal.

On the other hand, the results of the present study indicated that there were no statistically significant differences in specific growth rate (SGR), hepatosomatic index (HSI), viscerosomatic index (VSI), and relative gut length (RGL) among the treatment groups. However, these indices showed a numerical decline as the dietary garlic level increased from 3 to 6%, with the 3% garlic group recording the highest values among all garlic-supplemented diets. A similar trend was observed by **Zaefarian *et al.* (2017)**, who reported a decrease in both HSI and VSI in brown trout fed diets containing 10, 20, and 30g/ kg of garlic powder. Likewise, **Xu *et al.* (2020)** found that HSI in Japanese seabass initially increased with the addition of 5- 10g/ kg garlic powder, but declined when garlic supplementation was increased to 25g/ kg.

Regarding the body chemical composition of hybrid grouper fingerlings after nine weeks of feeding, no significant differences were observed in moisture and ash contents among the dietary treatments. However, fish fed the 6% garlic diet exhibited a significantly lower body protein content compared to those fed the 4.5% garlic diet. Additionally, the lowest body fat content was recorded in the 4.5% garlic group.

Xu *et al.* (2020) elucidated that in carnivorous species such as Japanese seabass, feeding high-protein diets led to increased body lipid accumulation. In their study, a higher inclusion level of garlic powder (25g/ kg) was associated with reduced feed intake and increased body fat, possibly due to its strong odor affecting feed palatability. Similarly, **Zaefarian *et al.* (2017)** found that brown trout fed diets containing 10– 30g/ kg garlic powder showed a slight increase in body fat content from 17.60 to 18.00%. In contrast, in the present study, hybrid grouper fingerlings showed increased feed intake and improved growth across all garlic-supplemented diets (1.5–6%) compared to the control. Moreover, the crude lipid percentage decreased as the garlic inclusion level increased from 3 to 6%, suggesting that moderate levels of garlic powder enhanced nutrient utilization, without negatively affecting feed palatability.

In general, the inclusion of garlic powder can influence feed palatability, growth performance, and body composition through its various bioactive compounds; however, the direction and magnitude of these effects vary among studies. Nevertheless, variations in the effects of garlic across different studies may be attributed to multiple factors, including fish species, experimental duration, environmental conditions, type and form of garlic (e.g., powder, extract, or in combination with other herbs), inclusion levels, feed formulation, and feeding strategies **(Xu *et al.*, 2020)**.

Histological assessments of the intestine and liver are valuable indicators for evaluating the digestive capacity and overall health status of fish. Although limited data are available on the effects of garlic powder on the tissue histology of hybrid grouper, particularly during the nursery phase, the present study provides initial insights into its potential impact at this critical developmental stage. Notably, during the feeding trial, fish displayed a greater feeding response toward garlic-supplemented diets compared to the control diet, suggesting increased palatability and feed attractiveness. This observation is supported by the higher feed intake recorded in garlic-fed groups relative to the control. It is well established that feed characteristics, including size, type, and quantity, can influence intestinal morphology, particularly in carnivorous species such as the hybrid grouper. These changes may manifest in variations in intestinal length and wall thickness, potentially reflecting adaptive responses to dietary components. Given these findings, further investigations are recommended to explore the long-term histological and functional impacts of garlic supplementation in hybrid grouper, especially under varying environmental and nutritional conditions.

In the present study, both outer and inner intestinal diameters showed a significant reduction with increasing levels of dietary garlic powder compared to the control. This effect may be attributed to bioactive compounds in garlic, particularly allicin, which possesses strong antimicrobial properties. These compounds could have altered the gut microbiota by suppressing certain beneficial microbial populations that play a role in intestinal development and health. Moreover, the improved palatability and increased feed intake observed in garlic-supplemented groups may have contributed to faster digestion and softer chyme texture, thereby reducing the mechanical stimulation necessary for intestinal elongation and expansion **(Adibmoradi *et al.*, 2006; Shalaby *et al.*, 2006; Adineh *et al.*, 2020)**.

The present results showed that villus length and width were the greatest in fish fed the control and 3% garlic-supplemented diets. Villi are small, finger-like projections that increase the intestinal surface area, thereby enhancing the efficiency of nutrient absorption into the bloodstream. An increase in the number, length, and width of villi generally indicates improved absorptive capacity, as these structures represent the primary sites for nutrient uptake following digestion **(Firdaus-Nawi *et al.*, 2013a)**.

The results of the current study revealed a gradual decrease in villus width with increasing levels of garlic powder, aligned with a significant reduction observed at the 6% inclusion level. Conversely, the number of villi showed a significant and progressive increase as garlic concentration increased. The observed enhancements in villus length and number may be associated with the increased feed intake recorded in garlic-supplemented groups. Garlic is known to be rich in flavonoids and certain vitamins, which may stimulate intestinal epithelial cells and promote villus development. Furthermore, improvements in nutrient metabolism and immune function may have contributed to the enhanced intestinal morphology observed in the garlic-fed groups **(Shalaby *et al.*, 2006)**.

The lamina propria is a layer of loose connective tissue that, together with the epithelial layer, forms the mucosal membrane and is characterized by a high concentration of lymphocytes **(Firdaus-Nawi *et al.*, 2013a)**. In the present study, the thickness of the lamina propria exhibited a non-linear response to dietary garlic supplementation, with a general reduction observed at higher garlic concentrations, except in the group fed 6% garlic. **Firdaus-Nawi *et al.* (2013a)** reported an inverse relationship between the number of intestinal villi and the thickness of the lamina propria, where an increase in villi number was associated with a decrease in lamina propria thickness. They also noted that juvenile tiger grouper (1-2 months old) displayed an increased lamina propria thickness, suggesting developmental changes during early growth stages.

The nutritional and health status of fish can be assessed through histological evaluation of the liver **(Huang *et al.* 2023)**. Under normal conditions, hepatocytes exhibit a round nucleus, and reticular fibers are distributed around the central vein, forming part of the hepatic connective tissue structure. In the present study, the inclusion of garlic powder in the diet did not induce any pathological alterations in liver tissue, such as inflammation, degeneration, or necrosis. Hepatosomatic index (HSI) and liver weight were numerically higher in fish fed the control diet, followed by those fed diets supplemented with 3, 1.5, 6, and 4.5% garlic powder, respectively. The central vein, which drains into the hepatic vein to facilitate the removal of toxins and metabolic by-products, showed a decrease in both diameter and length in fish fed garlic-supplemented diets (3- 6%) compared to the control. This reduction may be attributed to enhanced feed utilization and improved digestive efficiency, leading to lower metabolic waste production and thus a reduced burden on hepatic vasculature.

The hepatic duct is responsible for transporting bile from the liver. The common hepatic duct begins where the right and left hepatic ducts merge outside the liver and continues until it joins the cystic duct from the gallbladder to form the common bile duct. In the present study, a significant increase in both the length and diameter of the hepatic duct was observed in fish fed garlic-supplemented diets compared to those in the control group. This enlargement may be associated with the known therapeutic properties of garlic, which have been reported to support liver function and prevent biliary obstruction and fibrosis **(Mahmoud *et al.*, 2014)**. These findings suggest a potential role of garlic in maintaining hepatobiliary health in hybrid grouper fingerlings. A detailed understanding of the digestive physiology during the larval and nursery phases is essential for producing healthy fingerlings of high-value fish species, such as hybrid grouper, to ensure the sustainability of aquaculture operations **(Martínez-Lagos *et al.*, 2014)**.

In the present study, the activity of three key digestive enzymes, amylase, protease, and lipase, was evaluated in hybrid grouper fingerlings during the nursery phase, at a body weight ranging from 12 to 100g. The results demonstrated that dietary garlic supplementation significantly affected intestinal amylase activity. Specifically, fish fed 3 and 6% garlic diets exhibited significantly lower amylase activity compared to the control group. These findings contradict those of **Xu *et al.* (2020)**, who reported no significant changes in amylase activity in Japanese seabass fed diets containing 5- 25g/ kg of garlic powder. Protease activity in the stomach was not significantly affected by garlic supplementation. However, a gradual numerical decrease was observed as the garlic inclusion level increased from 3 to 6%, suggesting a potential dose-dependent trend.

Regarding lipase activity in the intestine, a significant increase was observed in fish fed 1.5% garlic compared to the control. However, a progressive decline in lipase activity was noted with higher garlic inclusion levels (from 1.5 to 6%). Despite this trend, no significant differences were found among the 3, 4.5, 6%, and control groups. These findings are consistent with those reported by **Xu *et al.* (2020)** in Japanese seabass (initial weight: 75 g) after 28 days of feeding on a diet supplemented with 10g/ kg garlic powder.

Overall, digestive enzyme activity is influenced by several factors, including developmental stage, diet formulation, and the levels of crude protein and lipid in the diet, particularly in carnivorous species during the early growth stages **(Martínez-Lagos *et al.*, 2014)**. Previous research has consistently highlighted the value of medicinal plants, particularly garlic, in enhancing fish growth, immunity, digestive enzyme activity, and disease resistance. However, despite the promising findings, further studies are still required, especially in hybrid grouper, to address the high mortality rates commonly observed during the early larval and nursery stages and to better understand the mechanisms through which garlic exerts its effects on fish health and physiology.

CONCLUSION

The present study demonstrated that dietary supplementation with garlic powder, particularly at a 3% inclusion level, had a positive impact on the growth performance, feed utilization, intestinal morphology, and certain digestive enzyme activities in hybrid grouper fingerlings during the nursery phase. Fish fed the 3% garlic diet exhibited the highest specific growth rate, protein efficiency ratio, and feed intake, along with improved villus structure and favorable histological features in the intestine and liver.

Although higher levels of garlic (4.5- 6%) resulted in some reductions in intestinal diameter and enzyme activity, no pathological alterations were observed in liver tissues, indicating the general safety of garlic supplementation within the tested range. Moreover, garlic-fed groups showed increased feeding response and potential improvements in nutrient absorption, suggesting enhanced digestive efficiency.

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