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EFFECTS OF DIETARY SUPPLEMENTATION OF LYSINE AND METHIONINE IN TEMPEH-BASED DIET ON GROWTH PERFORMANCE AND FEED UTILIZATION OF TIGER GROUPER, *Epinephelus fuscoguttatus* JUVENILES

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ABSTRACT

The potential of tempeh (TMP) with supplementation of methionine (Met) and lysine (Lys) as a substitute of fishmeal (FM) was evaluated based on the growth performance and feed utilization for tiger grouper (*Epinephelus fuscoguttatus*) juveniles. Three diets were formulated to replace FM with TMP at 0% (D1, control diet), 40% without essential amino acids (EAAs) supplementation (D2) and 40% with EAAs supplementation (D3, 0.5% of Met and 0.5% of Lys) and fed to triplicate groups of fish (22.90±0.48g) twice a day for 8 weeks. Weight gain (WG), specific growth rate (SGR) and feed intake (FI) of D1 group (114.31%, 1.59%, and 45.51 g fish^{-1}, respectively) were significantly higher than those fed with TMP-based diets (P<0.05). On the other hand, supplementation of EAAs in D3 was able to significantly improve the protein efficiency ratio (PER) and net protein utilization (NPU) (1.17% and 23.13%, respectively) compared to other diets (P<0.05). Supplementation of EAAs reduced whole-body lipid (P<0.05) but slightly increased whole body protein (P>0.05) of the fish. In the present study, results indicated that supplementation of EAAs was only able to improve feed utilization (PER and NPU) but not in growth performance (WG and SGR) of *E. fuscoguttatus* fed on TMP-based diets.

ABSTRAK

Potensi tempeh (TMP) yang ditambah dengan metionin (Met) dan lisin (Lys) sebagai pengganti tepung ikan (FM) dalam diet juvenil kerapu harimau (*Epinephelus fuscoguttatus*) telah diuji berdasarkan tumbesaran dan kecekapan permakanan. Tiga diet telah diformulasi untuk menggantikan FM dengan menggunakan TMP pada kadar 0% (D1, diet kawalan), 40% tanpa tambahan asid amino perlu (EAAs) (D2), dan 40% ditambah EAAs (D3, 0.5% of Met and 0.5% of Lys) dan diberi makan kepada kumpulan triplikat ikan (22.93±0.50g) dua kali sehari selama 8 minggu. Pertambahan berat (WG), kadar pertumbuhan spesifik (SGR) dan pengambilan makanan (FI) dalam kumpulan D1 adalah lebih tinggi secara signifikan (114.31%, 1.59%, dan 45.51 g ikan^{-1}) daripada diet berasaskan TMP yang lain (P<0.05). Sebaliknya, tambahan EAAs dalam D3 telah meningkatkan kadar kecekapan protein (PER) dan penggunaan protein bersih (NPU) (masing-masing 1.17% dan 23.13%) secara signifikan berbanding diet-diet lain (P< 0.05). Penambahan EAAs telah mengurangkan kandungan lipid badan (P<0.05) tetapi serba sedikit meningkatkan kandungan protein badan (P>0.05). Keputusan kajian ini menunjukkan bahawa penambahan EAAs hanya mampu meningkatkan kecekapan makanan (PER dan NPU) tetapi tidak dalam peningkatan tumbesaran (WG dan SGR) *E. fuscoguttatus* yang diberi diet berasaskan TMP.

Key words: *Epinephelus fuscoguttatus*, Tempeh, methionine, lysine, growth performance, feed utilization

INTRODUCTION

Tiger grouper, *Epinephelus fuscoguttatus* is a commercial high-value grouper species in Asia due to its high market demands, fast growing and the ability to adapt in captive environment (Giri *et al*., 2004; Sugama *et al*., 2012). In Malaysia, the retail price of the fish is RM45 kg^{-1} (DOF, 2014) and could further increase up to 2-3 folds in local seafood restaurants. The culture of this species has developed and spread rapidly since 2002 with the first success in breeding and larviculture by Krabi Coastal Fisheries Research and Development Centre in Thailand (Pierre *et al*., 2007). Being carnivorous

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species, the fish required high protein diet as studies have indicated that the fish required 47-50% dietary protein and 9-16% of dietary lipid to support optimum growth (Giri *et al*., 2004; Shapawi *et al*., 2014). The fish is commonly fed with trash fish or fishmeal-based diets in commercial farm. However, unstable fisheries landing, increasing price of trash fish, poor feed efficiencies and other disadvantages of relying on trash fish or fishmeal (FM) as sole dietary protein source urged the need to develop cost-effective compound feed for *E. fuscoguttatus* from other alternative protein sources (Sim *et al*., 2005).

Soybean is one of the most commonly used alternative protein sources in animal feeds owing to its reasonable price and consistent availability (Drew, 2007; FAO, 2014). Soybean protein has been successfully replaced FM at approximately 20 to 40% replacement level in several carnivorous fish species without reducing growth performance and nutrient utilization (Carter and Hauler, 2000; Rachmansyah *et al*., 2005; Deng *et al*., 2006; Shapawi *et al*., 2013). However, major FM replacement by plant proteins including soybean in the diets are normally hindered by imbalanced amino acids profile, poor palatability and the presence of various anti-nutritional factors (Francis *et al*., 2001; Drew *et al*., 2007, Lim *et al*., 2014). Fermentation of plant ingredients has been reported to improve nutrients availability and digestibility, improve soybean meal-induced morphological changes in intestine, reduce antinutrient factors, and may act as immuno-stimulants (Astuti *et al*., 2000; Ashida and Okimasu, 2005; Kim *et al*., 2009; Babu *et al.*, 2009; Yamamoto *et al*., 2010). In our previous study, the potential of locally available fermented soybean, tempeh (TMP) as an alternative protein source in the diet of *E. fuscoguttatus* were evaluated. *E. fuscoguttatus* juvenile exhibited better feed utilization but showed no growth improvement after fed with TMP compared to unfermented soybean meal (Chor *et al*., 2015), as reported in other fish species fed on fermented soybean products (Yamamoto *et al*., 2010). Poor palatability and imbalanced and imbalanced amino acids profile of TMP-based diets was identified as the main factors which hindered the normal fish growth (Chor *et al*., 2015).

Nowadays, amino acids are commonly supplemented in plant-based diets to improve the nutritional profile and the palatability. L-amino acids are used in many compound feeds as feeding stimulant for carnivorous species as the amino acids are present in all animal tissues including their prey, both vertebrate and invertebrate in nature (Mackie, 1987; Papatryphon *et al*., 2000). Among those amino acids, lysine (Lys) and methionine (Met) are the essential amino acids (EAAs) which were normally reported to be insufficient in plant-based diets (Alam, *et al*., 2005). Lys is particulary important because it contributes the highest concentration among EAA in the carcass of many fish species while Met is usually the first limiting EAA in aquafeed, especially those containing high levels of plant protein (Luo *et al*., 2005; Luo *et al*., 2006; Yuan *et al*., 2011). Both of the EAAs are important for metabolic functions besides supporting normal growth. Currently, information regarding on the optimum EAAs requirement or the effect of plant-based diet supplemented with EAAs for *E. fuscoguttatus* are rather limited. Hence, it is of our interest to investigate the effects of Met and Lys supplementation in TMP-based diet on growth performance and feed utilization of *E. fuscoguttatus* juveniles.

MATERIALS AND METHODS

Diet preparation

Table 1 shows the ingredients, feed formulation and proximate compositions of the experimental diets. Essential Amino Acids (EAAs) profile of experimental diets is shown in Table 2. Three diets were formulated to replace Fishmeal (FM) by Tempeh (TMP) at 0% (D1, control diet), 40% (D2, basal diet) according to the optimum TMP inclusion level by Chor *et al*. (2015) and 40% with EAAs supplementation (D3, 0.5% Lys and 0.5% Met). TMP was obtained from local supplier in Kota Kinabalu, Sabah (manufactured through fermentation of soybean using Raprima Tempe Inoculum). Fresh TMP was oven-dried at 40ºC for 6 hours before ground into fine powder form. In preparing experimental diets, all dried ingredients were mixed thoroughly followed by approximately 40% of water and lipid sources to become dough. Crystalline Met and Lys were dissolved in water before mixing with other ingredients. The dough was pelleted through a meat mincer with the die size of 3mm diameter. The pellet was then oven-dried at 40ºC for 6 hours. All diets were sealed in bags and stored at -20ºC until used.

Fish husbandry

The feeding trial was conducted in the Fish Hatchery of Borneo Marine Research Institute, Universiti Malaysia Sabah. Juvenile *E. fuscoguttatus* were obtained from a local fish farm. Upon arrival, the fish were conditioned in a 10-ton fiberglass tank and fed with commercial marine fish feed (Cargill Marine Fish Feed, Extruded Slow Sinking Type No. 3) containing 43% crude protein and 8% crude lipid for two weeks.

Table 1. Diet formulation and proximate composition of the experimental diets

	Diets*		
	D1	D2	D3
Ingredients (g kg^{-1})			
Fishmeal	65.40	39.24	39.24
Tempeh	0.00	38.42	38.42
Cod liver oil	1.28	1.52	1.52
Soybean oil	8.43	1.49	1.49
Carboxymethyl cellulose	1.50	1.50	1.50
[a]Vitamin Premix	3.00	3.00	3.00
[b]Mineral Premix	2.00	2.00	2.00
Dicalcium phosphate	1.00	1.00	1.00
Lysine	0.00	0.00	0.50
Methionine	0.00	0.00	0.50
Alpha cellulose	7.33	8.32	7.32
Tapioca	10.06	3.51	3.51
Proximate analysis (%)			
Protein	50.59	51.79	52.23
Lipid	15.75	16.19	16.92
Moisture	9.04	10.44	9.81
Ash	11.37	9.72	8.54

[a] Contained (as/g/kg): ascorbic acid, 45; inositol, 5; choline chloride, 75; niacin, 4.5; riboflavin, 1; pyridoxine. HCL, 1; thiamin mononitrite, 0.92; calcium d-panthothenate, 3; retinyl acetate, 0.6; cholecalciferol, 0.083; menadione sodium bisulphate, 1.67; DL-α-tocopheryl acetate (poweder 500IU/g), 8; d-biotin, 0.02; folic acid, 0.09; vitamin B12, 0.00135; cellulose, 854.11.

[b]Reagent grade. Contained (as g/kg): calcium phosphate. H_2O (MDCP), 397.65; calcium lactate, 327; ferrous sulphate.H_2O, 25; magnesium sulphate. 7H_2O, 137; potassium chloride, 50; sodium chloride, 60; potassium iodide, 0.15; copper sulphate. 5H_2O, 0.785; manganese oxide, 0.8; cobalt carbonate, 0.1; zinc oxide, 1.5; sodium selenite. 5H_20, 0.02.

*Three diets were formulated to replace Fishmeal (FM) by Tempeh (TMP) at 0% (D1, control diet), 40% (D2, basal diet) according to the optimum TMP inclusion level by Chor *et al*. (2015) and 40% with EAAs supplementation (D3, 0.5% Lys and 0.5% Met).

Table 2. Essential amino acid composition (g 100g^{-1}g dry diet) of experimental diets

Essential amino acids**	Diets		
(g 100g^{-1} g fry diet)	D1	D2	D3
Val	2.55	2.45	2.59
Met	1.55	1.15	1.67
Lys	3.08	3.00	3.18
Ile	2.17	2.20	2.34
Leu	3.71	3.70	3.89
Phe	2.05	2.19	2.49
His	0.87	1.00	1.13
Arg	2.96	3.23	3.66
Thr	2.16	2.01	2.24

** **Val**: Valine; **Met**: Methionine; **Lys**: Lysine; **Ile**; Isoleucine, **Leu**: Leucine; **Phe**: Phenylalanine; **His**: Histidine; **Arg**: Arginine; **Thr**: Threonine.

Experimental fish with a mean initial body weight of 22.90±0.48g were randomly distributed into 9 cylindrical floating cages (50 cm depth and 50cm diameter) with 15 fish per cage at the beginning of the feeding trial. Each diet was randomly assigned to triplicate cages and placed in 10-tones fiberglass tank. Tanks were supplied with continuous aeration and flow-through seawater (30L min^{-1}). The fish were fed to apparent satiation twice a day at 0800 hours and 1500 hours. Weight of feed fed and number of uneaten feed particle of each cage was recorded half an hour after feeding. The mean weight of each feed particle was calculated by weighing 3 X 50 particles of each diet. Feces and uneaten feed were siphoned out half an hour later. Water parameters including dissolved oxygen, pH, temperature, salinity were maintained at 5.3-5.7 mg L^{-1}, 6.68-7.90, 27.7-30.3ºC and 30.75-33.52 ppt, respectively. Each replicate was bulk-weighed biweekly to observe the growth patterns. The growth performances, feed utilization, survival of the fish were evaluated after 8 weeks of feeding trial.

Samples collection

At the end of feeding trial, the fish were starved for 24-hour before harvest. Total number of fish and initial individual body weight on in each cage were recorded. A sample of 3 fish at the beginning and 3 fish per cage at the end of feeding trial were sacrificed and stored frozen (-20ºC) for proximate whole body composition.

Chemical analysis

Proximate composition analysis (crude protein, crude lipid, moisture and ash) on feed ingredients, experimental diets and experimental fish were performed by the standard methods of AOAC (Association of Official Analytical Chemists, 1995). Dry matter of samples was determined by oven-drying (Memmert Modell 500) at 108ºC for 24 hours; protein was determined according to Kjeldahl method by acid digestion (Foss Tecator Digester, Foss Analytical, Sweden) followed by distillation and auto-titration (Foss Kjeltec 230 auto distillation unit, Foss Analytical, Sweden); lipid by ether extraction using Soxtec method (Soxtec TM 2043 Extraction unit, Foss Analytical, Sweden); and ash by incineration at 550ºC for 5-h in furnace (Carbolite CWF 1300).

Amino acid concentrations of diet samples were analyzed by using a high performance liquid chromatography (HPLC) system consisting Waters 1525 Binary HPLC Pump, 717 Plus auto sampler (Waters®) and Waters 2475 Multi λ Fluorescence detector (wavelength excitation 250nm, emission 395nm) after acid hydrolysis using 6N HCl in glass tubes at 105ºC for 24 hours. Performic acid hydrolysis was carried out before acid hydrolysis to determine Met and cystine content. Tryptophan content was not analyzed in this analysis.

Data collection and statistical analysis

The body weight gain (WG), specific growth rate (SGR), survival rate (SR), feed intake (FI), feed conversion ratio (FCR), protein efficiency ratio (PER), and net protein utilization (NPU) of fish fed experimental diets were calculated after the feeding trial ended. The equations for calculating these parameters were listed as footnotes in Table 3. All data were presented as mean±SD and subjected to one-way ANOVA using SPSS ver21. Duncan's new multiple range test was used to test the differences among individual means. The difference was regarded as significant when $P < 0.05$.

RESULTS

Crude protein, Met and Lys of D3 (52.23%, 1.67 and 3.18 g $100g^{-1}$g dry diet, respectively) were the highest among diets (Tables 1 and 2). Growth performances, feed utilization and survival rate of *E. fuscoguttatus* juvenile after 8-weeks feeding trial are shown in Table 3. The highest growth performances in terms of WG and SGR were observed in fish fed with D1 (114% and 1.59, respectively) (P<0.05). Similar trend was observed in feed intake in which D1 group fish fed almost 150% of those fed with TMP-based diets (P<0.05). Supplementation of EAAs in TMP-based diet slightly but not significantly improved growth performance of *E. fuscoguttatus* juvenile in the present study (P>0.05). The best FCR value yielded by D1 (1.43), followed by D3 (1.64) and D2 (1.84) (P<0.05). On the other hand, feed utilization on TMP-based diet in terms of PER and NPU were improved and comparable to D1 group after EAAs supplementation in D3 (1.17% and 23.13%, respectively) (P<0.05). Survival rate of experimental fish in the present study were not affected by dietary treatments, which ranged from 91.11-95.56%.

Table 4 shows the whole-body proximate composition (% wet weight) of *E. fuscoguttatus* after 8-weeks feeding trial. Whole-body protein of fish fed TMP-based diet was slightly but insignificantly improved with EAAs supplementation (P>0.05). Highest whole-body lipid was observed in D1 group (6.94%) while lowest in those fed with D3 (4.83) (P<0.05). Fish fed with D1 exhibited significantly

Table 3. Growth performance, feed utilization and survival rate of *E. fuscoguttatus* after 8 weeks feeding trial

Growth performances	Diet		
	D1	D2	D3
Initial body weight (g)	22.78±0.85	22.86±0.35	23.04±0.17
Final body weight (g)	55.59±2.12^a	40.36±1.70^b	42.00±2.20^b
Weight gain (%)	114.31±14.20^a	76.51±7.21^b	82.26±8.93^b
Specific growth rate	1.59±0.10^a	1.01±0.01^b	1.07±0.09^b
Survival rate (%)	93.33±0.00	91.11±3.85	95.56±7.70
Feed intake (g fish^{-1})	45.51±2.04^a	31.78±1.16^b	30.80±3.92^b
Feed conversion ratio	1.43±0.06^a	1.84±0.10^c	1.64±0.11^b
Protein efficiency ratio (%)	1.13±0.18^{ab}	0.76±0.32^b	1.17±0.37^a
Net Protein Utilization (%)	20.22±3.86^{ab}	14.55±6.75^b	23.13±7.22^a

Different superscripted letters in each row indicate significant differences (P<0.05). **Weight gain (%)** = 100 x [(final weight (g) - initial weight (g))/ initial weight (g)], **specific growth rate (%/d)** = 100 x [ln (final weight (g))-ln (initial weight (g))/days], **survival rate (%)** = 100 x [(initial fish number- final fish number)/ initial fish number], **total feed intake (g fish^{-1})** = total feed intake for 8 weeks/ fish, **feed conversion ratio** = dry feed consumed (g)/ wet weight gain (g), **protein efficiency ratio** = wet weight gain (g)/ total protein intake (g), **net protein utilization (%)** = 100 x [(final fish body protein- initial fish body protein)/ (total protein intake)]

Table 4. Whole-body proximate composition (% wet weight) of *E. fuscoguttatus* after 8 weeks feeding trial

Proximate analysis	Diet		
	D1	D2	D3
Protein (%)	17.37±0.36	17.64±0.60	18.00±0.57
Lipid (%)	6.94±0.20^a	5.89±0.48^b	4.83±0.59^c
Moisture (%)	69.04±0.36^a	70.79±0.60^b	70.74±0.54^b
Ash (%)	6.04±0.23	5.84±0.06	6.00±0.17

Different superscripted letters in each row indicate significant differences (P<0.05).

lower whole-body moisture (69.04%) compared to those fed with TMP-based diets (70.74-70.79%) ($P<0.05$). Whole-body ash (ranging 5.84-6.04%) of all experimental fish were not affected by dietary treatments ($P>0.05$).

DISCUSSION

Poor palatability of TMP is believed to be one of the major constraints restricting the full utilization of TMP-based diet in the present study as also observed in our previous study (Chor *et al*., 2015). Supplementation of Met and Lys did not improve the palatability of TMP-based diet for *E. fuscoguttatus* as both of the EAAs may not serve as feeding stimulant for this species. Kasumyan (2004) reported that most amino acids are more often to be indifferent taste substances rather than as stimulants for fish, and some might even serve as feeding deterrents for certain species.

On the other hand, TMP-based diet supplemented with Met and Lys in the present study was able to improve protein utilization of *E. fuscoguttatus* juvenile. Although Met and Lys content in D3 were higher than in the control diet (D1) and the recommended level in other grouper species (0.97-1.31% and 2.83% for Met and Lys, respectively) (Chen, 2001; Luo *et al.*, 2005, 2006; Marzuqi, 2006), supplementation of both EAAs in current study might not met the optimum requirement of *E. fuscoguttatus* as there is no information available to date regarding the optimum dietary requirement of Met and Lys of the species. Murai *et al*. (1989) suggested optimum supplemental level of crystalline Met for carp, *Cyprinus carpio* and possibly other warm water fish species maybe in an extremely narrow and excess Met may adversely effects the performance. Fin erosion and formation of cataracts due to deficiencies of Lys and Met, respectively as reported in some fish species were not observed in current study (Luo *et al*., 2005, 2006). On the other hand, several studies have reported that supplementation of excessive dietary EAA in the fish diet could negatively affecting the normal growth and feed utilization if not lethal to the fish (Murai *et al*., 1989; Luo *et al*., 2005, 2006). Depressed growth performances and lower feed efficiencies were observed in Atlantic salmon (*Salmo salar* L.), rainbow trout (*Oncorhynchus mykiss*), Indian major carp (*Labeo rohita*) and mrigal carp (*Cirrhinus mrigala*) when higher concentrations of dietary Lys were supplemented in the diets (Luo *et al*., 2006). In another study using common carp (*Cyprinus carpio*), supplementation of just 0.50% Met (only up to 130% of the requirement of carp) was determined to be excessive and the beneficial effects of supplemented Met were cancelled by the toxicity of methinone itself (Murai *et al*., 1989). Investigation on the EAAs requirement of *E. fuscoguttatus* should be taken in the future in order to avoid negative effects of deficiencies or excess of dietary EAAs.

Another reason for poor growth performance of D3 group could be partially related to the method of amino acids supplementation used in current study. Although feed utilization was improved after EAAs supplementation in the present study, several studies have suggested that the growth performances and feed utilization of fish fed with diet supplemented with crystalline amino acids were inferior compared to amino acids in intact-protein (Alam *et al*., 2005; Yuan *et al*., 2011). Free amino acids tend to leach sooner than intact-protein into the environment before being consumed, thus reduced the nutritional values of the diet. Besides, it has been shown that CAA are also absorbed from the digestible tract much faster than amino acids in protein-bound forms, which caused an imbalance in tissue amino acid profile and diverting the amino acids into catabolic rather than anabolic process, while part of them is excreted directly through the urine (Murai *et al*., 1989; Luo *et al*., 2006; Nwanna *et al*., 2012). Alam *et al*. (2005) suggested that dietary value of supplemented Met and Lys could be further improved by reducing the leaching rate and increased the utilization of absorbed amino acids for protein synthesis through coating techniques as reported in kuruma shrimp (*Marsupenaeus japonicas*), red seabream (*Pagrus major*) and yellowtail (*Seriola quinqueradiata*).

Fish fed with crystalline Met and Lys in D3 exhibited slight increase of whole-body protein compared to D2 in this study which were in agreement with reports of dietary Lys and/or Met supplementation for orange-spotted grouper (*E. coioides*) and Chinese sucker (*Myxocyprinus asiaticus*) (Luo *et al*., 2005, 2006; Yuan *et al*., 2011). However, decreased of whole-body lipid of *E. fuscoguttatus* fed diet supplemented with EAAs in the present study was contradicted to those reported on *E. coioides* and *M. asiaticus*. On the other hand, similar results were reported in other studies in which whole-body lipid decreased inversely with the increased of whole-body moisture when soybean meal inclusion increased in the diets of Japanese flounder (*Paralichthys olivaceus*) and saddled bream (*Oblada melanura*) (Deng *et al*., 2006; Antovic' *et al*., 2012). Supplementation of Met and Lys mixture were also reported to increase the nitrogen retention and decreased lipid retention in Indian major carp, *(Labeo rohita H.*) (Sardar *et al*., 2009), which in turn explained the improved PER and NPU, as well as increased whole-body protein and reduction of whole-body lipid in the present study. Supplementation of EAAs in current study did not affect the whole-body ash content as also shown in *E. coioides* (Luo *et al*., 2006).

In conclusion, supplementation of crystalline Met and Lys in TMP-based diets for *E. fuscoguttatus* juvenile in the present study was able to improve the protein utilization but with little effect in promoting growth. Insufficient knowledge of optimum EAAs requirement of *E. fuscoguttatus* and supplementation method were believed to be the main reasons which hindered the full potential of supplemented Met and Lys in the diet.

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