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Effects of dietary methionine levels and sources on performance, blood lipid profile and histopathology in Nile tilapia (*Oreochromis niloticus*)

Amr Abd El-Wahab, Abeer Aziza, Hebatallah Mahgoub and Marwa Ahmed

Abstract
The present study was aimed to evaluate the effects of feeding different levels and sources of methionine (DL-Methionine and methionine hydroxy analogue, MHA) on growth performance and histopathological changes in Nile tilapia. The experiment was carried out over 60 days divided into 7 groups as follow: One group was fed a basic level of Met in the diet (5 g/kg diet) without any supplementation. The other groups were fed with different Met levels (+1/ +2/ +3 g/kg diet) as DL-Met or as MHA. Transmission electron microscopy and immunological index were also performed. Fish fed +1 g Met/kg as DL-Met had significantly higher body weight (58.1 g ± 3.85) than those fed +2 or +3 g Met/kg as DL-Met. No significant effects were found in body weight for fish fed +1 or +2 g Met/kg as MHA. All treatments induced significant reduction in the muscle thickness of the anterior segment of the intestine.

Keywords: DL-Met, MHA, Performance, Histopathology, Nile tilapia

1. Introduction
Aquaculture is one of the fastest growing food producing activities in the world. Tilapia is one of the most important freshwater farm raised fish and has had an increasing role in the global aquatic food trade. The Nile tilapia (*Oreochromis niloticus*) is an economically important freshwater fish in Egypt. Tilapia exhibit their best growth rates when fed with a balanced diet that provides a proper levels of protein, carbohydrates, lipids, vitamins, minerals and fiber [11, 27].

Fish nutrition has advanced dramatically in recent years with the development of new, balanced commercial diets that promote optimal fish growth and health [7]. Recently, the prices of different protein sources are tremendously increased. Therefore, looking for the exact amino acids requirements is nowadays very essential. Plant proteins are less expensive than animal sources; however it's deficiency in methionine and lysine limits its use as a main protein source in fish feeds [35].

Methionine is contained in both animal and plant protein sources. The need for a plant protein source has increased recently due to the decline in fish meal and the prohibition on the use of animal protein sources (meat and bone meal) due to the risk of pathogenic contamination [15]. In addition, the use of by-products of seed oil as a feed ingredient has increased. Therefore, more methionine supplementation is required.

Methionine is an essential amino acid for fish [22] and it is usually the first limiting amino acid in many fish diets, especially those containing higher levels of soybean meal protein source [15]. The dietary methionine supplementation has been indicated mainly in diets based on soybean protein, which have methionine as the first limiting amino acid for Nile tilapia [14, 25]. The dietary methionine requirements have been estimated for several species of fish, ranging from 1.80 to 4.00 g/kg of dietary protein [36]. This amino acid acts as methyl donor [3] precursor of several substrates, including nucleic acids, proteins, phospholipids [23], carnitine and choline [32], cystine [40]. Thus, methionine supplementation is associated with improvements of fish growth and health [12, 16].

In general, a single methionine requirement level is recommended based on weight gain of catfish when fed diets containing 8 to 10% fish meal with 32 to 36% CP. There is an increasing need for least-cost formulated diets with minimal fish meal and maximal plant

proteins. Studies have shown that diets including large amounts of plant proteins contain antinutritional compounds [17, 27], which may limit bioavailability of amino acids. Methionine alone can generally meet the requirement for sulfur-containing amino acid (SAA) [2] and all-plant-protein diets are most limiting for SAA.

Several analogues of Met exist, but methionine hydroxy analogue (MHA) is the most common analogue used in animal diets. Its chemical structure is similar to that of Met with a hydroxy group substituted in the amino component [20].

The aim of the present study was to investigate the effects of different dietary surplus methionine levels and its sources (DL- Met and MHA) on growth performance, some blood lipid profile as well as histopathology of Nile tilapia fish.

2. Materials and methods

2.1 Fish, diets and housing

Four hundred and twenty unsexed fish were received and stocked in 21 glass aquaria (80 cm length, 35 cm width, 40 cm height), 20 fish in each. Each diet was fed to the fish in triplicate aquarium at 3% body weight [10] twice daily (9.00 h-13.00 h) for 60 days. Fish were subjected to a photoperiod regimen of 12-13 h light and 12-11 h dark/ day and the temperature during the experimental period ranged from 24-27 °C. Daily cleaning for each aquarium was carried out with partial replacement of water by previously stored (for 48 hours) dechlorinated tap water. During the experiment, the water quality was checked every week.

Seven isonitrogenous (32%) and isocaloric (3000 Kcal DE /kg) diets were formulated to satisfy the nutrition requirements of Nile tilapia (*O. niloticus*) according to [NRC, 2011]. Dietary ingredients were similar in seven diets except the level and source of methionine. Composition and the proximate analysis of the control basal diet are listed in Table (1). These seven experimental diets were:One group was fed a basic level of Met in the diet (5 g/kg diet) without any supplementation (control group) according to [NRC, 2011]. The other groups were fed with different Met levels (+1/ +2/ +3 g/kg diet) as DL-Met (containing 99% Met) or as methionine hydroxy analogue (MHA, containing 84% Met).

Daily feed consumption for each 2 weeks was recorded. Dietary allowances of each experimental group was adjusted bi-weekly, after weighing of fish, according to mean body weight, then placed in small plastic bags for each aquarium for daily feeding of fish.

Table 1: Composition (%) and chemical analysis (g/kg as fed) of diet fed to fish during experimental period

Ingredients (%)		chemical composition (g/kg)			
yellow corn	31	crude fat	54.7	Ca	19.5
soybean meal	24.5	crude fibre	27.7	P	11.2
fish meal	22	NfE	402	Na	3.60
wheat bran	16	starch	241	Cl	6.26
corn gluten	3.2	sugar	35.2	K	7.76
soybean oil	2	threonine	12.7	S	3.82
min. and vit. Premix[1]	1	tyrosine	12.0	microminerals (mg/kg)	
NaCl	0.3	cystine	4.78	Cu	30.3
chemical composition (g/kg)		methionine	5.18	Zn	337
dry matter	898	lysine	12.3	Fe	496
crude ash	82.4			Mn	326
crude protein	320	macrominerals (g/kg)		Se	0.75

[1]vitamin mixture supplies the following per kilogram of diet:: vit. A -1,200.000 IU; vit. D3 - 200,000 IU; vit. E - 12,000 mg; vit. K3 - 2,400 mg; vit. B1 - 4,800 mg; vit. B2 - 4,800 mg; vit. B6 - 4,000 mg; vit. B12 - 4,800 mg; folic acid - 1,200 mg; vit. C - 48,000 mg; biotin - 48 mg; choline - 65;000 mg; niacin - 24,000 mg; Fe - 10,000 mg; Cu - 600 mg; Mg - 4,000 mg; Zn - 6,000 mg; I - 20 mg; Co - 2 mg; Se - 20 mg

2.2 Sample collections

At the end of experimental period (60 days), random fish samples (3 fish/ aquarium) from all experimental groups were collected and minced for whole body proximate chemical composition. Body weight and gain were calculated bi-weekly. Similarly, the feed intake and feed conversion ratio (FCR) were calculated bi-weekly. Fish was watched daily for mortalities and any dead fish was discarded and weighed. Five fish were collected for determination of the whole body composition. Samples of whole fish body were ground in an electric meat grinder to obtain homogeneous samples.

2.3 Serum metabolites

Blood samples were taken from the caudal vein of 3 fish per aquaria. The plasma was obtained by centrifugation at 1200 g for 8 min at room temperature. After centrifugation, the plasma was used for analysis of total cholesterol, total lipids, triglycerides, low-density lipoprotein (LDL) and high-density lipoprotein (HDL). The levels of plasma triglycerides and cholesterol were determined using colorimetric enzymatic method and photometric enzymatic method, respectively. The HDL and LDL were determined according to [Elliott, 1975]. The chemical analyses of diets and whole body were determined following the methodology described by [Silva *et al*., 2002].

2.4 Histopathology

Intestine (anterior segment) collected from all experimental groups was processed for haematoxylin and eosin staining of tissue sections at the Histology Department, Faculty of Medicine, Mansoura University, Egypt. Tissue sections were examined and photos were taken using Apex practitioner light microscope. Goblet cells were counted alongside recording histopathological alterations.

2.5 Intestinal-image analysis

Using ImageJ software, http://rsb.info.nih.gov/ij/, the villous area and muscular thickness of the anterior segment of the intestine were calculated. The villous area was calculated as a ratio from the whole image area.

2.6 Transmission electron microscopy (TEM)

Five to 10 small pieces of the anterior segment of the intestine (1 x 1 mm in size) were collected from each experimental group and fixed in 5% glutaraldehyde immediately after dissecting for 24-48 h. The specimens processing for TEM was performed at Electron microscopy Unit, Assiut University, Egypt.

2.7 Immunological index
Total bacterial load of anterior segment of intestine (one cm of the anterior segment) from all experimental groups were separately crushed in sterile phosphate buffered saline, pH 7.4 (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 in one litre) and an aliquot was spread on nutrient agar, pH 6.8 (5 g peptone, 3 g yeast extract, 5 g NaCl, and 15 g agar in one litre water), incubated overnight at 37 °C, and counted.

2.8 Statistical analyses
The results were subjected to one-way ANOVA to test the influence of excess dietary of methionine on performance, lipid profile of Nile tilapia. Data were analysed using statistical SPSS V20 (SPSS Inc., Chicago, IL, USA). Differences between means were compared using Duncan's multiple range test at significance of differences (P<0.05) among dietary treatments. Histopathological scoring data were analysed using Statpages website (http://statpages.org/). All experimental groups were compared with the negative control group using one-way analysis of variance (ANOVA) Turkey HSD post-hoc test for pairwise comparison.

3. Results
3.1 Performance
The effect of different levels and source of Met on performance parameters of Nile tilapia fish is present in Table (2). Fish fed diet containing basal diet +1 g Met/kg as DL-Met had significantly (*P*<0.05) higher body weight (58.1 g ± 3.85) than those fed +2 g or +3 Met/kg as DL-Met (47.6 g ± 3.98 and 43.4 g ± 2.51, respectively). No significant effects were found between groups fed either +1 or +2 g Met/kg as MHA. However, fish fed diet supplemented with basal diet +1 g Met/kg as DL-Met or MHA had numerically (*P*>0.05) higher body weight (58.1 g ± 3.85 and 57.7 g ± 3.14, respectively) than other experimental groups. Fish fed +3 g Met/kg either as DL-Met or as MHA had significantly (*P*<0.05) the lowest body weight (43.4 g ± 2.51 and 45.2 g ± 3.51, respectively) than other experimental groups except (group fed +2 g Met/kg as DL-Met).

The body weight gain in groups fed diets containing +3 g Met/kg either as DL-Met or as MHA had significantly (*P*<0.05) the lowest body weight gain (33.5 g ± 0.49 and 34.7 g ± 0.28, respectively) compared to the other experimental groups (Table 2). The significant highest body weight gain was recorded for fish fed +1 g Met/kg diet either as DL-Met or MHA (47.8 g ± 1.11 and 48.2 g ± 0.50, respectively) compared to other groups. The lowest FCR (0.86) was recorded for fish fed +3 g Met/kg as MHA in comparison to other experimental groups (Table 2), while the highest FCR (1.33) was found for fish fed +3 g Met/kg as DL-Met.

The weight of whole body and some internal organs were shown in Table 3. The lowest significant (*P*<0.05) weights of whole body (38.8 g ± 9.58 and 40.9 g ± 10.2) were recorded for fish fed +3 g Met/kg either as DL-Met or as MHA in comparison to those fed (+1 g Met/kg either as DL-Met or as MHA).

No marked differences were found among the experimental groups regarding weight of spleen. However, the highest liver weight (1.07 g ± 0.15) was recorded for fish fed control diet in comparison to other experimental groups (Table 3).

Table 4 shows the effect of DL-Met and MHA levels on whole body composition. A moisture content was found to be significantly different (*P*<0.05) among fish given the experimental diets. Fish fed control diet or +1 g Met/kg as MHA had significantly the higher moisture content in the body compared to those fed +2 g Met/kg either as DL-Met or as MHA. The crude protein content was significantly (P<0.05) the lowest (40.4 ± 0.17) for fish fed control diet in comparison to experimental groups. Fish fed +3 g Met/kg as DL-Met or as MHA had significantly (*P*<0.05) the lowest lipid content (13.8% ± 0.36 and 13.1% ± 0.45, respectively) compared to other experimental groups (Table 4). A significant differences were found between fish fed 5 g Met/kg (control) and those fed +2 g Met/kg either as DL-Met or MHA.

Data in Table (5) representing means for some blood biochemical levels of fish given diets containing different levels of Met as DL-Met or MHA. Blood cholesterol content of fish given 5 g Met/kg (control) was significantly (*P*<0.05) the highest (38.9 ± 2.85 mg/dl ± 1.93) in comparison to other experimental groups. The lowest significant value (*p*<0.05) of cholesterol (14.1 mg/dl ± 0.85 and 15.6 mg/dl ± 2.85) were recorded for fish fed +3 g Met/kg either as DL-Met or as MHA than other experimental groups. However, the highest significant value of triglycerides (136.6 mg/dl ±4.2) was recorded for fish fed +3 g Met/kg than other experimental groups. While, fish fed control diet or +3 g Met/kg as MHA had significantly (*p*<0.05) the lowest triglycerides value (92.4 mg/dl ± 3.5 and 86.1 mg/dl ± 5.6) compared groups had surplus levels of DL-Met (Table 5).

Table 2: Growth performance of fish fed the experimental diets (mean ± SD)

Parameters	Dietary treatments						
	Control 5 g Met/kg	+1 g Met/kg as DL-Met	+2 g Met/kg as DL-Met	+3 g Met/kg as DL-Met	+1 g Met/kg as MHA	+2 g Met/kg as MHA	+3 g Met/kg as MHA
IBW	10.6±2.52	10.3±2.91	10.1±2.77	9.9±2.47	9.5±3.11	10.7±2.9	10.5±3.3
FBW	54.7^{a}±3.64	58.1^{a}±3.85	47.6^{b}±3.98	43.4^{b}±2.51	57.7^{a}±3.14	54.6^{a}±4.16	45.2^{b}±3.51
BW gain	44.1^{b}±1.18	47.8^{a}±1.11	37.5^{c}±0.50	33.5^{d}±0.49	48.2^{a}±0.50	43.9^{b}±0.54	34.7^{d}±0.28
FCR	0.88^{b}±0.092	0.92^{b}±0.092	1.10^{a}±0.091	1.33^{a}±0.110	0.86^{b}±0.034	1.01^{ab}±0.087	1.32^{a}±0.077

IBW=initial body weight, FBW= final body weight, FCR= feed conversion ratio ^{a,b}Means in the same row with different superscripts are significantly different (*p*<0.05)

Table 3: Weight (g, mean±SD) of whole body and some internal organs of 5 fish at end of the trial

Item (g)	Dietary treatments						
	Control 5 g Met/kg	+1 g Met/kg as DL-Met	+2 g Met/kg as DL-Met	+3 g Met/kg as DL-Met	+1 g Met/kg as MHA	+2 g Met/kg as MHA	+3 g Met/kg as MHA
Whole body	56.7^{ab}±12.7	59.4^{a}±11.2	53.6^{ab}±2.53	38.8^{b}±9.58	58.3^{a}±8.93	56.1^{ab}±7.66	40.9^{b}±10.2
Spleen	0.2160.111	0.250±0.095	0.210±0.052	0.223±0.119	0.232±0.081	0.238±0.021	0.218±0.048
Organo somatic index	0.381±0.243	0.421±0.273	0.391±0.115	0.574±0.205	0.397±0.211	0.424±0.057	0.533±0.125
Liver	1.07±0.15	0.876±0.211	0.786±0.125	0.776±0.231	0.600±0.218	0.595±0.021	0.610±0.153
GIT	1.76±0.420	1.25±0.191	1.16±0.152	1.43±0.625	1.32±0.187	0.810±0.183	0.850±0.531

^{a,b}Means in the same row with different superscripts are significantly different (*p*<0.05)

Table 4: Effects of dietary DL-Met and MHA on whole body composition for 5 fish/group (mean±SD)

Parameters, %	Dietary treatments						
	Control 5 g Met/kg	+1 g Met/kg as DL-Met	+2 g Met/kg as DL-Met	+3 g Met/kg as DL-Met	+1 g Met/kg as MHA	+2 g Met/kg as MHA	+3 g Met/kg as MHA
Moisture	73.3^{a}±0.35	72.4^{ab}±0.74	71.7^{b}±0.72	71.1^{b}±0.31	73.1^{a}±0.63	71.5^{b}±0.60	71.2^{b}±0.47
CP	40.4^{c}±0.17	42.6^{ab}±0.40	43.9^{a}±0.35	44.2^{a}±0.23	41.8^{ab}±0.17	42.9^{ab}±0.20	43.5^{a}±0.26
Ash	24.1^{a}±0.61	23.8^{a}±0.51	24.2^{a}±0.35	23.7^{a}±0.65	23.9^{a}±0.47	23.8^{a}±0.15	24.0^{a}±0.75
Lipid	19.1^{a}±0.49	17.3^{ab}±0.71	15.8^{b}±0.35	13.8^{c}±0.36	16.4^{ab}±0.39	14.8^{b}±0.15	13.1^{c}±0.45

^{a,b}Means in the same row with different superscripts are significantly different (p<0.05)

Table 5: Effects of dietary DL-Met and MHA on some blood lipid profile in Nile tilapia

Parameters (mg/dl)	Dietary treatments						
	Control 5 g Met/kg	+1 g Met/kg as DL-Met	+2 g Met/kg as DL-	+3 g Met/kg as DL-Met	+1 g Met/kg as MHA	+2 g Met/kg as MHA	+3 g Met/kg as MHA
Cholesterol	38.9^{a}±1.93	28.8^{b}±0.95	23.9^{c}±2.37	14.1^{d}±0.85	30.1^{b}±1.65	24.5^{c}±0.95	15. 6^{d}±2.85
Triglycerides	92.5^{c}±3.50	119.8^{b}±2.10	111.4^{b}±2.10	136.6 ^{a}±4.20	104.1^{cb}±5.31	104.1^{cb}±3.96	86.2^{c}±5.60
HDL	48.3^{ce}±3.21	54.2^{c}±2.68	68.4^{b}±1.89	74.5^{b}±1.61	56.3^{c}±1.61	89.5^{a}±2.68	91.2^{a}±1.07

^{a,b}Means in the same row with different superscripts are significantly different (p<0.05)

3.2 Intestinal histopathological alterations

Apart from shortening of the intestinal villi and the reduced thickness of the muscular layer, noticed with the highest doses of both treatments, clear vacuoles were seen in some samples of both treatments (Figure 1). In addition, hypercellularity of the villous lamina propria was noticed when the highest dose of DL-Met was used. Sometimes, hypercellularity was noticed in the intestinal epithelium, when the lowest dose of MHA was used (Figure 1E).

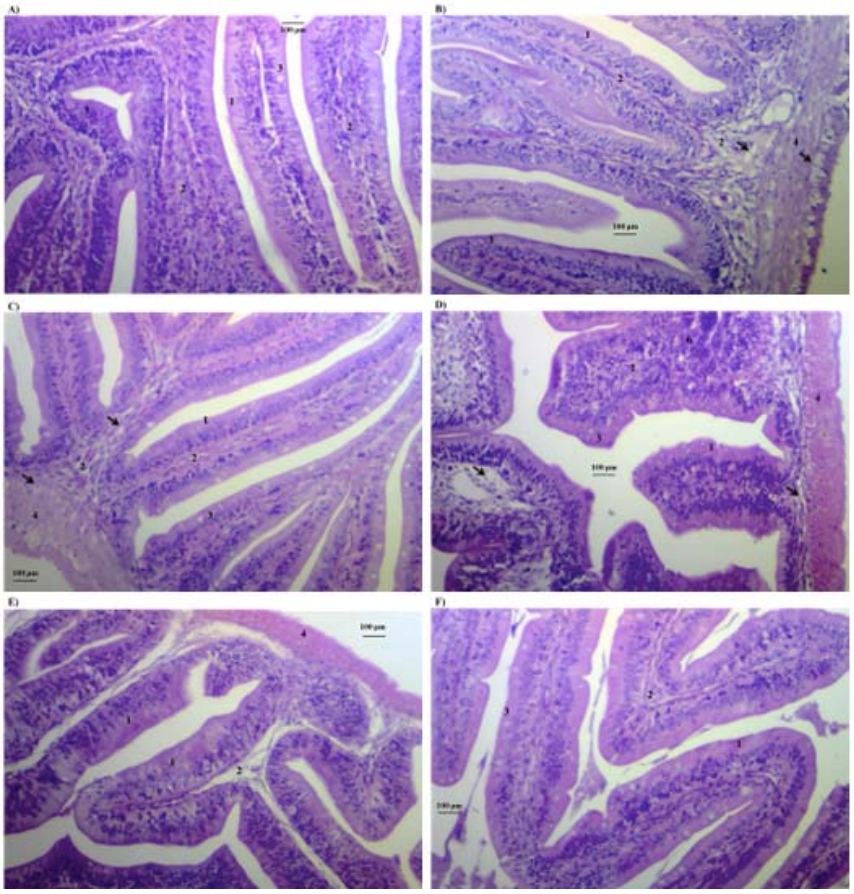


Fig 1: Histopathological examination of the anterior segment of the Nile tilapia intestine. A) Negative control group. B) Group that received DL-Met +1 g/kg diet. C) Group that received DL-Met +2 g/kg diet. D) Group that received DL-Met +3 g/kg diet. E) Group that received MHA +1 g/kg diet. F) Group that received MHA +2 g/kg diet. G) Group that received MHA +3 g/kg diet. 1: Villous intestinal epithelium; 2: lamina propria; 3: goblet cell; 4: muscular layer; 6: hypercellularity of the lamina propria. Arrows represent vacuolation noticed both in the lamina propria and muscular layer.

3.3 Villous area and muscular thickness

Compared to the control group, only groups treated with +3 g Met/kg of either DL-Met or MHA showed significant decrease in villous area (Figure 2). Similarly, all treatments induced significant reduction in the muscle thickness of the anterior segment of the intestine (Figure 3).

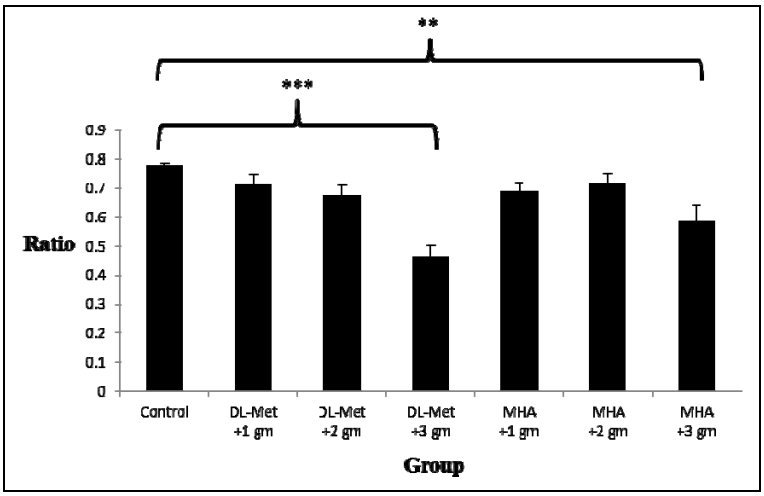


Fig 2: Image analysis of the villous area. The villous area was calculated as a ratio from the whole image area using ImageJ software. Results were statistically analysed using Statpages website. All experimental groups were compared with the negative control group using one-way analysis of variance (ANOVA) Turkey HSD post-hoc test for pairwise comparison. Error bars represent standard error of the mean (SEM). *=p< 0.05; ***=p<0.001.

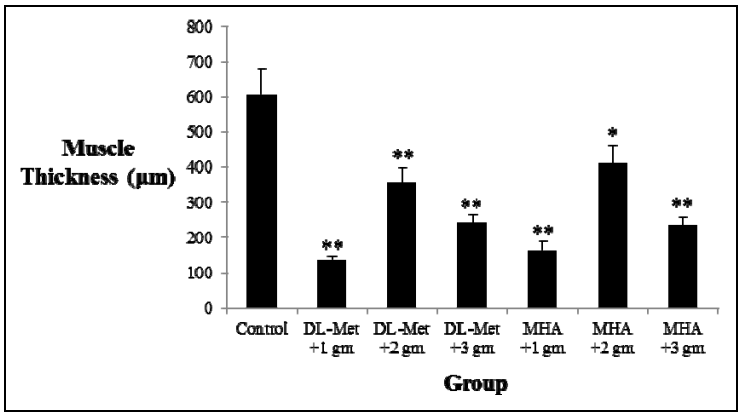


Fig 3: Image analysis of the intestinal muscle thickness. The muscle thickness was measured using ImageJ software. Results were statistically analysed using Statpages website. All experimental groups were compared with the negative control group using one-way analysis of variance (ANOVA) Turkey HSD post-hoc test for pairwise comparison. Error bars represent SEM. *=p< 0.05; **=p< 0.01.

3.4 TEM of the anterior segment of the intestine

The intestinal covering epithelium of the control group was formed of enterocyte and numerous goblet cells (Figures 4A and 4B). The enterocytes were elongated or tail cells, having oval nucleus situated at the base and the cell organelles, such as mitochondria which appear numerous and of variable shape and sizes, are mostly situated at the upper part of the cell. The cytoplasm also contains free ribosomes, rough endoplasmic reticulum (RER) and small electron-dense granules. The luminal surface of the enterocytes had long stretched microvilli. The goblet cells were few in number and filled with mucinous granules. The presence of lobulated leukocytes, in few number in-between the enterocytes, was also noticed.

The enterocytes of the intestine of group two (DL-Met +1 g Met/kg diet) showed moderate vacuolation and disintegration of most cellular organelles, accompanied with the presence of numerous electron-dense lysosomes (Figures 4C and 4D). The nucleus became rounded and having electron-dene nucleolus in some cells. Some cells were ruptured as a result of severe vacuolation. The goblet cells became small in size. The presence of polymorphonuclear leukocytes containing lysosomal granules and cell organelles was also recorded.

The lining epithelium of the intestine of group three (DL-Met +1 g Met/kg diet) showed severe vacuolation of most enterocytes, accompanied with shrinkage and increased electron density of the nucleus (Figures 4E and 4F). Condensation of the cytoplasm of some cells was also noticed. The mitochondria became more electron-dense with disintegration of its cristae. Disintegration of the other cell organelles occurred. Some areas of the enterocytes became more electron-dense and condensed, which seemed to be in a state of coagulative necrosis. Besides the vacuolation and disintegration of the cellular organelles, pyknosis of the nucleus and presence of small electron-dense granules and fat droplets, which appeared spherical and electron-dense, were observed. The goblet cells became hypertrophied and contained large amount of mucinous granules, which appeared light electron-dense.

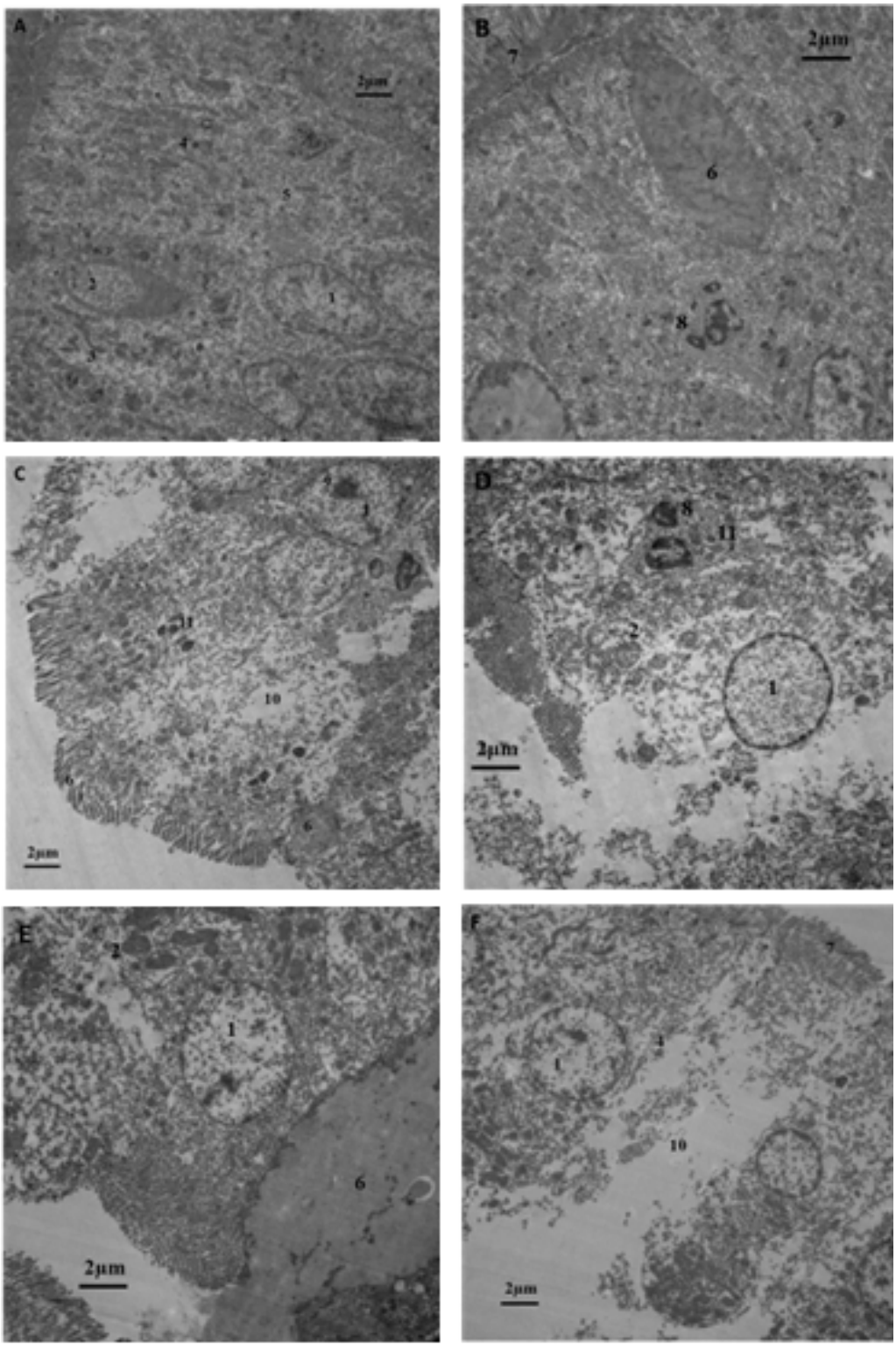
The epithelium of the intestinal mucosa of group four (DL-Met +3 g Met/kg diet) showed diffuse necrosis, which consisted of cellular debris and pyknotic or fragmented nucleus (Figure 4G).

The lining epithelium of the intestine of group five (MHA +1 g Met/kg diet) showed mild morphological changes (Figures 4H and 4I). The enterocytes appeared elongated, having oval nucleus situated at the base, and the mitochondria were crowded at the upper part of the cell, with presence of free ribosomes, RER, and small electron-dense granules. The

enterocytes contained numerous medium size electron-dense spherical fat globule. The luminal surface of the enterocytes contained stretched elongated microvilli. The subepithelial mucosa contained numerous cells which contained large amount of spherical electron-dense granules of variable sizes. These cells were similar to mast cell in mammals.

The changes of intestinal epithelium of group six (MHA +2 g Met/kg diet) were mostly similar to other experimental groups, with hypertrophy of goblet cells and the presence of numerous polymorphonuclear cells in-between the enterocytes (Figures 4J and 4K). Some enterocytes showed increased electron-density of their cytoplasm and became shrunk.

Most of the enterocyte of group seven (MHA +3 g Met/kg diet) became vacuolated, and the nucleus became condensed and electron-dense, with presence of numerous leukocytes (Figure 4L). The degenerated cells showed numerous fat globules (spherical in shape and electron-dense). The nucleus of some degenerated cells showed clumped nuclear chromatin at the periphery. Goblet cells were numerous and loaded with mucinous granules.



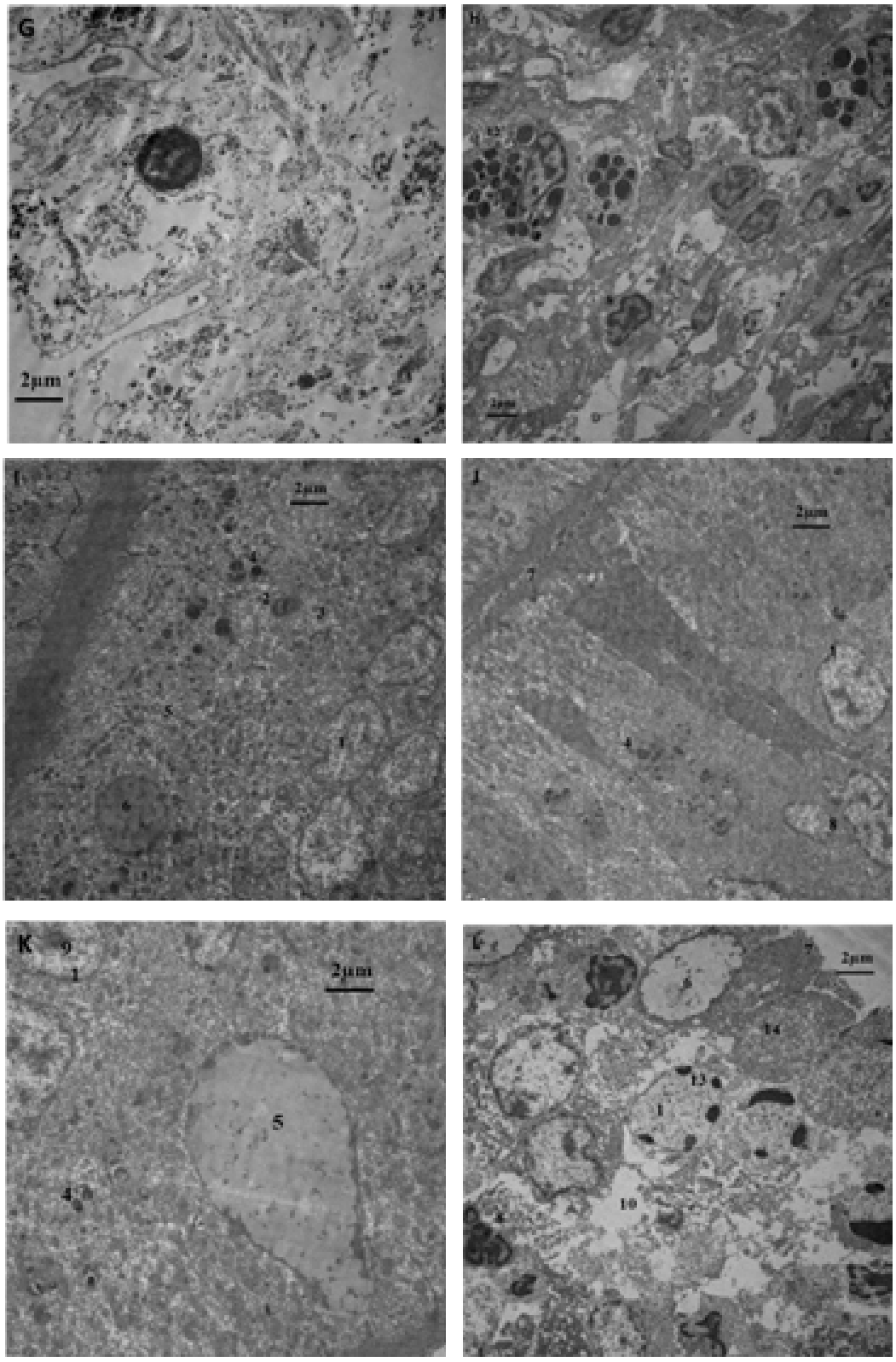


Fig 4: TEM of the anterior segment of the intestine. A and B refer to the control group. C and D refer to group two (DL-Met +1 g/kg diet). E and F refer to group three (DL-Met +2 g/kg diet). G refers to group four (DL-Met +3 g/kg diet). H and I refer to group five (MHA +1 g/kg diet). J and K refer to group six (MHA +2 g/kg diet). L refers to group seven (MHA +3 g/kg diet). 1= nucleus; 2= mitochondrion; 3= RER; 4= electron-dense granules; 5= ribosomes; 6= goblet cell; 7= microvilli; 8= polymorphonuclear leukocyte; 9= nucleolus; 10= vacuolation; 11= lysosomes; 12= mast cell-like cell; 13= clumped chromatin; 14= fat globules.

3.5 Total bacterial count of the intestine

Both the medium and highest levels of DL-Met increased the total bacterial load in the anterior segment of the intestine, compared to the control group (Figure 5). However, no significant alteration was detected in any other experimental group.

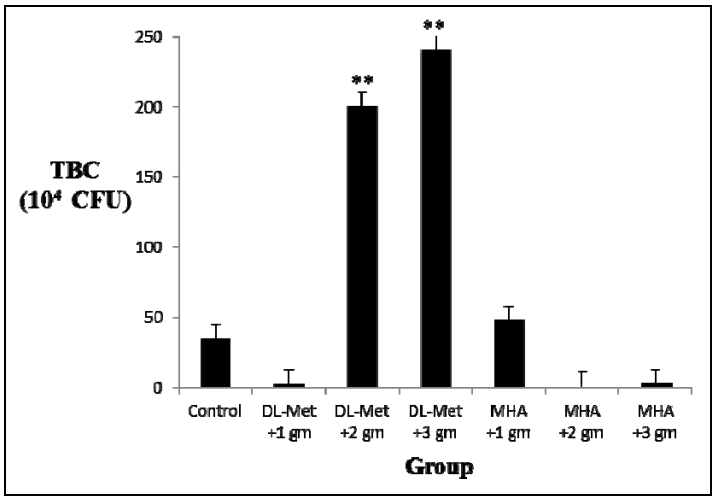


Fig 5: Total bacterial load of the anterior segment of the intestine. The presented results are to be multiplied by 10^4. Results were statistically analysed using Statpages website. All experimental groups were compared with the negative control group using one-way analysis of variance (ANOVA) Turkey HSD post-hoc test for pairwise comparison. Error bars represent SEM. **=$p< 0.01$.

3.6 Organosomatic index of the spleen

None of the experimental groups showed any significant variation, in the organosomatic index of the spleen, from the negative control group (Figure 6).

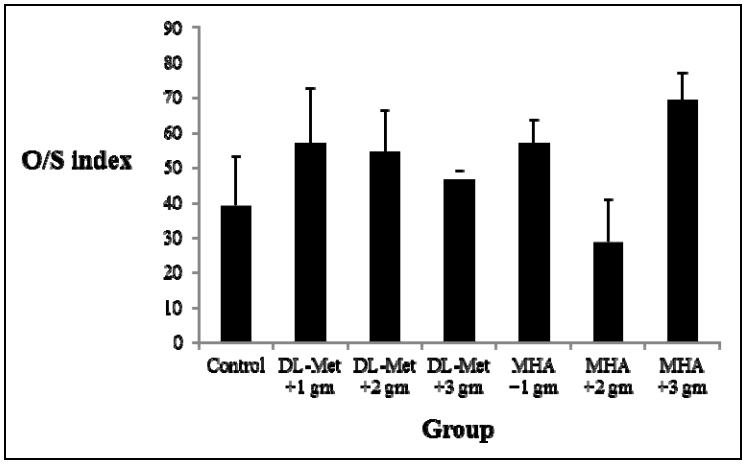


Fig 6: Organosomatic index of the spleen. Results were statistically analysed using Statpages website. All experimental groups were compared with the negative control group using one-way analysis of variance (ANOVA) Turkey HSD post-hoc test for pairwise comparison. Error bars represent SEM. O/S= organosomatic.

4. Discussion

Water quality parameters were consistently within acceptable ranges during the course of the study. The average values of temperature, dissolved oxygen and pH of the water cages were 27.20 ± 1.15ºC, 5.53 ± 0.18 mg/L and 7.24 ± 0.56, respectively. The data obtained are within the range recommended by [Sipauba-Tavares, 1994] for tropical freshwater fish.

Methionine is one of essential amino acids required by all animals including fish for protein synthesis. Methionine must be present as a source for sulphur as well as for methyl group donor [12] and plays a role in lipid metabolism [4]. The results suggested that increasing Met content (+3 g Met) in fish diets as DL-Met or as MHA had the lowest final BW. However, no significant differences were noted among control group and higher levels of Met (+1 g Met/kg diet). It means that higher Met supplementation did not improve fish performance further. The present findings are in agreement with [Cheng *et al.*, 2003] who concluded that MHA has a positive effect on fish performance when a diet deficient in methionine and cystinee is used. Some studies also reported that supplementing tilapia diets with crystalline essential amino acids did not improve Nile tilapia performance [34]. Nevertheless, positive effects of supplemental amino acids especially lysine and methionine have been demostrated [9].

Met requirements have been reported varying from 2% to 3.75% of dietary protein in different fish species [18].It has been reported that Met requirement of Nile tilapia was 4.90 g/kg with practice diet, which was higher than with semi-purified diet (4.30 g/kg) [24, 26]. It is important to consider that the requirements of methionine + cystine also have to be assessed separately in fish diets, as well as other non-ruminants that require a minimum of dietary methionine, considering that methionine can not be obtained from cystine (trans-sulfidation). Thus, based on the results of this study, a minimum of 5 to 6 g/kg of methionine is required for optimum performance and weight gain, in diets for the Nile tilapia. In this study, the highest levels of protein in the whole body are in accordance with the results obtained by [Yan *et al.*, 2007].

Moreover, the authors observed a reduction in the whole body fat linearly with increasing dietary Met level.

The highest concentration of protein in the whole body, in addition to lower levels of fat, are probably related to the functions of methionine on the synthesis of body protein and lipid metabolism. Methionine is an essential amino acid for normal growth of fish, because it participates in the protein synthesis and other metabolic functions [1, 33]. The reduction of the total cholesterol, total lipids and plasma triglycerides may be related to the different functions of methionine. Methionine affects the profile ofplasma amino acids in many species of fishas well as the metabolism of sulphur amino acids [12]. The S-adenosylmethionine,synthesized from methionine, originates compounds such as carnitine and choline [32]. Choline also acts as a lipotropic factor, enhances the synthesis of lipoproteins and prevents fatty liver [32]. The choline molecule has three methyl groups (-CH3), and reacts with acetyl coenzyme A, acting as a precursor of acetylcholine, a neurotransmitter [38] and phosphatidylcholine, which is a structural component of cell membranes acting also in the metabolism and transport of lipid-cholesterol [3]. It also acts as a lipotropic factor, and improves the synthesis of lipoproteins and prevents fatty liver [32]. This can resulted lower levels of whole body fat.

Met is the precursor of S-adenosyl methionine (SAM), and SAM is a substrate for de novo synthesis of choline [5]. Adequate Met in diet promotes choline and carnitine synthesis in hepatopancreas and then provided enough phospholipids and Acetyl-CoA for cholesterol and lipoprotein synthesis [39]. HDL-C is one of the serum lipoprotein, which can transport cholesterol from the surrounding tissue to liver for synthesis of bile acid then remove out of body. Because HDL-C particles are small, it can freely in and out of the arterial wall absorb the harmful material such as LDL-C and TG, then transport to liver decomposition and excretion. In the present study, plasma HDL-C increased with high dietary Met levels, which indicated that appropriate amount of dietary Met could promote cholesterol transported to hepatopancreas.

Histopathological examination of the anterior segment of the intestine revealed hyperculluarity in the lamina propria of fish treated with the highest dose of DL-Met. This observation coincided with the high bacterial load recorded in the same group. It could be possible that the high bacterial load was responsible for an immune response, that increased the cellularity of the lamina propria. However, this phenomenon could not be explained. In previous literature, adding MHA to drinking water of nursery pigs did not alter the total bacterial count in the caecum, but increased the villous height in the duodenum, jejunum and ileum and and the villous height:crypt depth ratio in the jejunum and ileum [19]. On the other hand, feeding DL-Met to chickens had enhancing effect on the duodenal crypt depth and villus height:crypt depth ratio [29]. In this study, dietary supplementation of either form of Met (DL-Met or MHA) decreased the villous area and muscle thickness of the anterior segment of the intestine. In contrast, previous literature indicated the enjancing effect of dietary intake of Met on crypt cell proliferation in the ileum and colon in an intestinal model of chemical carcinogenesis in rats [8]. Similarly, dietary Met restriction inhibited colon carcinogenesis, through reduction in aberrant crypt foci and colonic cellular proliferation,in an intestinal model of chemical carcinogenesis in rats [21]. Taken together, it seemed that overdoses with either DL-Met or MHA had deteriorating effect on intestinal health in Nile tilapia.

Dietary supplementation of either form of Met (DL-Met or MHA) failed to induce any alteration in the organosomatic index of the spleen. This could be attributed to lack of immune response induction by either form of Met. In an earlier study, Met dietary supplementation failed to stimulate chickens humoural immune response following various vaccines application [28].

5. Conclusion

Performance of fish fed surplus levels of Met (+1 g Met/kg) either as DL-Met or as MHA was not significantly differ from those fed control diet. It means that, in normal situations, MHA can be used as a source of Met without any negative effect on growth. MHA is economically available at the present time, although other hydroxy analogues may be more economical for more extensive supplementation in fish diets in the future. A positive effect on cholesterol level in blood was associated with dietary Met supplementation. Nevertheless, it seemed that overdoses with either DL-Met or MHA had histopathological deteriorating effect on intestinal health in Nile tilapia.

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7. References

1. Alam MS, Teshima S,Ishikawa M. Methionine requirement of juvenile Japanese flounder *Paralichthys olivaceus* estimated by the oxidation of radioactive methionine. Aquacult. Nutr. 2001; 7:201-209.
2. Baker DH. Construction of assay diets for sulfur-containing amino acids. Methods Enzymol. 1987; 143:297.
3. Bender DA. Nutritional biochemistry of the vitamins. 2ed Cambridge, USA: Cambridge University Press, 2003, 488.
4. Brosnan JT,Brosnan ME. The sulphur containing amino acids: an overview. J Nutr. 2006;136:1636-1640.
5. Chawla RK, Watson WH, Eastin CE, Lee EY, Schmidt J, McClain CJ. S-adenosylmethionine deficiency and TNF-a in lipopolysaccharide-induced hepatic injury. Am. J Physiol. 1998;275:125-129.
6. Cheng ZJ, Hardy RW, Blair M. Effects of supplementing methionine hydroxyl analogue in soybean meal and distiller's dried grain-based diets on the performance and nutrient retention of rainbow trout. Aquacult. Res. 2003; 34:1303-1310.
7. Craig S, Helfrich LA. Understanding Fish Nutrition, Feeds, and Feeding Department of Fisheries and Wildlife Sciences; Virginia Tech. 2002, 420-256.
8. Duranton B, Freund JN, Galluser M, Schleiffer R, Gossé F, Bergmann C, *et al*. Carcinogenesis, 1999; 20(3):493-497.
9. El-Dahhar AA,El-Shazly K.Effect of essential amino acids (methionine and lysine) treated oil in fish diet on growth performance and feed utilization of Nile tilapia, *Tilapia nilotica* (L.). Aquacult. Fish. Manage. 1993; 24:731-739.
10. Elliott JM. The growth rate of brown trout (*Salmo trutta* L.) fed on maximum rations. J Anim. Ecol. 1975; 44:805-821.
11. El-Sayed AFM, Teshima SI. Tilapia nutrition in

aquaculture. Reviews in aquatic sciences 1991; 5(3):247-265.
12. Espe M, Hevroy EM, Liaset B, Lemme B, El-Mowafi A. Methionine intake affect hepatic sulphur metabolism in Atlantic salmon, *Salmo salar*. Aquaculture, 2008; 274: 132-141.
13. Friedewald WT, Levi RI, Fredickson DS. Estimation of the concentration of low density lipoproteins cholesterol in plasma without use of the ultracentrifuge. Clinical Chemistry, 1972; 18:499-502.
14. Furuya WM, Silva LCR, Neves PR. Exigências de metionina + cistina para alevinos revertidos de tilápia do Nilo (*Oreochromis niloticus*), baseadas no conceito de proteína ideal. In: Reunião Da Sociedade Brasileira De Zootecnia, Piracicaba. Anais... Piracicaba: Sociedade Brasileira de Zootecnia, 2001, 1418-1419.
15. Furuya WM, Pezzato LE, Barros MM. Use of ideal protein concept for precise formulation of amino acid levels in fish-meal-free diets for juvenile Nile tilapia (*Oreochromis niloticus* L.), Aquaculture Research, 2004; 35:1110-1116.
16. Graciano TS, Furuya WM, Natali MRM. Metionina e colina em rações para juvenis de Tilápia do Nilo, desempenho produtivo, morfologia hepática e muscular. Pesquisa Agropecuária Brasileira, 2010; 45:737-743.
17. Grant G. Anti-nutritional effects of soybean: A review. Prog. Food & Nutr. Sci. 1989; 13:317.
18. Jia P, Xue M, Zhu X, Liu HY, Wu XF, Wang J, *et al*. Effect of dietary methionine levels on the growing performance of juvenile gibel carp. Acta Hydrobiol Sin. 2013;37:217-226.
19. Kaewtapee C, Krutthai N, Poosuwan K, Poeikhampha T, Koonawootrittriron S, Bunchasak C. Effects of adding liquid dl-methionine hydroxy analogue-free acid to drinking water on growth performance and small intestinal morphology of nursery pigs. J Anim. Physiol. and Anim. Nutr. 2010; 94:395-404.
20. Katz RS, Baker DH. Factors associated with utilization of the calcium salt of methionine hydroxy analogue by the young chick. Poult. Sci. 1975;54:584-591.
21. Komninou D, Leutzinger Y, Reddy BS, Richie Jr JP. Methionine Restriction Inhibits Colon Carcinogenesis. Nutrition and Cancer, 2006; 54(2):202-208.
22. Lovell RT. Nutrition and feeding of fish. Van Nostrand-Reinhold, New York, 1989, 260.
23. Mato JM, Alvarez L, Ortiz P. S-Adenosylmethionine synthesis: Molecular mechanisms and clinical implications. Pharmacology and Therapeutics-Elsevier, 1997; 73:265-280.
24. Nguyen TN. Total sulphur amino acid requirement and its application to practical diets for juvenile tilapia. Thesis for Doctor of Philosophy, Auburn, Alabama, USA, Auburn University, 2007.
25. Nguyen TN,Davis DA. Re-evaluation of total sulphur amino acid requeriment and determination of replacement value of cystine for methionine in semi-purified diets of juvenile Nile tilapia, *Oreochromis niloticus*. Aquacult. Nutr. 2009; 15:247-253.
26. Nguyen TN, Allen DD. Methionine requirement in practical diets of juvenile Nile tilapia. J World Aquaculture Soc. 2009; 40:410-416.
27. NRC, National Research Council. Nutrient requirements of fish. National Academy Press, Washington, DC, 2011.
28. Rubin LL, Canal CW, Ribeiro ALM, Kessler A,Silva I, Trevizan L, *et al*. Effects of Methionine and Arginine Dietary Levels on the Immunity of Broiler Chickens Submitted to Immunological Stimuli. Braz. J. Poult. Sci. 2007; 9(4):241-247.
29. Shen YB, Ferket P, Park I, Malheiros RD, Kim SW. Effects of feed grade l-methionine on intestinal redox status, intestinal development, and growth performance of young chickens compared with conventional dl-methionine. J Anim. Sci. 2015; 93:2977-2986.
30. Silva DJ,Queiroz AC. Análise de alimentos: métodos químicos e biológicos. 3.ed. Viçosa, MG: Universidade Federal de Viçosa, 2002, 235.
31. Sipauba-Tavares LH. Limnologia aplicada à aqüicultura. Jaboticabal: Funep, 1994; 70.
32. Swenson MJ, Reece WO. DUKES: Fisiologia dos animais domésticos. 11.ed. Rio de Janeiro: Guanabara Koogan, 1996; 856.
33. Teshima S, Alam MS, Koshio S. Assessment of requirement values for essential amino acids in the prawn, *Marsupenaeus japonicas*. Aquacult. Res. 2002; 33:395-402.
34. Viola S, Zohar G. Nutrition studies with market size hybrids of tilapia (*Oreochromis niloticus*) in intensive culture. Isr. J. Aquacult. 1984; 36:3-15.
35. Wilson RP. Amino acids and proteins. In: Fish Nutrition. Edited by J.E. Halver. 2nd Edition. Academic Press, San Diego. 1989, 111-151.
36. Wilson RP. Amino acids and proteins, In: Halver, J. E.; Hardy, R. W. (Eds.) Fish nutrition, 3ed New York: Academic Press, 2002, 143-179.
37. Yan Q, Xie S, Zhu X. Dietary methionine requirement for juvenile rockfish, Sebastes schlegeli. Aquacult. Nutr. 2007; 13:163-169.
38. Zeisel SH. Choline: an essential nutrient for humans. Nutrition, 2000; 16:669-671.
39. Zhan XA, Li JX, Xu ZR, Zhao RQ. Effects of methionine and betaine supplementation on growth performance, carcass composition and metabolism of lipids in male broilers. Br. Poult. Sci. 2006; 47:576-580.
40. Zhou QC, Wu ZH, Tan BP. Optimal dietary methionine requirement for juvenile cobia (*Rachycentron canadum*). Aquaculture, 2006; 258:551-557.