

# Effect of *Moringa oleifera* Leaf Meal Supplementation on Growth Performance, Morphological Indexes, Antioxidant Status and Resistance to *Streptococcus agalactiae* of Nile Tilapia (*Oreochromis niloticus*)

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## Article History

Received Jun 14, 2020  
Accepted Nov 17, 2020  
First Online Nov 18, 2020

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## Keywords

Antibacterial  
Antioxidant  
Lipotropic effect  
*Moringa oleifera*  
*Oreochromis niloticus*

## Abstract

An 8-week feeding trial was conducted to evaluate the effect of *Moringa oleifera* leaf meal (MOLM) on growth performance, antioxidant status and morphological indexes in Nile tilapia (*Oreochromis niloticus*). Resistance to *Streptococcus agalactiae* was also assessed based on a 192h challenge experiment after feeding trial. Fish (initial mean weight 4.05±0.05g) were held in 21 tanks (volume 300L, 30 fish/tank), with triplicate treatment groups being fed to apparent satiation three times each day on practical diets adding different levels of MOLM (0, 0.1, 0.5, 2, 5, 10 and 20%). Sampling procedure was performed after 24h of fasting. Growth metrics showed no significant differences among groups. Whole-body lipid content, viscerosomatic index and intraperitoneal fat ratio were decreased as dietary MOLM levels increasing. Total superoxide dismutase (T-SOD) and reduced glutathione content (GSH) were significantly influenced by MOLM. Moreover, the lowest cumulative mortality rate (CMR) after challenge experiment was noted in 10% group. Based on CMR and quadratic regression analysis of liver GSH content, it was concluded that dietary MOLM levels of 8.15-10.00% may be beneficial for enhancing resistance to *S. agalactiae* infection. Our results suggest that the maximum additive amount of MOLM reached 20% under the condition of the present study.

## Introduction

Soybean meal is a vital plant protein source and widely used as a feed ingredient for aquaculture species due to its relatively high nutritional value and digestibility (Puycha *et al.*, 2017). However, owing to its unstable prices and market supply, it is necessary to identify other plant resources to substitute dietary soybean meal (Kasiga & Lochmann, 2014). *Moringa oleifera*, a member of the family Moringaceae, is a fast-growing plant widely distributed in the tropics and subtropics (Richter *et al.*, 2003). It is initially treated as a plant with medical value and utilized as an alternative food source for resistance malnutrition in developing

countries (Rweyemamu *et al.*, 2015; Anwar *et al.*, 2007). Due to its leaf protein content reached 26%, stable market supply and contained some essential amino acids including methionine, cysteine, tryptophan and lysine (Makkar & Becker, 1996; Oyeyinka & Oyeyink, 2018), it has been treated as a plant protein source and applied into diet of some fish species, such as sea bass (*Lates calcarifer*) (Ganzon-Naret, 2014), African Catfish (*Clarias gariepinus*) (Hlophe & Moyo, 2014a), Nile tilapia (*Oreochromis niloticus*) (Bole, Mumba, Mupenda, & Kefi, 2016) and Bocourti's catfish (*Pangasius bocourti*) (Puycha *et al.*, 2017). Previous studies have shown that the maximum amount of *M. oleifera* leaf added to the diet was varied with fish species, and its value did not

exceed 12% under the promise of not negatively affecting the growth (Richter *et al.*, 2003; Puycha *et al.*, 2017). Moreover, fish meal, as the most urgently replaced protein source by producers, occupied an important proportion (31-40%) in the formula of these studies. However, in the case of low dietary fish meal, the maximum amount of *M. oleifera* leaves added in the diet is unclear.

Phytochemicals, such as carotenoids, vitamin C, vitamin E, sterols, glycosides, alkaloids, flavonoids and moringine, have been reported to exist in various organs of *M. oleifera* (Sreelatha *et al.*, 2011). Some of constituents mentioned above are considered to be beneficial for treating diseases, such as inflammation, cardiovascular and liver diseases (Limaye *et al.*, 1995; Rao & Misra, 1998; Gopalakrishnan *et al.*, 2016). Antioxidant property of *M. oleifera* was also found by evaluating oxidative stability of oil extracted from *M. oleifera* leaves (Anwar *et al.*, 2007; Park *et al.*, 2011). *Streptococcus agalactiae* infection has been recorded to cause some fish diseases, such as meningitis, sepsis, ascites and anorexia, which finally resulting in high mortality (Su *et al.*, 2016). It also negatively influenced antioxidant capacity of fish (Du *et al.*, 2020). Importantly, *S. agalactiae* is the main pathogen causing tilapia streptococcus disease in China. Whether the antioxidant and antibacterial properties of *M. oleifera* can help tilapia resist *S. agalactiae* infection is unknown. Besides, fat-degrade effect was observed in finisher pigs due to supplementation of *M. oleifera* leaf meal (Mukumbo *et al.*, 2014). To our knowledge, previous studies have mostly focused on effects of different forms and levels of *M. oleifera* on fish growth. However, research on fat-degrade effect and resistance to pathogen infection is limited. Therefore, the objective of the present research was to investigate the effect of graded levels of dietary MOLM on growth performance, morphometry indices, serum biochemical indexes, antioxidant enzymes activities and resistance to *S. agalactiae* infection in Nile tilapia.

## Materials and Methods

### Preparation of *M. oleifera* Leaf Meal and Experimental Diets

The method for making *M. oleifera* leaf meal was in accordance with that of Nkukwana *et al.* (2014) with minor modification. Fresh, green and undamaged mature *M. oleifera* leaves, obtained from South China Agricultural University and air-dried during the day with no direct sunlight exposure, with constant turning over to prevent fungal growth, were finely ground in a laboratory grinder and stored at room temperature before feed formulation. The nutrient and amino acid composition of MOLM were consistent with study reported by Chen *et al.* (2018) and shown as follows: crude protein, 26.6%; crude lipid, 4.3%; crude ash, 10.8%; arginine, 1.5%; histidine, 0.6%; lysine, 1.6%;

phenylalanine, 1.6%; leucine, 2.3%; isoleucine, 1.2%; methionine, 0.1%; valine, 1.4%; alanine, 1.7%; glycine, 1.4%; proline, 1.2%; serine, 1.1%; threonine, 1.2%. The amino acid composition of MOLM was analyzed by a Hitachi L-8900 automatic amino acid analyzer. The proximate analysis of diets and MOLM was based on the procedures of the Association of Official Analytical Chemists (AOAC) (1997) standard methods. Moisture was determined by putting samples in an oven at 105 °C until constant weight. The methods and instruments used to determine crude protein, crude lipid and ash content of dried diets were consistent with those reported by Gan *et al.* (2013). Crude protein (N × 6.25) was determined by the Kjeldahl method after acid digestion using an Auto Kjeldahl System (1030-Auto-analyzer, Tecator, Sweden). Crude lipid was tested by the ether-extraction method using a Soxtec System HT (Soxtec System HT6, Tecator, Sweden). Ash content was determined by incineration in a muffle furnace at 550 °C for 24 h.

The formulation and composition analysis of the experimental diets is given in Table 1. Fish meal, soybean meal, canola meal and wheat flour were used as dietary protein sources. Soybean oil was utilized as dietary lipid sources. The basal diet was formulated to contain about 30.1% crude protein and 6.4% crude lipid. Seven practical diets were formulated to contain MOLM at levels of 0, 0.1, 0.5, 2, 5, 10 and 20% diets. Various proportions of ingredients mentioned above were balanced with wheat flour. The method of manufacturing diet was consistent with the methods described by Gan *et al.* (2013). The dry ingredients were weighed and well-mixed by a Hobart mixer (A-200T Mixer bench Model unit, Russell Food Equipment, Ottawa, ON, Canada). Lipids and water were then added and thoroughly mixed. The 1.5 mm and 2.5 mm diameter pellets were cold-extruded using a pelletizer (Institute of Chemical Engineering, South China University of Technology, Guangdong, China), then air-dried to approximately 10% moisture, and saved at -20°C until used.

### Fish Management

Healthy juvenile Nile tilapia, obtained from a local commercial hatchery (Guangdong Tilapia Fry Farm, China), were reared in the feeding facility at the South China Agricultural University (Guangzhou, China) and fed to apparent satiation with a commercial diet (Zhuhai Shihai Feed Co., Ltd., China), and then acclimated to laboratory condition for two weeks. Afterwards, a total of 630 fish (initial mean weight 4.05±0.05g) were randomly distributed into 21 300-L tanks (inside diameter 80cm, height 60cm) at the end of the acclimatization period, and each dietary treatment was randomly assigned to triplicate tanks. Fish was fed the respective diets to apparent satiation three times a day (8:00, 12:00 and 17:00) at a feeding rate of 3 to 5% body weight during the 8-week feeding trial, and the amount

**Table 1.** Formulation and proximate composition of the experimental diets (% dry matter)

Ingredients	Dietary addition levels of MOLM						
	0%	0.1%	0.5%	2%	5%	10%	20%
Fish meal	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Soybean meal	32.00	31.95	31.75	31.00	29.48	26.95	21.90
Canola meal	22	22	22	22	22	22	22
Wheat flour	34.30	34.25	34.07	33.38	32.03	29.77	25.23
<i>Moringa oleifera</i> Lam (leaf meal)	0	0.1	0.5	2	5	10	20
Soybean oil	4.50	4.50	4.48	4.42	4.29	4.08	3.66
Monocalcium phosphate Ca (H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub>	2	2	2	2	2	2	2
Premix <sup>1</sup>	1	1	1	1	1	1	1
DL-methionine (≥98%) <sup>2</sup>	0.30	0.30	0.30	0.30	0.30	0.30	0.31
Choline chlorine (50%)	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Proximate composition (% dry matter)							
Crude protein	30.1	29.7	29.6	29.6	29.5	29.4	29.0
Crude lipid	6.4	6.4	6.3	6.4	6.3	6.2	6.0

Soybean meal, canola meal, fish meal, wheat flour and soybean oil were purchased from Zhuhai Shihai Feed Corporation Ltd.

Choline chlorine was purchased from Guangzhou Chengyi Aquatic Technology Ltd. <sup>1</sup>premix including (mg/kg premix): Zn, 7000; Fe, 6000; Mn, 750; Co, 100; I, 120; Cu, 500; Se, 35; thiamine, 600; vitamin B<sub>2</sub>, 850; vitamin A, 300,000 IU; vitamin E, 2,500; vitamin D<sub>3</sub>, 80,000 IU; vitamin K<sub>3</sub>, 400; vitamin B<sub>6</sub>, 600; vitamin C, 8,500; biotin, 12; calcium pantothenate, 1,500; nicotinamide, 3,000; inositol, 4,000 (Guangzhou Chengyi Aquatic Technology Ltd, Guangzhou, China). <sup>2</sup>DL-methionine consists of 98.5% DL-methionine (Novus International Inc.).

of feed fed everyday was recorded. During the feeding experiment, fish were weighed every two weeks, and the amount of the diet was adjusted according to fish weight. Fish was reared in indoor recirculation system tanks with continuous aeration. Uneaten feeds and fecal waste were removed by siphoning. The uneaten feeds were used to calculate amount of feed intake (FI) after drying. Water temperature and dissolved oxygen concentration were monitored daily using a portable oxygen meter (Taiwan, China, AZ8403), and water quality parameters were monitored using a HachTM water quality test kit. Water temperature and pH were kept at 29.0°C (average) and 7.4±0.2, respectively. Total ammonia nitrogen was lower than 0.05 mg/L, and dissolved oxygen was kept above 7.0 mg/L. Natural light-dark cycle was employed during the experiment, and the photoperiod was 12-h light/12-h dark.

#### Sample Collection and Chemical Analysis

All procedures implemented for fish followed handling guidelines of South China Agricultural University. At the end of the feeding trial, fish were starved for 24 h and anesthetized with MS-222 (Shanghai Reagent Corporation, China) before weighing and sampling. It is important to note that the fish used in the *S. agalactiae* infection experiment were not anesthetized. Four fish from each tank were randomly gathered for the analysis of the whole-body composition. Another six fish per tank were randomly selected for obtaining body length and weight of individual whole body, viscera, liver and intraperitoneal fat, and then calculating condition factor (CF), viscerosomatic index (VSI), hepatosomatic index (HSI) and intraperitoneal fat index (IPF). In addition, four fish from each tank were randomly sampled for collecting blood and liver. The serum was separated by centrifuging at 850 g for 10 min at 4 °C and then stored

at -80°C for testing serum biochemical indexes and antioxidant enzyme. Liver tissues were immediately dissected and placed into liquid nitrogen, and then stored at -80°C until used. Liver tissues were homogenized in the proportion of 1:9 (w/v) of ice-cold physiological saline solution and then centrifuged at 3,550 g for 20 min at 4°C. After that, the supernatant was collected and kept at -80°C for determining antioxidant enzyme. The proximate analysis of fish whole-body composition was based on the procedures of the AOAC (1997) standard methods.

#### *Streptococcus agalactiae* Infection Experiment

*S. agalactiae* challenge experiment was carried out according to the method reported by Yi *et al.* (2014) with some modifications. *S. agalactiae* THN strain was provided by the disease lab in College of Marine Sciences of South China Agricultural University (Guangzhou, China). The concentration of *S. agalactiae* infection was 0.1×10<sup>7</sup> CFU/mL obtained from a preliminary trial. Briefly, the bacteria were cultivated in BHI broth and incubated in a shaker (200 rpm) incubator at 2°C overnight and the concentration was adjusted to 0.1×10<sup>7</sup> CFU/mL. At the end of the feeding trial, fish were starved for 24 h, and then 10 fish from each tank without anesthesia were selected and each fish was intraperitoneally injected with 0.1mL culture. Each treatment group had triplicates, and mortality was monitored every 12 h for up to 192 h. All dead fish were immediately removed from the tanks.

#### Serum Biochemical Indexes and Antioxidant Parameters Assays

The content of low-density lipoprotein (LDL), high-density lipoprotein (HDL), triacylglycerol (TAG), cholesterol (CHO), total protein (TP) and activities of

alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by an automatic biochemical analyser (Hitachi 7170A; DAICHI, Tokyo, Japan). The serum was sent to a professional hospital for detection of indexes mentioned above (Traditional Chinese Medicine Hospital of Guangdong Province, China). The content of reduced glutathione (GSH) and activity of total superoxide dismutase (T-SOD) were determined by using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). GSH can react with dithiobisnitrobenzoic acid (DTNB) to form a yellow compound, which can be used for colorimetric quantitative determination of GSH content at 405 nm. In addition, T-SOD activity was determined by its ability to inhibit superoxide anion generated by a xanthine and xanthine oxidase reaction system. Absorbance of indexes mentioned above was tested by a microplate spectrophotometer Epoch (BioTek, USA).

### Statistical Analysis

Results were presented as means  $\pm$  standard error (standard error of the mean, SEM). One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was selected to assess significant differences among all groups by using SPSS Statistic 20.0 software (SPSS Inc., Chicago, IL, USA). The Kruskal-Wallis ANOVA test (non-parametric test) was utilized when inhomogeneous variance appeared, followed by pairwise comparisons using Kruskal-Wallis. Probability value of  $P < 0.05$  was considered statistically significant. In addition, quadratic regression method was used to evaluate the optimum level of MOLM supplementation in Nile tilapia according to Chen *et al.* (2019). Pearson correlation coefficient ( $r$ ) was used to evaluate the correlation between dietary MOLM levels and morphological parameters.

## Results

### Growth Performance, Morphometry Indices and Whole-Body Composition

Growth performance indexes of Nile tilapia are given in Table 2. FBW, WG, SGR, FI, FCR and SR did not vary considerably among experimental groups ( $P > 0.05$ ).

As shown in Figure 1, CF and IPF were markedly reduced as dietary MOLM levels increasing ( $P < 0.05$ ), and the lowest value was found in the 20% group. Although VSI showed a decreasing trend with the increase of dietary MOLM levels, there was no significant difference among groups ( $P > 0.05$ ). In addition, HSI showed no significant difference among all groups ( $P > 0.05$ ). According to Pearson correlation coefficient, a significantly negative correlation was existed between these parameters and dietary MOLM levels.

Whole-body composition is given in Table 3. Whole-body lipid content was markedly decreased with dietary MOLM levels increasing ( $P < 0.05$ ). Significant reduction of ash content was observed in 5% and 10% groups when compared with other groups (except for the 2% group) ( $P < 0.05$ ). However, no significant differences were noted in crude protein and moisture among all treatments ( $P > 0.05$ ).

### Serum Biochemical Indexes before *S. agalactiae* Infection

Serum biochemical indexes of Nile tilapia fed experimental diets are present in Figure 2. Dietary MOLM supplementation significantly reduced HDL in 0.1% and 2% groups and increased TAG in 0.5% group as compared with 0% group ( $P < 0.05$ ). TP, CHO and AST in 10% group was markedly lower than that of 0.5% group

**Table 2.** Effect of dietary MOLM levels on growth performance of Nile tilapia fed experimental diets

Parameters	Dietary addition levels of MOLM							P-value
	0%	0.1%	0.5%	2%	5%	10%	20%	
IBW	4.02 $\pm$ 0.05	4.06 $\pm$ 0.02	4.07 $\pm$ 0.00	4.06 $\pm$ 0.01	4.06 $\pm$ 0.01	4.02 $\pm$ 0.05	4.06 $\pm$ 0.03	0.786
FBW	74.7 $\pm$ 4.59	77.0 $\pm$ 3.19	77.3 $\pm$ 1.18	75.6 $\pm$ 0.67	69.0 $\pm$ 2.93	71.4 $\pm$ 2.28	70.9 $\pm$ 1.55	0.245
WG	1686.9 $\pm$ 73.3	1738.8 $\pm$ 45.7	1674.5 $\pm$ 58.8	1620.5 $\pm$ 49.7	1485.8 $\pm$ 89.4	1582.4 $\pm$ 67.0	1622.1 $\pm$ 39.7	0.191
SGR	5.21 $\pm$ 0.09	5.25 $\pm$ 0.07	5.26 $\pm$ 0.03	5.22 $\pm$ 0.01	5.05 $\pm$ 0.08	5.14 $\pm$ 0.08	5.11 $\pm$ 0.05	0.244
FI	7.60 $\pm$ 0.20	7.24 $\pm$ 0.10	7.21 $\pm$ 0.16	7.05 $\pm$ 0.01	6.98 $\pm$ 0.09	7.31 $\pm$ 0.07	7.73 $\pm$ 0.31	0.053
FCR	1.07 $\pm$ 0.03	1.00 $\pm$ 0.01	1.02 $\pm$ 0.02	1.02 $\pm$ 0.02	1.05 $\pm$ 0.02	1.06 $\pm$ 0.01	1.11 $\pm$ 0.03	0.059
SR	95.6 $\pm$ 1.13	96.7 $\pm$ 1.93	92.2 $\pm$ 2.22	91.1 $\pm$ 2.94	92.22 $\pm$ 1.11	94.5 $\pm$ 1.08	98.9 $\pm$ 1.11	0.077

IBW, initial mean body weight (g/fish);

FBW, final mean body weight (g/fish);

WG, weight gain (%) =  $100 \times (\text{FBW} - \text{IBW}) / \text{IBW}$ ;

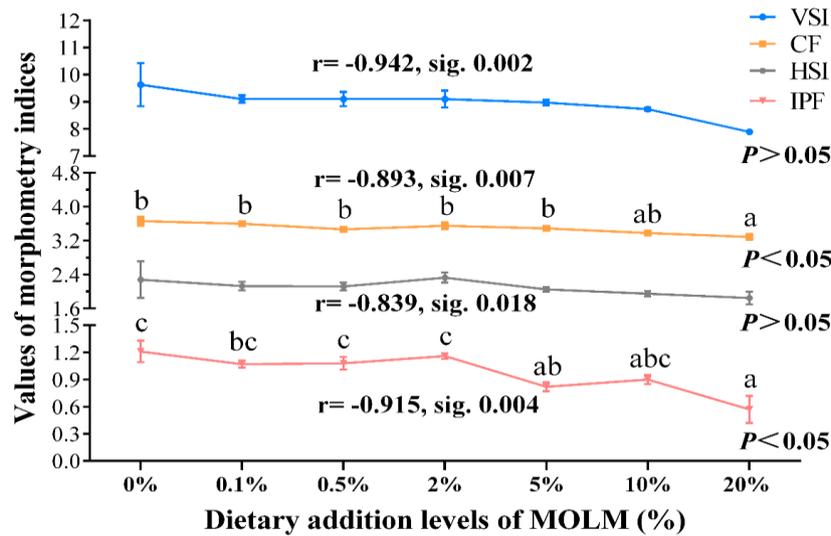
SGR, specific growth rate (%/day) =  $100 \times [\text{Ln}(\text{FBW}) - \text{Ln}(\text{IBW})] / 56$  days;

FCR, feed conversion rate = feed consumed (g, dry weight) / weight gain (g, wet weight);

FI, feed intake (%/d) = grams of dry feed consumed  $\times 100 / \sqrt{(\text{IBW} \times \text{Initial fish number}) \times (\text{FBW} \times \text{Final fish number} + \text{Dead fish body weight})} / 56$  days (The equation was consistent with the report of Gan *et al.* (2013)).

SR, survival rate (%) =  $100 \times (\text{final number of fish}) / (\text{initial number of fish})$

Data are presented as mean  $\pm$  SEM (n=3). Values with different letters in the same line indicate significant differences ( $P < 0.05$ ).

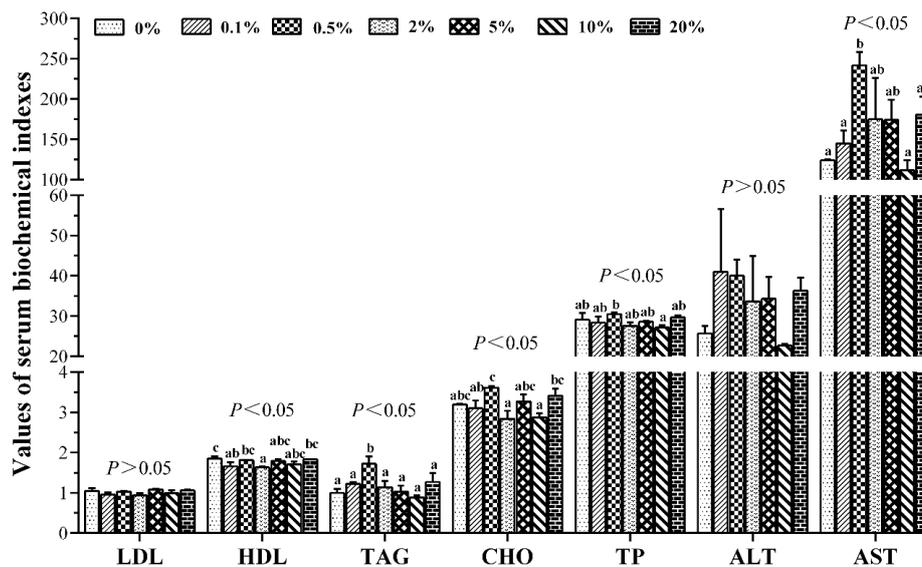


**Figure 1.** Morphometry indices of Nile tilapia fed experimental diets for 8-week. Data are presented as mean  $\pm$  SEM (n=3). The line with different letters indicates significant differences ( $P < 0.05$ ).  $r$ , Pearson correlation coefficient; sig., significance (for correlation analysis). CF, condition factor ( $\text{g}/\text{cm}^3 = 100 \times \text{body weight (g)} / \text{body length (cm}^3)$ ); VSI, viscerosomatic index ( $\% = 100 \times (\text{visceral weight}) / (\text{whole body weight})$ ); HSI, Hepatosomatic index ( $\% = 100 \times (\text{liver weight}) / (\text{whole body weight})$ ); IPF, intraperitoneal fat ratio ( $\% = 100 \times (\text{intraperitoneal fat weight}) / (\text{whole body weight})$ ).

**Table 3.** Effect of dietary MOLM levels on body composition of Nile tilapia fed experimental diets for 8-week

Parameters	Dietary addition levels of MOLM							P-value
	0%	0.1%	0.5%	2%	5%	10%	20%	
Whole body (% wet weight)								
Moisture	69.8 $\pm$ 0.84	69.0 $\pm$ 0.43	68.1 $\pm$ 0.38	69.4 $\pm$ 0.43	70.8 $\pm$ 0.95	70.2 $\pm$ 0.44	69.7 $\pm$ 1.03	0.235
Crude protein	16.3 $\pm$ 0.40	16.0 $\pm$ 0.54	16.2 $\pm$ 0.06	16.5 $\pm$ 0.45	15.8 $\pm$ 0.43	15.8 $\pm$ 0.86	15.7 $\pm$ 0.44	0.897
Crude lipid	9.05 $\pm$ 0.53 <sup>bc</sup>	9.52 $\pm$ 0.22 <sup>c</sup>	9.20 $\pm$ 0.31 <sup>c</sup>	8.60 $\pm$ 0.29 <sup>bc</sup>	7.88 $\pm$ 0.13 <sup>ab</sup>	8.43 $\pm$ 0.27 <sup>bc</sup>	7.16 $\pm$ 0.56 <sup>a</sup>	0.005
Ash	3.77 $\pm$ 0.11 <sup>b</sup>	3.79 $\pm$ 0.03 <sup>b</sup>	3.81 $\pm$ 0.08 <sup>b</sup>	3.60 $\pm$ 0.12 <sup>ab</sup>	3.41 $\pm$ 0.04 <sup>a</sup>	3.39 $\pm$ 0.15 <sup>a</sup>	3.86 $\pm$ 0.15 <sup>b</sup>	0.027

Data are presented as mean  $\pm$  SEM (n=3). Values with different letters in the same line indicate significant differences ( $P < 0.05$ ).



**Figure 2.** Serum biochemical indexes of Nile tilapia fed experimental diets for 8-week. Data are presented as mean  $\pm$  SEM (n=3). The column with different letters indicates significant differences ( $P < 0.05$ ). LDL (low-density lipoprotein, mmol/L), HDL (high-density lipoprotein, mmol/L), TAG (triacylglycerol, mmol/L), CHO (cholesterol, mmol/L), TP (total protein, g/L), ALT (alanine transaminase, U/L) and AST (aspartate aminotransferase, U/L).

( $P < 0.05$ ). However, ALT and LDL showed no significant difference among all groups ( $P > 0.05$ ).

#### Cumulative Mortality Rate after 192h *S. agalactiae* Challenge

As shown in Table 4, cumulative mortality rate (CMR) in 0%, 0.5%, 5%, 10% and 20% groups was increased with dietary MOLM levels increasing when stress time before 72 h, and maintained thereafter. 0.1% and 2% groups showed a similar trend, but the deadline for continuously increased CMR was changed to 120 h. After 192 h *S. agalactiae* challenge, the highest CMR was discovered in 0.1% group, and the lowest CMR was observed in 10% group.

#### T-SOD and GSH in Serum and Liver of Nile tilapia before and after 192 h *S. agalactiae* Challenge

T-SOD activity and GSH content in serum and liver of Nile tilapia before and after *S. agalactiae* challenge are given in Figure 3.

Before challenge, 0.1%, 5%, 10% and 20% groups had higher serum T-SOD activities when opposed to 0% group ( $P < 0.05$ ). Dietary MOLM supplementation significantly raised liver GSH content in 10% group and reduced the index in 0.5% group ( $P < 0.05$ ). After challenge, serum T-SOD activity in 10% and 20% groups and serum GSH content in 5% group were considerably higher than that in 0% group ( $P < 0.05$ ). 0.1%, 2% and 5% groups had higher liver GSH content when opposed to 20% group ( $P < 0.05$ ). However, the rest of parameters showed no significant difference among all treatments ( $P > 0.05$ ). The optimal level of dietary MOLM supplementation was estimated to be 8.15% diet based on liver GSH content after 192 h *S. agalactiae* challenge using a quadratic regression method ( $y = -0.3053x^2 + 4.9765x + 45.066$ ,  $R^2 = 0.6164$ ,  $P = 0.001$ ) (Figure 4).

#### Discussion

In this study, the results showed that the growth of fish fed with higher levels of MOLM (10% and 20%) was not considerably different with those of fish fed with control diet, suggesting that the maximum amount of MOLM added to diet is 20% without negative impact on growth. This value was greater than the results (maximum additional amount ranged from 7.27% to 10.0%) obtained from other fish species (Yuangsoi & Masumoto, 2012; Ganzon-Naret, 2014; Hlophe & Moyo, 2014b; Puycha *et al.*, 2017). Even for the same species, the results are different. Richter *et al.* (2003) found that high levels of dietary MOLM (exceeding 12%) led to the decrease of WG and FI in Nile tilapia. The main reason for these differences may be the composition of the formula. In most of the experiments mentioned above, the addition of fish meal exceeded 22%, while the addition in this experiment was only 3.5%. Besides, the

processing methods of leaves may be another factor contributing to the difference. In the study of Richter *et al.* (2003), *M. oleifera* leaves were freeze-dried and its phytic acid and saponins content is 2.3% and 6.4%, respectively. However, for air-dried leaves, the values were changed to 0.1% and 1.2%, respectively (Madalla *et al.*, 2013). Significantly, phytic acid and saponin have been reported to be antinutritional factors contributing to the poor growth performance in some fish species (Spinelli *et al.*, 1983; Dongmeza *et al.*, 2006). The present study suggested that under the condition of low fish meal, the addition of MOLM in the diet can reach 20% in Nile tilapia.

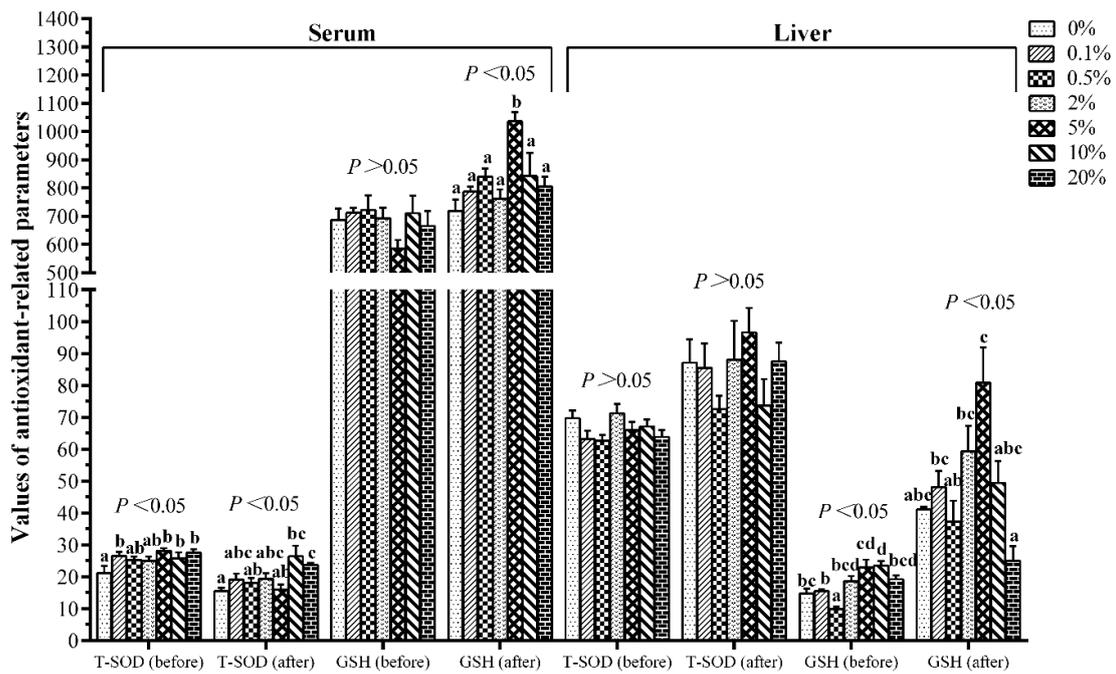
Oduro-Owusu *et al.* (2015) reported that supplementation of 5% MOLM was markedly reduced back fat thickness in pigs. It seems that *M. oleifera* has an effect on lowering body lipid content. In the present study, IPF and whole-body lipid content were significantly decreased as dietary MOLM levels increasing. Similar results related to whole-body lipid content were also observed in the research conducted by Richter *et al.* (2003). Reduction of body lipid content may be linked to poor feed intake which led to starvation, and finally mobilized reserved body lipid to meet energy requirements for body physical functions (Madalla *et al.*, 2013). However, feed intake in this experiment did not show a continuous decline with the increase of dietary MOLM levels. Feed intake did not seem to be the main cause of the decline in body lipid content. The decreased lipid content may be attributed to hypolipidaemic activity of bioactive agents (such as alkaloids and  $\beta$ -sitosterol) contained in the *M. oleifera* (Ghasi *et al.*, 2000; Atsukwei *et al.*, 2014). In addition, saponins existed in *M. oleifera* leaves may inhibit pancreatic lipase activity, which finally delayed intestinal absorption of dietary lipid (Han *et al.*, 2000). Further research is still needed to explore the potential mechanism of lowering body lipid.

Serum cholesterol (CHO), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL) and triacylglycerol (TAG) have been reported to be useful indicators for assessing lipid metabolism as well as predisposition of the heart to atherosclerosis and its associated coronary heart diseases (Yakubu *et al.*, 2008). Atsukwei *et al.* (2014) reported that ethanol leaf extract of *M. oleifera* were markedly increased serum HDL concentration and reduced total CHO and TAG concentrations in rats. The decrease of serum CHO in some MOLM-supplemented groups was also observed in the present study.  $\beta$ -sitosterol, as one of the main cholesterol-reducing components of the *M. oleifera* leaf, can reduce cholesterol levels by inhibiting cholesterol absorption in the intestines (Bordia & Verma, 1998). Another way for lowering CHO was related to the function of LDL which could carry cholesterol from the liver to the blood stream (Rana *et al.*, 2007). Similar trends of LDL and CHO seem to support the second view. In addition, chlorogenic acid and moringine contained in *M. oleifera* have been confirmed to decrease serum CHO

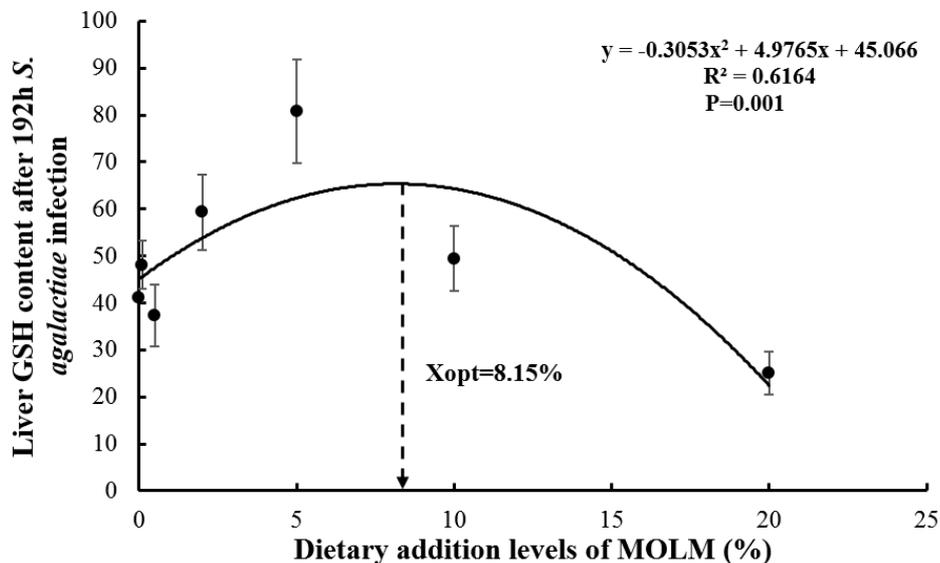
**Table 4.** Cumulative mortality rate of Nile tilapia treated with different dietary MOLM levels after 192 h *S. agalactiae* infection.

Time	Dietary addition levels of MOLM							P-value
	0%	0.1%	0.5%	2%	5%	10%	20%	
24h	3.33±3.3 <sup>a</sup>	20.0±0.0 <sup>b</sup>	13.3±3.3 <sup>ab</sup>	6.67±3.3 <sup>ab</sup>	0.00±0.0 <sup>a</sup>	0.00±0.0 <sup>a</sup>	3.33±3.3 <sup>a</sup>	0.021
48h	23.3±3.3	46.7±13.3	26.7±16.7	16.7±8.8	13.3±8.8	0.00±0.0	16.7±3.3	0.136
72h	33.3±3.3	46.7±13.3	33.3±23.3	20.0±11.5	20.0±10.0	3.33±3.3	20.0±5.8	0.238
96h	33.3±3.3	46.7±13.3	33.3±23.3	20.0±11.5	20.0±10.0	3.33±3.3	20.0±5.8	0.238
120h	33.3±3.3	50.0±15.3	33.3±23.3	23.3±8.8	20.0±10.0	3.33±3.3	20.0±5.8	0.199
144h	33.3±3.3	50.0±15.3	33.3±23.3	23.3±8.8	20.0±10.0	3.33±3.3	20.0±5.8	0.199
168h	33.3±3.3	50.0±15.3	33.3±23.3	23.3±8.8	20.0±10.0	3.33±3.3	20.0±5.8	0.199
192h	33.3±3.3	50.0±15.3	33.3±23.3	23.3±8.8	20.0±10.0	3.33±3.3	20.0±5.8	0.199

Data are mean ± SEM (n=3). Values with different letters in the same line indicate significant differences (P<0.05).



**Figure 3.** Activities of T-SOD and GSH in serum and liver of Nile tilapia treated with different dietary *M. oleifera* levels before and after 192 h *S. agalactiae* infection. Results are presented as the mean ± SEM (n=3). The column with different letters indicates significant differences (P<0.05). Serum, T-SOD (total superoxide dismutase, U/mL), GSH (reduced glutathione, μmol/L); Liver, T-SOD (U/mgprot), GSH (μmol/gprot).



**Figure 4.** Estimation of optimal dietary MOLM levels for Nile tilapia using the quadratic regression method.

(Cho *et al.*, 2010). These results may suggest that the reduction of cholesterol in Nile tilapia was influenced by several factors. Significant increment of TAG was observed in 0.5% group as compared with other experimental groups, which was inconsistent with the study in rat and may be related to increased lipolysis (Atsukwei *et al.*, 2014), suggesting that *M. oleifera* may exert different effects on TAG in rat and Nile tilapia.

Generally, AST and ALT are existed in liver with large quantities and high levels of these enzymes in serum may indicate hepatic dysfunction and damage (Soumendra *et al.*, 2010). In our study, MOLM supplementation enhanced serum AST and ALT activities, which was similar with the study in rat that methanolic extract from *M. oleifera* leaves enhanced these enzymes activities (Oyagbemi *et al.*, 2013). Sharma *et al.* (2002) reported that HgCl<sub>2</sub> caused an increase in serum AST and ALT activities, which may be related to change of permeability of the plasma membrane. The increase of serum AST and ALT activities in the present research might be related to the increase of permeability in the plasma membrane caused by MOLM, and then led to leakage of these enzymes into the blood stream. The present study may suggest that MOLM influenced the liver structure of Nile tilapia.

The healthy development of China's aquaculture industry has been plagued by outbreaks of diseases. *S. agalactiae*, as a member of a family of bacteria that affect the health of fish, is common in tilapia farming (Dong *et al.*, 2015). Due to the disadvantages of antibiotics against bacterial infection, more and more people focus on the application of herbal medicine (Ataguba *et al.*, 2018). Some herbal extracts, such as extracts of *Piper betle* and *Sophora flavescens*, have been reported to be beneficial for resisting *S. agalactiae* infection (Wu *et al.*, 2013; Ataguba *et al.*, 2018). Similar result was also observed in the present study. More importantly, adding 10% MOLM was the best in fighting bacterial infection. Based on in vitro antimicrobial screening methods, Busani *et al.* (2012) found that the acetone extract of *M. oleifera* leaves exhibited antibacterial activities against some pathogens (such as *Escherichia coli* and *Enterobacter cloacae*). High antioxidant and antimicrobial activities shown in *M. oleifera* were related to the presence of vitamin E, ascorbic acid, flavonoids, phenolics and carotenoids (Busani *et al.*, 2012; Jayawardana *et al.*, 2015; Das *et al.*, 2012; Qwele *et al.*, 2013). The reason why MOLM reduced mortality rate after 192h *S. agalactiae* challenge in this research seems to be explained in these studies, but the potential mechanism is still unclear. Previous study showed that *M. oleifera* extract could reduce lipid peroxidation caused by antitubercular drug treatment in rats (Kumar & Pari, 2003). Gupta *et al.* (2007) also demonstrated that *M. oleifera* seed powder could protect mouse from oxidative stress caused by arsenic. These results suggest that *M. oleifera* may participate in regulation of antioxidant status. Antioxidant enzymes, prevented biological

macromolecules from oxidative injury, are considered to be one of the most important components of the body's antioxidant system (Fontagné-Dicharry *et al.*, 2014). SOD plays a vital role in protecting cells against reactive oxygen species (ROS) by converting superoxide anions to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Qwele *et al.*, 2013). Increment of liver SOD activity was observed in diabetic rats treated with *M. oleifera* when compared with the untreated group (Jaiswal *et al.*, 2013). Similar result was observed in part of MOLM-supplemented groups after 192 h *S. agalactiae* infection. In addition, GSH has been reported to be an antioxidant in maintaining thiol-redox status and detoxicating of exogenous and endogenous reactive molecules (Oyedemi *et al.*, 2010). Antitubercular drug treatment regimens may lead to serious side effect-liver disease (Mahashar & Prabhudesai, 1991). The decrease in GSH content was observed in rats administered with an antitubercular drug. However, in rats co-treated with *M. oleifera* leaf extract, GSH level was increased, suggesting the protective role of *M. oleifera*. Similarly, dietary MOLM supplementation increased liver GSH content after pathogen infection. MOLM may have some beneficial role in improving antioxidant capacity, thereby decreasing mortality rate after *S. agalactiae* infection. Based on CMR and quadratic regression analysis of liver GSH content after 192 h *S. agalactiae* challenge, the optimal level of dietary MOLM supplementation was estimated to be 8.15-10.00%.

## Conclusion

This study demonstrated that *M. oleifera* leaf meal can be used as a feed ingredient and replace some proportion of dietary soybean meal. It also suggested that the maximum additive amount of MOLM was 20% under the conditions of this study. Moreover, dietary MOLM supplementation decreased body lipid. Appropriate level of MOLM supplementation may have a beneficial role in raising resistance to *S. agalactiae* infection and improving antioxidant status. Based on the results of the present study, optimum level of MOLM supplementation was estimated to be 8.58-10.00% diet in Nile tilapia with better pathogen resistance.

## Acknowledgement

The research was supported by Guangdong Marine Economy Promotion Projects (MEPP) Fund (GDOE [2019] A26), Guangdong Provincial Special Fund for Modern Agriculture Industry Technology Innovation Teams (2019KJ150), Department of Agriculture and Rural Affairs of Guangdong Province, Guangdong Forestry Science Technology and Innovation Commission (Grant No. 2018KJXC001) and Project of Department of Ocean and Fishery of Guangdong Province, China (A201701C03).

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