Accepted manuscript

Dietary DL-Methionyl-DL-Methionine (Met-Met) supplementation increased growth performance, antioxidant ability, the content of essential amino acids and improved the diversity of intestinal microbiota in Nile tilapia (*Oreochromis niloticus*)

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This peer-reviewed article has been accepted for publication but not yet copyedited or typeset, and so may be subject to change during the production process. The article is considered published and may be cited using its DOI

10.1017/S0007114519002289

The British Journal of Nutrition is published by Cambridge University Press on behalf of The Nutrition Society

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Abstract

The dipeptide DL-methionyl-DL-Methionine (Met-Met) has extremely low water solubility and better absorption than other methionine sources (such as DL-methionine and L-methionine) available in the market. Therefore, six diets (D1, D2, D3, D4, D5 and D6) containing 0 %, 0.07%, 0.15%, 0.21%, 0.28% and 0.38% Met-Met were formulated to investigate the effects of Met-Met on *Oreochromis niloticus*. The results indicated that percent weight gain and specific growth rate of fish fed with D2 and D3 diets were higher than those fed with D1, D4-D6 diets. The levels of total essential amino acid in whole body of fish fed with D3 and D4 diets were significantly higher than those fed the D1 diet. Superoxide dismutase activity and malondialdehyde content were no significant difference in fed the diet with or without Met-Met supplementation. Majority of reads derived from the fish intestines belonged to members of *Fusobacteria*, followed by *Bacteroidetes*, *Proteobacteria*. Diversity of intestinal microbiota and total antioxidant capacity in fish fed with D3 diet was significantly higher than that of other groups. Since due to the growth results the authors conclude the optimal proportion of Met was 0.61%, and the addition of Met-Met was 0.15% in *O. niloticus*.

Keywords: *Oreochromis niloticus*, DL-Methionyl-DL-Methionine, antioxidant capacity, intestinal microbiota, growth performance.

1. Introduction

Tilapia is a commercially important species widely distributed in over 100 countries. It has become the second most commonly cultured freshwater fish after carp in the world[1]. The Nile tilapia (*Oreochomis niloticus*) has dominated tilapia aquaculture due to its high nutritional value, high growth rate and yield. Because of its increasing market demands, *O. sniloticus* also has become the most extensively cultured freshwater fish in China since its introduced in 1978.

The combination of high nutritional value and increasing market demands has stimulated the development of the industry of tilapia-formulated feed. Dietary proteins are primary factors influencing fish growth and feed costs. Currently, fish meal remains the primary protein source of formulated fish feed. However, the market price of fish meal has risen by nearly 300% in the past decade because of the fishing restrictions of wild fish[2]. Undoubtedly, the growth of aquaculture production is fundamentally unsustainable if fish meal is still used as the main source of protein for aquafeeds. Therefore, plant protein derived from crops such as soybeans, peanut, corn or wheat has become a frequently-used alternative of fish meal in aquafeeds[3]. Large-scale application of plant protein in aquafeeds can reduce feed costs, but the application effect of plant protein is unsatisfactory due to nutritional constraints such as imbalance of amino acid composition, anti-nutritional compounds, palatability, and unknown nutritional limitations[4].

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Essential amino acids (EAAs) deficiency in formulated fish feed may cause poor growth, immunosuppression and poor diet efficiency[5]. Methionine (Met) is considered to be the first limiting EAA in most plant protein sources[6]. Supplementation of Met in diets containing higher levels of plant protein is becoming popular. Supplementation with Met in plant protein-based diets can improve growth performance, immune capacity, feed conversion efficiency and body composition have been reported in *Scophthalmus maximus*[7], *Pelodiscus sinensis*[8], *Sparus aurata*[9], *Oncorhynchus mykiss*[6], *Litopenaeus vannamei*[10], *Myxocyprinus asiaticus*[11], *Pseudosciaena crocea*[12]. However, the effects of Met, especially Met-Met products, on *O. niloticus* have not been reported. Currently, Met products used in aquafeeds mainly include DL-methionine (DL-Met), L-methionine (L-Met), or methionine hydroxy analogue calcium salt (MHA-Ca), which have also been proven to be utilized by aquatic animals[7,13,14]. The bioavailability of different forms of Met is also considered by nutritionists. Some researches in fish have shown that the bioavailability of methionine hydroxy analogue (MHA) is significantly lower than that of DL-Met[15,16]. However, Zhou et al. reported that DL-Met and MHA-Ca were equivalent in promoting growth, feed utilization efficiency and antioxidant enzyme activities of *P. sinensis*[8]. The difference of bioavailability of different Met products in aquatic animals may be related to different species or lack of sensitivity of the bioassays. Undoubtedly, it is necessary to study the bioavailability of different Met products to different aquatic animals.

The dissolution or leaching rate of Met additives in water is one of the important indexes for comparing different Met products. Meanwhile, the efficiency of Met additives to be absorbed and utilized by animals is also very important. The dipeptide DL-methionyl-DL-Methionine (Met-Met), developed by Evonik's aqua R&D group, has excellent physical and chemical properties, such as extremely low water solubility and better absorption than other Met products (such as DL-Met and L-Met)[5]. Met-Met additives can improve growth, feed conversion efficiency, intestinal immune function have been reported in *L. vannamei*[5,17] and *Ctenopharyngodon Idella*[18]. Met-Met is excreted mainly as sulphate and carbon dioxide, and its use as a feed additive for aquatic species does not represent a risk to the environment[19]. Met-Met has the potential to replace supplemental free amino acid Met in aquafeeds and is an effective source of the essential amino acid L-Met.

Until now, the effect of Met-Met products on *O. niloticus* has not been reported. The effect of Met-Met on intestinal microflora of fish has not been reported yet. Diversity of intestinal microflora plays a critical role in modulating the fish physiology[20]. Intestinal microflora can influence fish feeding, digestion, growth and energy homeostasis[21]. Therefore, the main purpose of this study was to evaluate the effects of Met-Met supplementation on growth performance, antioxidant capacity, body composition and intestinal microbiota of *O. niloticus*. In the present study, optimal Met-Met supplementation for *O. niloticus* was first reported. Our data contribute to improve the

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current understanding of the response relationship between Met and fish intestinal microflora. These results might provide a reference for formulated feed of *O. niloticus*.

2. Materials and methods

2.1 Diet Preparation

Met-Met were obtained from Evonik Degussa (China) Co., Ltd. Guangzhou Branch (Guangdong, China). Met-Met is a dipeptide whose production process is water-based and does not use organic solvents. The bioavailability of Met-Met was significantly better than that of conventional Met, so it is also considered as a new form of DL-methionine supplements[5,17].

Six isonitrogenous and isolipidic diets (D1, D2, D3, D4, D5 and D6) containing 0% (control group), 0.1%, 0.2%, 0.3%, 0.4% and 0.5% Met-Met were formulated, and proximate composition analysis of the diets were demonstrated in Table 1. The actual concentrations of Met and Met-Met in six experimental diets were showed in Table 2. The main protein source was derived from plant protein, such as soybean meal, rapeseed meal, wheat flour and rice. The requirement of lipid was satisfied by soybean oil and soybean lecithin. Six experimental diets were supplemented with amino acids, such as glycine, lysine, threonine, tryptophan, isoleucine, leucine, valine and histidine, to ensure that the formulation of the diets was nutritionally comprehensive for the fish.

Diets were prepared according to the method of Niu et al.[22]. Briefly, all dry ingredients were finely ground, accurately weighed, and thoroughly mixed and homogenized. Then the oil mix and distilled water were added to the mixture. The 2.5-mm-diameter puffed pellets were produced by using a puffing apparatus (Institute of Chemical Engineering, South China University of Technology, Guangdong, China). The pellets were then dried in a forced air oven at 40°C until the moisture was reduced to < 10% and stored at -20°C until used.

2.2 Fish and experimental set up

Juvenile *O. niloticus* was obtained from Guangdong Provincial Fishery Germplasm Conservation Center (Guangzhou, China). Fish were acclimated to the experimental conditions and fed the control group diet for two weeks prior to starting the experiments. A total of 540 fish with an initial body weight of about 17.15 g were randomly assigned into 18 fiberglass tanks (800 L, 3 tanks per diet, 30 fish per tank). Each tank was equipped with a water exchange system and uninterrupted oxygenation system. The conditions of water temperature (26 ± 1°C), dissolved oxygen (> 6.0 mg L^{-1}), ammonia-nitrogen (< 0.3 mg L^{-1}), and nitrite-nitrogen (< 0.10 mg L^{-1}) were ensured during the experiment period.

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During the experiment period, the fish were fed two times per day at 8:00 and 17:00, for 56 consecutive days, at 5% of their body weight. One hours after feeding, uneaten feed and faeces were siphoned from tanks. Uneaten feed particles were dried and weighed and used to calculate feed intake. Every morning before feeding, sorting out the dead fish, fish death amounts were recorded to calculate the survival rate.

2.3 Sample collection and growth performance analysis

At the end of the feeding trial, all fish were fasted for 24 h to enter a basic metabolic state and eliminate the dietary effect. Seventeen fish per tank were randomly collected and anesthetized with MS-222 (Sigma, St Louis, MO, USA), and then weighed, counted to calculate the percent weight gain (PWG), specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER). Four fish for analysis of whole-body composition and four fish for analysis of muscle composition, another four fish for analysis of whole-body amino acid composition. The whole-body and muscle of fish were sampled and stored at −20°C for body composition analysis. Then, five fish were collected and aseptically sacrificed in ice-bath, and their hepatopancreas and gut were rapidly frozen in liquid nitrogen and stored at −80°C for antioxidant enzymes activity and microbial flora analysis respectively. All the experiments were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). The study protocol and all experimental procedures were approved by Experimental Animal Ethics Committee of Sun Yat-sen University.

PWG, SGR, PER, FCR and survival were calculated according to the following equations:

$$PWG\ (\%) = 100 \times (W_f - W_i) / W_i$$

$$SGR\ (\%\ day^{-1}) = 100 \times (\ln W_f - \ln W_i)/t$$

$$Survival\ (\%) = 100 \times N_t / N_0$$

$$FCR = dry\ feed\ intake / (W_t - W_0)$$

$$PER = 100 \times (W_t - W_0) / (I \times C_{Nf})$$

where W_f and W_i were mean final and initial fish body weights; t is the duration of the experiment (56 days); N_t is number of fish at the end of the experiment and N_0 at the start; W_t (g) is total final body weight and W_0 (g) total initial body weight; C_{Nf} (%) is protein content in the feed; I (g) is total amount of the feed fed on a dry weight basis.

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2.4 Chemical analysis of feed and body composition

The experimental diets and fish samples were analyzed in triplicate for proximate composition. Crude protein, crude lipid, and ash were determined using the standard method of Association of Official Analytical Chemists (AOAC, 2012). Moisture was determined by drying in an oven at 105 °C for 24 h; crude protein content (N × 6.25) was determined by the Kjeldahl method after acid digestion (1030-Auto-analyzer, Tecator, Höganäs, Sweden); crude lipid was measured by ether extraction method by Soxtec System HT (Soxtec System HT6, Tecator, Sweden); crude ash was examined after combustion in a muffle furnace at 550°C for 24 h.

The amino acid composition of all samples was analyzed according to the method of Niu et al.[(5)]. Briefly, all samples were submitted to an acid hydrolysis, and then amino acid composition of all samples was analyzed using an automatic amino acid analyzer (Hitachi 835-50, Japan) with a column (Hitachi custom ion exchange resin no. 2619) by Evonik Degussa (China) Co. Ltd. Novel product, Evonik Degussa Gmbh.

2.5 Activity quantification of antioxidant enzymes

Hepatopancreas were homogenized 1:9 w/v in ice-cold 50 mM Tris-HCl buffer solution (pH 7.4), centrifuged at 12,000 g for 15 min at 4 °C, and the cold hepatopancreas supernatant was used for the determination of enzymatic activity. Superoxide dismutase (SOD) activity was determined by its ability to inhibit superoxide anion produced by xanthine and xanthine oxidase reaction system[(23)]. Malondialdehyde (MDA) contents were measured by the TBA method according to Miller and Aust[(24)]. Catalase (CAT) activity was determined by determining the initial amount of hydrogen peroxide decomposition at 240 nm[(25)]. Antioxidants could reduce Fe^{3+} to Fe^{2+}, the later could form solid clathrate with phenanthroline, and then the total antioxidant capacity (T-AOC) was measured according to the absorption value at 520 nm[(26)]. The activities of SOD and CAT, T-AOC, and the content of MDA were determined by using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's protocol.

2.6 DNA extraction and high-throughput sequence analysis

Total bacterial DNA of gut was extracted directly with the E.Z.N.A. stool DNA Kit (Omega Biotek, Norcross, GA, U.S.) according to manufacturer's instructions. The total DNA concentration and quality were measured by a NanoDrop spectrophotometer (ND-2000, Thermo Fisher Scientific, USA) and 1% agarose gel electrophoresis, respectively. The V4 domain of bacterial 16S rDNA were amplified using the primer pair 515 F (5' GTGCCAGCMGCCGCGGTAA 3') and 806 R (5' GGACTACHVGGGTWTCTAAT 3'). The reverse primer contained a 6-bp error-correcting barcode unique to each sample. The PCR reactions contained 100 ng DNA template, 1.5 μL of each forward and reverse primer (5 μM), 5 μL of 2.5 mM dNTPs, 1 μL of KOD Polymerase, and 5 μL of

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10 × KOD Buffer. Reaction conditions consisted of an initial 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s, with a final extension of 72 °C for 10 min. Sequencing was performed using the Illumina HiSeq platform by Biomarker (Beijing, China).

Raw reads were de-multiplexed, quality-filtered and analyzed using QIIME (Version 1.8.0, http:// qiime.org/)[27]. The low-quality reads were filtered through following rules: Sequences that were shorter than 150 bp, average Phred scores below 20, contained primer mismatches, ambiguous bases or mononucleotide repeats longer than 8 bp[28–30]. FLSAH[31] (Version 1.2.7, http:// ccb.jhu.edu/software/FLASH/) was used to merge the pairs of reads from the original DNA fragments. Analyze the reads and pick operational taxonomic units (OTUs) were done using QLIME software package (http://qiime.org/) and UPARSE pipeline (http://drive5.com/uparse/). The remaining high-quality sequences were assigned to OTUs at 97% sequence similarity by UCLUST[32], and then classified taxonomically using the Ribosomal Database Project (RDP) classifier[33]. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP190011).

Alpha diversity indices including Chao1 index (Chao1) and Shannon diversity index were calculated by QIIME (http://qiime.org/scripts/alpha_diversity.html). Shannon index and Chao1 were used to assess microbiota diversity and evenness. Beta diversity analysis was used to assess the composition variation of microbial communities among samples using UniFrac distance metrics and visualized via principal coordinate analysis (PCoA)[34].

2.7 Statistical analysis

All data are presented as means ± standard error (SE). Statistical analyses were conducted using SPSS 19.0 (SPSS, Chicago, IL, USA) and checked for normality and homogeneity of variance before analysis. The growth performance, activity of antioxidant enzymes, proximate composition (diets, whole-body and muscle), amino acid composition and Alpha diversity were subjected to one-way analysis of variance (ANOVA). The differences among treatment means were resolved using Duncan's new multiple range test[35]. P values < 0.05 were considered statistically significant.

3. Results

3.1 Growth performance

There was a significant difference in the growth performance of *O. niloticus* fed with different diets and was presented in Table 3. Results showed that survival rate was in the range of 71.11-100%, there was no significant difference in survival ($P > 0.05$). PWG and SGR of *O. niloticus* fed with D3 diet were significantly higher than those fed with D1, D4-D6 diets ($P < 0.05$) but without significant differences with *O. niloticus* fed the D2 diet ($P > 0.05$). *O. niloticus* fed with D3 diet

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showed the highest value of FBW and significantly higher than that of fish fed D6 diet ($P < 0.05$), but without significant differences with *O. niloticus* fed the D1, D2, D4 and D5 diets ($P > 0.05$). No significant differences were found in FCR and PER of *O. niloticus* among all diet treatments ($P > 0.05$).

3.2 Whole body and muscle composition

The proximate composition of whole body of *O. niloticus* among all diet treatments were shown in Table 4. No significant differences were found in protein, ash and lipid contents of whole body among all diet treatments ($P > 0.05$). Moisture contents of whole body of *O. niloticus* fed with D2 and D5 diets were significantly lower than those fed with D1 diet ($P < 0.05$) but without significant differences with *O. niloticus* fed the D3, D4 and D6 diets ($P > 0.05$).

The proximate composition of muscle of *O. niloticus* among all diet treatments were shown in Table 5. No significant differences were found in protein, ash and lipid contents of muscle among all diet treatments ($P > 0.05$). Moisture contents of muscle of *O. niloticus* fed with D4 diets were significantly lower than those fed with D1 and D3 diets ($P < 0.05$) but without significant differences with *O. niloticus* fed the D2, D5 and D6 diets ($P > 0.05$).

3.3 Whole body essential amino acid profile

The essential amino acid composition of whole body of *O. niloticus* among all diet treatments were shown in Table 6. No significant differences were found in tryptophan (Trp), isoleucine (Ile) and valine (Val) levels in whole body of *O. niloticus* among the dietary treatments ($P > 0.05$). There were significant differences in the composition of other essential amino acids in *O. niloticus* between the experimental groups. Met level in whole body of *O. niloticus* fed with D3 and D4 diets were significantly higher than those fed with D1 and D2 diets ($P < 0.05$) but without significant difference with *O. niloticus* fed the D5 and D6 diets ($P > 0.05$). Arginine (Arg) level in whole body of *O. niloticus* fed with D3, D4 and D6 diets were significantly higher than those fed with D1 and D2 diets ($P < 0.05$) but without significant difference with *O. niloticus* fed the D5 diet ($P > 0.05$). Lysine (Lys), histidine (His), Leucine (Leu) and phenylalanine (Phe) levels of *O. niloticus* fed with D3 and D4 diets were significantly higher than those fed with D1 diet ($P < 0.05$) but without significant difference with *O. niloticus* fed the D2, D5 and D6 diets ($P > 0.05$). Threonine (Thr) level of *O. niloticus* fed with D3 diet were significantly higher than those fed with D1 and D2 diets ($P < 0.05$) but without significant difference with *O. niloticus* fed the D4-D6 diets ($P > 0.05$). Total essential amino acid content in whole body of *O. niloticus* fed with D3 and D4 diets were significantly higher than those fed with D1 and D2 diets ($P < 0.05$) but without significant difference with *O. niloticus* fed the D5 and D6 diets ($P > 0.05$).

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3.4 Antioxidant capacity

Antioxidant capacity of *O. niloticus* were shown in Table 7. T-AOC was significantly higher in *O. niloticus* fed with D3 diet, than in *O. niloticus* fed with D1, D2 and D4-D6 diets ($P < 0.05$). The SOD activity and MDA content of *O. niloticus* fed with D2-D6 diets had no significant difference compared with *O. niloticus* fed the D1 diet ($P > 0.05$). No significant differences were found in CAT activity among all diet treatments ($P > 0.05$).

3.5 Composition of intestinal microbiota

A total of 5628254 high quality sequencing reads were obtained, and they were clustered into OTUs of ≥ 97% identity. The number of OTUs detected in each sample ranged from 431 to 456 (Table 8). Samples from the six diet groups were well separated. At the phylum level, *Fusobacteria* (53.44%-58.82%) was the dominant phylum in all the six dietary treatments, and then followed by *Bacteroidetes* (16.75%-30.99%) (Figure 1). *Bacteroidetes* in the D3 diet treatment was significantly lower than those from other diets treatments, however, *Firmicutes* in the D3 diet treatment was significantly higher than those from other diets treatments ($P < 0.05$). At the family level, *Fusobacteriaceae* (53.33%-58.69%) was the dominant family in all six diets treatments, and then followed by *Porphyromonadaceae* (16.71%-30.99%) (Figure 2). *Porphyromonadaceae* in the D3 diet treatment was significantly lower than those from other diets treatments, however, *Clostridiaceae_1* in the D3 diet treatment was significantly higher than those from other diets treatments, moreover, *Rhodobacteraceae* from D3-D6 diets treatments were significantly higher than those from D1 and D2 diets treatments ($P < 0.05$). At the genus level, *Cetobacterium* (55.50%-58.22%) was the dominant genus in all the six diets treatments, and then followed by *uncultured_bacterium_f_Porphyromonadaceae* (17.75%-20.74%) (Figure 3). No significant difference at genus level was found in all the six dietary treatments ($P > 0.05$).

3.6 Diversity analysis of intestinal microbiota

The alpha diversity metrics were calculated from the rarefaction curves at OTUs level for each experimental feed. Chao1, Shannon index and relative abundance of microbial community were shown in Table 8 and Figure 4. There were significant differences in Chao1 and Shannon index among the experimental groups (Table 8, $P < 0.05$). Chao1 in the D1 diet treatment was significantly higher than those from D2, D5 and D6 diets ($P < 0.05$) but without significant difference from D3 and D4 diets ($P > 0.05$). Shannon index in the D3 diet treatment was significantly higher than those from other diets treatments ($P < 0.05$). The results indicated that diversity of the microbial community in the D3 diet treatment was highest. Beta diversity was demonstrated by PCoA. All samples showed in PCoA using unweighted UniFrac distances were

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regularly distribute according to different treatment groups (Figure 5). PCoA showed separated clustering of the control group and the Met-Met supplementation groups, indicating that supplementation of Met-Met in feed could significantly alter structure of intestinal microbiota.

4. Discussion

To maintain the sustainable development of *O. niloticus* aquaculture, it is necessary to optimize the feed products used. A proper diet is essential to improve the growth performance and survival of the fish and to ensure a high-quality and sustainable product is available to consumer. Met-Met, as novel nutritional supplements for aquatic and terrestrial animals, have attracted much attention in recent years. Reports concerning aquatic and terrestrial animals have indicated that the dietary supplementation with Met-Met can improve the growth performance of organisms[(5,17,18,36)]. Su et al. indicated that dietary Met-Met supplementation increased PWG, SGR and feed efficiency (FE) in *C. Idella*[(18)]. Dietary Met-Met supplementation increased PWG and SGR was also reported in *Pagrus major* by Mamauag et al.[(37)]. Niu et al. demonstrated that supplementation of Met-Met in *L. vannamei* feed can improve growth performance (PWG and SGR) and efficiency of feed utilization (PER and FE)[(5)]. Similar results were also reported in *L. vannamei* by Xie et al.[(17)]. The results from the present study are similar to those described above. The present study indicated that the optimal Met-Met supplementation remarkably increased the PWG and SGR of *O. niloticus*. The results demonstrated that Met in dipeptide form can be efficiently absorbed and utilized by *O. niloticus*. Generally, the lower the dietary Met content, the worse the growth performance of fish. However, our results showed that the growth performance of *O. niloticus* was not improved when the dietary Met level was higher than 0.61 % or less than 0.51 %, but also no adverse effect could be detected. Espe et al. obtained different results in their research[(38)]. Their research indicated that dietary Met levels exceeding the requirements of *Atlantic salmon* have a negative impact on feed intake, growth performance and survival. Above results do not contradict the idea that supplementation of Met-Met in *O. niloticus* feed can improve growth performance. The difference in the effect of Met on the growth performance of aquatic animals may be related to the form and processing technology of Met and experimental conditions (supplementary level, species, and experimental period). Since due to the growth results the authors conclude the optimal proportion of Met was 0.61%, and the addition of Met-Met was 0.15% in *O. niloticus*.

The essential amino acid composition of whole body of *O. niloticus* were affected by the different dietary treatments. The present study indicated that dietary supplementation of Met-Met significantly increased the levels of Met, Lys, Thr, Arg, Leu, His, Phe and total essential amino acid in the whole-body of *O. niloticus*. Similar results were also reported in *L. vannamei* by Niu et al.[(5)] and Xie et al.[(17)]. Niu et al. indicated that the optimal Met-Met supplementation significantly

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increased the content of total essential amino acid in *L. vannamei*[5]. Nevertheless, Boonyoung et al. indicated that there was no significant difference in the essential amino acid composition of whole body of *Oncorhynchus mykiss* fed the diets with or without MHA supplementation[39]. Supplementation of coated Met did not show remarkably difference in the amino acid composition of whole body of *Marsupenaeus japonicus* was also reported by Alam et al.[40]. The present studies have shown that the effects of Met on amino acid composition of aquatic animals are not entirely consistent. This difference may be related to the form and processing technology of Met, proportion of amino acid and experimental conditions (supplementary level, feeding regime, species, and experimental period). There is no doubt that Met-Met, a new Met product, can be well absorbed and utilized by *O. niloticus*. Dietary Met-Met supplementation can promote the absorption and utilization of amino acids (Met, Lys, Thr, Arg, Leu, His and Phe), which may contribute to faster growth in *O. niloticus*.

Fish is the main source of animal protein and micronutrients in developing countries[41]. It is essential to provide adequate dietary amino acids for human health, growth, development and survival[42]. Amino acids play an important role in maintaining normal physiological function and nutritional status of the body[43]. For example, Arg plays a regulatory role in immune function, wound healing, hormone secretion, insulin sensitivity, protein synthesis and endothelial function[42,44]; Met can regulate the metabolic process, innate immune system and digestive function[45]. Therefore, human consumption of *O. niloticus* rich in essential amino acids is conducive to growth and health. Adding Met-Met to aquatic animal feed can improve the quality of aquatic products.

Oxidative burst is an important defense mechanism, but it can produce harmful substances, such as superoxide anion, hydrogen peroxide, hydroxyl radicals[46]. To reduce oxidative stress or damage caused by reactive oxygen species (ROS), cells have developed a set of antioxidant defense systems involving antioxidant enzymes. SOD and CAT are the main antioxidant enzymes and T-AOC can evaluate the antioxidant ability of organisms by non-enzymatic or enzymatic components[47]. MDA is a product of lipid peroxidation, which mainly evaluates the damage degree of cell structure and function by its content in serum or tissue[48]. In this study, the T-AOC was increased in fed the diet with Met-Met supplementation, however, the SOD and CAT activity and MDA content were no significant difference in fed the diet with or without Met-Met supplementation. The present results indicated that dietary Met-Met supplementation did not cause damage to hepatocytes and improved antioxidant capacity of *O. niloticus*. Feng et al. indicated that dietary MHA supplementation increased the SOD and CAT activity and reduced the MDA content in hepatopancreas of *Cyprinus carpio*[49]. Elmada et al. demonstrated that dietary supplementation of Met increased the SOD activity and the MDA content in serum of *Pelteobagrus fulvidraco*,

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however, the CAT activity was not significantly affected by the dietary Met levels[50]. The above results showed that the effects of different Met form on antioxidant enzymes activities in different species and tissues were not entirely consistent. This may be due to the different tolerance of different species to Met. These results do not contradict the idea that dietary supplementation of Met-Met or Met can improve antioxidant ability. The results also indicated that Met plays an important role in regulating immune function in aquatic animals.

The effect of Met-Met supplementation on intestinal microbiota of *O. niloticus* was first reported in this study. The present results indicated that *Fusobacteria* was the dominant phylum in *O. niloticus*, followed by *Bacteroidetes*, *Proteobacteria*, *Verrucomicrobia*, *Firmicutes* and *Actinobacteria*. However, Adeoye et al. showed that *Fusobacteria* was the dominant phylum in *O. niloticus*, followed by *Proteobacteria* and *Firmicutes*[51]. In our study, the second dominant phylum was *Bacteroidetes*. Our results are different from those of Adeoye et al.[51]. The main reason for the difference results may be attributed to the difference in feed formula. It is well known that the most important contribution of *Bacteroidetes* is the degradation of dietary fiber in human[52]. In our feed formula, the feed protein was all derived from plant protein and no fish meal was used as a feed protein source. Therefore, the dominance of *Bacteroidetes* may be related to the high fiber content in our feed.

The structure of intestinal microbiota is an extremely complex collective, which has proved to have a great positive impact on host immunity, such as improving innate immune response, preventing overgrowth of pathogenic microorganisms, and metabolizing exogenous substances[53–55]. In this study, alpha diversity (Chao1 and Shannon index) which reflects the microbiota richness and diversity evidence was significantly affected in the six experimental diets. The results indicated that dietary supplementation of Met-Met leads to alterations in the gut microbiota in *O. niloticus*, especially in D3 diet treatment. *O. niloticus* fed with D3 diet showed the lowest abundance of *Bacteroidetes* and the highest abundance of *Firmicutes*. *Bacteroidetes* and *Firmicutes* occupy different functional niches in the intestinal ecosystem, and their differences in relative proportions can lead to huge functional differences[56]. Mulder et al. indicated that there was a strong negative correlation between the abundance of *Firmicutes* and pathogenic bacterial populations in the intestine[57]. Mariat et al. also demonstrated that lower *Firmicutes*: *Bacteroidetes* ratios in the intestine could increase the sensitivity of infants and the elderly to pathogens[58]. In this study, dietary supplementation of 0.15 % Met-Met could significantly increase *Firmicutes*: *Bacteroidetes* ratios, so we infer that Met-Met supplementation could contribute to improving the resistance of *O. niloticus* to pathogens. The predominance of *Firmicutes* in intestinal microflora was also considered a characteristic of obese people[59]. Li et al. indicated that *Firmicutes* domination in high growth performance group and was likely beneficial to the growth of tilapia[60]. We also got similar results.

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High growth performance was obtained in *O. niloticus* fed D3 diet, meanwhile *Firmicutes* was dominant in intestinal microflora.

5. Conclusion

In this study, we evaluated the effects of Met-Met, a new source of Met product, on growth performance, antioxidant capacity and intestinal microbiota of *O. niloticus* for the first time. Dietary Met-Met supplementation increased growth performance, antioxidant ability and the content of essential amino acids in *O. niloticus*. Meanwhile, Met-Met supplementation alters intestinal microbiota composition in *O. niloticus*. Adding 0.15% Met-Met to feed can improve the diversity of intestinal microbiota of *O. niloticus*. The optimal proportion of dietary Met was 0.61%, and the addition of Met-Met was 0.15%, which correlated with the optimal growth performance and antioxidant ability in *O. niloticus*.

Acknowledgements

Thanks the funding of the Project from Evonik Degussa (China) Co., Ltd (03 87 16004_1), and Project of National Natural Science Foundation of China (31872580), and Project of Marine Fishery Science and Technology of Guangdong Province (A201601C11, GDME-2018C012), and Natural Science Foundation of Guangdong Province (2017A030313195), and Project of Science and Technology of Guangzhou City (201803020006) and Modern Agriculture and Marine Biological Industry of ShenZhen (20170428140437749).

Authors' contributions

The authors thank the participants who gave their time to the trial. Jin Niu, Yong-Jian Liu, Li-Xia Tian, Ju-Yun He and Karthik Masagounder designed the study. Tian-Yu Guo, Wei-Zhao, Jia-Jun Xie, and Shi-Yu Liao carried out the rearing work. Tian-Yu Guo and Wei-Zhao analyzed the results and wrote the paper with contributions from the other authors. There are no conflicts of interest.

Competing interests

Author Ju-Yun He was employed by company Evonik Degussa (China) Co., Ltd. Guangzhou Branch. Author Karthik Masagounder was employed by company Evonik Nutrition and Care GmbH. The authors declare that this study received funding from Evonik Nutrition and Care GmbH. The funder participated in the design of the experiment. All other authors declare no competing interests.

Consent for publication

Not applicable

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Ethics approval and consent to participate

All the experiments were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). The study protocol and all experimental procedures were approved by Experimental Animal Ethics Committee of Sun Yat-sen University.

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Table 1 Composition and nutrient levels of the experimental diets (%)

Items	D1	D2	D3	D4	D5	D6
Ingredients						
Soybean meal	29	29	29	29	29	29
Rapeseed meal	28	28	28	28	28	28
Wheat flour	22.02	22.02	22.02	22.02	22.02	22.02
Rice	12	12	12	12	12	12
Soybean oil	2.2	2.2	2.2	2.2	2.2	2.2
Soybean lecithin	1.2	1.2	1.2	1.2	1.2	1.2
$Ca(H_2PO_4)_2$	2	2	2	2	2	2
Vitamin premix[a]	0.5	0.5	0.5	0.5	0.5	0.5
Mineral premix[b]	0.5	0.5	0.5	0.5	0.5	0.5
Ascorbic phosphate ester	0.1	0.1	0.1	0.1	0.1	0.1
Choline chloride	0.2	0.2	0.2	0.2	0.2	0.2
AQUAVI® Met-Met	0	0.1	0.2	0.3	0.4	0.5
Gly	0.5	0.4	0.3	0.2	0.1	0
Lys	0.6	0.6	0.6	0.6	0.6	0.6
Thr	0.2	0.2	0.2	0.2	0.2	0.2
Trp	0.1	0.1	0.1	0.1	0.1	0.1
Ile	0.2	0.2	0.2	0.2	0.2	0.2
Leu	0.08	0.08	0.08	0.08	0.08	0.08
Val	0.2	0.2	0.2	0.2	0.2	0.2
His	0.4	0.4	0.4	0.4	0.4	0.4
Total	100	100	100	100	100	100
Nutrient levels[c]						
Moisture	9.35	9.73	9.54	9.49	9.46	9.63
Crude protein	30.5	30.54	30.76	30.42	30.38	30.52
Crude lipid	5.52	4.97	4.74	4.53	5.21	4.69
Ash	7.79	7.81	7.67	7.79	7.60	7.69

[a] Vitamin premix provides the following per kg of diet ：cholecalciferol 0.6 M.I.U., thiamin 3.6 g, riboflavin 7.2 g, pyridoxine HCL 6.6 g, cyanocobalamin 0.02 g, tocopherol 16.5 g, menadione 2.4

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g, nicotinic acid 14.4 g, calcium pantothenate 4 g, biotin 0.02 g, folic acid 1.2 g, inositol 30 g, ascorbic Acid 100 g, cellulose was used as a carrier.

[b] Mineral premix provides the following per kg of diet ：P 120 g, Ca 120 g, Mg 15 g, Fe 1.5 g, Zn 4.2 g, Cu 2.1 g, K 75 g, Co 0.11 g, Mn 1.6 g, Se 0.01 g, Mo 0.005 g, Al 0.025 g, I 0.4 g, cellulose was used as a carrier.

[c] Measured values.

Table 2 Amino acid composition of six experimental diets (% DM basis)

Items	D1	D2	D3	D4	D5	D6
Met-Met	0	0.07	0.15	0.21	0.28	0.38
Met	0.43	0.51	0.61	0.67	0.77	0.88
Cys	0.49	0.47	0.48	0.50	0.48	0.48
Met_Cys	0.92	0.99	1.08	1.17	1.25	1.37
Lys	1.94	1.96	1.97	1.96	1.95	1.97
Thr	1.30	1.26	1.27	1.28	1.28	1.29
Trp	0.48	0.46	0.46	0.45	0.45	0.46
Arg	1.78	1.83	1.84	1.83	1.83	1.85
Ile	1.35	1.37	1.37	1.37	1.35	1.40
Leu	2.06	2.05	2.07	2.06	2.05	2.07
Val	1.57	1.57	1.58	1.57	1.55	1.58
His	1.06	1.05	1.06	1.06	1.05	1.07
Phe	1.23	1.24	1.25	1.24	1.23	1.26
Gly	1.69	1.60	1.52	1.43	1.34	1.27
Ser	1.36	1.29	1.31	1.31	1.31	1.31
Pro	1.62	1.63	1.68	1.64	1.62	1.67
Ala	1.26	1.24	1.25	1.25	1.24	1.25
Asp	2.63	2.56	2.57	2.58	2.56	2.61
Glu	5.31	5.14	5.25	5.18	5.22	5.28

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Table 3 Growth performance of *Oreochromis niloticus* fed experimental diets for 56 days

Items	IBW/g	FBW/g	PGW/%	SGR/(%/d)	Survival/%	FCR	PER
D1	17.12±0.02	84.46±0.39ab	393±2.81a	2.85±0.01a	71.11±12.8	1.44±0.02	2.28±0.03
D2	17.18±0.06	87.94±0.38ab	412±4.03ab	2.92±0.01ab	95.57±1.13	1.42±0.03	2.30±0.04
D3	17.13±0.04	92.25±4.43b	448±19.6b	3.04±0.06b	78.89±9.09	1.37±0.03	2.38±0.05
D4	17.14±0.06	87.01±2.88ab	407±16.1a	2.90±0.06a	80.00±15.28	1.41±0.05	2.35±0.09
D5	17.18±0.08	86.91±2.49ab	406±13.8a	2.89±0.05a	98.89±1.11	1.38±0.03	2.38±0.06
D6	17.15±0.08	82.96±1.10a	384±5.16a	2.82±0.02a	100±0.00	1.45±0.02	2.27±0.04

Values are means ± SE of three replicates. The superscript small letters in the same column means the significant difference at $P<0.05$.

Table 4 Whole body composition (% dry matter) of *Oreochromis niloticus* fed experimental diets for 56 days

Items	D1	D2	D3	D4	D5	D6
Whole body						
Moisture	73.52±0.56b	71.71±0.52a	72.25±0.16ab	72.45±0.01ab	71.89±0.35a	72.11±0.63ab
Protein	55.05±1.73	55.59±1.01	55.56±0.83	56.56±2.47	56.03±0.65	55.971±0.63
Lipid	19.68±2.72	16.13±4.25	23.03±3.54	21.38±1.26	19.97±1.95	19.31±0.95
Ash	16.49±1.66	16.40±0.15	15.65±1.09	14.77±1.39	16.10±0.93	17.05±0.29

Values are means ± SE of three replicates. The superscript small letters in the same row means the significant difference at $P<0.05$.

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Table 5 Muscle composition (% dry matter) of *Oreochromis niloticus* fed experimental diets for 56 days

Items	D1	D2	D3	D4	D5	D6
Muscle						
Moisture	76.60±0.06b	76.37±0.15ab	76.67±0.30b	75.57±0.53a	76.47±0.24ab	76.00±0.25ab
Protein	86.53±0.07	88.00±0.21	87.57±0.12	87.13±0.95	87.03±0.35	87.17±0.15
Lipid	3.60±0.86	2.40±0.35	2.73±0.38	3.17±0.20	3.00±0.31	2.83±0.12
Ash	6.73±0.29	6.27±0.32	6.67±0.41	6.20±0.20	6.10±0.15	6.13±0.07

Values are means ± SE of three replicates. The superscript small letters in the same row means the significant difference at *P*<0.05.

Table 6 Essential amino profile (% dry matter basis) in the whole body of *Oreochromis niloticus* fed experimental diets for 56 days

Items	D1	D2	D3	D4	D5	D6
Met	1.39±0.02a	1.4±0.01a	1.51±0.05b	1.5±0.03b	1.44±0.03ab	1.45±0.03ab
Lys	4.03±0.16a	4.15±0.12ab	4.49±0.17b	4.54±0.17b	4.16±0.07ab	4.38±0.04ab
Thr	2.39±0.03a	2.43±0.03ab	2.58±0.06c	2.54±0.03bc	2.47±0.04abc	2.49±0.05abc
Trp	0.53±0.01	0.52±0.01	0.57±0.03	0.57±0.02	0.53±0.01	0.56±0.01
Arg	3.22±0.05a	3.26±0.07a	3.49±0.04b	3.53±0.07b	3.36±0.04ab	3.48±0.03b
Ile	2.30±0.07	2.32±0.05	2.47±0.07	2.47±0.08	2.32±0.04	2.42±0.01
Leu	3.77±0.09a	3.83±0.07ab	4.10±0.09b	4.08±0.14b	3.83±0.06ab	3.94±0.01ab
Val	2.65±0.04	2.67±0.08	2.82±0.12	2.82±0.04	2.75±0.08	2.78±0.06
His	1.3±0.01a	1.36±0.02ab	1.44±0.02b	1.45±0.03b	1.36±0.05ab	1.39±0.03ab
Phe	2.22±0.02a	2.33±0.06ab	2.45±0.04b	2.43±0.11b	2.27±0.05ab	2.36±0.03ab
ΣEAA	23.79±0.44a	24.27±0.22a	25.92±0.58b	25.94±0.69b	24.48±0.45ab	25.25±0.14ab

Values are means ± SE of three replicates. The superscript small letters in the same row means the significant difference at *P*<0.05.

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Table 7 Effect of experimental diets on antioxidant parameters (CAT, U mg^{-1} protein; SOD, U mg^{-1} protein; MDA, nmol mg^{-1} protein; T-AOC, mg protein ml^{-1}) of *Oreochromis niloticus* at the end of the trial (56 days).

	D1	D2	D3	D4	D5	D6
CAT	5.58±1.11	5.56±0.94	6.80±0.72	4.74±0.95	5.68±0.64	6.86±0.32
SOD	110.72±3.92ab	116.47±9.68b	103.85±3.14ab	92.85±4.49a	94.56±8.03a	105.88±5.23ab
MDA	1.25±0.07ab	1.40±0.07b	1.21±0.17ab	1.05±0.1ab	0.94±0.09a	0.95±0.14a
T-AOC	5.68±1.50ab	4.05±0.26a	13.30±1.71d	6.39±0.06ab	9.92±0.94c	8.08±0.13bc

Values are means ± SE of three replicates. The superscript small letters in the same row means the significant difference at $P<0.05$.

CAT, catalase; SOD, superoxide dismutase; MDA, malondialdehyde; T-AOC, total antioxidant capacity.

Table 8 Diversity index of gut bacteria of juvenile *Oreochromis niloticus* fed the six experimental diets for 8 weeks based on V4 sequences

Items	D1	D2	D3	D4	D5	D6
OTUs	456	431	454	456	435	431
Chao 1	443.9±5.86c	412.1±6.7ab	429.73±1.91bc	430.63±10.44bc	413.38±5.82ab	403.11±4.42a
Shannon	2.2±0.01b	2.07±0.01a	2.44±0.02c	2.21±0.06b	2.11±0.03ab	2.18±0.04ab

Values are means ± SE (n = 4 for OTUs and n = 10 for Chao1 and Shannon). The superscript small letters in the same row means the significant difference at $P<0.05$. OTU, operational taxonomic unit; Chao1, Chao 1 index; Shannon, Shannon diversity index.

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