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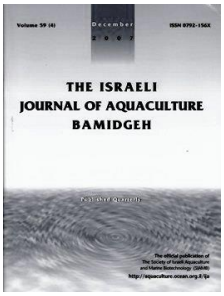


# Effects of Dietary Pantothenic Acid Supplement on Hepatic Antioxidative Abilities and Intestinal Microflora in Juvenile Golden Pompano (*Trachinotus Ovatus*)

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**Keywords**: *Trachinotus ovatus*; pantothenic acid deficiency; hepatic antioxidative abilities; intestinal microflora

**Abstract**

This study explored the effects of dietary pantothenic acid (PA) supplement on juvenile golden pompano (*Trachinotus ovatus*). Six isonitrogenous and isolipidic diets containing six graded levels of pantothenic acid (0, 16.4, 20.0, 26.0, 33.4 and 37.0 mg/kg) were formulated. Juvenile golden pompano (8.80±0.10g) were randomly assigned into six groups with three replicates in each group and 20 fish in each replicate. The fish were fed twice a day for 8 weeks. The results indicated that PA deficiency caused clubbed gills with interlamellar proliferative lesions. Survival rate increased significantly ($P<0.05$) when PA levels were increased. Dietary PA significantly increased the activities of hepatic acid phosphatase (ACP), alkaline phosphatase (ALP), peroxidase (POD) and glutathione reductase (GR) ($P<0.05$) and their activities reached the maximum in the Diet-P4 group. A diet supplemented with 20.0 and 26.0 mg/kg PA significantly decreased ($P<0.05$) the activity of hepatic MDA. Dietary PA also increased the diversity and abundance of intestinal microflora and inhibited the growth of harmful bacteria. In conclusion, PA deficiency caused lesions of gills and optimal PA supplement in diet increased hepatic antioxidative abilities and improved intestinal microflora of juvenile golden pompano.

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## Introduction

Pantothenic acid (PA) is a water-soluble vitamin, which has been demonstrated to be an essential dietary nutrient for fish (Raggi *et al.*, 2016). It plays an important role in the metabolic tricarboxylic acid (TCA) cycle (USA, 2011). PA-free diets have a negative influence on normal metabolism in cells which undergo rapid mitosis and high energy expenditure. PA deficiency can cause clubbed gills and severe hyperplasia of gill lamellae cells (USA, 2011). Our previous studies indicated that diets with 21.03 mg/kg PA can satisfy growth and enhance the blood serum response and activities of amylase, creatine kinase, and γ-glutamyl transpeptidase, in the intestines of juvenile golden pompano (Xun *et al.*, 2018). But the effects of PA on hepatic antioxidative abilities of juvenile golden pompano remain unknown. In fish, liver has the antioxidative and alexipharmic function (Zhang *et al.*, 2015) that enhances disease resistance of juvenile Jian carp (Wen *et al.*, 2010) and disease resistance related to antioxidative ability of fish (Yang *et al.*, 2010, Ai *et al.*, 2004). Intestinal microflora are sensitive to dietary changes. The secretory metabolites of intestinal microflora decompose organic matter in foods and enhance digestion and absorption of nutrients (Li *et al.*, 2015). Nevertheless, there are few reports on the effect of PA on hepatic antioxidative ability in fish.

As far as we know, there is only one report about the effect of PA on intestinal microflora in fish, that indicates that PA promotes growth and reproduction of beneficial bacteria and depresses harmful bacteria in juvenile Jian carp (Wen *et al.*, 2010). It is important to study the effects of PA on intestinal microflora in juvenile golden pompano. With the popularization of high-throughput sequencing technology, the study of intestinal microflora has reached the gene level, which can further help explore their mechanism of action. 16S/18S/ITS rDNA sequence consists of a conserved region and a hypervariable region. There is little difference between microbial species in the conserved region and the hypervariable region has the specificity of genus or species. Therefore, 16S/18S/ITS rDNA can be used as an indicator to reveal the characteristic nucleic acid sequence of biological species and its suitability for microbial phylogenetic development and taxonomic identification. (Wang, 2005)

This study was a part of a larger study to determine PA requirements (Xun *et al.*, 2018) and it investigates the characterization of PA deficiency and its effect on hepatic antioxidative ability and intestinal microflora of golden pompano, *T. ovatus*.

## Materials and Methods

*Experimental diets.*

Composition of the basal diet is presented in Table 1. Vitamin-free casein, soy protein concentrate, and fishmeal were used as dietary protein. Fish oil and soybean lecithin were used as lipid sources. The basal diet was formulated to contain 44.76% crude protein and 13.43% crude lipid. This formulation has enough nutritional value for the growth of golden pompano (Tan *et al.*, 2016, Lin *et al.*, 2015, Zhou *et al.*, 2015). PA was added to the diets in the form of calcium pantothenate to form six diets that were determined by liquid chromatography (GB/T 18397-2014) to contain 0, 16.4, 20.0, 26.0, 33.4, 37.0 mg/kg diet. Manufacturing process of the diets was the same as in our previous studies (Xun *et al.*, 2018). The diets were stored at -20℃ until used.

**Table 1.** Formulation and proximate analysis of the basal diet

| *Ingredient* | *Content/%* |
|---|---|
| Casein | 20.0 |
| Soy protein concentrate | 18.0 |
| Fishmeal | 16.0 |
| Wheat flour | 23.0 |
| Fish oil | 8.0 |
| Soybean Lecithin | 4.0 |
| Choline chloride | 2.0 |
| Antioxidant | 0.5 |
| Vitamin premix (PA free) † | 2.0 |
| mineral premix‡ | 1.0 |
| monocalcium phosphate | 1.0 |
| micro-cellulose | 3.5 |
| Attractant | 1.0 |
| proximate composition | |
| moisture | 7.97 |
| crude protein | 44.76 |
| crude lipid | 13.43 |
| ash | 6.44 |

†. Vitamin premix provided the following per kg of diet: $VB_1$ 25mg,$VB_2$ 45mg, $VB_{12}$ 0.1mg,$VK_3$ 10mg, inositol 800mg, nicotinic acid 200mg, folic acid 1.2mg, biotin 32mg, $VD_3$ 5mg, VE 120mg, VC 2.0g, choline chloride 2.0g, ethoxyquin 150mg, avicel 14.52mg;

‡. mineral premix provided the following per kg of diet: NaF 4 mg，KI 1.6 mg，$CoCl_2•6H_2O$(1%) 100 mg，$CuSO_4•5H_2O$ 20 mg，$FeSO_4•H_2O$ 160 mg，$ZnSO_4•H_2O$ 100 mg，$MnSO_4•H_2O$ 120 mg，$MgSO_4•7H_2O$ 2.4 g，$Ca(H_2PO_4)_2•H_2O$ 6.0 g，NaCl 200 mg，zeolite powder 30.90 g.

*Experimental procedure.*

Juvenile golden pompano (*T. ovatus*) fingerlings were obtained from Shenzhen Long Qizhuang Industrial Development Co., Ltd. Prior to the feeding trial they were acclimated to laboratory conditions for 2 weeks in polythene cages and were fasted for 24h, and thereafter were fed a commercial diet. A total of 360 golden pompano (initial weight 8.80±0.10 g) were randomly assigned to 18 floating cages (1×1×1.5 $m^3$; three cages per treatment) and 20 fish per cage. The juvenile golden pompano were fed twice a day at 6:30 and 17:00 until apparent satiation. The weight and number of dead fish and feeding quantity was recorded on a daily basis. Water temperature ranged from 26.7-29.0℃, salinity ranged between 15-20‰, and pH from 7.1-8.0. Dissolved oxygen was higher than 6.0 mg/L. Ammonia nitrogen was maintained lower than 0.05 mg/L.

*Sample collection and analysis.*

At the end of the feeding trial, fish were fasted for 24 h, then anesthetized with 100 mg/L Eugenol (Shanghai Medical Instruments Co., Ltd, Shanghai, China). Five fish with similar body weight from each cage were sacrificed, their gills dissected and placed in the Polyformaldehyde solution and then sent to Google Biotechnology Ltd. to prepare segments. The gill sections were examined for histological changes. Livers from the sampled fish were dissected and centrifuged together with sterilized physiological saline (0.86%, pH 7.4). Hepatic samples were homogenized by handheld homogenizer in an ice bath. The homogenate was then centrifuged for 20 min at 3000 r/min and the supernatant collected to quantify hepatic activities of acid phosphatase (ACP), alkaline phosphatase (ALP), total antioxidant capacity (T-AOC), peroxidase (POD), glutathione peroxidase (GPX), glutathione reductase (GR), and malondialdehyde (MDA) using an assay kit produced by Nanjing Jiancheng Bioengineering Institute (China). Intestines of the five fish were dissected, immediately placed in liquid nitrogen, and sent to Guangzhou JiRui Gene Technology Co. Ltd (China) for extraction and detection of DNA, PCR amplification by Illumina MiSeq Sequencing platform. After processing data, intestinal microflora diversity was analyzed by operational taxonomic unit (OUT) analysis and alpha diversity. Shannon-Wiener diversity index is defined as:

$$\mathrm{H} = -\sum_{i=1}^{s}(p\_i \log_2 p_i)$$ †

†: s is the number of OTUs; $p_i$ is the proportion of the community represented by OTU i.

*Statistical analysis.*

Results are expressed as the mean±S.D of three replicates following a one-way analysis of variance (ANOVA). *P*-value of <0.05 was considered significant, and Duncan's multiple range test was used to rank the treatments. (Duncan, 1955). All statistical analyses were performed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA) for Windows.

## Results

*Effect of dietary PA deficiency on histological changes of gills of golden pompano*.

Effect of dietary PA deficiency on gills histological changes is shown in Fig. 1. After 8 weeks, the fish fed PA-free diet exhibited clubbed gills with interlamellar proliferative lesions, which was the most apparent deficiency sign. Compared to the Diet-P1 (PA-free) group, there were lesions in the gills of fish fed with the supplemented diets.

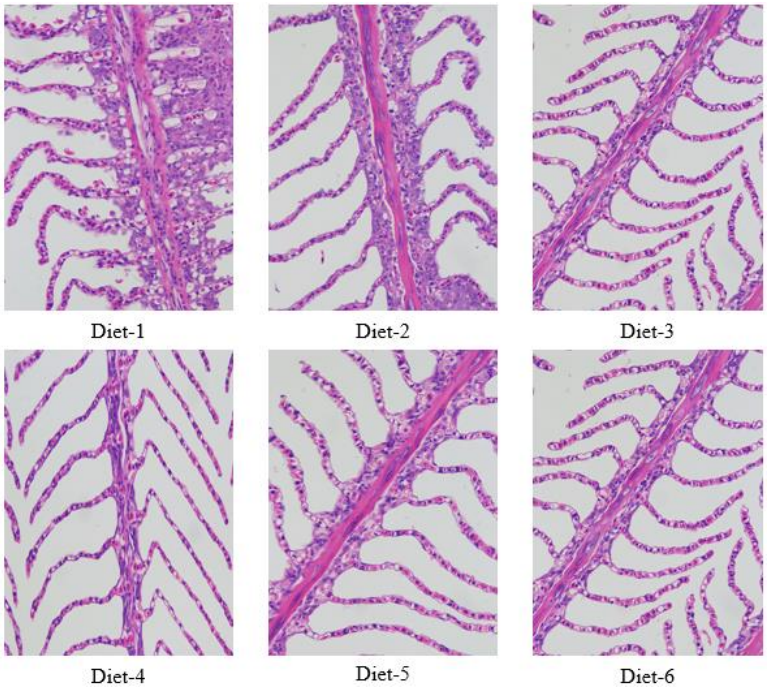


**Fig. 1.** Gills histological images (×400) of golden pompano fed on Diet-P1(PA-free), Diet-P2(16.4mg/kg), Diet-P3(20.0 mg/kg), Diet-P4(26.0 mg/kg), Diet-P5(33.4 mg/kg) and Diet-P6(37.0 mg/kg) for 8 weeks

*Effect of dietary PA level on the hepatic antioxidative ability of golden pompano.*

Effect of dietary PA deficiency on the hepatic antioxidative ability of golden pompano is shown in table 2. Dietary PA significantly increased the activities of ACP, ALP, POD and GR ($P<0.05$) and their activities reached a maximum or second maximum when the fish were fed Diet-P4 (26.0mg/kg). Dietary PA significantly decreased MDA activity ($P<0.05$). The minimum levels were recorded with Diet-P3 and Diet-P4. There were no differences on GPX and T-AOC activities.

**Table 2.** Effects of dietary PA on hepatic antioxidative abilities in golden pompano (*T. ovatus*) †

| Diets (Vitamin PA mg/kg) | Diet-P1 | Diet-P2 | Diet-P3 | Diet-P4 | Diet-P5 | Diet-P6 |
|---|---|---|---|---|---|---|
| | 0 | 16.4 | 20.0 | 26.0 | 33.4 | 37.0 |
| ACP activity (U/mg) | 246.69±4.53$^{a}$ | 236.94±29.57$^{a}$ | 381.42±50.19$^{b}$ | 352.59±23.6$^{b}$ | 251.34±35.47$^{a}$ | 247.17±0.02$^{a}$ |
| ALP activity (U/mg) | 22.83±5.72$^{a}$ | 26.91±10.17$^{a}$ | 34.60±0.00$^{a}$ | 51.81±0.98$^{b}$ | 23.93±6.83$^{a}$ | 27.38±12.71$^{a}$ |
| POD activity (U/mg) | 0.61±0.00$^{a}$ | 0.62±0.05$^{a}$ | 0.69±0.10$^{ab}$ | 0.74±0.02$^{b}$ | 0.74±0.02$^{b}$ | 0.76±0.09$^{b}$ |
| MDA content (nmol/mg) | 1.51±0.25$^{a}$ | 1.12±0.00$^{b}$ | 0.78±0.05$^{c}$ | 0.78±0.15$^{c}$ | 0.82±0.00$^{c}$ | 1.15±0.02$^{b}$ |
| GR activity (U/g) | 7.66±1.32$^{a}$ | 7.59±0.20$^{a}$ | 7.85±1.61$^{a}$ | 10.70±0.51$^{b}$ | 7.81±1.46$^{a}$ | 8.54±0.98$^{a}$ |
| GPX activity (U/g) | 64.19±4.17 | 67.56±17.69 | 73.97±2.36 | 69.62±2.27 | 69.15±0.00 | 65.70±4.98 |
| T-AOC (U/g) | 0.91±0.03 | 0.90±0.01 | 0.88±0.01 | 0.88±0.01 | 0.88±0.01 | 0.88±0.02 |

†ACP, acid phosphatase; ALP, alkaline phosphatase; POD, peroxidase; MDA, malondialdehyde; GR, glutathione reductase; GPX, glutathione peroxidase; T-AOC, total antioxidant capacity; Date represents mean±SD of three replicates and values in the same column with different letters are significantly different determined by Tukey's test ($P<0.05$).

*Effect of dietary PA level on the intestinal microflora of golden pompano.*

OTU number and Alpha diversity of intestinal microflora in golden pompano are shown in table 3.

**Table 3.** OTU number and Alpha diversity of intestinal microflora in golden pompano (*T. ovatus*)

| *Diets* | *Diet-P1* | *Diet-P3* | *Diet-P4* | *Diet-P6* |
|---|---|---|---|---|
| *(Vitamin PA mg/kg)* | *0* | *20.0* | *26.0* | *37.0* |
| Reads† | 59111 | 66589 | 64163 | 67496 |
| OTUs‡ | 31 | 32 | 41 | 35 |
| ACE§ | 66.12 | 66.43 | 80 | 73.90 |
| Chao1 | 64.5 | 65.5 | 80 | 71.5 |
| Shannon | 2.35 | 2.38 | 2.43 | 2.27 |
| Coverage | 100% | 100% | 100% | 100% |

† Optimized total number of sequences

† Optimized total number of sequences
‡The operational taxonomic units (OTU) were defined at the 97 % similarity level
§The richness estimators (ACE and Chao1), diversity indices (Shannon and Simpson) and coverage percentage (coverage) were generated with Qiime programme

Good's coverage was estimated for the completeness of sampling, by calculating the probability that a randomly selected amplicon sequence was already detected in the same sample (Li *et al*., 2015). Coverage reached 100% in this study, which suggests that all of bacterial phylotypes present in the samples were identified. This result reflects the true coverage of the intestinal microflora samples. Operational Taxonomic Units (OTU) represent the abundance of intestinal microflora. Analysis of biological information revealed that each sequence obtained by sequencing comes from a strain. In order to recognize the number of bacteria and genera in a sample, it is necessary to classify the sequence. By categorizing operations, sequences are grouped based on their similarity, and a group is an OTU. The number of OTUs first increased and then decreased when the PA-supplement was increased and the maximum OTUs were recorded in Diet-P4 group. The Chao1,ACE index can be used to estimate the number of OTUs in the community. This is commonly used in ecology to estimate the total number of species. The greater the value, the greater the total number of species (Shu *et al*., 2015). The result was similar to those of ACE and Chao1. The number of OTUs covered 47~51 and 48~51% of the richness estimated by the ACE and Chao1 indices, respectively. Shannon index reflects species diversity based on the number of species, and the greater the index, the more complex the community (Shu *et al*., 2015). Shannon analysis results showed the diversity of intestinal microflora which increased first and then decreased with increased PA-supplement. The maximum level was recorded in Diet-P4 group (Fig.2).The results of intestinal microflora composition are shown in Figs 3 & 4. Composition of intestinal microflora at the level of phylum included *Proteobacteria*, *Firmicutes,* and *Tenericutes*. They occupied 96~99% in all microflora (Fig.3). Composition of intestinal microflora at the level of genus included mainly *Exiguobacterium*, *Unclassified*, *Acinetobacter*, *Pseudomonas* and *Mycoplasma*. They occupied 96%~98% in all microflora (Fig.4).

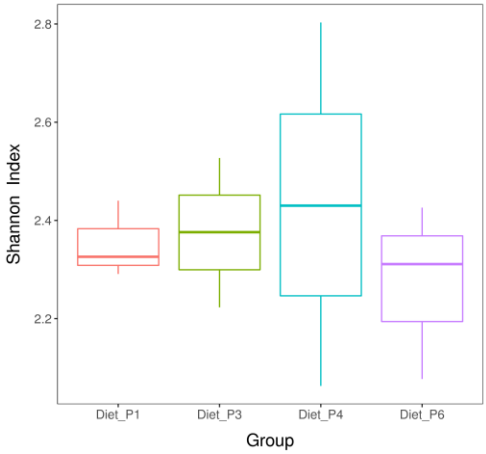


**Fig.2.** Inter-group difference box diagram based on Shannon analysis

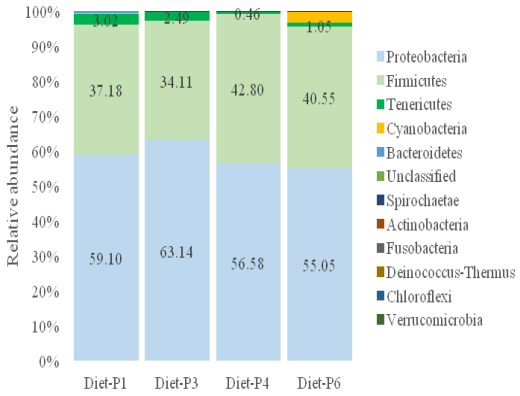


**Fig. 3.** Bacterial composition of the different communities (% of relative read abundance of bacterial phyla within each community).

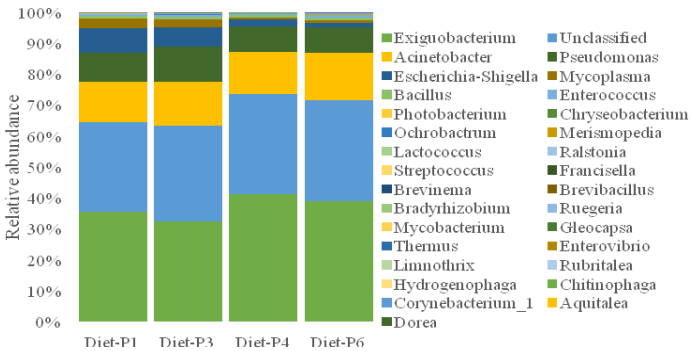


**Fig.4.** Bacterial composition of the different communities (% of relative read abundance of bacterial genus within each community).

## Discussion

The growth of fish is closely related to the health status of the gills (Liney *et al.*, 2006). Gills are a vital organ for breathing and also for the excretion of nitrogenous waste, osmoregulation, hormone production, and pH regulation (Herrero *et al.*, 2018). In the present study, PA deficiency disease appeared in the Diet-P1 group. Compared to the PA-supplemented groups, the Diet-P1 group exhibited clubbed gills with interlamellar proliferative lesions. Gill filaments of fish fed Diet-P1 were thicker than those fed the PA-supplement diets, but the survival rate of fish fed Diet-P1 group was significantly lower than those in the PA-supplemented groups. (see Fig 5). Clubbed gills decreased superficial area of gill filaments, resulting in reduced oxygen uptake probably responsible for low survival rate. The reports on the effects of PA on juvenile hybrid striped bass (Raggi *et al.*, 2016) and grouper, *Epinephelus malabaricus* (Lin *et al.*, 2012) supported our results.

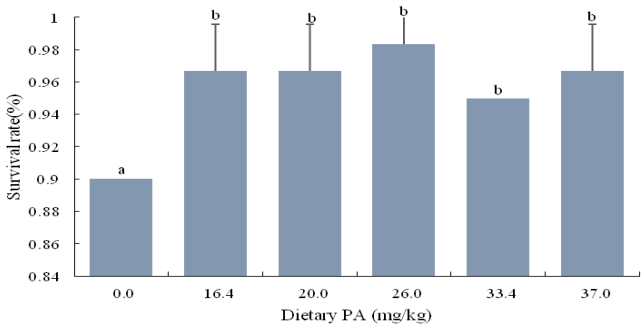


**Fig. 5.** Relation between the survival rate of golden pompano and dietary PA.

The liver is an important immune organ in fish. Hepatic antioxidative abilities are related to antibacterial compounds like ACP and ALP (Wu *et al.*, 2013). ACP plays a key role in decomposing phagocytosed bacteria (Puangkaew *et al.*, 2005, Boshra *et al.*, 2006) and its activity is an indication of macrophage activation (Dalmo *et al.*, 2010). ALP as a non-specific hydrolase that is widespread in liver. It plays a key role in growth, apoptosis and signal transduction pathways (Jin *et al.*, 2015). Hepatic antioxidative ability is closely correlated to POD, GR, GPX, MDA and T-AOC. In the present study, PA deficiency decreased ACP, ALP, POD and GR activities significantly, whereas optimal PA supplementation could reverse this tendency. PA deficiency increased MDA content in the liver of golden pompano. Oxidative damage may be associated with the decrease in free radical scavenging capacity. MDA indirectly reflects lipid peroxidation and the severity of free radical attacks on fish body cells (Li *et al.*, 2015). In reactive oxygen species scavenging is correlated with antioxidant systems, including non-enzymatic agents like glutathione and enzymatic antioxidants like POD, T-AOC, GPX, and GR. T-AOC reflected antioxidant capacity of fish. POD and GPX decompose $H_2O_2$ and protect organism from highly reactive hydroxyl radicals (Williams and Burk, 1990, Tan *et al.*, 2016). They play an indispensable role in scavenging ROS in the liver of fish (Li *et al.*, 2015). These results suggest that optimal PA supplementation improves antioxidative status by inhibiting free radical formation, reducing lipidic superoxide harm, increasing ACP, ALP, POD and GR activities and decreasing MDA content. There was no report on the effect of PA on liver antioxidative ability of fish. There are reports that PA can decrease MDA levels and increase GPX and GR activities in the gills and intestine of grass carp. These results are similar to our results (Li *et al.*, 2015, Li *et al.*, 2015).

In fish, intestinal microflora fulfill several roles. Digestion and absorption of nutrients is often related to intestinal microflora. In the present study, compared to Diet-P1, a diet supplemented with 26.0 mg/kg increased the diversity and abundance of intestinal microflora. The dominant bacteria of golden pompano belonged to three phyla: *Proteobacteria*, *Firmicutes,* and *Tenericutes* according to gut microbial analysis; this agreed with turbot (Xing, 2013). The maximum and minimum proportion of *Proteobacteria* appeared in the Diet-P3 and Diet-P6 groups, respectively, whereas the maximum and minimum proportion of *Firmicutes* appeared in Diet-P4 and Diet-P3 groups. Diet-P4 group had the smallest proportion of *Tenericutes* of all groups. *Mycoplasma* was regarded as pathogenic bacteria in many studies (Chaudhry *et al.*, 2016, Bürki *et al.*, 2016). In the present study, the greatest proportion of *Mycoplasma* was found in the Diet-P1 group, which suggests that PA-supplement could inhibit the number of *Mycoplasma* thus increasing the antioxidative abilities of golden pompano.

*Cyanobacteria* appeared only in the Diet-P1 and Diet-P6 groups. *Cyanobacteria* can produce and release microcystins, which can be harmful to the liver in aquatic life (Dong *et al.*, 2009, Qiao *et al.*, 2013). This suggests that both deficiency and excess of PA could increase growth of *Cyanobacteria* thus harming the liver of golden pompano.

## Conclusions

Three primary, novel, and interesting results from this study demonstrated that: (1) PA deficiency in *T. ovatus* can cause clubbed gills with interlamellar proliferative lesions and low survival rate; (2) PA deficiency significantly decrease hepatic antioxidative abilities of golden pompano; (3) A diet supplemented with 26.0 mg/kg PA could improve intestinal microflora of golden pompano by increasing the diversity and abundance of intestinal microflora, inhibiting the growth of harmful bacteria.

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