Effects of phosphatidic acid on growth and antioxidant capacity in juvenile turbot, *Scophthalmus maxius* L., fed with high plant protein-based diets

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Abstract
Replacement of fish meal with high plant protein meal leads to reduced growth performance and health issues in farmed fish. Supplementation of functional additives has been proposed as a potential strategy to alleviate the negative effect of plant protein replacement. In this study, the effects of phosphatidic acid (PA) on growth and antioxidant capacity in juvenile turbot fed with high plant protein-based diets were evaluated. An 11-week feeding trial was performed using diets containing 0 (CON group), 0.05, 0.1, or 0.5% PA. The results showed that adding 0.5% PA could significantly improve the growth performance of turbot without affecting feed intake. Moreover, supplementation of 0.5% PA significantly increased the mRNA level of *igf-1* in liver. The condition factor was also enhanced in PA addition groups. The activity of lipase was increased by PA supplementation, while no difference was observed in amylase and trypsin activity. Supplying PA significantly reduced the content of malondialdehyde. Furthermore, the mRNA levels of *superoxide dismutase* (*sod*), *glutathione peroxidase* (*gpx*), and *peroxiredoxin 6* (*prx6*) were enhanced in 0.1 and 0.5% PA groups. Our results showed that PA might be a...
INTRODUCTION

Although fish meal (FM) is an ideal protein source in aquafeed especially for carnivorous fish species because of its well-balanced amino acid profile and favorable palatability, the decline of global FM production, unstable supply, and the expansion of aquaculture industry make it hard to meet the market demand (Tacon & Metian, 2008). In recent years, much effort has been made to develop alternative protein sources including plant-based protein sources (Barros, Lim, & Klesius, 2002; Hernández, Martínez, Jover, & García, 2007; Takakuwa et al., 2020). However, as protein source, plant proteins can only replace a small amount of FM, especially for carnivorous fish’s diets. Numerous studies have found that the substitution of plant protein sources was compromised with reduced feed efficiency and fish growth (Penn, Bendiksen, Campbell, & Krogdahl, 2011; Takagi et al., 2010). Many limiting factors in plant protein such as high level of anti-nutritional factors (ANF), imbalanced content of amino acids, poor palatability, and increased cellulose could damage the growth performance, immunocompetence, and intestinal morphology of feeding objects (Dossou et al., 2018; Hansen, Rosenlund, Karlsen, Koppe, & Hemre, 2007). Moreover, Aksnes, Hope, Jónsson, Björnsson, and Albrektsen (2006) pointed out that the release amount and ratio of small molecular weight compounds of plant proteins during digestion were obviously different from those of FM, which could affect the absorption of protein sources in feeding objects.

It has been widely reported that high levels of plant protein diet disturbed the oxidative homeostasis of fish (Bian et al., 2017; Ostaszewska et al., 2010). Oxidative homeostasis is regulated by the generation and elimination of reactive oxygen species (ROS). The antioxidant system is a very important ROS scavenging system in aerobic organisms, including enzymatic and nonenzymatic antioxidants, such as superoxide dismutase (SOD) and catalase (CAT) (Lu et al., 2016). Due to the negative effects of plant protein source substitution, numerous studies focused on the use of additives, which was a kind of chemical that can promote growth performance, antioxidant capacity, and immune ability of aquatic animals (Awad & Awaad, 2017). To date, many additives have been already used in aquaculture, especially in plant protein-based diets, including resveratrol, lycopene, astaxanthin, and extractives from medicinal plants (Li, Liu, Xia, Wang, & Zhang, 2019; Sahin et al., 2014; Tan, Zhou, Wang, Mai, & He, 2019; Zheng et al., 2017).

Phosphatidic acid (PA) is a diacylglycerol phospholipid, in which two fatty acids and a phosphate group are covalently bonded to a glycerol molecule through ester linkages (Sobotik, Lee, Hagerman, & Archer, 2018). Previous studies have shown that PA is an important biological signaling molecule, whose function was mainly related to the activation of target of rapamycin complex 1 (TORC1) signaling pathway in animals (Fang, Vilella-Bach, Bachmann, Flanigan, & Chen, 2001; Joy et al., 2014; Wang et al., 2018). TORC1 regulates cell growth and metabolism by sensing the intracellular nutritional status (Laplante & Sabatini, 2012; Saxton & Sabatini, 2017). Meanwhile, TORC1 signaling network is wired to growth factor signaling via the insulin/insulin-like signaling system (Reiling & Sabatini, 2006). As an important hormone in vivo, circulating insulin-like growth factors I (IGF-I) is mainly produced in liver and involved in growth regulation. It has been suggested that the expression level of igf-1 might be a potential biomarker for monitoring fish growth performance as well as the efficacy of additives (Zheng, Liang, Yao, Wang, & Chang, 2012). Oral PA feeding stimulated protein synthesis in rodent skeletal muscle (Mobley et al., 2015). Oxidized PA enhanced the expression of antioxidant genes glutamate-cysteine ligase modifier subunit (GCLM) and NAD(P)H quinone oxidoreductase-1 (NQO1) in human endothelial cells (Jyrkkänen et al., 2008). In addition, PA could ameliorate stomach mucosal injury in mice (Tanaka et al., 2013), indicating that PA might improve the health of digestive track. It has been reported that postprandial total protease and amylase activity were reduced in the intestinal of rainbow trout.
and sea bream feed with plant protein based diet. (Santigosa et al., 2008), this might be partially due to the impaired digestion system. Whether supplementation of PA could restore these digestive enzymes activity is still unclear. Furthermore, evaluation of the use of PA was mainly observed in mammals, the effect of dietary PA supplement in aquaculture, especially its effects on the carnivorous fish species fed with plant-based diets, was rarely studied.

The replacement of FM with high levels of plant protein sources could cause inhibition of growth performance and decrease of immunity in turbot (*Scophthalmus maximus* L.), a widely cultured carnivorous fish species in Europe and Asia (Gu, Bai, & Kortner, 2017). In this study, we evaluated the effects of PA supplement in diets with high levels of mixed plant proteins on growth performance, *igf-1* gene expression, and antioxidant capacity of juvenile turbot. The findings of this study suggested that PA was a functional additive for juvenile turbot with beneficial effects.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

In this study, the animal care and treatment program used was approved by the Institutional Animal Care and Use Committee of Ocean University of China ( Permit coding: 20001001).

2.2 | Experimental diets

PA used in this experiment was obtained from Shenhe Chemical Co., Ltd. (Shangdong, China). FM, wheat gluten, soybean meal, corn gluten meal, beer yeast, and peanut meal were used as the major protein sources. Fish oil, phospholipid, and PA were used as the major lipid source and wheat flour was used as the carbohydrate source. Four iso-nitrogenic and iso-energetic experimental diets were formulated in this study. The diet with 50% of fishmeal replaced by plant protein mixture without PA was set as the control (CON) as described previously (Liu et al., 2014). Three PA-containing diets were made by adding 0.05%, 0.1%, and 0.5% dry weight of PA to the CON diet and named as PA-0.05%, PA-0.1%, and PA-0.5% group, respectively. Crystal amino acids methionine and lysine were added to meet the essential amino acids requirement in turbot based on the FM diet (Kaushik, 1998). All ingredients were ground to pass through a 180-μm mesh, then blended with oil and water, and made into pellets (4 mm in diameter) with pellet-making machine (F-26 [II], South China University of Technology). All the diets were dried in a ventilated oven at 50°C and were stored at −20°C. The detailed compositions of experiment diets are shown in Table 1.

2.3 | Test fish rearing and sampling

Juvenile turbot were obtained from a fish farm in Qingdao (Shandong, China). Fish were kept for 2 weeks for acclimatization and fed with commercial diets (Tianbang Biotech, 2# diet for juvenile turbot) before the trial started. After fasting for 24 hr, turbot with an average weight of 9.80 ± 0.02 g were distributed randomly into 12 tanks (water volume, 300 L) with 30 fish per tank. The fed diets were randomly assigned into three replicate tanks. The tanks were supplied with a recycling sea water system. Fish were hand-fed to apparent satiation twice daily at 8:00 and 18:00 for 77 days. The feeding trial was conducted on a natural light cycle from August to October in Qingdao, China. About 70% of the recycling water was replaced with fresh seawater daily after feeding finished to maintain water cleanliness. During the experimental period, water temperature and salinity were measured daily, the water temperature ranged from 16 to 19°C, salinity from 29.5 to 32.0‰, dissolved oxygen higher than 7 mg/L, and ammonia-nitrogen and nitrite lower than 0.1 mg/L.

At the end of the experiment, fish were starved for 24 hr. After anesthetized with eugenol (1:10000), total number and body weight of fish in each tank were measured. Four fish were randomly sampled from each tank and
### TABLE 1  Formulation and proximate composition of the experiment diets (%)

| Ingredients          | Amount of each treatment (dry matter basis) | Diet 1, CON | Diet 2, PA-0.05% | Diet 3, PA-0.1% | Diet 4, PA-0.5% |
|----------------------|-------------------------------------------|-------------|-----------------|----------------|----------------|
| Fish meala           |                                           | 30.00       | 30.00           | 30.00          | 30.00          |
| Phosphatidic acidb   |                                           | 0.00        | 0.05            | 0.10           | 0.50           |
| Wheat meala          |                                           | 12.51       | 12.51           | 12.51          | 12.51          |
| Soybean meala        |                                           | 19.60       | 19.60           | 19.60          | 19.60          |
| Corn gluten meala    |                                           | 10.00       | 10.00           | 10.00          | 10.00          |
| Wheat gluten meala   |                                           | 6.40        | 6.40            | 6.40           | 6.40           |
| Peanut meala         |                                           | 4.00        | 4.00            | 4.00           | 4.00           |
| Beer yeasta          |                                           | 2.50        | 2.50            | 2.50           | 2.50           |
| Vitamin premixc      |                                           | 2.00        | 2.00            | 2.00           | 2.00           |
| Mineral premixed     |                                           | 1.00        | 1.00            | 1.00           | 1.00           |
| Attractantb          |                                           | 1.00        | 1.00            | 1.00           | 1.00           |
| Taurine              |                                           | 0.25        | 0.25            | 0.25           | 0.25           |
| Methionine           |                                           | 0.32        | 0.32            | 0.32           | 0.32           |
| Lysine               |                                           | 0.92        | 0.92            | 0.92           | 0.92           |
| Fish oil             |                                           | 5.90        | 5.85            | 5.80           | 5.40           |
| Phospholipid         |                                           | 2.50        | 2.50            | 2.50           | 2.50           |
| Choline chloride     |                                           | 0.25        | 0.25            | 0.25           | 0.25           |
| Monocalcium phosphate|                                           | 0.40        | 0.40            | 0.40           | 0.40           |
| Phytase              |                                           | 0.20        | 0.20            | 0.20           | 0.20           |
| Yttrium oxide        |                                           | 0.10        | 0.10            | 0.10           | 0.10           |
| Calcium propionate   |                                           | 0.10        | 0.10            | 0.10           | 0.10           |
| Ethoxyquin           |                                           | 0.05        | 0.05            | 0.05           | 0.05           |
| Sum                  |                                           | 100         | 100             | 100            | 100            |

**Nutrient levels (dry matter)**

| Nutrient             | Diet 1, CON | Diet 2, PA-0.05% | Diet 3, PA-0.1% | Diet 4, PA-0.5% |
|----------------------|-------------|-----------------|-----------------|----------------|
| Dry matter           | 94.95       | 95.09           | 95.04           | 94.98          |
| Crude protein        | 52.37       | 52.43           | 52.67           | 52.87          |
| Crude lipid          | 10.85       | 10.18           | 10.29           | 10.51          |
| Gross energy (kJ/g)  | 20.03       | 20.05           | 20.07           | 20.01          |

Note: Control, replacement of 50% fish meal with plant protein; PA-0.05%, addition of 0.05% phosphatidic acid/kg based on CON diet; PA-0.1%, addition of 0.1% phosphatidic acid/kg based on CON diet; PA-0.5%, addition of 0.5% phosphatidic acid/kg based on CON diet.

aAll these raw materials are obtained from Tianbang Biotech (Qingdao, China), fish meal (% dry matter): crude protein 71.81, crude lipid 9.73; wheatmeal (% dry matter): crude protein 16.51, crude lipid 2.06; soybean meal (% dry matter): crude protein 54.29, crude lipid 1.96; corn gluten meal (% dry matter): crude protein 67.11, crude lipid 1.89; wheat gluten meal (% dry matter): crude protein 81.84, crude lipid 1.04; peanut meal (% dry matter): crude protein 53.96, crude lipid 3.01; beer yeast (% dry matter): crude protein 51.12, crude lipid 1.43.

bAll the phosphatidic acids are provided by Shenhe Chemical Engineer Corporation (Jinan, China).

cVitamin premix (mg/kg diet): cholecalciferol, 5; tocopheryl acetate, 240; ascorbic acid, 200; retinol acetate, 32; niacin, 200; folic acid, 20; menadione sodium bisulphite, 10; thiamin, 25; biotin, 60; cyanocobalamin, 10; riboflavin, 45; pyridoxine HCl, 20; inositol, 800.

dMineral premix (mg/kg diet): zeolite powder, 8485; calcium iodate, 60; CuSO₄•5H₂O, 10; ZnSO₄•H₂O, 50; CoCl₂•6H₂O, 50; Na₂SeO₃, 20; FeSO₄•H₂O, 80; MgSO₄•7H₂O, 1200; MnSO₄•H₂O, 45.

eAttractant (mg/kg diet): DMPT, 2; threonine, 2; glycine, 4; inosine-5' -diphosphate trisodium salt, 1; lactamine, 1.
stored at −20°C for whole body composition analysis. The fish was sacrificed by overdose of eugenol and cervical section. Four fish were randomly sampled from each tank to measure individual body weight, body length, visceral weight, and liver weight so as to calculate condition factor (CF), visceralosomatic index (VSI), and hepatosomatic index (HSI). Liver and intestine samples for enzyme activities assay and gene expression measurement were pooled into 1.5 ml tubes (RNase-Free; Axygen), frozen in liquid nitrogen, and then stored at −80°C (Xu et al., 2016).

2.4 | Diets and fish body composition analysis

All the ingredients, diets, and fish samples were using the standard methods (Association of Official Analytical Chemists, 1995) to analyze the dry matter, crude lipid, crude ash, and crude protein. Dry matter was analyzed by drying the samples to constant weight at 105°C for 24 hr. Crude lipid was measured after diethyl ether extraction by using the Soxhlet method (Buchi 36680, Switzerland). Crude protein was analyzed by using the Kjeldahl method (Kjeltec 8400 FOSS, Sweden) and estimated by multiplying nitrogen by 6.25. Gross energy was determined with a microbomb calorimeter (6100 Compensated Jacket Calorimeter; Parr Instrument Company, Moline, IL).

2.5 | Enzymes analysis

The intestines and livers were accurately weighed and then homogenized in phosphate buffer (50 mM, pH 6.8) in ice-cold distilled water. Following centrifugation (2,500 g, 10 min, 4°C), the supernatant was stored and kept at −80°C for enzymes analysis. Digestive enzymes were detected using intestinal tissue collected from samples and antioxidative enzymes were measured using liver tissue collected from samples. The activities of amylase, trypsin, lipase, malondialdehyde (MDA), SOD, and catalase (CAT) were measured using the colorimetric assay kits according to the instructions (Jiancheng Bioengineering Institute, China). Briefly, the samples were homogenized in PBS followed by centrifugation, and the supernatant was used for enzyme activity assay. The activity of trypsin and lipase was measured using benzoyl-L-arginine ethyl ester and triglyceride as substrate. Amylase starch hydrolysis followed by iodine staining was used to measure amylase activity. The SOD activity was determined using WST-1 assay. The activity of CAT was measured using H2O2 as substrate. Total protein level was determined using BCA assay. One unit of lipase and CAT activity is defined as the amount of enzyme required to transform 1 μmol of substrate per minute under the standard assay conditions. One unit of amylase activity is defined as the amount of enzyme required to transform 10 mg starch in 30 min at 37°C. One unit of trypsin activity is defined as the amount of enzyme required to increase the absorbance at 253 nm wavelength in 0.003 per min at 37°C, pH 8.0. One unit of SOD activity is defined as the amount of enzyme required to inhibit the formation of WST-1 formazan dye by 50%.

2.6 | Quantitative real-time PCR analysis

The mRNA expression of superoxide dismutase (sod), glutathione peroxidase (gpx), peroxiredoxin 6 (prx6), and insulin-like growth-factor-I (igf-1) in liver was determined by quantitative real-time PCR (qRT-PCR). The turbot β-actin gene (access no. EU686692.1) was used as the reference as described previously (Wang, Zhou, Wang, Mai, & He, 2019). The primer sequences of target genes used in the experiment are listed in Table 2. Total RNA of liver samples was extracted with TRIzol reagent (Invitrogen, USA) according to the manufacturer’s instruction. The quality and quantity of RNA were detected by electrophoresis on 1.2% denatured agarose gels, and then the Nano Drop®2000 spectrophotometer (Thermo Fisher Scientific, USA) was used to assess the concentration of RNA. 1 μg total RNA was used to synthesize cDNA using the PrimeScript RT reagent Kit with gDNA Eraser (Takara, Japan), and then it was diluted to 80 ng/μl using RNase-free water according to the manufacturer’s
instructions. According to method described previously (Song et al., 2016), the quantitative real-time PCR assays were conducted in a quantitative thermal cycler (Mastercycler ep realplex, Eppendorf, Germany) in a total volume of 25 μl containing 12.5 μl of Premix SYBR EX Tag (Takara, Japan), 2 μl of the cDNA product, 0.5 μl of each forward and reverse primers (10 mM), and 9.5 μl of RNase-free water. All the expression data of target genes were calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

### 2.7 Calculation and statistical analysis

The following calculations were performed:

- **Weight gain rate (WGR,%)** = (final body weight − initial body weight)/initial body weight × 100.
- **Specific growth rate (SGR,%)** = (Ln [final body weight] − Ln [initial body weight])/days × 100.
- **Feed intake (FI,%)** = dry feed intake (g)/([final body weight + initial body weight]/2)/days × 100.
- **Feed efficiency ratio (FER)** = wet weight gain (g)/dry feed intake (g).
- **Condition factor (CF,%)** = final body weight (g)/body length (cm) 3 × 100.
- **Hepatosomatic index (HSI,%)** = liver weight (g)/whole body weight (g) × 100.
- **Viscerosomatic index (VSI,%)** = viscera weight (g)/whole body weight (g) × 100.

SPSS 17.0 software for Windows was used for statistical analysis. All data were subjected to one-way analysis of variance followed by Tukey's multiple range test. Differences were regarded as significant when $p < .05$. Data were expressed as means ± SEM (SE of the mean).

## 3 RESULTS

### 3.1 Growth performance and feed utilization

As shown in Table 3, no significant difference in feed intake (FI) was observed among groups ($p > .05$). When 0.05 and 0.1% of PA (diet 2 and diet 3) were supplemented to the diet, no significant difference was found in final body

| Target gene | Forward primer (5’ to 3’) | Reverse primer (5’ to 3’) | Accession no. |
|-------------|---------------------------|---------------------------|---------------|
| sod         | AAACAATCGCCAACCTCTTG      | CAGGAGACAGTAAGCATGG       | HS029499.1    |
| gpx         | CCCTGATGACTGACCCAAAG      | GCACAAGGCTAGGAGTTTC       | HS032063.1    |
| prx6        | TCAGAGAGCGAGGAGATGAC      | CCGATGAGATAGACAGAGATTG    | GU56199       |
| igf-1       | GGTGGACGAGTGCTGCTTTC      | CTGCCCTGGGGTACTAACC       | FJ160587.1    |
| β-actin     | TGAACCCGACCCAGCACAGG      | CAGAGGATGACAGGACAGGAC    | EU686692.1    |

Note: sod, superoxide dismutase; gpx, glutathione peroxidase; prx6, peroxiredoxin 6; igf-1, insulin-like growth factor 1.
weight (FBW), weight gain rate (WGR), and feed efficiency ratio (FER) compared with the CON group (diet 1). However, PA-0.5% group (diet 4) significantly increased FBW, WGR, and FER of turbot compared with the CON group (\(p < .05\)). Specific growth rate (SGR) was significantly improved by the supplement level of PA compared with the CON group (\(p < .05\)), the highest level was observed in PA-0.5% group. We then detected the mRNA level of \(igf-1\), since it is an important biomarker for fish growth. As the results shown in Figure 1, supplying PA to the diet had a tendency to increase the expression of \(igf-1\), a significantly higher level of \(igf-1\) expression in PA-0.5% group was observed compared with the other groups (\(p < .05\)), while no significant effect was observed on CON, PA-0.05% and PA-0.1% group (\(p > .05\)).

### 3.2 Fish body composition

Fish body composition results are shown in Table 4, no significant differences were found in moisture, crude lipid, crude protein, crude ash, and energy (\(p > .05\)). As shown in Table 5, adding PA to the diet could significantly increase

| TABLE 3 Growth performance of turbot fed with diets containing graded levels of phosphatidic acid |

| Items | IBW\(^a\) (g) | FBW\(^b\) (g) | WGR\(^c\) (%) | SGR\(^d\) (%/day) | FI\(^e\) (%/day) | FER\(^f\) |
|-------|------------|------------|-------------|----------------|--------------|---------|
| Diet 1, CON | 9.83 ± 0.01 | 57.17 ± 3.68\(^a\) | 481.63 ± 36.98\(^a\) | 2.45 ± 0.09\(^a\) | 1.54 ± 0.03 | 1.31 ± 0.01\(^a\) |
| Diet 2, PA-0.05% | 9.78 ± 0.03 | 60.40 ± 2.70\(^ab\) | 517.26 ± 26.11\(^ab\) | 2.54 ± 0.06\(^b\) | 1.54 ± 0.01 | 1.33 ± 0.02\(^ab\) |
| Diet 3, PA-0.1% | 9.79 ± 0.02 | 61.49 ± 2.97\(^ab\) | 528.02 ± 28.88\(^ab\) | 2.56 ± 0.07\(^b\) | 1.55 ± 0.03 | 1.34 ± 0.01\(^ab\) |
| Diet 4, PA-0.5% | 9.78 ± 0.02 | 64.90 ± 1.96\(^b\) | 563.44 ± 21.12\(^b\) | 2.59 ± 0.05\(^b\) | 1.55 ± 0.02 | 1.36 ± 0.01\(^b\) |

Note: Treatment means representing the average values of three tanks per treatment. Data were expressed as means ± SE, values in the same column with different alphabets denote significant difference between experimental groups (\(p < .05\)).

\( ^{a}\)IBW: initial body weight.

\( ^{b}\)FBW: final body weight.

\( ^{c}\)WGR: weight gain rate = (final body weight – initial body weight)/initial body weight \(\times\) 100%.

\( ^{d}\)SGR: specific growth rate = (\(Ln\) [final body weight] – \(Ln\) [initial body weight])/days \(\times\) 100%.

\( ^{e}\)FI: feed intake = dry feed intake (g)/([final body weight + initial body weight]/2)/days \(\times\) 100%.

\( ^{f}\)Feed efficiency ratio (FER) = wet weight gain (g)/dry feed intake (g).

![Figure 1](image_url) Relative mRNA level of \(igf-1\) gene. The mRNA expression of \(igf-1\) in liver of turbot fed with diets containing graded levels of phosphatidic acid was measured by quantitative real-time PCR. All genes were standardized by \(\beta\)-actin. Data were expressed as means ± SE (\(n = 6\)). Different letters above the bars mean significant difference (\(p < .05\)).
the CF compared with the CON group \((p < .05)\), while the highest numerical value was appeared in PA-0.5% group. A significantly higher level of the HIS in PA-0.5% group was observed compared with the other groups \((p < .05)\). The VSI showed no significant differences among the different treatments \((p > .05)\).

### 3.3 | Activities of digestive enzymes in intestine

The activities of amylase, trypsin, and lipase in intestine were determined. As shown in Table 6, there were no significant differences in activities of amylase and trypsin among different groups \((p > .05)\). However, the activity of lipase in the intestinal was increased by the supplement of PA in a dose-dependent manner \((p < .05)\).

### 3.4 | Hepatic antioxidant capacity

The activities of MDA, SOD, and CAT content in turbot liver were determined. As shown in Figure 2(a), supplying PA to the diet could significantly decline the level of MDA \((p < .05)\). However, supplement of PA had no effect on the activities of SOD and CAT \((p > .05)\).

**TABLE 4** Whole body composition of turbot fed with diets containing graded levels of phosphatidic acid (wet weight)

| Items | Moisture (%) | Crude protein (%) | Crude lipid (%) | Crude ash (%wet weight) | Energy (MJ/kg) |
|-------|--------------|-------------------|-----------------|-------------------------|---------------|
| Diet 1, CON | 78.62 ± 0.29 | 15.04 ± 0.57 | 3.33 ± 0.37 | 3.24 ± 0.07 | 21.77 ± 0.24 |
| Diet 2, PA-0.05% | 78.81 ± 0.09 | 15.11 ± 0.41 | 3.28 ± 0.59 | 3.21 ± 0.06 | 21.81 ± 0.21 |
| Diet 3, PA-0.1% | 78.46 ± 0.42 | 15.14 ± 0.30 | 3.31 ± 0.34 | 3.27 ± 0.04 | 21.82 ± 0.11 |
| Diet 4, PA-0.5% | 78.54 ± 0.25 | 15.49 ± 0.46 | 3.27 ± 0.31 | 3.22 ± 0.08 | 21.77 ± 0.12 |

*Note: Treatment means representing the average values of three tanks per treatment. Data were expressed as means ± SE.

**TABLE 5** Condition factor (CF), hepatosomatic index (HSI), and viscerosomatic index (VSI) of turbot fed with diets containing graded levels of phosphatidic acid

| Items | CF\(^a\) (%) | HSI\(^b\) (%) | VSI\(^c\) (%) |
|-------|--------------|--------------|--------------|
| Diet 1, CON | 3.82 ± 0.05a | 0.76 ± 0.02a | 4.81 ± 0.11  |
| Diet 2, PA-0.05% | 4.17 ± 0.04bc | 0.72 ± 0.04a | 4.62 ± 0.04  |
| Diet 3, PA-0.1% | 4.11 ± 0.05bc | 0.75 ± 0.04a | 4.74 ± 0.17  |
| Diet 4, PA-0.5% | 4.29 ± 0.02c | 0.92 ± 0.03b | 4.71 ± 0.14  |

*Note: Treatment means representing the average values of three tanks per treatment. Data were expressed as means ± SE, values in the same column with different alphabets denote significant difference between experimental groups \((p < .05)\).

\(^a\)CF: condition factor = final body weight (g)/body length (cm)\(^3\) \times 100%.

\(^b\)HSI: hepatosomatic index = liver weight (g)/whole body weight (g) \times 100%.

\(^c\)VSI: viscerosomatic index = viscera weight (g)/whole body weight (g) \times 100%.
Table 6  Digestive enzymes activity in intestine of turbot fed with diets containing graded levels of phosphatidic acid

| Items | Amylase (U/mg prot) | Trypsin (U/g prot) | Lipase (U/g prot) |
|-------|---------------------|--------------------|-------------------|
| Diet 1, CON | 0.14 ± 0.03 | 29.77 ± 4.65 | 98.99 ± 4.06a |
| Diet 2, PA-0.05% | 0.12 ± 0.02 | 30.42 ± 4.01 | 114.42 ± 5.03b |
| Diet 3, PA-0.1% | 0.11 ± 0.01 | 28.95 ± 3.29 | 120.48 ± 3.89b |
| Diet 4, PA-0.5% | 0.13 ± 0.01 | 28.58 ± 3.36 | 142.46 ± 3.51c |

Note: Treatment means represent the average values of three tanks per treatment. Data were expressed as means ± SE, values in the same column with different alphabets denote significant difference between experimental groups (p < .05).

Figure 2  Hepatic antioxidant responses. (a) Content of malondialdehyde (MDA) and enzyme activities of superoxide dismutase (SOD) and catalase (CAT) in liver of turbot fed with diets containing graded levels of phosphatidic acid. (b) The mRNA expression of prx6, sod, and gpx in liver was measured by quantitative real-time PCR. All genes were standardized by β-actin. Data were expressed as means ± SE (n = 6). Different letters above the bars mean significant difference (p < .05). *p < .05, unpaired two-tailed t test

The transcriptional levels of antioxidant genes (prx6, sod, and gpx) in turbot liver were also determined in the experiment. As shown in Figure 2(b), the mRNA levels of prx6 in PA-0.1% and PA-0.5% groups were significantly higher than those in CON and PA-0.05% groups (p < .05). Dietary PA improved the expression levels of sod and gpx (p < .05), with threefold increase in sod mRNA levels observed in PA-0.5% group (p < .05).
4 | DISCUSSION

In the present study, we have evaluated the effects of PA on growth, antioxidant capacity, and igf-1 level in juvenile turbot feed with high plant protein diets. PA-0.5% diet supplementation significantly improved growth and feed utilization of turbot compared to CON group. However, there was no difference in feed intake among different groups. This observation was similar to previous findings in juvenile turbot whereby taurine supplementation induced a significant increase in feed utilization, but a decrease in feed intake (Yun et al., 2012). Thus, it is possible that the growth-promoting effects of PA may be mediated by feed utilization but not feed intake. The growth-promoting action of PA can be attributed to the improvement of feed utilization rate which paralleled with the findings of other feed additives (e.g., methionine) in southern catfish, Silurus meridionalis (Ai & Xie, 2005) and large yellow croaker, Larimichthys crocea (Mai et al., 2006). Meanwhile, a higher protein content of whole body was observed in PA-0.5 group. Previous studies have shown that protein anabolic processes are carried out under the control of the TOR pathway, and the activation of TORC1 signaling pathway promoting growth has also been confirmed in fish (Seiliez et al., 2011; Skiba-Cassy, Lansard, Panserat, & Médale, 2009). Wang et al. (2018) found that supplementation of PA, an important second messenger in signaling molecules, significantly enhanced cell proliferation and anabolism through the activation of TORC1 signaling in primary muscle cells of turbot. It suggests that PA plays a role in promoting the synthesis of proteins in the body by activating TOR signaling.

Morphological parameters are generally related to protein and fat changes. Liver is regarded as the main site of fat and glycogen deposition in fish body (Chatzifotis, Polemitou, Divanach, & Antonopoulou, 2008). In this study, CF significantly increased when PA was incorporated into the diets, and fish fed with the diet containing 0.5% PA had significantly higher HSI than other groups. Interestingly, the VSI was not changed in 0.5% PA group, indicating that PA might have potential effects on other organs. Therefore, significant changes of morphological parameters can indicate that PA plays a role in promoting growth of fish. Studying the activity of digestive enzymes contained in the intestines of fish can be used as an indicator to measure the absorption of nutrients in the feed. Although PA significantly increased the CF of fish body, no significant difference in the crude lipid content of whole body composition was observed. Lipase is an enzyme that catalyzes the hydrolysis of ester bonds in water-insoluble, lipid substrates and plays an essential role in the digestion, transport, and processing of lipids (Murray, Gallant, Perez-Casanova, Johnson, & Douglas, 2003). In this study, an enhancement of lipase activity in PA diets was observed in turbot intestine. The PA supplement groups might improve the ability of juvenile turbot to decompose fat, thereby promoting the absorption and utilization of fat sources in feed by the digestive tract. The protein-sparing effect by dietary lipid has been reported in several species (Akpinar et al., 2012; Vergara, Robainà, Izquierdo, & De La Higuera, 1996), and it might be a convincing strategy for reducing diet cost in aquaculture. Thus, it is worth further studying the effect of PA on lipid metabolism.

When reactive oxygen species (ROS) are increased in vivo, the activity of corresponding antioxidant enzymes and the amount of antioxidant synthesis are affected. Therefore, antioxidant enzyme activity can indirectly reflect the dynamic changes of free radical reactions and tissue damage in vivo (Johnson, 2002). As the main active substance of antioxidant enzymes in liver, SOD can catalyze the conversion of reactive oxygen anions into O$_2$ and hydrogen peroxide, and CAT can detoxify hydrogen peroxide into water. The content of MDA indicates the oxidation level of lipids and reflects the damage degree of free radical attack in the body (Matés & Sánchez-Jiménez, 1999). The effect of plant protein sources on the activity of fish antioxidant defense enzymes are complex. Although some antioxidants in plant proteins, such as flavonoids, isoflavone, and phenolic compounds, might be involved in ROS homeostasis and enhance the antioxidative capacity (Appelt & Reicks, 1999; Maqsood & Benjakul, 2010; Yao et al., 2004), some anti-nutritional factors in plant protein sources might lead to oxidative stress in fish (Zhang et al., 2013). In the present study, a severe depression of antioxidant enzyme activities in CON group was observed, which could be attributed to the excessive utilization of plant protein. PA supplementation could enhance the mRNA expression of prx6, sod, and gpx significantly. Furthermore, adding PA reduced the MDA content. However, we failed to detect the increase in the activity of SOD in PA supplement groups although an increase in trend was observed, and this might be due to the detection sensitivity used in this study. Our work
constitutes the first report on the positive effects of PA on antioxidant capacity of turbot, but the mechanism by which PA modulates oxidative stress needs further study. It has been reported that oxidized PA enhanced the expression of antioxidant genes GCLM and NQO1 in human endothelial cells (Jyrkkänen et al., 2008), whether PA could induce these genes' expression in fish is unknown.

Nutritional status strongly affects the IGF system, and IGF-I is an important hormone with multiple biological functions in teleost fish, including cell growth, metabolism, and embryonic development (Reinecke et al., 2005; Thissen, Underwood, & Ketelslegers, 1999). By binding to its receptors, igf-1 regulates body growth through phosphoinositide-3-kinase (PI3K)/protein kinase B (AKT) pathway, which integrates with TOR pathways at multilevel to modulate tissue growth (Sasai et al., 2010). Wang et al. (2018) indicated that the activation of TOR signaling by PA may be responsible for the development of muscle hyperplasia and muscle mass in turbot. Studies also indicated that high plant protein replacements could downregulate hepatic igf-1 expression levels in fish (Gómez-Requeni et al., 2004; Luo et al., 2013). In the present study, FBW, WGR, SGR, and hepatic igf-1 expressions were approximately correlated and the maximum values were observed in 0.5% PA group, the highest supplement group. Similar results were also found in tilapia, Oreochromis mossambicus (Uchida et al., 2003) and mud carp, Cirrhinus molitorella (Jiang, Zhang, Qiu, Lin, & Jiang, 2010), which all showed that liver igf-1 mRNA levels were approximately correlated with the results of growth performance. All the results demonstrated that 0.5% PA diet enhanced the gene expression of the hepatic IGF-I, thereby improving the growth of turbot. The action of igf-1 was not only mediated by circulating IGF secreted by liver, the local secreted igf-1 in specific tissues, for example, muscle, also play critical role in the organism growth (Wood, Duan, & Bern, 2005). Whether PA increases the IGF signaling in paracrine and autocrine manner needs to be further studied.

In summary, the results from this experiment demonstrated a positive effect on growth, feed utilization, and hepatic antioxidant capacity through dietary PA in juvenile turbot. Also, dietary PA supplementation could increase liver igf-1 mRNA expression. Therefore, PA might be an important functional additive to improve the utilization of high plant protein sources in turbot.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
Chengdong Liu and Gen He designed the research. Hao Jiang and Huihui Zhou conducted the research and analyzed the data. Xuan Wang provided technical assistance. Hao Jiang, Chengdong Liu, and Kangsen Mai wrote the manuscript. All authors read and approved the final manuscript.

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