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EVALUATION OF ZINC-BEARING PALYGORSKITE EFFECTS ON GROWTH PERFORMANCE, NUTRIENT RETENTION, MEAT QUALITY, AND ZINC ACCUMULATION IN BLUNT SNOUT BREAM *MEGALOBRAMA AMBLYCEPHALA*

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Abstract—Zinc (Zn) is widely known as an essential trace element for fish and new ways to supply it to them are needed. Palygorskite (Pal) is a natural silicate clay mineral and the palygorskite structure contains nano-channels, which are filled with water and exchangeable ions. Zn-bearing palygorskites (Zn-Pal) prepared using ion exchange have attracted attention due to the durable antibacterial properties that limit pathogens and as a potential new Zn source for livestock. The present study was conducted to evaluate the effects of Zn-Pal supplementation on the growth performance, nutrient retention, meat quality, Zn accumulation, and intestinal Zn transporter protein gene expression in blunt snout bream *Megalobrama amblycephala*. The fish were fed a basal diet without an exogenous Zn source and the basal diet was supplemented with 125 mg/kg Zn as Zn sulfate ($ZnSO_4$) or 35, 80, or 125 mg/kg Zn as Zn-Pal. Each diet was tested using three replicates for 7 weeks. The results showed that dietary Zn-Pal supplementation quadratically ($P<0.05$) increased growth performance, nutrient retention, total and Cu/Zn superoxide dismutase activity, Zn content in scales, and intestinal Zn transporter protein gene expression. The muscular cooking loss in blunt snout bream decreased with the optimum Zn-Pal Zn level of 35 mg/kg. Compared to the fish treated with $ZnSO_4$, the fish supplemented with 35 mg/kg as Zn-Pal exhibited similar growth performance and nutrient retention ($P>0.05$), increased mRNA expression of the metal-response element-binding transcription factor-1 in the intestine ($P<0.05$), and decreased cooking loss of muscle ($P<0.05$).The results suggested that 35 mg/kg Zn supplementation as Zn-Pal could improve the growth performance and body composition, increase nutrient retention and tissue Zn concentrations, enhance the muscle water-holding capacity, and enhance antioxidant status in blunt snout bream. The Zn-Pal was more efficient and could be used as an alternative Zn source to $ZnSO_4$ in the diet of blunt snout bream.

KeyWords—Blunt Snout Bream, Growth Performance, Meat Quality, Zinc-bearing Palygorskite.

INTRODUCTION

Zinc (Zn) has been demonstrated to be an essential trace element for fish (Halver and Hardy, 2002). Zn deficiency in fish is characterized by growth depression, high mortality, lens cataracts, and erosion of fins and skin (Halver and Hardy, 2002; Liang *et al*., 2012; Wu *et al*., 2015). Zn requirements vary among farmed species due to growth stage, season, and reproductive cycle (Carpene *et al*., 2003). A recent review reported that quantitative dietary Zn requirements for fish species (13 freshwater and 12 seawater species) ranged from 15 to 240 mg/kg (Prabhu *et al*., 2016). The amount of Zn used in aquaculture feeds is less than 200 mg/kg according to the regulations set by the Chinese government (AQSIQ, 2001). The new regulation 2016/1095 of the European Union sets an upper limit of 180 mg/kg Zn for a complete feed for Salmonids and 150 mg/kg for other fish (Panel, 2014).

Blunt snout bream (*Megalobrama amblycephala*) is a major herbivorous freshwater fish native to China, which belongs to the family Cyprinidae (Li*et al*., 1993). The tender flesh, high survival rate, fast growth, and high disease resistance make blunt snout bream widely favored in China aquaculture (Li *et al*., 2012). Liu *et al*. (2014) found that dietary Zn sulfate ($ZnSO_4$) supplementation could improve the growth performance of blunt snout bream and that the optimal growth performance was achieved when the dietary Zn level was 184.85 mg/kg. Jiang *et al*. (2016) reported that the optimum dietary Zn requirement of adult blunt snout bream was 52.1 mg/kg for maximum weight gain and 86.2 mg/kg for maximum whole body Zn content. The use of high concentrations of inorganic Zn has raised environmental concerns due to the high amounts of excreted fecal Zn (Case and Carlson, 2002; Apines-Amar *et al*., 2004; Carlson *et al*., 2004). Furthermore, a rapid increase in metal pollution has attracted more and more attention in aquatic environments (Nriagu, 1996; Förstner and Wittmann, 2012; Kraus *et al*., 2016). Thus, more effective Zn sources are urgently needed to reduce Zn excretion into the environment.

Palygorskite (Pal) is a natural fibrous silicate clay mineral, which has a structure with nano-channels (0.64×0.37 nm) filled with water and exchangeable

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ions (Leboda *et al*., 2006; Giustetto and Wahyudi, 2011). The large specific surface area, moderate cation exchange capacity, and excellent adsorption properties of Pal are of great benefit for its application in various fields as chemical, petrochemical, and agricultural carriers, and as environmental adsorbents, *etc*. (Galan, 1996; Fernandez *et al*., 1999). Pal is also utilized as an additive in animal feed and for sustained drug release (Galan, 1996; de Sousa Rodrigues *et al*., 2013). Recently, Zn-bearing clay minerals, which included montmorillonites and zeolites, were prepared using ion-exchange and have attracted considerable attention due to the durable antibacterial properties for pathogens and the potential use as a new Zn source in livestock (Hu *et al*., 2014; Jiao *et al*., 2015; Tang *et al*., 2015). Previous studies reported that Zn-bearing zeolite can increase endogenous digestive enzyme activity, nutrient retention, and even replace the antibiotics used in poultry production (Tang *et al*., 2014a, 2014b). In addition, Zn-bearing zeolite enhanced Zn retention in poultry, which was indicated by enhanced accumulation of Zn in tissues and expression of the Zn transporter gene in comparison to $ZnSO_4$ (Li *et al*., 2015; Tang *et al*., 2015). Yan *et al*. (2016) recently reported that Zn-bearing palygorskite (Zn-Pal) could be used as a good Zn source in broilers. Zn release from Zn-Pal in the intestine can in turn provide enough exchangeable cations (Yan, 2016). Yang *et al*. (2016) reported that Zn-Pal inclusion in the diet of broilers could improve meat quality and enhance the muscular antioxidant capacity. The use of Zn-Pal in aquaculture has been limited; the aim of the present study was, therefore, to evaluate Zn-Pal inclusion in the diet as an alternative to $ZnSO_4$ on growth performance, nutrient retention, body composition, meat quality, Zn accumulation, and gene expression of Zn-transporter proteins in blunt snout bream.

MATERIALS AND METHODS

Preparation of Zn-Pal

Natural Pal was provided by Jiangsu Sinitic Biotech Co., Ltd. (Xuyi, Jiangsu, China). Zn-Pal was prepared according to the method of Yan *et al*. (2016). The measured amount of Zn adsorbed by Pal was 28.19 mg/g using a PerkinElmer Optima 2100DV inductively coupled plasma mass spectrometer ICP-MS (PerkinElmer, Waltham, Massachusetts, USA). The chemical compositions of the Pal and Zn-Pal samples were measured using a PANalytical MiniPal 4 X-ray fluorescence spectrometer (PANalytical, Amelo, The Netherlands) and are listed in Table 1. The cation exchange capacities of Pal and Zn-Pal were determined using the NH_4Cl-NH_3 method as 25.60 mmol/100 g and 28 mmol/100 g, respectively (Jin and Dong, 2004). The X-ray diffraction (XRD) patterns were collected for Pal and Zn-Pal (Figure 1) using an X'pert PRO X-ray powder diffractometer equipped with a Cu-Kα radiation (0.1542 nm) source (40 kV, 40 mA) (PANalytical, Amelo, The Netherlands) and scanned from 3−60º2θ with a step interval of about 0.167º2θ. The characteristic Pal diffraction peaks at 8.37º2θ (110 plane), 13.71º2θ (200 plane), 16.38º2θ (130 plane), 19.81º2θ (040 plane), 21.42º2θ (121 plane), 24.16º2θ (240 plane), and 27.68º2θ (400 plane) were observed in the XRD patterns of raw Pal (Figure 1a), which were unchanged with Zn saturation (Figure 1b). This indicates that Zn was ion exchanged to Pal and no new crystal phase was formed during the preparation process. Quartz diffraction peaks at 26.7º2θ and 20.81º2θ were observed, which confirmed the presence of tiny amounts of quartz in both the raw Pal and Zn-Pal (Liu *et al*., 2013).

Fish and feeding trial

The experimental design and procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University. Blunt snout bream were obtained from the aquatic experiment station of Nanjing Agricultural University. Fish were reared in an indoor circulatory system for 2 weeks and acclimatized to the experimental conditions by feeding a basal diet (Table 2) prior to the experiment. After the acclimatization, similar size fish (initial weight, 6.58±0.13 g) were randomly distributed into 15 flow-through rectangular aquariums (400 L each) with 40 fish per tank. Fish were fed a basal diet without an exogenous Zn source and the basal diet was supplemented with 125 mg/kg Zn as $ZnSO_4$ or with 35, 80, or 125 mg/kg Zn as Zn-Pal. The measured Zn levels in the five diets (*i.e*. basal, 125 mg/kg Zn as $ZnSO_4$, and 35, 80, and 125 mg/kg Zn as Zn-Pal) were 50.35, 173.02, 89.24, 131.36, and 171.20 mg/kg, respectively. Each diet was tested using three replicates for 7 weeks. Fish were hand-fed to apparent satiation three times daily (08:00 h, 12:00 h, and 16:00 h). A 12:12 h light:dark regime (07:00−19:00 h, light period) was maintained using timed fluorescent lighting. Water temperatures varied from 24 to 26ºC and pH fluctuated between 7.0 and 7.5. Dissolved oxygen was maintained above 5.00 mg/L during the feeding trial.

Table 1. Chemical composition of the Pal[1] and Zn-Pal samples.

Composition	Pal (%)	Zn-Pal (%)
SiO_2	57.72	54.32
Al_2O_3	10.77	10.75
Fe_2O_3	7.69	7.07
MgO	7.65	7.31
CaO	1.38	1.28
K_2O	1.80	1.53
Na_2O	1.18	Not-detected
Zn	0.02	2.83

[1] Pal: palygorskite; Zn-Pal: zinc-bearing palygorskite.

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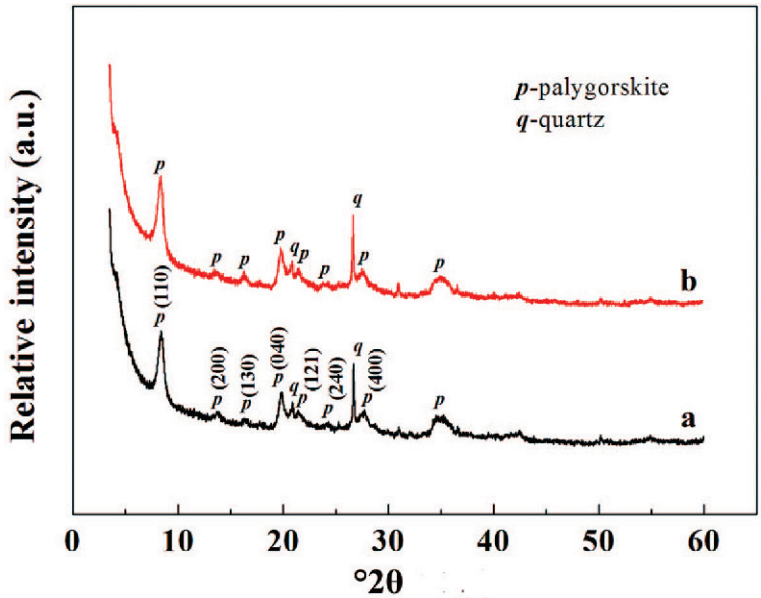

Figure 1. X-ray diffraction patterns of palygorskite samples Pal (a) and Zn-Pal (b).

Total NH_3 and nitrite N were maintained below 0.4 mg/L and 0.064 mg/L, respectively.

Sample collection

Samples of 20 fish at the beginning and 5 fish per tank at the end of the feeding trial were collected and stored at −20ºC for body composition analysis. At the end of the feeding trial, fish were starved for 24 h prior to sample harvest. Then, all the remaining fish were anesthetized in diluted (100 mg/L) MS-222 (tricaine methanesulfonate or tricaine mesylate, $C_{10}H_{15}NO_5S$, SigmaAldrich, St. Louis, Missouri, USA). The total number and weight of fish in each tank were determined. Nine fish were removed from each tank in triple resampling. Blood samples were rapidly taken from the caudal vessel, transferred into heparinized Eppendorf™ tubes, and stored at −20ºC until analysis. Fish were later sampled to analyze for the viscera/body ratio and the hepatosomatic index. Also, intestinal, individual back muscle, vertebra, and scale samples were quickly removed and stored at −80ºC for subsequent analysis.

Table 2. The formulation and proximate composition of the experimental diets (air-dry basis).

Ingredients	Basal diet (%)
Fish meal	5.00
Soybean meal	30.00
Rapeseed meal	16.00
Cottonseed meal	14.00
Wheat middlings	24.00
Wheat bran	5.80
Soybean oil	2.00
Calcium biphosphate	1.80
Sodium chloride	0.40
Premix[1]	1.00
Proximate composition (%)	
Moisture	12.91
Crude protein	30.12
Crude lipid	5.73
Ash	7.21
Energy (MJ kg^{-1})	17.90

[1]Supplied per kg diet the following minerals and vitamins: Cu, 5 mg; Fe, 120 mg; Mg, 100 mg; Mn, 20 mg; Se, 0.25 mg; I, 0.6 mg; Co, 0.07 mg; Vitamin A, 9,000 IU; Vitamin D, 2,000 IU; Vitamin E, 100 mg; Vitamin K_3, 2.2 mg; Vitamin B_1, 3.2 mg; Vitamin B_2, 10.9 mg; Niacin, 10 mg; Pantothenate, 20 mg; Vitamin B_6, 5.0 mg; Vitamin B_{12}, 0.016 mg; Vitamin C, 100 mg; Folic acid, 3 mg; Choline, 600 mg; Biotin, 0.15 mg, Inositol, 200 mg.

Growth performance

The fish were weighed individually before (initial body weight) and after (final body weight) the 7-week feeding experiment. The growth performance parameters were calculated as follows (Zhang *et al*., 2015; Wang *et al*., 2017):

$$\text{Percentage weight gain (PWG, \%)} = \frac{\text{final body weight (g)} - \text{initial body weight (g)}}{\text{initial weight (g)}} \times 100 \quad (1)$$

$$\text{Feed conversion ratio (FCR, g/g)} = \frac{\text{final body weight (g)} - \text{initial body weight (g)}}{\text{feed consumed (g)}} \times 100 \quad (2)$$

$$\text{Specific growth rate (SGR, \%day}^{-1}\text{)} = \frac{\ln(\text{mean final body weight}) - \ln(\text{mean initial body weight})}{\text{days}} \times 100 \quad (3)$$

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$$\text{Condition factor (CF, \%)} = W/L^3 \times 100 \quad (4)$$

where W = body weight (g), L = length (cm).

$$\text{Viscera/body weight ratio (VBR, \%)} = \frac{\text{viscera weight (g)}}{\text{wet body weight (g)}} \times 100 \quad (5)$$

$$\text{Hepatosomatic index (HSI, \%)} = \frac{\text{hepatopancreas weight (g)}}{\text{wet body weight (g)}} \times 100 \quad (6)$$

Body composition and nutrient retention

Diets and fish samples were analyzed for moisture, crude protein (CP), crude lipids (CL), and ash contents using the method described by AOAC (1995). Moisture was determined by oven drying at 105ºC until constant weight. The CP content (total N × 6.25) was determined by the Kjeldahl method using a Foss KT260 auto Kjeldahl system (Foss, Hillerød, Denmark), the CL content was determined by ether extraction using a Foss Soxtec™ 2050 Auto Soxtec system (Foss, Hillerød, Denmark), and ash content was determined by combustion at 550ºC for 5 h. The organic matter (OM, %) was calculated using the formula: (100-moisture (%) -ash (%)).

The calculation given below (equation 7) was used to determine the apparent dietary retention of OM, CP, and CL.

$$\text{Apparent retention of nutrient (\%)} = \frac{(\text{final body weight} \times \text{\% nutrient final body}) - (\text{initial body weight} \times \text{\% nutrient initial body})}{\text{feed intake} \times \text{\%nutrient diet}} \times 100 \quad (7)$$

Meat quality

The drip loss was determined as described by the modified method of Roth *et al*. (2010). In detail, the muscle samples were accurately weighed (W_1, g) using an analytical balance and then placed in inflatable plastic bags at 2−4ºC. After 24 h hanging, muscle samples were removed from their respective plastic bags, excessive moisture on the meat was absorbed using filter paper, and the samples were re-weighed ($W_{24\ h}$, g). The muscle samples were then placed back into the inflatable plastic bags at 2−4ºC for another 24 h. The drip losses after 24 and 48 h were calculated as follows:

$$\text{Drip loss}_{24\ h}\ (\%) = \frac{W_1 - W_{24}}{W_1} \times 100 \quad (8)$$

$$\text{Drip loss}_{48\ h}\ (\%) = \frac{W_1 - W_{48}}{W_1} \times 100 \quad (9)$$

The cooking loss was determined as described using the modified method of Castellini *et al*. (2002). Briefly, muscle samples were placed in plastic bags and incubated in a water bath (pre-heated to 80ºC) until an internal temperature of 70ºC was attained for 30 min. The cooking losses were measured as the percent weight of the incubated samples (cooled for 30 min to about room temperature and dried on the surface with a filter paper) relative to the raw samples.

Muscular antioxidant status assay

Approximately 0.2 g of muscle sample was homogenized (1:9, wt/vol) with ice-cold 154 mmol/L NaCl solution and then centrifuged at 3000 × *g* for 10 min at 4ºC. The supernatants were used to measure the activities of total superoxide dismutase (T-SOD), Cu/Zn superoxide dismutase (Cu/Zn-SOD), total antioxidant capacity (T-AOC), and the malondialdehyde (MDA) concentrations using the Diagnostic Reagent Kit (Nanjing Jiancheng Institute of Bioengineering, China) according to the commercial kit instructions. The MDA contents were measured using the barbiturate thiosulfate assay (Placer *et al*., 1966). The SOD activities were measured using the hydroxylamine method (Oyanagui, 1984). The spectrometric method was applied to evaluate T-AOC (Zhan *et al*., 2011). All results were normalized to the total protein concentration in each sample for inter-sample comparisons. Total protein concentrations were determined according to the method of Bradford (1976) using bovine serum album as the standard protein.

Zn accumulation

The Zn contents in the diet, blood, muscle, hepatopancreas, vertebra, and scales were determined using the electric heating method according to an earlier study (Zhang *et al*., 2015). Exactly 0.5 g of the samples (1 mL of blood) was weighed and digested with 10-mL of HNO_3:$HClO_4$ (4:1 vol/vol) acid mixture at 160ºC in a glass tube until the solution became clear. Then, the solutions were diluted to 25 mL with deionized water. The Zn concentrations were determined using a PerkinElmer Optima 2100 DV ICP-MS.

Messenger RNA quantification

Total RNA was extracted from intestine tissue using the RNAiso Reagent (Takara Bio, Inc., Dalian, Liaoning, China) following the manufacturer's instructions. The quality and quantity were measured using a NanoDrop ND-1000 UV spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). Then, the RNA samples were diluted in diethyl pyrocarbonate-treated water to a concentration of 500 ng/μL. Total RNA was reverse transcribed using the PrimeScript™ Realtime PCR kit (Takara Bio, Inc., Dalian, Liaoning, China). The house-keeping gene β-actin was used as an

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internal control (Ming *et al*., 2010). Primers (Table 3) for RT-PCR included metallothionein (MT), metal response element-binding transcription factor-1 (MTF-1), and the Zn-transporter proteins (ZnT-1, ZnT-5, ZnT-7, ZIP4, ZIP14), which were designed with reference to the known sequences of Cyprinidae. All primers were synthesized by Shanghai Sangon Biotechnology Co., Ltd. (China). Quantification of mRNA was performed using an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, California, USA) using the RT-PCR kit according to the instructions of SYBR Premix Ex Taq II (Takara Bio, Inc., Dalian, Liaoning, China). The optimized cycling conditions were performed as follows: 95ºC for 30 s followed by 40 cycles at 95ºC for 5 s, 60ºC for 31 s, a final dissociation stage at 95ºC for 15 s, 60ºC for 1 min, 95ºC for 15 s, and 60ºC for 15 s. The expression levels of relative genes were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) using SPSS statistical software (Version 20.0 for Windows, SPSS Inc., Chicago, Illinois, USA). Polynomial contrasts were used to determine the linear and quadratic effects of dietary Zn-Pal inclusion levels. The differences among treatments were examined using the Duncan test and treatments were considered significant at the $P<0.05$ level. The means and total means of the standard error (SEM) were presented.

RESULTS

Growth performance

As indicated in Table 4, dietary Zn-Pal supplementation quadratically increased the PWG (P=0.009), SGR ($P = 0.009$), and HSI ($P = 0.034$), and linearly ($P = 0.015$) and quadratically ($P = 0.003$) improved the FCR in blunt snout bream with the optimal Zn level from Zn-Pal of 35 mg/kg. The supplementation of $ZnSO_4$ or 35 mg/kg Zn as Zn-Pal significantly increased the FBW, PWG, FCR, SGR, and HSI of blunt snout bream when compared to the control group ($P<0.05$); the values of these parameters were similar between the two groups ($P>0.05$). Fish given a basal diet with 80 mg/kg Zn as Zn-Pal exhibited a lower CF in comparison to fish that received a diet supplemented with only 35 mg/kg Zn as Zn-Pal ($P<0.05$).

Nutrient retention

The retention of OM ($P = 0.001$; Table 5), CP ($P = 0.002$), and CL ($P = 0.060$) were quadratically increased by Zn-Pal supplementation and the optimum effect was observed with 35 mg/kg Zn supplement as Zn-Pal. The 35 mg/kg Zn supplement as Zn-Pal significantly improved the retention of OM, CP, and CL to the levels comparable to the $ZnSO_4$ group ($P>0.05$).

Body composition

Dietary Zn-Pal supplementation quadratically decreased the moisture contents ($P = 0.025$; Table 6), quadratically increased the OM level ($P = 0.025$), and

Table 3. Primer sequence of Cyprinidae mRNA used in real-time PCR assays.

Gene[1]	Genbank id	Primer sequence, sense/antisense	Product size, base pairs
β-actin	AY170122.2	GTGCCCATCTACGAGGGTTA GCTGTGGTGGTGAAGCTGTA	125
MT	NM_131075.1	GACTGGAGCTTGCAACTGTG AGGAATTGCCCTTACACACG	137
MTF-1	NM_152981.1	TCCACCTGTTCCTCTTCCTG AAGGAGGAGGTCTGTGACGA	116
ZnT-1	NM_200879.1	GCTGGGTGCTTTATCTGGAC ATTTGCTTGGGTACGGTTTG	121
ZnT-5	NM_001002322.1	CGCCTGTGAGGTTCTTCTTC TCCAAAAGTGTGGATCACGA	119
ZnT-7	NM_001100086.1	CCCTTCCTGAATGCTACCAA GCCACCAGGAGTTTGAGTGT	118
ZIP4	NM_001130777.1	CCTACGCTGATTCATGTGGA GCCCGATCTGGTCTTCATAA	121
ZIP14	XM_005171766.3	GCTCCTCGTCAGGTTAAACG GTTCGCTCATCCCGTACACT	122

[1]MT = metallothionein; MTF-1 = metal response element-binding transcription factor-1; ZnT-1, 5, 7=Zn-transporter proteins (SLC30A); ZIP4, 14=Zn-transporter proteins ZIP (SLC39A).

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Table 4. Effects of Zn-Pal[1] inclusion on the growth performance of blunt snout bream.

Item[2]	Control	$ZnSO_4$	– Zn as Zn-Pal (mg/kg) –			SEM	*P* values for Zn-Pal	
			35	80	125		Linear	Quadratic
FBW (g)	10.16^{b}	11.99^{a}	11.45^{a}	10.90^{ab}	11.66^{a}	0.22	0.054	0.518
PWG (%)	51.42^{b}	73.63^{a}	78.22^{a}	70.44^{a}	67.99^{a}	3.04	0.061	0.009
FCR (g/g)	0.26^{b}	0.40^{a}	0.40^{a}	0.35^{a}	0.34^{a}	0.02	0.015	0.004
SGR (%day^{-1})	0.84^{b}	1.13^{a}	1.18^{a}	1.09^{a}	1.06^{a}	0.04	0.055	0.009
CF (%)	1.96^{ab}	2.10^{ab}	2.16^{a}	1.76^{b}	1.91^{ab}	0.05	0.347	0.843
VBR (%)	16.83	17.17	17.13	15.83	15.96	0.27	0.127	0.929
HSI (%)	1.20^{b}	1.49^{a}	1.44^{a}	1.43^{ab}	1.32^{ab}	0.04	0.255	0.034

Means that do not share the same superscript in each column are significantly different ($P<0.05$).
[1] Zn-Pal: zinc bearing palygorskite.
[2] FBW: final body weight; PWG: percentage weight gain; FCR: feed conversion ratio; SGR: specific growth rate; CF: condition factor; VBR: viscera/body ratio, HSI: hepatosomatic index; SEM: means and total means of the standard error.

linearly (P = 0.049) and quadratically (P = 0.021) increased the CP level in the bodies of blunt snout bream. However, the highest Zn supplementation level as Zn-Pal (125 mg/kg) increased the moisture contents ($P<0.05$), but decreased the CP level in the body of blunt snout bream in comparison to the control group ($P<0.05$). Treatments did not affect the CL or ash levels in the body of blunt snout bream among groups ($P>0.05$).

Meat quality

Dietary Zn supplementation as Zn-Pal quadratically reduced the meat cooking loss in blunt snout bream (P = 0.047; Table 7) and the most pronounced effect was seen at 35 mg/kg Zn as Zn-Pal. Additionally, 35 mg/kg dietary Zn as Zn-Pal significantly decreased the meat cooking loss of blunt snout bream in comparison with either the control or the $ZnSO_4$ group ($P<0.05$). The 24-h and 48-h drip losses, however, were similar among treatments ($P>0.05$).

Muscular antioxidant status

The supplementation of Zn as Zn-Pal, especially at 35 mg/kg, quadratically increased the T-SOD (P = 0.021; Table 8) and Cu/Zn-SOD (P = 0.025) activities in the muscle of blunt snout bream. Similarly, the inclusion of 35 mg/kg dietary Zn as Zn-Pal also significantly improved muscular T-SOD activity of blunt snout bream in comparison with those fed the basal diet or the basal diet supplemented with 125 mg/kg Zn as Zn-Pal ($P<0.05$). In contrast, the T-AOC and Cu/Zn-SOD activities and MDA levels in the muscle of blunt snout bream did not differ among groups ($P>0.05$).

Tissue Zn accumulations

Zn accumulation in the hepatopancreas was linearly enhanced by increased levels of Zn-Pal ($P<0.05$, Table 9) and the highest Zn level in the hepatopancreas was found with 125 mg/kg Zn as Zn-Pal. The $ZnSO_4$ supplementation, however, did not alter Zn accumulation in the hepatopancreas in comparison to the control group ($P>0.05$). Compared to the $ZnSO_4$ group, 125 mg/kg dietary Zn as Zn-Pal produced higher Zn levels in the hepatopancreas than lower Zn concentrations ($P<0.05$).

Messenger RNA expression

Zn-Pal inclusion in the fish diets linearly increased the mRNA expression levels of intestinal Zn transporter proteins that included MT (P = 0.015; Table 10), ZnT-5 (P = 0.002), and ZIP14 (P = 0.010) and quadratically (P = 0.020) enhanced the mRNA abundance of MTF-1 in the intestine of blunt snout bream. The optimal Zn level as Zn-Pal was 35 mg/kg. In addition, the mRNA levels of MTF-1 in the intestines of blunt snout bream fed diets

Table 5. Effects of Zn-Pal[1] inclusion on nutrient retention by blunt snout bream.

Item[2]	Control	$ZnSO_4$	– Zn as Zn-Pal (mg/kg) –			SEM	*P* values for Zn-Pal	
			35	80	125		Linear	Quadratic
OM (%)	7.49^{b}	11.68^{a}	11.64^{a}	10.64^{a}	8.90^{b}	0.48	0.216	0.001
CP (%)	7.97^{c}	14.08^{a}	14.17^{a}	12.04^{ab}	9.13^{bc}	0.77	0.761	0.002
CL (%)	30.38^{b}	52.37^{a}	46.11^{a}	44.26^{a}	41.22^{ab}	2.43	0.149	0.060

Means not sharing the same superscript in each column are significantly different ($P<0.05$).
[1] Zn-Pal: zinc bearing palygorskite.
[2] OM: organic matter; CP: crude protein; CL: crude lipid; SEM: means and total means of the standard error.

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Table 6. Effects of Zn-Pal[1] inclusion on body composition of blunt snout bream.

Item[2]	Control	$ZnSO_4$	– Zn as Zn-Pal (mg/kg) – 35	80	125	SEM	*P* values for Zn-Pal Linear	Quadratic
Moisture (%)	74.46^{ab}	73.80^{b}	74.02^{ab}	73.38^{b}	75.05^{a}	0.20	0.532	0.025
OM (%)	22.53^{ab}	23.06^{a}	22.80^{ab}	23.38^{a}	21.84^{b}	0.18	0.335	0.025
CP (%)	12.66^{a}	12.89^{a}	12.92^{a}	12.92^{a}	11.89^{b}	0.13	0.049	0.021
CL (%)	7.38	7.52	7.03	7.61	7.34	0.09	0.623	0.834
Ash (%)	3.01	3.15	3.17	3.24	3.11	0.04	0.329	0.118

Means that do not share the same superscript in each column are significantly different ($P<0.05$).
[1] Zn-Pal: zinc bearing palygorskite.
[2] OM: organic matter; CP: crude protein; CL: crude lipid; SEM: means and total means of the standard error.

supplemented with 35 mg/kg Zn from Zn-Pal were higher than those fed the basal diet supplemented with Zn from $ZnSO_4$ ($P<0.05$).

DISCUSSION

Growth performance

Zn, an activator of enzyme systems, is involved in endocrine function, such as the synthesis and secretion of various hormones that include osteocalcin, testosterone, thyroid hormones, insulin-like growth factor-1, and insulin, and Zn plays a vital role in the growth and development of animals (Giugliano and Millward, 1984; Fukada *et al*., 2011). Dietary supplementation with Zn as $ZnSO_4$ has been reported to improve the FBW and FCR in blunt snout bream (Jiang *et al*., 2016). Likewise, the addition of dietary Zn regardless of sources improved the FBW, PWG, SGR, CF, FCR, and HSI of blunt snout bream in the present study. In addition, fish fed the basal diet supplemented with 35 mg/kg Zn as Zn-Pal achieved similar growth performance to those in the basal diet supplemented with 125 mg/kg Zn as $ZnSO_4$. This suggests that Zn-Pal is more effective than $ZnSO_4$. In contrast, Yan *et al*.(2016) found that Zn-Pal supplementation as an alternative to $ZnSO_4$ did not improve the growth performance of broilers even though it produced higher Zn retention. Yang *et al*. (2016) also reported that Zn-Pal inclusion did not affect the growth performance in broilers. These discrepancies regarding the consequences of Zn-Pal on the growth performance of animals may be due to the particular animal species, Zn level in the basal diet, the properties of Zn-Pal, and the dosage, which need further investigation.

Nutrient retention and body composition

Zn-bearing clay minerals, such as montmorillonite and zeolite, have been reported to improve nutrient digestibility in animals (Hu *et al*., 2012; Hu *et al*., 2014; Tang *et al*., 2014a). In the present study, Zn-Pal also increased OM and CP retention by blunt snout bream and 35 mg/kg Zn as Zn-Pal achieved optimal nutrient retention. Similarly, $ZnSO_4$ also significantly increased OM and CP retention by blunt snout bream in the present study. Several Zn-containing enzymes, which play important roles in digestion and absorption of proteins in the animal body (Vallee and Neurath, 1955; Halver and Hardy, 2002), may be improved by dietary Zn supplementation and then increase CP retention by blunt snout bream. A previous study reported that Zn could improve the activity of lipase and the intestinal structure in broilers (Tang *et al*., 2014a), which may also contribute to the improved nutrient retention of blunt snout bream. The present study showed that Zn-Pal inclusion quadratically decreased the moisture content, whereas the OM and CP contents in the whole body of blunt snout bream were increased. This is consistent with nutrient retention by blunt snout bream. McClung *et al*. (2007) demonstrated that Zn supplementation promoted the phosphorylation of proteins implicated in mTOR signaling, which is a key regulator in protein synthesis. This may help to explain the increased protein accumulation in blunt snout bream. Similarly, Wu *et al*. (2015)

Table 7. Effects of Zn-Pal[1] inclusion on meat quality of blunt snout bream.

Item		Control	$ZnSO_4$	– Zn as Zn-Pal (mg/kg) – 35	80	125	SEM	*P* values for Zn-Pal Linear	Quadratic
Drip loss (%)	24 h	7.85	6.64	5.92	6.76	7.17	0.37	0.678	0.154
	48 h	15.86	13.88	13.13	12.52	13.15	0.75	0.173	0.241
Cooking loss (%)		18.84^{a}	18.23^{ab}	15.40^{c}	16.15^{bc}	16.26^{abc}	0.42	0.087	0.047

Note: Means that do not share the same superscript in each column are significantly different ($P<0.05$).
[1]Zn-Pal: zinc bearing palygorskite; SEM: means and total means of the standard error.

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Table 8. Effects of Zn-Pal[1] inclusion on muscular antioxidant status of blunt snout bream.

Item[2]	Control	$ZnSO_4$	– Zn as Zn-Pal (mg/kg) –			SEM	*P* values for Zn-Pal	
			35	80	125		Linear	Quadratic
T-AOC (U/mgprotein)	0.56	0.68	0.44	0.58	0.71	0.05	0.163	0.798
MDA (nmol/mgprotein)	1.17	0.88	0.75	0.84	0.85	0.09	0.262	0.348
T-SOD (U/mgprotein)	50.42^{b}	54.12^{ab}	56.64^{a}	52.57^{ab}	51.12^{b}	0.82	0.960	0.021
Cu/Zn-SOD (U/mgprotein)	11.24	13.73	13.52	13.62	12.56	0.37	0.104	0.025

Means that do not share the same superscript in each column are significantly different ($P<0.05$).
[1] Zn-Pal: zinc bearing palygorskite.
[2] T-AOC: total antioxidant capacity; MDA: malondialdehyde; T-SOD: total superoxide dismutase; Cu/Zn-SOD: copper/zinc superoxide dismutase; SEM: means and total means of the standard error..

also found that Zn dietary supplementation increased the CP content in the muscle of young grass carp. In the present study, dietary 125 mg/kg Zn as Zn-Pal inclusion decreased OM retention by blunt snout bream. Jiang *et al*. (2016) reported that superfluous Zn in the diet impaired antioxidant function in blunt snout bream, which may have a negative effect on OM retention by blunt snout bream. Similarly, a previous study found that excessive amounts of Zn in the diets of sheep decreased pancreatic flow and amylase activity (Smith and Embling, 1984).

Meat quality and antioxidant status

Fish is usually consumed after cooking and preferably should not only be tender, but also juicy (Ofstad *et al.*, 1993). Muscle is the main constituent tissue of fish (Houlihan *et al.*, 1995). The water-holding capacity of raw and cooked meat has been related to such important organoleptic properties as tenderness and juiciness (Hamm, 1961). Drip loss and cooking loss are important indicators to evaluate the water-holding capacity (Honikel, 1987). Wu *et al*. (2015) reported that dietary Zn reduced cooking loss of muscle in young grass carp. Yang *et al*. (2016) found that dietary Zn-Pal supplementation reduced the cooking losses of breasts and thighs in broilers. In agreement with these findings, the present study also found that 35 and 80 mg/kg Zn dietary supplementation as Zn-Pal decreased muscular cooking losses of blunt snout bream. This in turn indicated that Zn-Pal could improve the meat quality of fish. A previous study found that the oxidation of lipids and proteins in muscles led to a reduction in water-holding capacity (Asghar *et al*., 1991). Zn is a necessary trace element for the structure and function of Cu/Zn-SOD, which is widely distributed and constitutes 90% of T-SOD. This protects the brain, lungs, and other tissues from oxidation (Noor *et al*., 2002). Zn is assumed to improve the antioxidant capacity and water-holding capacity of meat by increasing the Cu/Zn-SOD activity (Liu *et al*., 2011). The present study also found that Zn-Pal could enhance T-SOD and Cu/Zn-SOD activities in the muscle of blunt snout bream. The decreased cooking losses in the present study may be related to the fact that Zn-Pal improved the muscular antioxidant status. Similarly, Yang *et al*. (2016) also found that Zn-Pal enhanced the muscular antioxidant status to improve the meat quality of broilers.

TISSUE ZN ACCUMULATIONS

The XRD patterns of Pal and Zn-Pal indicated that modification of Pal did not affect the crystal structure as noted by Yan (2016), who reported that Zn was mainly attached in the pores and to the surfaces of Pal and could be released from Zn-Pal in *in vitro* research. Similarly, Malachová *et al*. (2011) reported that 60% of Zn in Zn-montmorillonite could be released by cation exchange with Na ions in NaCl solutions. In the present study, Zn

Table 9. Effects of Zn-Pal[1] on tissue Zn accumulation of blunt snout bream.

Item	Control	$ZnSO_4$	– Zn as Zn-Pal (mg/kg) –			SEM	*P* values for Zn-Pal	
			35	80	125		Linear	Quadratic
Blood (mg/L)	9.69	9.43	9.26	8.07	8.68	0.31	0.175	0.429
Muscle (mg/kg)	18.23	16.82	17.85	15.82	19.99	0.65	0.609	0.111
Hepatopancreas (mg/kg)	32.32^{b}	33.95^{b}	33.67^{b}	37.83^{ab}	41.82^{a}	1.10	0.005	0.581
Vertebra (mg/kg)	109.4	116.6	115.3	116.6	115.9	1.56	0.220	0.382
Scale (mg/kg)	131.0^{a}	147.0^{a}	146.3^{a}	148.0^{a}	125.7^{b}	3.01	0.737	0.007

Means that do not share the same superscript in each column are significantly different ($P<0.05$).
[1] Zn-Pal: zinc bearing palygorskite; SEM: means and total means of the standard error.

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Table 10. Effects of Zn-Pal[1] relative mRNA levels of Zn transporters in intestine of blunt snout bream.

Item[2]	Control	$ZnSO_4$	– Zn as Zn-Pal (mg/kg) –			SEM	*P* values for Zn-Pal	
			35	80	125		Linear	Quadratic
MT	1.00[b]	2.04[b]	1.69[b]	4.47[a]	2.66[ab]	0.36	0.015	0.099
MTF-1	1.00[b]	1.47[b]	3.86[a]	2.57[ab]	2.18[ab]	0.31	0.369	0.020
ZnT-1	1.00	1.51	1.59	2.56	2.30	0.33	0.153	0.583
ZnT-5	1.00[b]	3.05[ab]	1.90[ab]	4.13[a]	4.56[a]	0.47	0.002	0.826
ZnT-7	1.00	1.87	2.03	3.28	1.99	0.38	0.264	0.193
ZIP4	1.00	3.76	2.53	2.69	2.93	0.70	0.262	0.573
ZIP14	1.00[b]	3.12[ab]	3.17[ab]	3.38[ab]	3.78[a]	0.37	0.010	0.209

Means that do not share the same superscript in each column are significantly different ($P<0.05$).
[1] Zn-Pal: zinc bearing palygorskite.
[2] MT: metallothionein; MTF-1: metal response element-binding transcription factor-1; ZnT-1, 5, 7: Zn-transporter proteins (SLC30A); ZIP4, 14: Zn-transporter proteins ZIP (SLC39A); SEM: means and total means of the standard error.

contents in the hepatopancreas of fish displayed a linear response with increased Zn-Pal levels (0, 35, 80, and 125 mg/kg Zn) and Zn contents in the scales showed a quadratic response. The supplementation of 80 mg/kg Zn as Zn-Pal exhibited a higher efficiency than Zn as $ZnSO_4$ for Zn accumulation. A previous study showed that Pal could be used as a controlled-release carrier for drug molecules (de Sousa Rodrigues *et al*., 2013). The Zn ions released from Zn-Pal may either be adsorbed on Pal surfaces or suspended in the intestine after desorption and, thus, modify the rate, time, or sites of Zn release. This would improve the retention of Zn and, therefore, benefit Zn accumulation, nutrient retention, and growth of blunt snout bream.

Messenger RNA expression

MT, a widely studied protein modulated by Zn levels, helps to regulate intracellular levels of free Zn (Davis and Cousins, 2000). The present study revealed that Zn from Zn-Pal or $ZnSO_4$ increased the MT mRNA levels in the intestinal cells, which agrees with the previous finding that a Zn supplemented diet increased MT gene expression in broilers (Tang *et al*., 2015). The expression of intestine MT mRNA for the inclusion of 80 mg/kg dietary Zn as Zn-Pal was higher than those fed other levels of Zn-Pal (0, 35, and 125 mg/kg of Zn) or $ZnSO_4$ (125 mg/kg of Zn). These findings indicate that Zn-Pal enhanced the retention of Zn as evidenced by enhanced tissue Zn accumulation and Zn transporter gene expression in comparison to $ZnSO_4$. This is consistent with the fact that growth performance and nutrient retention of blunt snout bream are better than other groups when supplemented with 35 and 80 mg/kg Zn as Zn-Pal. The metal response element-binding transcription factor-1 (MTF-1) is an essential protein for metallothionein gene regulation and exhibits increased DNA binding activity after Zn treatment (Heuchel *et al*., 1994). The mRNA expression of MTF-1 exhibited a quadratic response with the increased diet Zn-Pal levels (0, 35, 80, and 125 mg/kg Zn) in the present study. The inclusion of 35 mg/kg dietary Zn as Zn-Pal exhibited higher MTF-1 mRNA levels than other groups, which is not consistent with the MT mRNA level and it may be due to the fact that the MT mRNA expression can also be regulated by other factors, such as the basic helixloop-helix upstream stimulatory factor-1 (Andrews *et al*., 2001).

Zn homeostasis is maintained by the activities of Zn transporters in the cell plasma membrane and intracellular organelles (Ho *et al*., 2012). Two families of Zn-transporter proteins, the ZnT (SLC30A) and ZIP (SLC39A), have been identified in mammals. The ZIP proteins have been shown to transport Zn to the cytosol either from organelles or from external sources. The ZnT proteins are involved in the compartmentalization of Zn into organelles or the extracellular space (Tako *et al*., 2005; Ho *et al*., 2012). The ZnT-5 protein is an exception and is possibly involved in Zn influx at the intestine (Cragg *et al*., 2005). In the present study, Zn supplements from Zn-Pal or $ZnSO_4$ increased the ZIP14 and ZnT-5 mRNA levels, which suggests that Zn-Pal like $ZnSO_4$ can be used as a potential Zn source for utilization by animals. Furthermore, dietary supplementation with 35 or 80 mg/kg Zn as Zn-Pal produced similar mRNA levels of Zn-transporter proteins as the $ZnSO_4$ group in blunt snout bream. This is in accordance with the finding of Yan *et al*. (2016) who found the Zn in Zn-Pal was more bioavailable in broiler diets than the Zn in $ZnSO_4$. As a Zn source, Zn-Pal may be more effective than $ZnSO_4$, which needs further study.

CONCLUSIONS

In conclusion, dietary Zn-Pal inclusion improved the growth performance, increased organic matter and crude protein retention, enhanced muscular T-SOD and Cu/Zn-SOD activity, decreased muscular cooking loss, and benefitted Zn accumulation and intestine Zn transporter protein gene expression in blunt snout bream. The optimal Zn level as Zn-Pal was 35 mg/kg. Zn-Pal was

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more efficient and can be used as an alternative Zn source to $ZnSO_4$ in the diet of fish.

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