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Effects of dietary dosage forms of copper supplementation on growth, antioxidant capacity, innate immunity enzyme activities and gene expressions for juvenile *Litopenaeus vannamei*

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ABSTRACT

The experiment was conducted to evaluate the effects of different dietary dosage forms of copper supplementation on growth performance, hematological characteristics, antioxidant capacity, immune responses and gene expressions related to innate immune of juvenile Pacific white shrimp *Litopenaeus vannamei*. Three isonitrogenous and isolipidic diets were formulated to contain three dosage forms of copper: copper sulfate (Diet I-Cu), copper sulfate + copper amino acid complex (1: 1, Diet M-Cu) and copper amino acid complex from Availa®Cu100 (Diet Availa-Cu), respectively. 360 Pacific white shrimp juveniles (initial weight 1.86 ± 0.03 g) were randomly allocated in 12 tanks corresponding to quadruplicate tanks of the three dietary treatments, and the 8-week feeding trail was conducted. The results indicated that percent weight gain (PWG) and specific growth rate (SGR) in shrimp fed M-Cu diet were significantly higher than that fed I-Cu diet. Survival, feed efficiency (FE), protein efficiency ratio (PER) of shrimp were not significantly different between all treatment groups. High contents of total protein (TP) and glucose (GLU) were found in shrimp fed the diet containing M-Cu, whereas contents of cholesterol (CHOL) and triacylglycerol (TAG) in shrimp fed M-Cu diet were significantly lower than that in I-Cu diet group. In hemolymph, shrimp fed M-Cu diet had high activities of phenoloxidase (PO), alkaline phosphatase (ALP) and acid phosphatase (ACP). While, Cu/Zn superoxide dismutase (Cu/Zn SOD), ceruloplasmin (CP) and lysozyme (LZM) in hemolymph were not significantly affected by different dietary dosage forms of copper. High activities of Cu/Zn SOD, ALP, ACP and LZM in hepatopancreas were observed in shrimp fed M-Cu diet. Shrimp fed diet supplemented with Availa-Cu showed a significantly higher gene expression levels of Cu/Zn *sod*, *alp*, *acp* and *lzm* in hepatopancreas than that fed I-Cu diet. This study indicated that copper amino acid complex was more effective than copper sulfate to improve growth performance and enhance antioxidant ability and innate immune system.

1. Introduction

Trace minerals are crucial components of hormones and enzymes, serve as cofactors and activators of a variety of enzymes as well as participate in a wide variety of biochemical processes [1]. Copper (Cu) is an essential trace element for humans and animals and acts as cofactors of specific proteins and enzymes such as ceruloplasmin, superoxide dismutase, lysyl oxidase, cytochrome *C* oxidase, ferroxidase and tyrosinase [2–5], which can be involved in a series of biological processes including scavenging free radicals, electron transport, and syntheses of hemoglobin and collagen [3]. For crustaceans, they utilize hemocyanin as the oxygen-carrying pigment which has an analogous role to hemoglobin in red-blooded animals [6,7]. It has been estimated that, on a fresh-weight basis, approximately 40% of the whole-body copper load in shrimp is found in hemocyanin, thereby indicating the tremendous significance of meeting copper requirements in shrimp [8]. Moreover, shrimp can obtain copper from ambient water or diets for physiological need. Nevertheless, studies showed that copper in the rearing water alone cannot meet the requirements and dietary copper supplementation is essential for shrimp to meet the need for optimal growth, tissue mineralization and reproduction [1,9–15].

It is known that the most common form of copper used in feeds for growth promotion is the sulfate salt ($CuSO_4$) [13,16–19]. As a new type of copper additive, organic forms of copper have also been found to be effective for some aquatic animals such as *A. gueldenstaedtii* [20], *C. auratus gibelio* [21], *L. japonicus* [22], *L. vannamei* [23], *O. mykiss* [24–26] and *S. schlegeli* [27]. Previous studies have indicated that the low bioavailability of inorganic copper results in over-fortification of

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diets and consequently causes environmental pollution in areas of intensive aquaculture production which may make aquaculture water copper excessive [1]. Furthermore, high levels of copper may be toxic and put pressures on the immune systems of aquatic animals [28–30]. Some studies have proved that dietary copper has a vital role in the innate immune response in crustaceans such as *E. sinensis* [31], *L. vannamei* [9] and *P. monodon* [12]. While, chemical forms of copper affect its absorption and utilization. Moreover, it was reported that organic forms of copper were more effective than $CuSO_4$ in some aquatic animals [24–26,32,33]. Organic trace minerals including copper are capable to bind with ligand such as amino acids, peptides and proteins [34], which are easier to get absorbed and better retained by the body than inorganic sources of trace minerals [35–37]. They are more stable in the digestive tract and less prone to interactions and antagonisms as they are bound to organic molecules and less accessible to interaction and binding [25]. Therefore, organic forms of copper may have a higher bioavailability which may be due to their higher absorption or additional biological effects compared to inorganic Cu salts [38]. Recently, few reports have demonstrated the higher bioavailability of dietary chelated copper in Pacific white shrimp [23,33]. However, limited information is available on the efficacy of dietary dosage forms of Cu from the inorganic and/or chelated sources in Pacific white shrimp. The choice of Cu compound with a high utilization rate in shrimp diet can also reduce the amount of dietary copper added and the level of copper excretion in feces.

The Pacific white shrimp, *L. vannamei*, is naturally distributed along the Pacific coasts of Central and South America [39]. Pacific white shrimp has become a worldwide specie for aquaculture because of its great economic value, rapid growth rate, and tolerance to a wide range of salinity (0.5–40 ppt) [40,41], and temperature (22–30 °C) [42,43]. However, with the development of intensive aquaculture and environmental deterioration, various diseases caused by bacteria, fungi, viruses and rickettsia-like organisms have frequently occurred in farmed Pacific white shrimp populations. Some studies have demonstrated that shrimp depend on innate immune system rather than on non-existent acquired immunity for protection against invading pathogens [44]. But So far, there is no information regarding the effects of dietary dosage forms of copper supplementation on antioxidant capacity, relative expressions of innate immune genes for *L. vannamei*. Therefore, the present study aims to evaluate the comparative effects of dietary dosage copper supplementation on growth performance, antioxidant capacity, immunity enzyme activities and gene expressions of juvenile Pacific white shrimp. The outcomes of this study will enhance our understanding on the antioxidant capacity and innate immune ability in Pacific white shrimp, and will also explore means to enhance innate immunity through nutritional modulation using organic copper in shrimp diets.

2. Materials and methods

2.1. Experimental diet preparation

Three isonitrogenous (42% crude protein) and isolipidic (7% crude lipid) experimental diets were formulated to contain different dosage forms of copper ($CuSO_4{\cdot}5H_2O$, $CuSO_4{\cdot}5H_2O$ + Availa®Cu100 = 1:1, and Availa®Cu100). The formulation and proximate composition of the diets are presented in Table 1. Fish meal, soybean meal, poultry by-product, krill meal and peanut meal were used as protein sources; fish oil and soybean oil (1:1) were used as the lipid sources; and wheat flour was used as the carbohydrate source. Three experimental diets were all formulated with $ZnSO_4{\cdot}7H_2O$ as the zinc sources. Availa®Cu100 (Cu content = 10%, Zinpro Corp., USA) and $CuSO_4{\cdot}5H_2O$ (Cu content = 25.45%, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) were used as dietary Cu sources. The diet ingredients, except the mineral premix, supplied with copper at the level of 10 mg Cu kg^{-1} diet (dry basis). Copper sulfate ($CuSO_4{\cdot}5H_2O$, Diet I-Cu) was added at the level of 30 mg Cu kg^{-1} diet; $CuSO_4{\cdot}5H_2O$ + Availa®Cu100 (1:1, Diet M-Cu) contained the same amount of copper from $CuSO_4{\cdot}5H_2O$ and Availa®Cu100, at levels of 15 mg Cu kg^{-1} diet, respectively. Copper amino acid complex (Diet Availa-Cu) had added at the level of 15 mg Cu kg^{-1} diet. The analyzed values of diet copper content were 40.64, 39.70 and 25.07 mg Cu kg^{-1} diet, respectively (Table 1). All ingredients were ground through 80-mesh and then weighed according to the formulation. The micro-components such as minerals and vitamins premix were mixed thoroughly by the progressive enlargement method and then added lipid and distilled water (35%, w/w). The ground ingredients were mixed in a Hobart type mixer and cold-extruded pellets produced (F-26, Machine factory of South China University of Technology, Guangzhou, China) with pellet strands cut into uniform sizes (1.5 mm and 2 mm diameter) (G-250, Machine factory of South China University of Technology, Guangzhou, China). Pellets were steamed for 30 min at 90 °C, and then air-dried to approximately 10% moisture, sealed in vacuum-packed bags and stored at −20 °C until use for the feeding trial.

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

Ingredient (g kg^{-1})	Dietary dosage forms of copper		
	I-Cu	M-Cu	Availa-Cu
Fish meal[a]	300.00	300.00	300.00
Soybean meal[b]	220.00	220.00	220.00
Poultry by-product[c]	60.00	60.00	60.00
Krill meal[d]	30.00	30.00	30.00
Peanut meal[e]	60.00	60.00	60.00
Wheat flour	276.70	276.70	276.70
Fish oil	10.00	10.00	10.00
Soybean oil	10.00	10.00	10.00
$Ca(H_2PO_4)_2$	15.00	15.00	15.00
Vitamin premix[f]	5.00	5.00	5.00
Mineral premix (Cu-free)[g]	10.00	10.00	10.00
Vitamin C	0.30	0.30	0.30
Choline chloride	3.00	3.00	3.00
Proximate composition[h] (g kg^{-1})			
Crude protein	426.30	427.90	427.20
Crude lipid	76.00	76.00	75.70
Moisture	106.10	94.20	101.30
Ash	91.30	91.00	90.80
Analyzed copper (mg kg^{-1})	40.64	39.70	25.07
Analyzed zinc (mg kg^{-1})	79.65	79.69	81.36

[a] Fish meal, crude protein, 710 g kg^{-1} dry matter; crude lipid, 108 g kg^{-1} dry matter.
[b] Soybean meal, crude protein, 527 g kg^{-1} dry matter; crude lipid, 19 g kg^{-1} dry matter.
[c] Poultry by-product, crude protein, 712 g kg^{-1} dry matter; crude lipid, 150 g kg^{-1} dry matter.
[d] Krill meal, crude protein, 797 g kg^{-1} dry matter; crude lipid, 36 g kg^{-1} dry matter.
[e] Peanut meal, crude protein, 540 g kg^{-1} dry matter; crude lipid, 34 g kg^{-1} dry matter.
[f] Vitamin premix were based on Xie et al. [45].
[g] Mineral premix (per kg diet): NaCl (99.5%), 0.767 g; KCl (99.5%), 1.916 g; $ZnSO_4{\cdot}7H_2O$, 80.230 mg; $MgSO_4{\cdot}7H_2O$ (99%), 6.145 g; $FeSO_4{\cdot}7H_2O$, 198.610 mg; $CoSO_4{\cdot}7H_2O$, 1.667 mg; $MnSO_4{\cdot}H_2O$, 30.731 mg; $Ca(IO_3)_2$ (0.99%), 0.077 mg; Na_2SeSO_3, 0.779 mg. The mineral premix does not supply Cu.
[h] Nutrient levels were measured values (dry matter basis). All values are as g kg^{-1} of diets except for copper and zinc which are in mg kg^{-1}.

2.2. Shrimp rearing and experimental conditions

Pacific white shrimp juveniles were obtained from Guangdong Evergreen Group (Zhanjiang, China). The feeding trial was conducted in South Marine Aquaculture Seed Base of National Program (863 Base). Prior to the start of the feeding trial, shrimp juveniles were

acclimated to laboratory conditions by feeding a commercial diet (40% dietary protein, 8% dietary lipid, purchased from Guangdong Evergreen Group Co. Ltd.) for two weeks. A total of 360 Pacific white shrimp juveniles (initial weight 1.86 ± 0.03 g) were randomly distributed into 300-L cylindrical fibre-glass tanks filled with 200 L of water at the stocking rate of 30 shrimp per tank. Each experimental diet was randomly assigned to four replicates. Shrimp were fed three times a day (daily ration was about 6–8% of biomass) at 6:00, 12:00 and 18:00, and total ration in the morning and evening was 60% of the total test diets. Shrimp in each tank were weighed once every 2 weeks and the feed's daily ration was adjusted accordingly. Dead shrimp were immediately removed, weighed and recorded. All tanks were cleaned daily by siphoning out the accumulated waste material and exuviae, and above 60% of the tank seawater were daily exchanged prior to the first feeding in the morning. Photoperiod was maintained on a natural cycle. Aeration was provided to maintain dissolved oxygen levels near saturation (6.0–6.7 mg L^{-1}). Temperature was 25.2–31.6 °C, pH was 7.6–7.8, salinity was 25–28 g L^{-1}, ammonia nitrogen was lower than 0.05 mg L^{-1}. Dissolved oxygen, pH, salinity and ammonia nitrogen were measured by YSI Proplus (YSI, Yellow Springs, Ohio, USA). The feeding trial lasted for 8 weeks.

2.3. Samples collection

At the end of feeding trial, shrimp in each tank were sampled 24 h after the last feeding. Shrimp in each tank were counted and weighed to determine the final body weight (FBW), percent weight gain (PWG), specific growth rate (SGR), survival, feed efficiency (FE) and protein efficiency ratio (PER). Hemolymph samples from eight shrimp in each tank were taken from the pericardial cavity using a 1-ml syringe, sorted into 1.5-ml Eppendorf tubes and centrifuged at 4 °C, 4000 r min^{-1} for 10 min by centrifuge (Eppendorf centrifuge 5810R, Germany). Then, the supernatant was collected, packaged and stored at −80 °C until analysis of hematological characteristics, antioxidant and immunity enzyme activities. Four shrimp per tank were used to analyze the whole body composition and Cu concentration. Muscle samples from four shrimp in each tank were collected by scalpel on ice and then stored at −20 °C until analysis of muscle composition and Cu concentration. Meanwhile, the length and hepatopancreas weight of shrimp were measured to calculate the condition factor (CF) and hepatosomatic index (HSI). Hepatopancreas samples were also collected and stored at −80 °C until further analysis of antioxidant and immune-related enzyme activities (four shrimp from each tank) and gene expressions (eight shrimp from each tank).

2.4. Proximate composition and mineral concentration analysis

Crude protein, crude lipid, moisture and ash content in diets as well as the whole body and the muscle of Pacific white shrimp were determined according to the methods of Association of Official Analytical Chemists [46]. Moisture content was determined by drying the samples to a constant weight at 105 °C. Crude protein (N × 6.25) was determined via the Dumas combustion methods with a protein analyzer (FP-528, Leco, USA). Crude lipid was determined by the ether extraction method using Soxtec System HT (Soxtec System HT6, Tecator, Sweden), and ash content was determined using a muffle furnace at 550 °C for 8 h. The whole body, muscle and diet were dried, ashed, acid digested and analyzed for copper and zinc concentrations using ICP-OES (Inductively Coupled Plasma Optical Emission Spectrometer, PE 2100DV, Perkin Elmer, USA) analysis in Ningbo Institute of Materials Technology and Engineering (Ningbo, China).

2.5. Assay of hematological characteristics

The hemolymph samples were disposed as described above. Total protein (TP), triacylglycerol (TAG), cholesterol contents (CHOL) and glucose (GLU) in hemolymph were assayed using an automatic biochemistry analyser (Hitachi 7600-110 Ltd., Japan) from a clinical laboratory in Ningbo University Hospital.

2.6. Antioxidant and immunity enzyme activity assays

Hepatopancreas samples were homogenized on ice in 9 vol (w:v) of ice cold physiological saline 8.9 g ml^{-1}, and then centrifugated at 10000 rpm for10 min at 4 °C. The supernatant was collected in a fresh sterile tube and used for further enzyme activity analysis. The enzymatic activities of Cu/Zn superoxide dismutase (Cu/Zn SOD), ceruloplasmin (CP), alkaline phosphatase (ALP), acid phosphatase (ACP) and lysozyme (LZM) were determined in hemolymph and hepatopancreas homogenates using the relevant diagnostic reagent kits (Nanjing Jiancheng Bioengineering Institute, China). Phenoloxidase (PO) activity was estimated spectrophotometrically using shrimp phenoloxidase ELISA kit (Cusabio, Wuhan, China).

Cu/Zn-SOD were assayed at 550 nm by use of the nitrite method as described by Ōyanagui [47]. One unit of Cu/Zn SOD activity was defined as the amount of SOD required to produce 50% inhibition of the rate of nitrite production at 37 °C in 1 min.

CP activity was determined according to the method of Martinez-Subiela et al. [48]. One unit of ceruloplasmin activity was defined as the amount of ceruloplasmin consuming 1 μmol of substrate (*o*-dianisidine dihydrochloride) at 37 °C in 1 min.

PO activity was estimated spectrophotometrically using shrimp phenoloxidase ELISA kit (Cusabio, Wuhan, China). The stop solution changes the color from blue to yellow and the intensity of the color is measured at 450 nm using a spectrophotometer. In order to measure the concentration of PO in the sample, this PO ELISA kit includes a set of calibration standards. The calibration standards are assayed at the same time as the samples and allow the operator to produce a standard curve of optical density versus PO concentration. The PO concentration in the samples is then determined by comparing the O.D. of the samples to the standard curve.

ALP and ACP activities were estimated spectrophotometrically using *p*-nitrophenyl phosphate (Sigma, USA) as substrate following the modified method described by Gonzalez et al. [49]. A 100-μL sample of the supernatant was incubated for 30 min at 37 °C with 2.0 mL of substrate (*p*-nitrophenyl phosphate in citrate buffer at pH 5 for ACP, and *p*-nitrophenyl phosphate in glycine-NaOH buffer at pH 9 for ALP). Then 2.9 ml of 0.1 N NaOH was added and the absorbance measured spectrophotometrically at 405 nm and the activity expressed as mg ml^{-1} *p*-nitrophenyl released.

LZM activity was conducted following methods of Minagawa et al. and Sotelo-Mundo et al. [50,51]. In brief, 500 ml of diluted haemolymph was centrifuged, and the precipitate was mixed with 1 ml (0.02%) of Micrococcus lysodeikticus (Sigma, USA). The reaction was carried out at room temperature and the absorbance at 530 nm was measured after 30 s and 4.5 min. A unit of lysozyme activity was defined as the amount of sample causing decrease in absorbance of 0.01 per min.

2.7. Total RNA extraction, reverse transcription and real-time PCR

Hepatopancreas were subsampled to 10–20 mg tissues prior to extraction. Total RNA of the hepatopancreas was extracted with TRIzol reagent (TaKaRa, Japan) following the manufacturer's protocol and the RNA was treated with RNase-Free DNase (TaKaRa, Japan) to remove DNA contamination. RNA was quantified at 260 and 280 nm, and RNA ratios (A260: A280) greater than 1.8 were used for further experiments, using a Nano Drop spectrophotometer (Thermo Scientific NanoDrop 2000, USA). Isolated RNA quality was determined to a 1.2% denaturing agarose gel through Molecular Imager® Gel Doc™ XR System (Bio-Rad, USA). Moreover, isolated total RNA was stored at − 80 °C until reverse transcription and quantitative real-time PCR analysis. The cDNA was

Table 2
Real-time quantitative PCR primers for antioxidant and immune related genes of Pacific white shrimp (*Litopenaeus vannamei*).

Gene	Nucleotide sequence (from 5′ to 3′)	Size (bp)	Accession no.
Cu/Zn sod[a]	F:GCCTTCCGAGGGTTCAGA R:TTTGGCAGCGTGTTGTCC	124	AY495084
alp[b]	F:AAAGGCACTGGGAAAATG R:CCTCCACCAAGGATGACC	127	KR534873
acp[c]	F:GAGGAGGTTCAGAGAGGA R:CAGATAAGGCACATAGGC	138	KR676449
lzm[d]	F:GCAAGAACGTCTGCAAAATCC R:CCAGCACTCTGCCATGTACTG	190	AF425673
β-actin	F:CCACGAGACCACCTACAAC R:AGCGAGGGCAGTGATTTC	–	AF300705

[a] *Cu/Zn sod*, Cu-Zn superoxide dismutase.
[b] *alp*, alkaline phosphatase.
[c] *acp*, acid phosphatase.
[d] *lzm*, lysozyme.

generated from 1000 ng of DNAase treated RNA and synthesized by a Prime Script™ RT Reagent Kit with gDNA Eraser (perfect realtime) (TaKaRa, Japan) according to the manufacturer's protocol, using Mastercycler nexus GSX1 PCR (Eppendorf, Germany).

The relative expression of the target genes in hepatopancreas were measured by qPCR. All primers were synthesized by BGI (The Beijing Genomics Institute, Shenzhen, China). The primers for qPCR were designed based on the target genes sequences, including Cu/Zn *sod* (AY495084), *alp* (KR534873), *acp* (KR676449), *lzm* (AF425673) and β-actin (AF300705) (Table 2). β-actin was used as a house-keeping gene and the stability of β-actin expression was confirmed. Primers of each gene were designed based on published *L. vannamei* cDNA using Primer Premier 5.0 software. The qPCR was carried out in a quantitative thermal cycler system (Roche, Light cycler 96, Switzerland) using SYBR Green I. The amplifications were performed in a 96-well plate in a 20 μL reaction volume containing 10 μL of 2 × conc SYBR Green I Master Mix (Roche, Switzerland), 1.0 μL (each) gene-specific forward and reverse primers which concentrations were 10 μM, 6 μL DEPC-water and 2 μL of cDNA. The quantitative PCR program was 95 °C for 2 min, followed by 45 cycles of 95 °C for 10 s, 58 °C for 10 s and 72 °C for 20 s. Each sample was run in duplication for each gene using shrimp β-actin gene as the internal control. Standard curves were generated using six different dilutions (in triplicate) and the amplification efficiency was analyzed as follows: $E = 10^{(-1/Slope)} - 1$. The amplification efficiencies of all genes were approximately equal and ranged from 91 to 99%. In this present study, we used the relative quantification method to analyze data from qPCR experiment. Expression levels of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen [52], and I-Cu diet group was used as the reference group, which the gene expression of the control diet group (I-Cu) were set at 1.

2.8. Calculations

The parameters were calculated as follows:

Percent weight gain (PWG, %) = 100 × [final body weight (g) − initial body weight (g)]/initial body weight (g);
Specific growth rate (SGR, % day^{-1}) = 100 × [Ln (final body weight) − Ln (initial body weight)]/days;
Survival (%) = 100 × (final number of shrimp)/(initial number of shrimp);
Feed efficiency (FE) = weight gain (g, wet weight)/feed consumed (g, dry weight);
Protein efficiency ratio (PER) = weight gain (g, wet weight)/total protein intake (g, dry weight);
Hepatosomatic index (HSI, %) = 100 × (hepatopancreas wet weight)/(body weight);

Table 3
Growth performance, feed utilization and morphological index of juvenile Pacific white shrimp (*Litopenaeus vannamei*) fed different dietary dosage forms of copper.

Parameter	Dietary dosage forms of copper		
	I-Cu	M-Cu	Availa-Cu
IBW[a] (g)	1.87 ± 0.01	1.87 ± 0.03	1.83 ± 0.04
FBW[b] (g)	12.55 ± 0.17[a]	13.26 ± 0.16[b]	12.61 ± 0.15[a]
PWG[c] (%)	570.22 ± 8.12[a]	609.74 ± 11.87[b]	590.57 ± 12.86[ab]
SGR[d] (% d^{-1})	3.34 ± 0.02[a]	3.44 ± 0.03[b]	3.39 ± 0.03[ab]
Survival (%)	80.00 ± 2.36	80.00 ± 1.36	78.34 ± 0.96
FE[e]	0.54 ± 0.02	0.55 ± 0.01	0.51 ± 0.00
PER[f]	1.12 ± 0.03	1.16 ± 0.03	1.07 ± 0.01
HSI[g] (%)	4.75 ± 0.03[b]	4.34 ± 0.02[a]	4.50 ± 0.14[ab]
CF[h] (g cm^{-3})	0.56 ± 0.01	0.56 ± 0.00	0.56 ± 0.00

Values are presented as the means ± SEM. (n = 4). Values in the same line with different superscripts are significantly different ($P < 0.05$).
[a] IBW, initial body weight.
[b] FBW, final body weight.
[c] PWG, percent weight gain.
[d] SGR, specific growth rate.
[e] FE, feed efficiency.
[f] PER, protein efficiency ratio.
[g] HSI, hepatosomatic index.
[h] CF, condition factor.

Condition factor (CF, g cm^{-3}) = 100 × body weight (g)/body $length^3$ (cm^3).

2.9. Statistical analysis

The results are presented as the means ± SEM of four replicates (n = 4). One-way analysis of variance (ANOVA) was used to test the main effect of dietary manipulation. Firstly, all the data were checked for normal distribution and homogeneity of variance. Then, the group means could be further compared using Tukey's multiple range test. All statistical analyses were performed using SPSS 22.0 (SPSS, Chicago, USA).

3. Results

3.1. Growth performance, feed utilization and morphological index

The results of the growth performance, feed utilization and morphological index of the juvenile Pacific white shrimp fed diets supplemented with different dosage forms of copper are presented in Table 3. Dietary dosage forms of copper supplementation significantly affected growth performance and morphological index ($P < 0.05$). Shrimp fed M-Cu diet had significantly higher percent weight gain (PWG) and specific growth rate (SGR) than that fed I-Cu diet ($P < 0.05$). While there were no significant differences in PWG and SGR between M-Cu diet group and Availa-Cu diet group ($P > 0.05$). Hepatosomatic index of the shrimp fed diet containing M-Cu was significantly lower than that fed diet supplemented with I-Cu ($P < 0.05$), but no statistical differences were found between M-Cu diet group and Availa-Cu diet group. However, survival, feed efficiency (FE), protein efficiency ratio (PER) and condition factor (CF) were not effected by different dietary dosage forms of copper ($P > 0.05$).

3.2. Hematological and biochemical parameters

The hematological and biochemical parameters of shrimp fed diets containing different dosage forms of copper are shown in Table 4. TP, TAG, CHOL and GLU in hemolymph were significantly affected by different dietary dosage forms of copper supplementation ($P < 0.05$). Shrimp fed M-Cu diet had significantly higher total protein (TP)

Table 4
Hematological and biochemical parameters of juvenile Pacific white shrimp (*Litopenaeus vannamei*) fed different dietary dosage forms of copper.

Parameter	Dietary dosage forms of copper		
	I-Cu	M-Cu	Availa-Cu
TP[a] (g L^{-1})	59.08 ± 4.84[a]	84.59 ± 6.74[b]	54.44 ± 2.50[a]
TAG[b] (mmol L^{-1})	0.72 ± 0.03[b]	0.57 ± 0.04[a]	0.68 ± 0.06[ab]
CHOL[c] (mmol L^{-1})	0.49 ± 0.04[b]	0.27 ± 0.02[a]	0.44 ± 0.03[b]
GLU[d] (mmol L^{-1})	0.28 ± 0.04[a]	1.39 ± 0.13[b]	1.05 ± 0.17[b]

Values are presented as the means ± SEM. (n = 4). Values in the same line with different superscripts are significantly different ($P < 0.05$).
[a] TP, total protein.
[b] TAG, triacylglycerol.
[c] CHOL, cholesterol.
[d] GLU, glucose.

concentration in hemolymph than that fed other diets ($P < 0.05$). Triacylglycerol (TAG) contents of hemolymph in M-Cu diet group were significantly lower than that in I-Cu diet group ($P < 0.05$). While, no differences were observed in TAG concentrations of hemolymph between M-Cu diet group and Availa-Cu diet group ($P > 0.05$). Moreover, shrimp fed M-Cu diet had significantly lower cholesterol (CHOL) concentrations in hemolymph than that fed other diets. Glucose (GLU) concentrations in hemolymph of shrimp fed M-Cu diet and Availa-Cu diet were significantly higher than that fed I-Cu diet ($P < 0.05$).

3.3. Hemolymph and hepatopancreas enzyme activities

The results of antioxidant and immune-related enzyme activities in hemolymph are presented in Fig. 1. The PO, ALP and ACP activities in hemolymph were significantly affected by different dietary dosage forms of copper ($P < 0.05$), but the activities of Cu/Zn SOD, CP and LZM were not significantly different among all treatments ($P > 0.05$). Shrimp fed M-Cu diet and Availa-Cu diet had significantly higher PO activity in hemolymph than that fed I-Cu diet ($P < 0.05$). The activity of ALP in hemolymph of shrimp fed M-Cu diet was significantly higher than that fed other diets ($P < 0.05$). Moreover, shrimp fed M-Cu diet had significantly higher ACP activity in hemolymph than that fed I-Cu diet ($P < 0.05$). While there were no statistical differences between M-Cu diet group and Availa-Cu diet group ($P > 0.05$).

The activities of Cu/Zn SOD, ALP, ACP and LZM in hepatopancreas of shrimp are shown in Fig. 2. Cu/Zn SOD, ALP, ACP and LZM activities in hepatopancreas of juvenile swimming crab were significantly affected by different dietary dosage forms of copper ($P < 0.05$). Shrimp fed M-Cu diet had significantly higher Cu/Zn SOD, ALP and ACP activities in hepatopancreas than that fed I-Cu diet, with no significant differences between M-Cu diet group and Availa-Cu diet group ($P > 0.05$). While, the highest value of LZM activities in hepatopancreas occurred in Availa-Cu diet group, followed by M-Cu diet group.

3.4. mRNA expression of antioxidant and immune-related genes

The expression of genes related to antioxidant and immunity including *Cu/Zn sod*, *alp*, *acp* and *lzm in* hepatopancreas of Pacific white shrimp juveniles are shown in Fig. 3. The expression levels of *Cu/Zn sod*, *alp*, *acp* and *lzm* in hepatopancreas were significantly affected by different dietary dosage forms of copper ($P < 0.05$). Shrimp fed Availa-Cu diet had significantly higher expression levels of *Cu/Zn sod*, *alp* and *acp* in hepatopancreas than that fed I-Cu diet ($P < 0.05$), with no statistical differences between M-Cu diet group and Availa-Cu diet group ($P > 0.05$). Moreover, M-Cu diet group and Availa-Cu diet group showed a significantly higher expression levels of *lzm* than I-Cu diet group ($P < 0.05$). Among all treatments, I-Cu diet group had the lowest levels of gene expression related to antioxidant and immunity.

3.5. Whole body and muscle composition

The whole body and muscle compositions of juvenile shrimp fed experimental diets for 8 weeks are shown in Table 5. The dry matter, crude protein, crude lipid and ash contents in whole body, and dry matter, crude protein and ash contents in muscle were not significantly affected by different dietary dosage forms of copper ($P > 0.05$). While, shrimp fed Availa-Cu diet and M-Cu diet had significantly higher crude lipid contents in muscle than that fed I-Cu diet ($P < 0.05$).

3.6. Copper concentration in whole body and muscle

Copper concentrations in the whole body and muscle of juvenile shrimp are shown in Table 6. Shrimp fed M-Cu diet and Availa-Cu diet had higher copper concentrations in the whole body and muscle than that fed I-Cu diet. However, it did not show any statistical differences among all the treatments.

4. Discussion

Growth, the deposition of new tissue, is the most commonly used response to evaluate modifications to the dietary content of a nutrient [1]. In the present study, Pacific white shrimp juveniles fed diets containing organic copper exhibited better growth performance than that fed diet containing inorganic copper alone. In this study, PWG ranged from 570.22 ± 8.12% to 609.74 ± 11.87%, which was much less than the result from a previous study about copper sources of Pacific white shrimp (PWG ranged from 2043% to 2566%) [33]. The difference in PWG may be due to the lower initial weight of the previous research in comparison with this study (0.39 g vs 1.86 g). Although, Katya's research showed a lower average initial weight than this study (0.6 g vs 1.87 g), the growth result had a similar PWG to this study [23]. It was notable that Katya's diet formulation did not contain fish meal, while the present study had fish meal at a level of 30% (dry matter). This may partially explain the difference on results of growth, since shrimp fed

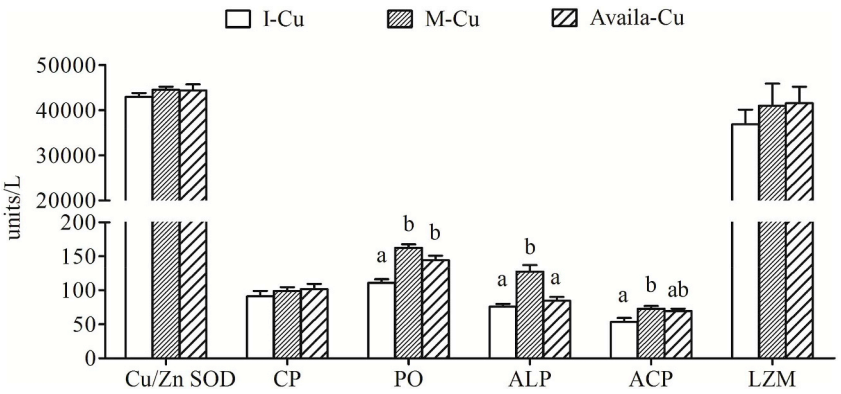

Fig. 1. Effects of different dietary dietary dosage forms of copper on antioxidant and immune enzyme activities in hemolymph of juvenile Pacific white shrimp (*Litopenaeus vannamei*). Values are means (n = 4), with their standard errors represented by vertical bars. Mean values for the same enzyme with different letters are significantly different ($P < 0.05$).

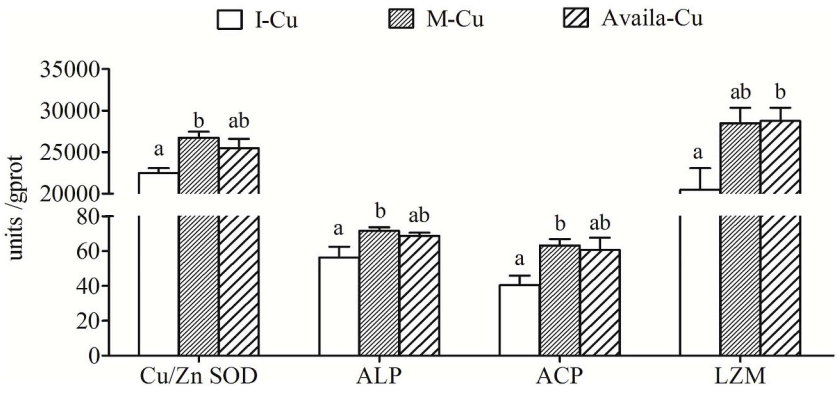

Fig. 2. Effects of different dietary dietary dosage forms of copper on antioxidant and immune enzyme activities in hepatopancreas of juvenile Pacific white shrimp (*Litopenaeus vannamei*). Values are means (n = 4), with their standard errors represented by vertical bars. Mean values for the same enzyme with different letters are significantly different ($P < 0.05$).

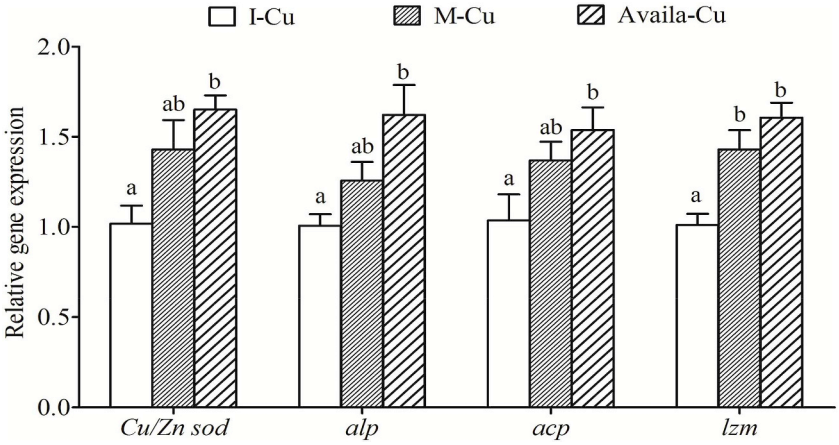

Fig. 3. Effects of different dietary dietary dosage forms of copper on relative mRNA expression levels of *Cu/Zn sod*, *alp*, *acp* and *lzm* in hepatopancreas of juvenile Pacific white shrimp (*Litopenaeus vannamei*). The gene expression of I-Cu diet group was set at 1. Values are means (n = 4), with their standard errors represented by vertical bars. Mean values for the same gene with unlike letters were significantly different ($P < 0.05$). *Cu/Zn sod*, Cu/Zn superoxide dismutase; *alp*, alkaline phosphatase; *acp*, acid phosphatase; *lzm*, lysozyme.

Table 5
The proximate composition (% wet weight) of whole body and muscle of juvenile Pacific white shrimp (*Litopenaeus vannamei*) fed different dietary dosage forms of copper.

Parameter	Dietary dosage forms of copper		
	I-Cu	M-Cu	Availa-Cu
Whole body (%)			
Dry matter	25.85 ± 0.52	25.70 ± 0.21	24.68 ± 0.38
Protein	19.11 ± 0.25	19.30 ± 0.22	18.74 ± 0.30
Lipid	0.96 ± 0.04	0.92 ± 0.01	0.91 ± 0.02
Ash	3.04 ± 0.05	3.04 ± 0.08	3.00 ± 0.10
Muscle (%)			
Dry matter	25.33 ± 0.19	25.99 ± 0.42	24.67 ± 0.73
Protein	21.23 ± 0.16	21.52 ± 0.36	21.09 ± 0.62
Lipid	0.45 ± 0.01[a]	0.51 ± 0.01[b]	0.57 ± 0.02[c]
Ash	1.62 ± 0.01	1.63 ± 0.02	1.60 ± 0.05

Values are presented as the means ± SEM. (n = 4). Values in the same line with different superscripts are significantly different ($P < 0.05$).

Table 6
The whole-body and muscle copper concentration (mg kg^{-1} dry matter) of juvenile Pacific white shrimp (*Litopenaeus vannamei*) fed different dietary dosage forms of copper.

Parameter	Dietary dosage forms of copper		
	I-Cu	M-Cu	Availa-Cu
Whole-body Cu concentration	12.36 ± 0.98	12.87 ± 0.79	12.61 ± 1.14
Muscle Cu concentration	14.31 ± 1.05	14.65 ± 0.69	15.04 ± 0.78

Values are presented as the means ± SEM. (n = 4). Values in the same line with different superscripts are significantly different ($P < 0.05$).

non-fish meal diet can not obtain optimum growth effect. Another concern was the level of different dietary dosage forms of copper. There were two studies on the lower requirement for chelated compared to inorganic copper to promote the similar growth performance in fish and crustaceans [32,33]. Shrimp fed diet containing chelated copper at a level of 15 mg/kg had higher PWG and SGR than that fed diet supplemented with 30 mg/kg copper from the inorganic source. In the present study, Cu in the form of organic showed more effective growth promotion than in $CuSO_4$ due to the high biological availability of chelated copper. The main factors influencing bioavailability included the supplemented levels and chemical sources and forms of the trace mineral, nutrient interactions which may be either synergistic or antagonistic, physiological and pathological conditions of the aquatic animal, waterborne mineral concentration [3]. Among these factors, chemical sources and forms are the most critical [1,3]. It was reported that the insoluble and non-absorbable substances which are thereby formed in the gastrointestinal tract of the animal may prevent the uptake, transport and metabolism of the mineral element [5]. Furthermore, worthy to note that, copper in the intestine was absorbed by epithelial cells in the form of Cu ions which could form insoluble or indigestible complexes with endogenous inhibitors (tricalcium phosphate, phytic acid and fiber) to prevent Cu absorption [53]. If an element is chelated by a compound that will release it in ionic form at the site of absorption or will be readily absorbed as the intact chelated, this form may greatly enhance the absorption of the element by preventing its conversion to insoluble chemical compounds in the intestine or by preventing its strong adsorption on insoluble colloids [26,54]. Some studies have proved that the bioavailability of organic chelated Cu is higher available than Cu sulfate in fish such as *A. gueldenstaedtii* [20], *C. auratus gibelio* [21], *E. malabaricus* [32], *I. punctatus* [36], *O. mykiss* [25,26], *P. major* [55], and crustacean, such as *L. vannamei* [23,33]. Therefore, higher bioavailability of copper amino acid complex may be due to its

higher absorption or additional biological effects compared to Cu sulfate, but the precise mechanism is not clear.

It is well known that hematological characteristics such as triglyceride, total protein, urea nitrogen and total cholesterol are important indicators of animal health [56]. There were only a few published reports about the effects of dietary trace minerals on hematological characteristics in aquaculture species [23,57]. Dietary copper dosage forms significantly influenced hematological characteristics of Pacific white shrimp. Similarly, Katya et al. [23] also pointed out that hematological performance was affected by the levels and sources of trace mineral premixes. Pacific white shrimp fed diet containing organic copper had a higher level of GLU content and a lower level of CHOL than that fed inorganic copper in these two studies. However, the specific reason was not known.

The antioxidant and immune indicators are always used to evaluate the long-term Cu nutritional status of animals [58]. Since crustaceans have no adaptive immunity memory cells to produce immunoglobulins, they mainly depend on innate immune systems for host defense [59]. In fact, some immune and physiological parameters such as total hemocyte counts, phenoloxidase activity, free radicals and superoxide dismutase activity have been used to as indicators for crustacean health and stress tolerance [60]. Copper is involved in the antioxidant system as it is an integral part of the enzymes Cu/Zn superoxide dismutase (Cu/Zn SOD) and ceruloplasmin molecules [23]. To protect against oxidative damage, organisms have developed a variety of antioxidant defenses that include metal sequestering proteins and specialized antioxidant enzymes. The most important antioxidant defence mechanism is the antioxidant enzyme of SOD which catalyze the dismutation of superoxide radicals (O_2^-) into O_2 and hydrogen peroxide (H_2O_2) as the first line of enzymatic antioxidant defense [61–63]. It has two forms according to the type of metal associated in the enzyme, i.e., copper-zinc (Cu/Zn) SOD and manganese (Mn) SOD [13]. As a copper-dependent enzyme, the Cu/Zn SOD has been shown to be a good indicator for the copper nutrition status in aquatic animals such as *E. malabaricus* [13,32], *E. sinensis* [31], *H. discus hannai* [64] and *I. punctatus* [65]. Some studies showed that minerals were mainly stored in bone and liver [66–68]. Therefore, in the present study, it is speculated that the reason for the higher Cu/Zn SOD activity in hepatopancreas of shrimp fed diet supplemented with copper amino acid complex might be due to the higher effective copper concentration in hepatopancreas. Recent studies suggested that the specific enzymatic activity of ceruloplasmin is a more sensitive indicator of Cu status than either serum Cu and ceruloplasmin or erythrocyte superoxide dismutase [69]. And ceruloplasmin is the major Cu transport protein to deliver Cu to cells, and it is also an enzyme, which possess oxidase activity [13]. In the present study, we did not find the statistic differences in Cu/Zn SOD and ceruloplasmin activities in hemolymph among all the treatments, but Cu/Zn SOD and ceruloplasmin activities in hepatopancreas were influenced by dietary copper dosage forms. This may be due to the higher sensitivity to copper of hepatopancreas, and more work were needed to explain the result. As a kind of immune system in crustaceans [70,71], the proPO system is the most important owing to the phenoloxidase (PO) which is a sensitive indicator to reflect the immune status of invertebrates, it is also a defense enzyme, and serves as a non-self recognition system in host defense reactions [72,73]. In invertebrates, PO existed in haemolymph as an inactive proenzyme, prophenoloxidase, which can be transformed to the active form (phenoloxidase) by several microbial polysaccharides, environmental factors and metal ions [74–76]. The results of present study indicated that shrimp fed diets supplemented with organic copper had higher PO activity in hemolymph than that fed copper sulfate. This may be due to the activation of Cu ions. However, there was little information available on the effect of copper sources on the prophenoloxidase system in shrimp. More workings are required to clarify the role of copper amino acid complex in the prophenoloxidase system of crustacea. It was reported that ALP and ACP participate in degradation of foreign proteins, carbohydrates and lipids as well as catalyse the hydrolysis of various phosphate-containing compounds and act as transphosphorylases at alkaline and acid, respectively [77–79]. Acid phosphatases act as marker enzymes for the detection of lysosomes in cell fractions and can be altered by the presence of xenobiotics [80], whilst alkaline phosphatases are intrinsic plasma membrane enzymes found on the membranes of almost all animal cells. Both enzymatic activities have been studied in several organisms [81]. All highly purified alkaline phosphatases have proved to be Zn (II) metalloenzymes, and the role of this metal as activator was related to the saturation of Zn (II) binding sites [82]. An interaction between dietary copper and zinc has been observed in some animals [83,84]. An elevated level of one component reduced the bioavailability of the other; however, such interactions have not been well established in fish and crustacea [85–87]. It is speculated that copper amino acid complex could increase the bioavailability of zinc. However, the specific mechanism was not clear, and it required more studies to illuminate the result. Lysozyme was also an important mediator of the innate immune response, attacking the peptidoglycan layer of bacterial cell walls by splitting the β-(1,4)-glycosidic bonds between the N-acetylmuramic acid and N-acetylglucosamine, resulting in the lysis of bacterial cells [88–90]. Lysozyme was abundant in various biological fluids and tissues, including skin, saliva, tears, liver blood and lymphatic tissues of humans and other animals [91,92]. Apart from the antibacterial activity, lysozyme had many other functions, including antiviral, immune modulation, anti-inflammatory and antitumor [93–96]. Some studies have reported that excessive copper could lead to irreversible inactivation of lysozyme [97]. Thus, the direct interaction between copper and lysozyme might induce the conformational and functional alterations of lysozyme [98]. However, due to the lack of supporting information on the effects copper amino acid complex on the lysozyme of shrimp, it was difficult to conclusively explain why increasingly trend was observed in diet supplemented with chelated copper in the present study. Overall, Cu performs several functions in the immune system through the specific mechanism of action which is not clear.

In the present study, there is a positive correlation between mRNA expression levels and enzymatic activities of Cu/Zn SOD, ALP, ACP and LZM in hepatopancreas, these results may suggest these enzymes may be regulated at a transcriptional level to a certain extent. Shrimp fed diet supplemented with chelated copper alone had the highest expression levels of Cu/Zn *sod*, *alp*, *acp* and *lzm* among all treatments. However, the activities of Cu/Zn SOD, ALP and ACP in hepatopancreas of shrimp fed diet supplemented with mixed copper were higher than that fed diet supplemented with copper amino acid complex alone. The mismatch between the gene expression and the enzyme activity may be involved in time-lag effect between transcription and translation, or RNA stability, as suggested by Zheng et al. [99,100]. Moreover, it should be noted that in the current study, we perhaps ignore the interaction between the trace minerals viz. Cu and Zn. This is because the organic minerals are generally considered less sensitive to the inhibitory action of other compounds due to different absorption pathways [32,89]. An in-depth study are needed to clarity the relationship between copper amino acid complex and innate immune response in Pacific white shrimp.

In the present study, proximate composition in whole body and muscle were not significantly influenced by dietary copper sources, except crude lipid content in muscle, which was similar to Wang's study [20]. In addition to antioxidant and immune indicators, tissue Cu deposition is also used to evaluate the long-term Cu nutritional status of animals [101]. Although, there were no significantly differences on copper concentration in whole body and muscle, the whole body and muscle of shrimp fed M-Cu and Availa-Cu had a higher Cu content than that fed I-Cu. The differences may be more and more significant if the feeding time last for longer time. And this would help us improve the further experiment.

In conclusion, the results of present study demonstrated that copper

amino acid complex could be more effective compared to inorganic sources to promote the growth performance as well as to improve the function of anti-oxidation and enhance non-specific immune system in Pacific white shrimp. Further studies and explorations concerning the relationship between growth response and dietary copper forms of Pacific white shrimp are needed, which can strengthen the exploitation of organic copper in aquatic feed and contribute to optimize aquatic feeds when considering selection of optimal copper sources.

Acknowledgments

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