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Effects of dietary cottonseed meal protein hydrolysate on growth, antioxidants and immunity of Chinese mitten crab *Eriocheir Sinensis**

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Abstract One hundred and sixty crabs (average initial weight: 51.32±0.08 g) were fed with four experimental diets containing cottonseed meal protein hydrolysate (CPH) at 0% (CPH0, control), 0.3% (CPH0.3), 0.6% (CPH0.6), and 1.2% CPH (CPH1.2). The experiment results show that no difference was observed in specific growth rate and survival rate of crabs fed with CPH diet. Moisture content of crabs fed with CPH0.6 diet was significantly reduced than that of the CPH0 group. Superoxide dismutase and catalase activities of crabs fed with CPH0.6 diet were significantly increased and the difference was not significant between the CPH0.3 and CPH0.6 groups. Malondialdehyde content of CPH0.3 group was significantly lower than that of the CPH0 group. Lysozyme, alkaline phosphatase, and acid phosphatase activities of CPH0.3 diet crabs were significantly higher than that of the CPH0 group. Glutamic-pyruvic transaminase activity of crabs fed with CPH0.3 diet was significantly decreased compared to the CPH0 group. The relative expression levels of *Toll1*, *Toll2*, *MyD88*, *LITAF*, and *ILF-2* of crabs fed with CPH0.3 diet were significantly higher than that of the CPH0 group. The expression level of *SOCS2* showed an opposite pattern. After CPH perfusion, the expression levels of *SOCS2* and *Toll1* in intestine at time 3 h and *SOCS2* in hepatopancreas at time 18 h increased significantly to the highest value. The expression level of *Toll2*, *MyD88*, *LITAF*, decreased at times 6 h, 6 h, 12 h, respectively, then increased gradually. Therefore, supplementation of dietary CPH could improve antioxidant capacity and immune function; the appropriate supplement dosage of CPH for crab could be 0.3%–0.6%. Furthermore, the short-term CPH stimulation could significantly increase or decline the expression levels of immune-related genes at different times after CPH perfusion.

Keyword: *Eriocheir sinensis*; cottonseed meal protein hydrolysate; growth; body composition; immunity; antioxidant

1 INTRODUCTION

Chinese mitten crab *Eriocheir sinensis* is one of the most economically valuable freshwater crabs in Asia (Rudnick et al., 2003). It was reported that world aquaculture output of Chinese mitten crab reached 796 622 tons in 2014 (Pauly and Zeller, 2017). However, with the development of aquaculture practice, a variety of diseases caused by viruses (Zhang and Bonami, 2012) and bacteria (Wang and Gu, 2002) may emerge and affect the production of Chinese mitten crab. When bacteria or viruses infect crabs, it would affect the health of crabs and consequently their growth, thus result in a burst of death (Fu et al., 2017). Therefore, it is important to strengthen the resistance of crabs. In the past few years, to reduce the risk of bacterial and viral infections in crabs, many researches carried out studies to enhance the antioxidant capacity and immune response of crabs, for example, supplying

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nutrient suitably of feed (Sun et al., 2013b; Wei et al., 2016), and adding small bioactive peptides (Guo et al., 2017) and immune-enhancers moderately (Sun et al., 2013a; Hong et al., 2017; Jia et al., 2017). However, most of the researchers concentrated on juvenile *Eriocheir sinensis*, and research on adult ones remains poor. As crustaceans, it is necessary to work more to enhance or regulate the immunological response of adult crab in order to avoid alteration of the immune system (Sánchez et al., 2001).

Plant protein as an ingredient of aquaculture feed is less cost compared with animal protein, especially fish meal. However, the utilization of plant proteins (e.g., cottonseed meal, rapeseed meal) is limited due to their antinutritional factors such as gossypol (Jiang et al., 2018). These antinutritional factors can cause negative effect on growth and health of aquatic animals (Figueiredo-Silva et al., 2015; Jiang et al., 2018). Plant protein hydrolysate is produced using processes such as hydrolytic enzymes (Colla et al., 2014; Muranova et al., 2017). Studies has proven that these hydrolysates are ideal nutrient sources, they could increase weight gain, improve immunity and antioxidant capability of aquatic animals when added within a suitable range (Gibbs et al., 2004; Kotzamanis et al., 2007; Möller et al., 2008; Gui et al., 2010; Yimit et al., 2012; Song et al., 2014).

A previous study by Liu (2005) suggested that cottonseed meal protein hydrolysate (CPH) had the best effect on the growth and immunity of fish compared with those hydrolysates of rapeseed meal, soybean meal, and peanut meal. Thus, we chose cottonseed meal (CM) to produce CPH. The proportions of small molecule peptide and free amino acid consisting in CPH were significantly improved compared with CM. The peptide content of CPH was 13.4% (180–1 983 Da), whereas CM was only 1.9% (180–1 983 Da) (Gui et al., 2010). CPH can improve feed ingredient palatability as an attractant of Chinese mitten crab (Cheng et al., 2019), and boost immunity of *Alogynogenetic crucian* carp (Liu, 2005); it can also improve the activities of serum lysozyme and alkaline phosphatase in the intestines of *Cyprinus carpio* var. jian (Xia et al., 2012). However, presently, there is no available information on the effects of dietary CPH supplementation on immunity of Chinese mitten crab. Thus, the main aim of our study is to explore the effects of dietary CPH at different levels on growth, antioxidant capability and immune response as well as the mRNA expression levels of immune-related genes of Chinese mitten crab.

Table 1 Formulation and proximate composition of the experimental diets

Ingredient (g/kg)	CPH0	CPH0.3	CPH0.6	CPH1.2
Fish meal	275.8	275.8	275.8	275.8
Soybean meal	223.1	223.1	223.1	223.1
CPH	0	3	6	12
α-starch	200	197	194	188
Peanut meal	145.5	145.5	145.5	145.5
Rapeseed meal	38.5	38.5	38.5	38.5
Soybean oil	14.6	14.6	14.6	14.6
Fish oil	9.7	9.7	9.7	9.7
$Ca(H_2PO_4)_2$	21.3	21.3	21.3	21.3
Attapulgite clay	9.7	9.7	9.7	9.7
Zeolite powder	9.7	9.7	9.7	9.7
Blood powder	19.4	19.4	19.4	19.4
Premix [a]	10	10	10	10
Mixture [b]	22.7	22.7	22.7	22.7
Proximate analysis (% dry weight)				
Dry matter	89.93	90.03	89.32	89.74
Crude protein	39.70	39.78	39.83	39.95
Crude lipids	5.98	5.83	5.93	6.09
Crude ash	11.87	12.02	11.95	11.81

Fish meal obtained from Tech-Bank Co., Ltd. (Ningbo, China). Soybean meal obtained from Zhengchang Feed Industry Co., Ltd. (Huaian, China). Peanut meal obtained from Liuhe Feed Industry Co., Ltd. (Qingdao, China). Rapeseed meal obtained from Zhengchang Feed Industry Co., Ltd. (Huaian, China). Premix[a] supplied the following minerals (g/kg) and vitamins (IU or mg/kg): $CuSO_4 \cdot 5H_2O$, 2 g; $FeSO_4 \cdot 7H_2O$, 25 g; $ZnSO_4 \cdot 7H_2O$, 22 g; $MnSO_4 \cdot 4H_2O$, 7 g; Na_2SeO_3, 0.04 g; KI, 0.026 g; $CoCl_2 \cdot 6H_2O$, 0.1 g; Vitamin A, 900 000 IU; Vitamin D, 200 000 IU; Vitamin E, 4 500 mg; Vitamin K3, 220 mg; Vitamin B1, 320 mg; Vitamin B2, 1 090 mg; Vitamin B5, 2 000 mg; Vitamin B6, 500 mg; Vitamin B12, 1.6 mg; Vitamin C, 10 000 mg; pantothenate, 1 000 mg; folic acid, 165 mg; choline, 60 000 mg; biotin, 100 mg; myoinositol 15 000 mg. Mixture[b] feed includes the following ingredients (%): choline chloride 4.21%; antioxidants 1.26%; mildew-proof agent 2.09%; salt 21.03%; Lvkangyuan 63.15%; biostimep 8.26%.

2 MATERIAL AND METHOD

2.1 Experimental ingredients and diets

CPH was produced by the method of Xia et al. (2012). Cottonseed meal protein was hydrolysated by AS1.398 protease in water bath (5 h, 45°C, pH 7.0). Four different CPH dosages were added to the basic diets to make the experimental diets: 0% (CPH0 group), 0.3% (CPH0.3 group), 0.6% (CPH0.6 group), and 1.2% (CPH1.2 group). Ingredients and proximate composition of the experimental diets are shown in Table 1. Fish meal, soybean meal, peanut meal, and rapeseed meal were supplemented as protein sources. Soybean oil and fish oil were supplemented as lipid

sources, and α-starch was supplemented as carbohydrate source. All diets were prepared in laboratory. Feed ingredients were ground into fine powder, and then they mixed with oil and water to be dough. After that, the dough was extruded through a single-screw meat grinder extruder. These feeds were air-dried and then were cut to appropriate sizes. Last, they were stored in sealed bags at -20°C.

2.2 Crab and experimental design

Crabs were obtained from a local feeding farm in Pukou, Jiangsu Province, China. In the beginning, crabs were fed a basal diet twice a day to adopt experimental conditions for 2 weeks. Then, 160 crabs (initial weight: 51.32±0.08 g, mean±SEM) were randomly assigned into 20 cement pools (width×length×height, 2.0 m×2.0 m×0.8 m) at a density of 8 crabs per cement pool, five replicates was contained in each group. Crabs were fed twice daily (07:00 am and 05:00 pm) for 60 days. The number of deaths crabs was recorded daily. During the experimental period, water temperature ranged from 23 to 30°C, pH fluctuated between 7.2 and 7.4, and dissolved oxygen was maintained above 5.0 mg/L.

2.3 Sample collection

At the end of the feeding trial, one crab from each cement pool was sampled at random for analysis of the whole body composition. Three crabs from per replicate were anesthetized by an MS-222 (tricaine methanesulfonate, Sigma, USA) at a concentration of 150 mg/L. After that, they were weighed, respectively. Hemolymph was obtained using syringes from each crab's second last pair of walking legs 1:1 with precooling anticoagulant solution (citrate, 26 mmol/L; glucose, 100 mmol/L; citric acid, 30 mmol/L; NaCl, 450 mmol/L; EDTA, 15 mmol/L; pH=7.2) (Li et al., 2014), with that, hemolymph was centrifuged immediately at 3 500 r/min, 4°C for 20 min. The supernatant serum was sucked up in the centrifuge tube, and then they were stored at -80°C for subsequent analysis. After that, every crab was dissected, and then obtained hepatopancreas were put in a liquid nitrogen container. Finally, they were stored at -80°C for subsequent analysis.

2.4 Perfusion experiment

Another group of crabs (mean weight 85 g) were acclimated for 2 weeks with commercial diet at 28°C in one 200-L tank with circulating water system. Crabs were starved for 24 h before perfusion.

Six crabs were randomly collected and anesthetized by an MS-222 (tricaine methanesulfonate, Sigma, USA) at a concentration of 150 mg/L, and after that, they were dissected, obtained hepatopancreas and intestine, which were used for time 0. Then, 30 crabs were divided into two groups and weighted respectively. Crabs in two groups were perfused intragastrically with either saline solution (10 mL per kg crab body weight) or CPH solution (10 mL per kg crab body weight), which a saline solution (0.9%) containing 5 mg CPH per mL. The two groups were labeled as Sham group and CPH group. After that, crabs were transferred quickly to 10 tanks (100 L each) at a density of three crabs per tank. After perfusion, crabs in each group were sampled at 3, 6, 12, 18, and 24 h, respectively. One tank was used for each sampling time so as to minimize the stress during sampling. Hepatopancreas and intestine were sampled and then were kept at -80°C for subsequent analysis of gene expression.

2.5 Parameters measurement and methods

2.5.1 Crabs growth performance

The body weight of each crab was determined at the start and end of the feeding experiment. In addition, the specific growth rate (SGR) and survival rate (SR) were determined using the following formula:

$$\text{Specific growth rate (SGR, \%/d)} = 100 \times (\ln W_f - \ln W_i)/t,$$

Survival rate (SR, %)=(initial crab number−final crab number)×100/initial crab number,

where W_f represented final body weight (g), W_i represented initial body weight (g), t represented the total experimental days.

2.5.2 Proximate analysis of the experimental diets and body composition

The diets and the crabs were analyzed to determine their proximate composition according to the procedures detailed by the AOAC (1984). The moisture contents of diets and crabs were estimated by using drying oven to a constant weight; ash was determined by combustion at 550°C for 4–6 h; crude protein was determined using an Auto Kjeldahl System (1030-Auto-analyzer, Tecator, Hoganas, Sweden) by using the micro-Kjeldahl method; crude lipid was determined using a Soxtec System HT (Soxtec System HT6, Tecator, Hoganas, Sweden) by solvent extraction; the energy levels of diets and crabs were determined using a Bomb Calorimeter (Parr 1281, Parr Instrument Company, Moline, IL, USA).

Table 2 Nucleotide sequences of the primers used to assay gene expression by real-time quantitative PCR

Target gene	Primer type	Sequence (5′→3′)	References
Toll1	Sense	5′-CTCCTTCACCTGCCCTAACTGCT-3′	Yu et al., 2013
	Anti-sense	5′-CTCCAGTTTGTATTGCTGTGCGAAA-3′	
Toll2	Sense	5′-CATTGATTGCTCTTACCTGAACCTA-3′	Yu et al., 2013
	Anti-sense	5′-CTGCAAGCTATCTAGGCTCGTTAAG-3′	
My D88	Sense	5′-GCAACAGGTGGACTTTGAGGAGTG-3′	Huang et al., 2014
	Anti-sense	5′-CACGGACAAACCACGACTAAACC-3′	
LITAF	Sense	5′-CAGGAGTAGTGTCGGGATTTGC-3′	Li et al., 2014
	Anti-sense	5′-AGTTGTTGGAGCAGCACCTTG-3′	
ILF-2	Sense	5′-CCGATGGAGACGACCGATAAC-3′	Yang et al., 2011
	Anti-sense	5′-GCACTGTGTGGCACTACTTGC-3′	
SOCS2	Sense	5′-CACAGCCAAAGACACACGCCAGAGTTCA-3′	Zhang et al., 2010
	Anti-sense	5′-AATCTTGGACTGACTGGGCTGACCTCTG-3′	
β- Actin	Sense	5′-GCATCCACGAGACCACTTACA-3′	Jia et al., 2017
	Anti-sense	5′-CTCCTGCTTGCTGATCCACATC-3′	

2.5.3 Analysis of antioxidant capabilities

Hemolymph and hepatopancreas were used to estimate the antioxidant capability of crabs. The hepatopancreas was weighed accurately and homogenized in an ice bath with nine volumes (v/w) of chilled saline in a tissue homogenizer, and then the extract was centrifuged at 9 000 r/min at 4°C for 15 min. Finally, all the supernatant was then stored at -20°C for subsequent analysis.

Total superoxide dismutase (*t*-SOD) activity in hemolymph was detected according to the method described by Wang and Chen (2005). Superoxide dismutase (SOD) activity in hepatopancreas was detected using the Ransod Kit (Randox, Crumlin, UK) by the ability of inhibiting superoxide radical-dependent reactions. Catalase (CAT) activity was measured according the method described by Lygren et al. (1999). Malondialdehyde (MDA) content was estimated using the thiobarbituric acid test (Satoh, 1978).

2.5.4 Immune parameters in hemolymph

Hemolymph was used to measure the activities of lysozyme (LZM), acid phosphatase (ACP) and alkaline phosphatase (ALP). The activity of LZM was assayed according to LZM kit protocol at 530 nm according to Yuan et al. (2007). The activities of ACP and ALP were measured by the disodium phenyl phosphate hydrate method (Chen et al., 2016).

2.5.5 GPT and GOT activities in hemolymph

The activities of glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) in hemolymph and their standard curves were measured by the spectrophotometer at 520 nm, then the activities of GPT and GOT were calculated according to the standard curves (Bergmeyer et al., 1978).

2.5.6 Analysis of gene expression

Total RNA was extracted using RNAiso Plus (TaKaRa, Dalian, China). The total RNA purity was detected using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The RNA samples were used for the subsequent analysis only when the A260/A280 ratio between 1.8 and 2.0. The reverse transcription-PCR reaction of PrimeScript™ RT Master Mix (TaKaRa, Dalian, China) was: 15 min at 37°C, 5 s at 85°C, and after that, 4°C thereafter. The obtained cDNA was kept at -20°C.

Real-time quantitative PCR (RT-qPCR) primers for immune-related genes are presented in Table 2. RT-qPCR was performed on a real-time PCR detection system (ABI USA-7500) using a SYBR Premix Ex Taq II kit (TaKaRa, Dalian, China). The RT-qPCR reactions were performed in a final 20-μL volume, including 10 μL SYBR Premix Ex TaqII (2x), 0.4 μL primer (10 μm), 0.4 μL ROX, 6.8 μL DEPC water, as well as 2 μL of cDNA template. The reactions were initially denatured at 95°C for 10 min and then 40

cycles at 95°C for 15 s, followed by annealing at 60°C for 34 s. The relative mRNA levels were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), then, β-Actin was acted as the endogenous control to normalize the mRNA expression of the immune-related genes.

2.6 Statistical analysis

The data on feeding trial was subjected to One-way analysis of variance (One-way ANOVA) followed by Turkey's test using the SPSS version 22.0 software (SPSS Inc., Chicago, IL, USA). The data regarding gene expression levels in the CPH perfusion and Sham perfusion were analyzed by Two-way ANOVA for significant differences among treatment means based on sampling time, perfusion type and their interaction. If the interaction were significant (P<0.05) differences, each factor was further analyzed separately by One-way ANOVA. Specifically, data among the different treatments at each sampling time was analyzed by One-way ANOVA. Data among different sampling time within each treatment was analyzed by t-test. The data are presented as mean±S.E.M.

Table 3 Effects of dietary CPH on growth performance of crabs

Diets	IW (g)	FW (g)	SGR (%, /d)	SR (%)
CPH0	51.24±0.12	103.72±2.15	1.26±0.04	87.50±3.95
CPH0.3	51.42±0.24	102.40±2.97	1.23±0.05	92.50±3.06
CPH0.6	51.20±0.11	106.82±1.09	1.31±0.02	90.00±2.50
CPH1.2	51.43±0.15	101.17±1.67	1.21±0.03	85.00±2.50

Values are means±S.E.M of five replicates. Means in the same column with different superscripts are significantly different (P<0.05).

Table 4 Effects of dietary CPH on body composition and energy level (wet weight) of crabs

Diet	Moisture (%)	Crude protein (%)	Crude lipid (%)	Crude ash (%)	Energy (KJ/g)
CPH0	73.67±0.47^{a}	12.17±0.09	3.28±0.25	8.41±0.63	4.33±0.26b
CPH0.3	71.82±0.52^{ab}	11.79±0.34	3.23±0.54	9.39±0.50	5.32±0.28a
CPH0.6	70.94±0.38^{b}	12.60±0.40	3.15±0.35	9.34±0.51	4.88±0.16ab
CPH1.2	71.72±0.86^{ab}	12.23±0.87	2.91±0.29	8.93±0.81	4.44±0.20ab

Values are means±S.E.M of five replicates. Means in the same column with different superscripts are significantly different (P<0.05).

3 RESULT

3.1 Growth performance

As can be seen from Table 3, no difference (P>0.05) were seen in FW, SGR and SR in crabs fed with CPH diet compared to the CPH0 group.

3.2 Whole body composition

As shown in Table 4, crude protein, lipid, and ash content all shows no differences (P>0.05) among all the groups. However, moisture content was significantly reduced (P<0.05) in crabs fed with CPH0.6 diet compared to the CPH0 group, but no difference (P>0.05) was observed among other supplemented diet. Similarly, energy content was significantly higher (P<0.05) in crabs fed with CPH0.3 diet compared to the CPH0 group, but no noticeable difference (P>0.05) was observed among others.

3.3 The activities of SOD, CAT and the content of MDA of Chinese mitten crab

As shown in Table 5, SOD and CAT activities in hemolymph and hemolymph of crabs fed with CPH0.6 diet were significantly (P<0.05) higher than that of the CPH0 group whereas the CPH0.3 and CPH0.6 groups was not different significantly (P>0.05). MDA content in hemolymph of crabs fed with CPH0.3 diet was significantly lower than those of the other groups in terms of dietary CPH levels (P<0.05). MDA content in hepatopancreas of crabs fed with CPH0.3 diet was significantly (P<0.05) lower than that of the CPH0 group whereas the

Table 5 Effects of dietary CPH on CAT and SOD activities and MDA content of crabs

Diet	Hemolymph			Hepatopancreas		
	t-SOD (U/mL)	CAT (U/mL)	MDA (nmol/mL)	SOD (U/mg prot)	CAT (U/g prot)	MDA (nmol/mg prot)
CPH0	307.91±14.61^{b}	10.64±0.56^{b}	4.77±0.11^{a}	59.02±3.22^{b}	4.58±0.15^{bc}	17.60±0.63^{a}
CPH0.3	333.72±15.39^{ab}	11.27±0.27^{ab}	2.15±0.10^{c}	65.17±1.52^{ab}	4.44±0.23^{ab}	6.03±0.39^{c}
CPH0.6	373.89±18.43^{a}	12.81±0.30^{a}	3.07±0.18^{b}	70.44±3.44^{a}	5.25±0.18^{a}	6.91±0.31^{bc}
CPH1.2	332.52±16.72^{ab}	12.58±0.39^{a}	5.02±0.38^{a}	59.41±1.89^{b}	3.73±0.16^{b}	8.04±0.36^{b}

Values are means±S.E.M of five replicates. Means in the same column with different superscripts are significantly different (P<0.05).

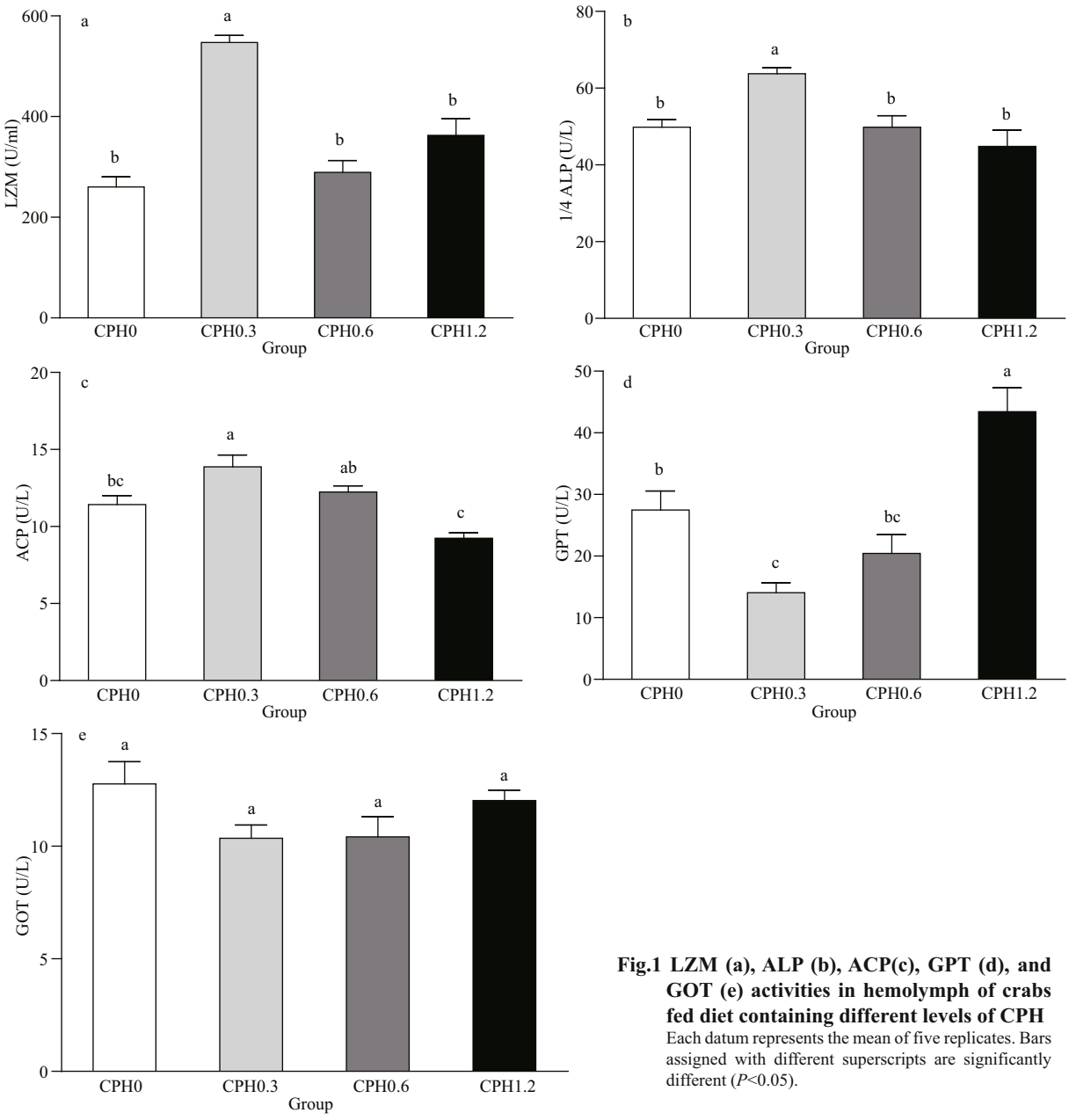

Fig.1 LZM (a), ALP (b), ACP(c), GPT (d), and GOT (e) activities in hemolymph of crabs fed diet containing different levels of CPH
Each datum represents the mean of five replicates. Bars assigned with different superscripts are significantly different (P<0.05).

CPH0.3 and CPH0.6 groups was not different significantly (P>0.05).

3.4 The activities of LZM, ALP, ACP, GPT and GOT in hemolymph of Chinese mitten crab

As shown in Fig.1: LZM activity in hemolymph of crabs fed with CPH0.3 diet was significantly (P<0.05) higher than those of the other groups in terms of dietary CPH levels; ALP had the same tendency with LZM, and it was significantly (P<0.05) higher than those of the other groups in terms of dietary CPH levels; ACP activity in hemolymph of crabs fed with CPH0.3 diet was significantly (P<0.05) higher than the CPH0 group whereas the CPH0.3 and CPH0.6 groups was not different significantly (P>0.05). GPT activity in hemolymph of crabs fed with CPH0.3 diet was significantly (P<0.05) lower than that of the CPH0 group, although there was no significant difference between CPH0.3 and CPH0.6 groups (P>0.05). There was no significant difference in GOT activity among all groups (P>0.05).

3.5 The relative expression levels of *Toll1*, *Toll2*, *MyD88*, *LITAF*, *ILF-2*, and *SOCS2*

As shown in Fig.2, the relative expression level of *Toll1* of crabs fed with CPH0.3 diet was significantly

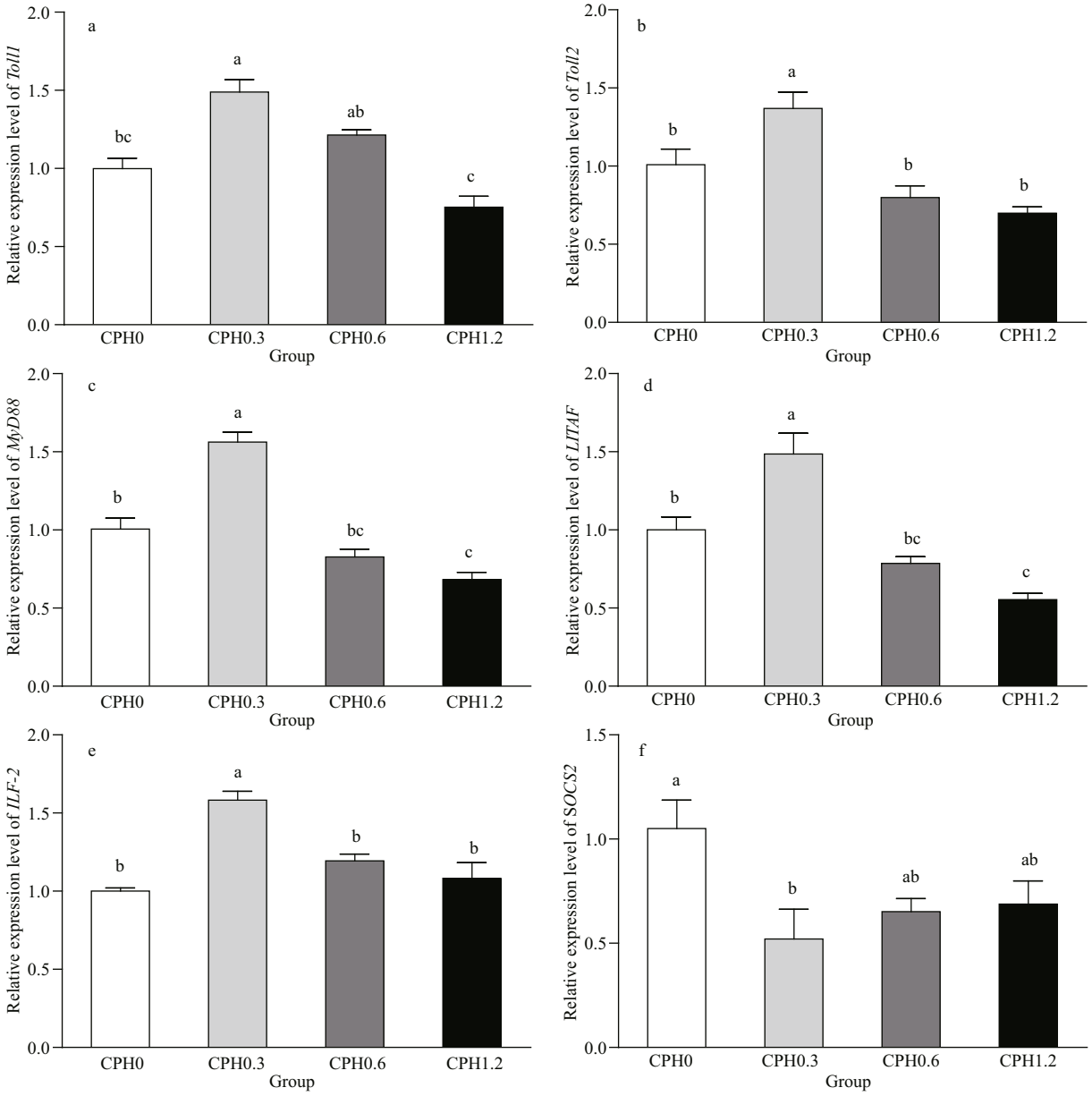

Fig.2 Relative expression levels of *Toll1* (a), *Toll2* (b), *MyD88* (c), *LITAF* (d), *ILF-2* (e), and *SOCS2* (f) in hepatopancreas of crabs fed diet containing different levels of CPH

Each datum represents the mean of five replicates. Bars assigned with different superscripts are significantly different (P<0.05).

(P<0.05) higher than that of the CPH0 group and CPH1.2 group. The relative expression level of *Toll2* of crabs fed with CPH0.3 diet was significantly (P<0.05) higher than those of the other groups in terms of dietary CPH levels. The relative expression levels of *MyD88* and *LITAF* of crabs fed with CPH0.3 diet were significantly (P<0.05) higher than those of the other groups in terms of dietary CPH levels. Similarly, the relative expression level of *ILF-2* of crabs fed with CPH0.3 diet was significantly (P<0.05) higher than those of the other groups in terms of dietary CPH levels. The relative expression levels of *SOCS2* of crabs fed with CPH0.3 diet was significantly (P<0.05) lower than that of the CPH0 group.

3.6 The relative expression levels of *Toll1*, *Toll2*, *MyD88*, *LITAF*, *ILF-2*, and *SOCS2* after CPH perfusion

As shown in Figs.3 & 4, after CPH perfusion, the relative expression levels of *Toll1*, *Toll2*, *MyD88*, *LITAF*, *ILF-2* in intestine and hepatopancreas were all significantly (P<0.01) affected by perfusion type, time and their interaction; *SOCS2* expression level was significantly (P<0.001) affected by time and the

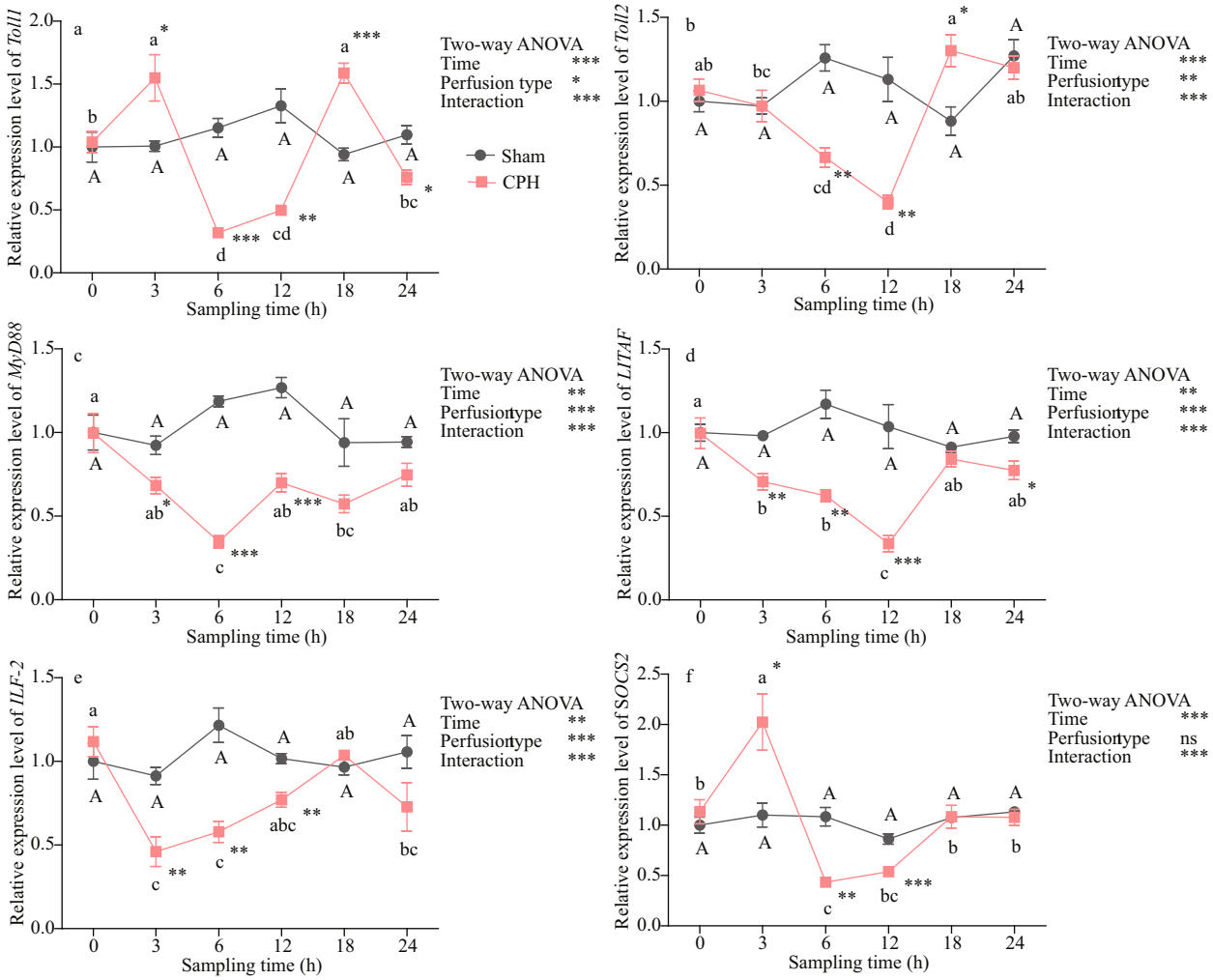

Fig.3 Relative expressions of *Toll1* (a), *Toll2* (b), *MyD88* (c), *LITAF* (d), *ILF-2* (e) and *SOCS2* (f) in intestine of crabs after CPH perfusion

Data are referred to the value (relative units, RU) obtained in the corresponding tissue of the sham treatment at 0 h. Each data represents the mean of three replicates. Significant differences (P<0.05) among sampling times within each treatment are indicated by different letters (lower-case letters for CPH, capital letters for sham). * represents a significant difference (P<0.05) between the two groups at each sampling time. *P<0.05, **P<0.01, ***P<0.001; ns: not significant.

interaction of time and perfusion type. The CPH treatment resulted in the expression levels of *Toll1* at times 3 h, 18 h and *Toll2* at times 18 h, 24 h in intestine significantly (P<0.05) increase after perfusion. However, they declined significantly (P<0.01) at other times. After the CPH treatment, *Toll1* and *Toll2* expression in hepatopancreas and *MyD88* expression in intestine and hepatopancreas decreased significantly (P<0.05) with the lowest value at 6 h. *LITAF* expression in intestine and hepatopancreas decreased significantly (P<0.05) with the lowest value at 12 h. The expression level of *ILF-2* in intestine at time 3 h and in hepatopancreas at times 6 h decreased significantly (P<0.05) to the lowest value. After that, they increased gradually until basal level was reached compared to Sham group. The expression level of *SOCS2* in intestine at time 3 h and in hepatopancreas at times 18 h increased significantly (P<0.05) to the highest value. The expression levels of all these genes did not difference at all times after sham treatment.

4 DISCUSSION

The growth of animal is affected by feed utilization rate and the nutrients deposition of animal is greatly influenced by the nutritional composition of feed (Sheridan and Mommsen, 1991; Duan and Plisetskaya, 1993). It is well known that crabs grow quickly only after molting (Hartnoll, 2001; He, 2005). The molting period is influenced by temperature, nutrition, and breeding environment (Chen et al., 1995; Saucedo et al., 2004; He, 2005). The above could explain why there was no significant difference in SGR and SR of

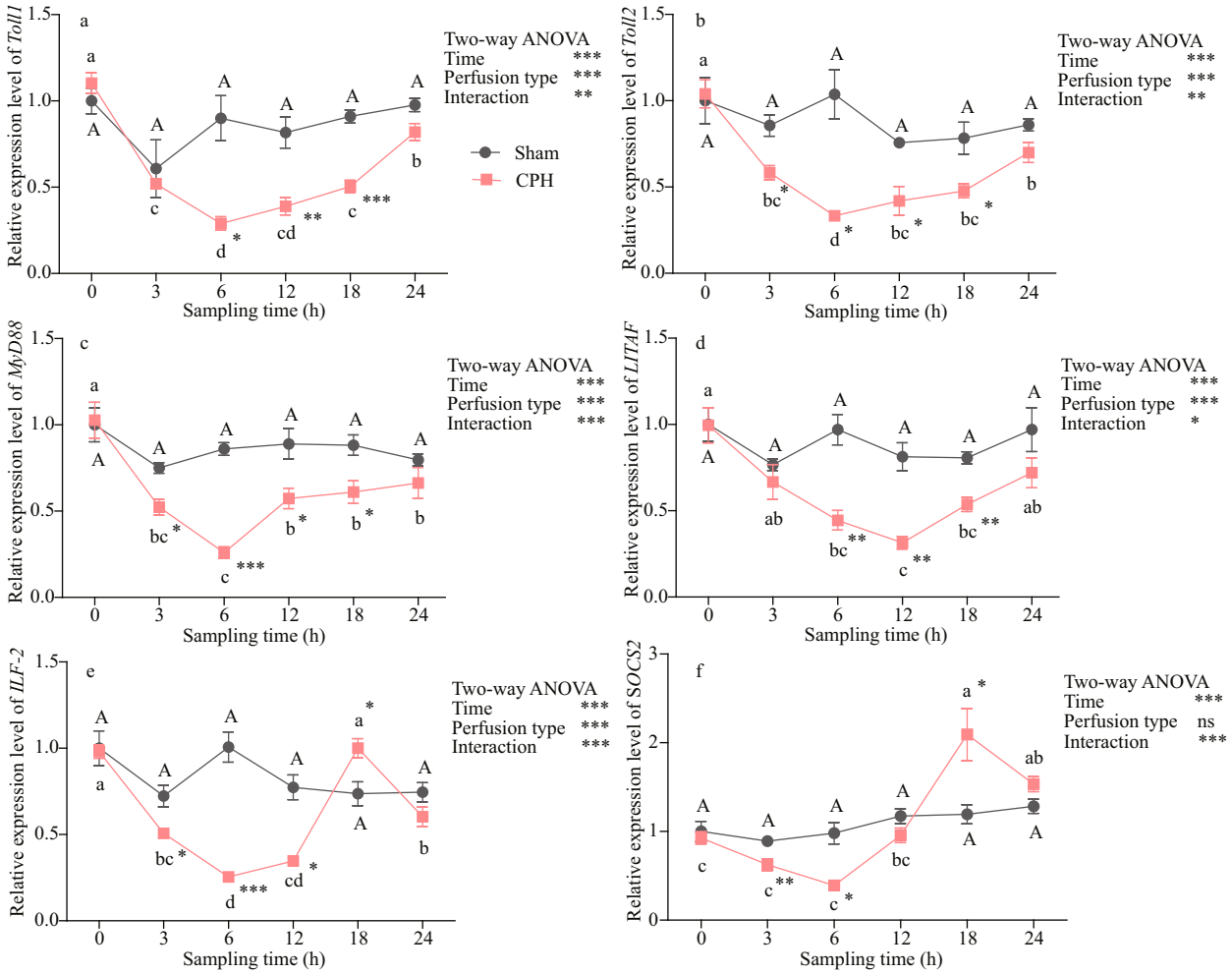

Fig.4 Relative expressions of *Toll1* (a), *Toll2* (b), *MyD88* (c), *LITAF* (d), *ILF-2* (e) and *SOCS2* (f) in hepatopancreas of crabs after CPH perfusion

Data are referred to the value (relative units, RU) obtained in the corresponding tissue of the sham treatment at 0 h. Each data represents the mean of three replicates. Significant differences ($P<0.05$) among sampling times within each treatment are indicated by different letters (lower-case letters for CPH, capital letters for sham). * represents a significant difference ($P<0.05$) between the two groups at each sampling time. *$P<0.05$, **$P<0.01$, ***$P<0.001$; ns, not significant.

crabs. However, Gui et al. (2010) found that supplement of dietary CPH could increase the body weight of crucian carp (*Carassius auratus gibelio*), it may be that crustacean growth only occurs during molting (He, 2005).

It has been reported that the differences in dietary nutrients would possibly cause the differences in body composition (Hansen et al., 2007). In the present study, dietary supplement with 0.6% CPH could decrease the moisture content of crabs, whereas the opposite was true for crude protein content, indicating that CPH may promote the accumulation of organic matter. This result was possible that the lower molecular weight, small peptides, and free amino acids in CPH could promote protein synthesis by activating TOR signaling pathway, this needs further study. Our study also found that the energy content of crabs fed CPH0.3 diet increased significantly, it might be that absorbed dietary amino acids and peptides were processed to increase energy content of total dry matters in crabs. Overall, our study suggested that CPH might have no impact on the puberty molt, but it could promote the deposition of nutrients of crabs.

The body cells produce reactive oxygen species (ROS) under normal physiological conditions (Zhang et al., 2013). However, the imbalance of generation and elimination of ROS will result in oxidative stress (Shen et al., 2010; Wu et al., 2013), then, SOD and CAT would be treated as the first line of the antioxidant defense system against ROS produced in the organism (Li et al., 2007). In the study, enhancing SOD and CAT activities and reducing MDA content were seen in crabs fed diets containing CPH, suggesting that the CPH could improve the antioxidant capacity of crabs.

Previous study has reported that small peptides could stimulate and activate the antioxidant capacity of animal body and increase the activities of antioxidant enzymes (Chen et al., 1998; Moure et al., 2006). So, the results of our study were probably related to the rich concentration of small peptides in CPH. Similarly, Wang et al. (2017) studied that fermented cottonseed meal could increase serum antioxidant enzymes activities in broilers. Fermented mushroom bran hydrolysate replacement fishmeal could significantly decrease the content of MDA in serum of the allogynogenetic crucian carp (Zhang et al., 2017). Adding soy protein hydrolysates in diets enhanced SOD activity and decreased MDA content in serum of juvenile starry flounder (Song et al., 2014). However, in the present study, high dietary CPH inclusion level might provoke oxidative stress. It may be that high level CPH could stimulate the ROS release largely resulting in the imbalance of generation and elimination. Similarly, it was reported that prawn fed with a high fermented soybean meal level diet might induce oxidative stress (Matozzo et al., 2011). Overall, our study suggested that appropriate supplement CPH could promote the antioxidant capability of crabs.

LZM is regarded as an important non-specific immune response indicator of crustaceans and has bactericidal activity in many studies (Yano et al., 1996; Fu et al., 2017). ACP and ALP also play an important role in crustacean non-specific immune defence (Matozzo et al., 2011). In the present study, the activities of LZM, ALP, and ACP of crabs in hemolymph were significantly increased when dietary supplement with 0.3% CPH. This indicated that an optimum administration of CPH might improve the immune function of crabs. It might be that the small molecule peptide consisting in CPH had an important role in boosting immunity. It was similar with our results that the appropriate amount of CPH could improve the non-specific immunity of the *Cyprinus carpio* var. jian (Xia et al., 2012). Previous similar studies showed that fermented cottonseed meal could enhance immune function of yellow-feathered broilers (Tang et al., 2012), and increase serum immunoglobulin level of broilers (Wang et al., 2017). Another similar study shown that fish fed with soy protein hydrolysates diet could increase LZM activity significantly (Song et al., 2014).

GPT and GOT can be widely found in the mitochondria of many tissues and organs, particularly in the liver cells (Zheng and Pu, 1997). In the present study, we found that the activity of GPT in the hemolymph of crabs declined as dietary CPH was 0.3%. It indicated that appropriate supplementation of CPH could protect the hepatopancreas from damage, implying that CPH had a protective mechanism to the hepatopancreas of crabs. It may be that CPH can inhibit harmful bacteria and viruses infections. Nevertheless, high concentration of CPH significantly increased the activity of GPT, it might be that the hepatic cells were injured, and then the GPT transported into blood, resulting the increase of GPT activities in hemolymph (Wang et al., 2005). The reason might be that high CPH level might be harmful to hepatic cells by enhancing amino acid metabolism and producing more metabolic wastes. A similar study result appeared in juvenile starry flounder after feeding with soy protein hydrolysates (Song et al., 2014). Therefore, our study indicated that supplementation of moderate CPH was useful to protect hepatopancreas of crabs. This may be that the CPH could enhance immune function of crabs. Moreover, the SR of crabs in different groups was consistent with these result, this further suggested that dietary CPH improved the resistance of crab by the CPH-induced immune responses.

Next, we examined the relative expressions of *Toll1*, *Toll2*, *MyD88*, *LITAF*, *ILF-2*, and *SOCS2* in the hepatopancreas of crabs. Tolls play an important role in eliminating extracellular invaders, and they can be regarded as the bridge linking the innate and adaptive immunity (Lin et al., 2012). LITAF is a key transcription factor in downstream of Toll signaling pathway, and it is contacted with the transcriptional regulation of cytokines dependent on MyD88 (Li et al., 2014). SOCS2 could be induced by bacteria challenge and played a key role in the immune defense response of crabs (Zhang et al., 2010). In the present study, we found that the relative expression levels of *Toll1* and *Toll2* were significantly increased after feeding CPH0.3 diet. It suggested that Toll1 andToll2 might be potential receptor of CPH to regulate the downstream transcription factors. It was known that Tolls might participate in antifungal and antibacterial immune responses, and it was beneficial to the ability of crabs to start a suitable immune response against different pathogen-associated molecular patterns (Yu et al., 2013). The relative expression level of *MyD88* increased significantly after feeding CPH0.3 diet. This could be conjectured that the moderate supplement of CPH could promote the activation of the downstream signal factors by MyD88 in crabs. It

was reported that MyD88 play a key role in activating the immune system of crabs to resist the bacterial infections (Huang et al., 2014). Dietary supplement with 0.3% CPH could increase the expression level of *LITAF* of crabs, the result indicated that feeding CPH0.3 diet might have a stimulating role to the downstream cytokines of Toll signaling pathway. The relative expression level of *LITAF* was consistent with *MyD88*, suggesting that the immune function of CPH on Toll signaling pathway may be shown by affecting its downstream cytokines through MyD88. The relative expression level of *ILF-2* increased when dietary CPH level was 0.3%, suggesting that ILF2 might induce all kinds of immune-related gene expression (Wang et al., 2006). However, the expression of *SOCS2* declined after feeding CPH0.3 diet. It may be that SOCS2 was only induced after bacteria challenge. These results further suggested that dietary CPH could improve the immunity of crabs.

After that, we performed a perfusion experiment to explore further the effect of short-term CPH stimulant on immune gene expression of crabs. In intestine, the highest expression level of *Toll1* and *SOCS2* were obtained at time 3 h, indicating that the immune function of Toll1 and SOCS2 were firstly activated by CPH, in hepatopancreas, the highest expression level of *SOCS2* was obtained at time 18 h, suggesting that the immune response in intestine was faster than in hepatopancreas, a probably reason is that the CPH first acted on the intestine. Moreover, the expression level of *SOCS2* was affected by time and the interaction of time and perfusion type, which suggested that SOCS2 could be induced by CPH and probably played an important role in immune defense responses of crabs. Evidence suggests that the transcripts encoding SOCS proteins in cells are usually at low or undetectable levels, but they can induced rapidly by cytokines and pathogen components widely in *M. musculus* (Zhang et al., 2010). Our results were similar to those discoveries. However, other genes expression levels declined firstly and then increased gradually, suggesting that these genes could not rapidly respond to CPH stimulation. The expression level of these genes decreased at CPH stimulation might indicate that this period belonged to the convalescence of crab after CPH stimulation. It took least 6 h for these genes to reanimate and then produced effect in innate immune responses. This is similar to the findings that the expression level of *SpToll* in hemocytes of *Scylla paramamosain* dealt with poly I:C. or *V. parahemolyticus* (Lin et al., 2012) and the expression level of *FcToll* of *Fenneropenaeus chinensis* in the early period after exposure to bacteria (Yang et al., 2008). Another reason we speculated that these genes may have additional functional roles. It was reported that certain genes were only expressed when the epithelial cells in 'mature zone' of the organ was damaged (Hauton et al., 2006). However, further studies should be performed to explore the reason at a deeper level.

5 CONCLUSION

In summary, this experiment showed that dietary supplementation of CPH has beneficial effects on the accumulation of organic matter, antioxidant capacity, and immune response that might via the activation of immune-related genes such as *Tolls* and *MyD88*; the appropriate supplement dosage of CPH for Chinese mitten crab could be 0.3%–0.6%. However, the precise mechanism requires further study.

6 DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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