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Effects of dietary supplementation with icariin on growth performance, antioxidant capacity and non-specific immunity of Chinese mitten crab (*Eriocheir sinensis*)

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ABSTRACT

We investigated the effects of icariin (ICA) on growth performance, antioxidant capacity and non-specific immunity in Chinese mitten crab (*Eriocheir sinensis*). A total of 200 healthy crabs (average weight: 33.58 ± 0.05 g) were randomly assigned to four treatments with five replicates, each with ten individuals per pool. There were four dietary treatments: the control group (fed with the basal diet), the ICA 50 group, the ICA100 group, and the ICA 200 group (fed with the basal diet supplemented with 50, 100, and 200 mg/kg ICA, respectively). These diets were provided for 8 weeks. Results indicated that ICA100 crabs had higher weight gain (WG), specific growth rate (SGR) and survival rate (SR) than the controls. Protein carbonyl content (PCC) and malondialdehyde (MDA) concentrations in the haemolymph and hepatopancreas of ICA100 crabs were significantly lower than in the control group, while the superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were significantly higher. The activities of PO, LZM, ACP and AKP were significantly enhanced with ICA supplementation at 50 and 100 mg/kg, yet decreased subsequently at 200 mg/kg. Furthermore, supplementation of 100 mg/kg ICA up-regulated the mRNA expression of prophenoloxidase (*proPO*), catalase (*CAT*), mitochondrial manganese superoxide dismutase (*mtMnSOD*), thioredoxin-1 (*Trx1*) and peroxiredoxin 6 (*Prx*6), while the mRNA expression of toll like receptors (*TLRs*), NF-κB-like transcription factor *Relish* and lipopolysaccharide-induced TNF-α factor (*LITAF*) were down-regulated in the hepatopancreas ($P < 0.05$). These findings indicate that dietary ICA supplementation at an optimum dose of 100 mg/kg may be effective in improving growth performance, antioxidant capability and non-specific immunity of Chinese mitten crab.

1. Introduction

The Chinese mitten crab, *Eriocheir sinensis*, is an important commercial aquaculture species in China owing to its desirable taste, nutritional value and high economic value [1]. Culture of *E. sinensis* has increased substantially over the past decade, with annual aquaculture production reaching 812,103 metric tons in 2016 [2]. However, under intensive aquaculture, *E. sinensis* are susceptible to environmental stressors such as hypoxia [3] and ambient nitrite [4], and increasing cases of pathogenic infections and viral infections have been reported in pond culture of *E. sinensis*, such as tremor disease [5], hepatopancreatic necrosis disease [6] and white spot syndrome [7]. Unsuitable artificial pond rearing conditions will result in low immunity and poor oxidative stress response of crabs, leading to culture failure and catastrophic economic losses. Therefore, certain antioxidants and immunopotentiators are used to enhance antioxidant capacity and activate innate immune responses to alleviate oxidative damage and prevent pathogenic infections. Many previous studies have confirmed that dietary supplementation with exogenous antioxidants and immunoactivators can successfully enhance growth performance, immunity, antioxidant status and disease resistance in aquatic animals. These agents include pigments [8,9], vitamins [10–12], minerals [13] and synthetic antioxidants [14]. Natural plant extracts are also increasing used and have become a major feed additive in aquaculture due to their high efficiency and minimal toxicity. These mainly include polysaccharides [15], saponins [16], flavonoids [17] and polyphenols [18].

Epimedium is the dried leaves of medicinal plant *Epimedium*

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grandiflorum, which is well established in traditional Chinese medicine for its efficacy with minimal side effects in clinic. Icariin is its primary natural active flavonoid glucoside [19]. Numerous studies have demonstrated that icariin possesses an extensive spectrum of pharmacological and biological activities, including effects on reproductive function in rat ovarian granulosa cells [20], osteoprotective effects in late postmenopausal women [21], neuroprotective effects in cell culture and animal models of brain disorders [22] and anti-cancer effect in hepatocellular carcinoma-initiating cells [23], and further revealed were the molecular mechanisms for the treatment of related diseases in pharmacological experiments [24–26]. The anti-inflammation [27] and antioxidant bioactivities [28] of icariin have become the focus of recent clinical studies, with some indicating that icariin has the potential to inhibit H_2O_2-induced oxidative stress. In mouse embryo neuron culture models, icariin reduced production of reactive oxygen species (ROS) and promoted the expression and activity of cellular antioxidant enzymes including catalase (CAT) and peroxiredoxin 1 (Prx1) [29]. In lung tissue and macrophages cells, icariin was found to suppress the lipopolysaccharide (LPS)-induced production of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) via deactivation of nuclear factor-κB (*NF-κB*) phosphorylation and activation of the *PI3K/Akt* pathway [30].

However, immune response patterns in invertebrates are different from vertebrates. Most crustacean species, including *E. sinensis*, lack true adaptive immunity system and depend on non-specific immunity including cellular and humoral immune responses [31]. The humoral immunity of crustaceans depends mainly on enzymes such as phenoloxidase (PO) [32], lysosome (LZM) [33], acid phosphatase (ACP) [34] and alkaline phosphatase (AKP) [35], which can be used as the specific indices for evaluating the non-specific immunity conditions of crustaceans [36]. Among these, the LZM plays an important role in eliminating microorganisms through phagocytosis in the haemocytes and hepatopancreas of crustaceans. The central enzyme PO of the prophenoloxidase-activating system (proPO-AS) is considered the most important component of the immune system in crustaceans. The cellular immunity of crustaceans depends mainly on haemocytes, which are involved in the recognition and elimination of the invading pathogens by phagocytosis, encapsulation, melanisation, nodule formation and cell agglutination [37]. With the exception of haemocytes, the fixed phagocytes and epithelial cells of the hepatopancreas play a vital role in the initiation of the immune response and the clearance of pathogens in crustaceans [38], and most antioxidant and immune-related proteins including mitochondrial manganese superoxide dismutase (*mtMnSOD*) [39], thioredoxin-1 (*Trx-1*) [40] and NF-κB-like transcription factor (*Relish*) [41] are mainly expressed in the hepatopancreas.

There has been limited study on the antioxidant and innate immunity effects of dietary icariin in Chinese mitten crab. Hence, we investigated the effects of supplementing the *E. sinensis* diet with various doses of icariin to determine an optimum concentration to improve the growth, antioxidant capacity and non-specific immunity of haemocytes and the hepatopancreas. We also examined the corresponding gene expression in the hepatopancreas with the aim of developing a new antioxidant and immunopotentiator for supporting healthy *E. sinensis* cultivation and providing a basis for its application in future production.

2. Materials and methods

2.1. Experimental diets

Icariin of at least 98% purity was purchased from Nantong Feiyu Biological Technology Co., Ltd, (Nantong, China). The icariin was supplemented in the diet at 50, 100 and 200 mg/kg, respectively, (designated as ICA 50, ICA 100 and ICA 200, respectively), and the diet without icariin (CON) is used as control diet (Table 1). All dietary dry ingredients were ground passed through a 250 μm sieve and thoroughly mixed, then added to lipid sources and manually mixed to homogeneity. Distilled water was added at 30% of the ingredient weight and mixed for another 15 min. The feed was then processed through a 2.5 mm diameter die of a single-screw meat grinder extruder, air-dried in the ventilation room for 24 h, ground and sieved to appropriate sizes (2 cm length and 2 mm diameter) for crabs, and stored at −20 °C in sealed plastic bags until use.

Table 1
Ingredients and proximal composition of the control diet.

Ingredients (% dry matter)	Concentration (% dry matter)
Fish meal	32
Soybean meal	20
Peanut meal	13
Rapeseed meal	2
Blood powder	4.5
α-Starch	16.6
Fish oil	2.4
Soybean oil	2.4
$Ca(H_2PO_4)_2$	2
Attapulgite	0.9
Zeolite	0.9
Premix[a]	1.0
Mixture[b]	2.3
Proximate analysis (% dry weight)	
Dry matter	89.13
Crude protein	41.96
Crude lipids	7.03
Ash	13.62

[a] Premix 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, 4500 mg; Vitamin K_3, 220 mg; Vitamin B_1, 320 mg; Vitamin B_2, 1090 mg; Vitamin B_5, 2000 mg; Vitamin B_6, 500 mg; Vitamin B_{12}, 1.6 mg; Vitamin C, 10,000 mg; Pantothenate, 1000 mg; Folic acid, 165 mg; Choline, 60,000 mg; Biotin, 100 mg; Myoinositol 15,000 mg.

[b] Mixture includes the following ingredients (%): choline chloride 4.75%; antioxidants 1.72%; mildew-proof agent 2.35%; salt 22.06%; Lvkangyuan 59.30% and biostimep 9.51%.

2.2. Crab management and ethics

All experimental procedures involving animals care were conducted according to the Guidance of the Care and Use of Laboratory Animals in China. This present study was approved by the Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, China) (permit number: SYXK (Su) 2011–0036). Intermolt Chinese mitten crabs were collected from a local farm in Pukou, Jiangsu Province, China. Crabs were fed a commercial diet (Haipurui Feed Co., Ltd., Jiangsu, China) three times a day and acclimated to experimental conditions for 2 weeks. A total of 200 healthy crabs (average weight: 33.58 ± 0.05 g) were randomly assigned to four treatments with five replicates, each with ten individuals per cement pool (1.0 × 1.0 × 0.8 m, L: W: H). During the experimental period, water temperature, pH and dissolved oxygen were monitored and recorded daily as 26 ± 2 °C, 8.5–8.6 and 5 mg/L, respectively.

2.3. Sample collection and procedures

At the end of 8 weeks feeding trial, crabs were deprived of food for 24 h, and a state of hypothermia anesthesia was induced by placing them on ice for 10 min. Each individual was weighed to calculate growth performance parameters before sampling. Weight gain was calculated as: (WG) = 100 × (FW-IW)/IW; Where IW represented the initial weight at the beginning of the experiment and FW represented the weight at the end of the experiment. Specific growth rate was calculated as: (SGR, %/d) = 100 × (ln FW-ln IW)/T, where IW and FW were as above and T was the number of days in the feeding period. Crab

survival rate (SR, %) = 100 × (final number)/(initial number). Following this, four crabs were selected randomly from each pool. Haemolymph was collected using sterile 1-mL syringes from each crab's third paraeiopod, mixing 1:1 with precooling anticoagulant solution (100 mmol L^{-1} glucose, 26 mmol L^{-1} citrate, 30 mmol L^{-1} citric acid, 450 mmol L^{-1} NaCl, 10 mmol L^{-1} EDTA, pH = 7.2) [49] and immediately centrifuged at 9000 r/min, 4 °C for 20 min. The supernatant was collected and stored at −20 °C prior to use in determining immune indicators and antioxidant indicators. In addition, the hepatopancreas was dissected aseptically quick-frozen in liquid nitrogen and stored at −80 °C for RNA isolation and subsequent analysis.

2.4. Measurement of antioxidant enzyme activity in the haemolymph and hepatopancreas

2.4.1. The preparation of hepatopancreas homogenate

Approximately 0.3 g hepatopancreas tissue was used to prepare the whole hepatopancreas homogenate. The minced hepatopancreas tissue was homogenised in ice-cold 0.86% stroke-physiological saline solution (w/v, 1:9) using an Ultra-Turrax homogeniser (Tekmar Co., Cincinnati, OH, USA) and centrifuged at 5000 r/min at 4 °C for 10 min to obtain the supernatant for further analysis. The total protein content of the haemolymph supernatant and hepatopancreas was measured using the Biuret method [42].

2.4.2. Protein carbonyl content

PCC was evaluated using a method based on the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) to form a 2,4-dinitrophenylhydrazone [43], which can eventually be detected and quantitated using spectrophotometry with a commercial kit purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China), according to manufacturer's instructions.

2.4.3. Malonaldehyde concentration

MDA in the haemolymph and hepatopancreas samples was estimated using thiobarbituric acid based on its reaction with malondialdehyde and other aldehydes at low pH and 95 °C for 60 min to form a complex with maximum light absorption at 534 nm [44]. MDA content is expressed as nmol/mL in the haemolymph supernatant and nmol/mg protein in the hepatopancreas.

2.4.4. Total superoxide dismutase activity

Total superoxide dismutase (t-SOD) activity in the haemolymph and hepatopancreas was measured using a simple microplate WST-1 method as described by Zhou and Prognon [45]. In brief, superoxide radical O^{2-} is produced by the xanthine-xanthine oxidase system, and both SOD and WST-1 catalyse the dismutation to hydrogen peroxide (H_2O_2) and oxygen (O_2). The presence of SOD reduces the WST-1 formazan formation, and the absorbance of formazan can be measured using a microplate reader at 450 nm. Therefore, this competing assay yields an indirect measurement of SOD activity. A WST-1 working solution, enzyme working solution and SOD standard solution were all obtained in a detection kit (Nanjing Jiancheng Bioengineering Institute, China). The activities of haemolymph t-SOD were expressed as units (U/mL), and the activities of t-SOD in the hepatopancreas were expressed as U/mg protein.

2.4.5. Glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was determined following the procedures described by Bjarte et al. [46], using H_2O_2 in the presence of glutathione (GSH). GPx activity was determined using a UV/visible-6100 Spectrophotometer (Shanghai Precision Instrument Co., Ltd., Shanghai, China) according to manufacturer's protocols from the Institute of Biological Engineering of Nanjing Jiancheng (Nanjing, China).

2.5. Haemolymph and hepatopancreas immune-related parameters

2.5.1. Haemolymph phenoloxidase activity assay

PO activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) as Ashida [47], where 10 μL 0.01 M dihydroxyphenylalanine solution, 300 μL phosphate buffer (0.1 M, pH 6.0) and 10 μL sample were added to a 96-well microplate and the absorbance was determined every 2 min at 490 nm. An increase of 0.001 every minute in the OD490 was defined as one unit of activity.

2.5.2. Haemolymph and hepatopancreas lysozyme, acid phosphatase and alkaline phosphatase activity

LZM activity was determined in the haemolymph and hepatopancreas using a turbidimetric method described as Yin et al. [48], using *micrococcus lysodeikticus* lyophilised powder as a substrate (Institute of Biological Engineering of Nanjing Jiancheng). AKP activity was determined using disodiumphenyl phosphate-4-aminoantipyrine-potassium ferricyanide and ACP was determined using disodium phenyl phosphate method both using a UV/visible-6100 Spectrophotometer (Shanghai Precision Instrument Co., Ltd., Shanghai, China) as Jia [49]. AKP and ACP analysis kits were obtained from the Institute of Biological Engineering of Nanjing Jiancheng (Nanjing, China).

2.6. Antioxidant gene expression in the hepatopancreas

Total RNA was isolated using RNAiso Plus (Takara Co. Ltd. Dalian, China) from frozen hepatopancreas samples following the manufacturer's protocol. RNA integrity was tested using 1% agarose gel electrophoresis. RNA concentration and purity were determined based on OD 260/280 readings (ratio > 1.8) using a NanoDrop ND-1000 UV Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Following this, 1 μg total RNA was immediately reverse-transcribed into cDNA using a Perfect Real Time SYBR Prime Script TM RT Reagent Kit (TaKaRa Biotechnology, Dalian, China) according to manufacturer's instructions and stored at −20 °C. Real-time quantitative PCR (RT-qPCR) was carried out using an ABI StepOnePlus TM RealTime PCR system (Applied Biosystems, Grand Island, NY) according to the manufacturer's instructions. The primer sequences for the target and

Table 2
Nucleotide sequences for real-time PCR primers.

Target gene	Forward (5′-3′)	Reverse (5′-3′)	References
CAT	ATCAAGTGTCATTCCTCTTCTCTG	CCTTCCCTTCTTTGTTCACCA	[52]
mtMnSOD	AAGGTTCTGGTTGGGGCT	AACATTCTTGTACTGCAG	[39]
Trx1	TCGAGACTACATCGCTAAGTACAAA	AAACTCCACTCCGAGCATCC	[40]
Prx6	ACCCATCGGACTACACCCCAG	GGACCAATGACAAAGACAGCA	[53]
proPO	CCATCCCTTCCTGCTTACCA	CTCCATCACAAACCCTAACGACTT	[54]
TLRs	CTCCTTCACCTGCCCTAACTGCT	CTCCAGTTTGTATTGCTGTGCGAAA	[49]
Relish	TCTCCCTACTCTGACCATTCC	TTCCCACCATCTCACTCTTGT	[41]
LITAF	CAGGAGTAGTGTCGGGATTTGC	AGTTGTTGGAGCAGCACCTTG	[55]
S27	GGTCGATGACAATGGCAAGA	CCACAGTACTGGCGGTCAAA	[51]

reference genes (prophenoloxidase (*proPO*); *mtMnSOD*; *Trx1*; *Prx6*; toll like receptors (*TLRs*); *Relish*; lipopolysaccharide-induced TNF-α factor (*LITAF*) and *S27* are listed in Table 2. Briefly, the reaction mixture was prepared using 2 μL cDNA, 0.4 μL forward primer, 0.4 μL reverse primer, 10 μL SYBR Premix Ex TaqTM (TaKaRa, Dalian, Liaoning, China), 0.4 μL ROX Reference Dye (TaKaRa, Dalian, Liaoning, China), and 6.8 μL double-distilled water. Each sample was tested in duplicate. PCR consisted of a pre-run at 95 °C for 15 min and 40 cycles of denaturation at 95 °C for 15 s, followed by a 60 °C annealing step for 34 s. The melting curve analysis of 5 s per step from 65 to 95 °C was performed at the end of each PCR thermal profile to assess the specificity of each amplicon. The relative levels of mRNA expression were calculated using the $2^{-\Delta\Delta CT}$ method [50], normalised to the reference mRNA level of *S27* [51]. The values of *E. sinensis* fed the basal diet without icariin supplementation were used for comparison.

2.7. Statistical analysis

All data were expressed as treatment means with the pooled SEM of eight replicates. Tukey's Honestly Significant Difference Test (Tukey's HSD) and one-way ANOVA were used to analyse differences among the four groups, and a *P*-value < 0.05 was considered significant. All statistical analyses were performed using SPSS 20.0 software (Chicago, USA; Version 20.0).

3. Results

3.1. Growth performance

Growth performances of *E. sinensis* subjected to the different treatments were presented in Table 3. Average final weight (FW), weight gain rate (WGR), SGR and SR were significantly increased in crabs fed ICA100 diet compared to crabs fed the control, ICA50 and ICA200 diets ($P < 0.05$). Meanwhile, compared to the control group, no significant differences were found in FW, WGR, SGR and SR of crabs both either ICA50 or ICA200 diets ($P > 0.05$).

3.2. Haemocytes and hepatopancreas antioxidant capabilities

Haemocytes and hepatopancreas antioxidant capabilities of *E. sinensis* were both presented in Fig. 1. PCC and MDA contents in both haemocytes and hepatopancreas decreased with ICA supplementation levels increasing from 50 to 100 mg/kg, and carbs fed ICA100 diet showed significantly lower PCC and MDA contents compared to the control group ($P < 0.05$). SOD and GPx activities in the haemocytes and hepatopancreas of crabs fed ICA100 diet were higher than those in control group ($P < 0.05$). Meanwhile, there were no significant differences in PCC or MDA contents, or activities of SOD and GPx in crabs fed ICA200 diet compared to controls.

3.3. Immune parameters in the haemocytes and hepatopancreas

As shown in Fig. 2, the activities of PO, LZM, ACP and AKP in haemocytes were increased in the ICA50 and ICA100 groups compared to the control ($P < 0.05$). In the hepatopancreas, higher LZM and ACP activities were observed in ICA100 crabs compared to the control ($P < 0.05$). The activity of AKP was increased in both ICA50 and ICA100 groups ($P < 0.05$).

3.4. Analysis of antioxidant and immune related genes expression in hepatopancreas

As shown in Fig. 3, ICA100 crabs showed greater expression of *CAT*, *mtMnSOD*, *Trx1*, *Prx6* and *proPO*, and depressed expression of *TLRs*, *Relish* and *LITAF* in the hepatopancreas compared to controls ($P < 0.05$). Meanwhile, ICA50 supplementation induced greater mRNA expression of *proPO*, but lower expression of *TLRs* and *Relish* in the hepatopancreas ($P < 0.05$). Contrastingly, there were no significant differences between controls and ICA200 crabs in the mRNA expression levels of *CAT*, *mtMnSOD*, *Trx1*, *Prx6*, *proPO, TLRs, Relish* and *LITAF*.

4. Discussion

4.1. Growth performance

This report is the first to describe the effects of ICA on the growth, antioxidant, and non-specific immune response of *E. sinensis*. Medicinal plant extracts and phytogenic products have been demonstrated as growth promoters for aquatic animals directly through enhancing digestive enzymes activity and feed utilisation efficiency [56,57], or indirectly through boosting the antioxidant capacity and immunity hence raised growth prospects and survival [58,59]. In the present study, crabs fed diets supplemented with 100 mg/kg ICA showed higher FW, WG, SGR and SR compared to control group, suggesting that ICA could improve the growth and survival of *E. sinensis*. This improved growth performance and SR could be ascribed to the enhanced antioxidant and nonspecific immunity function of *E. sinensis*. Previous studies have indicated that the improved growth performance of aquatic animals by various plant extracts can be attributed to enhanced antioxidants levels and immune enzymes activities, which suppresses pathogenic microbial infection and reduces oxidative stress [60–62]. This may indirectly lead to a greater feed utilisation, resulting in enhanced growth of aquatic animals. But due to the fact that relevant literature is quite limited, the mechanisms underlying this process are largely unknown, and warrant further investigation to elucidate this.

4.2. Antioxidant capability

Under normal physiological conditions, animal cells produce reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{-\cdot}$), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2). Excessive ROS can cause damage to biomolecules including protein, DNA and lipid, ultimately lead to lipid peroxidation and protein carbonylation. Carbonyl groups including aldehydes and ketones are produced on protein side chains (especially of Pro, Arg, Lys, and Thr) when they are oxidised, PCC is actually the most established biomarker of protein oxidation [63]. MDA, the main ending products of lipid peroxidation, is highly cytotoxic and will damage the cell structure and function [64]. Meanwhile, the burden of ROS production is largely counteracted by a

Table 3
Growth performance of *E. sinensis* fed different levels of dietary ICA.

Diets	IW/g	FW/g	WGR/%	SGR/%	SR/%
CON	33.63 ± 0.11^{a}	64.88 ± 0.58^{b}	92.93 ± 1.14^{b}	1.1 ± 0.01^{b}	86.35 ± 3.30^{b}
ICA50	33.68 ± 0.08^{a}	68.06 ± 1.52^{b}	102.10 ± 4.15^{b}	1.17 ± 0.03^{b}	90.50 ± 9.10^{ab}
ICA100	33.4 ± 0.09^{a}	72.93 ± 0.35^{a}	118.36 ± 0.49^{a}	1.3 ± 0.00^{a}	91.83 ± 2.03^{a}
ICA200	33.6 ± 0.14^{a}	65.48 ± 0.84^{b}	94.86 ± 2.06^{b}	1.11 ± 0.02^{b}	88.4 ± 4.19^{ab}

Note: IW, average initial weight; FW, average final weight; WGR, weight gain rate; SGR, specific growth rate; SR, survival rate. Values are means ± S.E.M of 8 replicates. Means in the same column with different superscripts are significantly different ($P < 0.05$).

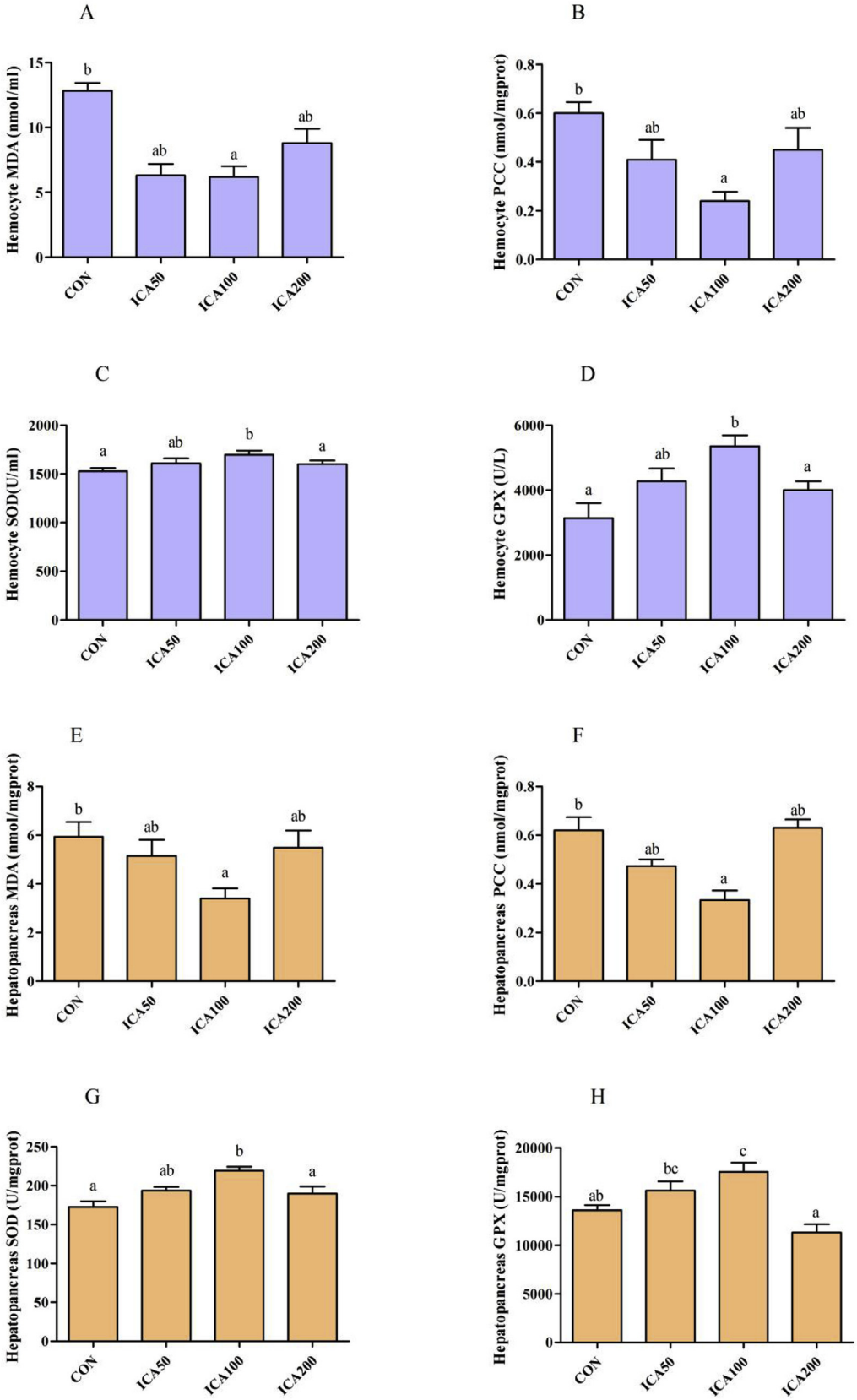

Fig. 1. The contents of MDA (A,E) and PCC (B,F), and activities of SOD (C,G) and GPx (D,H) in *E. sinensis* haemocytes (A-D) and hepatopancreas (E-H) subjected to different levels of dietary ICA. Each datum represents the mean of eight replicates. Bars assigned with different superscripts are significantly different ($P < 0.05$).

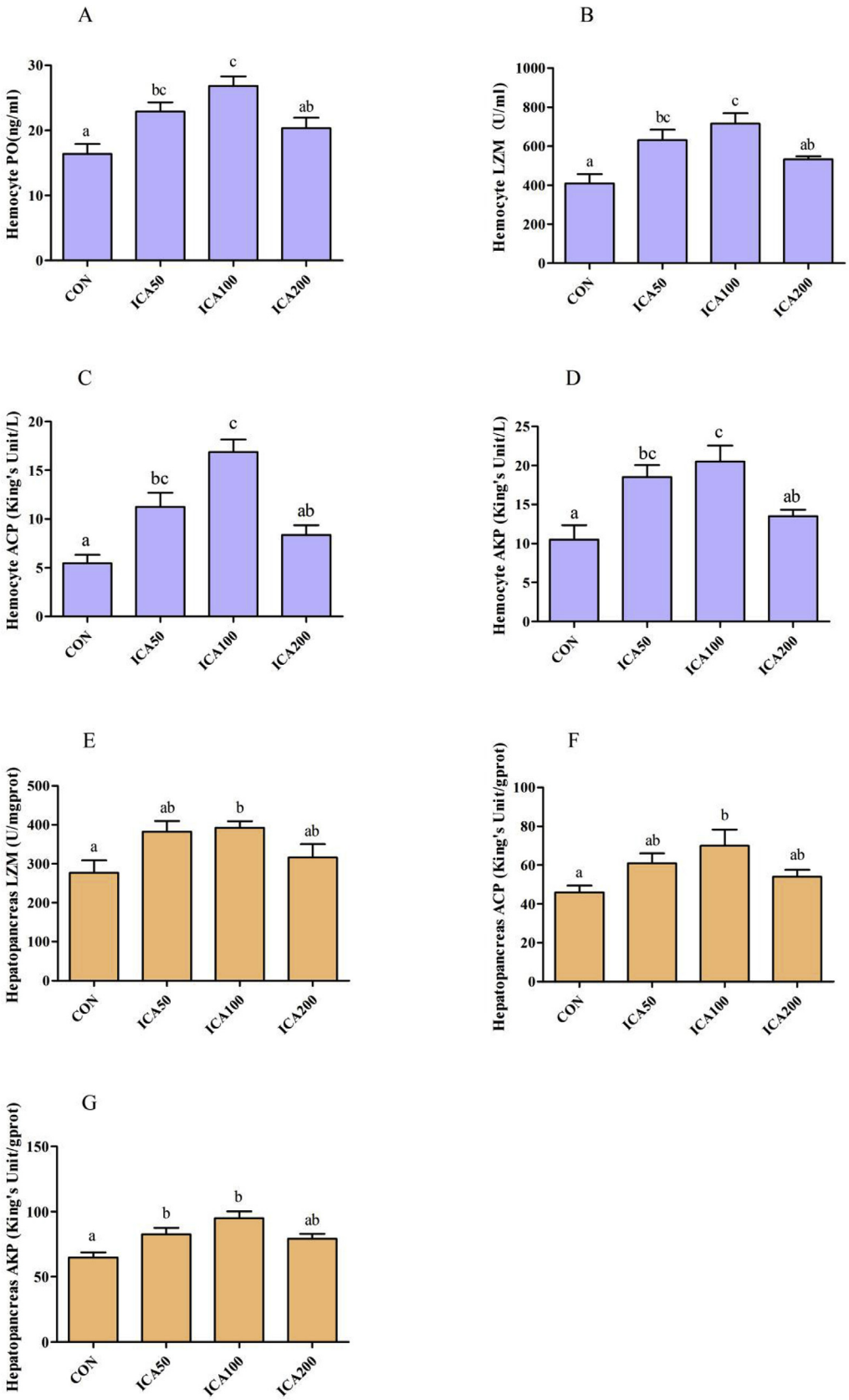

Fig. 2. The activity of PO (A), LZM (B, E), ACP (C, F) and AKP (D, G) in *E. sinensis* haemocytes (A-D) and hepatopancreas (E-G) subjected to different levels of dietary ICA. Each datum represents the mean of eight replicates. Bars assigned with different superscripts are significantly different ($P < 0.05$).

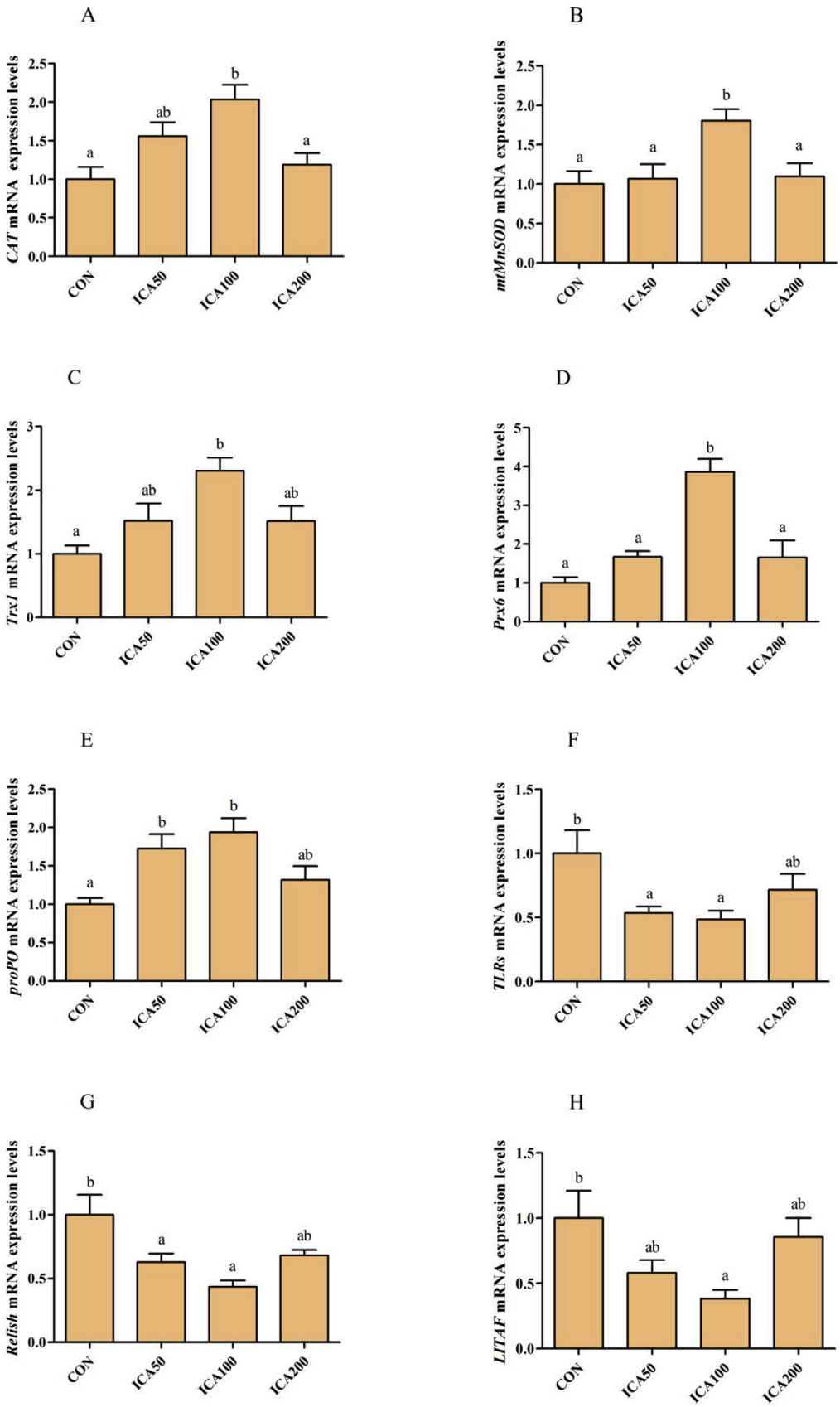

Fig. 3. Relative expressions of *CAT* (A), *mtMnSOD* (B), *Trx1* (C), *Prx6* (D), *proPO* (E), *TLRs* (F), *Relish* (G) and *LITAF* (H) in crabs subjected to different levels of dietary ICA in hepatopancreas. Each datum represents the mean of eight replicates. Bars assigned with different superscripts are significantly different ($P < 0.05$).

sophisticated antioxidant defense system including the enzymatic scavengers SOD, CAT and GPx. SOD specifically catalyzes the dismutation reaction of superoxide radicals to hydrogen peroxide and oxygen, and then CAT and GPx exert complementary roles in catalyzing the decomposition of hydrogen peroxide into water and oxygen [65]. In the present study, notably enhanced SOD and GPx activities, and reduced MDA and PCC contents both in haemocytes and hepatopancreas were observed in crabs fed with 100 mg/kg ICA. These findings indicate that ICA supplementation is beneficial in protecting haemocytes and hepatopancreas tissues from lipid peroxidation and protein oxidative carbonylation as evidenced by reduced production of MDA and PCC, and improved antioxidant enzymatic activities of SOD and GPx. Similarly, other studies in vivo and in vitro have showed that ICA can alleviate the oxidative stress caused by duck hepatitis A virus by increasing SOD and GPx activities and reducing the MDA contents [66], and ICA pre-treament for 24 h significantly ameliorates cisplatin-induced oxidative stress by reducing levels of MDA and ROS in HEK-293 cells [67].

CAT and *mtMnSOD* are well documented as efficient antioxidant enzymes with potential involvement in the innate immune response of *E. sinensis* [39,52]. *Trx1* has also been shown to be critical in regulation of cellular redox homeostasis in a physiological context and is more potent than GSH in its antioxidant capacity [40]. Meanwhile, the peroxiredoxins are superfamily of antioxidative proteins that play important roles in protecting organisms against ROS toxicity, *Prx*6 belongs to the 1-Cys peroxiredoxin subgroup with its highest expression level in the hepatopancreas [53]. The present study demonstrated that dietary 100 mg/kg ICA supplementation induced expression of *CAT*, *mtMnSOD*, *Trx1* and *Prx*6 in the hepatopancreas. Similarly, ICA appears to inhibit H_2O_2-induced neurotoxicity by up-regulating *SIRT1*-dependent mRNA and protein expression of the antioxidant enzymes CAT and Prx1 in E16-17 mouse embryo primary cortical neurons [29]. The present study indicated that ICA can enhance the antioxidant capacity of *E. sinensis* by regulating the expression of certain antioxidant genes in the hepatopancreas, supported by similar activities of antioxidant enzymes in both haemocytes and hepatopancreas.

4.3. Immune response

Most crustacean species including the *E. sinensis* lack true adaptive immune system, they depend on the non-specific immune responses. Certain specific enzymes including PO, LZM, ACP and AKP play leading roles in crustacean immunity and have been recognized as the specific indices for evaluating the non-specific immunity condition of crustaceans. LZM arises from neutrophils and macrophages, and is secreted into blood and mucus to eliminate the pathogenic bacteria by activating the complement and phagocytes both in haemocytes and hepatopancreas of crustaceans [68]. As a typical lysosomal enzyme, ACP activity expectedly has a trend similar to that of LZM [69]. AKP can enhance recognition and phagocytosis of the pathogen by altering their surface structures [35]. In this study, the activities of LZM, ACP and AKP in haemocytes were significantly increased by ICA supplementation at 50 and 100 mg/kg, and in hepatopancreas, higher LZM and ACP activities were observed in the same group. AKP activities increased at both 50 and 100 mg/kg ICA supplementation. Furthermore, the higher PO activities of haemocytes in both ICA50 and ICA100 groups indicated an enhanced immune response of *E. sinensis*, due to the fact that PO produced from proPO system in haemocytes plays an important role in the immune defense of crustaceans [70]. Similar dramatic increases in PO, LZM, ACP and AKP activities have also been noted after the application of immunostimulants including taurine and fructooligosaccharides, or Chinese herb extracts such as honeysuckle stem ethanol extract, *Angelica sinensis* polysaccharide, anthraquinone extract from *Rheum officinale Bail* in *E. sinensis* [71,72], Pacific white shrimp (*Litopenaeus vannamei*) [73,74] and Wuchang bream (*Megalobrama amblycephala*) [75].

In this present study, the expression of *proPO* significantly increased along with PO activity in haemocytes. This result suggests that ICA may activate the proPO system involved in cell adhesion, cellular responses to inflammatory cytokines and environmental stresses. *TLRs* and *LITAF* are important components of the *NF-κB* pathway activating the expression of multiple genes involved in immune response [49]. *Relish* is the NF-κB-like transcription factor of *E. sinensis*, which is potentially involved in the immune response against fungi and bacteria [41]. *LITAF* can directly interact with the signal transducer and translocates into the nucleus, where it binds to the promoter regions of *TNF-α* and other inflammatory cytokines, thereby transcriptional regulating their expression in response to various endotoxin challenges [55]. The present study indicated that ICA supplementation at 50 and 100 mg/kg suppressed the mRNA expression levels of *TLRs* and *Relish* in the hepatopancreas, while only 100 mg/kg ICA supplementation suppressed the expression of *LITAF*. These results may be directly due to the anti-inflammatory effect of ICA. An increasing number of studies suggest that ICA can effectively reduce phosphorylation and nuclear translocation of NF-κB, as well as mRNA expression and secretion of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β in LPS-stimulated inflammatory mouse models [30,76] and mouse models of depression [77]. This study demonstrated that dietary ICA at suitable levels could enhance non-specific immunity in *E. sinensis* by up-regulating the expression of *proPO,* while suppressing *TLRs*, *Relish* and *LITAF* in the hepatopancreas. This theory is supported by similar activities of immune parameters in both haemocytes and hepatopancreas.

In conclusion, this study demonstrated that dietary ICA supplementation at an optimum dose of 100 mg/kg can be effective in improving growth performance, antioxidant capability and non-specific immunity in *E. sinensis*. In addition, ICA could be developed as a new antioxidant and immunopotentiator for the future culturing of *E. sinensis*. However, further study on different cell models and animal models are required to completely evaluate the anti-oxidative and immunopotentiator capabilities and exact molecular mechanisms of ICA.

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