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Research Article

Effects of Iron and Vitamin C on Growth Performance, Iron Utilization, Antioxidant Capacity, Nonspecific Immunity, and Disease Resistance to *Aeromonas hydrophila* in Chinese Mitten Crab (*Eriocheir sinensis*)

Ying Song,[1] **Xinyu Cai**,[1] **Xianyong Bu**,[1] **Shubin Liu**,[1] **Mingqi Song**,[1] **Yiwen Yang**,[1] **Xiaodan Wang**,[1] **Qingchao Shi**,[2] **Jianguang Qin**,[3] **and Liqiao Chen**[1]

[1]*Laboratory of Aquaculture Nutrition and Environmental Health, School of Life Science, East China Normal University, Shanghai 200241, China*
[2]*Key Laboratory of Sichuan Province for Fishes Conservation and Utilization in the Upper Reaches of the Yangtze River, Neijiang Normal University, Sichuan 641100, China*
[3]*College of Science and Engineering, Flinders University, Adelaide, SA 5001, Australia*

Correspondence should be addressed to Xiaodan Wang; xdwang@bio.ecnu.edu.cn and Liqiao Chen; lqchen@bio.ecnu.edu.cn

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Iron is an essential trace element with an abroad physiological function, and iron deficiency can impair animal health. Vitamin C (VC) has the potential to release iron from diets and increase iron uptake. This study evaluates the effects of dietary iron and vitamin C on the growth performance, iron utilization, antioxidant capacity, and nonspecific immunity of the Chinese mitten crab (*Eriocheir sinensis*). Juvenile *E. sinensis* (1.14 ± 0.01 g) were fed six diets supplemented with three levels of iron (41.40, 92.25, and 143.00 mg/kg), and each iron level supplemented either 4.33 mg/kg or 700.90 mg/kg of vitamin C for 8 weeks. Crabs fed 41.40 mg/kg of iron diet had the lowest weight gain (WG) and specific growth rate (SGR) and had the highest feed conversion rate (FCR) regardless of dietary vitamin C levels. Dietary 700.90 mg/kg of vitamin C improved WG and SGR but decreased FCR when the diets were supplemented with 41.40 and 143.00 mg/kg of iron ($P < 0.05$). Dietary 700.90 mg/kg of vitamin C significantly improved the expression levels of divalent metal ion transporter 1, ferroportin, and iron regulation protein in the intestine and hepatopancreas when supplemented with 92.25 and 143.00 mg/kg of iron ($P < 0.05$) and enhanced the transferrin content in hemolymph and iron deposition in the hepatopancreas when supplemented with 92.25 and 143.00 mg/kg of iron ($P < 0.05$). Crabs fed the diet supplemented with 41.40 mg/kg of iron had the highest malondialdehyde content and the lowest superoxide dismutase activity in the hepatopancreas. Dietary 700.90 mg/kg of vitamin C supplementation also increased the acid phosphatase and bacteriolytic activity when diets were supplemented with 41.40 and 143.00 mg/kg of iron ($P < 0.05$). In addition, crabs fed 143.00 mg/kg of iron and 700.90 mg/kg of vitamin C diet had the highest survival rate after the *Aeromonas hydrophila* challenge. This study demonstrates that dietary 700.90 mg/kg of vitamin C could promote iron absorption and utilization, thereby increasing the growth, immunity, and disease resistance of *E. sinensis*.

1. Introduction

Fish meal is a quality protein source widely applied in aquaculture [1]. With the rapid development of aquaculture, the demand and price for fishmeal have increased dramatically [2]. However, the fishmeal supply has been affected by the decline of fishery resources [3, 4]. Therefore, plant proteins such as soybean and cottonseed meals have been widely used in aquaculture feed [5]. Nevertheless, antinutritional factors in dietary plant protein such as phytic acid in soybean meal and gossypol in cottonseed meal can negatively affect the absorption and utilization of mineral nutrients [6].

Generally speaking, mineral deficiency such as iron, zinc, and manganese would damage animal growth performance and health [7–9]. Iron is an essential trace element which has abundant physiological functions, including DNA synthesis and repair [10], mitochondrial biooxidation [11], and ecdysone synthesis [12]. The antinutritional factors combine with iron to form insoluble chelates, whereas vitamin C can compete with antinutritional factors for iron to form soluble chelates. As a result, dietary vitamin C can reduce the chelation of antinutritional factors [13]. Meanwhile, as a reductant, vitamin C can reduce the Fe^{3+} to Fe^{2+} to increase iron absorption through the divalent metal ion transporter 1 (*dmt1*) [14]. Dietary 200 mg/kg of vitamin C enhanced iron absorption six times in human subjects. Even dietary 25 mg/kg of vitamin C also can improve iron absorption [15]. Moreover, the carcass iron content of growing chicks fed a diet supplemented with moderate iron and vitamin C levels was significantly enhanced compared with a diet without vitamin C [16].

Vitamin C can reduce the risk of oxidative stress as an antioxidant [17]. For example, dietary vitamin C increased the antioxidant enzyme activity of the fingerling *Channa punctatus* [18]. Furthermore, the appropriate vitamin C level can also improve the resistance of coral trout (*Plectropomus leopardus*) to *Vibrio harveyi* [19]. In addition, vitamin C plays a vital role in crustacean shell hardening [20], and vitamin C could promote iron absorption through their interactions. However, it is uncertain that vitamin C can promote the absorption of dietary iron in crustaceans owing to scarce information on livestock and aquaculture. With rich nutritional value and unique taste, the Chinese mitten crab (*Eriocheir sinensis*) has become China's important economic crustacean species in the aquaculture industry. However, diseases have always been a threat to *E. sinensis*. Therefore, based on the role of iron and vitamin C in immunity mentioned above, this study was conducted to investigate the effects of dietary iron and vitamin C on iron metabolism and nonspecific immunity of juvenile *E. sinensis*, aiming to provide a reference for developing functional diets to increase disease resistance in aquaculture.

2. Materials and Methods

2.1. Preparation of the Experimental Diets. Our study formulated six diets containing 420 g/kg crude protein and 80 g/kg total lipid (Table 1). Ferrous sulfate ($FeSO_4{\cdot}7H_2O$, Sangong Biotech, Ltd., Shanghai, China, purity > 99%) was used as an iron source to add 0, 50, and 100 mg/kg of iron. The average level of analyzed iron was 41.40, 92.25, and 143.00 mg/kg, respectively. L-Ascorbate-2-phosphate (Hangzhou Tiannong Bio-nutrition Technology Co., Ltd., Hangzhou, China, purity 35%) was used as a vitamin C source to add 0 and 700 mg/kg of vitamin C [21]. The average level of analyzed vitamin C was 4.33 and 700.90 mg/kg, respectively. The diets were prepared as described by our previous study [22].

2.2. Crabs and Rearing Conditions. Healthy *E. sinensis* were purchased from the Liu Aquaculture Farm in Chongming (Shanghai, China), and the feeding trial was conducted at the East China Normal University for 56 days. Healthy crabs (720 individuals) with an initial weight of 1.14 ± 0.01 g were randomly distributed to 24 tanks (300 L) with 30 individuals each in four replicates per group. As our previous study had determined the administration of the feed trial and the water-rearing condition [23], the crabs were fed with diets at 4% of the body mass daily at 06:30, 15:00, and 21:00 for 8 weeks. The residual feed and feces were siphoned out 2 h after feeding, and one-third of the water in each tank was changed daily. During the feeding trial, dead crabs were immediately removed from the tank daily. The present study was carried out according to the Committee on the Ethics of Animal Experiments at East China Normal University (f20201001).

2.3. Sampling. At the end of the feed trial, all the crabs were fasted for 24 h before being counted and weighed. Five crabs per tank were randomly collected for proximate composition analysis. Hemolymph samples were extracted from the crab appendages and centrifuged at 3500 g (4°C) for 10 min. The supernatant of the hemolymph sample was gathered and stored at -80°C until used. The hepatopancreas samples were homogenized with 0.85% saline solution in 9 volumes (*v*/*w*) and centrifuged at 3500 g (4°C) for 10 min. Then, the supernatant of the homogenate was collected. The samples were stored at -80°C until used.

2.4. Proximate Composition Analysis. The proximate composition in diets and the whole body of crabs was determined according to the standard methods [24]. The moisture, total lipid, crude protein, and ash content were determined as described by Song et al. [25]. The iron concentration of body samples, hepatopancreas, muscle, and rearing water samples was also measured according to our previous study [26]. The iron concentration of tap water was under 0.01 ppm.

2.5. Biochemical Assay. The activity of phenoloxidase (PO) in the hemolymph was determined as described by Ashida [27]. The hemocyanin concentration was determined by diluting it with distilled water 100 times at 335 nm [28]. The activities of GSH-Px (No. A005-1), SOD (No. A001-3), T-AOC (No. A015-2), MDA (No. A003-1), and total protein (No. A045-4) in the hepatopancreas were determined using reagent kits (Nanjing Jiancheng Bioengineering Institute). The activities of ACP (No. A060-2), AKP (No. A059-2), and iron content (No. A039-1-1) in hemolymph were measured by reagent kits (Nanjing Jiancheng Bioengineering Institute). The hemolymph transferrin (No. ml89624-J) was determined via diagnostic kits (Shanghai Enzyme-linked Biotechnology Co., Ltd.).

According to the previous study, bacteriolytic activity in hemolymph was determined [29]. Briefly, the *Micrococcus lysodeikticus Fleming* freeze-dried powder (Shanghai Yuanye Bio-Technology Co. Ltd., Cat. No. S24857) was activated and purified. The purified *Micrococcus lysodeikticus Fleming* was inoculated into the Luria-Bertani (LB) medium and cultured at 28°C for 20 hours. PBS was used to resuspend and adjust the OD_{600} of *Micrococcus lysodeikticus Fleming* between 0.3 and 0.4. Afterwards, 1 mL of bacterium liquid was put into an ice bath for 10 minutes. We then added

Table 1: Formulation and chemical composition of six different experimental diets (g/kg dry basis).

Ingredients	Content (g/kg dry basis)					
Iron (mg/kg)	41.40		92.25		143.00	
Vitamin C (mg/kg)	4.33	700.90	4.33	700.90	4.33	700.90
Casein[a]	360.00	360.00	360.00	360.00	360.00	360.00
Gelatin[b]	90.00	90.00	90.00	90.00	90.00	90.00
Corn starch	250.00	250.00	250.00	250.00	250.00	250.00
Fish oil : soybean oil (1 : 1)	60.00	60.00	60.00	60.00	60.00	60.00
Vitamin premix[c]	20.00	20.00	20.00	20.00	20.00	20.00
Mineral premix[d]	40.00	40.00	40.00	40.00	40.00	40.00
Choline chloride[e]	5.00	5.00	5.00	5.00	5.00	5.00
Cholesterol[e]	5.00	5.00	5.00	5.00	5.00	5.00
Betaine[e]	30.00	30.00	30.00	30.00	30.00	30.00
Lecithin[f]	10.00	10.00	10.00	10.00	10.00	10.00
Cellulose	110.00	108.00	109.75	107.75	109.51	107.51
Carboxymethyl cellulose	20.00	20.00	20.00	20.00	20.00	20.00
$FeSO_4 \cdot 7H_2O$[e]	—	—	0.25	0.25	0.49	0.49
L-Ascorbate-2-phosphate[g]	—	2.00	—	2.00	—	2.00
Proximate composition (g/kg)						
Moisture	149.96	139.32	139.27	141.21	138.39	136.08
Crude protein	421.65	418.68	406.68	414.09	406.14	408.41
Total lipid	71.24	79.01	79.03	78.19	74.05	80.92
Ash	70.42	70.04	71.01	71.26	72.58	72.17
Iron (mg/kg)	43.40	38.80	89.10	95.40	140.70	145.30
Vitamin C (mg/kg)	5.10	691.50	3.20	706.90	4.70	704.30

[a]Gansu Hualing Dairy Co., Ltd. [b]Henan Boyang Biotechnology Co., Ltd. [c]Vitamin premix (per 100 g premix): retinol acetate, 0.043 g; thiamin hydrochloride, 0.15 g; riboflavin, 0.0625 g; Ca pantothenate, 0.3 g; niacin, 0.3 g; pyridoxine hydrochloride, 0.225 g; para-aminobenzoic acid, 0.1 g; biotin, 0.005 g; folic acid, 0.025 g; cholecalciferol, 0.0075 g; α-tocopherol acetate, 0.5 g; menadione, 0.05 g; inositol, 1 g. All ingredients are filled with α-cellulose to 100 g. [d]Mineral premix (per 100 g premix): $CaCO_3$, 38.25 g; KCl, 14.6 g; $MgSO_4 \cdot 7H_2O$, 10.0 g; $ZnSO_4 \cdot 7H_2O$, 0.511 g; $MnSO_4 \cdot 6H_2O$, 0.143 g; Ca $(IO_3)_2$, 0.058 g; $CuSO_4 \cdot 5H_2O$, 0.562 g; $CoCl_2 \cdot 6H_2O$, 0.176 g. All ingredients are diluted with α-cellulose to 100 g. [e]Sangong Biotech, Ltd., Shanghai, China, purity > 99%. [f]Shanghai Taiwei, Ltd., Shanghai, China. [g]Hangzhou Tiannong Bio-nutrition technology Co., Ltd., Hangzhou, China, purity 35%.

Table 2: Primer pair sequences and product size of the genes used for quantitative real-time PCR.

Gene	Position	Primer sequence	Length	Reference
irp	Forward	TCACTACCTCCAGCACTCCG	20	Yang et al. [30]
	Reverse	TGACCAATGGGCTCCTTCTC	20	
dmt1	Forward	ACCTATGTGGGACAGTTTGC	20	Song et al. [25]
	Reverse	CATGATGACGTTGAGGAGGT	20	
fpn	Forward	CCTCACTGTCATCATCCTCG	20	Song et al. [25]
	Reverse	GCTGTAAAGTAGTCCGGTCG	20	
β-Actin	Forward	TCGTGCGAGACATCAAGGAAA	21	KM244725.1
	Reverse	AGGAAGGAAGGCTGGAAGAGTG	22	

irp: iron regulation protein; *dmt1*: divalent metal ion transporter 1; *fpn*: ferroportin.

50 μL hemolymph and mixed homogeneously. The mixture was measured at 600 nm and noted as A_0. Then, the mixture was put into a water bath at 37°C for 90 min and an ice bath for 10 min. At last, it was measured at 600 nm and noted as A. The calculation formula is $U = (A_0 - A)/A$.

2.6. mRNA Expression Analysis. The isolation of total RNA from crabs was performed using RNAiso Plus (Takara, Dalian, China), and RNA quality was determined via the ND-2000, Nanodrop Technologies. The RNA sample was reversely transcribed via PrimeScript™ RT Reagent kits (Takara, Dalian, China). The RT-PCRs were performed in the CFX96 Real-Time PCR system (Bio-Rad) using the ChamQ Universal SYBR qPCR Master Mix (Q711-02/03, Vazyme Biotech Co., Ltd.) according to the instructions. The primers used are shown in Table 2. β-Actin was used as a reference gene. The relative expression of RNA was calculated through the 2-$\Delta\Delta$CT method [31].

2.7. Aeromonas hydrophila Challenge. The *Aeromonas hydrophila* was available in our laboratory. According to the preliminary experiment, medial lethal concentration was determined at 2.1×10^6 CFU/mL. The purified *Aeromonas hydrophila* was inoculated into the Luria-Bertani (LB) medium and cultured at 28°C for 20 hours. PBS was used to adjust the concentration of *Aeromonas hydrophila* to 2.1 $\times 10^6$ CFU/mL. Twenty healthy juvenile crabs were randomly selected from each group, and each crab was injected with a 20 μL bacteria solution. The survival rate was recorded every 12 hours until 96 hours.

2.8. Statistical Analyses.

$$\begin{aligned}
\text{Weight gain (WG, \%)} &= 100 \times , \\
\text{Specific growth rate } \left(\text{SGR, \%day}^{-1}\right) &= 100 \times \frac{\ln\ (\text{final body weight, g}) - \ln\ (\text{initial body weight, g})}{56 \text{ days}}, \\
\text{Hepatosomatic index (HSI, \%)} &= 100 \times \frac{\text{wet hepatopancreas weight, g}}{\text{wet body weight, g}}, \\
\text{Feed intake (FI, \%/d)} &= 100 \times \frac{\text{dry feed intake}}{(\text{final body weight} + \text{initial body weight})/2 \times 56 \text{ days}}, \\
\text{Feed conversion ratio (FCR)} &= \frac{\text{dry feed intake}}{\text{wet weight gain}}.
\end{aligned} \tag{1}$$

The calculation formulas can be found in the supplementary material. Statistical analysis was performed using SPSS 21.0 (SPSS, Chicago, IL, USA). Two-way ANOVA was performed to compare dietary iron and vitamin C levels. An independent-sample *t*-test was used to determine the significant difference between crabs fed 4.33 and 700.90 mg/kg of vitamin C diets with the same iron levels. At the same vitamin C level, one-way ANOVA and Duncan's test were performed to analyze the significant difference between crabs fed 41.40, 92.25, and 143.00 mg/kg of iron diets. The Cox regression method was used to evaluate the effects of different diets on the survival of *E. sinensis* after *Aeromonas hydrophila* challenge [32]. $P < 0.05$ indicates a significant difference.

3. Results

3.1. Growth Performance and Whole-Body Proximate Composition. The growth performance and whole-body proximate composition of crabs are shown in Figures 1 and 2, respectively. Crabs in the 143.00 mg/kg of the iron group had the highest final body weight (FBW), weight gain (WG), and specific growth rate (SGR) regardless of vitamin C levels ($P < 0.05$). Dietary 700.90 mg/kg of vitamin C significantly improved the FBW, WG, and SGR when diets were supplemented with 41.40 and 143.00 mg/kg of iron ($P < 0.05$). The hepatosomatic index (HSI) and survival rate (SR) had no significant difference ($P > 0.05$). The feed conversion ratio (FCR) of 143.00 mg/kg of iron groups decreased significantly regardless of vitamin C levels ($P < 0.05$). Moreover, the FCR in the 700.90 mg/kg vitamin C group was lower than that in the 4.33 mg/kg vitamin C group ($P < 0.05$).

There were no interactions between dietary levels of iron and vitamin C in the whole-body proximate composition ($P > 0.05$). Furthermore, the hepatopancreas vitamin C content in 700.90 mg/kg of vitamin C groups was significantly enhanced compared with that in 4.33 mg/kg of vitamin C groups, and the vitamin C content was not affected by dietary iron ($P > 0.05$).

3.2. Iron Absorption, Transportation, and Iron Content in the Tissues. The mRNA levels of divalent metal ion transporter 1 (*dmt1*), ferroportin (*fpn*), and iron regulation protein (*irp*) are presented in Figure 3. The mRNA levels of hepatopancreas *dmt1*and *fpn* in the hepatopancreas of 143.00 mg/kg of iron groups markedly increased when compared to those of the 41.40 mg/kg of the iron group regardless of vitamin C levels ($P < 0.05$). The mRNA levels of hepatopancreas *irp* in the 143.00 mg/kg iron group were higher than those in the 41.40 mg/kg iron group when supplemented with 4.33 mg/kg of vitamin C, and the mRNA levels of *irp* of crabs fed diets with 92.25 and 143.00 mg/kg of iron were higher than those with 41.40 mg/kg of iron when supplemented with 700.90 mg/kg of vitamin C ($P < 0.05$). Dietary vitamin C markedly enhanced the mRNA levels of *dmt1*, *fpn*, and *irp* in the hepatopancreas when supplemented with 92.25 and 143.00 mg/kg of iron ($P < 0.05$).

The expression levels of *dmt1* and *fpn* in the intestine of crabs fed 143.00 mg/kg of iron diet markedly improved compared with those fed 41.40 mg/kg of iron diet regardless of vitamin C levels ($P < 0.05$). The mRNA level of *irp* in the intestine of crabs fed 143.00 mg/kg of iron diet was higher than those fed 41.40 and 92.25 mg/kg of iron diets when supplemented with 4.33 mg/kg of vitamin C. The mRNA levels of *irp* in 92.25 and 143.00 mg/kg of iron groups were higher than those in the 41.40 mg/kg of the iron group when supplemented with 700.90 mg/kg of vitamin C ($P < 0.05$). Dietary vitamin C markedly enhanced the mRNA levels of *dmt1*, *fpn*, and *irp* in the intestine of crabs fed diets supplemented with 92.25 and 143.00 mg/kg of iron ($P < 0.05$).

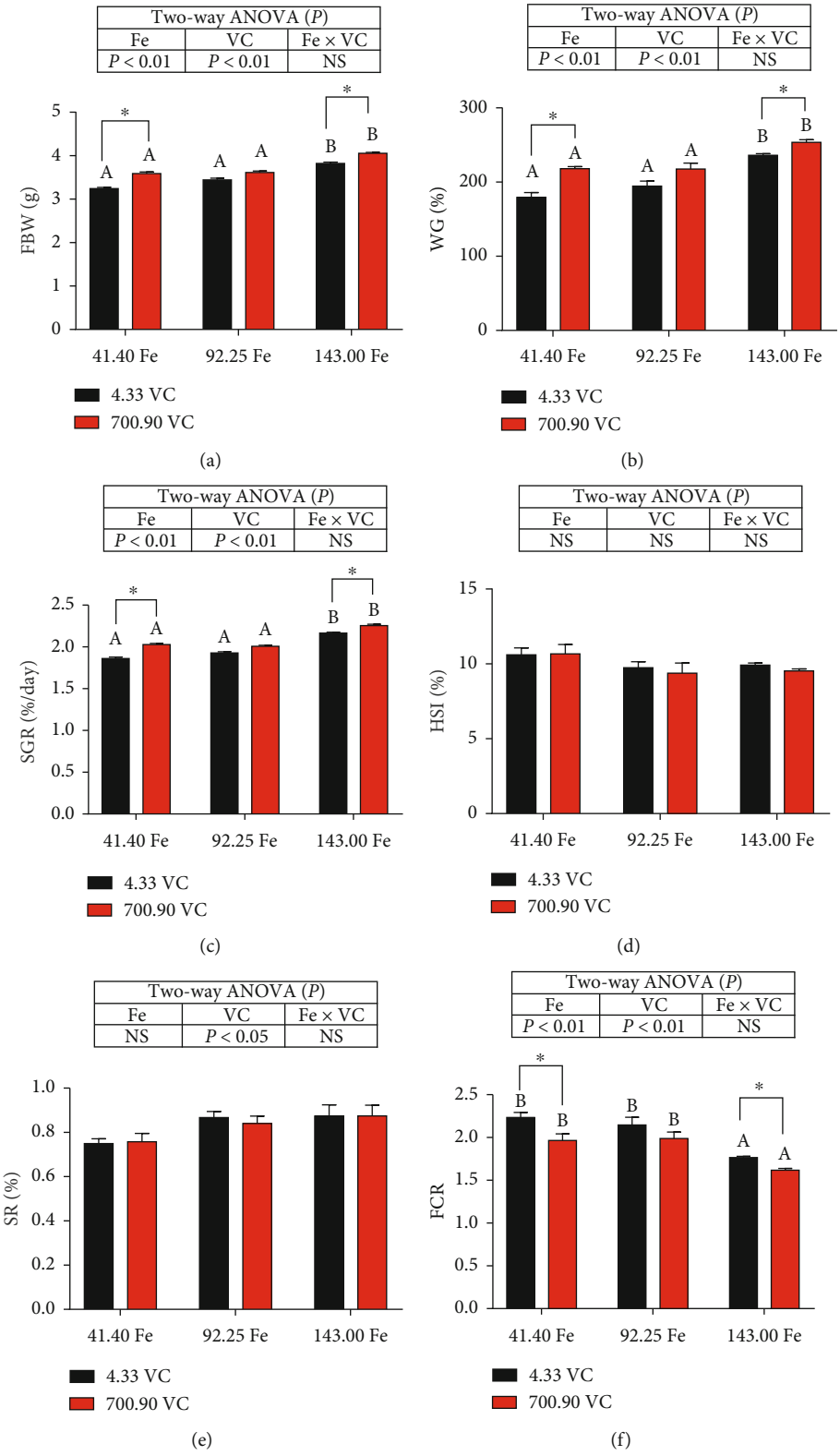

Figure 1: Continued.

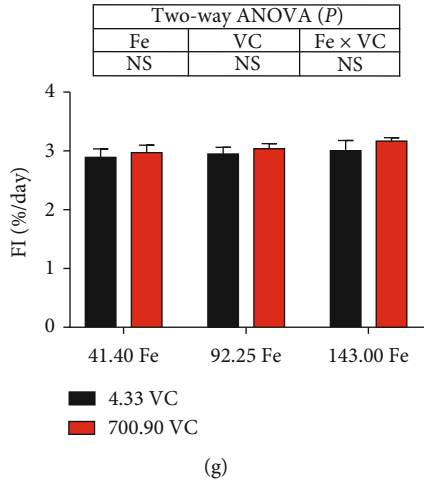

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Figure 1: Effects of dietary levels of iron and vitamin C on growth performance, HSI, and feed utilization of *Eriocheir sinensis*. (a) FBW: final body weight; (b) WG: weight gain; (c) SGR: specific growth rate; (d) HSI: hepatopancreas index; (e) SR: survival rate; (f) FCR: feed conversion rate; (g) FI: feed intake. Values are means ($n = 4$ replicate tanks) with standard errors represented by vertical bars. Different letters indicate significant differences between different iron levels in the same vitamin C levels ($P < 0.05$). * indicates significant differences between different vitamin C levels in the same iron levels ($P < 0.05$). The calculation formula of each parameter is in the supplemental materials.

The hemolymph iron content, transferrin, and iron content in different tissues are shown in Figure 4. The hemolymph iron and transferrin content in 92.25 and 143.00 mg/kg iron groups was significantly higher than that in the 41.40 mg/kg iron group when supplemented with 4.33 mg/kg of vitamin C ($P < 0.05$). The hemolymph iron content in 92.25 and 143.00 mg/kg of iron groups was markedly higher than that in the 41.40 mg/kg of the iron group when supplemented with 700.90 mg/kg of vitamin C ($P < 0.05$). Dietary 700.90 mg/kg vitamin C markedly improved the iron and transferrin content in the 92.25 and 143.00 mg/kg of iron groups compared with those fed 4.33 mg/kg of vitamin C diets ($P < 0.05$).

The whole-body and hepatopancreas iron content of crabs fed 143.00 mg/kg of iron diet markedly improved compared with those fed 41.40 mg/kg of iron diet regardless of vitamin C levels ($P < 0.05$). However, vitamin C levels significantly affected the hepatopancreas iron content when crabs were fed 92.25 and 143.00 mg/kg of iron diets ($P < 0.05$).

3.3. Hepatopancreas Antioxidant Enzyme Activities. The hepatopancreas antioxidant enzyme activities are shown in Figure 5. The MDA content in the 143.00 mg/kg of the iron group was markedly lower than the 41.40 and 92.25 mg/kg of iron groups when supplemented with 700.90 mg/kg of vitamin C ($P < 0.05$). Dietary 700.90 mg/kg of vitamin C markedly improved the T-AOC activity when crabs were fed a diet with 143.00 mg/kg of iron ($P < 0.05$). The SOD activity in the 143.00 mg/kg of the iron group was higher than that in the 41.40 mg/kg of the iron group when supplemented with 700.90 mg/kg of vitamin C, and the vitamin C markedly affected the SOD activity when fed with 143.00 mg/kg of iron ($P < 0.05$).

3.4. The Nonspecific Immunity Enzyme Activities in Hemolymph. The ACP and bacteriolytic activities in 92.25 and 143.00 mg/kg of iron groups were significantly enhanced compared with those in the 41.40 mg/kg of the iron group when supplemented with 4.33 mg/kg of vitamin C ($P < 0.05$, Figure 6). The ACP activity in the 92.25 and 143.00 mg/kg of iron groups was significantly increased than that in the 41.40 mg/kg of the iron group when supplemented with 700.90 mg/kg of vitamin C ($P < 0.05$). Furthermore, the bacteriolytic activity in the 143.00 mg/kg of the iron group was significantly increased compared with that in the 41.40 mg/kg of the iron group when supplemented with 700.90 mg/kg of vitamin C ($P < 0.05$). Dietary 700.90 mg/kg of vitamin C significantly increased ACP and bacteriolytic activity with 92.25 and 143.00 mg/kg of iron ($P < 0.05$). In addition, there was a significant difference in the PO level between 4.33 and 700.90 mg/kg of vitamin C with 92.25 mg/kg of iron ($P < 0.05$).

3.5. Survival Rate after Aeromonas hydrophila Challenge. The diets containing different iron and vitamin C levels affected the survival rate (Figure 7). The control group was the diet with 41.40 mg/kg of iron and 700.90 mg/kg of vitamin C. After the *Aeromonas hydrophila* challenge for 96 hours, the crabs in the 92.25 Fe+700.90 VC group, 143.00 Fe group+4.33 VC group, and 143.00 Fe+700.90 VC group had higher survival rates than those in the control group ($P = 0.012$, $P = 0.024$, and $P = 0.003$, respectively).

4. Discussion

Iron is an essential microelement for almost all creatures, and it plays a vital role in various activities involved in the synthesis and repair of DNA [33], biooxidation [34], and ecdysone synthesis [12]. Furthermore, crustaceans including

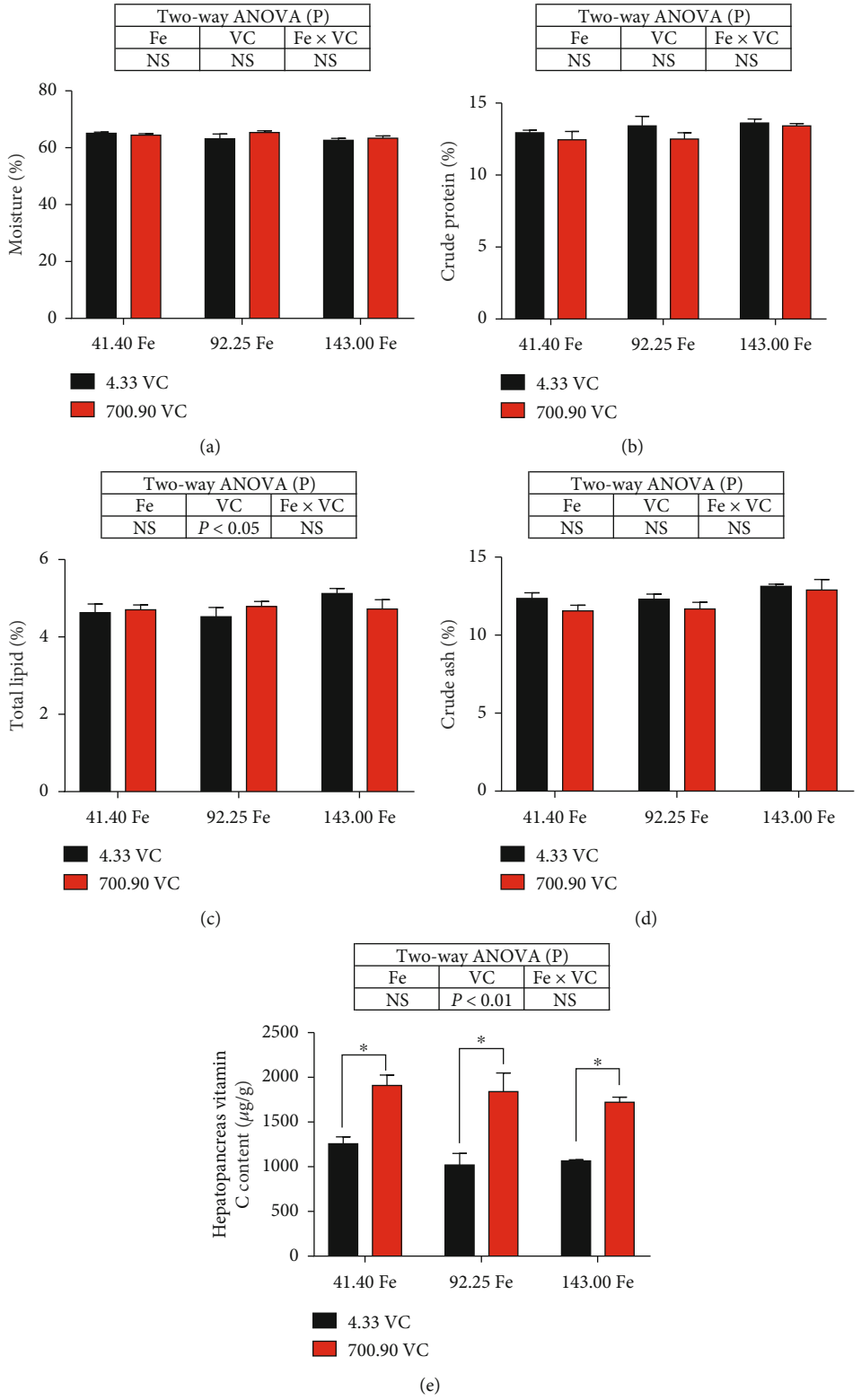

Figure 2: Effects of dietary levels of iron and vitamin C on proximate compositions and the hepatopancreas vitamin C content of *Eriocheir sinensis*. (a) Moisture, (b) crude protein, (c) total lipid, (d) ash, and (e) hepatopancreas vitamin C content. Values are means ($n = 4$ replicate tanks) with standard errors represented by vertical bars. Different letters indicate significant differences between different iron levels in the same vitamin C levels ($P < 0.05$). * indicates significant differences between different vitamin C levels in the same iron levels ($P < 0.05$).

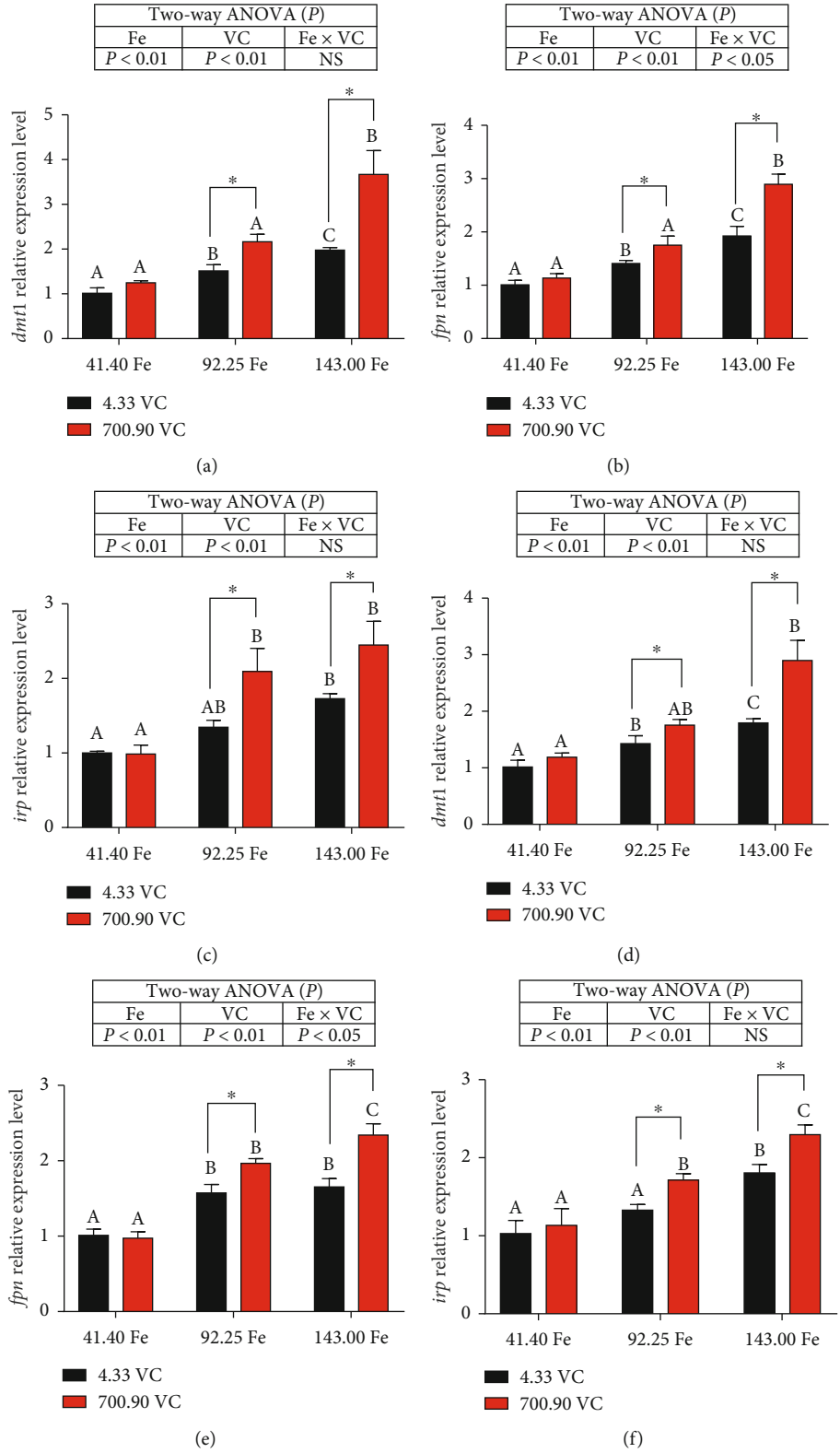

Figure 3: Effects of dietary levels of iron and vitamin C on the expression of genes involved in iron metabolization in hepatopancreas (a–c) and intestine (d–f) of *Eriocheir sinensis*. (a, d) *dmt1*: divalent metal ion transporter 1; (b, e) *fpn*: ferroportin; (c, f) *irp*: iron regulation protein. Values are means ($n = 4$ replicate tanks) with standard errors represented by vertical bars. Different letters indicate significant differences between different iron levels in the same vitamin C levels ($P < 0.05$). * indicates significant differences between different vitamin C levels in the same iron levels ($P < 0.05$).

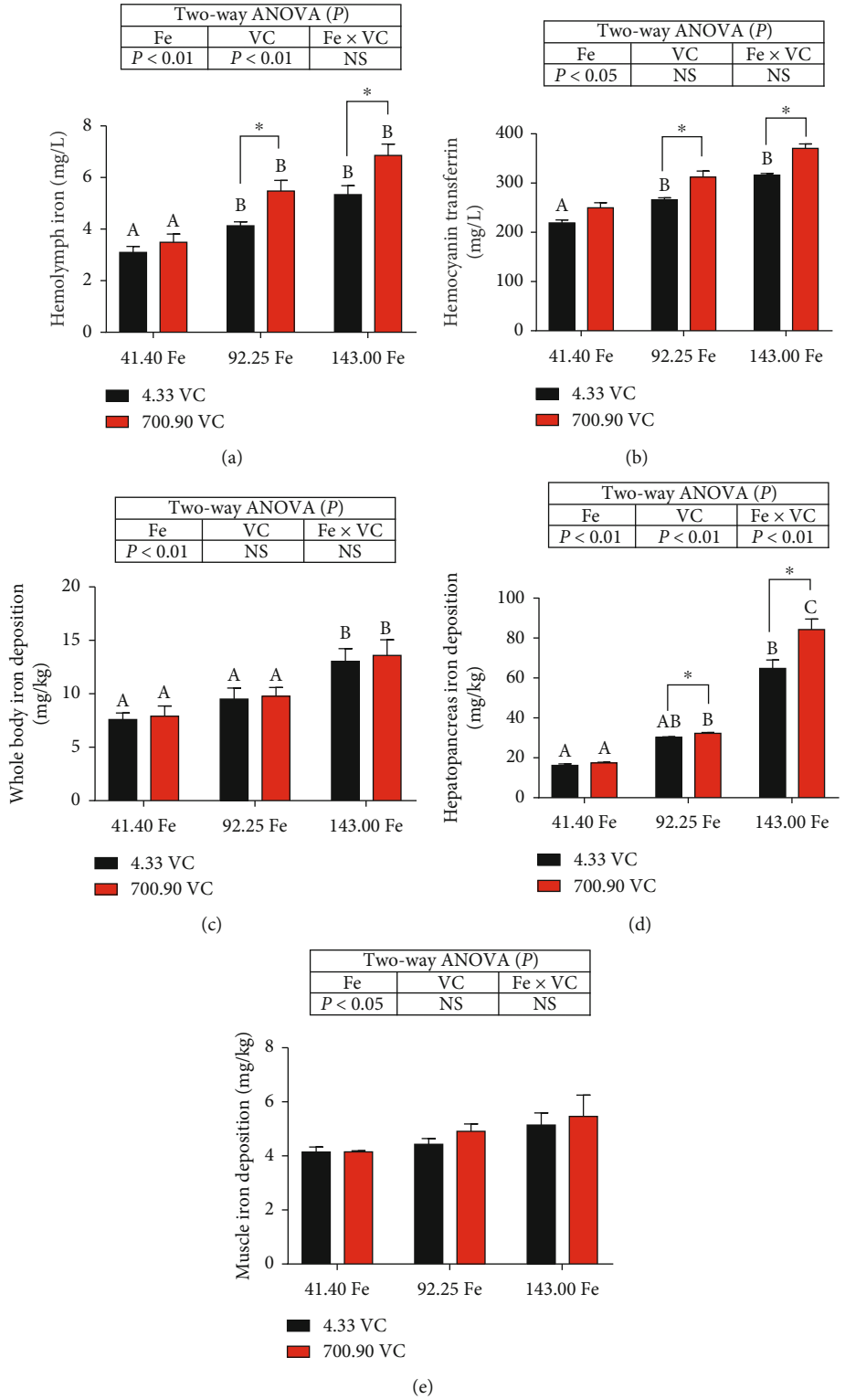

Figure 4: Effects of dietary levels of iron and vitamin C on hemolymph iron content and tissue iron content of *Eriocheir sinensis*. Values are means ($n = 4$ replicate tanks) with standard errors represented by vertical bars. Different letters indicate significant differences between different iron levels in the same vitamin C levels ($P < 0.05$). * indicates significant differences between different vitamin C levels in the same iron levels ($P < 0.05$).

E. sinensis require ecdysone involved in molt to support growth [35]. In the present study, dietary-supplemented 41.40 mg/kg of iron reduced the growth performance of juvenile *E. sinensis*. Dietary iron deficiency may inhibit ecdysone synthesis. Previous studies reported that dietary iron deficiency also reduced the growth of stinging catfish

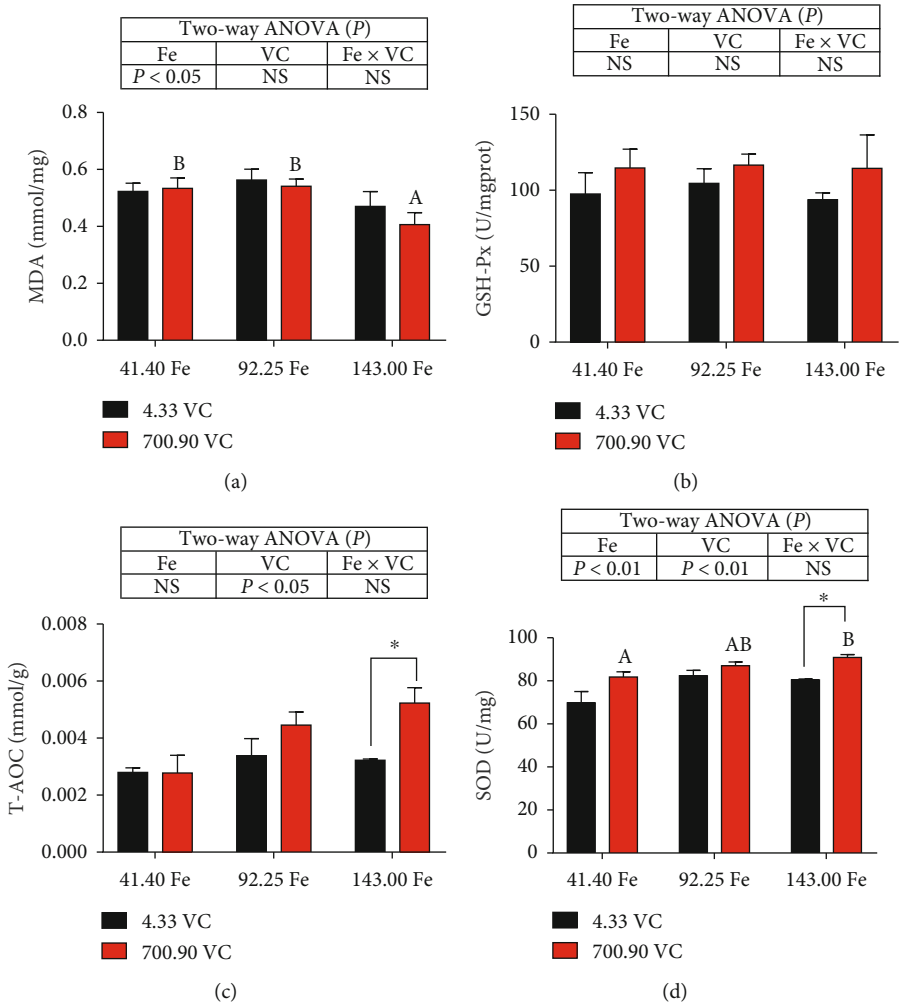

FIGURE 5: Effects of dietary levels of iron and vitamin C on antioxidation state of *Eriocheir sinensis*. (a) MDA: malondialdehyde; (b) GSH-Px: glutathione peroxidase; (c) T-AOC: total antioxidant capacity; (d) SOD: superoxide dismutase. Values are means ($n = 4$ replicate tanks) with standard errors represented by vertical bars. Different letters indicate significant differences between different iron levels in the same vitamin C levels ($P < 0.05$). * indicates significant differences between different vitamin C levels in the same iron levels ($P < 0.05$).

(*Heteropneustes fossilis*) [36], bighead carp (*Aristichthys nobilis*) [37], and yellow catfish (*Pelteobagrus fulvidraco*) [38]. However, the present results showed that dietary 700.90 mg/kg of vitamin C improved crab growth when crabs were fed 41.40 mg/kg of iron diets. Vitamin C is involved in synthesizing benzoquinone, a component of the crustacean shell [20]. In other words, vitamin C may facilitate the shell hardening of *E. sinensis* to promote growth performance. In other aquatic animals, dietary supplementation with appropriate vitamin C improved the growth performance of fingerling Bloth (*Channa punctatus*) [18], coral trout (*Plectropomus leopardus*) [19], and juvenile hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂) [39]. Therefore, dietary iron and vitamin C are indispensable nutrients for the growth of *E. sinensis*.

Generally, iron absorption was affected by many factors [40]. As dietary iron is supplied mainly as ferric iron, it must be reduced to ferrous iron to enter the intestinal epithelial cells through DMT1 [41]. Previous studies demonstrated that vitamin C could reduce ferric iron to ferrous iron, thus facilitating iron absorption [42, 43]. In the present study, the *dmt1* expression in the hepatopancreas and intestine was increased when supplemented with 700.90 mg/kg of vitamin C. The DMT1 is regulated by the iron regulatory protein-iron regulatory element (IRP-IRE) [44]. IRE, a fragment of the mRNA untranslated region, can bind to IRP to ensure the translation of DMT1 [44]. The *irp* expression increased when supplemented with vitamin C in the present study. It demonstrated that dietary vitamin C might promote the reduction of ferric iron in the hepatopancreas and intestine and stimulate the expression of *irp* to guarantee sufficient DMT1 for iron absorption.

Hemolymph is an important media for iron transportation in *E. sinensis*, absorbing iron from the intestine and transporting iron to the target organs. In the present study, vitamin C may facilitate iron absorption by reducing ferric iron to ferrous iron in the intestine. Accordingly, the iron content in hemolymph was increased in *E. sinensis*. Iron is mainly stored in the liver or hepatopancreas of animals. Once in demand, the cell iron could be transferred to the hemolymph through

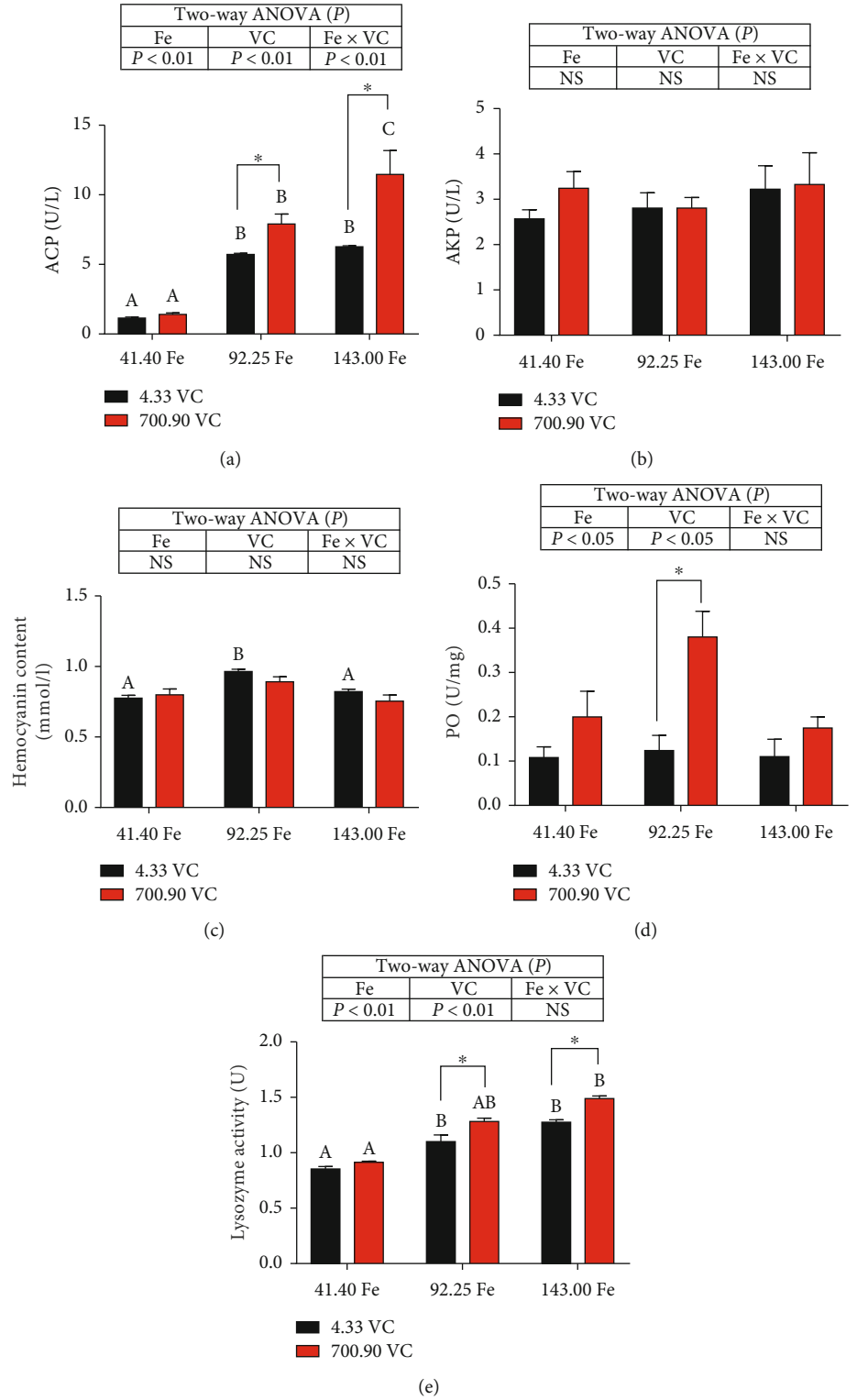

Figure 6: Effects of dietary levels of iron and vitamin C on the nonspecific immunity of hemolymph of *Eriocheir sinensis*. (a) ACP: acid phosphatase; (b) AKP: alkaline phosphatase; (c) hemocyanin; (d) PO: phenoloxidase; (e) bacteriolytic activity. Values are means ($n = 4$ replicate tanks) with standard errors represented by vertical bars. Different letters indicate significant differences between different iron levels in the same vitamin C levels ($P < 0.05$). * indicates significant differences between different vitamin C levels in the same iron levels ($P < 0.05$).

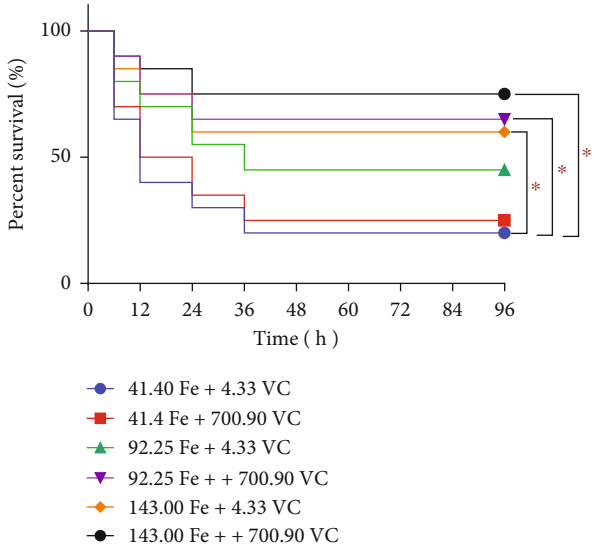

Figure 7: Effects of dietary levels of iron and vitamin C on the survival rate of *Eriocheir sinensis* after *Aeromonas hydrophila* challenge for 96 h.

ferroportin. Subsequently, the iron in the hemolymph would be transported to target cells by transferrin [45]. Previous studies showed that vitamin C could accelerate the iron released from transferrin in cells and increase cell iron uptake [46]. Besides, vitamin C can induce ferritin synthesis, the main storage protein of iron, to improve cell iron storage [47]. In the present study, the transferrin content in the hemolymph and the iron content in the hepatopancreas of crabs were increased when diets were supplemented with vitamin C, suggesting that dietary vitamin C can promote cell iron deposition. Dietary vitamin C significantly enhanced iron deposition in the liver of channel catfish (*Ictalurus punctatus*) with a diet with 300 mg/kg of iron [48]. This may account for the increased absorption and storage of iron in the liver by vitamin C. Dietary vitamin C improved the carcass iron of growing chicken compared with diets without vitamin C [16]. However, vitamin C does not affect the whole body's iron content. Iron is mainly stored in the hepatopancreas, while other tissues including muscle and carapace have less iron content, resulting in indistinctive changes in the whole-body iron. Further studies are needed to elucidate the role of vitamin C in iron metabolism.

In the present study, dietary-supplemented 41.40 mg/kg of iron decreased the antioxidant capacity of *E. sinensis*. Iron participated in the synthesis of antioxidant enzymes to defend lipid peroxidation in the organism [49]. Therefore, it suggested that iron deficiency affects the activities of antioxidant enzymes and decreases the antioxidant capacity. It is well known that vitamin C is a good antioxidant [50], and it is an indispensable ingredient in feed [51]. A previous study reported that appropriate dietary vitamin C increased the activities of antioxidant enzymes and reduced the MDA content to improve the antioxidant capacity of red swamp crayfish (*Procambarus clarkii*, Girard) [52]. However, in the present study, vitamin C did not affect the antioxidant enzyme activities when crabs were fed the same iron level. A study also showed no significant differences in glutathione content, oxidized glutathione content, and glutathione peroxidase activity of African catfish (*Clarias lazera*) fed different vitamin C/iron ratio diets [53]. This study hypothesized that the sensitivity of GSH metabolism to vitamin C was low, and the antioxidant state of *E. sinensis* was influenced by age or the maturation process. So far, the mechanism of the dietary levels of iron and vitamin C on antioxidant capacity has not been elaborated thoroughly. Therefore, further studies are needed to determine the effect of the interaction between iron and vitamin C on the antioxidant capacity.

Previous evidence reported that iron could regulate animal immunity, including macrophage polarizations, neutrophil recruitment, and NK cell activity [54]. Dietary iron deficiency aggravated the enteritis of grass carp (*Ctenopharyngodon idellus*) [55]. In the present study, the immune enzyme activities of crabs decreased when fed diets without iron supplementation, suggesting that iron deficiency could inhibit the nonspecific immunity of crabs. Conversely, the dietary vitamin C enhanced the nonspecific immunity and the resistance to *Aeromonas hydrophila* of *E. sinensis*. On the one hand, dietary vitamin C supplementation may promote iron absorption through the increased *dmt1* mRNA expression level, as mentioned above, to improve the nonspecific immunity of *E. sinensis*. On the other hand, vitamin C also has positive effects on the health of animals [56]. In other aquatic animals, dietary supplementation with appropriate levels of vitamin C reduced the mortality of red swamp crayfish (*Procambarus clarkii*, Girard) after the white spot syndrome virus (WSSV) challenge [52]. In addition, it activated the immune responses in the hemolymph cell of the swimming crab (*Portunus trituberculatus*) to resist *Mesanophrys* sp. by increasing the enzyme activities of PO, lysozyme, and SPs and activating the immune-related signaling pathways [57]. A high level of vitamin C (3000 mg/kg) stimulated the macrophage chemotaxis; however, different levels of iron and vitamin C had no significant effects on the resistance of channel catfish (*Ictalurus punctatus*) to *Edwardsiella ictaluri* [48]. These results suggested that a delicate balance exists between the need for vitamin C to stimulate immune responses and the need for iron and vitamin C for host defense mechanisms. The results differed from our findings, which may be because of different species or feed stages. Therefore, it requires further studies to explore the effects of dietary iron and vitamin C on immunity and resistance to pathogens.

5. Conclusion

This study suggested that dietary iron deficiency impaired the growth performance, antioxidant capacity, and immunity of *E. sinensis*. Therefore, dietary 92.25 mg/kg of iron and 700.90 mg/kg of vitamin C are recommended to improve crab growth performance and nonspecific immunity. Meanwhile, when crabs were fed 92.25 and 143.00 mg/kg of iron diets, the dietary vitamin C enhanced the expression of *dmt1* and *fpn* in the hepatopancreas and intestine and increased the iron content and transferrin content in the hemolymph and hepatopancreas iron deposition to facilitate the iron absorption and utilization of crabs. In

addition, dietary iron and vitamin C can effectively enhance the resistance of crabs against *Aeromonas hydrophila*. Therefore, dietary vitamin C may be a vital nutrient to enhance the immunity of *E. sinensis* by promoting iron absorption. Furthermore, it provides a reference for developing functional diets to increase disease resistance in aquaculture. Nonetheless, further studies are needed to explore the possible pathway of dietary iron and vitamin C on growth performance, iron metabolism, antioxidant capacity, and immunity in *E. sinensis*.

Data Availability

The sequencing data used to support the findings of this study are available from the corresponding authors upon request.

Conflicts of Interest

There is no potential conflict of interest.

Authors' Contributions

Ying Song, Xiaodan Wang, and Liqiao Chen initiated the research ideas and designed the experiments. Ying Song, Xinyu Cai, Xianyong Bu, Shubin Liu, Mingqi Song, and Yiwen Yang performed the experiments. Qingchao Shi provided experimental materials and analysis tools. Ying Song drafted the manuscript. Jianguang Qin, Xiaodan Wang, and Liqiao Chen revised the manuscript.

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