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Effects of microcystin-LR on the immune dysfunction and ultrastructure of hepatopancreas in giant freshwater prawn *Macrobrachium rosenbergii*

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ABSTRACT

Microcystins (MCs), produced by cyanobacteria, can strongly inhibit the activity of protein phosphatase, and exhibit strong hepatotoxicity. *Macrobrachium rosenbergii* is an important aquaculture economic species. Cyanobacterial blooms occur frequently during the culture of *M. rosenbergii*. However, the effects of MCs on the *M. rosenbergii* immune function have not been studied. In the present study, *M. rosenbergii* were exposed to environment-related concentrations of MC-LR type (0.5 and 5 μg/L) for 3 weeks. Hepatopancreatic histology was investigated, and antioxidant enzymes activity, acid phosphatase, alkaline phosphatase and lysozyme activity in hepatopancreas were also analyzed. Results showed that MC-LR could damage *M. rosenbergii* hepatopancreas, induce hepatopancreatic apoptosis and antioxidant dysfunctions. The expression profiles of major immune-related genes after MC-LR exposure were also detected. Some genes with antibacterial functions were suppressed, and the expression of apoptosis-related genes were up-regulated. After MC-LR exposure, the cumulative mortality of *M. rosenbergii* infected with *Vibrio vulnificus* and *Aeromonas hydrophila* were much higher than the control in a time-dose dependent manner. These results indicated the potential negative influence of MC-LR on the immune function of *M. rosenbergii*.

1. Introduction

With the accumulation of nitrogen and phosphorus in water environment, once the temperature and light reach appropriate conditions, the cyanobacteria blooms occur frequently and seasonally [1]. The outbreak of cyanobacteria produces a variety of toxins such as peptides and alkaloids [2]. Among these toxins, microcystins (MCs) are the most frequently occurring, widely distributed and endangered class of cyclic peptide toxins [3]. To date, approximately 90 species of MCs have been identified and isolated, while microcystin-LR (MC-LR) is the most widely distributed and abundant in the environment [4]. During the nature water blooms, the concentration of MCs could be generally between 0.1 and 10 μg/L, while it could reach as high as tens μg/L in the high season of algae. Ye et al. [5] showed that the maximum MCs concentration reached 2.75 ± 0.27 μg/L in the bloom period in the northern lake areas. Li et al. [6] showed that MC-LR was 0.97 μg/L in the slight discoloration pond water, 4.91 μg/L in the discoloration pond water, and 50.84 μg/L in the large discoloration pond water. However, there are obvious cyanobacterial blooms in the culture ponds of *M. rosenbergii* during the production process, which could result in reduced yield and frequent diseases.

MCs can inhibit the activity of protein phosphatase and have strong hepatotoxicity. Till present, the hepatotoxic effects and mechanisms of MCs have been extensively studied among mammals, fish and some invertebrates [7–9]. MCs can disrupt ROS balance in animals, leading to oxidative stress and damage to animal cells [10]. After intraperitoneal injection of MCs into tilapia *Oreochromis niloticus*, the ROS levels in the hepatopancreas and gill changed significantly [11]. Some studies have demonstrated that MC-LR could damage the cell DNAs at time-dose dependence, and inhibit the repair activity of DNA [12,13]. They could also induce the apoptosis in cells by regulating the expression of apoptosis-related genes *bcl2*, *bax* and *p53* [14,15]. MCs exhibit multi-organ toxicity and cause different degrees of damage to the animal's organs. At present, several reports focus on the effects of MCs on animal immune system [16]. MCs can induce the decrease of lymphocyte count in mice, inhibit the activity of T and B cells, reduce the phagocytic ability of macrophages, and directly damage the DNA of lymphocytes [17,18]. However, at present, most of the exposure experiments on the immune system are concentrated on mammals. Few studies on aquatic invertebrates that are more affected by MCs, have been reported.

M. rosenbergii is a kind of arthropod phylum, belonging to *Genus Macrobrachium*, which is the largest freshwater prawn around the

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world. Due to its fast growth, easy domestication, wide appetite, strong adaptability and short growth cycle, *M. rosenbergii* has high economic efficiency in breeding, and already developed into a special aquaculture species with large aquaculture and development prospects [19]. In the past few years, the *M. rosenbergii* farming industry has developed rapidly in China and achieved good economic benefits [20]. However, there are obvious cyanobacterial blooms in the culture ponds of *M. rosenbergii* during the production process, which could result in reduced yield and frequent diseases [21]. The increase in the amount of cyanobacteria could produce a large amount of MCs. As reported in other studies, the crustacean's immune system is relatively simple, mainly based on innate immune defense [22]. However, there are few reports on the immune effects of MCs on *M. rosenbergii*. For the hepatopancreas and hemolymph are generally considered as the main target organ of prawn, in the present research, we studied the damage of the hepatopancreas of *M. rosenbergii* exposed to environment-related concentration MC-LR, determined the effect of MC-LR on the expression of major immune-related genes and immune related enzyme activity in hepatopancreas. These results could not only reveal the effect of MC-LR on the immune function of *M. rosenbergii*, but also have important significance for assessing the damage of MCs to aquatic organisms.

2. Materials and methods

2.1. Animal treatments and sample collection

Five months old *M. rosenbergii* were purchased from a *M. rosenbergii* farm in Gaoyou city (Jiangsu Province, China). They were raised in glass tanks with dechlorinated tap water (28 ± 0.5 °C) with a 14 h/10 h light/dark cycle and fed with *M. rosenbergii* special feed twice a day. After 2-weeks acclimation, the prawns (11.31 ± 1.05 g) were randomly selected and exposed to MC-LR (Yuanye Biological Technology Co., Shanghai, China) in glass tanks (about 1 L water/g shrimp) for 1, 2 and 3 weeks in triplicate, 9 prawn per tank. The exposure dose of MC-LR (0, 0.5 and 5 μg/L) was selected based on its concentration in aquatic environment [4,5]. During exposure, half of the exposure water in each tank was replaced daily with fresh dechlorinated tap water and dosed with the appropriate amount of MC-LR. The prawns were sacrificed after 1, 2 and 3 weeks exposure. Following dissection, hepatopancreas were weighed for calculation of the hepatosmatic index (HSI, HSI = 100% * hepatosmatic weight/body weight). The precipitate is used to extract RNA. Hepatopancreatic tissue were fixed with Bouin's solution for histopathological examinations, and fixed with 2.5% glutaraldehyde solution for transmission electron microscopy (TEM) analysis. Samples for quantitative real time PCR (qPCR) and enzyme activity analysis were snap frozen in liquid nitrogen and stored at −80 °C.

2.2. RNA extraction, reverse transcription and quantitative real-time PCR (qPCR)

Total RNA of hepatopancreas (9 samples per treatment, 3 samples per replicate) were isolated using trizol one-step method. RNA integrity was checked by analyzing 28S ribosomal RNA (rRNA) and 18S rRNA ratios with 1% agarose gel electrophoresis. The concentration and purity of isolated RNA were assessed by the spectrophotometric method with a nanodrop spectrophotometer (Thermo Electron Corporation, USA). The cDNAs were synthesized from total RNA with M-MLV reverse transcriptase with gDNA wiper (Vazyme), random primer and oligo $(dT)_{18}$ primer in a 20 μL final reaction volume.

qPCR was applied to evaluate mRNA expression profiles of immune related genes. These genes were including *Crustin 1*, *Crustin 3*, *lysozyme (i-type)*, *prophenoloxidase (proPO)*, *hemocyanin 1*, *anti-lipopolysaccharide factor (ALF1, ALF4)*, *Caspase 3*, and *Apoptosis-inducing factor 1 (AIF1)*. 18S rRNA and eukaryotic translation elongation factor 1 alpha (*ef1a*) were selected as the reference genes in the present study. The primers were listed in Table 1. The qPCR efficiency (E) of each PCR reaction was calculated, and the E values were all between 90 and 110%. The relative transcript changes under MC-LR exposure were calculated using $2^{-\triangle\triangle}C_q$ method with formula $F = 2^{-\triangle\triangle}C_q$, C_q = ($C_{q,\ \text{target gene}}$ - $C_{q,\ \text{mean value of reference gene}}$) MC-LR - ($C_{q,\ \text{target gene}}$ - $C_{q,\ \text{mean value of reference gene}}$) control.

Table 1
Primers used for qRT-PCR.

Gene	Name	Sequence
18S	F	TGACGAAAAATAACAATGCGG
	R	GCGGGACACTCGGTAAAGA
ef1a	F	AGGAAGATTGAACGCAAGAGTG
	R	GAGCAAAGTTGACCACCATACC
Crustin1	F	CAGTGTGGGATGTCGGAGGT
	R	GCTGTCATGGCATTTGGCA
Crustin3	F	CGTCTCTGCCACTGGGTTAG
	R	GGTTTGGAGCATAGCACATTG
Hemocyanin 1	F	AGAACAAGAGTTGAACGGAAAT
	R	CTGGTGGACTGTGGAGCA
Lysozyme-i	F	TGGGTGTGCATCGCTCAGTA
	R	CCAGGCTTCCGTGTCTTTGA
proPO	F	CGTGGCTGGAGGCGATGA
	R	CCAATGAGGGCAAGAGGGG
ALF1	F	GGCAGTCCAAGATTTCCT
	R	TACCTTTGTAACGGCTCA
ALF4	F	CAACGGCACAATCAAGAG
	R	TGAGGACACCGAGACGAG
Caspase 3	F	CCGTGGAGTAACTTGTAC
	R	CCAATAAGTGGAGGATTA
AIF1	F	TATTGTTGACTCATCCCTACC
	R	CTGCCATCTGTTCTGTTTCT
SOD	F	ACCAACCAACAGAGCCAATA
	R	TGCTGACAAGCCACAACC
GSH	F	CCACCCAAGGCACCATCA
	R	GCAGGAAAGGGCAAGCAG
POD	F	CCACCCAAGGCACCATCA
	R	GCAGGAAAGGGCAAGCAG

2.3. Effects of MC-LR on immune-related enzyme activity in hepatopancreas of M. rosenbergii

Hepatopancreas (9 samples per treatment, 3 samples per replicate) tissues were collected from different shrimp at different time points. The enzyme activities of antioxidant enzyme, including superoxide dismutase (SOD), glutathione peroxidase, and peroxidase, were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute). The immune-related acid phosphatase and alkaline phosphatase, and lysozyme activities were also measured according to commercial kit (Nanjing Jiancheng Bioengineering Institute). After homogenization in ice bath, and centrifugation at 3500*g* for 10 min, the supernatant was used to determine the activity of these enzymes. The protein concentrations of supernatant were measured by BCA commercial protein assay kit (Beyotime Biotechnology). The final data were expressed as enzyme unit per protein (U/mg). For each sample, three independent experiments were conducted as the replications.

2.4. Histopathological examinations

For paraffin sections analysis, prawns (6 hepatic samples per treatment, 2 samples per replication) were fixed with Bouin's solution at 4 °C for 48 h, and then dehydrated through ascending grades of alcohol series, decolorization with xylene and embedded in paraffin. Six μm-thick paraffin sections were cut with a rotary microtome (Leica). And stained by haematoxylin eosin methods. Microscopic examination was carried out using CHC binocular microscope (Olympus). For TEM analysis, after fixed in glutaraldehyde overnight, the hepatic tissue was then washed in phosphate buffer (PBS) and post-fixed in 1% osmium tetroxide at. After dehydrated in a graded series of alcohol and

embedded with LR white resin, the blocks were cut on microtome (ULTRACUT, Leica Microsystems Ltd). Ultrathin sections, 80 nm thick, were contrasted with uranyl acetate and lead citrate, and examined with a transmission electron microscope (HT7700, Hitachi).

2.5. The accumulated mortality rate of shrimp with bacterial infection after long-term MC-LR exposure

After 2-weeks acclimation, the prawns (11.31 ± 1.05 g) were randomly selected.

To further validate the long-term exposure of MC-LR to the immunity of shrimp, we conducted bacterial infection experiment. After 0.5 and 5 μg/L MC-LR exposure for 3 weeks, *M. rosenbergii* was infected with *Vibrio vulnificus* and *Aeromonas hydrophila*, respectively. After acclimation for 2-weeks, the prawns (6.22 ± 0.53 g) were selected to measure the accumulated mortality rate. The bacteria strains were isolated and stored by our lab. After overnight culture, the bacteria were collected and washed by sterile PBS for three times, and re-suspended by PBS at 1×10^7 cfu/mL. Each shrimp was intramuscularly injected with 100 μL bacterial solution. Each group had 30 different individual shrimp. The *M. rosenbergii* without MC-LR exposure was used as a control group. Then the different groups were breeding in the same environmental conditions. After 1 week bacterial challenges, the cumulative mortality among different groups were counted. Each group had three replications.

2.6. Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). Data were tested for normality of distribution (Shapiro-Wilk test) and homogeneity of variance (Levene's test) prior to analysis. Data that did not meet normality and homoscedasticity were transformed (lg) and then analyzed by one-way ANOVA. In the data sets for which there was a significant difference, a Tukey post hoc test was carried out.

3. Result

3.1. The MC-LR exposure change the relative expression profiles of immune-related genes

To further understand the effects of MC-LR exposure on *M. rosenbergii* immunity, some immune-related effector molecules in hepatopancreas were also detected. These immune-related effector molecules include *Crustin 1*, *Crustin 3*, *lysozyme-i*, *prophenoloxidase (proPO)*, *hemocyanin*, *anti-lipopolysaccharide factor (ALF1, ALF4)*, *Caspase*, and *Apoptosis-inducing factor 1 (AIF1)*. As shown in Fig. 1, compared with control, both 0.5 μg/L and 5 μg/L MC-LR could suppress the expression of *Crustin 1, Crustin 3, lysozyme-i, proPO, ALF1* and *hemocyanin*. And with the increase concentration of MC-LR, the inhibition effect was more obvious. Some apoptosis-related genes such as *Caspase* and *AIF1* were also detected. As shown in Fig. 1H and I, at the initial 1 and 2 week, 0.5 μg/L MC-LR did not significantly induce the expression of apoptosis genes, while the expression of the two genes were up-regulated in week 3. Different from the low concentration, 5 μg/L MC-LR could significantly up-regulate the expression of the two genes, inducing cell apoptosis, which was consistent with the followed microscope observations (results 3.3 part).

3.2. Effect of chronic infection of MC-LR on enzyme activities and genes expressions

Some anti-oxidase enzymes including SOD, glutathione peroxidase and peroxidase, had been detected in *M. rosenbergii* hepatopancreas after MC-LR exposure. As shown in Fig. 2A, 2B and 2C, in a short time 1w, the exposure of MC-LR could lead to a decrease in these antioxidant enzyme activities in shrimp. However, with the prolongation of time, these enzymes activities became higher than that of control, and showed a certain upward trend. At different concentrations, the impact of decline and upward trend induced by 5 μg/L were greater than that of 0.5 μg/L, showing a dose-dependent effect.

The alkaline phosphatase (ALP) and acid phosphatase (AP) activities were also detected after MC-LR exposure. As shown in Fig. 2D and E, the activities could be significantly changed by MC-LR. Under the 0.5 μg/L exposure, the activities of ALP and AP were both enhanced and reached maximum at 2 w, but significantly decreased at 3 w. However, under 5 μg/L MC-LR exposure, the activity of ALP was significantly inhibited by MC-LR at all time points. Lysozyme was an enzyme that played a direct immune function in crustaceans. In the present study, 0.5 and 5 μg/L MC-LR could affect the activities of lysozyme and reduce the functions of lysozyme, and this inhibitory effect showed a dose-dependent effect.

In hepatopancreas tissues, the expression profiles of antioxidase genes had been detected after different MC-LR exposure. Three different antioxidase genes including *SOD, GSH* and *POD* had been selected. From the results, as shown in Fig. 3, 0.5 μg/L and 5 μg/L MC-LR could significantly up-regulate the expression levels of these genes. Higher concentrations of MC-LR exposure had a greater effect on gene expression. These results indicated MC-LR could cause oxidative damage repair disorder in *M. rosenbergii.*

3.3. The observation of MC-LR on the hepatopancreas microstructure and ultrastructure

From the results, 5 μg/L MC-LR showed strong damage to the hepatopancreas of *M. rosenbergii*: the connective tissue between the hepatic tubules disappeared, the wall of the tubules dissolved, and this toxic effect was aggravated with time (Fig. 4). After week 1 and 2, low concentration MC-LR had no obvious damage to the hepatopancreatic structure, when the exposure time was extended to 3 weeks, the connection between the hepatopancreatic tubules in the low concentration group was also destroyed and the spacing between the tubules increases (Fig. 4).

Fig. 5 shows the ultrastructure of the hepatopancreas from the control and 5 μg/L MC-LR exposure groups. It is clearly shown that the microvilli of the hepatopancreas in the normal group are relatively straight and arranged neatly, while the microvilli of the treatment group were curved and arranged disorderly (Fig. 5A and D). Compared to the control groups, the endoplasmic reticulum of the treatment group proliferated significantly (Fig. 5C, F, G and I). Mitochondria were swollen and the number is significantly increased in MC-LR exposed group (Fig. 5C and I). Meanwhile, the condensed hepatocyte nucleus was also found in MC-LR exposure groups, and indicated apoptosis was induced by MC-LR (Fig. 5B and E). In addition, lots of lamellar bodies were observed in the MC-LR treated hepatopancreas (Fig. 5H).

3.4. Chronic exposure of MC-LR affected the body weight, hepatosmatic weight and HSI of M. rosenbergii

After 1 and 2 weeks' exposure, MC-LR didn't show any significant influence on the body weight and HSI. At week 3, high MC-LR (5 μg/L) significantly decreased the body and hepatosmatic weight, while low MC-LR (0.5 μg/L) significantly decreased HSI (Table 2).

3.5. The accumulated mortality rate of M. rosenbergii after bacterial infections

After exposure by MC-LR, the *M. rosenbergii* in different groups were injected with *V. vulnificus* and *A. hydrophila*, which were the common pathogens during the breeding. From the results, as shown in Fig. 6A, the accumulated mortality in "0.5 μg/L + *V.v*" and "5 μg/L + *V.v*" groups were much higher than "Control + *V.v*" group. Meanwhile, the accumulated mortality in "5 μg/L + *V.v*" group was higher than in

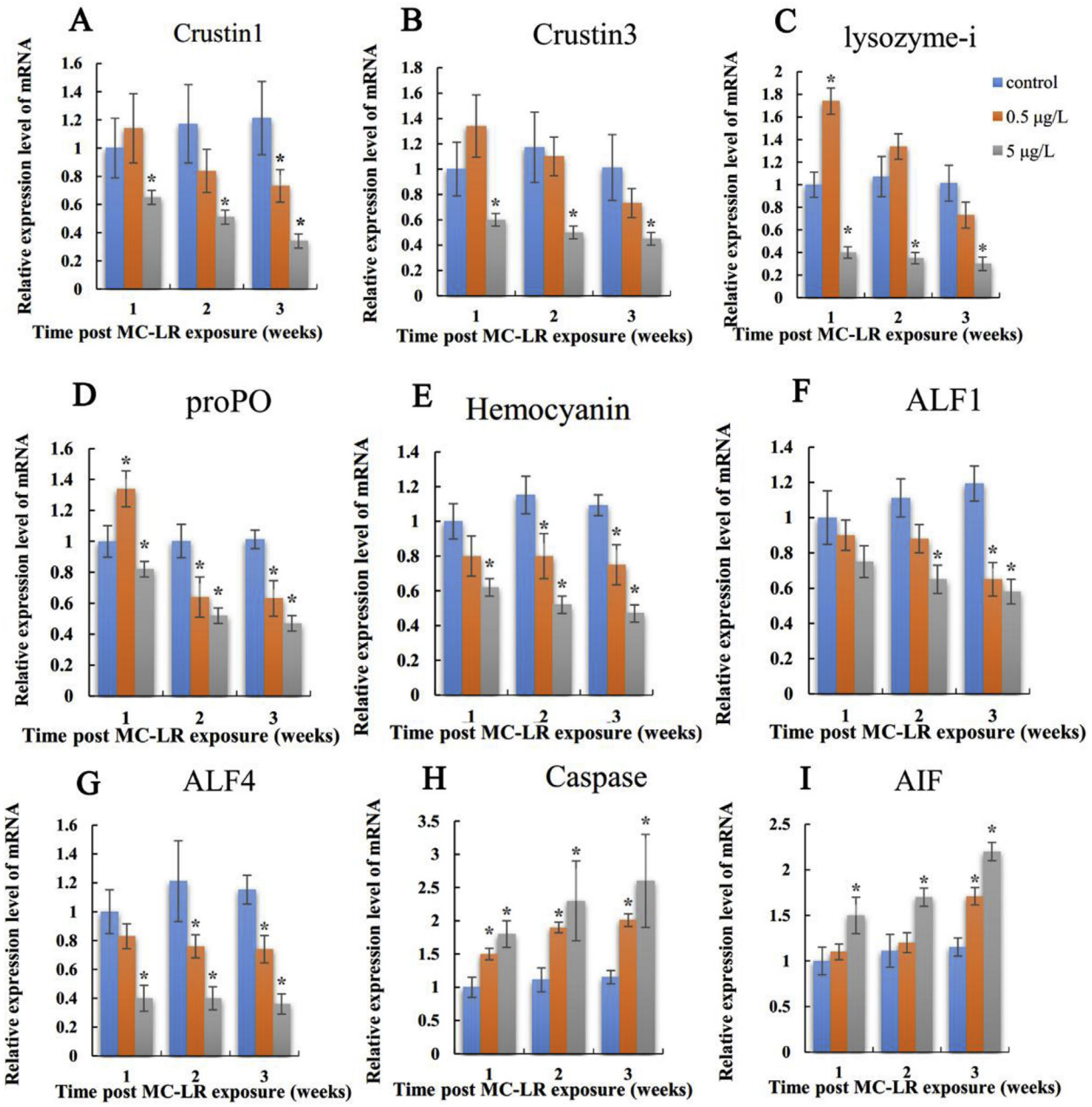

Fig. 1. Hepatopancreatic immune-related gene expression profiles in response to MC-LR exposure. The X axis represents the MC-LR exposure time and the Y axis represents the relative expression of the gene (relative to the internal reference *ef1α* and 18S). All data were expressed as mean ± SEM (n = 3). Bars with asterisk * indicated significantly difference ($p < 0.05$).

"0.5 μg/L + *V.v*" group. The uninfected groups showed lower accumulated mortality than the infected groups. As shown in Fig. 6B, under *A. hydrophila* infections, the overall trend was consistent with Fig. 6A. After 5 μg/L MC-LR exposure for 3 weeks, the shrimp showed lower resistance to *A. hydrophila* infections, resulting in an increased mortality. The "5 μg/L + *A.h*" group showed the highest accumulated mortality among all the groups. These results indicated that after MC-LR exposure, the cumulative mortality of *M. rosenbergii* infected with *V. vulnificus* and *A. hydrophila* were much higher than the control in a time-dose dependent manner.

4. Discussion

MCs are widely distributed in freshwater. The World Health Organization (WHO) has set a provisional guideline of 1 μg/L MCs in water destined for human drinking water, but the content could be several to tens μg/L, even as high as 2.5 mg/L in cyanobacterial blooms occur waters [4,5]. At present, a lot of reports have studied the toxicity mechanism of MCs, mainly focusing on liver damage, cytoskeletal disruption, reproductive toxicity and oxidative stress [15,23–26], while little studies focused on the immune damage of MCs. Nowadays, *M. rosenbergii* has become an important freshwater economic shrimp in China [27], but in the process of breeding, obvious cyanobacterial blooms in the culture ponds. However, there are still no reports in the long-term effects of different MC-LR concentrations on *M. rosenbergii* immune functions. So in the present study, to discuss the potential effects of cyanobacterial outbreaks on the production of *M. rosenbergii* in culture ponds, we have studied the effects of environmentally relevant concentrations of MCs on *M. rosenbergii* in production, focusing on its the growth and immunization influence.

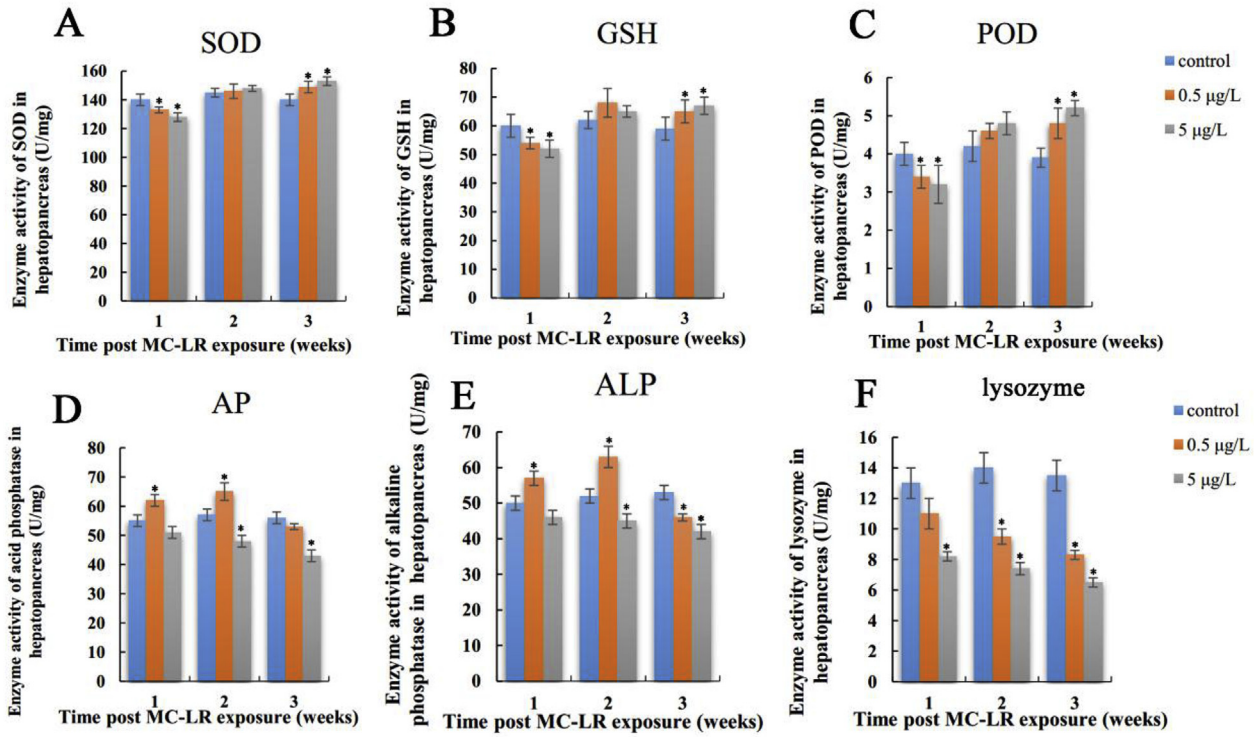

Fig. 2. The Hepatopancreatic enzymes activities in response to MC-LR exposure. SOD (A), glutathione peroxidase (B), peroxidase (C), alkaline phosphatase (D), acid phosphatase (E) and lysozyme (F)were included. All data were expressed as mean ± SEM (n = 3). Bars with asterisk * indicated significantly difference ($p < 0.05$).

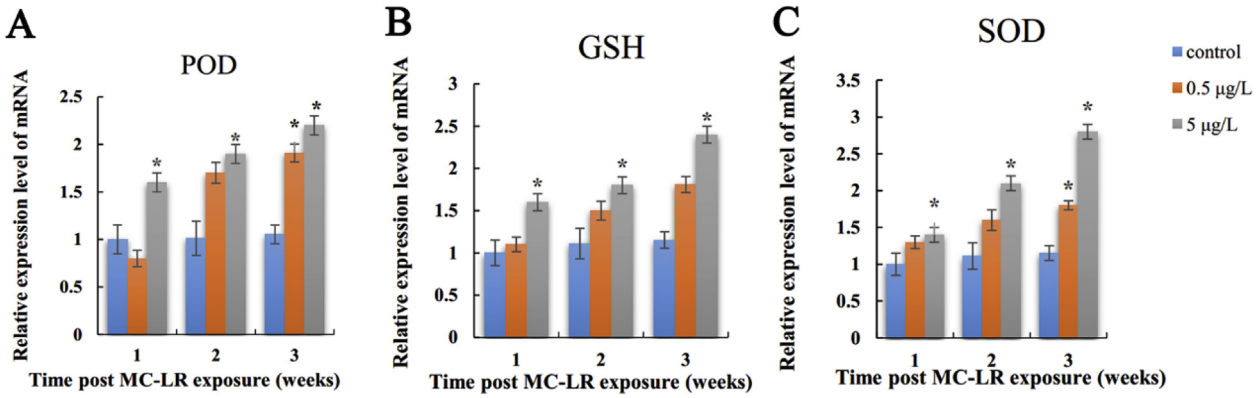

Fig. 3. Hepatopancreatic antioxidase genes expression profiles in response to MC-LR exposure. The X axis represents the MC-LR exposure time and the Y axis represents the relative expression of the gene. All data were expressed as mean ± SEM (n = 3). Bars with asterisk * indicated significantly difference ($p < 0.05$).

Crustaceans like *M. rosenbergii* mainly depend on their innate immune system to defense against the invading pathogens. The hepatopancreas and hemolymph play important roles in the immune system for the enrichment of immune effectors and pattern recognition proteins (PRPs) [27,28]. The healthy development of the hepatopancreas is essential for the growth of *M. rosenbergii*. In the present study, the body weight of *M. rosenbergii* could be affected by MC-LR, accompanied by a decrease in the weight of hepatopancreas. The HSI is used to measure the liver function of fish and shrimp during the production [29,30]. Many studies have demonstrated HSI are rough measurements of the energy reserved nutritional state of fish and related to the growth situation [31,32]. Nutritional research often uses the HSI to indicate the health status of animals [33,34]. Some toxicological studies also use the HSI to compare the health status of shrimp [35]. The acute poisoning of MCs mainly leads to hepatic enlargement, congestion, hepatic hemorrhage and altered HSI [36]. From the results, the HSI was significantly reduced after MC-LR exposure, which could finally affect their immune functions. Meanwhile, the hepatopancreas is the main target organ of MCs. The hepatotoxicity of MC-LR has been reported in many aquatic animals [37,38]. The liver is thought to be the main target organ of MCs. MCs could specifically inhibit the activity of serine and the protein phosphatidic acid synthase 1 and 2A, thereby affecting intracellular protein phosphorylation and dephosphorylation, and destruction of the cytoskeleton, which ultimately leads to apoptosis and cell necrosis [39–41]. In the present study, the obvious hepatotoxicity of *M. rosenbergii* was found, and the molecular mechanism should be similar to the previous studies on the liver injury toxicity.

Apoptosis is an important means for body to clear aging cells and maintain the balance of body [42]. A lot of exogenous substances including MC-LR have been reported to induce apoptosis in the body [15]. Till present, a lot of researches have studied the mechanism of MCs inducing apoptosis in different cells of various animals [43,44]. In the present study, observing the hepatopancreatic ultrastructure after MC-LR exposure, it showed that the hepatopancreatic cell nucleus was highly shrunk and apoptotic bodies were produced. Moreover, the endoplasmic reticulum of the treatment group proliferated significantly

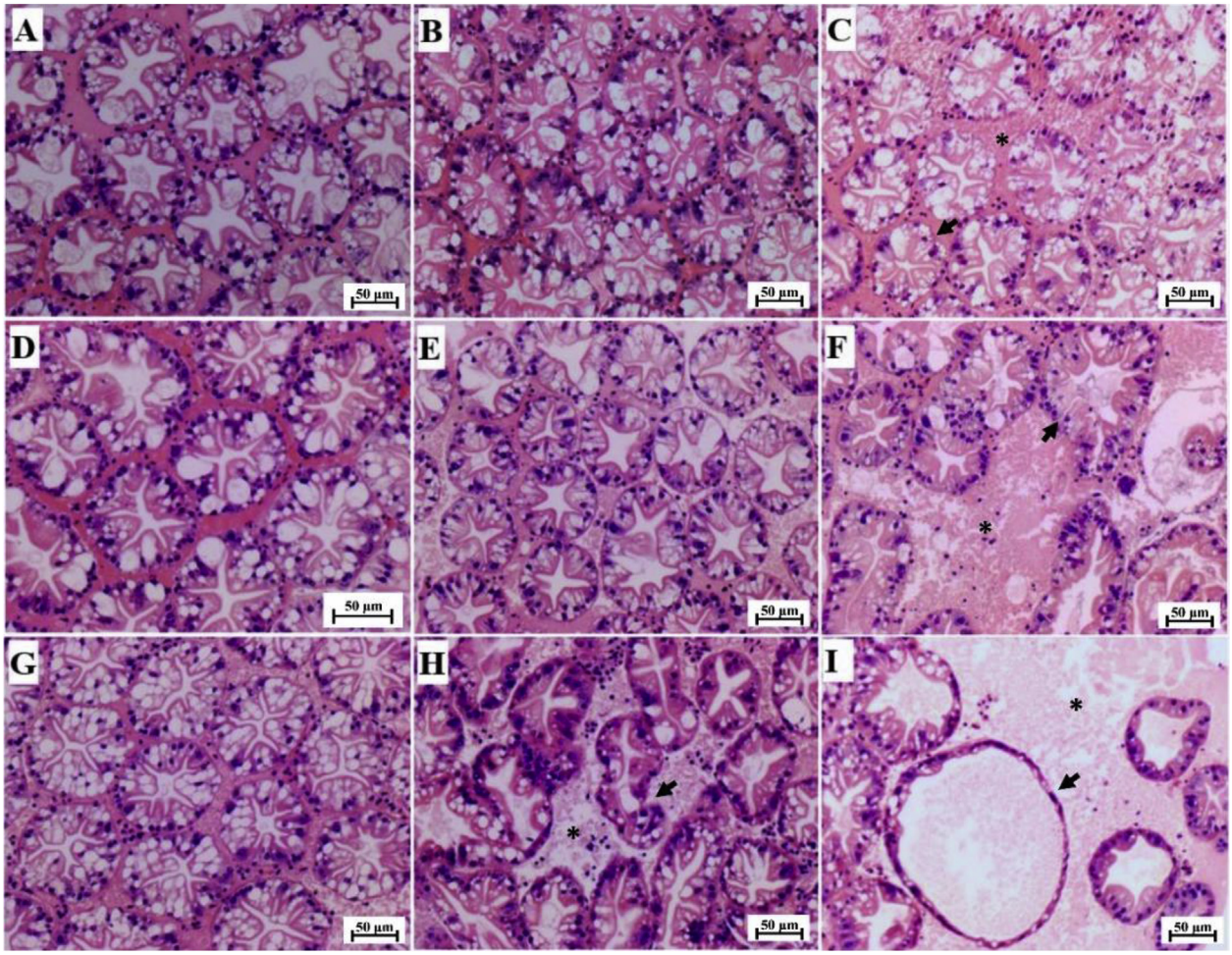

Fig. 4. Change of hepatopancreas microstructure following MC-LR exposure. A: 1w Control; B: 1w 0.5 μg/L MC-LR; C: 1w 5 μg/L MC-LR; D: 2w Control; E: 1w 0.5 μg/L MC-LR; F: 1w 5 μg/L MC-LR; G: 1w Control; H: 1w 0.5 μg/L MC-LR; I: 1w 5 μg/L MC-LR. The asterisk indicates that the connective tissue between the hepatic tubules is dissolved. The arrow indicates that the hepatic tubule wall is gradually thinning and disappearing.

and mitochondria were swollen. It was accordance with previous study that mitochondria dysfunction was induced by MC-LR in mice [45]. The endoplasmic reticulum stress could also be induced by MC-LR [46]. Lamellar bodies (LBs) are laminate structures composed of concentric phospholipid bilayers as the anion section [47]. It is supposed that these structures are synthesized in dictyosomes, at first usually in a form of multivesicular bodies [48]. Some studies revealed that lamellar body is secreted in response to barrier perturbation [49]. The LBs also contain proteolytic enzymes and antimicrobial peptides, which when secreted along with lipid contribute to permeability barrier function [50]. The function of LBs in liver or hepatopancreas is not very clear, but considering its antibacterial effect in skin, we suspected that LBs observed in hepatopancreas may have the similar functions [51]. It is interesting that this is the first study to report that LBs in hepatopancreas could be induced by MC-LR exposure. But the specific functions of LBs during the toxicity stress need to be further studied.

SOD, GSH, and POD are the main antioxidant enzymes in body, which play important physiological roles in scavenging reactive oxygen species ROS and preventing biomolecular damage. Due to various external factors and internal factors of cells, it will lead to an increase in active oxygen in the body, or a decrease in the body's ability to scavenge reactive oxygen species, and then oxidative stress will appear in the body [52]. Studies have shown that MC can increase the level of ROS in rat stem cells in a time- and dose-dependent manner, and the high concentrations of MC could significantly inhibit the activity of SOD [53,54]. From the results, MC-LR can significantly affect the antioxidant enzyme activity in shrimp and disturb the antioxidant function in body. But this effect has a time and dose-dependent effect. In a short period of low concentration (0.5 μg/L), MC-LR could induce an increase in the activity of antioxidant enzymes in body to maintain the homeostasis of free radicals in the internal environment. However, with the increase of induction time and induction dose, MC-LR inhibited the activity of antioxidant enzymes and weaken the ability of antioxidant enzymes to scavenge reactive oxygen species. Acid phosphatase and alkaline phosphatase are not only important detoxifying enzymes in crustaceans, but also they play an active role in the body's immune system as an important component of lysosomal enzymes [55]. Studies have shown that low concentrations of MC can increase phosphatase activity *in vivo*, while high concentrations inhibit its activity. At the same time, the activity of phosphatase decreases with the prolongation of MC exposure time [56,57]. MC-LR affects the host's antioxidant capacity in a variety of ways, but the specific mechanism between immunity and antioxidant needs far more studied. Lysozyme is an important component of the innate immune system in crustaceans and is effective in removing foreign bodies from the body [22]. The present study indicated that MC-LR could inhibit the lysozyme activity *in vivo*. These results are consistent with previous studies [58]. These results indicate that MC-LR could cause antioxidant function disorder and inhibit the activity of immune-related enzymes in *M. rosenbergii*.

Some immune-related genes were selected to analyze the expression profiles after MC-LR exposure. In crustaceans, the immune effector molecules play a direct role in combating pathogenic microorganisms.

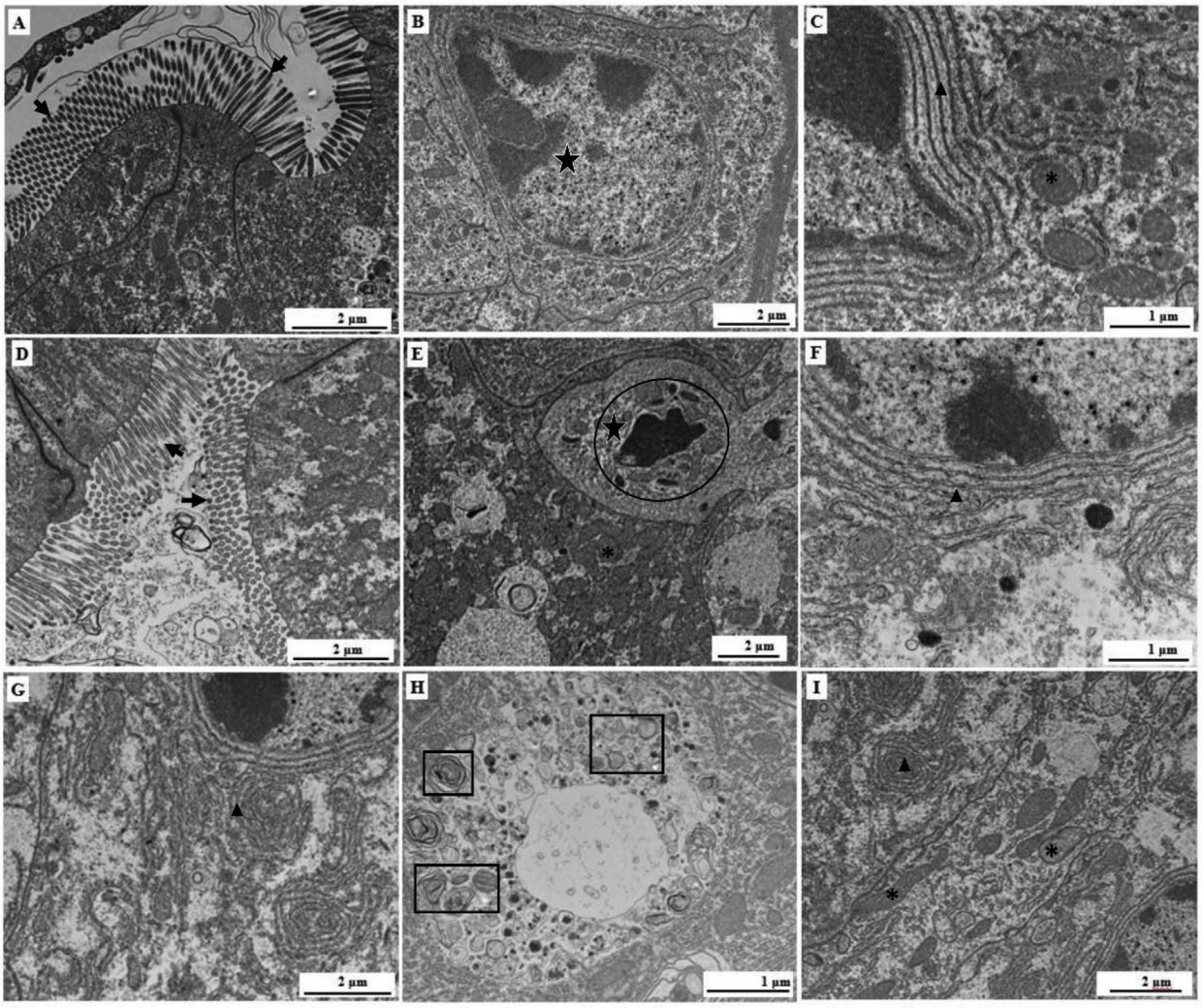

Fig. 5. Change of hepatopancreas ultrastructure following MC-LR exposure. A–C: TEM image from the control hepatopancreas; D–I: TEM image from the 5 μg/L MC-LR exposed hepatopancreas. Arrows indicate liver microvilli, pentagrams indicate hepatocyte nucleus, the circle indicates highly concentrated hepatocyte nuclei and apoptotic bodies, triangles indicate endoplasmic reticulum, and asterisks indicate mitochondria and black box indicate lamellar corpuscles. Exposure to MC-LR results in hepatopancreatic micro-villi confusing, hepatocyte apoptosis, endoplasmic reticulum hyperplasia, and increase in the number of lamellar bodies.

Table 2
Effect of MC-LR on the body weight, hepatopancreas weight and HSI of *M. rosenbergii*. All data were expressed as mean ± SEM. The different letters above the numbers indicate the significantly different ($p < 0.05$).

		control	0.5 μg/L	5 μg/L
Body weight (g)	1W	12.84 ± 0.88	13.70 ± 1.29	12.53 ± 1.00
	2W	12.03 ± 0.85	13.76 ± 0.71	13.62 ± 0.90
	3W	16.01 ± 1.01[a]	17.58 ± 0.72[a]	13.56 ± 0.64[b]
Hepatopancreas weight (g)	1W	0.54 ± 0.07	0.59 ± 0.06	0.49 ± 0.06
	2W	0.47 ± 0.03	0.57 ± 0.06	0.52 ± 0.05
	3W	0.62 ± 0.04[a]	0.55 ± 0.03[ab]	0.47 ± 0.05[b]
HIS (%)	1W	4.14 ± 0.25	4.17 ± 0.24	4.22 ± 0.21
	2W	3.92 ± 0.18	3.87 ± 0.25	3.85 ± 0.13
	3W	3.66 ± 0.11[a]	3.13 ± 0.12[b]	3.53 ± 0.13[ab]

To a certain extent, their expression levels reflect the immune status of the host. The functions of crustins, ALFs, lysozymes, proPO and hemocyanin especially defensing against bacteria have been well reported [22]. The crustins, ALFs and lysozymes are the classical antimicrobial peptide (AMPs) in crustaceans, and exhibit a broad spectrum of bactericidal activity and certain antiviral activity, which are the important effector molecules in the shrimp immune system [59,60]. proPO could active the humoral immunity of melanization, and plays an important role in the invertebrate immune system [61]. Hemocyanin also exhibit the antibacterial activity in shrimp [62]. From these results, MC-LR could stimulate the *M. rosenbergii* immune response, however, as the concentration increased or the exposure time prolonged, the expressions of most genes were inhibited, indicating the limited immune defense ability. Caspase 3, and Apoptosis-inducing factor 1 participated in the regulation of the apoptotic process in body. The high expression of these genes induced by MC-LR suggested the occurrence of apoptosis in the body. It was consistent with the microscope observations. Therefore, in the case of this immune function limitation, when *M. rosenbergii* was infected with harmful bacteria, the mortality rate becomes high. And it could be seen from the results that this effect was time and dose dependent.

In conclusion, in the present study, we examined the immunological effects of two environmentally relevant dose MC-LR on *M. rosenbergii*. Through histological sections and submicroscopic observations, MC-LR has a dose-dependent effect on hepatopancreas toxicity and can significantly induce hepatocyte apoptosis and antioxidant dysfunction. MC-LR exposure represses the immune response of *M. rosenbergii*, and affects the expression of immune-related genes. Under MC-LR exposure, bacterial infection could lead to a higher mortality of *M. rosenbergii*. The

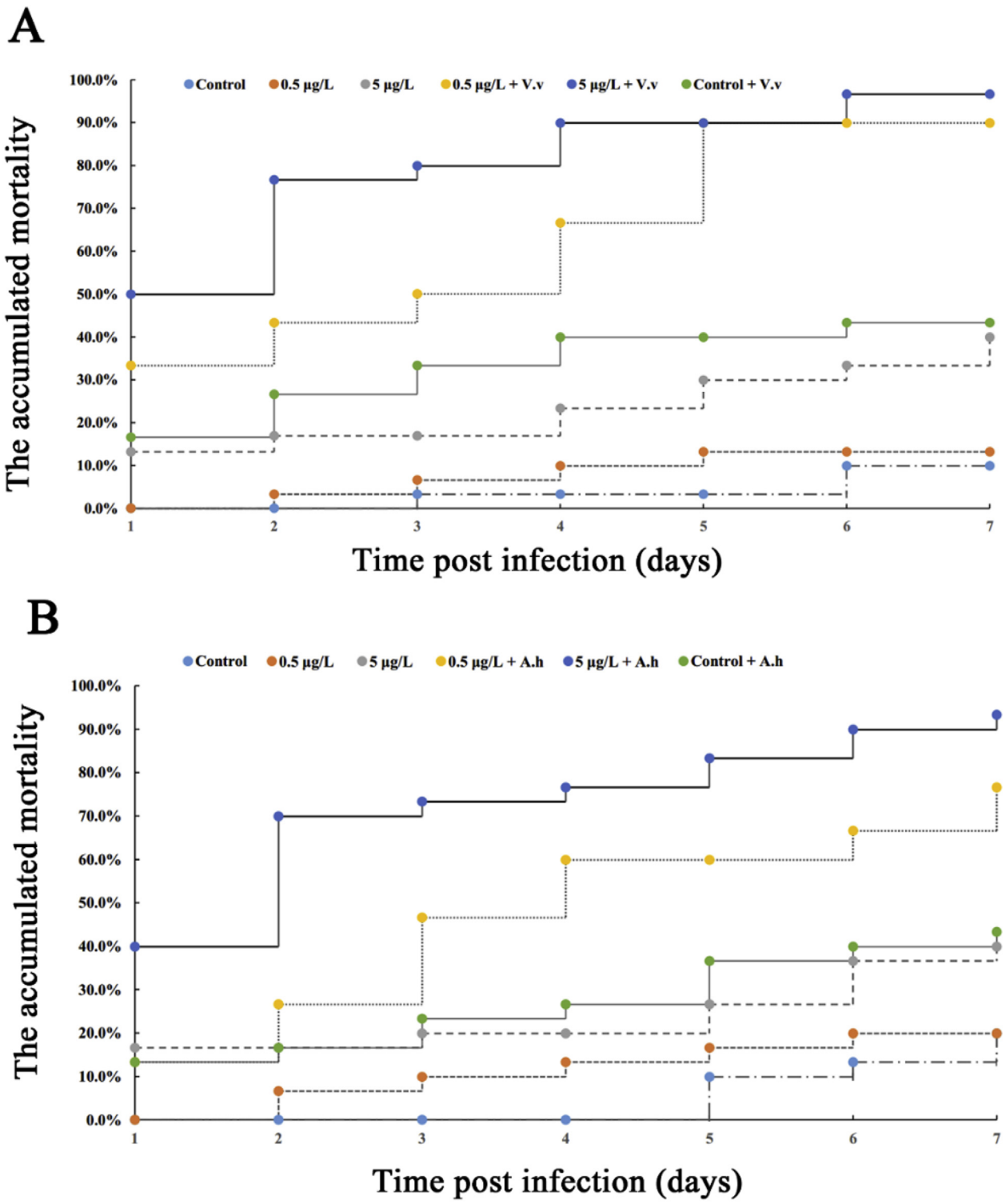

Fig. 6. The accumulated mortality rate of *M. rosenbergii* after *V. vulnificus* (A) and *A. hydrophila* (B) infections. Each group had three replications. All data were expressed as mean ± SEM (n = 3).

results of present study suggested that MC-LR had potential negative influence on the immunity of *M. rosenbergii*.

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