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Effect of pure microcystin-LR on activity and transcript level of immune-related enzymes in the white shrimp (*Litopenaeus vannamei*)

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Abstract Microcystins (MCs) in freshwater and marine waters released by toxin-producing cyanobacteria have negative impacts to the aquatic environment. This study aimed to investigate the effect of pure microcystin-LR on activity and transcript level of immune-related enzymes in the white shrimp *Litopenaeus vannamei*. After exposed to varying concentrations of pure microcystin-LR (MC-LR) for 30 days, the activity of superoxide dismutase (SOD), lysozyme (LZM), glutathione peroxidase (GPx), peroxidase (POD), acid phosphatase (ACP), alkaline phosphatase (AKP) and transcript level of *cMn-sod*, *lzm*, *gpx* were investigated in the hepatopancreas of white shrimp (*L. vannamei*). Immune-related enzyme activities responded differently to MC-LR exposure. SOD, GPx, and POD activity in the hepatopancreas were activated in a concentration-dependent manner while LZM activity was significantly inhibited in the treatment groups. ACP and AKP activity showed an increase, followed by a decrease. The transcript levels of *cMn-sod, lzm*, and *gpx* were consistent with changes in their encoding enzyme activity. These results demonstrated that sub-chronical exposure to MC-LR induced the alteration of immune-related enzymes and corresponding genes in the hepatopancreas, which may help explain the presence of detoxification mechanisms in crustaceans and how they were protected from MC-LR stress for a long period of time.

Keywords Microcystin-LR · Immune-related enzymes · Transcript level · *Litopenaeus vannamei*

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Introduction

Eutrophication in shrimp ponds frequently leads to rapid cyanobacterial growth during warm temperatures due to nutrient imbalances, which can diminish dissolved oxygen in water, elevate pH, and subsequently affect the growth of aquatic animals. Some genus of cyanobacteria release toxic microcystins (MCs), which may affect the growth of aquatic animals (Gonçalves–Soares et al. 2012).There are some cases where shrimps are more vulnerable to diseases (Zha et al. 2007; Peng et al. 2011). A shrimp culture usually takes several months to process; therefore, the investigation into the chronic effects of cyanotoxins on the immunocompetence of shrimps is imperative. One particular investigation of the verification of cyanotoxins revealed low concentrations in the muscle tissue of fish and crustaceans lasted 11 months in succession (Magalhães et al. 2003).

Cyanobacteria can produce hepatotoxins, lipopolysaccharides, neurotoxins, dermatotoxins, and other secondary metabolites (Han et al. 2001). MCs are the most widely distributed hepatotoxins, with over 100 variants (Carmichael 1997; Zastepa et al. 2015). MCs have been widely investigated as they have been found to accumulate

and disorder the organ functions of aquatic animals, including the hepatopancreas, heart, gill, kidney, intestine, and gonad (Best et al. 2001; Chen and Xie 2005; Hauser–Davis et al. 2015). The absorption of MCs is done by direct contact (skin and gill), digestion of toxic cyanobacteria, and the accumulation of MCs in plants and animals (Saqrane et al. 2007; Zhang et al. 2009a). Previous studies have shown evidence of MCs excretion mechanism in animals and plants. However, the elimination process of MCs from an organism is limited (Zhang et al. 2009b; Bieczynski et al. 2014; Hauser–Davis et al. 2015). It is difficult to purify water that is laden with high toxin levels since the toxins are easily bioaccumulated through the trophic chain (Xie et al. 2005; Ferrão–Filho and Kozlowsky–Suzuki 2011; Sabatini et al. 2015).The uptake of MCs in an animal's body not only results from the consumption of food containing toxins, but also from toxins dissolved in water (Sieroslawska et al. 2012). Moreover, the toxic effect of MCs on aquatic animals has a close correlation with the cell density of cyanobacterial blooms, toxin content, and the gender and age of animals (Li 2007).

The accumulation of MCs in the tissues of aquatic animals not only inhibits protein phosphatases PP1 and PP2A specifically and potently, but also frequently causes the generation of reactive oxygen species (ROS) (Lankoff et al. 2003; Li et al. 2003; Bouaicha and Maatouk 2004; Jiang et al. 2011). Antioxidant enzymes are susceptible to MCs, and can effectively eliminate ROS induced by MCs; however, the altered enzymes are often used as indicators of immunity (Ding et al. 1998; Amado and Monserrat 2010; Sabatini et al. 2011). Several studies have found that oxidative stress is one of the MC's toxicity mechanisms (Botha et al. 2004; Jos et al. 2005; Amado and Monserrat 2010). Usually, a high level of MCs causes oxidative stress in a time-dependent manner (Cazenave et al. 2006; Hou et al. 2015). Franco et al. (1999) implied that oxidative stress is positively correlated to the antioxidant enzyme mRNA expression. The alteration in the transcription of antioxidant enzymes may play an important role in neutralizing the toxic effects induced by MCs (Xiong et al. 2010).

The hepatopancreas in crustaceans is the vital detoxicated center of xenobiotics, and it plays a significant role in their immune system (Vogt 1994; Söderhall and Cerenius 1998; Zhou et al. 2009). Some studies have recorded visible damage in the liver or hepatopancreas of organisms exposed to MCs (Fischer and Dietrich. 2000; Li et al. 2008; Ferreira et al. 2010). There are three types of uptake of MCs into an aquatic animal's body, including ingestion of toxic algae, uptake of dissolved MCs by the skin or gills and ingestion of other aquatic organisms that had accumulated MCs in their bodies by the food chain (Song and Chen 2009).

There is no immunoglobulin existing in shrimp's humor, but plenty of its humoral factors can recognize xenobiotics and defend against pathogens in different ways (Wang and Wu 2000). In previous works on acute immune response, up-regulation for glutathione S-transferase(GST) isoform (ΩGST, μGST, and mGST2 isoform), and increases in the total GST and catalase (CAT) enzyme activity were detected after a 48-h injection of extraction from the toxic *Microcystis aeruginosa* (Gonçalves–Soares et al. 2012). The expression of antimicrobial peptide genes (*Penaeidin 3* and *ALF* was significantly affected in *L. vannamei*, which was inoculated with the dilution of pure MC-LR (Li et al. 2014).

Most laboratory experiments were conducted by the administration of MCs into the bodies at high concentration or fodder mixing with cyanobacteria. However, immersion in water containing MCs at low concentration is seldom used for assessing the sub-chronic toxic effects on an experimental subject. The aim of the our present study was to assess the defense mechanism by investigating the response of immune-related enzymes and their corresponding genes induced by MCs in the hepatopancreas of white shrimp.

Materials and methods

MC-LR exposure experiment

The exposure experiment was carried out at the Donghai Island Marine Biological Research Base in Zhanjiang, China (110°32′22.07″ and 21°01′33.12″). MC-LR (purity ≥ 95%) was supplied by Technology Co. Ltd, Taiwan and dissolved in the ultrapure water before use. Larval *L. vannamei* with initial body length from 2.5 to 3.5 cm were purchased from the Donghai Shrimp Hatchery of the Yuehai Feed Group (Zhanjiang, China). The size of the shrimp used in our experiment was similar to those at the early stage of shrimp culture when MC-LR level was undetectable. *L. vannamei* were acclimated to the indoor laboratory condition for 2 weeks before experiment. The shrimps were grown in 300 L containers with constant aeration and a temperature of 30 ± 2 °C. The containers were covered with a black sunshade net to prevent the MC-LR in the water was destroyed by ultraviolet light. Shrimps were fed three times a day with a formulated pellet (No.2 white shrimp feed, manufactured by Yuehai Feed Group, Zhanjiang, China).

A total of 630 *L. vannamei* of similar body weight (0.04 ± 0.01 g) were randomly divided into seven groups, including one control group and six treatment groups, and every group had 90 shrimps in triplicates. Shrimps were immersed in fresh seawater containing 0, 0.50, 1.03, 1.47, 2.11, and 3.00 μg/L MC-LR for 30 days. Half of the water was replaced by fresh water containing the corresponding MC-LR concentration every 3 days. The level of MCs in the

water was examined with a MC-LR ELISA Kit every 5 days.

Quantification of MC-LR in shrimp pond water

After air pump filtration with a 0.45 μm ultrafiltration membrane, water samples from three grow-out shrimp ponds were measured using a microcystin-LR ELISA Kit (Qingchuang, Beijing, China). The results were read at 450 nm with a microplate reader (Thermo Fisher Scientific, USA).

Sampling

After the *L. vannamei* were killed with a spike, the hepatopancreas were immediately removed and placed into 1.5 ml centrifuge tubes with RNAlater and 1.5 mlcentrifuge tubes without RNAlater. The former were kept in the crushed ice, and the latter were kept in liquid nitrogen. Samples were then taken back to the laboratory and analyzed for enzyme activity and transcript level.

Enzyme activity assays

The hepatopancreas were defrosted and washed in ice-cold 0.86% saline (1:9 m/v), and were then weighted and homogenized with 10 volumes of 0.86% saline. The homogenates were centrifuged at 4000 r for 10 min at 4 °C, and the supernatants were used for enzyme activity assays. Total proteinwas measured following Bradford's (1976) method with BSA as a standard. The activities of SOD, GPx, POD, AKP, ACP, and LZM were determined using kits purchased from Nanjing Jiangcheng Bioengineering Institute (Nanjing, China). Each enzymatic assay was performed in triplicate. All enzymatic activities are expressed in U/mg prot.

Gene expression analysis

The total RNA of hepatopancreas sections was isolated using a RNA General Isolation Kit (Dongsheng Biotech, Guangzhou, China), quantified with a SimpliNano microspectrophotometer (GE Healthcare). Next, the purified total RNA (1 μg) was reverse-transcribed to cDNA using a PrimeScript RT Reagent Kit and kept at −20 °C.

Primers used for RT-qPCR were designed by Primer Premier 5.0, according to the gene sequences of *L. Vannamei* from GenBank. The accuracy of each pair of primer (*cMn-sod*, *gpx, lzm* and *β-actin*) was detected with PCR before RT-qPCR (the primers used in the RT-qPCR are shown in Table 1). The RT-qPCR analysis was carried out on a CFX Connet Real-Time System (Bio-Rad, USA) with the use of a SYBR Premix ExTaq II Kit (TaKaRa Bio). The final reaction volume of 20 μL contained 1 μL cDNA, 10 μL of 1 × SYBR Premix Ex Taq, 1 μL of each primer (10 μM), and 7 μL of ddH2O. The reactions were performed under the following conditions: 95 °C, 4 min; 95 °C, 20 s; 60 °C, 30 s (40 cycles). A melting curve analysis was performed to access amplification of specific products. The *β-actin* gene was used as an endogenous control. The *cMn-sod*, *gpx* and *lzm* transcript levels were normalized to *β-actin* and reported as relative expression values (*cMn-sod* /*β-actin*, *gpx* /*β-actin* and *lzm* /*β-actin*) using the $2^{-\Delta\Delta Ct}$ method. Shrimps from MC-LR-detected ponds served as positive control, and shrimps from the control group served as negative control. Analysis was performed in triplicate for each sample.

Table 1 Primers used for quantitative real time PCR

Genes	Sequences (5′–3′)	Product size /bp
cMn-sod	F: TGCCACCTCTCAAGTATGATTTC	130
	R: TCAACCAACTTCTTCGTAGCG	
gpx	F: AAGATGGTTATGTTCGGCAAAG	147
	R: GCAGACAGGTGTCCAAATGAT	
lzm	F: TATTCTGCCTGGGTGGCTTAC	132
	R:CTAGAACATAGAGCTCGAAGTGGTC	
β-actin	F: AGTAGCCGCCCTGGTTGT	183
	R: AGGATACCTCGCTTGCTCT	

Results

Effects on enzyme activities in hepatopancreas

No mortality occurred during theexposure experiment. Direct observations showed that all shrimp individuals in both control and treatment groups behaved normally and similarly during the experiment.

The activity of antioxidant enzymes after the exposure to MC-LR are shown in Figs. 1a–c. SOD, GPx, and POD activity rapidly increased in a dose-dependent manner, and reached a maximum at the concentration of 3.00 μg/L MC-LR. SOD, GPx and POD activities in the treatment group were 1.66-fold, 1.86-fold and 1.88-fold greater than the control group, respectively ($P < 0.05$).

ACP and AKP activity in the hepatopancreas exposed to MC-LR is shown in Figs. 1d–e. Both ACP and AKP activities showed similar changes, with, with a downward trend after the first rise to the peak at a concentration of 0.72 μg/L of MC-LR. Both ACP and AKP activities in the treatment group were 1.65-fold and 1.41-fold greater than the control group. There were no increases in ACP and AKP at a MC-LR concentration of 3.00 μg/L ($P < 0.05$).

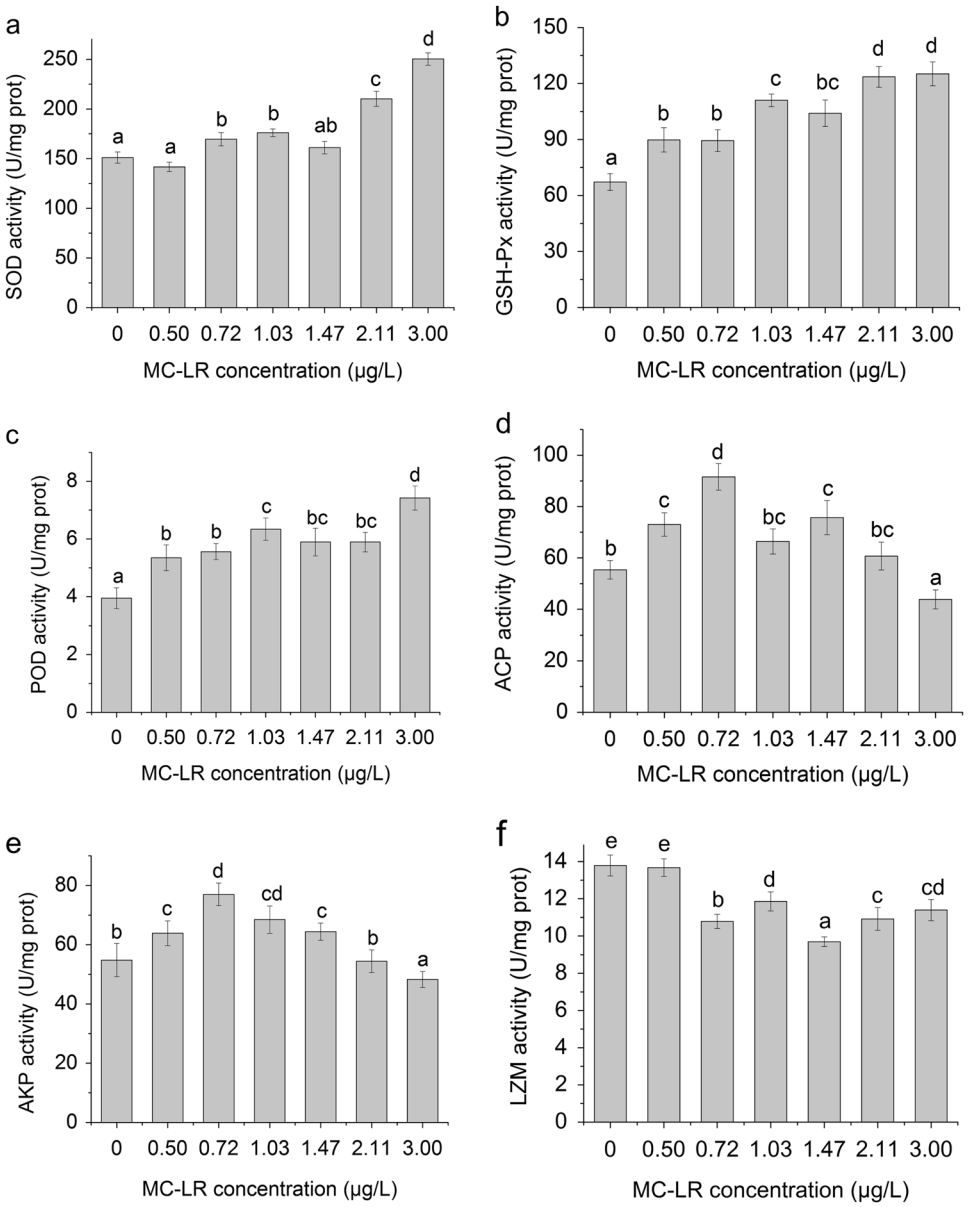

Fig. 1 Superoxide dismutase (SOD) **a**, selenium-dependent glutathione peroxidase (GPx) **b**, peroxidase (POD) **c**, alkaline phosphatase (AKP) **d**, acid phosphatase (ACP)**e**) and lysozyme (LZM) **f** enzyme activities in the hepatopancreas of *L. vannamei* treated with different concentrations of MC-LR. Values are expressed as the mean ± SD ($n = 9$). Different letters above bars indicate significant differences between different groups ($P < 0.05$)

A marked decrease in LZM activity was observed after MC-LR exposure (Fig. 1f). MC-LR had significant effects on the LZM activity, resulting in a downward trend, LZM activity in the 1.47 μg/L of MC-LR treatment group was only 0.70-fold greater than the control ones.

Effects on transcript level of corresponding enzymes in hepatopancreas

The changes in *cMn-sod*, *gpx*, and *lzm* transcript levels are shown in Fig. 2a. By the end of the experiment, the *cMn-sod* and *gpx* transcript level was significantly up-regulated in *L. vannamei*, which was exposed from 1.03 to 3.00 μg/L of MC-LR. However, the MC-LR had no significant effects on the *cMn-sod* and *gpx* transcript level at concentrations from 0 to 0.72 μg/L of MC-LR. The *lzm* transcript level was significantly down-regulated in *L. vannamei* over the concentration of 0.72 μg/L of MC-LR, while there were no significant changes in the transcript level exposed to 0–0.50 μg/L of MC-LR.

Discussion

Fischer and Dietrich (2000) showed that low concentrations of MCs could damage the hepatopancreas and kidney in carp (*Cyprinus carpio*). To a certain extent, shrimps that are directly exposed to MCs after the death and lysis of cyanobacterial cells are vulnerable to MCs and likewise by organic anion transporting polypeptides (OATP) (Fischer

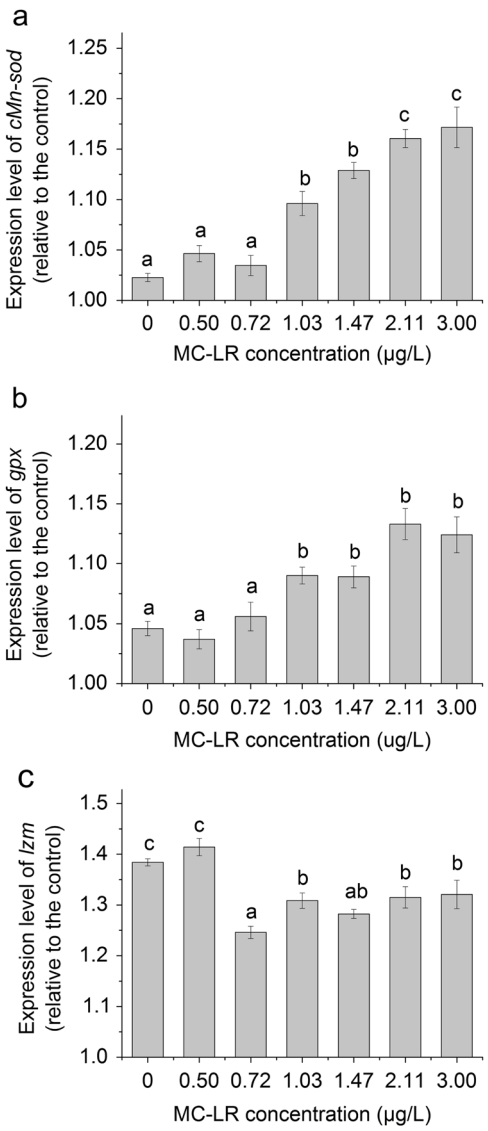

Fig. 2 Relative expression of *cMn-sod* **a**, *gpx*, **b** and *lzm* **c** gene transcripts in the hepatopancreas of *L. vannamei* treated with different concentrations of MC-LR. The data are presented as an expression ratio for each gene. Values are expressed as mean ± SD ($n = 9$). Different small letter superscripts indicate significant differences among up—or down-regulated genes

et al. 2005). Cao et al. (2011) showed a survival rate reduction and growth inhibition in *L. vannamei* larvae exposed to *Oscillatoria* sp. Furthermore, several works assumed that the mortality of shrimp may be explained due to ingestion of the toxic algal cells during foraging (Zimba et al. 2001; Li et al. 2004; Xie et al. 2004). Chen et al. (2004) and Lankoff et al. (2004) indicated that there was a suppressive toxic effect of MC-LR on the immune function of organisms. In general, acute experiment with high dose of MC-LR rapidly causes significant changes in enzyme activity within days (Atencio et al. 2008; Gonçalves-Soares et al. 2012). However, in some studies, it took weeks to affect the enzyme activity in MCs sub-chronic experiment (Jos et al. 2005; Sabatini et al. 2015), suggesting that the changes in enzyme activity is closely related to the dose of MCs and time.

In crustaceans, the hepatopancreas is a crucial metabolic center for eliminating excess ROS and plays a key role in the immune system. However, MCs may cause the imbalance between ROS and the antioxidant defense system (Pandey et al. 2003). Antioxidants were divided into antioxidant enzymes and non-enzymatic antioxidants. Changes in antioxidant enzyme activity mirror the level of oxidative damage in organisms (Livingstone 2001). Antioxidant enzymes are an important part of protecting organisms from oxidative damage by inactivating the ROS and repairing the oxidized biological molecules (Halliwell and Gutteridge 2015). In some cases, chronic toxic effects of MCs induce the elevation in antioxidant enzyme activity. Lenartova et al. (1997) and Di Giulio (1991) indicated that the increase in antioxidant enzyme activity may be an indication that organisms are adapting to the oxidative stress induced by MCs, which was a compensating mechanism to overcome negative factors caused by the external environment. In previous studies, Sabatini et al. (2015) reported SOD activity in the hepatopancreas of the crab (*Neohelice granulata*), which when feeding on *Microcystis aeruginosa*, were elevated from week 3 to 7 during the exposure experiment. Jos et al. (2005) showed that after *Oreochromis sp*. fed on commercial pellets mixed with crushed *Microcystis aeruginosa*, the SOD, CAT, and GPx activity in the liver significantly increased after a 21-day experiment. In the present study, the concentration of MC-LR dissolved in water waslow in the experiment that lasted 30 days; therefore, the low MC-LR lever may induce chronic toxicity in shrimp. A significant increase was observed in SOD, GPx, and POD activity. Shrimps exposed to MCs sub-chronically experienced an increasing trend in SOD, GPx, and POD activity in the hepatopancreas in a concentration-dependent manner, suggesting persistent and vigorous responses of antioxidant enzymes in detoxifying MC-LR. Results of similar enhancements were reported in SOD, CAT, and GPx activity in the loaches (*Misgurnus mizolepis)* fed *Microcystis* sp. for 28 days (Li et al. 2005), as well as in the SOD, GST, and GPx activity in zebrafish (*Danio rerio*) exposed to MCs at lower concentrations (≤5.0 μg/L) (Pavagadhi et al. 2012). The elevation of antioxidant enzyme activity showed that MCs can induce oxidative stress in *L. vannamei*, which was closely related to the toxic effect on the hepatopancreas. Moreover, the increase in antioxidant enzyme activity may eliminate excess ROS to maintain normal function of hepatocytes. In the present study, changes in *cMn-sod* and *gpx* transcript levels were consistent with changes in the SOD and GPx activity after exposure to MC-LR, suggesting that the alteration of SOD

and GPx activity were closely associated with their corresponding transcript levels. Similar results were observed in other studies. Galanti et al. (2013) and Xiong et al. (2010) indicated that MCs-induced ROS can regulate antioxidant enzymes by changing their activities and gene expression. In addition, there was a positive relationship between the transcription levels of antioxidant enzyme genes and changes in the oxidative damage of proteins in a study of Franco et al. (1999). Zegura et al. (2011) showed that MC-LR can upregulate oxidative stress-responsive genes (GPx1 and SOD1) in human peripheral blood lymphocytes. In a recent study, MC-LR exposure induced enzymatic activities and up-regulated the transcript level of SOD and GPx in the low dose group (50 μg MC-LR/kg), and quickly recovered after a slight injury in the liver of zebrafish (*Danio rerio*). However, in a high dose of MC-LR (200 μg MC-LR kg^{-1}), the enzymatic activities were inhibited, and the transcript levels of SOD and GPx were down-regulated, with severe hepatic injury (Hou et al. 2015). However, Yuan et al. (2016) reported that GPx was activated by longer MC-LR exposure in the male red swamp crayfish (*Procambarus clarkii*), and *gpx1* mRNA expression showed uncoordinated regulation pattern compared with its enzyme. In the present study, the increases in antioxidant enzyme activity and the up-regulation of corresponding genes in *L. vannamei* suggested an adaptation to oxidative stress caused by the chronic exposure to MC-LR. However, it would adversely influence the immune system by affecting the alteration of environmental factors and diseases if *L. vannamei* were in a stressful state for a long period of time.

ACP and AKP are involved in a series of physiological metabolic activities, such as molecule permeability, growth and cell differentiation, and the digestion, absorption and transport of some phosphides and other nutrients. They not only effectively detoxify pollutants and toxicants invading the crustaceans, but also play a positive role in the immune system as parts of the lysosomal enzyme, which is of great significance to the growth and survival of crustaceans (Mazorra et al. 2002). Pathologically damaged liver tissues usually lead to alterations in enzyme activity for the leaks after the destruction of the structure of the hepatocytes (Malbrouck and Kestemont 2006). Lankoff and Kolataj (2001) showed that MC-YR can weaken the stability of the lysosomal membrane by inhibiting some proteases activity, and accelerate cell apoptosis, leading to serious damage of liver function. Molina et al. (2005) reported that ACP and AKP activity increased significantly in the liver and kidney of tilapia (*Oreochromis* sp.) that were sub-chronically exposed to *Microcystis aeruginosa*, in comparison with the control group. In freshwater snail (*Bellamya aeruginosa*) fed *Microcystis aeruginosa*, the ACP and AKP activity in the hepatocytes were enhanced, compared to those fed *Scenedesmus quadricauda* (non-toxic green algae) (Zhang et al. 2009b). However, Sieroslawska et al. (2012) showed that AKP activity remained stable in common carp (*Cyprinus carpio* L.) during exposure to microcystin-containing cyanobacterial extract. And similar data were also reported in *C. carpio* L. gavaged with 50 μg MC-LR/kg *Microcystis* daily (Li et al. 2004). Although there were different results in ACP and AKP activity according to the studies above, Zhang et al. (2009b) assumed that the alteration in ACP and AKP activity was in concurrence with the changes in the concentration of MCs and treated time. In the present study, ACP and AKP activity showed a biphasic change with a downward trend after the rise of activity in the hepatopancreas. The increase in ACP and AKP activity might be due to the leak of the lysosomal enzyme (including ACP, AKP and so on) because permeability of the lysosomal membrane was enhanced in a state of oxidative stress. The lysosomal enzyme was activated after entering the cytosol (Leist and Jäättelä 2001; Guicciardi et al. 2004). The decrease in ACP and AKP activity might be due to the organism being beyond the ability to regulate above a certain concentration of MC-LR, and due to the stress response system being out of order because of high-intensity cellular stress, which leads to the lysosome destabilization and rapid cell necrosis (Alverca et al. 2009).

LZM, an alkaline protein in the acetyl amino polysaccharide of the mucopeptide in the cytoderm of gram-positive bacteria, was released after the hydrolyzation of LZM. It destroys and eliminates the invasion of xenobiotics by forming a hydrolytic enzyme system. This process plays an important role in defensive system of shrimp (Mukherjee et al. 2016). Sieroslawska et al. (2012) suggested that LZM plays a major role in defending against pathogens and oxidative stress, and it has been used indicator for the detection of environmental pollutants. It mainly stems from neutrophils, monocytes and macrophages, and acts as the first line of defense in the immune system (Murray and Fletcher 1976). The LZM of aquatic animals is different from that of terrestrial animals, since it can dissolve both gram-negative bacterium and gram-positive bacterium (Xu et al. 2011). Sieroslawska et al. (2012) reported that LZM activity of the common carp (*Cyprinus carpio* L.) after immersion exposure to MCs was half of that of the control group. Li et al. (2013) showed that LZM activity increased at the earlier stages of exposure but decreased at the late stages in the serum, hepatopancreas, and spleen of common carp (*Cyprinus carpio* L.) treated with chlorpyrifos. In freshwater fish (*Carassius auratus*) exposed to crowding stress, LZM activity decreased significantly, and the fish were more sensitive to pathogens, in comparison to the control group (Wang et al. 2004). In the present study, significant decreases at 0.72–0.30 μg/L MC-LR were absorbed in LZM activity, as well as in the down-regulation of *lzm*; this trend was inconsistent with LZM activity,

suggesting that MC-LR treatment may disturb the normal structure and function of LZM in the hepatopancreas of *L. vannamei*. This agrees with the decreased LZM activity in rainbow trout (*Oncorhynchus mykiss*) exposed to transportation and water pollution (Möck and Peters 1990).

Conclusions

In the present study, SOD, GPx, and POD levels were activated, while LZM in white shrimp was suppressed after sub-chronical exposure to MC-LR. Their corresponding genes showed coordinated changes when compared with their enzyme activity. In addition, a biphasic change with a downward trend after the rise was shown in ACP and AKP activity. Therefore, to avoid harm to both cultivated organisms and humans, shrimp farming should be monitored regularly for the levels of MCs.

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Author contributions X.H. and C.L. designed the experiment. Y.C. performed the experiment. Y.C. and J.W. prepared and revised the manuscript. X.H. and C.L. reviewed the initial and final version of the manuscript.

Compliance with ethical standards Research involving animals and informed consent

Conflict of interest The authors declare that they have no competing interests.

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