Toxicity of the microcystin-producing cyanobacterium *Microcystis aeruginosa* to *Litopenaeus vannamei*

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Abstract

Microcystis aeruginosa is a common kind of harmful bloom algae, which was also frequently found as a dominant microalgae specie in shrimp breeding ponds. And it was found that blooms always induced massive death of shrimp, but the toxic effects of *M. aeruginosa* on *Litopenaeus vannamei* are still not completely understood. In this paper, the toxicity of *M. aeruginosa* cells to *L. vannamei* was examined, and the toxic components in the cells were analyzed through high-pressure liquid chromatography (HPLC). In addition, the immune response of shrimp to the microalgal extract was assessed by measuring the activity of immune-related enzymes, as well as the transcription of the relevant genes. Overall, both *M. aeruginosa* cells and the algal extract resulted in a 100% mortality rate in shrimp, whereas the cell-free culture medium was ineffective. And HPLC analysis results revealed the presence of microcystin-LR (MC-LR) at a concentration of 190.40 mg/kg of cells. In addition, the activity and gene transcription of two immune related enzymes, SOD and LZM, were both significantly reduced in shrimp hepatopancreas ($p < 0.05$) after injection with cell extract. However, reduced glutathione (GSH) content was slightly increased, but the ratio of GSH to GSSG was down, and the transcription of *gst* gene function as detoxification, was significantly downregulated ($p < 0.05$). The results demonstrated that *M. aeruginosa* cell extract was highly toxic to *L. vannamei*, and exerted a negative effect on shrimp immunity including reduction of antioxidant capacity, antibacterial activity and detoxification activity, due to microcystin-LR.

Introduction

Waters in shrimp breeding ponds are always rich in nitrogen and phosphorus, which are beneficial to microalgal growth. The algae can provide dissolved oxygen, food, and a comfortable environment to aquatic animals, which is beneficial for production (Cao et al., 2014). However, the overpopulation of harmful cyanobacteria may pose a great threat to aquatic animals (Wu et al., 2017; Wood, 2016; Zi et al., 2018). There are clear indications that cyanobacterial blooms may have adverse effects on fish (Atencio et al., 2008). However, under natural conditions, it is extremely complicated to attribute the mortality of aquatic organisms to specific cyanobacteria, as the low level of dissolved oxygen, elevated pH, and toxic inorganic nitrogen may exacerbate the toxicity of cyanobacteria. Controlled laboratory experiments may allow to investigate the effect on aquatic organisms of individual species of toxic cyanobacteria. Previous reports showed that aqueous cyanobacterial extracts may exert acute or chronic toxic effects on growth, survival, and reproduction of aquatic animals (Padmavathi and Veeraiah, 2009). Moreover, exposure to cyanotoxins was reported to affect the immune system in fish. For instance, cell apoptosis and liver damage were observed in fish after exposure to pure MC-LR (Djediati et al., 2011). Furthermore, the levels of immune-related molecules such as superoxide dismutase (SOD), catalase (CAT), and GSH are also affected by cyanotoxins (Ren et al., 2014). Yeal et al. reported that cyanotoxins suppress lymphocyte response to lipopolysaccharide (LPS) in carp, indicating impaired defense against gram-negative bacteria (Zhang et al., 2019).

L. vannamei is a euryhaline organism, which is reared worldwide. In China, *L. vannamei* has been reared in brackish water widely in recent years, and its production reached 1862 million kg in 2020 (Bureau of Fisheries under the Ministry of Agriculture and Rural Affairs, 2021). However, various diseases and decreased immunity have caused enormous losses to the shrimp industry (Dastidar, 2013; Xiong et al., 2018). As an invertebrate, *L. vannamei* only has innate immunity as a shield against pathogen infection (Wu et al., 2017). It is reported that the antioxidant system of shrimp plays an essential role not only in protecting cells from superoxide anions, but as a mechanism of innate defense. SOD, is important antioxidant enzyme that eliminates excess levels of reactive oxygen species (ROS) from oxidative damage (Okado-Matsumoto and Fridovich, 2001). In addition, Li et al. found that the injection of *V. alginolyticus* rapidly reduces SOD activity in shrimps, suggesting SOD implication in shrimp immunity (Li et al., 2013). GSH, another vital antioxidant, decomposes H_2O_2 into water and oxygen, thereby reducing oxidative stress (Deponte, 2012). GSH also acts as a detoxification enzyme, counteracting microcystin toxicity with the help of glutathione S-transferase (GST) (Jiang et al., 2011; Ming et al., 2019). Wang et al. demonstrated that GSH supplementation in the diet increases the total hemocyte count and decreases cumulative mortality in shrimp after challenge with *Vibrio parahaemolyticus*, indicating that GSH promotes shrimp immune response (Xia and Wu, 2018). In invertebrates, humoral immunity plays an important role in innate defense, and a wide array of antimicrobial peptides (AMPs) contribute to shrimp defense from various pathogens. Lysozyme (LZM) is one of the most important AMPs in crustaceans, and protects these organisms from a broad spectrum of bacteria (Duan et al., 2019).

Microcystis aeruginosa, belonging to the Cyanophyta phylum, is known as a harmful cyanobacteria, and was a specie of dominant microalgae in breeding ponds frequently (Peng et al., 2011). *M. aeruginosa* cells produce microcystins (MCs), which are probably the most prevalent cyanotoxins released by cyanobacteria in water. MCs are highly hepatotoxic and display cancer-promoting activity (Meriluoto and Spoof, 2008; Pavagadhi and Balasubramanian, 2013). More than 200 MC variants have been identified in cyanobacteria blooms and cultures (Carmichael, 2017), among which microcystin-LR, RR, and YR are the most common (Bláha et al., 2009; Briand et al., 2003). Uptake of cyanobacterial toxins in fish may occur by oral ingestion of toxins or cyanobacterial cells (Bury et al., 1998). However, considering the feeding habits of shrimps, toxin uptake via the gill epithelium may be the predominant way. The injection of *M. aeruginosa* cells (which have a similar size to bacteria, 3-7 μm) or extract is a suitable strategy to study acute toxic effects of this microalgae in shrimp, avoiding the interference of other environmental factors such as pH or the concentration of dissolved oxygen (Leulier et al., 2003).

To date, few studies have explored the effects of *M. aeruginosa* on *L. vannamei* breeding. The aim of this study was to evaluate acute toxicity of *M. aeruginosa* cells in *L. vannamei*, including intracellular and extracellular metabolites. And the toxins of *M. aeruginosa* cells were analyzed by high-efficiency liquid chromatography. In addition, the enzyme activity and gene transcription of SOD, GSH, and LZM were investigated in shrimp hepatopancreas to characterize the immune response of *L. vannamei* to toxic substance of *M. aeruginosa* cells.

Materials And Methods

Cyanobacteria

The *M. aeruginosa* strain used in this study was isolated from shrimp breeding ponds with cyanobacteria blooms in Guangdong province, China, and stored in the South China Sea Fisheries Research Institute (SCSFRI). Cyanobacteria were cultured in BG11 medium at pH 8.0 and 28 °C, with illumination at a photon flux density of 1000 lux under 12 h light/12 h dark cycles.

Shrimp

Healthy *L. vannamei*, with a mean body weight of 3.5 ± 0.5 g, were obtained from shrimp ponds of the Shenzhen Experimental Station of the SCSFRI, Shenzhen, China. The shrimp were acclimated in tanks containing aerated brackish water which was filtrated with sand (salinity 4, pH 8.2, dissolved oxygen 5.5-6.5 mg/L) at 28 ± 1 °C for one week prior to the experiment. During the acclimation period, one-half of the water in each tank was replaced daily, and the shrimp were fed with specific shrimp feed three times daily. Only apparently healthy shrimp were used for the study.

Preparation of the fractions

Algae cells and cultured medium were separated by centrifugation (10 min, 4000 ×g), then the cell-free medium was collected, while the cells were rinsed 3 times with phosphate buffer solution (PBS) and dissolved in PBS (cell density 1×10^8 cells/mL). Cell sample was subjected to ultrasonication, with 60% pulse and 30% power, at 0-4 °C for 20 min. After ultrasonication, the supernatant (crude extract) was collected by centrifugation (10 min, 8000×g). Finally, both the medium and cell extract were passed through a 0.22-μm pore filter for subsequent experiments. The samples were stored at 4 °C before the toxicity test.

The remaining aliquot of *M. aeruginosa* cells, weighing 0.54 g, was diluted to 10 mL with 70% methanol, homogenized for one min, and then subjected to ultrasonication as above. Cell breakage was confirmed by microscopic observation, and the supernatant was collected by centrifugation (10 min, 8000× g), and then loaded into a C18 cartridge that had been previously activated with 100% methanol and deionized water. The cartridge was rinsed with 3 mL of 20% methanol and eluted with 3 mL of 100% methanol, and the eluates were dried by using a Zymark LV TurboVap® evaporator (Biotage, Charlotte NC) at 60 °C, with N₂. The samples were reconstituted with 5 mL of deionized water and filtered with 0.45-μm PVDF membranes prior to analysis.

Acute toxicity assays in *L. vannamei*

A total of 360 *L. vannamei* were randomly divided into four groups, including one control group and three treatment groups, and every group had 90 shrimps in triplicates. The shrimps were kept in 400-L tanks at 28 ± 1 °C, pH 8.2, at a concentration of dissolved oxygen of 5.5-6.5 mg/L. Three treatment groups of shrimps were injected intramuscularly with 50 μL of cultured medium, *M. aeruginosa* cells, and cell

extract (cell density 1×10^8 cells/mL), respectively, while control shrimps were injected with 50 μ L of PBS. The intra-muscular injection into the shrimp was performed with an insulin syringe in the lateral region of the animal's body between the second and third abdominal segment and the collection of shrimp. During the trial, the shrimps were fed three times daily, and uneaten food was removed one hour later, while water was replaced daily. The shrimps were observed in situ, and the death rate was recorded.

HPLC analysis of microcystins

Microcystins in cyanobacterial extract were analyzed by HPLC with diode-array detection (HPLC-DAD). Specifically, we used an Agilent 1100 Series HPLC system with Supelcosil ABZ + Plus 150 mm $\times$ 4.6 mm 5- μ m columns (Supelco) and a mobile phase consisting of (A) 0.1% (v/v) TFA, (B) acetonitrile with 0.1% (v/v) TFA (gradient elution: 20-59% B in 0-30 min), at a flow rate of 1 mL/min, a temperature of 30 $^{\circ}$ C, with an injection volume of 25 μ L. UV-spectra between 200 and 300 nm were collected and chromatograms evaluated at 238 nm. Identification of the MCs peaks was based on the comparison of retention times and UV spectra with the standard MCs. The concentration of MCs in extract was calculated by the standard curve equations, and the standard curves were drawn according to the MC-RR/YR/LR standards (USA, Sigma) dissolved in methanol, the concentration of which were 1, 0.5, 0.2, 0.1 and 0.05 μ g/mL, respectively (Vesna et al., 2007).

Effect of *M. aeruginosa* extract on the immune response of *L. vannamei*

A total of 750 *L. vannamei* were randomly divided into three groups, including two control groups and one treatment group, and each group had 250 shrimps in 5 parallels. *L. vannamei* was treated with 50 μ L of a median lethal concentration of extract (cell density was 1×10^7 cells/mL, based on the results of preliminary experiments) in treatment group, 50 μ L of MC-LR (the concentration was 1.69 μ g/mL) in positive control group and 50 μ L of PBS in negative control group, respectively. At 0, 8, 24, 48, 72, 96, and 120 h post-injection, 10 shrimps were dissected. The hepatopancreas of each shrimp was used for further assay, and a part of organs were used for analysis of enzymes activity, while the rest were stored in RNAlater (Life technology, USA) for gene transcription analysis. All samples were immediately stored at -80 $^{\circ}$ C until use.

Immune-related enzyme activity assay

The activity of SOD, GSH, and LZM was measured according to the manufacturer's instructions. Briefly, hepatopancreas samples stored at -80 $^{\circ}$ C were weighed and homogenized for 30 s in phosphate buffer (0.125 M, pH 7.2, containing 0.05 M Na_2EDTA , 2-4 $^{\circ}$ C). After centrifugation at 3000 g for 10 min at 4 $^{\circ}$ C, the supernatant was separated and analyzed by protein assay. The enzyme activity and protein assay were all determined using a relevant Detection Kit (Jian Cheng Bioengineering Ltd., Nanjing, China). SOD assay was based on the ability of SOD to inhibit WST-1 auto-reduction by superoxide anion ($\text{O}_2^{\cdot-}$). SOD activity was monitored by measuring the absorbance at 450 nm and expressed as unit/mg protein (U/mg protein). The GSH content was evaluated by incubation with 6 mM 5, 5'-dithiobis (2-nitrobenzoic acid)

(DTNB), and measurement of the absorbance at 310 nm. Lysozyme (LZM) assay was based on the ability of LZM to lyse the *Micrococcus lysodeikticus* cells. During incubation of the lysozyme sample with the substrate, the reaction was followed by monitoring the decrease of the absorbance at 530 nm.

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

Total RNA was extracted from hepatopancreas using a RNeasy Plus Mini kit (Qiagen, Germany). RNA quantity, purity, and integrity were evaluated by spectrophotometry (A260/A280) and electrophoresis on 1% agarose gels. cDNA was synthesized from 2 µg of total RNA by using the Prime-Script™ Real-time PCR Kit (TaKaRa, Dalian, China). The expression of target genes (*sod*, *gst*, *lzm*) and of the internal control gene (β -actin) was measured by qPCR. Primer premier 5.0 was used to design the fluorescent primers before gene synthesis performed (PREMIER Biosoft International, Palo Alto, CA, USA) based on the published *L. vannamei* mRNA sequences (Table 1), and each primer pair was designed and tested for specificity. Primer efficiency was determined by performing serial dilutions of reference cDNA. Quantitative real-time PCR (qPCR) was carried out with a LightCycler480 384-well Real-Time PCR System (Roche, Switzerland). The amplification was performed in a total volume of 10 µL, containing 5 µL of SYBR Premix Ex Taq II (Takara), 1 µL of the diluted cDNA, 0.5 µL of each primer, and 3 µL ddH₂O. The qPCR program was 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The relative quantification of transcripts was performed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical analysis

Mortality rate was calculated as: [number of deaths /total number of shrimp]] ×100

All data presented are means of three independent sets of experiments ± standard error (SE). Significant differences between control and exposed shrimp were determined using a one-way ANOVA followed by multiple comparison via Dunnett's test. p values < 0.05 were considered to be statistically significant. Statistical computations were performed with SPSS 13.0 for Windows (SPSS Inc.).

Results

Cumulative mortality in *L. vannamei* after treatment with *M. aeruginosa*

The acute toxicity of *M. aeruginosa* in shrimp was assessed by mortality of *L. vannamei*, which was evaluated after injection of *M. aeruginosa* cells, cell extract, substrate in cell-free medium and PBS, respectively (fig.1). The microalgal extract exerted rapid effects, and the shrimp mortality during the periods 0-6 h, 6-12 h, and 12-24 h was 8.5%, 79.8%, and 10.0% respectively, while total mortality was 98.3%. On the other hand, shrimp injected with cells did not die until 24 h post-injection, and the mortality during the periods 24-36 h, 36-48 h, 48-60 h, and 60-72 h was 15.5%, 26.7%, 33.4%, and 24.4% respectively, while the total mortality was 100%. In the group injected with cell-free medium substrate, the mortality at the end of trial was only 1.7%, while no mortality was observed in the PBS-injected group. The

results showed that the toxic effect of extract was much more rapidly than cells, while the mortality of shrimp injected with extract or cells was similarly high.

MC determination in the microalgal extract

HPLC is the preferred method for the separation of microcystins and can be used to identify and quantify specific variants. Microcystin standards (MC-RR/YR/LR) and the extract of *M. aeruginosa* cells were analyzed by HPLC. As shown in fig. 2A, MC-RR/YR/LR exhibited three independent peaks at 8.9 min, 9.4 min and 11.2 min, respectively, while the extract produced a single peak at 11.2 min (fig. 2B), indicating that it contained MC-LR and no MC-RR/YR. A standard curve based on MC-RR/YR/LR was drawn, and the curve fitting coefficient is all above 0.999 (fig. 3). According to the relevant standard curve equation (fig. 3C), the concentration of MC-LR in *M. aeruginosa* cell extract was 190.40 mg/kg (wet cells).

Effect of *M. aeruginosa* extract on enzyme activities in the hepatopancreas of *L. vannamei*

In order to examine the impact of *M. aeruginosa* extract on immune-related enzymes, the activity of SOD, GSH, and LZM was analyzed. As shown in fig. 4, SOD activity in the hepatopancreas of *L. vannamei* dramatically decreased after extract injection. Specifically, the value fell from 16.8 U/mg to 5.3 U/mg one day post-injection, representing an almost 70% decline. During the whole trail, the activity of SOD in extract injection group was lower compared with that of negative group, while the SOD activity of positive group was higher than that of negative group ($p < 0.05$).

Compared with negative group, the content of GSH in the hepatopancreas decreased one day after extract injection and MC-LR injection respectively. However, GSH restored at the end of trail, and reached 200 $\mu\text{mol/L}$. GSSG is the oxidation form of GSH, and the GSH/GSSG usually presents redox state of body. As shown in fig. 4, an obvious reduction of the GSH/GSSG ratio in hepatopancreas followed extract injection and MC-LR injection compared with that in the PBS treatment group ($p < 0.05$).

LZM is an important enzyme involved in the defense against exogenous bacteria. As shown in fig. 4, after extract injection, LZM activity in the hepatopancreas was substantially inhibited, dropping from the basal value of 28 U/mg to approximately 2 U/mg post-injection. The activity of LZM also dropped after MC-LR injection, and the value reached the bottom, which was 7.35 U/mg. Besides, LZM activity was significantly lower in the extract-injected group compared to the PBS treatment group, throughout the trial ($p < 0.05$).

Transcription of immune-related genes following extract injection

The transcription of *sod*, *gst*, and *lzm* in the hepatopancreas of shrimp was measured by real-time PCR after treated with the extract. As shown in Fig.5, the expression of *sod*, *gst*, and *lzm* was all downregulated after extract injection. The transcript level of *sod* and *gst* reached the bottom at 8 h post-injection, which was 4 fold and 25 fold lower than initial level, respectively. while the transcription of *lzm* was also downregulated after 48 hs post-injection, and the transcript level was 9 fold lower than the basal level. The transcription of *sod*, *gst*, and *lzm* was also depressed after injection with MC-LR, however,

they showed different trends during the trial, the transcript level of *sod* and *lzm* significantly increased at 24 hrs and 48 hrs post-injection ($p < 0.05$), while the transcription of *gst* was downregulated after injection, but restored at 48 hrs post injection with MC-LR.

Discussion

M. aeruginosa is one of the most common microalgae species in shrimp breeding ponds, and cell densities above 10^7 cells/L have been reported (Cao et al., 2014), resulting in deleterious effects in aquatic animals (Shen et al., 2018; Zhao et al., 2019). This study showed that injection of *M. aeruginosa* cells or cell extract caused high death rate in shrimp, whereas the cell-free culture medium of *M. aeruginosa* was harmless. Therefore, we speculated that the detrimental effects of *M. aeruginosa* were mainly due to intracellular toxic metabolites. This could explain why high mortality in aquatic animals was always observed immediately after the collapse of *M. aeruginosa* blooms (Christoffersen & Kaas, 2000).

Cyanobacteria deleterious effects are mainly attributed to the production of potent toxins, including hepatotoxic microcystins, neurotoxic saxitoxins, and cylindrospermopsin (Martin and Hans, 2006; Kenefick et al., 1993). In this study, the MC-LR was detected in *M. aeruginosa* cell extract at a concentration of 190.40 mg (MC-LR)/kg (cells). That was similar to the result of a previous study that 1.90 µg MC-LR per liter was detected in a *M. aeruginosa* bloom waters (Peng et al., 2011). MC-LR is a very common byproduct of cyanobacteria, and has been found in many species of algae like *Microcystis wesenbergii*, *Cyanodictyon imperfectum*, *M. aeruginosa* (Atencio et al., 2008; Herrera et al., 2014; Karlsson et al., 2005; Shen et al., 2018). Moreover, MC-LR is one of the most toxic cyanotoxins which exerts a strong toxicity in both mammals and aquatic animals (Djediati et al., 2011; Rymuszka and Adaszek, 2013). Martins et al. found that the injection of pure MC-LR leads to pathological changes in rat liver, and that the LD₅₀ of MC-LR is 36-122 µg/Kg (Martins et al., 2019). Furthermore, Li et al. reported that MC-LR induced hepatocyte apoptosis in silver carp, with an LD₅₀ of 524.4 µg/Kg (Li et al., 2019).

Apart from acute toxic effects, previous reports showed that cyanotoxins could exert chronic toxic effects on immune system of aquatic animals (Padmavathi and Veeraiah, 2009). The increasing reactive oxygen species (ROS) level was always observed in fish after treatment with cyanobacterial toxins (Li et al., 2003), which means they were stated in uncomfortable environment (Prasannaraj and Venkatachalam, 2017; Valko et al., 2007; Chen et al., 2016). To protect organisms from excess ROS, the anti-oxidase system will make response immediately (Ren et al., 2014). Chen et al. reported that SOD activity and gene transcription are both increased in the hepatopancreas after a 30-day exposure of white shrimp to pure MC (Chen et al., 2017). However, opposite results were reported in tilapia, as oral exposure to cyanobacterial cells decreased SOD activity in the examined organs (Prieto et al., 2007). In the present study, SOD activity and mRNA expression were both decreased in the hepatopancreas of *L. vannamei* after injection with *M. aeruginosa* cell extract. Decreased activity and transcription of SOD implied weaker antioxidant capacity and immunity of *L. vannamei* (Pang et al., 2019). GSH was another effective antioxidant and reported to play a vital role against cyanobacterial toxicity (Carneiro et al.,

2017). It was reported that exposure to MC or cyanobacteria increased the GSH content in the liver of both mammals and aquatic animals (Gehring et al., 2004; BLÁHA et al., 2004). However, in this study, the level of GSH in hepatopancreas of *L. vannamei* was slightly decreased. GSSG is the oxidation form of GSH and the GSH/GSSG ratio well reflects the intracellular redox state (Schmitt et al., 2015). This study showed that the GSH/GSSG ratio declined after shrimp injection with the extract. Most likely, the decreased GSH/GSSG ratio was found in the livers of *Cyprinus carpio* L. exposed to microcystin-LR (Jiang et al., 2011). The decreased value indicated an oxidative stress in shrimp treated with the *M. aeruginosa* cell extract. The increasing oxidative stress and unbalance of GSH/GSSG usually observed in the body that infected by pathogens or stated in uncomfortable environment (Prasannaraj and Venkatachalam, 2017; Valko et al., 2007; Chen et al., 2016).

GSH plays important roles in protecting organisms from toxic effect of cyanotoxins not only as an antioxidant eliminating reductant ROS, but also as an essential antidote that directly takes part in detoxification with the help of Glutathione S-transferase (GST), a phase II detoxification enzyme (Deponte, 2012). Thus, changes in GST transcript level are useful indicators of the detoxification ability of marine animals (Hannam et al., 2009). Le et al. reported a significant GST increase after a 24-h exposure of fish to MC-LR (Le et al., 2009). Gonçalves et al. reported that MCs caused up-regulation for *gst* in the hepatopancreas of *L. vannamei* (Gonçalves-Soares et al., 2012). However, the GST response may vary depending on the exposure scenarios. Li et al. showed that MC-LR inhibited *gst* transcription in the liver and kidney of goldfish (Li et al., 2008). The present study revealed that shrimp injection with MC-LR-containing *M. aeruginosa* cell extract inhibited *gst* expression in the hepatopancreas. The different responses of GST might the result of different concentration of toxins, or different tissue sample (Martins et al., 2019). In addition, the different effects of pure MCs and cyanobacterial extracts on immunity response were also found in fish, which could be attributed to differences in composition of the pure MCs and extracts containing MCs and other toxins like LPS (Rymuszka and Adaszek, 2013). Atencio et al. reported that decreased *gst* transcription limited the ability of the organism to clear specific toxic substances from the body (Gehring et al., 2004).

In the immune system of shrimp, antimicrobial peptides (AMPs) play an important role in combating pathogenic microorganisms (Li et al., 2013). Lysozyme, as one of the most important AMPs, is associated with the first line of immune defense (Duan et al., 2019), and its activity is a biomarker of environmental contamination (Bols et al., 2001). Decreased LZM activity in shrimp has been reported after intoxication by MC-LR (Zhang et al., 2019). Moreover, Qiao et al. reported that in carp LZM activity is clearly increased fed with low dose of cyanobacteria lyophilized powder but a sharp decrease in high dose group (Qiao et al., 2013). In the present study, both LZM activity and transcription were inhibited by the *M. aeruginosa* extract, and decreased LZM activity may therefore weaken nonspecific defense in shrimp, making *L. vannamei* more susceptible to pathogens, further confirming microalgal-induced immunotoxicity in shrimp.

Conclusions

The result of the present study revealed that *M. aeruginosa* has great toxic effect on *L. vannamei*. High dose of *M. aeruginosa* cells would exert great mortality in *L. vannamei*, and low dose depressed the immunity of shrimp, including antioxidant, detoxification and antimicrobial activity. Furthermore, the toxic substance may mainly belongs to intracellular metabolites, which contains 190.40 mg/kg (wet cells) of MC-LR. Considering that MCs can accumulate in tissues of aquatic animals, the density of *M. aeruginosa* should be regularly monitored and kept to a low level during shrimp farming, to preserve the health of cultivated organisms, as well as humans. In recent years, studies on using algicidal bacteria to control harmful algal blooms have received increasing attention, as algicidal bacteria have specificity against harmful algae and lack secondary pollution (Hu et al., 2019; Xu et al., 2019)

Declarations

Data availability

The datasets supporting the conclusions of this article will be available in the Open Science Framework repository.

Author contributions

All authors contributed to this work. Y.C. Cao formulated the project idea, Y. Xu performed the experiments and wrote the paper, W.J. Xu and X.J. Hu analyzed the data, H.C. Su created figures and tables, G.L. Wen and K. Yang provided editorial advice. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Consent to participate All authors consent to participate.

Consent for publication All authors consent for publication.

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Tables

Table 1. Primers used for quantitative real time PCR

Target gene name	Primer name	Sequences(5'-3')
<i>sod</i>	<i>sod-F</i>	GCCAATGACGTAAGCGCAAT
	<i>sod-R</i>	TCGTCAATGGCTTGTGCAAC
<i>gst</i>	<i>gst-F</i>	CCGGAGACAAGCTAACCTACATC
	<i>gst-R</i>	CCTCGAGATCCTCAAACCTCTT
<i>lzm</i>	<i>Lzm-F</i>	GTCTAGGCTCAACCATCGCA
	<i>Lzm-R</i>	GAAATTCCTGAGCCGAAGTGC
<i>β-actin</i>	<i>β-actin-F</i>	CTCCTCGCTGGAGAAGTCCTA
	<i>β-actin-R</i>	ACAGGTCCTTACGGATGTCCA

Figures

Figure 1

The cumulative mortality rate of shrimp (%) injected with *M. aeruginosa* cells, extract and media substrate, having sterile phosphate buffer solution (PBS) as a comparison. Each bar represents the mean value from three replicate tanks with the standard error. Means ($\pm$ S.E.) with a different letters indicates $p < 0.05$.

Figure 2

HPLC analysis of microcystins. The MC-RR/YR/LR peaks are showed as a standard reference in the analysis (A). Chromatogram showing the results of liquid chromatography (LC) analysis from extract containing microcystin-LR (MC- LR) (B).

Figure 3

The calibration curve of a certified reference standards of microcystin-RR (A), microcystin-YR (B), microcystin-LR (C) measured with HPLC. The concentration of microcystin- RR/YR/LR was 0, 0.1, 0.2, 0.5 and 1.0 µg/mL.

Figure 4

SOD activity, GSH content, GSH/GSSG and LZM activity in hepatopancreas of *L. vannamei* inoculated with sterile *M. aeruginosa* cell extract, MC-LR and PBS for 0, 1, 2, 3, 4 and 5 days. Each bar represents the

mean value from five replicate tanks with the standard error. Means ($\pm$ S.E.) with an asterisk indicates $p < 0.05$, and with two asterisks indicate $p < 0.01$.

Figure 5

The transcript level of immune-related genes *sod*, *gst* and *lzm* in hepatopancreas of *L. vannamei* injected with sterile *M. aeruginosa* cell extract, MC-LR and PBS for 0, 8, 24, 48, 72, 96 and 120 hours. Each bar represents the mean value from five replicate tanks with the standard error. Means ($\pm$ S.E.) with an asterisk indicates $p < 0.05$, and with two asterisks indicate $p < 0.01$.