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Dietary resveratrol attenuates oxidative stress in Pacific whiteleg shrimp, *Litopenaeus vannamei*, in response to ammonia stress

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Abstract

To study the effect of resveratrol on the antioxidant system of *Litopenaeus vannamei*, resveratrol (0, 40, 80, 120 and 180 mg/kg) was added to the feed. After 7 days of culture, 10 tails of *L. vannamei* were randomly selected and exposure to ammonia stress at the sublethal concentration of ammonia (20 mg/L) for 24h. The results showed that the malondialdehyde (MDA) level in serum of shrimp fed with resveratrol decreased significantly compare to the control group ($p< 0.05$). After the ammonia stress, the MDA levels in serum in the Res 120 group and the Res 160 group was lower significantly than that in the control group ($p< 0.05$). The activities of superoxide dismutase (SOD) in the hepatopancreas and the serum, and glutathione peroxidase (GPX) in the serum of shrimps fed with resveratrol showed a decrease trend before the ammonia stress. Conversely, the activities of SOD and the total antioxidant competence (T-AOC) levels in the hepatopancreas in the Res 80 group and Res 120 group were significantly higher than that in the control group after the ammonia stress ($p<0.05$). Dietary resveratrol decreased oxidative damage, increased antioxidant enzyme activities under the ammonia stress. Our results demonstrated that resveratrol attenuated oxidative stress and improved the resistance to the ammonia stress.

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Introduction

Shrimp culture has been become an important part of aquaculture, and the Pacific whiteleg shrimp, *Litopenaeus vannamei*, is the most important farmed shrimp specie worldwide with a total of 1.82 million tons production in China in 2019 (Ministry of Agriculture and Rural Affairs of PRC, 2020). However, with the expansion of demand for shrimp production, the cultivation scale and the cultivation density are increasing. Shrimp subjects to serious environmental stress, such as ammonia nitrogen, nitrite and unnormal pH, which could increase oxidative stress and the susceptibility of shrimp to pathogens, and lead to the outbreak and epidemic of diseases (Tseng et al., 2004; Li et al., 2008; Liu et al., 2015a; Liang et al., 2016; Wang et al., 2020a). Ammonia nitrogen (ammonia), a common environmental stressor for aquatic animals, originates from excretion of the animals and decomposing organic wastes such as residual feed and feces in the water. Ammonia exposure induced oxidative stress, endoplasmic reticulum stress and apoptosis in hepatopancreas, inhibited the expressing of some immune response genes, and increased the malonyldialdehyde (MDA) level (Liang et al., 2016; Lu et al., 2016). Furthermore, ammonia could inhibit growth and resistance to pathogens, cause mortality of shrimp (Liu et al., 2004; Rostami et al., 2019).

Shrimp, just like any other organism, has a strong antioxidant system including antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), glutathione peroxidase (GPx) and some non-enzymatic antioxidants such as reduced glutathione, metallothionein, ascorbic acid, caratenoids. The antioxidant system maintains a balance of the generation and clearance of reactive oxygen species (ROS) in organism (Xu et al., 2012; Chen et al., 2020; Ebadi et al., 2020; Yu et al., 2020). Supplemental exogenous antioxidant can increase the antioxidant capacity. Therefore, some preventive and remedial additive agents were studied and applied in shrimp farming (Chien et al., 2003; Kitikiew et al., 2015; Shan et al., 2019; Chen et al., 2020). Resveratrol (trans-3,4,5-Trihydroxystilbene) is a natural polyphenol found in particularly high concentrations in red wine as well as numerous plants, such as grapes, peanuts, and other groundnuts. It has been attracted intense interest for its potentially beneficial effects and the mechanism of action (Robb, et al., 2008; Wang et al., 2019). It exhibits anti-oxidant, anti-inflammatory, anti-aging and anti-tumorigenic effects (Robb et al., 2008; Kim et al., 2018; Wang et al., 2019). Previous studies have reported that resveratrol could promote growth (Wilson et al., 2015), attenuate the oxidative damage (Liu et al., 2015b), increase antioxidant capability (Wang et al., 2020b), improve lipid and glucose metabolism (Zhang et al., 2017), protect from liver injury, inflammation and immunotoxicity induced by H_2O_2 (Jia et al., 2019) in aquaculture. However, whether resveratrol affects the oxidative status of shrimp has not yet been reported. Thus, we evaluated the effects of resveratrol on oxidative stress and resistance to ammonia by investigating oxidant stress status and antioxidant capacity with the aim of understanding the mechanism of action.

Materials and methods

Experimental animal and Diets

The study was performed using a batch of apparently healthy shrimps (*L. vannamei*) from a shrimp farm at Donghai Island, Zhanjiang, Guangdong province. The average body weights of the shrimp were 11.09±0.51 g. Shrimps were acclimated to experimental conditions in an indoor water system for 7 d before the experiment began.

In this study, Yuehai brand commercial shrimp diet was used as the fundamental diet. The diet contains 40% of crude protein, 4.0% of crude fat, 5.0% of crude fibre, 16.0% of ash and 12% of moisture. Resveratrol (98%) was bought from Harveybio (Beijing) Gene Technology Co., Ltd. Five different diets containing different resveratrol levels (0, 40, 80, 120 and 160 mg kg-1, as Diet 1, Diet 2, Diet 3, Diet4 and Diet 5) were prepared based on the commercial diet. The feed was covered with 1% feed adhesive (Hainan Zhuoyue Biology Co., Ltd). Diet without resveratrol was used as control diet.

Experimental design

The experiment was carried out in an indoor rearing system. The experiment was divided into two phases: a 7 d feeding trial and a 24 h ammonia stress trial. At the beginning of the feeding trial, 450 healthy shrimps were randomly assigned into 15 plastic tanks (300 L) with 200 L water at a density of 30 shrimps per tank. Each treatment has three replicates. The shrimps in control group, Res 40 group, Res 80 group, Res 120 group and Res 160 Group were fed with Diet 1, Diet 2, Diet 3, Diet 4 and Diet 5, respectively. Shrimps was fed daily at 6:00A.M., 14:00 P.M., and 20:00 A.M. at normal day-night illumination. Residual diets and faces were removed by siphoning, and then about 15-30% of the total water volume was renewed daily to supplement the water siphoned off. During the feeding trial, the water was aerated continuously; the pH, salinity and temperature of the water in tanks during the trial were 8.1±0.1, 28.0 ppt and 28.0 °C, respectively. The natural filtered seawater was used.

After the feeding trial, ammonia stress trial was performed. At the end of the feeding trial, 10 shrimps in the intermoult per tank were collected for the ammonia stress trial. Shrimps exposed to the sublethal concentration of ammonia (20 mg/L total ammonia) for 24h. NH_4Cl was added to adjust to the ammonia concentration. During ammonia stress trial, the water wasn't aerated and renewed to ensure the stability of the ammonia concentration and minimize environmental impacts.

Sample collection

All animal sampling and handling procedures were followed in accordance with the standard guidelines and regulations on the use of animals for scientific research and purpose. To estimate oxidative stress, 5 individuals were sacrificed on ice for haemolymph and tissue collection for the analysis of antioxidant parameters at the end of the feeding trial and the ammonia stress trial, respectively. Haemolymph samples were rapidly collected from the sternal sinus located at the ventral part of cephlathorax with a syringe, and transferred to a sterile microcentrifuge tube, then kept at 4 °C for 12 h. The serum was collected by centrifuging at 3000 g for 15 min at 4°C, and then stored at -80 °C. The excised hepatopancreas tissues were homogenized in Tris-HCl buffer (pH 7.4) at 4°C. The supernatant fluids were collected after centrifuging at 4000 g for 10 min at 4°C for antioxidant parameters analysis.

Analytical methods

The levels of MDA and T-AOC, the activity of SOD, CAT and GPX were monitored by measuring the optical density (OD) value at 523nm, 520nm, 550nm, 405nm and 412nm, respectively, using commercial kits (Nanjing Jiancheng Bioengineering Institute, China) according to the instructions of the manufacturers.

Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA) and Duncan's multiple range tests using the SPSS 21 statistical software (SPSS Inc., USA) and EXCEL 2016 (Microsoft, USA). With respect to the results of Duncan's multiple range test, different lowercase letters indicate significant differences between the different treatments groups at each sampling point.

Result

MDA concentration in different tissue

The MDA levels were investigated in the hepatopancreas and serum of shrimps at the end of the feeding trial and ammonia stress trial (**Figure 1**). Prior to the ammonia stress trial, the MDA Levels in the hepatopancreas of shrimps fed with resveratrol had decreased by 5.2%, 7.1%, 17.1%, 19.0%, but no significant differences were observed. The MDA levels in the serum of shrimps fed with resveratrol diet were significantly lower than that in the control group ($p < 0.05$). The MDA level of shrimps fed with diet 4 containing 120 mg kg^{-1} resveratrol had decreased significantly by 57.45% (**Figure 1A**).

As shown in **Figure 1B**, the MDA levels in the serum in the Res 120 group and the Res 160 group after ammonia stress were significantly lower than that in the control group (p

< 0.05). Surprised, the MDA levels in the hepatopancreas in the Res 120 group was higher than that in the control group ($p < 0.05$), while those in the other groups were lower than that in the control group. After ammonia stress, the MDA levels in the hepatopancreas show an increase trend compare to those before ammonia stress trial, conversely, those in the serum show a decrease trend.

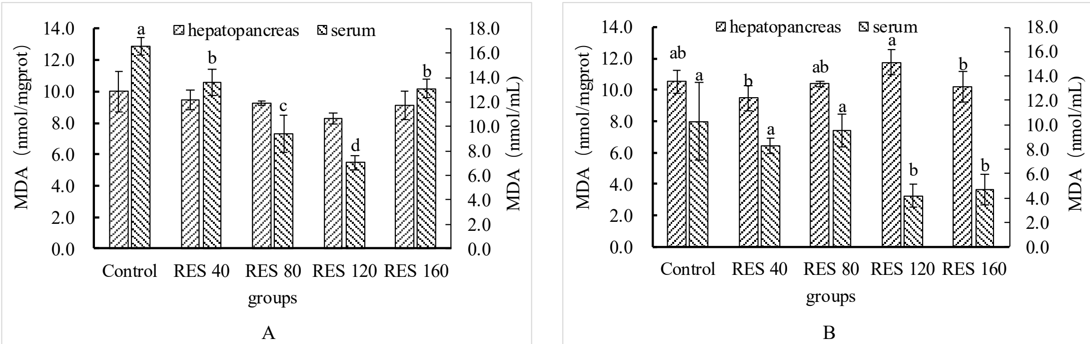

Figure 1 Effect of resveratrol on the MDA levels in different tissues of *L. vannamei* before (A) and after (B) the ammonia-N stress.
Notes: Different lower-case letters indicate significant differences between groups (the same as below). The primary Y axis is used for the MDA levels in the hepatopancreas. The second Y axis is used for the MDA levels in serum.

Activity of SOD in different tissue
As showed in **Figure 2A**, the activities of SOD in the hepatopancreas of shrimps fed with resveratrol diets were significantly lower than that in the control group ($p<0.05$). The activities of SOD in the serum of shrimps fed with 120 mg kg^{-1} resveratrol diet was also significantly lower than that in the control group ($p<0.05$).
After ammonia stress, the activities of SOD in the hepatopancreas of shrimps fed with resveratrol diets show an increase trend compare to that before ammonia stress trial (**Figure 2B**). The activities of SOD in the hepatopancreas in the Res 40 group, Res 80 group and Res 120 group were significantly higher than that in the control group ($p<0.05$). There were no significant differences in the activities of SOD in the serum between all groups.

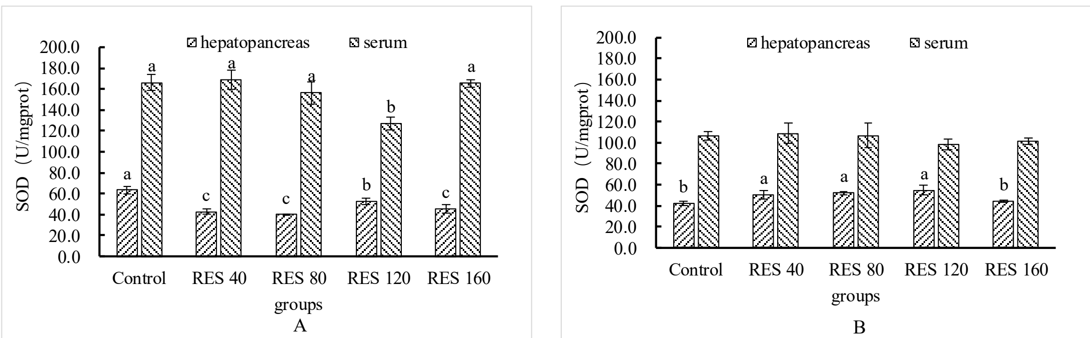

Figure 2 Effect of resveratrol on the activities of SOD in different tissues of *L. vannamei* before (A) and after (B) the ammonia-N stress.

Activity of GPX in different tissue
The activity of GPX of in the hepatopancreas and serum of the shrimps at the end of the feeding trial and ammonia stress trial were shown in **Figure 3**. The activities of GPX in all tissues of shrimps were affected significantly by resveratrol ($p<0.05$). The activities of GPX in the hepatopancreas in the Res 40 group and Res 80 group were significantly lower than that in the control group ($p<0.05$). Meantime, the activities of GPX in the serum in the Res 40 group, Res 120 group and Rev 120 group were significantly lower than that in the control group ($p<0.05$) (**Figure 3A**).

As showed in **Figure 3B**, the activities of GPX in the serum of the shrimps showed the same change trend compare to that before the ammonia stress. The activities of GPX in the serum in the control group and the Rev 80 group were higher than those in the Res 40 group and Res 120 group ($p<0.05$). Compare to that before the ammonia stress trial, the activities of GPX in the hepatopancreas and serum of shrimps in all group showed a decreased trend.

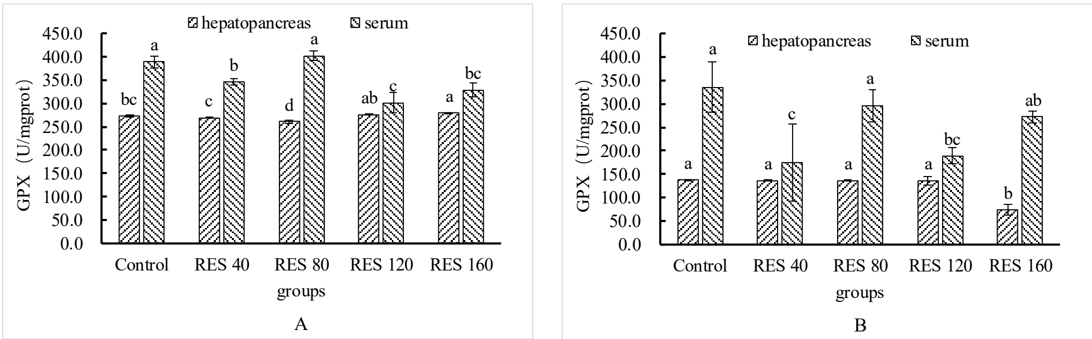

Figure 3 Effect of resveratrol on the activities of GPX in different tissues of *L. vannamei* before (A) and after (B) the ammonia-N stress.

Activity of CAT in different tissue

The activities of CAT in the hepatopancreas and serum of shrimps were shown in **Figure 4**. The activities of CAT in the serum in the Res 120 group was significantly higher than that in the control group ($p<0.05$). Although large difference in the activities of CAT in the hepatopancreas and serum were found, but no significantly differences were observed after the ammonia stress ($p>0.05$).

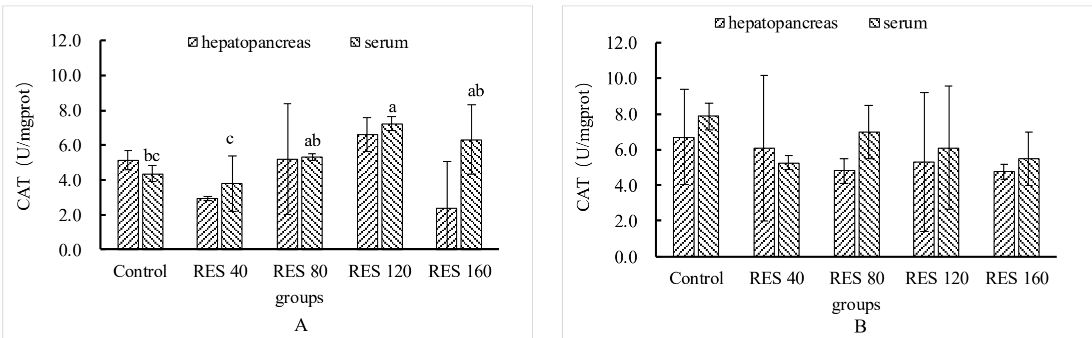

Figure 4 Effect of resveratrol on the activities of CAT in different tissues of *L. vannamei* before (A) and after (B) the ammonia-N stress.

T-AOC level in different tissue

The T-AOC levels in all tissues of shrimps were affected significantly by dietary resveratrol before the ammonia stress trial ($p<0.05$) (**Figure 5A**). The T-AOC levels in the hepatopancreas of the shrimps fed with resveratrol were significantly lower than that in the control group before the ammonia stress ($p<0.05$). However, the T-AOC levels in the serum in the Res 120 group were significantly higher than that in the control group ($p<0.05$). After the ammonia stress, the T-AOC levels in the hepatopancreas in the Res 80 group, Res 120 group and Res 160 group were significantly higher than that in the control group ($p<0.05$). There is no significant difference in the serum between all groups after the ammonia stress.

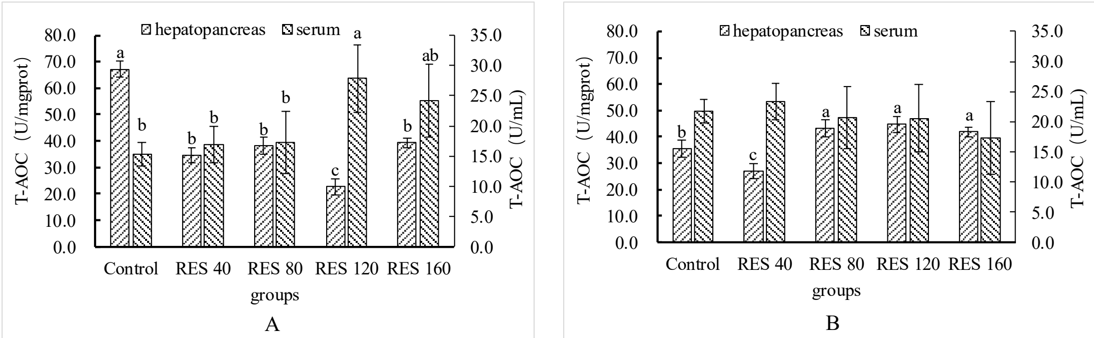

Figure 5 Effect of resveratrol on the T-AOC levels in different tissues of *L. vannamei* before (A) and after (B) the ammonia-N stress.
Notes: The primary Y axis is used for the T-AOC levels in the hepatopancreas. The second Y axis is used for T-AOC levels in serum.

Discussion

Effects of dietary resveratrol supplementation on MDA

Resveratrol is a naturally occurring antioxidant, and has been proved to have antioxidant effects in mammals. Nevertheless, there is a few reports about its effect on aquatic animals, especially shrimp. Prior studies have examined the effects of some remedial addition agents, such as probiotics and prebiotics (Kitikiew et al., 2015; Chen et al., 2020), feed organisms (*Ampithoe* sp.) (Shan et al., 2019), astaxanthin (Chien et al., 2003; Yu et al., 2020), vitamin C and vitamin E (Ebadi et al., 2020) on oxidative stress and antioxidant capacity in shrimp. Various parameters, MDA and protein carbonyl, are used as the biomarkers of oxidative damage. The MDA is one of the final products of lipid peroxidation, and is positively correlated with oxidative stress and lipid peroxidation. Astaxanthin contributed to dose-dependent protection against the oxidative damage induced by oxidized fish oil in *L. vannamei* (Yu et al., 2020). In this study, the MDA levels in the hepatopancreas and serum decreased compare to that in the control group. Moreover, the MDA level in the hepatopancreas in shrimp fed with resveratrol were significantly lower than that in the control groups before ammonia stress ($p<0.05$). Briefly, the higher MDA levels revealed that the shrimps suffered more oxidative stress. These results demonstrated that resveratrol decreased the degree of the environmental-induced increase of lipid peroxidation and the oxidative stress in shrimp. Our results are in accordance with other findings. Resveratrol treatment reduced significantly the increase of ROS and oxidative damage with age in a short-lived fish (*Nothobranchius guentheri*) (Liu et al., 2015b). Resveratrol also enhanced growth, increased antioxidant capacity, and limited protein and lipid damage in *Paralichthys lethostigma* (Wilson et al., 2015).

Hepatopancreas is as the metabolic center for reactive oxygen species production in crustacean, and is more sensitive to ROS (Han et al., 2018). After the ammonia stress, an increase trend of the MDA levels in the hepatopancreas in all groups was observed. Liang et al. reported that ammonia exposure could induce oxidative stress, and cause increase of the MDA level in *L. vannamei* (Liang et al., 2016). Dietary antioxidant, such as glutathione, reduced dose-dependently the MDA level in *L. vannamei* by the glutathione supplement antioxidant (Xu et al., 2012). In this study, the MDA levels in the serum in the Res 120 group and the Res 160 group were significantly lower than that in the control group after the ammonia stress ($p < 0.05$). This result indicated that pre-treatment with resveratrol attenuated the oxidative damage by ammonia stress in *L. vannamei*. Which was consistent with the study that resveratrol suppressed the elevation of MDA in serum and liver in *Oreochromis niloticus* (Jia et al., 2019).

Effects of dietary resveratrol supplementation on antioxidant capacity

Under normal conditions, antioxidant system maintain a balance of the generation and clearance of reactive oxygen species in organism. However, cultured shrimp often suffers various environmental stresses, such as stocking density, physicochemical parameters (e.g., ammonia, nitrite, unnormal temperature and unnormal pH) and pathogens.

Organisms have a certain capacity to adapt these stresses, but excess stress affect the antioxidant capacity and the immune capacity (Marcadenti et al., 2015). Various antioxidant enzymes play an important role in their environmental adaption and antioxidant defense. Endogenous antioxidant enzymes, such as SOD, GPX and CAT, are the important and major enzymes used to balance out free radicals, eliminate these potentially hazardous and prevent from oxidative damage (Ifeanyi, 2018). The nuclear erythroid 2-related factor 2 (Nrf2) is a critical transcription factor, which activates the expression of the genes containing the antioxidant response element (ARE), and increase the activities of antioxidant enzymes (Dong et al., 2008). Resveratrol is an Nrf2 activator and an effective antioxidant that scavenges ROS and modulates physical performance preventing oxidative stress (Salomão et al., 2019; Farkhondeh et al., 2020). Resveratrol decreased the levels of ROS, elevated the activities of CAT, SOD and GPX, and exerted resveratrol increased protective effects on activities of antioxidant enzymes in *N. guentheri* (Liu et al, 2015). Dietary resveratrol upregulated the mRNA expressions of Cu/Zn-SOD, CAT and GPx, and improve immunity, antioxidant capability and lipid metabolism of blunt snout bream (*Megalobrama amblycephala*) (Jia et al., 2019). Resveratrol also enhanced significantly the mRNA expression of *SOD*, *GPX* and the activity of SOD in turbot (Tan et al., 2019). Conversely, in this study, the activities of SOD and GPX, and the T-AOC levels in the hepatopancreas and serum of shrimps fed with resveratrol showed decreased trends before ammonia stress compare to that in the control group. The activities of SOD and the T-AOC levels in the hepatopancreas of shrimps fed with resveratrol diets were significantly lower than that in the control group ($p<0.05$). Considering the lower MDA levels, we speculated that the decreased enzyme activity of SOD and GPX of shrimps fed with resveratrol might be an adaptive response to lower oxidative stress. Fish (*Hyphessobrycon callistus*) fed with carotenoid had lower SOD and GPX (Wang et al., 2006). Dietary astaxanthin also reduced the SOD activity in Tiger prawn (*Penaeus monodon*) (Chien et al., 2003). The activities of SOD and CAT, and the MDA levels of *Clarias gariepinus* in clean region from Orontes river were higher than those in the polluted regions (Turan et al., 2020). The SOD activity of *L. vannamei* injected *Vibro parahaemolyticus* at 3h, 6h and 12h were higher than that in the control group (Pang et al., 2019). These results indicated that organism with lower oxidative stress had a reduced need for endogenous antioxidant enzymes.

As mentioned above, hepatopancreas is more sensitive to ROS. In this study, an increase trend in the activities of SOD in the hepatopancreas of shrimps fed with resveratrol diets was observed compare to that before ammonia stress trial. The T-AOC levels in the hepatopancreas in the Res 80 group, Res 120 group and Res 160 group were also higher than that before ammonia stress. The increased activity of SOD and the T-AOC level of shrimp fed with resveratrol might be an adaptive response to the elevated oxidative stress, suggesting that the antioxidant capacity of shrimp was improved by dietary resveratrol. The expression of the SOD, and prophenoloxidase genes in shrimp fed with hydrolyzed yeast and/or *Bucillus licheniformis*up-regulated after ammonia challenge (Chen et al., 2020). Dietary astaxanthin improved the antioxidant capacity of *L. vannamei* by increasing the CAT activity (Yu et al., 2020). Consequently, the present research hypothesizes that resveratrol might play an important role in increase antioxidant capacity and decrease oxidative damage by environmental stress in shrimp.

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