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RESEARCH PAPER

Effects of Dietary Vitamin C on the Physiological Responses and Disease Resistance to Ph Stress and *Aeromonas Hydrophila* Infection of *Megalobrama Amblycephala*

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Abstract

We evaluated the effect of vitamin C (Vit C) supplementation on the resistance of *Megalobrama amblycephala* to *Aeromonas hydrophi1a* infection under pH stress. Fish were randomly divided into six groups: a control group (fed with a basal diet) and five treatment groups (fed with basal diet supplemented with 33.4, 65.8, 133.7, 251.5 and 501.5 mg/kg Vit C, respectively). After 90 days, fish were exposed to combined stressors, first pH 9.5 followed by *A. hydrophila* infection. The results showed that 133.7 mg/kg vit C improved complement 4 (C4), anti-superoxide anion free aradical (ASAFER) and heat shock protein (HSP) 70 compared with the control group; and 251.5 mg/kg vit C enhanced complement 3 (C3), HSP60, HSP70 and HSP90 compared to the control group before stress. After pH stress and *A. hydrophila* infection, hemoglobin, ASAFER and HSP60, HSP70, HSP90 in the groups fed with 133.7 and 251.5 mg/kg vit C were still significantly higher, while serum cortisol in the group fed with 251.5 mg/kg vit C was lower compared to the control group 15 days after pH stress. The cumulative mortality of the control group was higher than that of the five treatment groups at 12, 24 h after *A. hydrophila* infection. The results of this study suggest that 133.7- 251.5mg/kg vit C in a diet could have potential to stimulate immune response, and enhance resistance against high pH stress and *A. hydrophila* infection of Wuchang bream.

Keywords: *Aeromonas hydrophila*; pH stress; Vitamin C; Physiological response; *Megalobrama amblycephala*.

Introduction

Wuchang bream (*Megalobrama amblycephala*) is a principal species in Chinese freshwater polyculture systems. Its production in China was approximately 0.72 million tons in 2012 (Ministry of Agriculture of the People's Republic of China, 2013). However, as one of the most widely cultured freshwater fish in China, Wuchang bream has faced an increasing disease outbreak caused by pathogenic bacteria. This has been resulted in a considerable economic loss in China (He *et al*. 2006).The main reason could be due to adverse environmental stress factors (including pH), which may affect the metabolism of aquatic animals (Wendelaar 1997), osmotic capacity (Pan *et al*. 2007), gill function and morphology (Wilkie *et al*.1994) and immune system (Yin *et al*.1995; Rotlant *et al*.1997; Ndong *et al* 2007 ; Le Moullac *et al*. ,2000).This may render fish vulnerable to pathogenic bacterial infection and lead to the outbreaks of infectious diseases followed by economic losses in fish farming industry (Ojolick *et al*.1995; Lavilla-Pitogo *et al*.1998; Li and Chen 2008).

Aeromonas spp. is ubiquitous bacteria, native to aquatic environments and consists of two major groups, the psychrophilic and mesophilic groups (Monfort *et al*.1990; Topić Popovic *et al*. 2000). In particular, *Aeromonas hydrophila* has been reported as an important pathogen for humans and for lower vertebrates, including amphibians, reptiles and fish (Janda and Abbott 1998). *A. hydrophila* is responsible for hemorrhagic septicemia and causes high levels of mortality and significant economic loss in fish culture (Kozinska *et al*. 2002; Vivas *et al*. 2004; Ogara *et al*.1998; Wang and Silva1999).

Vitamin C (vit C), also known as L-ascorbic acid, is an essential micronutrient for normal growth and physiological function of fish. Previous studies demonstrated that vit C have been proved to play key roles in enhancing health and growth performance of fish (Ai *et al*. 2006; Eo and Lee 2008; Tewary and Patra 2008), improving reproduction (Emata *et al*. 2000; Lee and Dabrowski 2004), modulating stress (Özkan *et al*. 2012; Ming *et al*. 2012; Barrosa al. 2014), elevating immune capacity (Sobhana *et al*. 2000; Ortuno *et al*. 2003; Ai *et al*. 2006; Nayak *et al*. 2007; Eo and Lee, 2008; Tewary and Patra 2008), and

enhancing disease resistance (Sobhana *et al*.2000; Barrosa al. 2014).

In our previous study, feed supplemented with 251.5 mg/kg -501.5 mg/kg vit C improved the growth performance and non-specific immunity of *M. amblycephala* (Wan *et al*., 2013). At present, the effect of vit C on the physiological responses under the combined stress of high pH level and *A. hydrophila* infection of *M. amblycephala* has been hardly found in research reports. Our objective was to further evaluate the effect of dietary vit C supplementation on the non-specific immune responses to pH stress and bacterial infection in *M. amblycephala*. Firstly fish were fed diets with or without vit C, and secondly fish were exposed to high pH stress condition. Then fish were challenged with *A. hydrophila* and measured haematological parameters [white blood cell (WBC), red blood cell (RBC), and hemoglobin (HGB)], serum physiological parameters [cortisol, complement 3 (C3), and complement 4 (C4)) and hepatic oxidization indices (superoxide dismutase activity (SOD), antisuperoxide anion free radical (ASAFR), malondialdehyde content (MDA)], and three hepatic heat shock proteins (HSP60, 70, 90) mRNA expressions. Our results provide insight in to the physiological responses and molecular mechanisms underlying the protective effect of vit C in *M. amblycephala* under pH stress and *A. hydrophila* infection.

Materials and Methods

Fish, Vitamin C, and Diets

The use of the experimental fish was according to the recommendations of the Guidelines for the Care and Use of Laboratory Animals of China. Healthy juvenile *M. amblycephala* (average weight 6.40±0.05 g) were provided by freshwater fisheries research institute of Jiangsu province, China. A total of 450 fish were stocked in 18 round fiberglass tanks (φ820×700mm, N= 25 fish/tank). Prior to the experiment, Wuchang bream were acclimated with the commercial diet (Wuxi Tongwei Feed Co., Ltd., China) in the tanks for 22 days. After that, fish were randomly divided into six groups (N=3 tanks / group): one control and five treatment groups. Triplicate groups of *M. amblycephala* (3 tanks, 25 individuals per tank) were fed with the basal diet (See Table 1) and the basal diet supplied with 33.4, 65.8, 133.7, 251.5 and 501.5 mg/kg vit C, respectively.

The basal practical diet was formulated to contain about 32.12% crude protein and 6.68% lipid. Six diets were formulated to contain 0.0, 30.0, 60.0, 120.0, 240.0 and 480.0 mg ascorbic acid per kg diet, respectively. Coated-ascorbic acid (CAA) (95% ascorbic acid equivalent, Roche, Swiss) was used as the vit C source. However, the analyzed ascorbic acid levels were 0.2, 33.4, 65.8, 133.7, 251.5 and 501.5 mg kg^{-1} diets. Various feedstuffs were separately pulverized and screened through 60 mesh size sieve; and first mixed calcium dihydrogen phosphate, soy lecithin, choline chloride, ethoxyquin, mineral and vitamin premix (vitamin C free) and then evenly mixed with vit C; and at last evenly mixed bulk feed ingredients. The diets were prepared at the research facilities of Fishery Machinery and Instrument Research Institute with 1.0 mm granular wet pellet. The moisture of feed was about 10% and was kept at -20℃ until used.

Fish Husbandry

Fish were cultured in a tank with automatic thermo-regulated recirculating system. The tanks were supplied with aerated and recycled water at a rate of 2 L min^{-1}. During the experimental period, fish were hand fed three times a day (8:00-9:00, 11:00-12:00, and 15:00-16:00) at a feeding rate of 2.0%-4.0% body weight. The tanks were supplied with continual oxygen. During the experiment, water temperature was measured twice a day (at 8:00 and 16:00), and other water quality parameters were checked once a week. The mean water quality indices were: water temperature ranged 26- 28℃, dissolved oxygen (DO) > 6 mg L^{-1}, NH_3-N < 0.05 mg L^{-1}, pH 7.20 – 7.60. The amount of feed was adjusted every two weeks to account for increasing body weight. After 90 days, fish from each tank were counted and weighed.

Challenge Experiment

The Ph Challenge Experiment

At the end of the rearing experiment and according to a previously described method (Li and Chen 2008), after first sampling (before stress, 0 d), the rest fish from six groups (3 tanks/group) were reared for pH stress (high pH level: 9.5) for 15 days in the fiberglass tanks (φ820×700mm) with running water and the flow rate was 2 L/min. The mean water quality indices were: water temperature ranged 27±1℃, DO > 6 mg/L, and NH_3-N <0.05 mg/L. The water pH level was adjusted by adding 4N NaOH twice a day (8:00, 16:00).

A. Hydrophila Challenge Experiment

After 15d pH stress (mentioned on the above), after second sampling (15 d pH stress) 18 fish from each group (3 tanks/group) were challenged with *A. hydrophila*. The seven day LC_{50} was determined by intraperitoneal injection of 48 fish with graded concentrations of *A. hydrophila* (10^5,10^6,10^7,10^8 and 10^9 CFU/ml) at 24℃, and the result showed that the LC_{50} on day 7 was about 1×10^7 CFU/ml. According to our previous study's method (Liu *et al*. 2012), *A. hydrophila* was activated twice and diluted with sterile normal saline to a final concentration of 1×10^7

Table 1. Formulation and composition of experimental diet

Ingredients	(%)	Proximate composition	(%)
Casein (vitamin C free)	27.50	Crude protein	32.12
Gelatin	6.50	Crude lipid	6.68
Calcium dihydrogen phosphate	2.75	Nitrogen-free extract [c]	37.91
Soybean oil	6.00	Lysine	2.26
Soy lecithin	1.00	Methionine	0.79
Choline chloride (50%)	0.15		
Vitamin premix(vitamin C free) [a]	0.50		
Mineral premix [b]	0.50		
Dextrin	10.00		
α-starch	25.00		
Microcrystalline cellulose	9.05		
Carboxyl-methy cellulos	11.00		
Ethoxyquin	0.05		
Total	100.00		

[a] Vitamin premix (IU or per kg premix): Vitamin A, 900000 IU; Vitamin D, 250000 IU; Vitamin E,4500 mg; Vitamin K3, 220mg; Vitamin B1,320 mg; Vitamin B2,1090 mg; Vitamin B6,5000 mg; Vitamin B12,116 mg; Pantothenate,1000 mg; Folic acid,65 mg; Choline,60000 mg; Biotin,50 mg; Inositol,15000 mg; Niacin acid,2500 mg.

[b] Mineral premix (per kg premix): $CuSO_4 \cdot 5H_2O$,2.5g; $FeSO_4 \cdot 7H_2O$,28g; $ZnSO_4 \cdot 7H_2O$,22g; $MnSO_4 \cdot 4H_2O$,9g; Na_2SeO_3,0.045g; KI,0.026g; $CoCl_2 \cdot 6H_2O$,0.1g.

[c] Nitrogen-free extract,%=100%-(Moisture + CP + EE + CF+ Ash)%, and the others are measured according to Feed Industry Standard of China.

CFU/mL. The bacterial suspension (1.0 mL, per 100 body weight) was injected into the abdominal cavity, and then mortality was checked at 0h, 12h and 24h after challenge.

Serum and Liver Sample Collection and Measurement

Serum and liver samples were collected from 9 individuals in each group (3 fish/tank) prior to stress (0 d) and 15 d after high pH stress, and 1d after the challenge, respectively. At each sampling point, fish were rapidly netted and placed into the dose of 150 mg/L of MS-222. The blood from three fish randomly sampled from each tank was collected by caudal venipuncture using 1 mL medical syringes. After collection, 20 μl whole blood was used for analyzing blood WBC, RBC and HGB. The remaining whole blood was allowed to clot at 4℃ for 1-2 h. Following centrifugation (3000 × g, 10 min, 4℃), the serum was removed and frozen at -20℃ until used. The abdominal cavity of fish was immediately cut open after blood collection. About 0.1 g liver was frozen in liquid nitrogen and stored at -80℃ for determination of gene expression. Another piece of liver was stored at -20℃ for the analysis.

Measurement of Blood and Liver Samples

Blood WBC, RBC and HGB Measurement

Blood WBC, RBC and HGB were directly measured using an Auto Hematology Analyzer (BC-5300Vet, Mindray, P.R. China) with a test kit from Shenzhen Mindray Medical International Co. Ltd. (P.R. China) using a previously described method (Cui *et al*. 2013).

Serum Cortisol, C3 and C4 Measurement

The levels of cortisol were measured by the automatic chemiluminescence immunoassay analyzer MAGLUMI 1000 (Shenzhen, China) using assay kits purchased from Shenzhen New Industries Biomedical Engineering Co., Ltd, China, following a previously described method (Cui *et al*.2013; Zhou *et al*. 2013). Serum C3 and C4 activities were measured using the immunoturbidimetric method and the kits were purchased from Zhejiang Yilikang Biotech Co., Ltd (P.R. China), following a previously described method (Cui *et al*.2013).

Hepatic SOD, ASAFR and Malondialdehyde Measurement

Hepatic samples were homogenized in ice-cold phosphate buffer (1:10 dilution) (phosphate buffer saline: 0.064 M, pH 7.4). The homogenate was then centrifuged for 10 min (4℃, 4000 × g) and aliquots of the supernatant were used to quantify hepatic SOD, ASAFR and MDA. Hepatic SOD activity, ASAFR activity and MDA content were measured using the xanthine oxidase method (Granelli *et al*. 1995), xanthine oxidase method (Kong *et al*. 2004) and barbituric acid colorimetry (Drape *et al*.1993), respectively. We measured the hepatic protein content using the Folin method with bovine serum albumin as a standard. These kits for the detection were purchased from Nanjing Jiancheng Bioengineering Institute of China.

Real-time PCR Measurement of Hepatic HSP60, HSP70 and HSP90

We used *M. amblycephala* cDNA sequences in GenBank to design the primers for HSP60, HSP70,

HSP90 and beta-actin (Table 2). All primers were synthesized by Shanghai Biocolor, BioScience & Technology Company, China.

The total RNA was extracted from liver tissue of 50-100 mg using Trizol reagent (Dalian Takara Co. Ltd., China). Generally, the purified RNA had OD_{260}/OD_{280} ratio of 1.8-2.0. RNA samples were treated with RQ1 RNase-Free DNase (Dalian Takara Co. Limited, China) to avoid genomic DNA amplification. We generated cDNA from 500 ng DNase-treated RNA using ExScript™ RT-PCR Kit (Dalian Takara Co. Ltd., China). The reverse transcription PCR reaction solution consisted of 500 ng RNA, 2 μL 5×Buffer, 0.5 μL dT-AP Primer (50 mM), 0.25 μL ExScript ™ RTase (200 U μL^{-1}), and DEPC H_2O, up to a final volume of 10 μL. The reaction conditions were as follows: 42 ℃ for 40 min, 90 ℃ for 2 min, and 4 ℃ thereafter.

We used real-time quantitative PCR to determine mRNA levels with an SYBR Green one fluorescence kit, following a previously described method (Liu *et al*., 2012). Real-time quantitative PCR was performed in a Mini Opticon Real-Time Detector (Bio-Rad, USA). The fluorescent quantitative PCR reaction solution consisted of 12.5 μL SYBR® premix Ex Taq™ (2×), 0.5 μL PCR Forward Primer (10 μM), 0.5 μL PCR Reverse Primer (10 μM), 2.0 μL RT reaction mix (cDNA solution), 9.5 μL dH_2O. The reaction conditions were as follows: 95℃ for 10s, followed by 45 cycles consisting of 95℃ for 5s, 62℃ for 15s, 72℃ for 10s, plate read, and final step at 72℃ for 3 min. After the program finished, the C_t values of the target genes (three HSPs) and a chosen reference gene (beta-actin) were obtained from each sample. We measured the standard equation and correlation coefficient by constructing a standard curve using a serial dilution of cDNA; HSP60: Y=-0.310x+10.65, R^2=0.991; HSP70: Y=-0.361x+13.38, R^2=0.995; HSP90: Y=-0.314x+10.29, R^2=0.996; Beta-actin: Y=-0.304x+9.817, R^2=0.990; Y is the logarithm of the starting template to base 10, x is the C_t values. The relative expression level of gene could be calculated by double-standard curves method (Tang and Jia 2008).

Data Statistics and Analysis

We used SPSS (version 11.5) software followed by Turkey's-b test and Independent-Samples t-tests to determine the differences. Diverse little letters above histogram bars show the significant differences ($P<0.05$) among different dosage groups of each sampling point in Turkey's-b test. Significant differences ($P<0.05$) between values obtained before and after stress or infection are marked by asterisks above histogram bars in Independent-Samples t-tests. All the results were expressed as means ± standard error of means ($\overline{X}$ ± SEM).

Results

Effect of vit C on Survival of *M. Amblycephala*

The cumulative mortality was calculated for 24 h (Figure 1). At the conclusion of the experiment (24 h post challenge), the total accumulated percentages of mortalities were 100% in the control, and 61.11%, 61.11%, 44.44%, 38.34% and 55.56% in the group of 33.4, 65.8, 133.7, 251.5 and 501.5 mg/kg vit C, respectively. Higher cumulative mortality was observed in the control group compared to the five treatment groups at 12 and 24 h after *A. hydrophila* infection *(*$P<0.05$, Figure 1). Relatively, small number of death occurred in the groups fed with 133.7 and 251.5 mg/kg vit C 24 h after *A. hydrophila* infection compared to the other groups.

Effects of vit C on blood WBC, RBC and HGB in *M. Amblycephala*

The effects of vit C on blood WBC, RBC and HGB in fish are shown in Figure 2. Before stress, the blood WBC count was significantly reduced in the groups of 133.7 mg/kg vit C compared with the control group ($P<0.05$, Figure 2A). After pH stress, significantly higher blood WBC count was measured in the groups of 65.8, 133.7, and 251.5 mg/kg vit C than pre-stress level ($P<0.05$, Figure 2A). After infection, blood WBC count was only significantly reduced in the group of 501.5 mg/kg vit C compared with pre-stress level ($P<0.05$, Figure 2A).

Before stress, there was no significant effect on the blood RBC count and HGB content between the five treatment groups and the control group ($P>0.05$, Figure 2B, 2C). After pH stress, the groups of 65.8, 133.7, 251.5 mg/kg vit C had significantly higher

Table 2. Primer sequences for RT-PCR analysis of HSP and β-actin genes

Genes	Primer sequences (5'→3')	Product size (nt)	Gene accession
Beta- actin	(F) TCTGCTATGTGGCTCTTGACTTCG (R) CCTCTGGGCACCTGAACCTCT	132	AY170122.2
HSP60	(F) 5'-TGCTGTCTACTGCTGAAGCCGTTGT-3' (R) 5'-CCATCACTCAGTTTCGGCAGGTTT-3'	213	KC521465
HSP70	(F) 5'-CGACGCCAACGGAATCCTAAAT-3 (R) 5'-CTTTGCTCAGTCTGCCCTTGT-3'	92	EU884290.2
HSP90	(F) 5'-TGCGGGACAACTCCACCAT-3' (R) 5'-TCCAATGAGAACCCAGAGGAAAGC-3'	98	KC521466

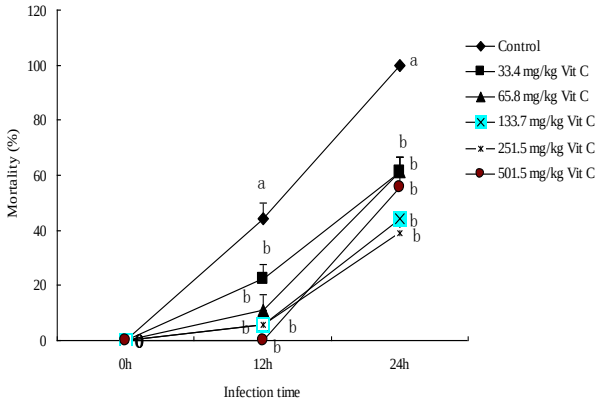

Figure 1. Effects of vit C on disease resistance again *A.hydrophila* infection of *M.amblycephalain.*
Note: Data are expressed as means ± SEM (*n* =3). Diverse little letters show significant differences (P<0.05) in different dosage groups of each sampling point in Turkey's-b test.

RBC count and HGB content compared with the pre-stress level (P<0.05, Figure 2B, 2C). The groups of 133.7 mg/kg vit C had significantly higher RBC count compared with the control group 15d post-stress (P<0.05, Figure 2B, 2C). In addition, the groups of 133.7, 251.5 mg/kg vit C had significantly higher HGB content compared with the control group 15d post-stress (P<0.05, Figure 2B, 2C). After infection, significantly lower RBC count was measured in the 65.8 mg/kg vit C group compared with its pre-infection level (P<0.05, Figure 2B), while HGB content was significantly lower in the groups of 33.4, 251.5 and 501.5 mg/kg vit C compared with pre-infection level (P<0.05, Figure 2C).

Effects of Vit C on Serum Cortisol, C3 and C4 in *M. Amblycephala*

The effects of vit C on the serum cortisol, C3 and C4 concentrations in fish are shown in Figure 3. Before stress, there was no significant effect on the serum cortisol concentration in the five treatment groups compared to the control group (P>0.05, Figure 3A). After pH stress, serum cortisol concentration increased and it was significantly higher in the control and the group of 33.4 mg/kg vit C 15 d post pH stress than pre-stress level (P<0.05, Figure 3A). In addition, serum cortisol concentrations were significantly lower in the treatment group of 251.5 mg/kg vit C 15d post-stress compared with the control group (P<0.05, Figure 3A). After infection, serum cortisol concentration was significantly improved in the group of 33.4 mg/kg vit C compared with its pre-stress level (P<0.05, Figure 3A).

Before stress, serum C3 concentration was significantly improved in the group of 251.5 and 501.5 mg/kg vit C compared with the control group (P<0.05, Figure 3B). After pH stress, serum C3 concentration was significantly lower than pre-stress level in the control and all the treatment groups 15d post-stress (P<0.05, Figure 3B). Serum C3 concentration was significantly improved in the group of 33.4 and 133.7 mg/kg vit C compared to the control group 15d post-stress (P<0.05, Figure 3B). After infection, there was no significant effect on serum C3 concentration in the five treatment groups compared to the control group (P< 0.05, Figure 3B).

Before stress, serum C4 concentration was significantly improved in the group of 133.7 mg/kg vit C compared with the control group (P<0.05, Figure 3C). After pH stress, serum C4 concentration was significantly lower than pre-stress level in the control and all treatment groups post-stress (P<0.05, Figure 3C). After infection, serum C4 concentration was also significantly lower than pre-infection level in the treatment groups of 33.4, 65.8 and133.7 mg/kg vit C 1d after infection (P<0.05, Figure 3C). In addition, the serum C4 concentration was significantly higher in the group of 501.5 mg/kg vit C than that of the group of 33.4, 65.8 and 133.7 mg/kg vit C 1d after infection (P<0.05, Figure 3C).

Effects of Vit C on Serum SOD, ASAFR and MDA in *M. Amblycephala*

We examined the effect of vit C on the hepatic anti-oxidization capacity in fish, and the results are shown in Figure 4. Before stress, SOD activity was significantly elevated in the group of 251.5 mg/kg vit C compared with the control group. After pH stress, SOD activity was significantly lower than pre-stress levels in the control group and all treatment groups 15d post-stress (P<0.05, Figure 4A). Furthermore, SOD activity in the group of 133.7 mg/kg vit C was significantly higher than the control group (P<0.05, Figure 4A). After infection, there was no significant effect on the serum SOD activity in the five treatment groups compared to the control group (P>0.05, Figure 4A). However, SOD activity was significantly lower than pre-infection levels in all treatment groups 1d after infection (P<0.05, Figure 4A).

Before stress, the groups of 65.8, 133.7 mg/kg

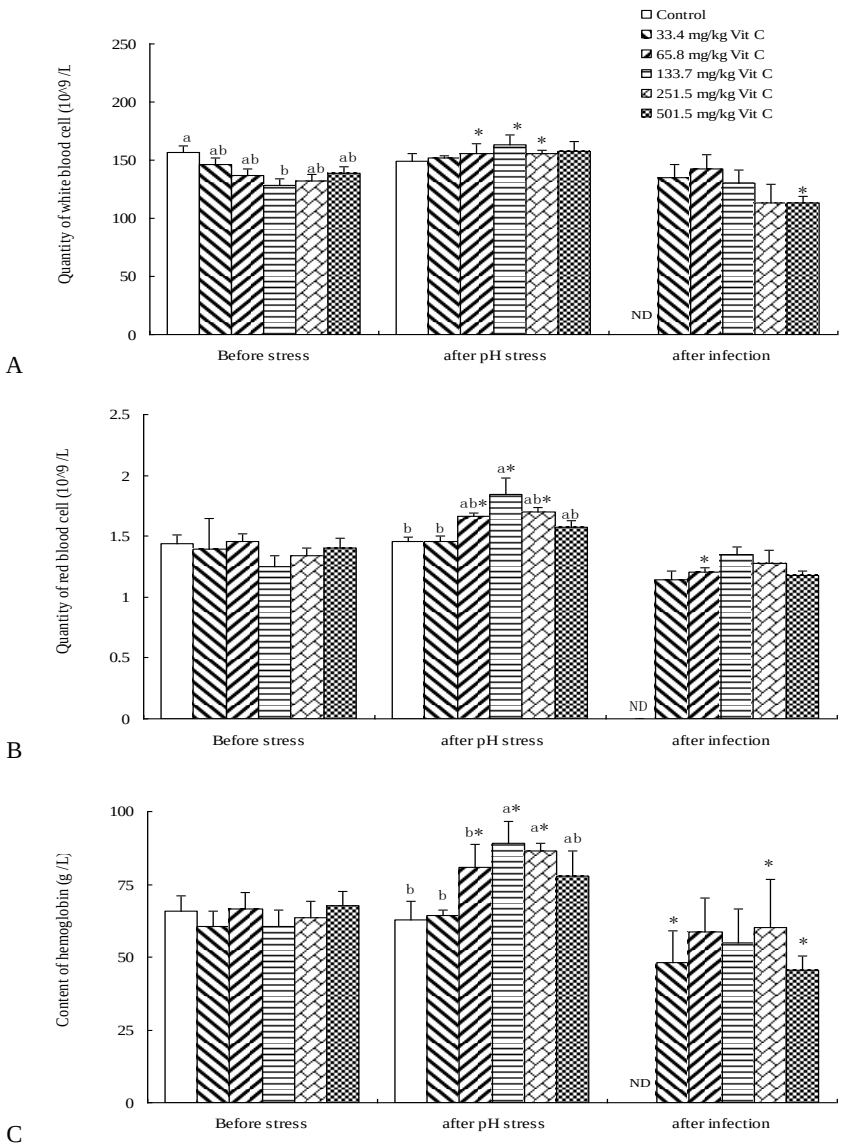

Figure 2 Effects of various levels of vit C on the WBC (A), RBC (B) and HGB (C) of *M.amblycephala* after pH stress and *A. hydrophila* infection.

Note: Data are expressed as mean ± SEM (n = 9). Letters indicate significant differences (P<0.05) in different dosage groups of each sampling point in Turkey's-b test. Asterisks indicate significant differences (P<0.05) between values obtained pre-stress and post-stress or post-infection in t-test. "ND"shows all the fish died 24 h after infection.

vit C had significantly higher ASAFR concentrations compared to the control group (P<0.05, Figure 4B). After pH stress, ASAFR concentration was significantly lower than pre-stress levels in the control group and the treatment groups of 33.4, 65.8, 133.7, 251.5 mg/kg vit C (P<0.05, Figure 4B). Furthermore, all the treatment groups except the group of 33.4 mg/kg vit C had also significantly higher ASAFR concentrations compared to the control group 15 d after pH stress (P<0.05, Figure 4B). After infection, ASAFR concentration was significantly lower than pre-infection levels in all treatment groups 1d after infection (P<0.05, Figure 4B).

Before stress, there was no significant difference in MDA content between the treatment group and control group (P>0.05, Figure 4C). After pH stress, there was no significant difference in MDA content yet (P>0.05, Figure 4C).After infection, MDA content was significantly higher than pre-infection level in the group of 33.4 mg/kg vit C 1d after infection (P<0.05, Figure 4C). In addition, MDA content was significantly lower in the group of 65.8 mg/kg vit C than that of the group of 33.4 mg/kg vit C (P<0.05, Figure 4C).

Effects of Vit C on the Relative Level of Hepatic HSP60, HSP70 and HSP90 Mrna in *M. Amblycephala*

We also examined the effect of vit C on hepatic HSP60, HSP70 and HSP90 mRNA expressions in fish (Figure 5). Before stress, the expression level of HSP60 mRNA was significantly higher in the treatment group of 251.5 mg/kg vit C than that of the

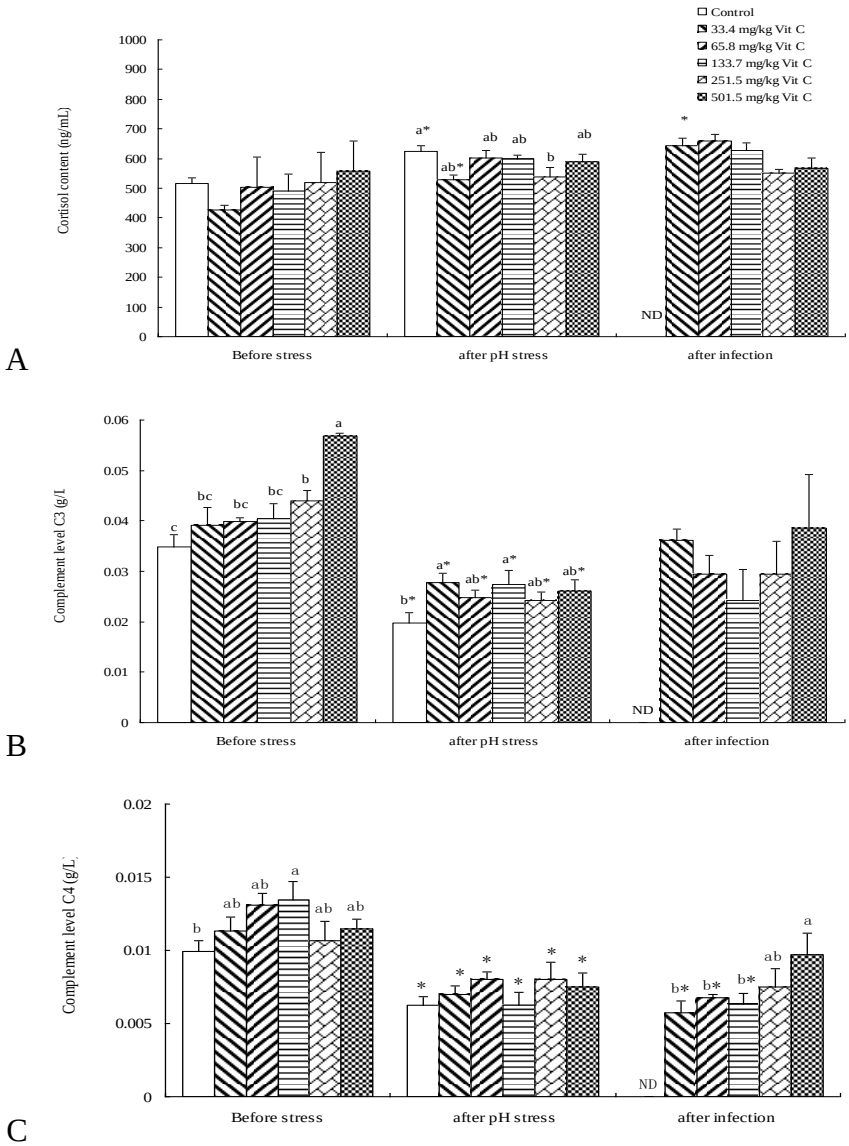

Figure 3. Effects of various levels of vit C on the serum on serum cortisol (A), C3 (B) and C4 (C) of *M.amblycephala* after pH stress and *A. hydrophila* infection.
Note: Data are expressed as mean ± SEM (n = 9). Legends are the same as Figure 2. "ND"shows all the fish died 24 h after infection.

control group (P<0.05, Figure 5A). After pH stress, HSP60 mRNA expression was significantly higher than pre-stress levels in the control group and the group of 33.4, 65.8 mg/kg vit C 15d after pH stress (P<0.05, Figure 5A). Furthermore, hepatic HSP60 mRNA expression was significantly enhanced in the group of 65.8, 133.7, 251.5 and 501.5 mg/kg vit C compared to the control group (P<0.05, Figure 5A). After infection, HSP60 mRNA expression was significantly higher than pre-infection level in the group of 33.4, 65.8 and 501.5 mg/kg vit C 1d after infection (P<0.05, Figure 5A). In addition, the group of 251.5 mg/kg vit C improved the HSP60 mRNA expression compared with the others group of 33.4, 65.8, 133.7and 501.5 mg/kg vit C 1d after infection (P<0.05, Figure 5A).

Before stress, the expression level of HSP70 mRNA was significantly higher in the treatment group of 133.7, 251.5 and 501.5 mg/kg vit C than that of the control group (P<0.05, Figure 5B). After pH stress, HSP70 mRNA expression was significantly higher than pre-stress level in the control group and the group of 33.4,133.7 and 501.5 mg/kg vit C 10d after pH stress (P<0.05, Figure 5A). Furthermore, hepatic HSP70 mRNA expression was significantly higher in the group of 133.7, 251.5 and 501.5 mg/kg vit C compared to the control group (P<0.05, Figure 5B). After infection, HSP70 mRNA expression was significantly higher than pre-infection stress level in the treatment groups 1d after infection (P<0.05, Figure 5B). In addition, HSP70 mRNA expression was significantly higher in the group of 251.5 mg/kg vit C than that of the others group of 33.4, 65.8, 133.7and 501.5 mg/kg vit C (P<0.05, Figure 5B).

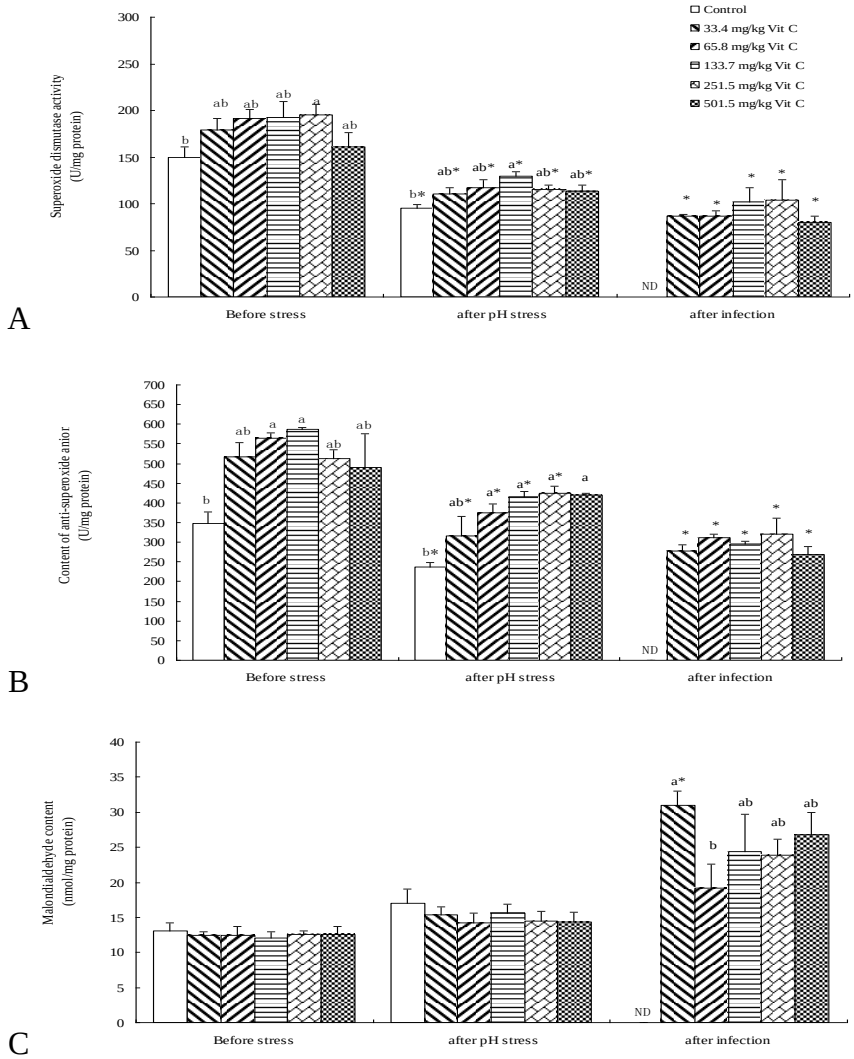

Figure 4. Effects of various levels of vit C on the serum SOD (A), ASAFR (B) and MDA (C) levels of *M.amblycephala* after pH stress and *A. hydrophila* infection.
Note: Data are expressed as mean ± SEM (*n* = 9). Legends are the same as Figure 2 "ND"shows all the fish died 24 h after infection.

Before stress, the expression level of HSP90 mRNA was significantly higher in the treatment group of 251.5 mg/kg vit C than that of the control group (P<0.05, Figure 5C). After pH stress, HSP90 mRNA expression was significantly higher than pre-stress level in the control group and the group of 33.4, 133.7 and 501.5 mg/kg vit C (P<0.05, Figure 5A). Furthermore, hepatic HSP90 mRNA expression was significantly higher in the entire treatment groups compared with the control group 15d after pH stress (P<0.05, Figure 5B). After infection, HSP90 mRNA expression was only significantly higher than pre-infection level in the treatment group of 133.7 mg/kg vit C 1d after infection (P<0.05, Figure 5C).

Discussion

In aquaculture aquatic animals are consistently affected by various stress factors such as ambient pH and temperature, stocking density, and so on. Besides, low or high pH has been shown to affect the growth of aquatic animal and reduce the resistance against pathogen such as *Vibrio alginolyticus* (Li and Chen, 2008), *Enterococcus* (Cheng and Chen 1998), *Lactococcus garvieae* (Cheng *et al*. 2003). Dietary vit C reduced disease susceptibility in Mrigal or Asian catfish (Sobhana *et al*., 2002; Kumari and Sahoo, 2005). Earlier studies in our laboratory also suggested that resistance against *A. hydrophila* infection could be enhanced in *M. amblycephala* by the supplementation of dietary vit C or Chinese herb extracts (Ming *et al*., 2012). Herein, we found a similar phenomenon in the same species, being more susceptible to *A. hydrophila* infection when the fish were reared at a high pH (9.5), and the total accumulated percentages of mortalities of *M. amblycephala* in the control was significantly higher

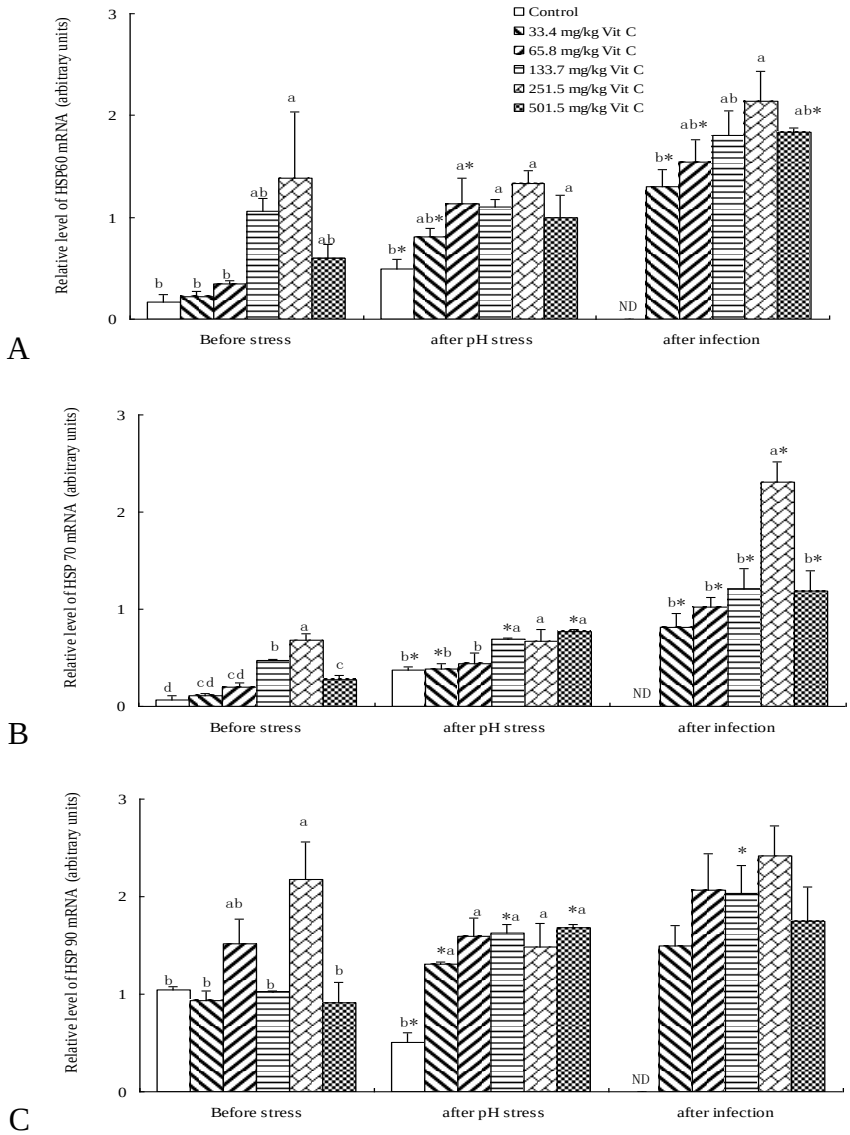

Figure 5. Effects of various levels of vit C on the relative expression levels of liver HSP60 (A), HSP70 (B) and HSP90 (C) mRNA of *M.amblycephala* after pH stress and *A. hydrophila* infection.
Note: Data are expressed as mean ± SEM (n = 9). Legends are the same as Figure 2 "ND"shows all the fish died 24 h after infection.

than the vit C treatment groups at 12, 24 h after *A. hydrophila* infection. Furthermore, all fish died in the control group when fish were exposed to the combined stresses of 15d pH 9.5 and 1d *A. hydrophila* infection. It indicated that the supplementation of 33.4, 65.8, 133.7, 251.5 and 501.5 mg/kg vit C in the diets for *M. amblycephala* had certain effect on resistance to pathogenic bacteria. These facts also indicated that changes in environmental parameters (high pH) might trigger disease outbreaks by reducing the immune defense mechanisms of the host.

With respect to the immune parameters, pH change might lead to immunodepression. For example, Cheng and chen (2000) and Cheng *et al*. (2003) reported that total haemocyte count and phenoloxidase activity of *M. rosenbergii* decreased when prawns were transferred to pH 5.0 and pH 9.5. The related immune parameters such as total haemocyte count, phenoloxidase activity, respiratory burst, superoxide dismutase activity, glutathione peroxidase activity, and lysozyme activity of *Litopenaeus vannamei* significantly decreased when shrimps were transferred to seawater at pH 6.8 (Lin *et al*. 2010). In the present study, Wuchang breams were exposed to the combined stresses of 15d pH 9.5 and 1d *A. hydrophila* infection and it showed that decreasing trends of serum C3, C4 in the group of 33.4, 65.8, 133.7 mg/kg vit C, and hepatic SOD, ASAFR in in all group and increasing trends of serum cortisol, hepatic HSPs gene expression. Furthermore, WBC, RBC, HGB, C3, C4, SOD, ASAFR in some groups were also lower than the pre-stress level, while serum cortisol, MDA, and hepatic HSPs gene expression were higher than the pre-stress level in

some groups. Therefore, the immunity of *M. amblycephala* decreased when fish was subjected to the combined stresses of high pH and bacterial infection.

However, the blood WBC count was significantly reduced in the groups of 133.7 mg/kg vit C compared with the control group before stress, and the group of 133.7 mg/kg vit C had significantly improved blood RBC count and HGB content compared to the control group after pH stress. Similarly, Lin *et al*. (2010) demonstrated that 200-600 mg/ L *Spirulina platensi* extract reduced total haemocyte count of *L.vannamei* under low pH stress. Yeh *et al*. (2010) also reported that 600 mg/L *Gracilaria tenuistipitata* extract could reduce the total haemocyte count, hyaline cells, granular cells under combined stresses of *Vibrio alginolyticus* and temperature change compared to those of control shrimp. These evidences suggested that vit C as an immunostimulant could impact on the hematological parameters such as blood RBC, WBC and HGB and enhance immune capacity.

Cortisol is secreted in response to stressors to mobilize energy stores and is generally thought to have a negative effect on the immune system, thereby increasing disease susceptibility (Trip *et al*.1987; Steinhagen 1989). Earlier studies in our laboratory found that serum concentrations of cortisol significantly increased under high temperature and pathogenic infection in Wuchang bream (Liu *et al*. 2010; Liu *et al*. 2012) and high dose of 700 mg/kg vit C reduced the serum cortisol concentrations (Ming *et al*. 2012). In the present study, serum cortisol concentrations were significantly reduced in the treatment groups of 251.5 mg/kg vit C after pH stress compared with the control group. Some similar results were also observed in gilthead seabream (*Sparus aurata* L.) under crowding stress (Montero *et al*.1999) and a multiple stress (Ortuno *et al*. 2003).

Serum alternative complement activity can be severely depressed by various stress conditions in fish (Ortuno *et al*. 2002; Boshra *et al*. 2006), and may be a good indicator of fish immunocompetence in stressed animals (Tort L *et al*., 1996). In conformity with these reports, this study showed that serum C3 and C4 concentrations in all the groups at 15d post-stress were significantly lower than pre-stress level. In addition, serum C4 concentration in the treatment groups of 33.4, 65.8 and 133.7 mg/kg vit C at 1d after infection was also significantly lower than pre-infection level. However there was no effect on serum C3 concentration after infection compared to pre-stress level. This indicated that analysis of C3 might not necessarily be susceptibility of bacteria for fish. Therefore further studies will be needed to address the effect of vit C on the specific pathways and complement components under combined stresses of high pH and *A. hydrophila* infection of *M. amblycephala*.

In addition, the serum complement concentration of fish has been reported to be significantly enhanced by oral administration of vit C-supplemented diets (Chen *et al*. 2003; Ortuno *et al*. 2003). Similarly, in the present study, 251.5 and 501.5 mg/kg vit C in the diet elevated the serum C3, and 133.7 mg/kg vit C in the diet elevated the serum C4 concentration before stress. The supplementation of 33.4 and 133.7mg/kg vit C in the di*et al*so improved the serum C3 concentration compared to the control group after pH stress. The serum C4 concentration was significantly higher in the group of 501.5 mg/kg vit C than that of 33.4, 65.8, 133.7 mg/kg vit C. These evidences indicated that dietary vit C can increase the C3 and C4 concentrations following high pH stress and bacterial infection. These findings indicate that the role of vit C in stress and disease resistance in fish.

Some stress factors and pathogenic infection is often associated with an increase in free radical content, which may lead to an increase in lipid peroxidation content and lipid peroxidation injury. Decreases in respiratory bursts and SOD activity were observed in white shrimp exposed to pH 6.5, and pH 10.1 (Li and Chen, 2008). Similarly, SOD activity was significantly decreased under the combined stresses of 9.0-9.2 pH stress and *Fenneropenaeus chinensis* (Ha *et al*. 2009). In rat, vit C enhanced plasma glutathione, superoxide dismutase, and glutathione peroxidase levels, reduced MDA content, and prevented diabetic rats from oxidative stress (Aksoy *et al*. 2005). In fish, dietary supplementation with vit C significantly enhanced SOD and catalase activities, and reduced MDA content (Ming *et al*. 2012; Wan *et al*. 2013). Consistent with these studies, 251.5 mg/kg vit C significantly improved the SOD activity, and the dose of 65.8-133.7 mg/kg vit C also significantly improved ASAFR concentrations compared to the control group before stress. Furthermore, 65.8, 133.7 mg/kg vit C significantly enhanced the SOD activity and ASAFR concentration after pH stress. In addition, MDA content was significantly lower in the group of 65.8 mg/kg vit C than that of 33.4 mg/kg vit C. Taken all together, our results suggest that the dose of 133.7- 251.5 mg/kg vit C reduces the potential for oxidative damage following pH stress and *A. hydrophila* infection in *M. amblycephala*. In addition, ethoxyquin is allowed in fish feed as a fat stabilizer and prevent the oxidation of lipid and vit C can also improve the antioxidant capacity of fish. The relationship between vit C and ethoxyquin for fish feed remains to be further investigated.

Heat shock proteins (HSPs) are conserved proteins induced by heat and numerous noxious stimuli, including high temperature, viruses, and pathologic stresses (Lindquist and Graig, 1998; Basu *et al*.2002). HSP60, HSP70 or HSP90 has a number of functions, including the maintenance of cellular homeostasis and the protection of an individual following stress or pathogenic stress in aquatic animals (Deane *et al*. 2004; Cellura *et al*. 2006;

Rungrassamee *et al*. 2010; Gao *et al*. 2008; Qu *et al*. 2011). In fish, Ming *et al*. (2012) found that dietary vit C enhanced the expression levels of HSC70 and HSP70 mRNA before or after high temperature stress. In rat, Han *et al*. (2011) reported that dietary vit C also enhanced the expression levels of HSP70 mRNA. In the present study, the expression levels of HSP60, HSP70 and HSp90 mRNA in the treatment group of 251.5 mg/kg vit C were significantly higher than those in the control group before stress. After stress, the group of 133.7, 251.5and 501.5 mg/kg vit C still improved the expression levels of HSP60, HSP70 and HSp90 mRNA compared to the control group. Additionally, HSP60 and HSP70 mRNA expressions were significantly higher in the group of 251.5 mg/kg vit C than that of the group of 33.4 mg/kg vit C after 1d infection. Thus, our results suggest that the dose of 133.7- 251.5mg/kg vit C could elevate the HSPs gene expression and enhance tolerance to stressors by inducing the cellular stress response following pH stress and *A. hydrophila* infection in *M. amblycephala*.

Conclusions

Supplementation of diet with 133.7- 251.5mg/kg vit C was associated with less mortality compared to the control fish when Wuchang breams were transferred to pH 9.5water, and then challenged with *A. hydrophila*. Supplementation of diet with 133.7-251.5mg/kg vit C increased blood RBC, HGB, C3, C4, and hepatic SOD, ASAFR and HSP60, 70, 90 gene expressions. Furthermore, we noted a decrease in serum cortisol content in fish fed with 251.5mg/kg dietary vit C. All in all, our results suggest that the dose of 133.7- 251.5mg/kg vit C in the diet can increase non-specific immune and anti-oxidation capacities, and enhance resistance against high pH stress and pathogenic bacterial infection in Wuchang bream.

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