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Effects of antimicrobial peptides on serum biochemical parameters, antioxidant activity and non-specific immune responses in *Epinephelus coioides*

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A R T I C L E I N F O

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A B S T R A C T

Antimicrobial peptides (AMPs) are small proteins showing broad-spectrum antimicrobial activity that have been known to be powerful agents against a variety of pathogens (bacteria, fungi and viruses). In this study, the effects of AMPs from *Bacillus subtilis* on *Epinephelus coioides* were examined. *E. coioides* were fed with diets containing AMPs (0, 100, 200, 400 or 800 mg/kg) for four weeks. Results showed that the levels of total protein (TP), albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and blood glucose (GLU) and lipopolysaccharide (LPS) in the serum of *E. coioides* changed than those of the control group; compared to the control group, the levels of total antioxidant capacity (T-AOC), superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA) and lysozyme (LZM) levels in *E. coioides* fed with different dosages AMP diets were also different; in addition, the mRNA expression of tumor necrosis factor alpha (TNF-α), interleukin-1-beta (IL-1β), and heat shock protein 90 (Hsp90) in the tissues of *E. coioides* were measured, the three genes in the tissues examined were significantly upregulated. The results demonstrated that diets containing AMPs can enhance the antioxidant capacity and innate immune ability of *E. coioides*, indicating that AMPs might be a potential alternative to antibiotics in *E. coioides*.

1. Introduction

With the continuous improvement of people's living standards, the food types of people are changing mainly from mainlands to water [1]. Fish, especially marine teleosts, are an important food source for humans worldwide now [2]. To satisfy human need, a large number of fish are farmed. Due to increasing high density cultivation, more and more various diseases in fish farm have broken out: *Aeromonas caviae* causes a high mortality of infected animals [3]; *Streptococcus agalactiae* is an important pathogeny in tilapia [4]; outbreak of a novel disease associated with *Citrobacter freundii* infection occurred in *Potamotrygon motoro* [5]; parasites *Cryptocaryon irritans* and *Ichthyophthirius multifiliis* causes white spot disease of fish and can kill large numbers of fish in a short period of time [6]); and various viruses infected cultured fish etc. [7]. To sure the health of fish, various antibiotics and other chemicals are widely used in aquaculture to expedite growth and control diseases [8]. However, the overuse of these drugs has brought about a number of serious problems: the enhancement of drug resistance, antibiotic residues and environmental pollution and possible threat to public health [9]. Therefore, health strategies response to fish disease in aquaculture are very important for the future.

Antimicrobial peptides (AMPs) are gene-encoded small proteins showing broad-spectrum antimicrobial activity that have been known to be powerful agents against a variety of pathogens (bacteria, fungi

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and viruses) [10,11]. The first-discovered AMP from *Lactococcus lactis* was a 34-residue peptide [12]. Subsequently, hundreds of AMPs have been isolated and identified in all kingdoms including various viruses, bacteria, fungi, animals and plants (http://aps.unmc.edu/AP/main.php) [13]. AMPs are known to be essential components of the innate immune response, providing the first line of defense against a wide range of pathogens [14]. Compared to antibiotics, AMPs have a broader spectrum, no drug residues, more rapid killing action, low resistance potential and highly selective toxicity [14,15]. Therefore, AMPs are an interesting field of research for the identification of potential drug candidates [11]. The use of AMPs in aquaculture for enhancing growth, antioxidant capacity, immunity and resistance to infections has been studied in shrimp *Litopenaeus vannamei*, Nile tilapia *Oreochromis niloticus*, Koi carp *Cyprinus carpio koi*, triploid crucian carp *Carassius auratus*, and common carp *Cyprinus carpio* etc. [16–18]. However, few studies have been conducted to explore the effects of AMPs on the immune-related gene expression of fish.

Epinephelus coioides is an important cultured marine fish in southern China [6]. Nevertheless, high-density *E. coioides* culture has led to various diseases, which have impacted *E. coioides* production and increased costs. Therefore, the aim of this study was to clear the effects of various levels of dietary AMPs on biochemical parameters, antioxidant activities, and immune responses in *E. coioides* in order to evaluate the effectiveness of AMPs as substitutes for antibiotics.

2. Materials and methods

2.1. Peptides

AP1 (Guangdong Institute Of Microbiology, Guangzhou, China) were the AMPs from *Bacillus subtilis* B06 used in this study. The determination of the value of antibacterial potency (Arbitrary Units, AU/g) and the method of measuring were conducted according to the instructions “Determination of Antimicrobial Activity of Peptide-Agar Diffusion Method” which provided by the Guangdong Institute of Microbiology. The purified AP1 displayed broad-spectrum antibacterial potency against pathogens, with a minimum inhibitory concentration (MIC) of less than 172 mg/ml. AP1 had a molecular mass of ~4.5 kDa and an activity of more than 20,000 AU/g producing a zone of inhibition diameter > 8.9 mm. The peptide was tested in this study to explore the regulative and protective effects on *E. coioides*. The nucleotide and amino acid sequences of the peptide have been not published due to a patent application.

2.2. Diet preparation and experimental design

The formulation and chemical composition of the basal diet (control) are presented in Table 1. In the experimental groups, the AMP was added to the basal diet as an additive at different concentrations: 0 mg/kg (G1), 100 mg/kg (G2), 200 mg/kg (G3), 400 mg/kg (G4) and 800 mg/kg (G5) of AMP. All the ingredients were ground (< 0.375 mm) and blended thoroughly with an additional 100 ml of water per 1 kg of diet in a food mixer. Then, the feed was extruded as pellets that were 1.5 mm in diameter. The moist pellets were air-dried at ambient temperature until the moisture content was less than 10%. Finally, the dry pellets were stored in plastic bags and frozen at 4 °C for use.

2.3. Experimental fish and feeding

Healthy grouper *E. coioides* (weighing 7.48 ± 1.8 g) from the Hainan *E. coioides* Farm (Hainan, China) were maintained in 0.5 t tanks with salinity 30, continuous aeration and continuously circulated seawater for 2 weeks before experimentation in our laboratory.

The groupers were randomly separated into 5 groups in triplicate with 30 fish held in each tank for a 4-week feeding trial. The fish were fed twice daily (at 9:00 and 16:00) at a rate of 5% body weight. The feed intake and the weight of dead fish were recorded daily.

Table 1
Formulation and approximate nutrient content of the basal diet (dry diet).

Ingredients	Proportion (%)	Nutrients composition	Proportion (%)
Wheat middling	15	Moisture	8.89
Soybean meal	24.7	Crude protein	34.27
Fish meal	52		
Fish oil	5		
Soybean lecithin	1		
Krill meal	0.3		
Vitamin premix[a]	1		
Mineral premix[b]	0.5		
Choline chloride	0.5		
Total	100		

[a] Vitamin premix: V_A (IU/kg): 350,000, V_{D3} (IU/kg): 210,000, V_E (g/kg): 6, V_{K3} (mg/kg): 500, V_{B1} (mg/kg): 450, V_{B2} (mg/kg): 900, V_{B6} (mg/kg): 600, V_{B12} (mg/kg): 2, V_C (mg/kg): 14000, nicotinamide (mg/kg): 3500, D-calcium pantothenate (mg/kg): 2000, folic acid (mg/kg): 160, biotin (mg/kg): 8.

[b] Mineral premix: Mg (mg/kg): 5000, Fe (mg/kg): 5250, Mn (mg/kg): 1000, I (mg/kg): 168, Cu (mg/kg): 788, Zn (mg/kg): 4200, Se (mg/kg): 25, Co (mg/kg): 131.

2.4. Growth measurements

The weight and length of each grouper at the beginning and end of the trial were measured. The growth performance of the fish was calculated using the following formula:

Weight gain (g/fish) = Wt - W0

Specific growth rate (SGR) = 100 × (lnWt - lnW0) /t

Relative growth rate (%) = (Wt - W0)/ W0 × 100

Feed conversion ratio (FCR) = FI / (Wt -W0)

Here, Wt and W0 are the final and the initial weight, ‘t’ is the duration of feeding (day), and FI is feed intake.

2.5. Sample collection and analytical methods

After four weeks of the feeding trial, six fish per tank were randomly selected after 24 h of starvation. Blood was collected from the caudal vein using a 1 ml syringe, stored in 1.5 ml sterilized tubes at 4 °C overnight, and centrifuged at 1500 g for 10 min. The supernatant was collected, and stored at −20 °C until use. Subsequently, the samples gill, head kidney, spleen and trunk kidney from each individual were collected and immediately frozen in liquid nitrogen for further research.

Serum biochemistry parameters including the contents of total protein (TP), albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and blood glucose (GLU) were measured at the Jining No.1 People's Hospital using a Vitec compact automatic biochemical analyzer (Mérieux, France). The total antioxidant capacity (T-AOC), superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), lysozyme (LZM) and serum lipopolysaccharide (LPS) were analyzed by kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) according to the manufacturer's instructions. The relative expression levels of immune-related genes including tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), and heat shock protein 90 (Hsp90) in the gill, spleen, liver, head kidney, and trunk kidney were examined with real-time quantitative PCR (RT-qPCR).

Total RNA was isolated using with TRIzol Reagent (Invitrogen, Canada), and digested with DNase I (Promega, USA). The concentration

Table 2
Primers used in this study.

Primer	Sequence (5′ to 3′)
TNF-α-F	CCTGGTGATGTGGAGATG
TNF-α-R	GTCCGACTTGATTAGTGCTT
IL-1β-F	CTCCACCGACTGATGAGGATATG
IL-1β-R	GGCTGTTATTGACCCGAACTAAG
Hsp90-F	ACCCACTCCAACCGCATCTACAG
Hsp90-R	AGGGGAGGAATCTCATCTGGGAC
β-actin-F	TGCTGTCCCTGTATGCCTCT
β-actin-R	CCTTGATGTCACGCACGAT

was measured spectrophotometrically, and RNA integrity was examined using 1% agarose gel electrophoresis. The first-strand cDNA was synthesized using ReverTra Ace-α reverse transcriptase following the manufacturer's instruction manual (Toyobo, Japan) and was used as a template in subsequent polymerase chain reaction (PCR) (TakaRa, Japan) and quantitative real-time PCR (qPCR) (TakaRa). The gene β-actin was chosen as the reference gene for sample normalization. The primers were are presented in Table 2. Real time PCR was performed with SYBR Green Real-Time PCR Master Mix (Toyobo) on a Roche LightCycler 480 Real-Time PCR Detection System (Roche). The PCR cycling conditions were as follows: one cycle of 94 °C for 2 min, followed by 40 cycles of 94 °C for 15 s, 58 °C for 15 s, and 72 °C for 20 s. The specificity of the PCR products was confirmed with a melting-curve analysis and sequencing. Each sample was amplified in triplicate. The mRNA expression of the target gene relative to that of the reference gene was calculated with the $2^{-\triangle\triangle Ct}$ method.

2.6. Challenge test

Ten fish per group were randomly selected, and used to assess disease resistance by challenge with *Vribro alginolyticus* LP01. Each *E. coioides* was injected intraperitoneally with the bacteria at a dose of 10^5 CFU in 100 μl PBS. Meanwhile, fish was injected intraperitoneally with 100 μl PBS as a blank control. Comulative survival was recorded daily for 14 days after inoculation.

2.7. Statistical analysis

All data were analyzed by one-way ANOVA using SPSS for windows version 17.0 (SPSS, Inc., USA) and expressed as the means ± standard errors. Duncan's test was applied to determine significant differences between all groups. The significance level was considered at a value of $P < 0.05$. The same letter between groups indicates no significant difference, while different letters indicate significant differences.

3. Results

3.1. Growth performance

The growth performance of *E. coioides* in the trial is shown in Table 3. The final weight and specific growth rate of *E. coioides* fed AMP diets (G2, G3, G4, and G5) were higher than those of the fish fed the control diet (G1) ($P < 0.05$). Feed conversion ratios between each treatment were analyzed, and compared to those of the G1 group (control group), the feed conversion ratios in the G2, G3, and G4 groups were lower than that of the control ($P < 0.05$), while the feed conversion ratio of the higher in G5 group was higher than that of the control ($P < 0.05$).

3.2. Biochemical parameters of serum

The serum biochemistry parameters of *E. coioides* are shown in Table 4. Compared to those levels in the control group, the levels of TP and AST in the G3, G4, and G5 groups were higher ($P < 0.05$); the levels of ALB and LPS with G2, G3, G4, G5 were higher ($P < 0.05$); the higher levels of LDL-C occurred at G4 and G5 groups ($P < 0.05$); the levels of TG decreased, and HDL-C was slightly increased. However, there was no significant difference in the levels of ALT and GLU in any of the groups ($P > 0.05$).

3.3. Antioxidant activity and non-specific immunity

As shown in Table 5, compared to those of the control group, the T-AOC and LZM activities in *E. coioides* fed AMP-diets were significantly higher ($P < 0.05$); the SOD and CAT activities of *E. coioides* with AMP diets were also higher; LPS levels appeared to decrease with increasing doses of AMPs; except the MDA levels in the G1 and G3 have no significantly difference ($P > 0.05$), lower level of MDA occurred in the G2, G4 and G5 ($P < 0.05$).

3.4. Expression of immune-related genes

To examine the effect of AMP-supplemented diets on further immune responses, the expression of three cytokines IL-1β, TNF- α, and Hsp90 were measured at the end of the 4-week feeding trial using real-time quantitative PCR. As shown in Fig. 1, the expression of the three genes (IL-1β, TNF- α, and Hsp90) was up-regulated in all the examined tissues of the fish fed with AMP diets compared to that in the tissues of the control group: the mRNA expression of IL-1β was highest in the head kidney (66.4 times, G4 group) ($P < 0.05$), followed by the trunk kidney (56.2 times, G4 group), the spleen (26.1 times, G5 group), the liver (16.3 times, G4 group) and the gill (6.8 times, G4 group); likewise, the highest expression of TNF- α occurred in the head kidney (63.3 times, G4 group) ($P < 0.05$), followed by the trunk kidney (48.3 times, G5 group), the spleen (24.1 times, G5 group), the liver (6.3 times, G5 group), and the gill (4.9 times, G5 group); finally, the highest

Table 3
Effect of antimicrobial peptide (AMP) contents on growth performance in *Epinephelus coioides*.

Growth parameters	G1	G2	G3	G4	G5
Final weight (g)	13.11 ± 0.61^{a}	18.35 ± 0.82^{b}	20.72 ± 0.58^{bc}	21.20 ± 0.16^{cd}	15.04 ± 0.35^{b}
Specific growth rate (SGR) (%)	3.90 ± 0.03^{a}	5.10 ± 0.04^{b}	5.53 ± 0.02^{cd}	5.62 ± 0.01^{de}	4.39 ± 0.02^{bc}
Relative growth rate (%)	197.95 ± 16.5^{a}	317.05 ± 22.8^{b}	370.91 ± 21.0^{cd}	381.82 ± 11.7^{de}	241.82 ± 12.9^{bc}
Feed conversion ration (FCR)	1.48 ± 0.07^{b}	1.29 ± 0.04^{a}	1.33 ± 0.06^{a}	1.31 ± 0.04^{a}	1.79 ± 0.10^{c}

Note: Values are means ± SE (n = 3). Values in the same row with different superscript letters are significantly different ($P < 0.05$).
Weight gain (g/fish) = Wt - W0.
Specific growth rate (SGR) = 100 × (lnWt - lnW0)/t.
Relative growth rate (%) = (Wt - W0)/W0 × 100.
Feed conversion ratio (FCR) = FI/(Wt -W0).
Here, Wt and W0 are the final and the initial weight, 't' is the duration of feeding (day), and FI is feed intake.

Table 4
Effect of AMP contents on serum biochemical parameters of *Epinephelus coioides*.

Parameter	G1	G2	G3	G4	G5
TP (g/L)	18.5 ± 1.58^{a}	19.7 ± 0.78^{a}	21.3 ± 1.32^{b}	24.5 ± 1.29^{c}	26.3 ± 0.64^{d}
ALB (g/L)	2.11 ± 0.03^{a}	2.43 ± 0.02^{b}	2.57 ± 0.07^{c}	2.72 ± 0.06^{d}	2.70 ± 0.01^{d}
ALT (U/L)	19.3 ± 1.43	18.5 ± 1.72	18.4 ± 1.5	18.7 ± 1.21	16.8 ± 1.33
AST (U/L)	61.4 ± 5.6^{bc}	76.9 ± 16.7^{c}	46.3 ± 7.1^{a}	48.8 ± 1.6^{ab}	36.6 ± 1.2^{a}
TG (mmol/L)	2.48 ± 0.10^{e}	1.65 ± 0.14 ^{bc}	1.98 ± 0.13^{cd}	0.99 ± 0.16^{a}	0.98 ± 0.08^{ab}
HDL-C (mmol/L)	0.01 ± 0.07^{a}	0.06 ± 0.01^{b}	0.19 ± 0.06^{c}	0.02 ± 0.06^{ab}	0.02 ± 0.022^{ab}
LDL-C (mmol/L)	0.01 ± 0.03^{a}	0.05 ± 0.02^{ab}	0.17 ± 0.04^{ab}	0.07 ± 0.02^{b}	0.03 ± 0.02^{c}
GLU (mmol/L)	< 0.00 ± 0.02^{ab}	0.06 ± 0.04^{a}	0.07 ± 0.07^{a}	0.06 ± 0.04^{ab}	< -0.03 ± 0.02^{ab}

TP: the contents of total protein; ALB: albumin; ALT: alanine aminotransferase; AST: aspartate aminotransferase; TG: triglycerides; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; GLU: glucose.

expression of Hsp90 was observed in the trunk kidney (59.8 times, G5 group) ($P < 0.05$), the head kidney (17.1 times, G4 group), the gill (7.4 times, G4 group), the spleen (6.7 times, G5 group) and the liver (4.1 times, G4 group). In contrast, the expression of the three genes in the G1 (control) and G2 groups was not significantly different ($P > 0.05$).

3.5. Challenge test

The cumulative survival of the *E. coioides* fed the experimental diets and then challenged with *V. alginolyticus* LP01 is shown in Fig. 2. No *E. coioides* died in the unchallenged control group (PBS group) during the trial. Within 14 days post challenge, the *E. coioides* in the control group challenged with *V. alginolyticus* had significantly lower survival compared to *E. coioides* in groups with AMP diets. In the end of the trial, the survival of *E. coioides* in the control group were 40%, survival rate of *E. coioides* in G2 (50%), G3 (60%), G4 (60%), and G5 (70%) were higher than that of the control group, respectively. The results demonstrated that all *E. coioides* in the AMP-diet groups had significantly higher survival compared to the control *E. coioides*.

4. Discussion

Fish are a major component of the aquatic fauna, and are also a major food source for humans with high densities of fish culture and an increasing number of fish disease outbreaks, and antibiotics have been chosen to control various diseases. However, the overuse of antibiotics has led to serious problems in aquaculture, such as drug resistant pathogens, environmental hazards and food safety problems [19]. It is necessary to find new methods to control aquatic diseases. Recently, it was demonstrated that AMPs play major roles in the innate immune system, and protect against a wide variety of bacterial, fungal, viral, and other pathogenic infections [20]. In this study, the effects of an AMP from *Bacillus subtilis* on the antioxidant activity and immunity of *E. coioides* were investigated.

4.1. Growth performance

The growth performance of *E. coioides* in the trial was examined, and the results are shown in Table 3. The final weight, SGR and relative growth rate of *E. coioides* with AMP diets were higher than those fed on the control diet, which are similar to the *C. carpio* fed on an AMP diet [16]. Compared to that of the control group, the feed conversion ratios in the G2, G3, and G4 groups were lower, while the feed conversion ratio in the G5 group was higher ($P < 0.05$). The results indicated that AMP diets could improve the final weight, SGR and relative growth rate of *E. coioides*. However, compared to the groups between *E. coioides* with the AMP diets, excess AMP in diets decreased the finial weight, SGR, and relative growth rate in group G5 (Table 3). Compared to that of the control group (G1), the FCR in G2, G3 and G4 was lower, but the FCR in G5 was significantly higher ($P < 0.05$), which further indicates that only appropriate concentrations of AMPs in diets for fish can increase growth performance [16].

4.2. Effect of AMPs on serum biochemical parameters

Serum biochemical characteristics, such as protein levels, enzymes, and electrolytes, are very useful in the assessment and management of animal health condition, which could provide information on internal organs, nutritional status, metabolic state, etc. [21]. Serum TP is an index to evaluate the nutritional and metabolic status of animals, and indirectly reflects the level of health and immunity; TP measurements are based on dietary protein content, liver metabolism and even protein loss caused by some lesions, and a higher TP serum level can promote the development of tissues [22]. In this study, Higher TP level in serum with AMP-supplemented diets (G3, G4 and G5 groups) was found ($P < 0.05$), which is similar to the results in tilapia *Oreochromis niloticus* [23]. Serum ALB has important applications in many clinical indications, which is responsible for targeting and transporting the ligands form reversible bindings in varying degrees, exhibiting superb bioeffects such as free radicals scavenging, maintenance of blood colloid osmotic pressure, inhibition of platelet aggregation and anticoagulation, as well as influence in the transport of nutrients and drugs [24]. This study demonstrated that the serum ALB level in fish with AMP diets were higher than that of the fish fed the control diet ($P < 0.05$), and the results of the TP and ALB suggested that AMP-supplemented diets could enhance the protein synthesis and immunity of grouper. ALT and AST are one of liver-specific enzymes in humans and animals, and widely exists in the liver and heart, but when liver or heart disease causes cell damage, a lot of ALT and AST enter the serum

Table 5
Effect of AMP contents on antioxidant activity and non-specific immunity of grouper *Epinephelus coioides*.

Parameter	G1	G2	G3	G4	G5
T-AOC(U/ml)	22.53 ± 0.54^{a}	33.09 ± 0.26^{bc}	35.22 ± 2.90^{c}	31.83 ± 1.19^{b}	44.03 ± 3.42^{d}
SOD (U/ml)	0.32 ± 0.09^{a}	0.51 ± 0.05^{c}	0.43 ± 0.08^{ab}	0.52 ± 0.04^{c}	0.37 ± 0.02^{ab}
CAT(U/ml)	5.32 ± 0.32^{a}	5.81 ± 0.12^{b}	6.93 ± 0.25^{c}	8.96 ± 0.13^{d}	7.63 ± 0.23^{e}
MDA (nmol/ml)	0.18 ± 0.02^{d}	0.16 ± 0.03^{c}	0.17 ± 0.08^{cd}	0.15 ± 0.08^{b}	0.08 ± 0.04^{a}
LZM(μg/ml)	3.78 ± 0.05^{a}	4.56 ± 0.31^{b}	5.92 ± 0.06^{c}	7.35 ± 0.04^{c}	5.42 ± 0.08^{d}
LPS (pg/ml)	8.18 ± 0.14^{d}	5.37 ± 1.53^{c}	4.89 ± 1.42^{c}	3.86 ± 0.66^{b}	2.66 ± 0.61^{a}

T-AOC: total antioxidant capacity; SOD: superoxide dismutase; CAT: catalase; MDA: malondialdehyde; LZM: lysozyme; LPS: lipopolysaccharide.

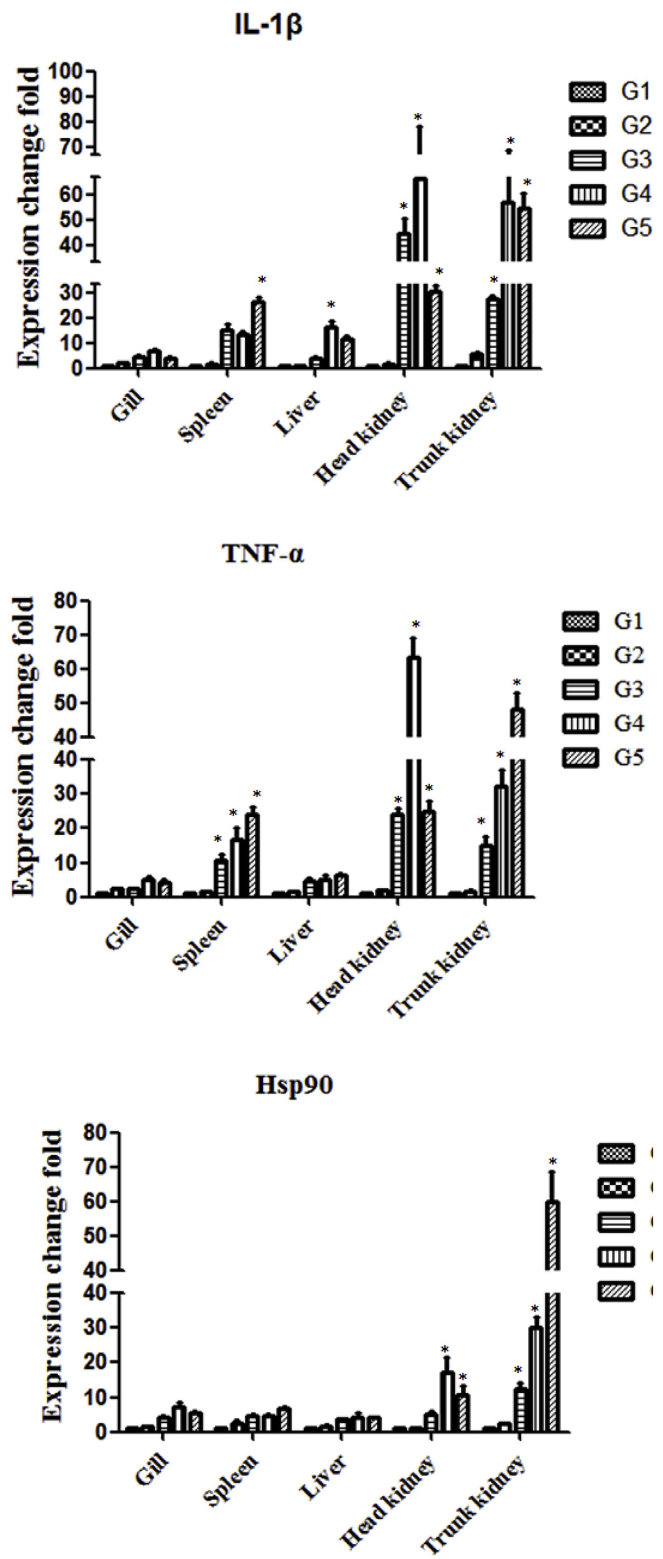

Fig. 1. Effects of AMP-supplementation on the relative expression levels of interleukin-1-beta (IL-1β), tumor necrosis factor alpha (TNF-α), and heat shock protein 90 (Hsp90) in the gill, spleen, liver, head kidney and trunk kidney of *Epinephelus coioides*. Significant differences of the genes expression between groups is indicated with * (significant increase, $P < 0.05$). All data are presented as Mean ± SE, N = 3. The horizontal dotted line is indicated the 1.

which leads to rapid increases of the two kinds of enzyme activity in the serum, so ALT and AST are important indicators of liver, and heart function [21,25]. Some studies have showed that AMPs can decrease

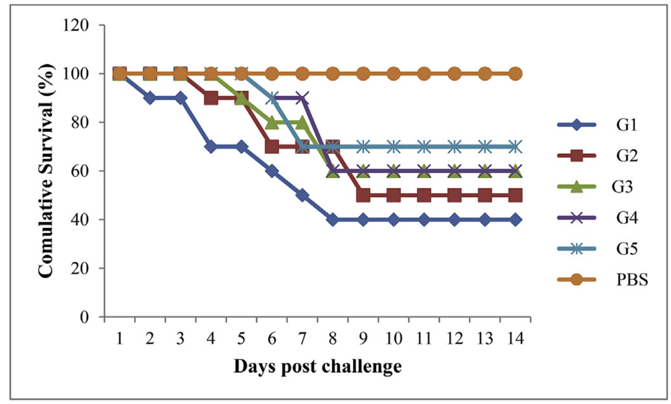

Fig. 2. Cumulative survival of *E. coioides* challenged with *Vribro alginolyticus* LP01 via intraperitoneal injection.

the AST level in *megalobrama amblycephala*, and chickern, while some studies have found that AMPs have no effect on the ALT and AST of serum in pigs [26]. Here, the levels of ALT were not significantly different between the AMP-diet groups and the control group, and the AST in the G3, G4, G5 groups were lower than those of the G1 and G2 groups. The levels of serum HDL-C, LDL-C and TG are vital indices that reflect lipometabolic status. HDL-C, one of the good cholesterol, can prevent the occurrence of atherosclerosis, and decreased HDL-C was an independent risk factor of cardiovascular disease in metabolic syndrome [27]. Here, AMP supplementation had an impact on the HDL-C content in grouper serum. LDL particles are highly sensitive to oxidative damage and oxidative modification of native LDL particles, which are heterogeneous with respect to the amount of cholesterol, makes them highly pathogenic, immunogenic and atherogenic, and LDL-C are a major cause and accelerator of atherogenesis [28,29]. Additionally, serum GLU activity could be a useful marker of hepatitis progression and increased degradation of proteoglycans in diabetes [30]. The GLU content was not affected by the dietary dosages of AMPs.

4.3. Effect of AMPs on antioxidant activity and non-specific immunity

Fish can live in multiple water environments and has its own unique immunity systems against pathogens around them [31,32]. The immune parameters were always related to the antioxidant abilities. To avoid or repair the damage, the compounds may cause in tissues, organisms possess adequate protection systems such as key enzymatic antioxidant defenses (i.e., SOD and MDA) [33]. T-AOC is an index to assess the functional state and indirectly reflect the health status of an organism; SODs are antioxidant enzymes that can catalyze the conversion of reactive superoxide anions into hydrogen peroxide and molecular oxygen [34]. The present study showed that the T-AOC and SOD in fish with AMP-supplements diets were higher than that of the fish with control diets, which reflected that immune ability of fish with AMP-supplements diets was stronger. CAT, one of the major enzymes in the cellular antioxidant defense system, participates in the breakdown of hydrogen peroxide to water and oxygen, deters oxidative stress and is involved in cellular homeostasis [35]. CAT activity in fish treated with dimethoate was increased significantly, and the increased activity of CAT in different tissues is the physiological response of the antioxidant defense system to the production of hydrogen peroxide during detoxification [36]. Higher activity of CAT in fish with AMP diets was found in this study. MDA is the product of lipid peroxidation; high MDA level can enhance the production of free radicals and damage to cell membranes [37]. Fish treated with dimethoate alone or combined with Bacilar, showed a significant increase in MDA level [36]. The present study demonstrated that MDA levels in all AMP-treated groups were lower than that of the control group, which is similar to the results obtained in *O. niloticus* [23]. Lysozyme, a small protein, has gained great attention due to its unique characteristics against a wide range of bacteria [38]. In this study, the level of lysozyme in the groupers with AMP diets was higher than that of the groupers with no-AMP diet. LPS, a component of the outer membrane of gram-negative bacteria, enters the bloodstream by way of intestinal absorption, bacteria that are released into the blood under the occurrence of sepsis, and exogenous injection [23]. This study demonstrated that AMP diets could decrease the LPS levels of *E. coioides.* In brief, the results of this study clearly demonstrated that AMP-diets could enhance the antioxidant activity and non-specific immunity of *E. coioides*.

4.4. Effect of AMPs on expression of immune-related genes

To evaluate the immune responses of grouper with AMP diets, expression profiles of three immune related genes (IL-1β, TNF-α, and Hsp90) were determined using real-time quantitative PCR. IL-1β, a major coordinator in immune response of fish, can stimulate the immune responses by inducing the release of cytokines [33,38]. As shown in Fig. 1, IL-1β was detected in all the examined grouper tissues, and expression level of IL-1β was up-regulated in *E. coioides* with AMP-diets. The increased IL-1β would active cytokine IL-1β production, and subsequently up-regulated a number of inflammation-linked molecules in fish [33,39]. TNF-α, a cell signaling protein, could induce inflammation and cell apoptosis [33]. TNF-α expression in different fish species and different fish status was different mainly due to the fish species and pathogen species [33]. Similar to IL-1β, TNF-α in all the examined groups was detected, and expression level was up-regulated in *E. coioides* with AMP-diets. Hsps family were involved in chaperone activity, and participate in the regulation of the RNA polymerase synthesis of viral replication [40]. Besides, Hsp90 also participates in the immune response, and is activated by numbers of stressors such as heat or cold shock, hyperosmotic stress, food-deprivation, reduced oxygen level, arsenates, heavy metals and various pathogens etc. [41–44]. In this study, Hsp90 was detected in all examined grouper tissues, and higher expression levels were found in all AMP-diets than that of control group (Fig. 1). The present results demonstrated that the diets with AMP significantly up-regulated the expression of the examined genes IL-1β, TNF-α, and Hsp90 post 4 weeks of feeding (Fig. 1), suggesting that antimicrobial peptides can modulate the immune-related genes. Similar results were found in tilapia, *Carassius auratus* et al. [45]. But the mechanism need to further research.

4.5. Challenge test

To evaluate the effect of AMPs on *E. coioides*, *E. coioides* were challenged with *V. alginolyticus*. Within 14 days post challenge, the *E. coioides* in the control group had significantly higher mortalities (60%) compared to the *E. coioides* in the groups with AMP diets (50–70%). The results demonstrated that AMP could significantly improve the survival rate of *E. coioides* post challenge, and the tested AMP has immune enhancement effects on *E. coioides*.

In conclusion, dietary including AMP supplementation can increase the levels of TP, ALB, GLB and HDL-C in grouper serum, improve antioxidant capacity and non-specific immunity, and regulate expression of immune related genes to enhance positive immune responses for disease resistance in *E. coioides*. Fish fed with AMP diets had a significantly improved survival rate post *V. alginolyticus* challenge. However, excess AMPs in diets also affected growth performance. The results indicated that concentration of AMPs range from 200 mg/kg to 400 mg/kg was optimal in the feed, and therefore provide an important reference for further studies in this area.

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