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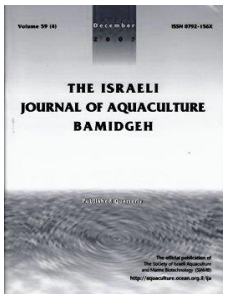

Effects of Antibacterial Peptide Extracted from *Bacillus subtilis* fmbJ on the Growth, Physiological Response and Disease Resistance of *Megalobrama amblycephala*

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Key words: *Megalobrama amblycephala*, Antibacterial peptide, Immunity, *Aeromonas hydrophila*, Growth

Abstract

The effects of an antibacterial peptide obtained from *Bacillus subtilis* fmbJ on growth, serum lysozyme complements 3 and 4, total protein content, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total antioxidative capacity, superoxide dismutase (SOD) activity, malondialdehyde (MDA) content, and disease resistance of Wuchang bream (*Megalobrama amblycephala*) were examined. Fish were randomly divided into five groups: a control group which was fed a basic diet, and four groups fed the basic diet supplemented with 0.1%, 0.2%, 0.4%, or 0.8% antibacterial peptide. At eight weeks, *M. amblycephala* fed the diet containing 0.2% antibacterial peptide had higher serum lysozyme activity, complement 3 and 4 contents, and SOD activity than the control fish, but lower serum MDA content and AST activity. Fish fed the 0.4% diet had higher weight gain rate, serum lysozyme activity, complement 4 content, total antioxidative capacity, and total protein than the control, and lower serum ALT activity. Feed conversion ratios of fish fed the 0.2% or 0.4% diets were lower than those of control fish. Artificial infection with *Aeromonas hydrophila* resulted in 93% cumulative mortality in the control group, and 61-84% in the groups fed the 0.2% or 0.4% diets. The present study suggests that feed supplementation with 0.2-0.4% antibacterial peptides can stimulate immunity, increase resistance to pathogenic infection, and promote growth in *M. amblycephala*.

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Introduction

Megalobrama amblycephala, also known as blunt snout or Wuchang bream, are highly prized in China as a delicacy. For this reason, *M. amblycephala* is currently the most widely cultivated freshwater fish in China. This species (together with other species of Cyprinidae) is commercially important for its value as a food source. However, *M. amblycephala* cultured in China as well as in other parts of the world, have suffered from serious viral and bacterial diseases, causing significant economic losses (Feng 2010; He et al., 2006).

Antibiotics used to control diseases have been combined with feed additives in intensive aquaculture facilities, to promote health and enhance disease prevention (Darwish and Hobbs, 2005). The abuse of antibiotics has however been consistently identified as a major risk factor since it results in an increase in the number of antibiotic-resistant bacteria. This also impacts on the food safety of humans who consume these fish (Cabello, 2006; Rodriguez et al., 2007). As a result, the widespread use of various antibiotic feed additives was banned by the European Union in 1997 (Acar et al., 2000). It is therefore necessary to search for alternative methods of disease control, such as vaccines, immunostimulants, and natural therapeutic compounds from plants (Sun et al., 2012; Verschuere et al., 2000).

Antimicrobial peptides are key components of the immune systems of most multicellular organisms (Andreu and Rivas, 1998). These peptides have antimicrobial properties but no resistance potential; they have the potential to act as alternatives to antibiotics (Zasloff, 2002). There is increased interest in the pharmacological application of antimicrobial peptides to treat infection. Efforts are currently underway to increase the potency and specificity of these peptides so that they are toxic to microbes and not to aquatic animals (Noga et al., 2011). They have the capacity to scavenge oxygen free radicals (Jung et al., 2007) and are highly potent against bacteria (Brogden et al., 2005).

Ingestion of apidaecins from honeybees can improve growth performance and induce positive modulation in the immune response of common carp, *Cyprinus carpio* (Zhou et al., 2008). Recent *in vitro* studies carried out in our laboratory have also indicated that an antibacterial peptide from *Bacillus subtilis* fmbJ can be administered to prevent and control aquatic animal diseases associated with infection by *Aeromonas hydrophila* (Yang et al., 2011). There are however very few reports on the effects of antimicrobial peptides on hemolymph metabolites or the effects of antioxidization enzymes on aquatic animals (Zhou et al., 2008). The objectives of the present study were to evaluate the effect of an antibacterial peptide, obtained from *B. subtilis* fmbJ, on the growth and non-specific immune parameters of *M. amblycephala,* with the purpose of using antimicrobial peptides to provide a theoretical basis for the prophylaxis of *M. amblycephala* diseases.

Materials and Methods

Fish, antibacterial peptide, and diets. Megalobrama amblycephala were supplied by a fish farm associated with the Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences in China. These fish were reared in our experimental fish farm. They were transferred to 15 concrete tanks (2.50 ×1.50 × 1.00 m) and acclimatized for 15 days after which they were randomly divided into five groups: one control and four treatment groups, in triplicate, (50 fish with an average body weight of 38.21 ± 0.14g, per tank). After acclimation, the control group and the four experimental groups were fed with the basic diet (Table 1), or with the basic diet supplemented with 0.1%, 0.2%, 0.4% or 0.8% of antibacterial peptide from *B. subtilis* fmbJ, respectively.

Production, extraction and determination of the antibacterial peptide. Bacillus subtilis fmbJ was inoculated into a 500 mL flask containing 100 ml of Landy medium, cultivated at 33^0C, and shaken at 180 rpm for 36 h. The cells were then removed by centrifugation at 11,000 × *g* for 15 min. The supernatant was treated with 6 M HCL to adjust to pH 2.0 and kept overnight. The culture was centrifuged at 11,000 × *g* for 15 min and the supernatant was removed. The substratum precipitate was extracted with methanol and then readjusted to pH 7.0. The content of the antibacterial peptide reached 4.3 g/l assayed by reversed-phase high-performance liquid chromatography (HPLC). A

small mincing machine was used to prepare the feed with 2.0 mm granular wet pellets. The pellets were dried in a forced air oven at 40°C to a moisture content of 10% and stored at -20°C until use.

Table 1. The basal diet and nutrition levels of *M. amblycephala*

Ingredients	(%)	Nutrition levels	
Rapeseed meal	25.0	Dry matter (%)	88.68
Soybean meal	20.0	Crude protein (%)	30.89
Peanut meal	8.0	Ether extract (%)	4.58
Wheat middling	17.5	Calcium (%)	1.15
Rice bran	10.0	Total phosphorous (%)	1.24
Cotton meal	8.0	Available phosphorous (%)	0.84
Fish meal	4.0	Methionine + Cystine (%)	1.13
Fish oil	2.0	Lysine (%)	1.64
Calcium dihydrogen phosphate	2.0	Gross energy (kJ/g)[a]	17.68
Vitamin premix[b]	1.0		
Mineral premix[b]	1.0		
Attapulgite	1.0		
Salt	0.4		
Choline chloride	0.1		

Note: [1] Gross energy (GE) kJ/g: protein 23.64kJ/g, fat 39.54k J/g, carbohydrate 17.15k J/g; and the others, are measured in the nutrition levels.
[2] Vitamin and mineral additives were provided by Wuxi Hanove Animal Health Products Co.,Ltd, China.

Rearing management. M. amblycephala were acclimatized in concrete tanks for 15 days and handfed on the trial diet at a feeding rate of 2.0%-4.0% body weight, three times a day at 8:00-8:30, 12:00-12:30, and 18:00-18:30. Water from an underground water source was oxygenated day and night using an aerator. Feces and debris were siphoned out daily and one third of the water was changed in each tank once a week. Water temperature was measured daily and water quality checked once a week. During the test period the average water quality parameters were: water temperature: 27.5± 2.32°C; DO > 5 mg/l; NH_3< 0.05 mg/l; H_2S < 0.1mg/l; pH 7.8-8.0. Fish were weighed fortnightly and feeding levels were increased accordingly. On completion of the test period of 56 days, blood samples were collected and weight gain measured.

Infection experiment. At the end of the trial, a sample of 30 fish from the five groups (n = 10 fish/tank) was challenged with the bacterial pathogen *A. hydrophila*, Ah, strain BSK-10, provided by the Zhejiang Provincial Freshwater Fisheries Research Institute, China. Using the method described by Immanuel et al (2004), *A. hydrophila* was activated twice and diluted with sterile normal saline to a final concentration of 1×10^8 CFU cells/ml. The bacterial suspension (1.0 ml/100g body weight) was injected into the abdominal cavity and mortality checked 0, 24, 48, 72, 96, 120 and 144h after the challenge.

Serum sample collection. Serum samples were taken from nine fish in each group at the end of weeks 2, 4, and 8, after the initiation of feeding. Fish were netted and immediately placed into a 200 mg/l solution of MS-222 to induce immediate deep anesthesia. Serum samples were then taken from the caudal vein, refrigerated at 4°C for 1-2 hours, and then centrifuged at 3000 x *g* for 10 minutes. The supernatant was then removed and preserved at -20°C until further analysis.

Measurement of serum immune and anti-oxidization parameters. The serum lysozyme activity, complement 3, and complement 4 contents were determined by test kits for lysozyme activity, purchased from Nanjing Jiancheng Biological Engineering Research

Institute, China and Zhengjiang Elikan Biological Technology Co., Ltd., China. The serum total anti-oxidative capacity, superoxide dismutase activity, and malondialdehyde content were determined by test kits (also from aforementioned institute) for total anti-oxidative capacity, superoxide dismutase activity, and malondialdehyde content. Serum total protein content, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) activities were determined by the colorimetric method of Liu et al.(2010) using a test kit from Shanghai Fudan Zhangjiang Bio Medical Co., Ltd., China in a Beckman Cx-4 type Auto Bio-chemical Analyzer (Beckman Coulter, USA).

Data analysis. Duncan's multiple range tests (SPSS v11.5) were used to determine differences between groups. Significant differences were expressed at a significance level of $P<0.05$. All results were expressed as means ± standard error.

Results

After eight weeks, weight gain rate and specific growth rate of the group fed 0.4% antimicrobial peptide were significantly higher, and feed conversion ratio was significantly lower than that of the control (Table 2).

Table 2 Effects of antimicrobial peptide on growth performance of *M. amblycephala*

Content of antimicrobial peptide (%)	Initial avg wt(g)	Final avg wt (g)	Weight gain rate (%)	Specific growth Rate (%)	Feed conversion ratio
0	38.14±0.03	77.38 ± 2.67^{b}	102.87 ±6.85^{b}	1.26±0.06^{b}	2.47±0.02^{a}
0.1	38.15±0.05	80.79 ± 1.46^{ab}	111.75±3.84^{ab}	1.34±0.04^{ab}	2.39±0.01^{ab}
0.2	38.05±0.03	82.82 ± 0.83^{ab}	117.69±2.27^{ab}	1.38±0.02^{ab}	2.28±0.06^{b}
0.4	38.64±0.58	86.53 ± 2.47^{a}	123.98±5.85^{a}	1.44±0.05^{a}	2.30±0.04^{b}
0.8	38.06±0.04	83.65 ± 2.36^{ab}	119.77±6.39^{ab}	1.40±0.09^{ab}	2.37±0.02^{ab}

Data are expressed as the mean of triplication of every group ± SEM (n=3), and compared at the same index of different doses using Duncan multiple range tests (SPSS, Ver.11.5). Significant differences ($p< 0.05$) from the control are marked by different letters.
Weight gain rate (WGR, %) = 100 × (Final average weight–Initial average weight) /Initial average weight;
Specific growth rate (SGR, %) = 100 × [(Final average weight)–(Initial average weight)]/test days;
Feed conversion ratio (FCR) = Feed consumption / Weight gain;

Serum lysozyme, C3, and C4. After 2, 4 and 8 weeks serum lysozyme activity in the groups supplemented with 0.1-0.4% antibacterial peptide was higher than that of the control. After 4 weeks serum C4 concentrations were significantly higher than the control, and only after 8 weeks were serum C3 concentrations significantly higher than those of the control in the groups treated with 0.2% antibacterial peptide (fig. 1C).

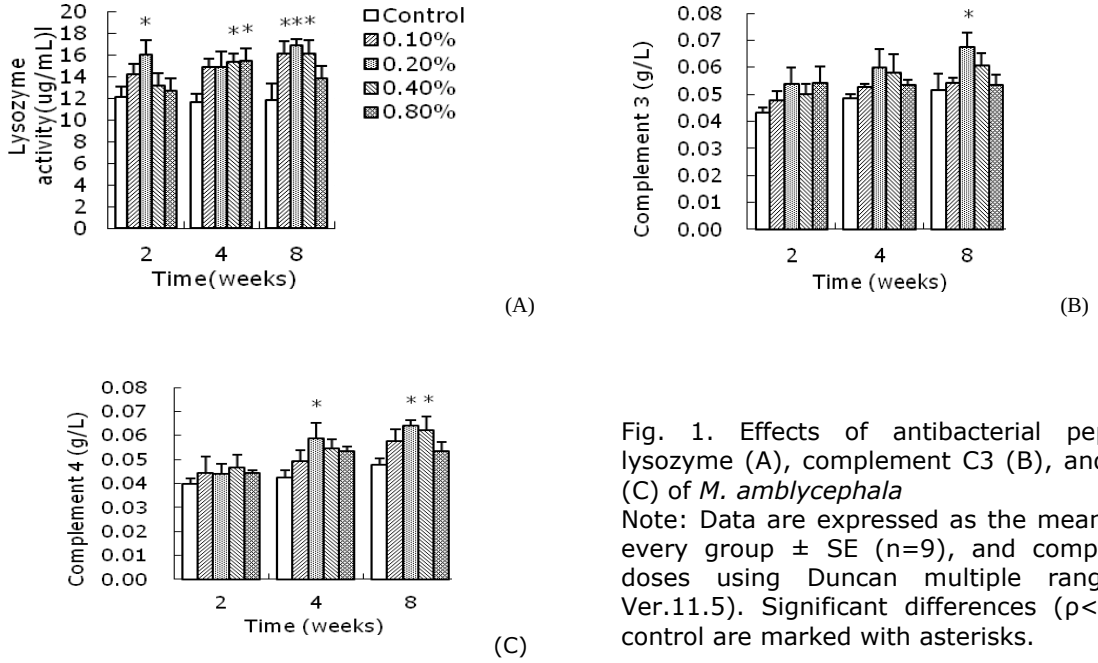
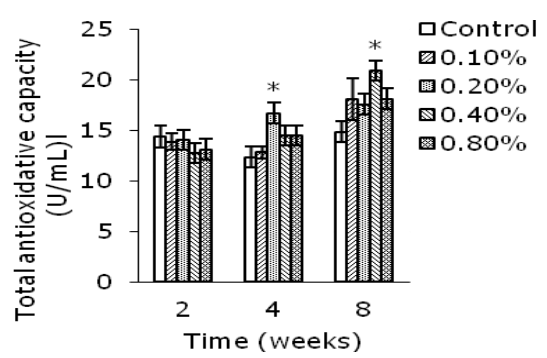
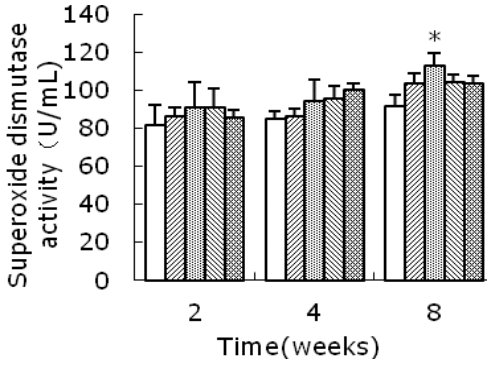


Fig. 1. Effects of antibacterial peptide on serum lysozyme (A), complement C3 (B), and complement C4 (C) of *M. amblycephala*
Note: Data are expressed as the mean of triplication of every group ± SE (n=9), and compared at different doses using Duncan multiple range tests (SPSS, Ver.11.5). Significant differences ($p< 0.05$) from the control are marked with asterisks.

Only after 8 weeks was a significant increase in total serum anti-oxidative capacity noted in groups treated with 0.2% antibacterial peptide (AP) for 4 weeks, or 0.4% AP for 8 weeks (Fig. 2A). Prior to 8 weeks there were no significant differences in this parameter in any of the treated groups compared to the control. Serum superoxide dismutase activity significantly increased compared with the control in the groups fed with 0.2% AP for 8 weeks (Fig. 2B). Serum malondialdehyde content significantly decreased in the group fed with 0.8% AP for 4 weeks or with 0.2% AP for 8 weeks compared with the control. No significant differences in this parameter were noted between the treated and control group after 2 weeks (Fig. 2C).



(A)



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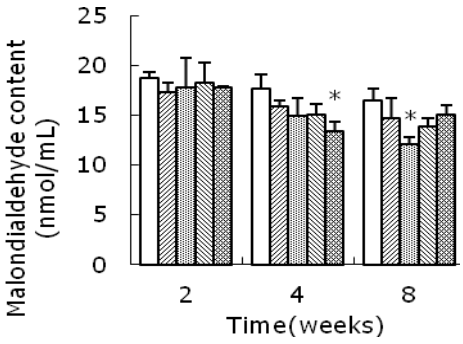


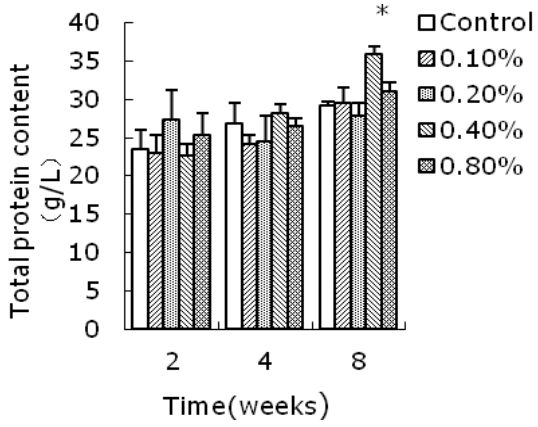
(C)

Fig. 2. Effects of antibacterial peptides on serum total antioxidative capacity (A), superoxide dismutase activity (B), and malondialdehyde content (C) of *M. amblycephala*

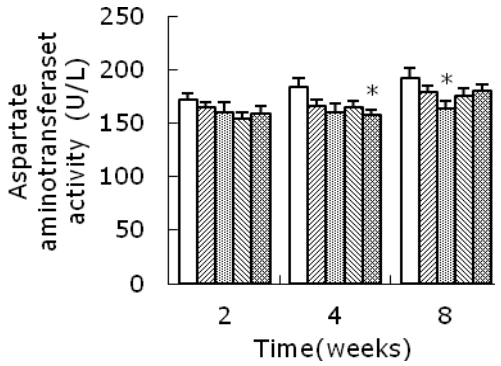
Note: Data are expressed as the mean of triplication of every group ± SE (n=9), and compared at different doses using Duncan multiple range tests (SPSS, Ver.11.5).

Significant differences ($\rho < 0.05$) from the control are marked with asterisks.

Serum total protein, AST and ALT activity. Significant differences in the serum total protein content were noted only in the groups fed with 0.4% antibacterial peptide (AP) for 8 weeks compared with the control (Fig. 3A). Serum AST activity significantly decreased in the group fed 0.8% AP for 4 weeks or 0.2% AP for 8 weeks compared to the control. ALT activity significantly decreased after 2 weeks in all groups fed AP compared to the control (Figs. 3B, 3C).



(A)



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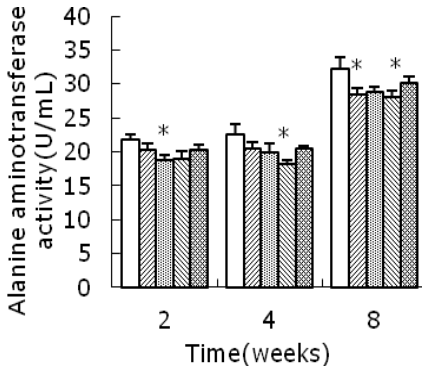

(C)

Fig. 3. Effects of antibacterial peptides on total protein (A), aspartate aminotransferase activity (B) and alanine aminotransferase activity (C) of *M. amblycephala*
Note: Data are expressed as the mean of triplication of every group ± SE (n=9), and compared at different doses using Duncan multiple range tests (SPSS, Ver.11.5).
Significant differences ($p < 0.05$) from the control are marked with asterisks.

Pathogenic infection. After challenging *M. amblycephala* with *A. hydrophila* infection following 8 weeks of feeding antibacterial peptide (AP), accumulative mortality percentages six days post challenge indicated that supplements of 0.2%-0.4% AP could enhance disease resistance (Fig. 4).

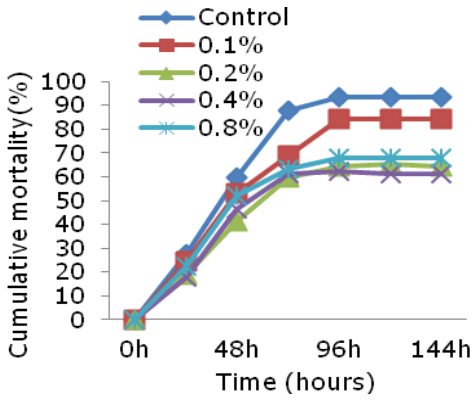

Fig. 4. Effects of antibacterial peptides on cumulative mortality after *A.hydrophila* infection of *M. amblycephala*
Note: Data are expressed as the mean of triplication of every group ± SE (n=3).

Discussion

This study was conducted to evaluate how different concentrations of antibacterial peptide affect growth performance, serum metabolites, and non-specific immune parameters, as well as disease resistance in *M. amblycephala*. In aquaculture, the innate immune system is a fundamental defense mechanism of fish and the main parameters of the innate system are commonly divided into physical parameters and cellular, and humoral factors (Magnadóttir, 2006). Immunostimulants and natural therapeutics from plants can stimulate the innate immune system by activating or increasing the activity of granulocytes and macrophages, increasing the number of phagocytes, or activating alternative complementary metabolic pathways in aquatic animals (Yeh et al., 2009; Liu et al., 2010). Antimicrobial polypeptides consisting of peptides and small proteins with antimicrobial activity are an integral component of innate immunity. Their potent properties, and widespread prevalence in fish suggest that devising methods of manipulating their levels has considerable potential for maintaining or improving fish health (Noga et al., 2011). In the present study we showed that the total accumulated mortality percentages in the control group were higher than those of the antibacterial peptide groups.

Lysozyme is an important component of the protective enzyme system and is responsible for breaking down the polysaccharide wall of bacteria, thus contributing to protection from infectious pathogens (Rø et al., 2002). Lysozyme activity in fish increases after being fed with probiotic supplements (Panigrahi et al., 2004). A number of such supplements have been reported by other researchers, for example anthraquinone extract (Liu et al., 2010). In a previous study on common carp (*C. carpio* var. Jian), enhanced lysozyme activity was observed when fish were fed with apidaecin antimicrobial peptide (Zhou et al., 2008). Results from our experiment indicate that serum lysozyme activity in the group supplemented with 0.1% antibacterial peptide for 2 weeks after the feed intake, 0.2% and 0.4% antibacterial peptide for 6 weeks and 0.1%, 0.2%, 0.4% antibacterial peptide for 8 weeks was significantly higher than that observed in the control. The increase of lysozyme activity within a certain range will stimulate the immune response of fish which may contribute to host resistance against infectious pathogens (Ye et al., 2011; Sun et al., 2012; Misra et al., 2006).

Complement pathways are an essential part of the innate immune system, involving about 35 soluble and membrane-bound proteins. The complement pathway constitutes the humoral component of natural defense against infections, which can operate without antibody participation (Ellis, 2003). In common carp, the alternative complement pathway activity increased after feeding 15 mg/kg or 30 mg/kg apidaecin antimicrobial peptide. This increased activity was not observed in the control group (Zhou et al., 2008). In tilapia, dietary intake of 0.01%-0.1% doses of *E.alba* aqueous extract significantly increased serum complement activities, when compared to such activity in control group (Christybapita et al., 2007). Correspondingly, in the present study, the serum C3 concentration, after 2 or 4 weeks of feeding, was significantly higher in the group treated with 0.2% antibacterial peptide compared to that in the control. Serum C4 concentration in the groups supplemented with 0.2% antibacterial peptide for 4 weeks, and 0.2% and 0.4% antibacterial peptide for 8 weeks, was significantly higher than that of the control.

The non-specific defense mechanisms of fish include neutrophil activation, the production of peroxidase, oxidative free radicals, and initiation of other inflammatory factors (Ainsworth et al., 1991). Aquatic animals maintain complex systems of multiple types of antioxidants, such as glutathione, catalase, superoxide dismutase, and various peroxidases. Activation of non-specific defense mechanisms in fish is indicated by increased levels of total anti-oxidative capacity, glutathione peroxidase activity, reactive oxygen, and nitrogen species (Itou et al., 1996), which can prevent the oxidation of other molecules, and protect the organism from oxidative damage as well as reducing levels of lipid hydroperoxides. In fish, dietary intake of Chinese herbal extracts also can significantly enhance antioxidant ability (Rao et al., 2004). In the present study the serum total anti-oxidative capacity in the group treated with 0.2% antibacterial peptide

for 4 weeks or 0.4% antibacterial peptide for 8 weeks after feed intake increased significantly. In addition to this, a significant increase in serum SOD activity in the group fed with 0.2% antibacterial peptide for 8 weeks was also noted. However, the serum MDA content decreased significantly in the group fed with 0.8% antibacterial peptide for 4 weeks or with 0.2% antibacterial peptide for 8 weeks, compared to that measured in the control. Thus 0.2%-0.4% antibacterial peptide can, to some extent, reduce the harm caused by lipidic superoxide.

Serum proteins are involved in maintaining normal osmotic pressure and constant pH levels, and in the transport of lipid acid, bilirubin, cholesterol, and phosphatide. The hemolymph protein content is used as an immune parameter indicating whether the prawn is healthy or not (Bachère et al., 1997). When under stress, rainbow trout treated with fructose as an immunostimulant indicated that fructose in the diet can cause increased plasma protein content (Rotlant et al., 1997). Improved levels of total protein content in hemolymph were noted in abalone that had been fed with traditional Chinese medicine (Xue et al., 2008). In the present study we showed that serum total protein significantly increased in the group treated with 0.4% antibacterial peptide for 8 weeks after feed intake, compared to that of the control.

ALT and AST— ubiquitous aminotransferases found in the mitochondria of fish — are important indices for diagnosis of hepatopancreas function and damage. AST and ALT activities in *Labeo rohita* significantly decreased when given 0.05% *Achyranthes aspera* compared with control fish (Rao et al., 2006). AST and ALT activity in *Macrobrachium rosenbergii* fed on a diet containing anthraquinone extract under high temperature stress were significantly lower than those of control fish (Liu et al., 2010). In the present study, we found that serum AST activity significantly decreased in the group fed with 0.8% antibacterial peptide for 4 weeks or 0.2% antibacterial peptide for 8 weeks compared with that in the control. Serum ALT activity significantly decreased in the group fed with 0.2% antibacterial peptide for 2 weeks, 0.4% antibacterial peptide for 4 weeks, and 0.1% and 0.4% antibacterial peptide for 8 weeks.

Antimicrobial peptides which encompass a wide variety of structural motifs have improved fish health (Noga et al., 2011). These compounds have a high capacity to scavenge oxygen free radicals (Jung et al., 2007) and also contain anti-bacterial properties (Brogden et al., 2005). They can thus indirectly contribute to increased growth of aquatic animals (Zhou et al., 2008). In the present study, the results showed that the dose of 0.2%-0.4% antimicrobial peptides could improve serum lysozyme activity, C3 and C4 activity, and also increase anti-oxidation activity. *M. amblycephala* treated with these substances were also found to have higher resistance to *A. hydrophila* infection and improved weight growth, but reduced feed conversion ratios.

The results from these experiments indicate that a diet supplemented with 0.2%-0.4% antimicrobial peptide can result in increased levels of serum protein, lysozyme, C3, C4, and total anti-oxidative capacity (or SOD) and decreased levels of serum AST, ALT or MDA in fish. The present study indicates that a dose of 0.2%-0.4% antimicrobial peptide can increase immune and anti-oxidation capability, enhance resistance to pathogenic infection, and promote the growth performance of *M. amblycephala*.

Acknowledgements

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References

Acar J., Casewell M., Freeman J., Friis C. and H. Goossens, 2000. Avoparcin and virginiamycin as animal growth promoters: a plea for science in decision making. *Clin. Microbiol. Infec.*, 6:477-482.

Ainsworth A.J., Dexiang C. and P.R.Waterstrat, 1991. Changes in peripheral blood leukocyte percentages and function of neutrophils in stressed channel catfish. *J. Aquat. Anim. Health.,* 3: 41-47.

Andreu D. and L.Rivas, 1998. Animal antimicrobial peptides: an overview. *Biopolymers,* 47: 415-433.

Bachère E., Mialhe E. and J. Rodriguez, 1997. Identification of defence effectors in the haemolymph of Crustaceans with particular reference to the shrimp *Penaeus japonicus* (Bate): prospects and applications. *Fish & Shellfish Immunol.*, 5:597-612.

Brogden K.A., 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria. *Nat. Rev. Microbiol.,* 3:238-250.

Cabello C.F., 2006. Heavy use of prophylactic antibiotics in aquaculture, a growing problem for human and animal health and for the environment. *Environ. Microbial.,* 8:1137-1144.

Christybapita D., Divyagnaneswari M. and R.D. Michael, 2007. Oral administration of *Eclipta alba* leaf aqueous extract enhances the non-specific immune responses and disease resistance of *Oreochromis mossambicus*. *Fish Shellfish Immunol.,* 4: 840–852.

Darwish A.M. and M.S. Hobbs, 2005. Laboratory efficacy of amoxicillin for the control of Streptococcus iniae infection in blue tilapia. *J. Aquat. Anim. Health,* 17:197–202.

Ellis A.E., 2003. Immunity to bacteria in fish. *Fish Shellfish Immunol.,* 4:291-308.

Feng D.Y., 2010.Survey and evaluation of diseases found in freshwater fishes in 2009. *Chinese J. of Fish,* 23:60-65(In Chinese).

He L.J., Liao L. K., Yuan J.F., Tang H.T., Wu Q. and G.W.Zhang, 2006. Pathological observation of bacterial septicemia in *Megalobrama amblycephala*. J*ournal of Southwest University,* 3:483-490 (In Chinese).

Immanuel G., Vincybai V. C., Sivaram V., Palavesam A.and M.P. Marian, 2004. Effect of butanoic extracts from terrestrial herbs and seaweeds on the survival, growth and pathogen (*Vibrio parahaemolyticus*) load on shrimp *Penaeus indicus* juveniles. *Aquaculture,* 236:53-65.

Itou T., Lida T.and H. Kawatsu, 1996. Kinetics of oxygen metabolism during respiratory burst in Japanese eel neutrophils. *Dev. Com. Immunol.,* 20:323–330.

Jung W.K., Qian Z.J.and S.H.Lee, 2007. Free radical scavenging activity of a novel antioxidative peptide isolated from in vitro gastrointestinal digests of *Mytilus coruscus*. *Med. Food*, 10:197-202.

Liu B., Xie J., Ge X.P., Xu P., Wang A.M., He Y.J., Zhou Q.L., Pan L.K.and R.L.Chen, 2010. Effects of anthraquinone extract from *Rheum officinale* Bail on the growth performance and physiological responses of *Macrobrachium rosenbergii* under high temperature stress. *Fish Shellfish Immunol.*29:49-57.

Magnadóttir B, 2006. Innate immunity of fish (overview). *Fish Shellfish Immunol.* 20:137-151.

Misra C.K., Das B.K., Mukherjee S.C and P. Pattnaik, 2006. Effect of multiple injections of β-glucan on non-specific immune response and disease resistance in *Labeo rohita* fingerlings. *Fish Shellfish Immunol.,* 20, 305-319.

Noga E.J., Ullal A.J., Corrales J. and J.M.O. Fernandes, 2011. Application of antimicrobial polypeptide host defenses to aquaculture: Exploitation of down-regulation and upregulation responses. *Comp. Biochem. and Physiol., Part D,* 6:44-54.

Panigrahi P.A., Kiron V., Kobayshi T., Puangkaew J., Satoh S. and H. Sugita, 2004. Immune responses in rainbow trout *Oncorhynchus mykiss* induced by a potential probiotics bacteria *Lactobacillus rhamnosus* JCM 1136. *Vet. Immunol. Immunopathol.* 102:379-388.

Rao Y.V., Das B.K., Jyotyrmayee P. and R. Chakrabarti, 2006. Effect of *Achyranthes aspera* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila. Fish Shellfish Immunol.,* 3: 263-273.

Rø K.H., Fevolden S.E. and K.T.Fjalestad, 2002. Disease resistance and immune characteristics in rainbow trout (*Onchorhyncus mykiss*) selected for lysozyme activity. *Aquaculture,* 209:91-101.

Rodriguez J.C., Pastor E., Ruiz M., Flores E and G. Royo, 2007. Antibiotic resistance during therapy: mechanisms and means of control. *Infect. Dis. Drug Targets,* 7:43-45.

Rotlant J., Pavlidis M., Kentouri M., Abad M.E. and L. Tort, 1997. Non-specific immune responses in the red porgy *Pagrus pagrus* crowding stress. *Aquaculture,* 156: 279-290.

Sun J, Wang Q., Qiao Z., Bai, D., Sun J., and X. Qiao, 2012. Effect of Lipopolysaccharide (LPS) and Outer Membrane Protein (OMP) Vaccines on Protection of Grass Carp (*Ctenopharyngodon idella*) against *Aeromonas hydrophila*. *Isr. J. Aquacult. Bamidgeh,* IIC:63.2011.655 8 pages

Verschuere L., Rombaut G., Sorgeloos P.and W. Verstraete, 2000. Probiotic bacteria as biological control agents in aquaculture. *Microb. Mol. Biol. Rev.*, 64:655-671.

Xue J.P., Xu Y.P., Jin L.J., Liu G. Sun , Y.X., Li S.Y. and J.C. Zhang, 2008. Effects of traditional Chinese medicine on immune responses in abalone, *Haliotis discus hannai* Ino. *Fish Shellfish Immunol.,* 6:752-758.

Yang L.L., Lu F.X., Bie X.X., He Y.J., Wang T. and Z.X. Lu, 2011. Study on the Anti-*Aeromonas hydrophila* effect in vitro of Lipopeptide from *Bacillus subtilis* fmbJ. *J. Food Sci.*, 32:193-198(in Chinese).

Ye Ji-Dan, Wang K., Li F-D, Sun Y-Z, 2011. Effects of long-term dietary administration of β-glucan on tissue enzyme activity and disease resistance in common Carp, *Cyprinus carpio*, 6 pages, *Isr. J. Aquacult. - Bamidgeh,*IIC:63.2011.664 6 pages.

Yeh R.Y., Shiu Y.L., Shei S.C., Cheng S.C., Huang S.Y., Lin J.C. and C.H. Liu, 2009. Evaluation of the antibacterial activity of leaf and twig extracts of stout camphor tree, *Cinnamomum kanehirae*, and the effects on immunity and disease resistance of white shrimp, *Litopenaeus vannamei*. *Fish Shellfish Immunol.,* 27:26-32

Zasloff M., 2002. Antimicrobial peptides of multicellular organisms. *Nature,* 415:389-395.

Zhou X.X., Wang Y.B. and W.F. Li, 2008. Effect of feeding apidaecin on common carp (*Cyprinus carpio*) growth performances and immune function. *Aquaculture,* 279:108-112.