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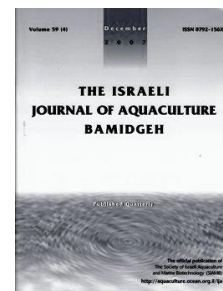
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## Immunomodulation with Probiotics Against *Aeromonas veronii* in Grass Carp (*Ctenopharyngodon idellus*)

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**Keywords:** grass carp *Ctenopharyngodon idellus*; probiotics; immune response; *Aeromonas veronii*

### Abstract

*Aeromonas* comprise a wide range of opportunistic bacteria that cause septicemia (blood poisoning) in fish. With expanding aquaculture, myriad methods have been used to combat these pathologies including probiotics and plant extracts which can potentially address these diseases without harming the environment. In this study, four strains of opportunistic pathogens were isolated from diseased fish, then molecular identification of 16SrDNA was performed. Results identified the four strains as *Aeromonas veronii*, *Shewanella sp.*, *Bacillus cereus*, and *Aeromonas salmonicida*. Experimental trials revealed that *A. veronii* was the primary strain. Multiple virulence-related factors were discovered, including aerolysin (*Aer*), cytotoxic enterotoxin (*Act*), and the outer membrane protein (*OmpAII*). These were found only in *A. veronii*. LC50 in grass carp was  $3.6 \times 10^4$  CFU/mL over 96 h, whereas the safe concentration was  $9.11 \times 10^3$  CFU/mL. Grass carp were challenged with 3 different concentrations of *A. veronii*. Following a 48 h incubation period they were treated for 24 h, 48 h, and 72 h with  $8.1 \times 10^6$  CFU/mL concentrations. The expression of Interleukin-8 (IL-8) and Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ) was up-regulated in the kidney, liver, spleen, and heart using qRT-PCR. Simultaneously, enzyme activity involving Pyruvate Kinase (PK), Alkaline Phosphatase (AKP), Alanine aminotransferase (ALT), Succinate Dehydrogenase (SDH), and Lysozyme (Lsz) in serum and liver tissues was significantly up-regulated. We found that probiotics enhanced growth of grass carp, improved health status, and was beneficial in reducing the prevalence of this disease. Subsequent to feeding mixed probiotics, IL-8 and TNF- $\alpha$  gene expression was significantly lower ( $p > 0.05$ ) than in the infected group, at 16 and 31 days. Expression of these genes in the liver, spleen, and intestines of healthy grass carp fed probiotics was significantly up-regulated in contrast to grass carp fed a basal diet ( $p < 0.05$ ). Our results suggest that use of probiotics as immune stimulator may improve the control of fish diseases in aquaculture.

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## Introduction

Aquaculture constitutes the fastest-growing food production system in the world. For several decades, aquaculture has provided one-third of the global supply of fish. Grass carp (*Ctenopharyngodon idellus*) is the primary freshwater aquaculture fish in China (Chi *et al.* 2016). With growing intensification and diversification of aquaculture, fish diseases have caused serious economic losses, and this has led to intensive research in recent years (Sun *et al.* 2016). Fish septicemia can spread rapidly, is the most common widespread disease in aquaculture, and is the most significant constraint to further development (Rahman *et al.* 2002).

The primary and most significant impact of disease in aquaculture is related to intensification, which leads to stressful conditions, poor water quality and the proliferation of pathogenic bacteria (Yang *et al.* 2017, Armstrong *et al.* 2005). Concurrently, aquaculture production is affected by opportunistic pathogens, which induce innate immune responses in grass carp (Becker *et al.* 2015). The common pathogenic bacteria involved in septicemia are *Aeromonas veronii*, *Aeromonas hydrophila* and *Aeromonas salmonicida*. Inflammatory cytokines are particularly useful when investigating the host responses of fish following pathogenic bacteria infection. Interleukin-8 (IL-8) plays a critical role in immune regulation, hematopoietic stem cells, and inflammation, whereas Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ) is a major pro-inflammatory cytokine that plays a vital role in pathogenic invasion and antimicrobial defense. This is considered an excellent health indicator and biomarker of vaccine efficacy in teleosts (Nguyen *et al.* 2017). In mammals, IL-8 is generated during infection by many cell types, such as macrophages, monocytes, epithelial cells, neutrophils, and fibroblasts, or in response to stimulation by other cytokines, such as IL-1 $\beta$  and TNF- $\alpha$  (Tafalla *et al.* 2013). Hence, we aimed to identify TNF- $\alpha$  and IL-8 during pathogenic infection in grass carp to evaluate their importance as health-related biomarkers.

Likewise, virulence factor genes (Aerolysin (*Aer*), cytotoxic enterotoxin (*Act*), and the outer membrane protein (*OmpAII*)) serve as excellent biomarkers for the identification of pathogenicity in fish (Nawaz *et al.* 2010). A wide range of putative virulence factors have been detected and studied in several *Aeromonas spp.* (Faggio *et al.* 2015). Virulence-encoded *Aeromonads* genes secrete a variety of toxins that increase the severity of many infections by irreversibly combining with cells, which leads to host death (González-Serrano *et al.* 2002). *OmpAII* is an important adhesion factor and protective antigen that is intimately related to the virulence of bacteria (Sen *et al.* 2004). Since little is known about the virulence mechanisms involved in *A. veronii*, we endeavored to investigate whether these isolates contained disease-related virulence factors (Debmalya *et al.* 2016).

The introduction of medications into aquaculture may result in the emergence of antibiotic resistant genes. Selective pressures on bacteria in the natural environment may potentially have negative impacts on human health (Kumar *et al.* 2013). Recently, immune stimulators such as probiotics have been developed as new feed additives to enhance immune system, feed efficiency, and growth performance of fish (Beaz-Hidalgo and Figueras 2013). Although these bacteria comprise a new line of alternative antibiotics for the effective prevention of disease, they must survive and colonize the gastrointestinal tract to confer their functional properties and health benefits (Guo *et al.* 2016). Therefore, the selection of appropriate probiotics culture is crucial. For this study, probiotics were fed to grass carp in culture, after which their effects on immune stimulation were investigated. The use of natural immune stimulants is considered safe for both human health and the environment.

## Materials and methods

### *Ethics statement*

For the current study, fish tissues were obtained through routine methods. Fish were anesthetized and killed according to the guidelines of the China Law for Animal Health Protection and Instructions for Granting Permits for Animal Experimentation for Scientific Purposes (ethics approval no. SCXK (YU) 2005-0001). All surgeries were performed under 3-Aminobenzoic acid ethyl ester methanesulfonate (MS-222) anesthesia, and every effort was made to minimize suffering.

*Experimental fish and diet preparation.*

Diseased grass carp (average body weight: 20±5g, body length: 13±2 cm) were procured from a local fish farm. Healthy grass carp (av. weight: 20±5g) were procured from Kaifeng County, Henan Province. The grass carp for this study were held in indoor tanks equipped with a recirculating freshwater system at 25°C and fed with commercial food pellets. The fish were acclimatized for at least one week prior to being introduced into the experimental tanks. One third of the water was replaced with dechlorinated tap water twice per week.

Diet preparation: All dietary compositions were prepared in the laboratory (see Table 1). A 3% addition of probiotics was introduced into the regular feed and mixed evenly. The dietary ingredients were thoroughly mixed and moistened with the addition of 50% (w/v) water, converted to pellets, then air-dried at 35°C for 24 h, and stored in a freezer.

**Table 1.** Proximate compositions of feeds

Food	Amount (%)	Proximate compositions	Basal diet
soybean meal	20.0	Dry matter (%)	93.2 ± 0.05
vegetable meal waste	10.0	Ash (%)	9.18 ± 0.46
corn starch	5.0	Total phosphorous ( µg/g fish feed) <sup>a</sup>	1942 ± 478
cereal meal waste	15.0	Total Kijedal nitrogen ( µg/g fish feed) <sup>a</sup>	2299 ± 232
fish meal	10.0	Protein (%)	31.1 ± 3.36
meat meal waste	20.0	Lipid (%)	13.3 ± 1.81
bone meal	8.0	Fiber (%)	10.1 ± 0.63
fish oil	2.5	Carbohydrates (%)	24.2
Soybean oil	2.5	Energy (kJ/g diet)	16.6
Salt	2.5	P/E (mg/kJ) <sup>b</sup>	1898
other food waste	4.5		

<sup>a</sup> Total phosphorous and total Kijedal nitrogen were analyzed following the methodology of APHA (1998).

<sup>b</sup> Protein to energy (P/E) (mg/kJ) = crude protein (%) / Energy.

*Experimental probiotics*

*Bulgarian lactobacillus*, *bacillus* and *clostridium butyrate* were obtained from the National Collection of Industrial Microorganisms and provided by the Microbiology and Genetic Laboratory, Henan Normal University. A 3% addition of probiotics at a concentration of  $1.82 \times 10^5$  CFU/mL was sprayed on ordinary commercial feed, mixed evenly, and then fed to the fish twice daily at a ratio of 5% of their total biomass.

*Isolation and identification of pathogenic bacteria.*

The abdominal cavities of dead diseased fish were surgically exposed following surface sterilization with 70% ethanol. Tissue samples were aseptically extracted from the liver, kidney, and skin, and then streaked onto a LB plate and incubated at 35°C for 24 h. Subsequent to incubation, bacterial colonies were selected and purified and then stored at -80°C with 30% glycerol, prior to identification.

Bacterial genomic DNA from each sample was extracted using a DNA kit (OMEGA, USA) in accordance with the manufacturer's instructions. 16S rDNA sequence amplification was performed using the general primer 27 F-1492 R; the specific primers using Primer 6.0 were designed to amplify the designed primer (Table 2). Polymerase chain reaction (PCR) amplification was carried out in a 25 µl reaction volume, which contained 2 x Easy Taq PCR Supermix (Transgen Biotech, Beijing, China) 12.5 µl, forward and reverse primer 1 µl, 9.5 µl ddH<sub>2</sub>O water, and 1 µl DNA templates. The reaction conditions and cycle index were 94°C for 3 min., followed by 34 cycles at 94°C for 1 min, 52°C for 1 min. 30 s, and 72°C for 1 min. 30s. The amplified products were sequenced through purification, which were then compared with nucleotide sequence homology in the NCBI Gen Bank database. The construction of phylogenetic trees was conducted using MEGA4 according to Zhang et al. (2011).

**Table 2** List of primer sequences and amplicon size in this study

Genes	Primer sequence(5'-3')	PCR product (bp)	GenBank ID or reference
27F-1492R	F:AGAGTTTGATCMTGGCTCAG	1500	AB128892.1
	R:TACGGMTACCTTGTTACGACTT		
Aer	F:CCTATGGCCTGAGCGAGAAG	431	Mohamed et al.2010
	R:CCAGTTCCAGTCCCACCACT		
Act	F:AGAAGGTGACCACCACCAAGAACA	232	Mohamed et al.2010
	R:AACTGACATCGGCCTTGAAGTC		
OMP II	F:GCTGAATTCATGAAACTCAAATGGCTC	1001	KC297682.1
	R:GCGAAGCTTTTACTGTTGTAAGTTC		
$\beta$ -actin	F:GATGATGAAATTGCCGCACTG	135	M25013.1
	R:ACCGACCATGACGCCCTGATGT		
IL-8	F:GGTGTAGATCCACGCTGTGC	156	JN663841.1
	R:GTGAGGGCTAGGAGGGTAGAG		
TNF- $\alpha$	F:ACGCTCAACAAGTCTCAG	237	JQ670916.1
	R:CTGGCTGTAGACGAAGTAA		

#### Challenge assays

To confirm the pathogenicity of the purified strains, grass carp were injected intraperitoneally with 200  $\mu$ l of bacterial suspension in 0.68% saline (control),  $1.82 \times 10^5$ ,  $2.81 \times 10^6$ ,  $1.125 \times 10^7$ ,  $1.82 \times 10^8$  CFU/mL according to Choi et al. (2014), respectively. Following 96 hours of feeding, the overall characteristics of the challenged fish were recorded. Furthermore, the pathogens were re-isolated from the experimentally infected fish, that also underwent physiological and biochemical characteristics analysis.

The concentration curve (OD600) of *A. veronii* was measured in each test tube, from which we derived one bacterium concentration and its corresponding standard OD value curve.

**Detection of virulence genes.** PCR assays for the amplification of *OMP II*, *Act*, and *Aer* were performed with the template DNA of the isolates. Primers for the amplification of the virulence and related genes are listed in Table 2. The thermocycling program was optimized as follows: initial denaturation at 95°C for 2 min, then 34 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 1 min., and extension at 72°C for 30 min. Following the final extension at 72°C for 10 min., the tubes were cooled to 4°C.

#### Detection of LC50 and sample collection.

For intraperitoneal introduction, 200  $\mu$ l of an *A. veronii* suspension was injected (pre-experiment duration was 96 h) to determine the minimal lethal and maximal sub-lethal concentrations, followed by the establishment of formal experimental groups of grass carp (thirty fish per group). These were intraperitoneally injected as follows: 0.68% saline (control),  $1.82 \times 10^5$ ,  $7.28 \times 10^5$ ,  $2.81 \times 10^6$ ,  $1.125 \times 10^7$ ,  $4.5 \times 10^7$ , and  $1.82 \times 10^8$  CFU/mL, respectively. LC50 was recorded with in 96 h. The LC50 and safe bacterial concentrations in the grass carp were obtained using a modified Koch method to calculate acute toxicity with the formula:  $\lg LC_{50} = \bar{X}_m - i(\sum p - 0.5)$ . The formula for safety concentrations were:  $Sc = LC_{50}(48h) \times 0.3 / [LC_{50}(24h) / LC_{50}(48h)]^2$ .

Grass carp were injected with 200  $\mu$ l of an *A. veronii* suspension at a concentration of 0.68% saline (control),  $1.82 \times 10^5$ ,  $2.81 \times 10^6$ ,  $1.82 \times 10^8$  CFU/mL, and RNA extraction within 48h, respectively. Simultaneously, they were injected at 24 h, 48 h, 72 h with  $8.1 \times 10^6$  CFU/mL, after which the RNA from a variety of organizations was isolated and reverse transcribed. Samples of heart, liver, skin, gill, spleen, swim bladder, kidney, and intestines were collected from five fish at -80°C for RNA extraction. This study was carried out in strict accordance with the recommendations provided by the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The use of experimental fish was approved by the Animal Ethics Committee of Henan Normal University.

#### *Probiotics on the immune response in fish*

The experimental group was infected with  $1.82 \times 10^5$  CFU/mL of *A. veronii* bacterium and fed with probiotic fermentation feed, and three repetitions were established (50 fish  $\times$  3 replicates = 150 fish). Total RNA was extracted from tissues on the 1st, 16th, and 31st days, and health status and mortality rates of the grass carp were recorded.

The effects of the probiotics on the mRNA expression of IL-8 and TNF- $\alpha$  were detected using qRT-PCR. Serum and liver tissues samples were collected on the 1st, 16<sup>th</sup> and 31st days to detect changes in each immunological index of the grass carp.

#### *RNA extraction and real-time PCR analyses of gene expression*

Total RNA was extracted from the samples using TRIzol Reagent (Cwbio, Beijing, China). All containers used in the experiment were treated with a 1% DEPC solution. DNA was synthesized using the Prime Script RT reagent Kit (TaKaRa, Otsu, Japan) following the manufacturer's instructions. IL-8 and TNF- $\alpha$  gene expressions were detected using qRT-PCR, where the mRNA of the healthy fish tissues served as the control, and  $\beta$ -actin was used as the house-keeping gene. Reaction conditions and cycle index were 95°C for 10 min. followed by 40 cycles at 95°C for 15 s, 60°C for 60 s, 95°C for 15 s, 60°C for 15 s, and 37°C for 30 s. The data from these samples were analyzed by the  $2^{-\Delta\Delta Ct}$  method, where the  $\Delta Ct$  value was determined by subtracting the average  $\beta$ -actin Ct value from the average target gene Ct value ( $\Delta\Delta Ct = (Ct(\text{target}) - Ct(\beta\text{-actin}))_{\text{target}} - (Ct(\text{target}) - Ct(\beta\text{-actin}))_{\text{control}}$ ). Significant differences were set at the level of  $P < 0.05$ , and three PCR replicates were performed.

#### *Enzyme activity assay*

Grass carp were intraperitoneally injected as follows: 0.68% saline (control),  $1.82 \times 10^5$ ,  $2.81 \times 10^6$ ,  $1.125 \times 10^7$ , and  $1.82 \times 10^8$  CFU/mL at 24 h, 48 h, 72 h, and 96 h for enzyme activity assay, respectively, to quantify the Pyruvate Kinase (PK), Alkaline Phosphatase (AKP), Alanine aminotransferase (ALT), Succinate Dehydrogenase (SDH), and Lysozyme (Lsz) activity in the serum and liver tissues.

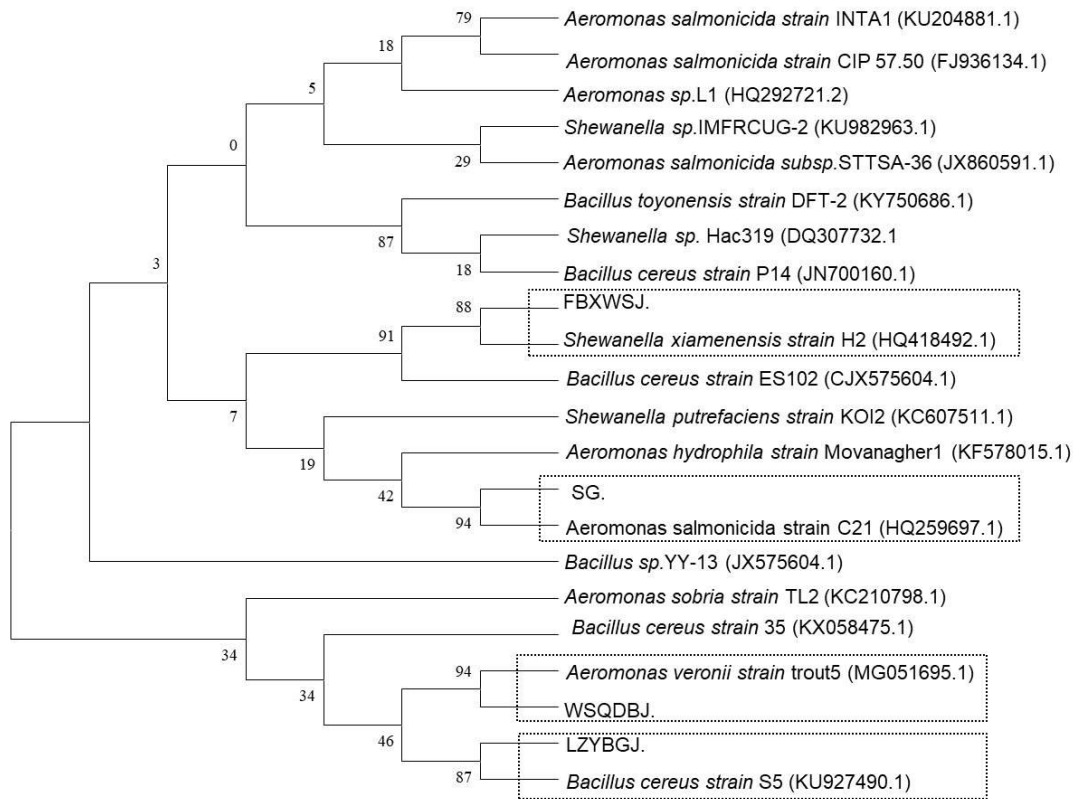
Nine fish (three fish  $\times$  three replicates = nine fish) were collected from each group for immunological assays. Blood samples were collected from the caudal veins of the fish using syringes and then centrifuged at 4,000 rpm for 8 min, after which the supernatant was collected. The liver tissue was subsequently separated. Determination of each immunological index was carried out with an assay Kit (Jiancheng, Nanjing, Jiangsu, China) according to the manufacturer's instructions.

#### *Statistical analysis.*

The test data were statistically analyzed using SPSS 20.0 software. The significance difference test was carried out by means of single factor variance analysis (one-way ANOVA), whereas the variances between the different times and concentrations were analyzed through Multivariate Analysis of Variance (MANOVA). Each group of experiments was conducted in triplicate, and the data were expressed as a mean  $\pm$  SEM.

## **Results**

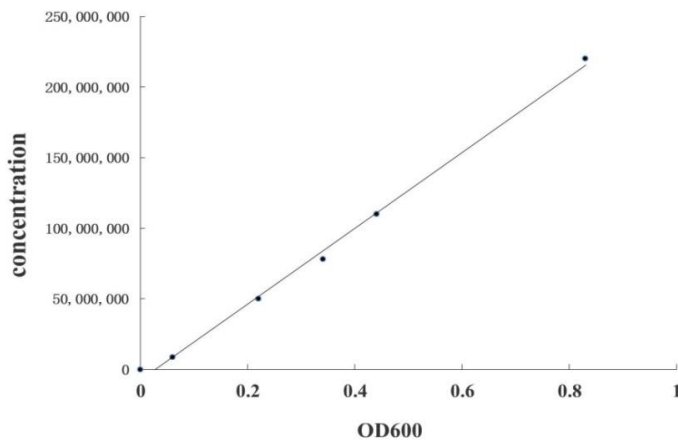
*Phylogenetic and homology analysis.* To study molecular evolution and compare sequence homologues, all of the known and predicted bacterial sequences in GenBank were selected to construct a phylogenetic tree (Figure 1). These results revealed that *WSQDBJ*. had the highest identity with *A. veronii*, *SW*. had the highest identity with *Shewanella sp.*, *LZYBGJ*. had the highest identity with *Bacillus cereus*, and *SGQDBJ*. had the highest identity with *Aeromonas salmonicida*.



**Figure 1:** Phylogenetic tree based on partial 16S rDNA sequences

#### Concentration curve of *A. veronii*.

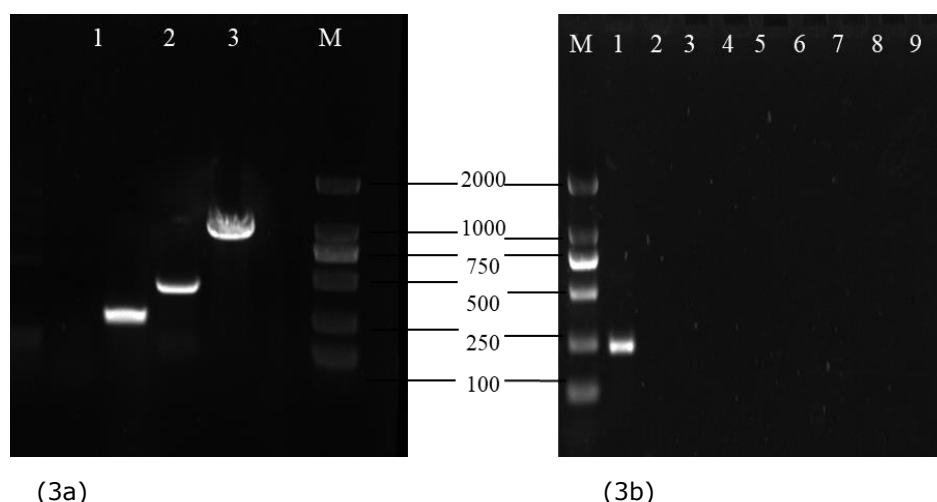
The concentration curve (OD600) of *A. veronii* was measured, with the results shown in Figure 2. The standard curve formula of the bacteria number and absorbance in OD600 was:  $y=249500000.00x \quad R^{2.00}=0.99$



**Figure 2:** Concentration curve of bacterial cell number (OD600) of *A. veronii*.

#### Detection of virulence genes.

The PCR amplification of the DNA of the four pathogenic bacteria was carried out using three specific types of primers. The results indicated that three virulence genes of *A. veronii* were expressed (Figure 3), and the size of the amplified fragment was consistent with the expected results.



**Figure 3:** Agarose gel electrophoresis of 1% agarose in the amplification products of virulence genes from the four pathogens. M, Trans2K DNA Maker; 3a, *A. veronii*; 1, Act; 2, Aer; 3, OMP II; 3b, 1-3, *Shewanella sp.* OMP II, Aer, Act; 4-6, *Salmonella aeruginosa* OMP II, Aer, Act; 7-9, *Aeromonas salmonicida* OMP II, Aer, Act.

#### *Acute toxicity test of A. veronii to healthy fish.*

The grass carp were intraperitoneally injected with different concentrations of *A. veronii*, and death was statistically analyzed to determine the toxicity of the bacterium, with the results reported in Table 3. The safe concentrations of the tested bacteria on the grass carp are shown in Table 4.

**Table 3:** Determination of LC50 in grass carp

concentration (CFU/mL)	Number(tail)	12h	24h	48h	72h	96h
$1.82 \times 10^5$	30	0	6	6	9	9
$7.28 \times 10^5$	30	0	6	9	12	12
$2.81 \times 10^6$	30	3	6	12	18	18
$1.15 \times 10^7$	30	6	12	12	18	27
$4.50 \times 10^7$	30	6	13	21	27	30
$1.82 \times 10^8$	30	9	15	24	30	30
death rate		30%	50%	80%	100%	100%

**Table 4:** LC50 and safe concentrations

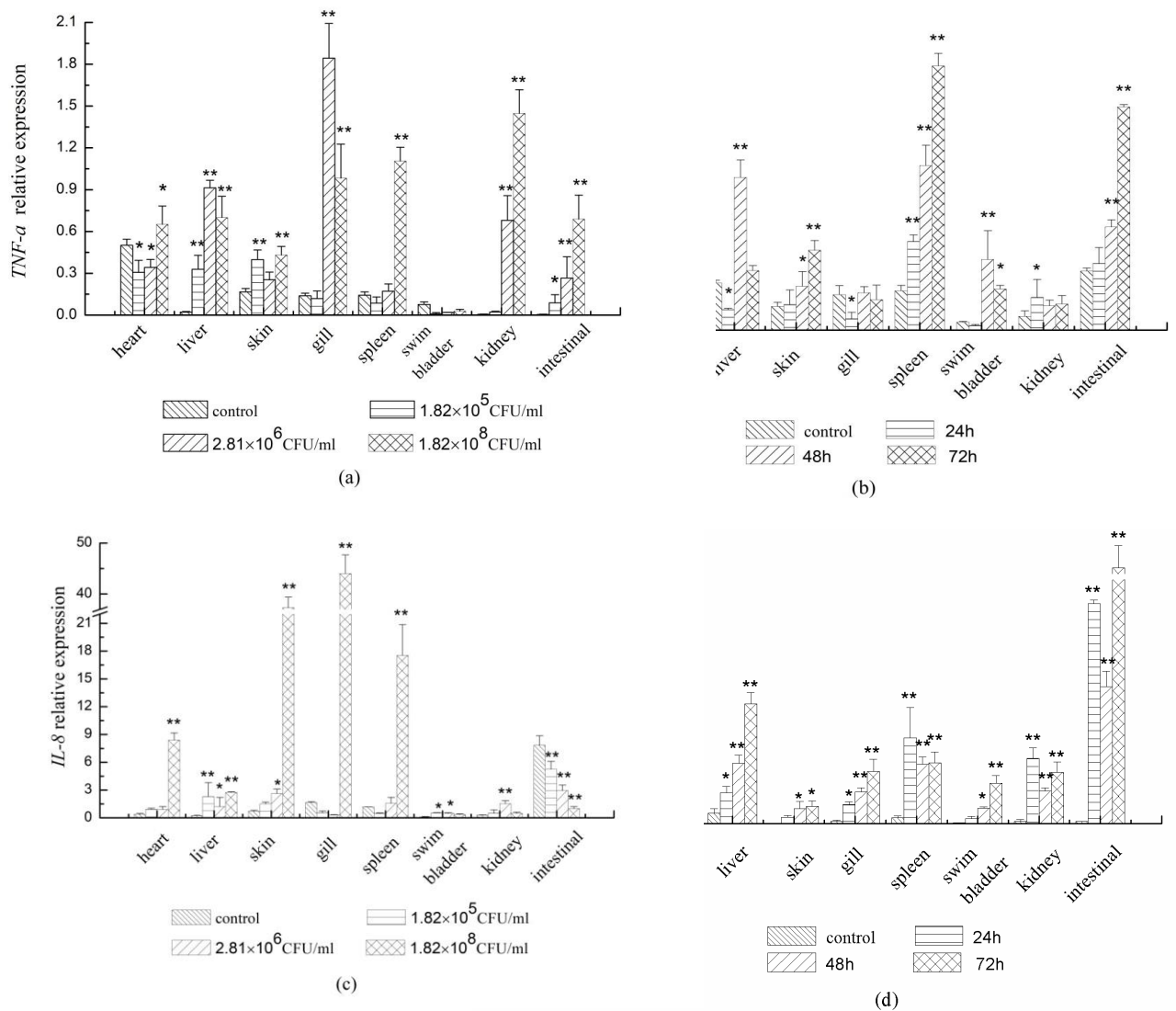
LC50(CFU/mL)					SC(CFU/mL)
12h	24h	48h	72h	96h	
$1.20 \times 10^8$	$2.63 \times 10^7$	$7.59 \times 10^6$	$1.90 \times 10^6$	$3.60 \times 10^4$	$9.11 \times 10^3$

SC=safe concentration; LC50= semi-lethal concentrations.

#### *Effects of A. veronii on the expression of IL - 8 and TNF - $\alpha$ in grass carp.*

As depicted in Figure 4, shows that the mRNA expression of IL-8 and TNF- $\alpha$  genes was detected. Over time, the mRNA expression of IL-8 and TNF- $\alpha$  increased in most tissues following the pathogenic infection. The mRNA level of the genes was significantly higher than in the control group at 24 h, 48 h, and 72 h ( $P < 0.05$ ). The effect of concentration on IL-8 and TNF- $\alpha$  mRNA expression in each tissue was significant ( $P < 0.05$ ). For the TNF- $\alpha$  gene, the expression of the infection concentration increased significantly ( $P < 0.05$ ) except for the swim bladder. The expression of the IL-8 gene in the intestinal tissue was markedly decreased with the infection concentration ( $P < 0.05$ ), while for the other groups, it was significantly higher ( $P < 0.05$ ).





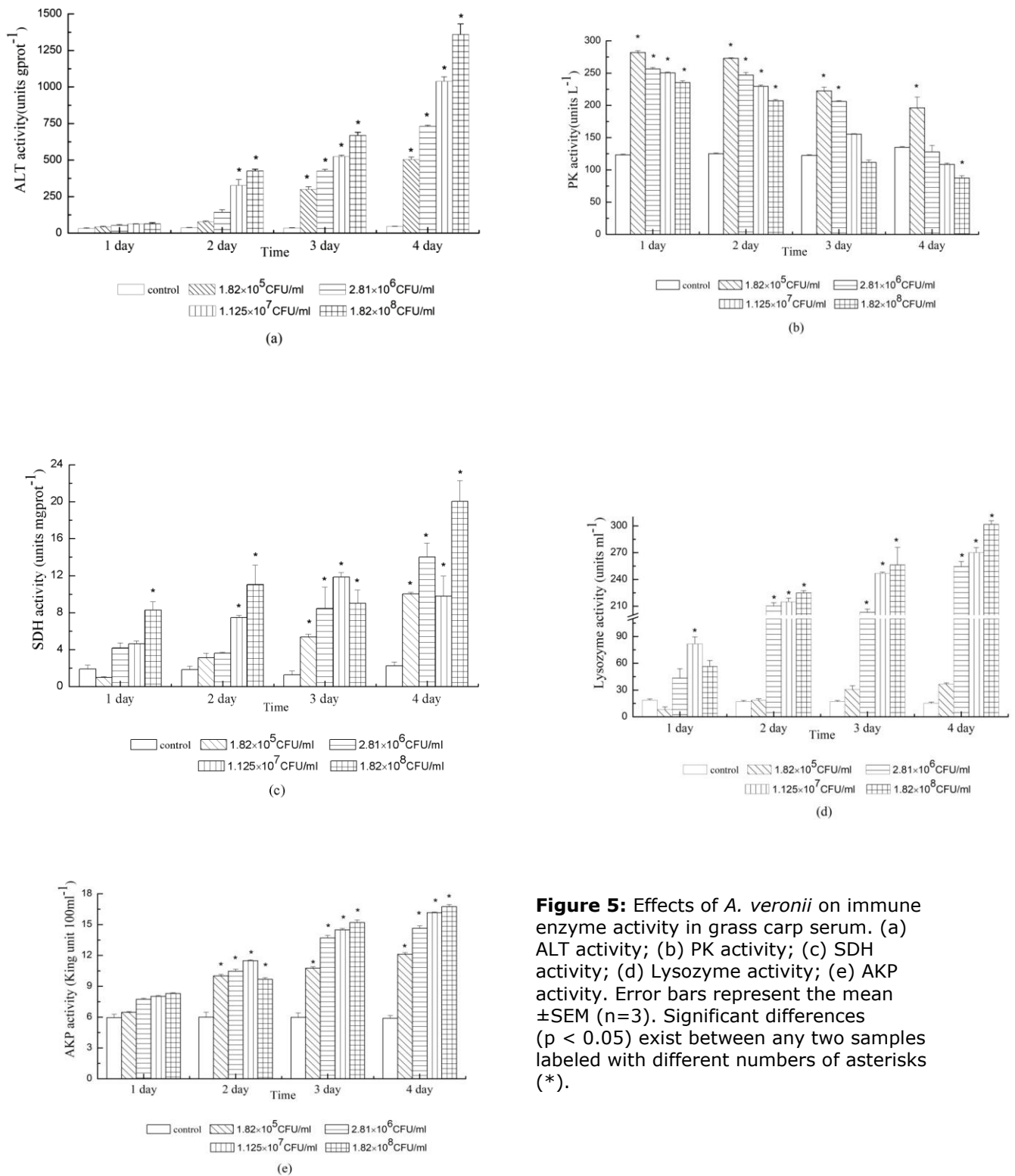
**Figure 4:** Relative expression of immune-related genes in infected grass carp tissues. (a) Expression of the TNF- $\alpha$  gene in different concentrations; (b) Expression of the TNF- $\alpha$  gene at different times under the same concentrations; (c) Expression of IL-8 gene in different concentrations; (d) Expression of IL-8 gene at different times at the same treatment concentration; Error bars represent the mean  $\pm$  SEM ( $n=3$ ). Significant differences ( $p < 0.05$ ) exist between any two samples labeled with different numbers of asterisks (\*)

#### *Determination of five immune enzymes in serum.*

ALT and AKP enzyme activity in serum, as shown in Figures 5 (a) and (e), showed no significant difference ( $p > 0.05$ ) between the concentrations following 24 h of infection. Following 48 h at high concentrations, the infection was observed to be significantly higher than that of the control group ( $P < 0.05$ ). At 72 h and 96 h the infection worsened, and infection concentration increased significantly ( $P < 0.05$ ).

SDH and Lyz activity in the serum showed no significant variation at 24 h ( $p > 0.05$ ), where the activity of Lyz was significantly higher than that of the control group ( $p < 0.05$ ). SDH and Lyz activity, as shown in Figures 5 (c) and (d), increased significantly with the prolongation of the infection time, and the increase of the infection concentration ( $p < 0.05$ ).

PK enzyme activity in serum, as depicted in Figure 5 (d), was significantly higher in the control group at 24 h and 48 h following infection ( $p < 0.05$ ), and there was no significant difference ( $p > 0.05$ ) in the high concentration infection group at 72 h ( $p < 0.05$ ).



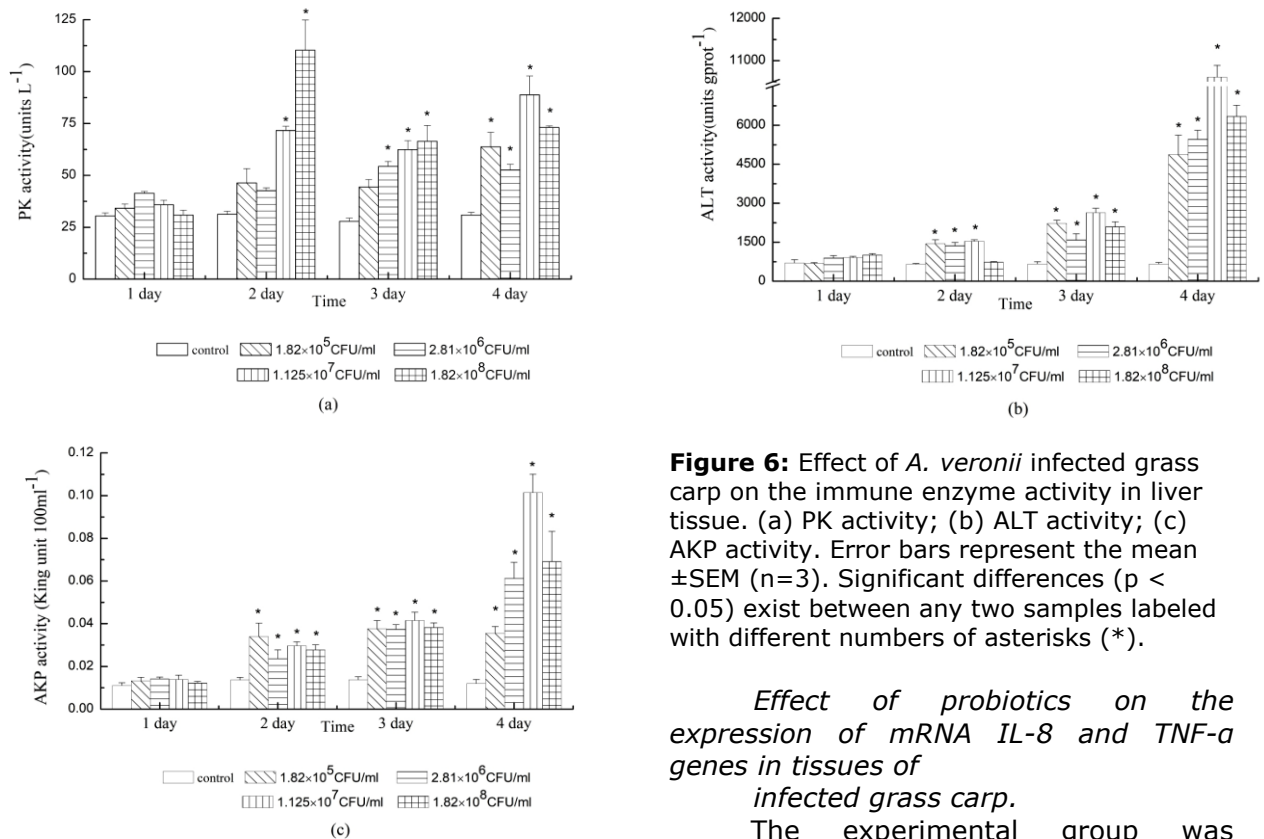
**Figure 5:** Effects of *A. veronii* on immune enzyme activity in grass carp serum. (a) ALT activity; (b) PK activity; (c) SDH activity; (d) Lysozyme activity; (e) AKP activity. Error bars represent the mean  $\pm$ SEM (n=3). Significant differences ( $p < 0.05$ ) exist between any two samples labeled with different numbers of asterisks (\*).

*Determination of immune enzyme activity in liver tissue.*

The enzymatic activities of PK, ALT, and AKP reflected the level of immunity in the organisms. PK activity in liver tissue was not significantly higher than in the control group at 24 h ( $p > 0.05$ ), as shown in Figure 6 (a); however it was significantly increased in the

infection group at 48 h under a high concentration ( $p < 0.05$ ), whereas a significant increase in PK enzyme activity under high infection concentration ( $p < 0.05$ ) was observed at 72 h and 96 h.

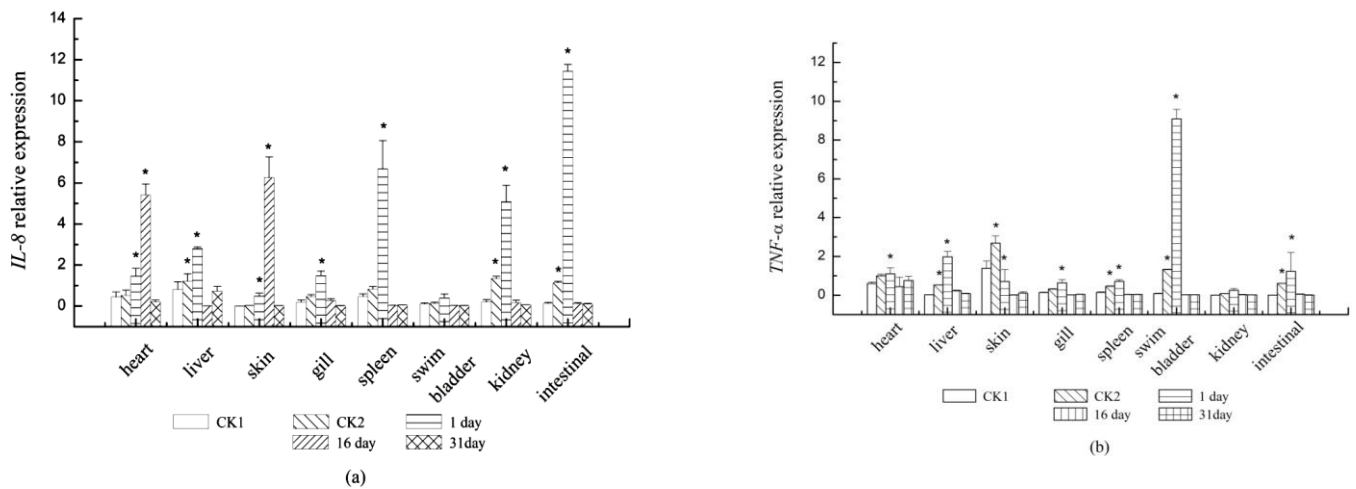
ALT and AKP activity in liver tissue showed no significant difference at 24 h and 48 h following infection, as shown in Figures 6 (b) and (c) ( $p > 0.05$ ). Further, ALT activity was significantly increased at 72 h and 96 h ( $p < 0.05$ ). AKP enzyme activity showed no significant difference in contrast to the control group at 24 h ( $p > 0.05$ ). There was a significant difference in the PK enzyme activity at 48 h, 72 h, and 96 h when compared with the control group ( $P < 0.05$ ).



**Figure 6:** Effect of *A. veronii* infected grass carp on the immune enzyme activity in liver tissue. (a) PK activity; (b) ALT activity; (c) AKP activity. Error bars represent the mean  $\pm$  SEM ( $n=3$ ). Significant differences ( $p < 0.05$ ) exist between any two samples labeled with different numbers of asterisks (\*).

*Effect of probiotics on the expression of mRNA IL-8 and TNF- $\alpha$  genes in tissues of infected grass carp.*

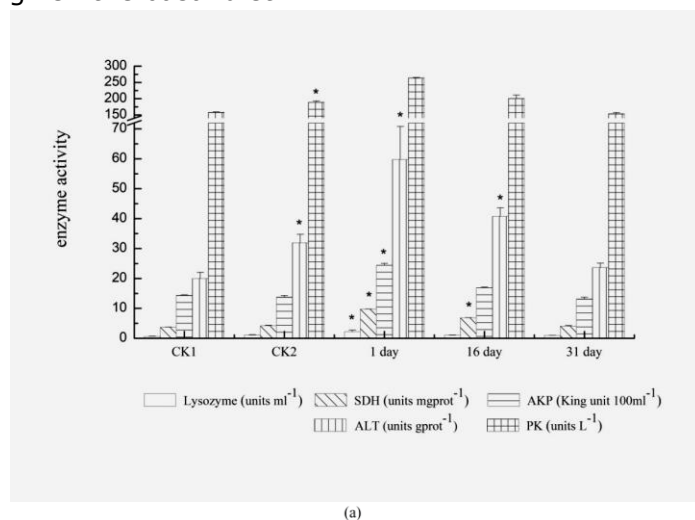
The experimental group was infected with  $1.82 \times 10^5$  CFU/mL of bacterium and fed with probiotic fermentation feed. Figures 7 (a) and (b) reveal that the expression of IL-8 and TNF- $\alpha$  genes were altered after feeding with probiotics at 1, 16, and 31 days. At 24 h the mRNA gene level was increased significantly ( $p < 0.05$ ) in contrast to the control group. Expression level of the genes was decreased when compared with the control after the fish were fed with probiotics mixed fermentations at 16 days and 31 days ( $p > 0.05$ ). Further, the expression of IL-8 in the liver, kidneys, and intestines of healthy grass carp were fed probiotics was higher than those fed the basal diet ( $p < 0.05$ ). TNF- $\alpha$  expression in the liver, skin, spleen, and intestines of the healthy grass carp that were fed probiotics was higher than in those fed the basal diet ( $p < 0.05$ ).



**Figure 7:** Effect of probiotics on the expression of mRNA IL-8 and TNF- $\alpha$  genes in the tissues of infected grass carp. (a) Expression of the TNF- $\alpha$  gene (b) Expression of the TNF- $\alpha$  gene; CK1 represents the basal diet for uninfected healthy grass carp; CK2 represents the probiotic feeds for infected grass carp. Error bars represent the mean  $\pm$ SEM (n=3). Significant differences ( $p < 0.05$ ) exist between any two samples labeled with different numbers of asterisks (\*).

*Effects of probiotics on immune indices in the serum of grass carp.*

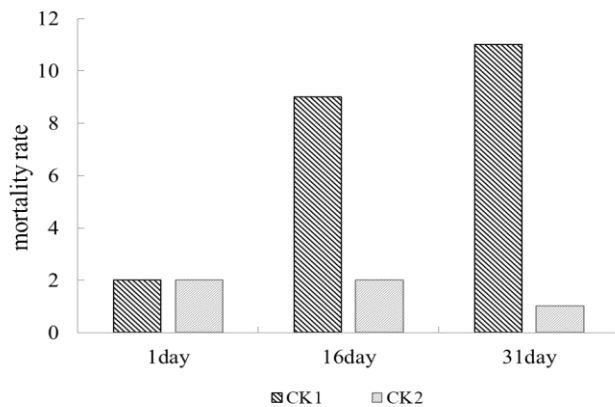
The effect of *A. veronii* on PK, AKP, ALT, SDH, and Lyz was measured on the 1st, 16<sup>th</sup>, and 31st days in serum (Figure 8). The activity of AKP in the serum was significantly higher ( $P < 0.05$ ) than that of the control group at 1, 16, and 31 days, and longer feeding times had not significant ( $p > 0.05$ ) compared to the control group. At the same time, the ALT and PK activities under the probiotics diet increased significantly ( $p < 0.05$ ) in contrast to those given the basal diet.



**Figure 8:** Effect of probiotics on immune enzyme activity in infected grass carp. CK1 represents the basal diet for uninfected healthy grass carp; CK2 represents the probiotic feeds for infected grass carp. Error bars represent the mean  $\pm$ SEM (n=3). Significant differences ( $p < 0.05$ ) exist between any two samples labeled with different numbers of asterisks (\*).

*Effect of probiotics on infected grass carp mortality.*

The mortality of fish was recorded following the injection induced infection, (Figure 9). Survival rate of the fish given the probiotic fermentation feed was higher in all groups, other than the control.



**Figure 9:** Effect of probiotics on infected grass carp mortality. CK1 represents the basal diet for uninfected healthy grass carp; CK2 represents the probiotic feeds for infected grass carp.

### Discussion

Grass carp comprises one of the most important commercial fish, and fish septicemia is the most common widespread disease in Chinese aquaculture. With increasing intensification and diversification of aquaculture, septicemia diseases occur often and inflict serious economic losses; hence, it is of great significance to reduce the virulence of pathogenic bacteria in grass carp (Mo *et al.* 2016). *A. veronii* is commonly found in aquatic environments, wherein virulence genes serve as excellent biomarkers that can identify the pathogenicity of specific bacteria. *OMP11*, *Act*, and *Aer* genes were present in *A. veronii*, and have been shown to be closely associated with the pathogenicity of *Aeromonas*. Namba *et al.* (2008) reported that *OMP11* was an adhesion factor of *A. veronii*, which was isolated from the intestinal tract of carp (Reverter *et al.* 2014). According to the pathogen virulence genes, it appears possible to establish an *A. veronii* virulence gene detection technique that is based on a common and effective strategy.

When the body of a fish is compromised by external factors, physiological or pathological changes are reflected in the blood and liver indices. Infection by pathogenic bacteria can stimulate the expression of several non-specific immune-related genes (Harikrishnan *et al.* 2010). IL-8 is a member of the chemokine subfamily, which can protect against pathogen invasion by promoting the migration of neutrophils, whereas TNF- $\alpha$  serves as a general stimulus that is involved in inflammation, apoptosis, cell proliferation, and immune system pro-inflammatory cytokines (Zhang *et al.* 2008). Chen *et al.* (2009) revealed that LBP can activate NF- $\kappa$ B and AP-1 transcription factors (using RAW264.7 macrophage cells), while inducing TNF- $\alpha$  and IL-1 $\beta$  mRNA expression. The expression of a variety of extracellular toxins may effectively stimulate neutrophils in the body, as well as induce the expression of innate immune-related cytokines, which coincided with a previous study that explored the suppressive effects of *B. subtilis* PB6 on inflammatory disease (IBD) in mice (Plaza-Diaz *et al.* 2014).

Transaminase is an enzyme that can be found in serum, and traverses the membranes of liver cells, for instance. Only a small number of enzymes are released into the blood when the body is suffering from external environmental stress. With stress-induced cell membrane permeability changes, abundant ALT can be released into the serum, leading to rapid increase in serum aminotransferase activity, which affects the body's normal metabolic function. AKP is an important metabolic regulatory enzyme that is present in almost all tissues and fluids in the human body. Lyz is an important reference indicator of nonspecific immunity (Nandi *et al.* 2017). Concomitantly, we found that the activity of PK in serum and liver tissue was significantly less than in the control group, with a high concentration in the infected group at 72 h ( $p > 0.05$ ). The activity of highly concentrated infection was significantly lower than that of the control group ( $p < 0.05$ ). Excessive or prolonged stress response may cause harm to an organism and be manifest as decreased immune function and increased morbidity and mortality.

In the quest for aquaculture vaccines, a successful commercialized product has not yet been realized. In this study, probiotics, as a natural immune stimulant in feed for infected grass carp, may significantly improve their growth and survival rates. The rationale may be as follows: First, the microbial fermentation of the feed altered macromolecular substances that are more susceptible to the cultivation and biological absorption of small molecules

(Foligne et al. 2012). Second, the fermentation of feed contains a vast number of viable beneficial bacteria, which enter the intestinal tract to improve the digestion and absorption of nutrients (Geissler et al. 2017). Third, the fermentation feed is rich in small peptides, amino acids that can enhance immunity and resistance pathogens (Faramarzi et al. 2011). In this study we found that probiotics could be a promising contribution in the field of aquaculture, as they are biodegradable, biocompatible, and safe for human health and the ambient environment.

Our results indicated that immunogenic genes, as well as the general health status and survival rates of grass carp improved significantly by using probiotic feed. Although the exact kinetics of probiotics on the innate immune system of the fish was not clear, it may be summed up by stating that probiotics had a significantly positive role for enhancing immunity in grass carp. The proposed use of probiotics as an immune stimulator may improve the control of aquaculture diseases (Zamfir et al. 2012). It might even be possible to formulate a suitable polyvalent vaccine against *A. veronii* infection and test it by actively challenging fish with pathogens.

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