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Histopathological Observation of *Aeromonas hydrophila* Infection and Influences on Immune-related Enzyme Activity Indexes in *Carassius auratus indigentiaus* subsp. Nov.

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Abstract

Bacterial sepsis caused by *Aeromonas hydrophila* infection is one of the most common infectious diseases of *Carassius auratus indigentiaus* subsp. Nov. It has characteristics of quick onset, high morbidity, and high mortality. Bacterial sepsis has become an important constraint against the industrialization of aquaculture of *Carassius auratus indigentiaus* subsp. Nov. In this study, *Carassius auratus indigentiaus* subsp. Nov. were immersed in 1.0×10^9 CFU/mL *Aeromonas hydrophila* for infection, the cumulative mortality at 1d, 3d, 5d, 7d and 14d were 10%, 26.67%, 40%, 40% and 40%, respectively. After being challenged with *Aeromonas hydrophila*, hemorrhage focus and suppuration were observed on the body surface, pelvic fin, anal fin, and tail fin base of *Carassius auratus indigentiaus* subsp. Nov. Dissecting *Carassius auratus indigentiaus* subsp. Nov., blackening of the spleen and hemorrhage at the liver were discovered at 3d after being infected with *Aeromonas hydrophila*. Through tissue section observation, liver tissue was found to have to hemorrhage focus, and vacuolated liver cells, accompanied by inflammatory cell penetration. Inflammatory cell infiltration was also observed in spleen tissues and was most serious at 3d day after infection. After being infected with *Aeromonas hydrophila*, the gill filaments of *Carassius auratus indigentiaus*

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subsp. Nov. were deformed and shortened, gill filament cells fell off, intestinal villi are shortened and mucus cells are increased. After *Aeromonas hydrophila* infection to *Carassius auratus indigentiaus* subsp. Nov., the lysozyme content and activities of catalase, alkaline phosphatase, and total superoxide dismutase (T-SOD) in the liver began to increase significantly after the first day and reached a peak on the third day. However, they began to decrease gradually on the fifth day. The acid phosphatase (ACP) activity increased dramatically after the first day, peaked on the fifth day, and then decreased on the seventh day. It is speculated that such changes were attributed to the following two aspects: 1) On the one hand, *Aeromonas hydrophila* infection stimulates the nonspecific immune system of *Carassius auratus indigentiaus* subsp. Nov. 2) On the other hand, lesion of tissues occurs after *Aeromonas hydrophila* infection of *Carassius auratus indigentiaus* subsp. Nov. reaches a certain extent, thus influencing lysozyme content and the catalase activities, ACP, alkaline phosphatase and T-SOD in the liver.

Introduction

Aeromonas hydropila belongs to the genus *Aeromonas*, *Aermonadaceae*, it is widely distributed in various water bodies, aquatic animals, plants, and aquatic products (Daskalov, 2006; Yang et al., 1995). It is divided into pathogenic and non-pathogenic strains. The former can cause disease outbreaks in several freshwater aquaculture species and damage Mammalia, livestock, and poultries.

Aeromonas hydropila attracted people's attention first as a pathogenic bacterium that causes human infection and diarrhea, and it is a common human-animal-fish pathogenic bacterium. Since the 1980s and 1990s, *Aeromonas hydrophila* has caused disease outbreaks and high mortality in several freshwater aquatic organisms. It can infect various aquaculture species, such as *Ctenopharyngodon idellus*, *Hypophthalmichthys molitrix*, *Crucian carp*, *Megalobrama amblycephala*, *Carp cyprinoid*, *Oreochromis niloticus*, *Pelteobogrus fulvidraco*. *Aeromonas hydropila* has emerged as one of the most serious pathogens, causing disease threats of the freshwater aquaculture industry and attracting great attention to researchers (Tian et al., 2010; Zhang et al., 2008; Gao Han et al., 1995).

Carassius auratus indigentiaus subsp. Nov. was discovered by Yang et al. during a fish resource survey in Dongting Lake in 2002, and it was named for its indigo body surface (Yang, 2002). Due to its tender meat, fresh taste and rich nutrients, *Carassius auratus indigentiaus* subsp. Nov. is popular among consumers. With the rapid development of *Carassius auratus indigentiaus* subsp. Nov. aquaculture industry in recent years, various fish diseases have occurred frequently while promoting large-scaled high-density intensive aquaculture mode. The bacterial sepsis caused by *Aeromonas hydrophila* infection is one of the most common infectious diseases of *Carassius auratus indigentiaus* subsp. Nov. It has characteristics of a short time of onset, high morbidity, and mortality.

Therefore, bacterial sepsis has become an important constraint on the industrialization of aquaculture of *Carassius auratus indigentiaus* subsp. Nov. Recently, many scholars have isolated *Aeromonas hydrophila* pathogens from affected tissues of fishes with sepsis. It has been proved that *Aeromonas hydrophila* is one of the bacteria that cause diseases in fishes and the pathogen of sepsis in fishes. However, there have been a few studies concerning the influences of *Aeromonas hydrophila* on the tissue pathology of *Crucian carp* (Zeng, 2015). Lysozyme (LSZ), catalase (CAT), alkaline phosphatase (AKP), ACP, and total superoxide dismutase (T-SOD) are significant humoral immune factors found in fish tissues that can reflect in vivo tissue damages indirectly. They are major indexes to evaluate nonspecific immunity in animals (Fang et al., 2015; Zhang et al., 2018). Infection caused by pathogens can influence the activity of immune factors in fish bodies. Therefore, the health immunity of

fish bodies can be assessed by activity changes of immune-related enzymes in tissues. Nevertheless, influences of *Aeromonas hydrophila* on immune-related enzymes in the liver of *Carassius auratus indigentiaus* subsp. Nov. have not been reported yet.

In this study, experimental groups of fish were infected with *Aeromonas hydrophila* by immersion, and death rates were calculated. Anatomy and immune-related tissue section observations were examined in the samples—meanwhile, the activity changes of immune-related enzymes in the liver of *Carassius auratus indigentiaus* subsp. Nov. were tested. The histopathological changes and variation laws of immune-related enzymes before and after the *Aeromonas hydrophila* infection were investigated. Research conclusions provide some references to disease control and healthy aquaculture of *Carassius auratus indigentiaus* subsp. Nov., as well as further, disclose the disease-resistance mechanism.

Materials and Methods

Experimental fish

200 *Carassius auratus indigentiaus* subsp. Nov. (50±2g) was collected from the Shangjun Aquaculture Elite Breeding Farm in An'xiang County, Hunan University of Arts and Sciences. Before sampling, samples were cultured in the recirculating aquaculture system in the laboratory, during which oxygen was supplied 24 h/day to ensure enough dissolved oxygen in the water. The dissolved oxygen (DO) content was 6.0±0.2 μg/mL, and the water temperature was set 26±2 °C. Meanwhile, an appropriate amount of floating baits for adult fish was supplied. *Aeromonas hydrophila* was provided by the Microbiology Laboratory of Hunan University of Arts and Sciences.

Challenge with Aeromonas hydrophila

The *Aeromonas hydrophila* was inoculated into the LB fluid media and cultured for 24 h on a shaker (28 ℃), followed by centrifuging at the rate of 4000 rpm for 10 min. After supernatant was eliminated, the *Aeromonas hydrophila* was diluted with sterile PBS solution, and the concentration of bacteria after dilution was tested by the plate viable count method. Then, the bacterial concentration was diluted to 1.0×10^9 CFU/mL for later use. The experimental group set three parallel groups and each group had 30 samples of *Carassius auratus indigentiaus* subsp. Nov. Experemental groups of fish were immersed in aquaculture boxes with 1.0×10^9 CFU/mL *Aeromonas hydrophila* for 30 min and then transferred into aquaculture buckets with oxygen supply. Healthy *Carassius auratus indigentiaus* subsp. Nov. without *Aeromonas hydrophila* infection was chosen as the control group. The death rates on the first, third, fifth, seventh and fourteenth day of infection were calculated.

Anatomy and HE dyeing

The morbidity of the experimental group on the first, third and fifth day were observed. Fish with typical disease symptoms were selected. After MS-222 anesthesia, blood samples were collected from tail veins by an injection syringe. Following that, anatomical observation was performed. Meanwhile, the liver, spleen, gills, and intestine in size of 3-5 mm were fixed in Bouin's solution and processed for paraffin embedding according to routine procedures. The sections were stained with hematoxylin and eosin. Micro-pictures were taken.

Immune-related enzyme activity in liver

On the first, third, fifth, seventh and fourteenth day of infection, three samples were collected from the experimental and control groups, respectively. All samples had MS-222 anesthesia, and blood samples were collected from tail veins by injection syringe. Later, some livers were collected quickly, and water on liver samples was eliminated using filter paper. Liver samples were weighted 0.2 g by an analytical balance. Later, liver samples were homogenized in an ice bath, and normal saline, which is nine times of sample weight, was added to prepare tissue homogenate. After centrifuging at the rate of 2500 rpm for 10 min at 4 ℃, the organ extracting solution was gained after eliminating sediments. Supernate was stored in a refrigerator at 4 ℃. The kit purchased from the Nanjing Jiancheng Institute of

Biological Engineering was used to test lysozyme content (Lys), activities of T-SOD, CAT, ACP and AKP, as well as protein content in livers.

Data processing

Data analysis was implemented using SPSS15.0 and EXCEL. $P<0.05$ indicates significant differences, $P<0.01$ denotes extremely significant differences and $P>0.05$ indicates insignificant differences.

Results

Pathological changes in the body surface, liver, and spleen after infection

After *Carassius auratus indigentiaus* subsp. Nov. was infected with 1.0×10^9 CFU/mL *Aeromonas hydrophila*, the cumulative death rates on the first, third, fifth, seventh, and fourteenth days were 10%, 26.67%, 40%, 40%, and 40%, respectively (**Figure 1**). On the first day, hemorrhageappeared on the body surface, pelvic fin, anal fin, and tail fin base (**Figure 2**). The tail fin and body surface had festered on the third day, while the tail fin base had hemorrhaged. On the fifth day, suppuration was observed on the tail fin (**Figure 2**). The body surface was in perfect condition on the seventh day, but there was red swelling at the anus (**Figure 2**). After the third day, the fish underwent a complete necropsy, blackening of the spleen, and hemorrhagein the liver were discovered (**Figure 3**).

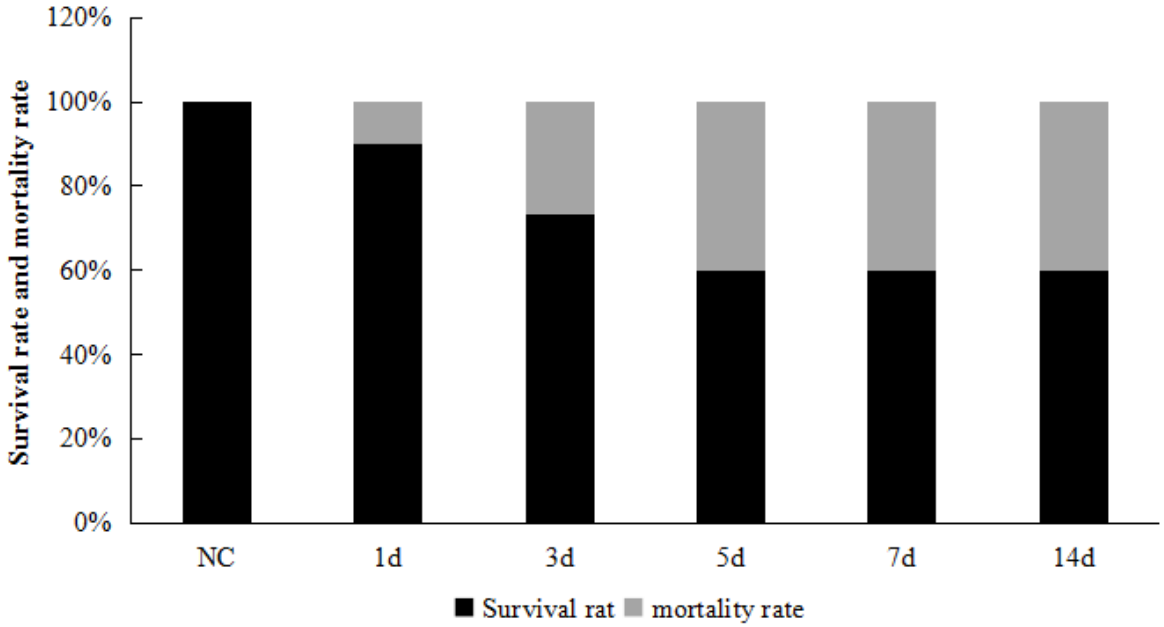

Figure 1 Survival rate and death rate of *Carassius auratus indigentiaus* subsp. Nov. after *Aeromonas hydrophila* infection

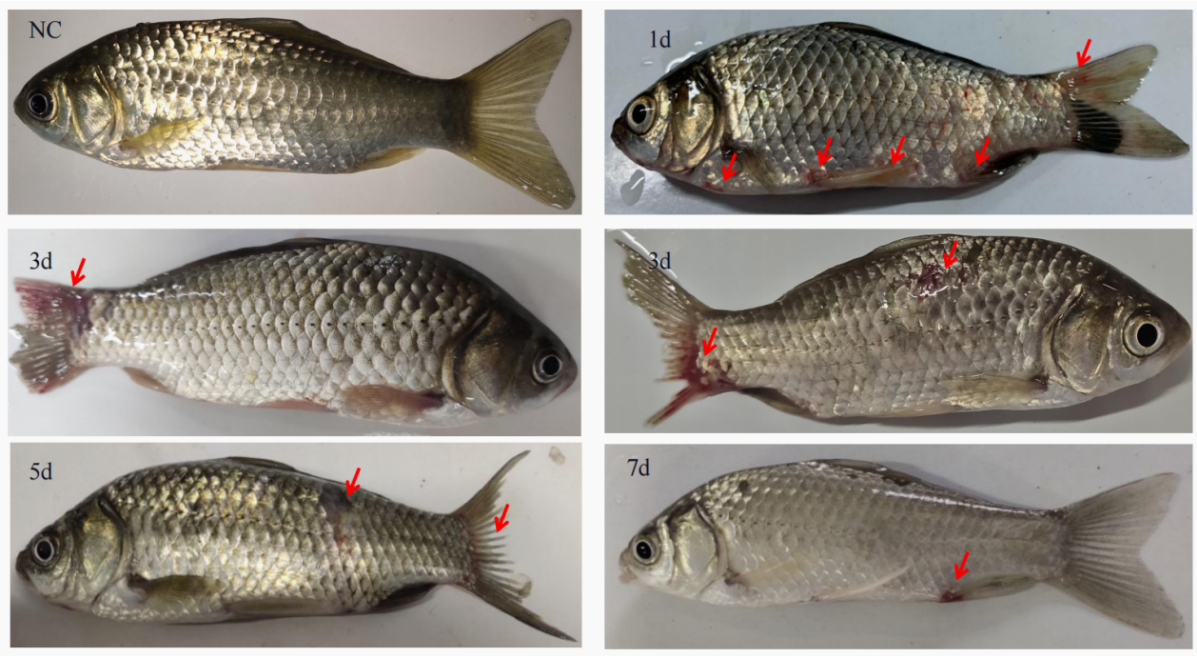

Figure 2 Signs of *Aeromonas hydrophila* infection were found on fish body. Red arrows indicate hemorrhage points

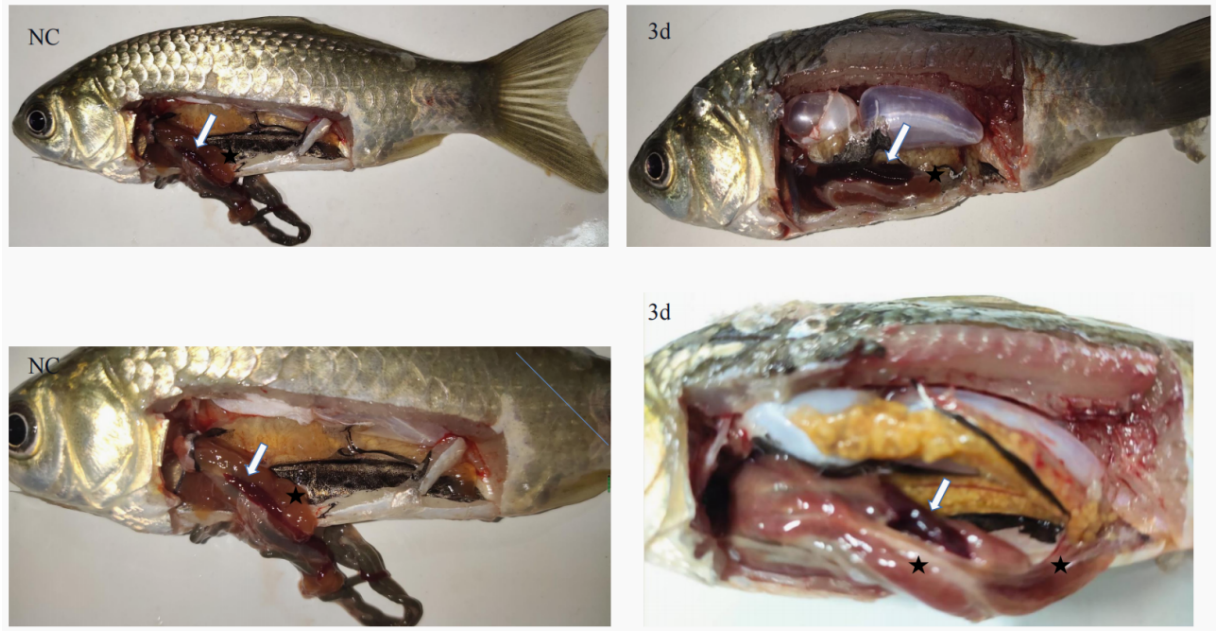

Figure 3 Liver and spleen of *Carassius auratus indigentiaus* subsp. Nov. after *Aeromonas hydrophila* infection. Spleen: white arrow; Liver: black stars

Histopathological changes in liver, spleen, gill, and intestinal tissues

The liver tissues of *Carassius auratus indigentiaus* subsp. Nov. had hemorrhage after *Aeromonas hydrophila* infection (**Figure 4**). On the third day, liver cells developed vacuoles, accompanied by inflammatory cell infiltration (**Figure 4**). There were bleeding points on the liver tissues on the fifth day, but the number of hemorrhage focus was lower than on the first day (**Figure 4**). On the first, third, and fifth days, inflammatory cell infiltration was observed

in spleen tissues, with the third day exhibiting the most serious inflammatory cell penetration (**Figure 5**). On the first day, the gill filaments were deformed and shortened. On the third day, the gill filament cells fell off, and the gill filaments were shortened to their maximum extent. On the fifth day, gill filaments were shortened (**Figure 6**). The intestinal villi were shortened, and the mucous cell increased on the third day (**Figure 7**)

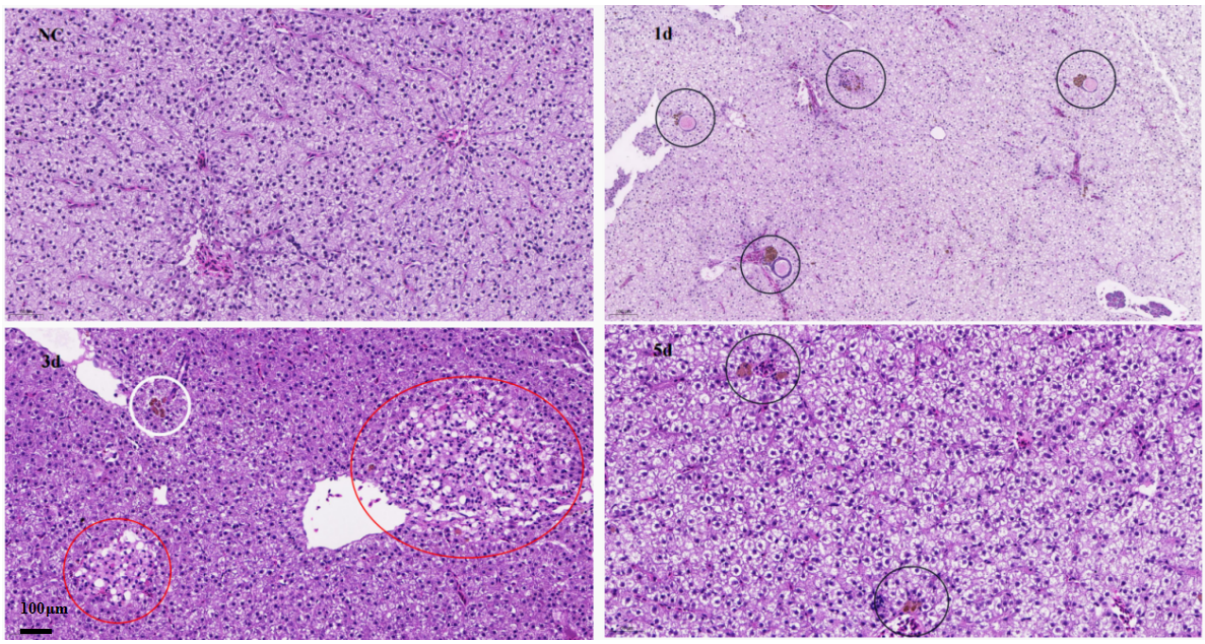

Figure 4 The liver of *Carassius auratus indigentiaus* subsp. Nov. after *Aeromonas hydrophila* infection. Black circles represent haemorrage focus; red circles represent vacuoles of liver cells; white circles represent inflammatory cell infiltration.

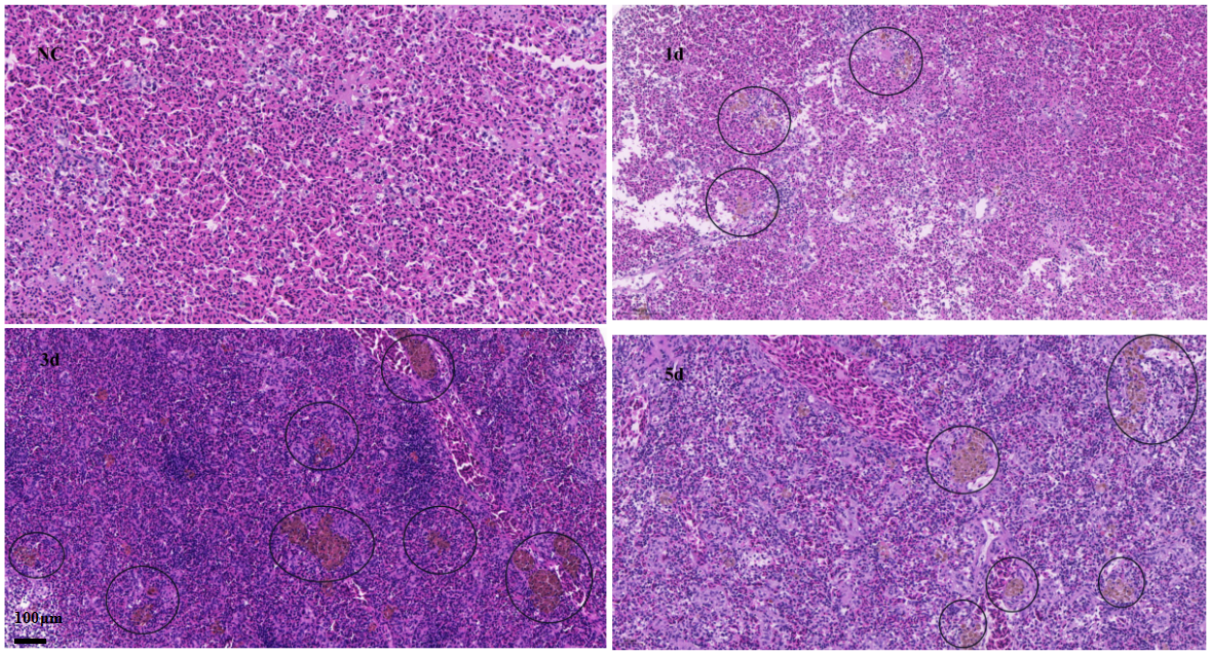

Figure 5 The spleen of *Carassius auratus indigentiaus* subsp. Nov. after *Aeromonas hydrophila* infection. Black circles indicate inflammatory cell penetration.

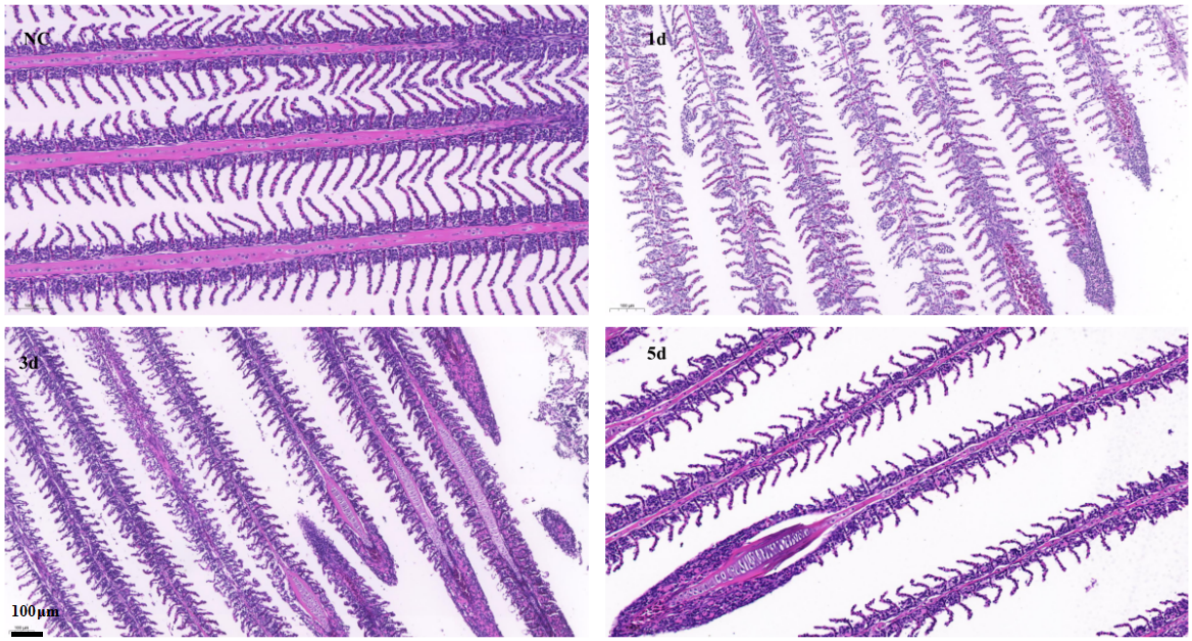

Figure 6 The gills of *Carassius auratus indigentiaus* subsp. Nov. after *Aeromonas hydrophila* infection

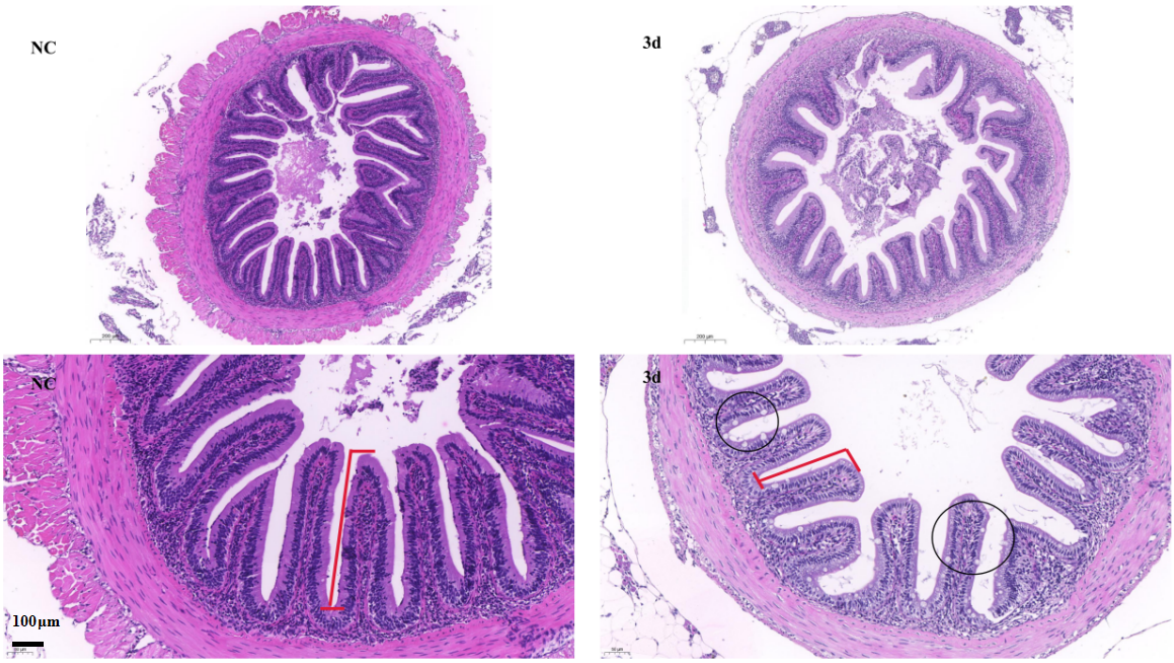

Figure 7 The intestines of *Carassius auratus indigentiaus* subsp. Nov. after *Aeromonas hydrophila* infection. Black circles indicate goblet cells.

Changes in immune-related enzyme activity indexes in the liver after Aeromonas hydrophila infection

After *Aeromonas hydrophila* infection to *Carassius auratus indigentiaus* subsp. Nov., the lysozyme content and activity of CAT, AKP, and T-SOD in the liver increased significantly after the first day and peaked on the third day. However, they decreased gradually on the fifth day.

The activity of ACP significantly increased after the first day, peaked on the fifth day, and then declined on the seventh day (**Figure 8**).

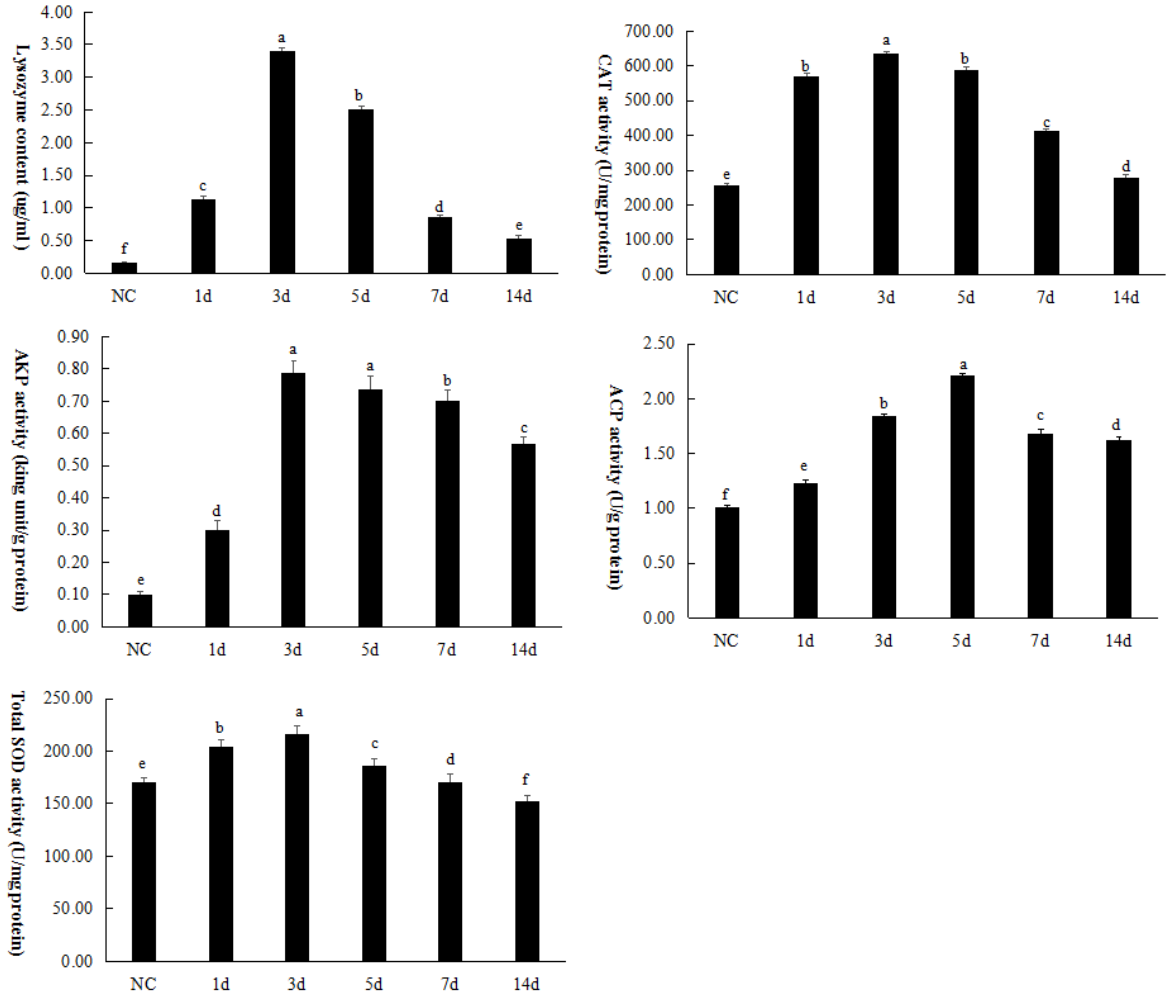

Figure 8 Changes of lysozyme content and activities of CAT, ACP, AKP and T-SOD in the liver after infection

Discussion

Aeromonas hydrophila can induce sepsis and visceral hemorrhage in fishes. It can cause serious tissue degeneration and necrosis through extracellular proteases, quickly decrease bodies' immunity, and eventually cause the death of fish. After *Aeromonas hydrophila* infection, different degrees of hemorrhage were observed in the head-kidney, kidney, intestine, and skin of *Crucian carp*, accompanied by pathological changes of degradation and necrosis of tissue cells (Zeng, 2015). Wang et al. found that *Aeromonas hydrophila* can cause sepsis and local infection of aquatic animals, such as tortoise, turtle, bullfrog, and calm (Wang et al., 2002). Jiang et al. (2012) used *Aeromonas hydrophila* which was isolated and identified from *Pelteobagrus fulvidraco* to infect *Ctenopharyngodon idellus* and discovered that pathological changes of liver and spleen tissues in juveniles were similar to symptoms of *Crucian carp* after artificial infection of *Aeromonas hydrophila* (Jiang et al., 2012; Zeng, 2015). After *Aeromonas hydrophila* infection, fishes mainly manifested as cell swelling and degeneration, demonstrating that *Aeromonas hydrophila* and its toxic components might damage membrane structures directly. Such damage can destroy the lysosome membrane, causing hydrolase to

flow over and cause considerable cell autolysis, eventually leading to cell necrosis or vacuolation (Lu et al., 1996; Yang et al., 1998; Wang et al., 2002). Lesions in the kidney, spleen, and liver after artificial injection of *Aeromonas hydrophila* to *Crucian carp* were exhibited as cytomembrane lysis at lesions and reduction of internal cells, thus influencing the normal physiological metabolism of *Crucian carp* (Zeng, 2015). In this study, *Carassius auratus indigentiaus* subsp. Nov. was infected by *Aeromonas hydrophila*, and lesions were found in the liver, spleen, gill, and intestines. Many pancreatic tissues were mixed up with the liver tissues of *Crucian carp*. The liver of *Crucian carp* is not only the most potent digestive gland but also one of the metabolism organs with the most diverse functions. It not only can secrete several digestive enzymes, but it also participates in metabolism, detoxification and phagocytosis defense. *Carassius auratus indigentiaus* subsp. Nov. produced liver cell cytoclasis and vacuoles after *Aeromonas hydrophila* infection, along with liver hemorrhage and tissue destruction. These can weaken the detoxification ability of the liver.

After *Aeromonas hydrophila* infection of *Plecoglossus altivelis*, there were obvious hemorrhage in the spleen, and a large number of lymphocytes and plasmocytes were developed in the spleen, showing inflammatory responses (Li et al., 2011). The pathological characteristics of *Carassius auratus indigentiaus* subsp. Nov. after *Aeromonas hydrophila* infection were similar with lesions of *Plecoglossus altivelis*, manifested by apparent dam. The spleen is one of the immune organs of fishes. Spleen tissues demostrates hemorrhage after *Aeromonas hydrophila* infection, thus lowering the hematopoiesis and immunity. This might be one of the major reasons for the rapid deaths of *Carassius auratus indigentiaus* subsp. Nov. after *Aeromonas hydrophila* infection.

After *Aeromonas hydrophila* infection of *Plecoglossus altivelis*, the cilia on the gill surface of the infected fish were damaged, and the small piece structures of gill were collapsed to form vacuoles, resulting in a severe respiratory disorder of the affected fish. In addition, abundant goblet cells were produced in the recess and epithelium of the intestinal mucosa, which were mainly to secrete mucins. These mucins cover the mucous membrane surface, not only protecting it from bacterial infection but also secreting lysozyme for sterilisation (Li et al. 2011). After *Aeromonas hydrophila* infection, the intestines and gill of *Carassius auratus indigentiaus* subsp. Nov. also displayed noticeable pathological changes. Specifically, gill filaments fell off and were shortened, intestinal villi were shortened, and goblet cells increased. This reflects that sepsis caused by *Aeromonas hydrophila* not only destroys the immune system of *Carassius auratus indigentiaus* subsp. Nov. but also weakens the respiratory and digestive systems.

The specific immunity mechanism of fish is not perfect yet, and the nonspecific immune system still plays an essential role. Various enzymes in fish tissues, including LSZ, ACP, AKP, SOD and CAT, are significant humoral immune factors and serve important defence functions in fish bodies. They can improve disease resistance and stress resistance in fish bodies to different extents (Fang et al., 2015; Zhang Zhao et al., 2018). At 3 h after injection of *Aeromonas hydrophila* to catfish, the c-type lysozyme expression in the kidney was upregulated significantly, but it subsequently began to increase at a stable rate (Pridgeon et al., 2013). The expression of g-type lysozyme in stomach, spleen and protonephros increased at 72 h following injection of Seaweed vibrio into *Epinephelus coioides* (Yin et al., 2003). After injection of *Aeromonas hydrophila* and lipopolysaccharide (LPS) into *Carassius auratus* in Qihe River, mRNA expressions of Ca-clys and Ca-glys in liver and gill were upregulated, but their expressions in spleen, kidney and head-kidney presented different variation laws (Wang et al., 2016). After the Av X005 infection of *Ctenopharyngodon idellus*, expressions of g-type and c-type lysozymes in the liver and spleen increased significantly within 3 h and then dropped sharply (Chen, 2020). After *Aeromonas hydrophila* infection to *Carassius auratus indigentiaus* subsp. Nov., the lysozyme activity in the liver increased significantly on the first day and peaked on the third day and decreased on the fifth day. This was consistent with variation laws of lysozyme expressions in the liver and spleen after Av X005 infection to

Ctenopharyngodon idellus (Chen, 2020). It is speculated that such changes are attributed to the following two aspects: 1) On the one hand, *Aeromonas hydrophila* infection stimulates *Carassius auratus indigentiaus* subsp. Nov. 2) On the other hand, the autoimmune system of *Carassius auratus indigentiaus* subsp. Nov. is partially destroyed after infection, thus influencing lysozyme expression and decreasing the lysozyme content.

As an important component of the lysosomal enzyme, ACP is viewed as a marker enzyme of macrophage lysosomes in higher animals. At 24 h after *Aeromonas hydrophila* infection, ACP activity and AKP activity in spleen, liver and kidney of Monopterus albus were strengthened, indicating that the nonspecific immunologic functions were enhanced (Fang et al., 2015). After *Aeromonas hydrophila* infection to *Carassius auratus indigentiaus* subsp. Nov., ACP activity increased gradually and peaked on the fifth day. The activities of AKP, SOD and CAT increased gradually after the first day and peaked on the third day. This reveals that in the early stage of *Aeromonas hydrophila* infection, nonspecific immunity was strengthened, and it plays an important role in the early stage of resisting pathogenic infection. At 24 h after *Aeromonas hydrophila* infection, ACP activity in *Monopterus albus* declined gradually while AKP activity decreased after 48 h (Fang et al., 2015). After five days, AKP activity, SOD activity and CAT activity of *Carassius auratus indigentiaus* subsp. Nov. decreased gradually, while ACP activity began to decline after seven days. It is speculated that as the infection time prolongs, lesions develop in tissues, weakening nonspecific immunity of *Carassius auratus indigentiaus* subsp. Nov. This is consistent with the findings of histopathological sections.

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The authors declare no conflict of interests.

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