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Effectiveness of traditional Chinese herbal medicine, San-Huang-San, in combination with enrofloxacin to treat AHPND-causing strain of *Vibrio parahaemolyticus* infection in *Litopenaeus vannamei*

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ABSTRACT

The effects of oral administration of enrofloxacin (ENR) and San-Huang-San (SHS), singly or in combination, on the survival performance, disease resistance, and immunity of *Litopenaeus vannamei* were investigated. After challenge with an AHPND-causing strain of *Vibrio parahaemolyticus* (VP_{AHPND}), shrimp were immediately fed a drug-free diet, diets containing only ENR (20 mg·kg^{-1}) or SHS (500 mg·kg^{-1}) or diets containing low-dose (10 mg·kg^{-1} ENR + 250 mg ·kg^{-1} SHS), medium-dose (20 mg·kg^{-1} ENR + 500 mg ·kg^{-1} SHS), and high-dose (40 mg·kg^{-1} ENR + 1000 mg ·kg^{-1} SHS) drug combinations for 5 days. The cumulative shrimp mortality over 5 days after injection of VP_{AHPND} in the ENR + SHS combination groups was significantly lower than that in the ENR or SHS alone groups ($p < 0.05$). Immune parameters, including the vibrio density, total hemocyte counts (THCs), hemocyanin (HEM) concentration, antibacterial activity, activity levels of lysozyme (LZM), acid phosphatase (ACP), alkaline phosphatase (AKP), and phenoloxidase (PO) in cell-free hemolymph, and the expression levels of the immune-related genes anti-lipopolysaccharide factor (ALF), cathepsin B (catB), crustin, lectin (Lec), lysozyme (LZM), and Toll-like receptor (TLR) in hemocytes were determined in the shrimp. The results showed that the shrimp in drug combination groups cleared more VP_{AHPND} than that in the ENR or SHS group in the same time. The values for other immune parameters in the drug combination groups were higher than those in the ENR or SHS group ($p < 0.05$). Finally, in the histological examinations, the histological structural alignment and integrity of the hepatopancreatic tubules in the drug combination groups were better than that in the ENR and SHS groups. Under the experimental conditions, compared with ENR or SHS used alone, the combination use of ENR and SHS could improve immunity and disease resistance in shrimp after VP_{AHPND} infection, and could reduce the use of ENR when the better therapeutic effect was achieved.

1. Introduction

The Pacific white shrimp, *Litopenaeus vannamei*, which is native to the Pacific coast, from Mexico to Peru, has become the main shrimp species cultured worldwide (including China) because of its high yield, fast growth, and wide adaptability to culture conditions, accounting for over 42% of the total shrimp production [1]. With the development of intensive culture and the deterioration of the ecological environment, some environmental stressors and highly virulent bacteria or viruses have caused frequent outbreaks of diseases that result in huge economic losses of shrimp. Acute hepatopancreatic necrosis disease (AHPND), a newly emerging shrimp disease that can cause mass mortalities (up to 100%), is known can be caused by a unique strain of the bacterium *Vibrio parahaemolyticus* carrying a toxin-producing plasmid [2]. *V. parahaemolyticus* AHPND (VP_{AHPND}) can release pirA- and pirB-like toxins, which result in severe cellular damage and necrosis of the hepatopancreas in *L. vannamei* [3].

To date, many strategies have been proposed and applied in shrimp aquaculture to prevent and control diseases, among which chemotherapeutic drugs have long been the main method [4]. However, with excessive use of these drugs, issues such as the emergence of drug-resistant microbial strains, environmental pollution, and drug residue accumulation have exacerbated [5,6]. Increasing public awareness of the side effects caused by overexposure to chemotherapeutic drugs has

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led to a search for ecofriendly and safe medicines. Therefore, medicinal herbs have attracted a lot of attention, because they are safer to aquatic animals and environments relative to the chemotherapeutic drugs [7,8]. Furthermore, they are rich in resources and easy to prepare, which contributes to their application [8,9]. The combinations of chemotherapeutic drugs and herbal medicines, with differing modes of actions, have potential for application in the treatment of many diseases. Combinations of medicines can substantially reduce the probability of evolving drug resistance and improve efficacy [10]. Thus, to achieve better preventive and treatment effects, many researchers have focused on the combined utilization of traditional Chinese herbs and chemotherapeutic drugs.

Enrofloxacin (ENR) is a member of the 6-fluoro-7-piperazinyl-4-quinolone family, which acts by inhibiting the DNA gyrase enzyme, and is active against a wide range of gram-negative and gram-positive pathogens. This antibiotic has also been used to control certain intracellular pathogens [11–13]. It is among the most frequently used antibacterial compounds in veterinary applications [14]. ENR has been licensed for use in aquaculture in China, and it has become an essential strategy to confront and alleviate infectious disease and reduce aquatic animal mortality [15,16]. ENR has received growing attention because of its potential efficacy and safety for the treatment of diseases in aquaculture [17–20]. San-Huang-San (SHS) was developed on the basis of the traditional Chinese medicine formula San-Huang-Xie-Xin-Tang, and it consisted of Rhei Rhizoma (rhizomes of *Rheum officinale* BAILL; RR; the main chemical composition is anthraquinone), Scutellariae Radix (roots of *Scutellaria baicalensis* Georgi; SR; the main chemical composition are baicalin and wogonoside), Cortex Phellodendri (dried bark of *Phellodendron chinensis Schneid*; CP; the main chemical composition is alkaloid), and Folium isatidis (dry leaves of *Isatis indigotica*; FI; the main chemical composition are indirubin and indigo). SHS has been reported to possess a variety of pathophysiological and pharmacological properties, including anti-inflammatory [21,22], anti-oxidant [23], anti-apoptotic [24], and anti-hypertensive activities [25]. In addition, SHS also has immunomodulatory [26] and hepatoprotective effects [27]. Currently, SHS is widely used to prevent and treat aquatic animal diseases such as in fish and shrimp [28,29]. Both ENR and SHS play important roles in disease prevention in aquatic animals. However, whether the combination use of the two drugs can improve the disease resistance of shrimp compared with the use of ENR or SHS alone, and whether combined with SHS can reduce the use of ENR remains unknown.

Therefore, the present study was undertaken to evaluate the effects of the combination of ENR and SHS on the survival rate, immunological parameters, and hepatopancreas histology of VP_{AHPND} infected *L. vannamei*. Through this study, we hope to provide a reference for applying combinations of traditional Chinese medicines and antibiotics in the prevention and treatment of bacterial diseases in actual production.

2. Materials and methods

2.1. Drugs and experimental diets

The ENR (purity > 98%) used in the preparation of the medicated feed was purchased from Solarbio Company (Beijing, China). SHS (containing 30% RR, 30% SR, 30% CP and 10% FI) was commercial product obtained from Beijing Yujing Biotechnology Co., Ltd., China.

Various drug-containing (ENR and SHS) diets and the control diet for experimental shrimps were prepared according to the feed formulation shown in Table 1. Briefly, the basal diet was formulated to contain approximately 430 g kg^{-1} crude protein and 73 g kg^{-1} crude fat, which are sufficient to support the optimal growth of *L. vannamei*. ENR and SHS were supplemented separately into the basal diet at the expense of cellulose to obtain the final drug dose for the different experimental groups: control (0 mg·kg-1), only infected (0 mg·kg^{-1}), ENR (20 mg·kg^{-1}), SHS (500 mg·kg^{-1}), low-dose combination (10 mg·kg^{-1} ENR + 250 mg ·kg^{-1} SHS), moderate-dose combination (20 mg·kg^{-1} ENR + 500 mg ·kg^{-1} SHS), and high-dose combination (40 mg·kg^{-1} ENR + 1000 mg ·kg^{-1} SHS) groups. The ingredients were ground and sieved through a 200-μm mesh to obtain a fine powder. The powder was then mixed thoroughly with fish oil and then tap water was gradually added until a stiff dough was obtained. Later, the dough was extruded through a mincer, ripened at 90 °C for 30 min, air-dried in the dark, and then sieved into pellets. The feed pellet was stored in plastic bags at −20 °C until use.

2.2. Bacterial culture

VP_{AHPND} strains (no. 20130629002S01) separated from AHPND-infected *L. vannamei* were kindly provided by the Mariculture Disease Control and Pathogenic Molecular Biology Laboratory, Yellow Sea Fisheries Research Institute, Qingdao, China. The bacteria were grown overnight at 28 °C in tryptic soy broth (TSB) supplemented with 2% NaCl. A single VP_{AHPND} bacterial colony was picked, inoculated into TSB containing 2% NaCl, and cultured by incubation with shaking at 200 rpm and 28 °C for 16 h. The density of bacterial cell (cfu·ml^{-1}) was performed by bacterial plate count on thiosulfate-citrate-bile salts-sucrose (TCBS) agar. The VP_{AHPND} stock solution was gradient diluted with a sterile potassium phosphate buffer (0.1 M, pH = 7.4) to obtain a VP_{AHPND} solution with a concentration of 10^7 cfu ml^{-1}.

2.3. Shrimp and culture conditions

White shrimp (*L. vannamei*; body length, 9.72 ± 0.23 cm and body weight, 9.05 ± 0.17 g) used in this study were obtained from HaiFeng Fisheries Science and Technology Company (Changyi, China). They were acclimated at 24 ± 0.5 °C, pH 7.8–8.2, and salinity of 32% in 200-L polyvinyl chloride polymer tanks for 1 week before the experiments. The shrimp were fed the basal diet for a week to acclimate them to the experimental diet and conditions.

2.4. Experimental design

Approximately 1260 healthy mature shrimp were divided into three batches. In the first batch, 350 shrimps were divided into seven groups (control, only infected, ENR, SHS, low-dose combination, moderate-dose combination, and high-dose combination groups). Except the control group, the other six groups were challenged with 15 μl of 10^7 cfu·ml^{-1} VP_{AHPND} between the second and third body parts of the abdomen of *L. vannamei*. Then, all the groups were immediately given the corresponding feeds with different drug compositions (as shown in Table 1) for five days after infection. The experiment was performed with three replicates. The cumulative mortality and protection ratio (RPS) were recorded. The second batch of 105 shrimps were also divided into seven groups (control, only infected, ENR, SHS, low-dose combination, moderate-dose combination, and high-dose combination groups), and were treated in the same way as the first batch, and after five days of administration, three shrimps were randomly selected from each group, and the selected shrimps were carefully dissected and the hepatopancreas were immediately fixed in Davidson's solution for 24 h for histological analysis. The third batch of 105 shrimps were divided into the same seven groups as the first batch with 15 shrimps per group, and were used to investigate changes of VP_{AHPND} density in the hepatopancreas and changes in immune parameters in hemolymph of *L. vannamei*. This batch was treated in the same way as the first batch, and the immune parameters in the shrimp were evaluated at 0, 0.5, 1, 3, and 5 days after infection, three shrimps were randomly sampled in each group at each time point; day 0 was considered as the control in each group. 600 μl hemolymph of each shrimp was drawn directly from the cardiocoelom using sterile syringes containing of 600 μl anticoagulant solution. 300 μl of anticoagulant hemolymph was used for total hemocyte counts. The other 900 μl of anticoagulant hemolymph was

Table 1
Formulation and chemical proximate composition of the experimental diets.

	Groups						
	Control	Only infected	SHS	ENR	Low-dose combination	Mid-dose combination	High-dose combination
Ingredients							
Fish meal[a] (g/kg)	200.0	200.0	200.0	200.0	200.0	200.0	200.0
Wheat glutens[a] (g/kg)	300.0	300.0	300.0	300.0	300.0	300.0	300.0
Wheat meal[a] (g/kg)	200.0	200.0	200.0	200.0	200.0	200.0	200.0
Cellulose (g/kg)	180.0	180.0	179.5	179.98	179.74	179.48	178.96
Fish oil (g/kg)	25.0	25.0	25.0	25.0	25.0	25.0	25.0
Soybean oil (g/kg)	25.0	25.0	25.0	25.0	25.0	25.0	25.0
Soybean phospholipids (g/kg)	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Gelatin (g/kg)	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Choline chloride (g/kg)	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Vitamin mix[b] (g/kg)	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Mineral mix[c] (g/kg)	10.0	10.0	10.0	10.0	10.0	10.0	10.0
SHS (mg/kg)	0	0	500	0	250	500	1000
ENR (mg/kg)	0	0	0	20	10	20	40
Proximate nutrient composition (as fed)							
Crude protein (g/kg)	431.0	431.0	431.0	431.0	431.0	431.0	431.0
Crude fat (g/kg)	73.0	73.0	73.0	73.0	73.0	73.0	73.0
Crude ash (g/kg)	68.0	68.0	68.0	68.0	68.0	68.0	68.0
Total energy (kJ/g)	16.44	16.44	16.44	16.44	16.44	16.44	16.44

[a] Fish meal: crude protein, 689.9 g/kg dry matter, crude fat, 78.1 g/kg dry matter; wheat glutens: crude protein, 790.5 g/kg dry matter; crude fat, 1.8 g/kg dry matter; wheat meal: crude protein, 165.0 g/kg dry matter; crude fat, 15.8 g/kg dry matter.
[b] Vitamin mixture (mg/kg diet): riboflavin, 45.0 mg; thiamine, 25.0 mg; vitamin K3, 10.0 mg; inositol, 800.0 mg; pyridoxine hydrochloride, 20.0 mg; vitamin B12, 0.1 mg; calcium pantothenate, 60.0 mg; biotin, 1.2 mg; vitamin A, 32.0 mg; vitamin D, 5.0 mg; nicotinic acid, 200.0 mg; folic acid, 20.0 mg; vitamin E, 120.0 mg.
[c] Mineral mix (mg/kg diet): KI, 0.8 mg; NaF, 2.0 mg; $Fe2(SO_4)_3$, 80.0 mg; $ZnSO_4$, 50.0 mg; $CoCl_2{\cdot}6H2O$, 50.0 mg; $CuSO_4{\cdot}5H_2O$, 10.0 mg; $MgSO_4$, 200.0 mg; NaCl, 100.0 mg; $Ca(H_2PO_4)2$, 3000.0 mg.

centrifuged at 3000 r/min for 10 min at 4 °C to separate the supernatant fluid (plasma) and hemocytes. The plasma was stored at −20 °C for hemocyanin concentration, antibacterial activity and immune enzyme activities measure; while the hemocytes dissolved Trizol Reagent was stored at −80 °C for RNA extraction.

2.5. Survival performance

The number of dead shrimp after the infection in each group was recorded to calculate the cumulative mortality (%) and RPS (%) as follows:

Cumulative mortality (%) = Cumulative number of dead shrimp / initial number of shrimp × 100%

RPS = (1 - cumulative mortality in each group / cumulative mortality in the only infected group) × 100%

2.6. Determination of VP_{AHPND} density in the hepatopancreas

Hepatopancreases from three shrimp in each sampling time point post-injection (0, 0.5, 1, 3, and 5 d) were sampled to count the VP_{AHPND} density. The hepatopancreas were dissected, mixed, and crushed with 1 ml phosphate-buffered saline (PBS, pH 7.0, 0.1 M) solution under aseptic conditions. One hundred microliters of the mixture was coated on a TCBS agar plate and incubated for 24 h at 30 °C to count the colony forming units of VP_{AHPND}.

2.7. Determination of immune parameters

2.7.1. Total hemocyte counts

Total hemocyte counts (THCs) were determined using a hemocytometer with a light microscope. The anticoagulant hemolymph was mixed with the same volume of 10% formaldehyde in a 1.5 mL centrifuge tube, fixed for 30 min, and then the mixed suspension was placed on a hemocytometer plate to observe and count the number of hemocytes (expressed as cells ml^{-1} hemolymph).

2.7.2. Determination of hemocyanin concentration

To determine the hemocyanin (HEM) concentration, the absorbance of 100 μl of anticoagulant hemolymph mixed with 900 μl of sterile water was measured at 335 nm using Multiskan spectrum (Thermo, USA), and the HEM concentration was calculated using an extinction coefficient of 17.26 [30]. The calculation formula of HEM concentration is: E_{335nm} (mM) = 17.26 × $O.D._{335.}$

2.7.3. Determination of antibacterial activity

Antibacterial activity was determined as described by Hultmark et al. [31]. Three hundred microliters of bacterial suspension and 10 μl of cell-free hemolymph sample were pipetted into a 96-well ELISA plate and the plate was put into a microplate reader and shaken for some time. Then absorbance was read at 570 nm and recorded as A0. Then, the plate was incubated in the microplate reader in dark at 37 °C for 30 min and absorbance at 570 nm was recorded (A). The antibacterial activity, defined as Ua, was calculated as follows: $U_a = \sqrt{(A0 - A)/A}$.

2.7.4. Determination of immune enzyme activities in cell-free hemolymph

Phenoloxidase (PO) activity was measured spectrophotometrically according to the procedure described by Ji et al. by using L-3,4-dihydroxyphenylalanine (L-DOPA, Sigma) as a substrate [32]. Mix 150 μl of 3 mg ml^{-1} L-DOPA solution with 50 μl of plasma in a 96-well microtiter plate and immediately place the microtiter plate into a microplate reader. The optical density (O.D.) value of samples in the microtiter plate was measured at 490 nm every 2 min in the microplate reader for 30 min, and using anticoagulant instead of plasma as a control. One unit of PO activity was defined as an increase in absorbance of 0.001 min^{-1} ml^{-1} cell-free hemolymph.

The activities of LZM, ACP, and AKP in cell-free hemolymph were measured by using commercial enzyme detection kits purchased from Jiancheng Bioengineering Institute (Nanjing, China) according to manufacturer's protocols. The protein content of hemolymph sample was measured by the Bradford method using a total protein quantification kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) with bovine serum albumin as the standard.

Table 2
Primers used for qPCR in the experiment.

Primer	Primer sequence (5′–3′)	GenBank accession number
ALF-F	TGTTCCTGGTGGCACTCTTC	GQ227486.1
ALF-R	GTCTCCTCGTTCCTCCACAG	
catB-F	CCTCTGTGGTTTTGGATGTA	GU571199.1
catB-R	GATGCTGTATGCTTTGCCTC	
Crustin-F	AACCAGAGACACCTGTTGGC	AY488497.1
Crustin-R	AGAATGAGGGAGGCTTGCAC	
Lec-F	CGGGATCCATGAAGTTCCTAGCGCCG	EF583939.1
Lec-R	CGCTCGAGTATATTTCTTGAGGCAAAT	
LZM-F	TCGAGTCGTCCTTCAACACG	AF425673.1
LZM-R	AGACGTTCTTGCCGTAGTCG	
TLR-F	TGAGAGATGCCCACTGCCTG	DQ923424.1
TLR-R	CGCTTGAAGGTTTGTGAGGGAG	
β-actinF	AGTAGCCGCCCTGGTTGT	AF300705.2
β-actinR	AGGATACCTCGCTTGCTCT	

2.7.5. Expression of immune-related genes

Total RNA was extracted separately from hemocytes and hepatopancreas by using Trizol Reagent (Invitrogen, USA) following the manufacturer's protocol and then treated with RNase-free DNase I (Promega, USA) to remove genomic DNA contamination. The quality of the isolated RNA was checked by gel electrophoresis on a 1.5% agarose gel. The first strand of cDNA was synthesized from 1 μg of RNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Japan).

Expressions of the target genes were determined using quantitative real-time PCR (qPCR). Primers for qPCR were designed using Primer 5 software (Table 2). The efficiency of qPCR was in the optimal range of 90–110% (slope of standard curves 3.1–3.6) for all of the primer pairs used. qPCR was conducted with SYBR Premix Ex Taq (Takara, Japan) on the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, USA) according to the manufacturer's instructions. β-Actin rRNA (GenBank accession number: AF300705) of *L. vannamei* was used as an internal control to normalize the expression level and all experiments were performed in triplicate. The PCR program was 95 °C for 30 s, then 40 cycles of 95 °C for 5 s, and 60 °C for 34 s, followed by 1 cycle of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The relative quantitative method ($2^{-\triangle\triangle}$CT) was used to calculate the fold change in the expression levels of the target genes.

2.8. Histological observation

Shrimp were carefully dissected and the hepatopancreas was immediately fixed in Davidson's solution for 24 h, before being transferred to 70% ethanol. After being dehydrated with alcohol at a series of concentrations, tissues were embedded in paraffin. Sections 5 μm in thickness were obtained with a conventional microtome and stained with hematoxylin and eosin (H.E.). Histological changes were observed using a light microscope (Olympus BX60 microscope).

2.9. Statistical analysis

All data in this experiment were subjected to one-way analysis of variance using the SPSS software (SPSS version 17.0 for Windows). Differences between means were assessed by Duncan's multiple-range test and effects with a probability of $p < 0.05$ were considered significant.

3. Results

3.1. Survival and drug protection ratio

Shrimp from each group were injected with VP_{AHPND} at a dose of 10^7 cfu·ml^{-1}, and all the groups were immediately given the corresponding feed with different drug compositions. The cumulative mortality rates and protection ratio after the 5-day feeding period were determined. The control group showed no obvious disease during the experiment. The shrimp fed with diets supplemented with ENR and SHS alone or in combination, tended to have better survival than that of the only infected group ($p < 0.05$; Table 3). Survival was better and drug protection ratio was greater in the ENR + SHS combination groups than those in the ENR and SHS groups ($p < 0.05$). Furthermore, the results improved with an increase in the dose of the combined treatment.

Table 3
Cumulative mortality and drug protection rates of *L. vannamei* in different groups after infection with VP_{AHPND} (Mean ± SD, n = 3).

Groups	Cumulative mortality (%)	Protection ratio (%)
Control	0^a	100^a
Only infected	77.33 ± 4.16^b	0.00 ± 5.38^b
ENR	38.00 ± 2.00^c	50.86 ± 2.59^c
SHS	44.67 ± 2.31^d	42.24 ± 2.99^d
Low-dose combination	31.33 ± 3.06^e	59.48 ± 3.95^e
Moderate-dose combination	17.33 ± 1.15^f	77.59 ± 1.49^f
High-dose combination	9.67 ± 0.58^g	87.50 ± 0.75^g

Note: The different letters indicate significant differences between the groups ($p < 0.05$).

3.2. Clearance of VP_{AHPND} from infected hepatopancreases of shrimp

Approximate 10^7 cfu·ml^{-1} of VP_{AHPND} was injected intramuscularly into each group at the beginning of the experiment, respectively. The density of VP_{AHPND} infecting the hepatopancreas were decreased by about 21.23%, 34.50%, 27.39%, 42.07%, 50.07%, and 53.73% after 0.5 d, and by 42.77%, 54.65%, 47.72%, 62.53%, 68.95%, and 74.74% after 1 d in the only infected, ENR, SHS, low-dose combination, moderate-dose combination, and high-dose combination groups, respectively. At 3 d after infection, the density of VP_{AHPND} in the hepatopancreas had decreased to 31.25×10^5 cfu·ml^{-1}, 19.82×10^5 cfu·ml^{-1}, 27.13×10^5 cfu·ml^{-1}, 11.55×10^5 cfu·ml^{-1}, 8.54×10^5 cfu·ml^{-1}, 6.13×10^5 cfu·ml^{-1}, and 5.85×10^5 cfu·ml^{-1} in the only infected, ENR, SHS, low-dose combination, moderate-dose combination, and high-dose combination group, respectively. At 5 d, the VP_{AHPND} infecting the hepatopancreas were almost cleared completely in the drug combination groups (Fig. 1).

3.3. Variation in THCs, HEM concentrations, and antibacterial activity in the shrimp

The THCs of shrimp infected with VP_{AHPND} decreased to the minimum value at 0.5 days after infection during the experiment.

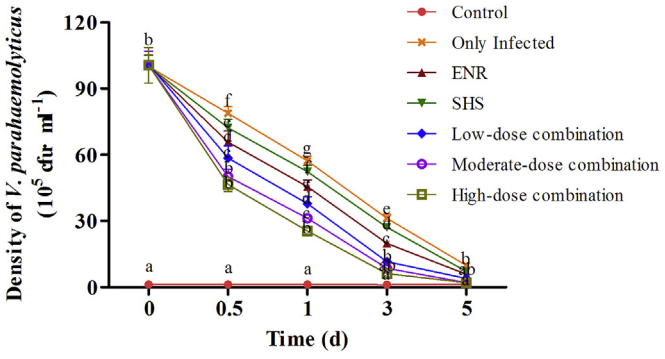

Fig. 1. The density of residual VP_{AHPND} in the hepatopancreas of shrimp in different groups after injection with VP_{AHPND}. Different letters indicate the significant differences between the different groups at the same time point ($p < 0.05$).

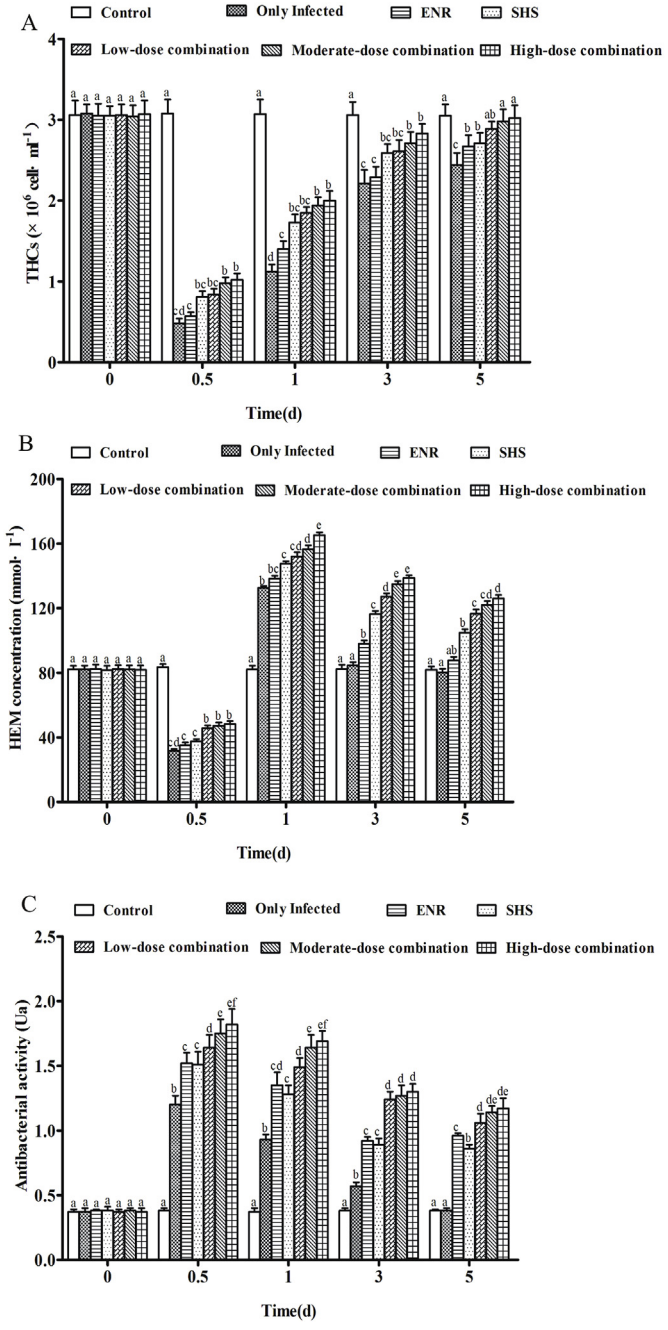

Fig. 2. The total hemocyte counts (THCs) (A), hemocyanin (HEM) concentration (B), and antibacterial activity (C) of shrimp after injection with VP_{AHPND}. Different letters indicate the significant differences between the different groups at the same time point ($p < 0.05$).

Afterward, THCs increased gradually to the normal level in the drug combination groups until day 5 but did not increase up to the normal level in the only infected, ENR and SHS groups. In drug combination groups, the THCs were significantly higher than those in only infected, ENR and SHS groups at days 0.5–5 ($p < 0.05$) (Fig. 2A). The HEM concentration in the shrimp first increased and then decreased, with the concentration being the highest at 1 day after the infection. The HEM concentrations in the combination groups were higher than those in the only infection, ENR, and SHS groups ($p < 0.05$). The HEM concentrations in the combination groups increased with an increase in the combined dose (Fig. 2B). The antibacterial activities peaked at 0.5 days in all the groups. The antibacterial activities in the combination groups were higher than those in the ENR and SHS groups ($p < 0.05$) (Fig. 2C).

3.4. Variation of immune enzyme activities in cell-free hemolymph of shrimp

After infection, PO activity decreased in each treatment group and reached a minimum on the third day. The PO activity in the only infected, ENR, SHS, low-dose combination, moderate-dose combination, and high-dose combination groups were approximately 0.43-, 0.58-, 0.57-, 0.67-, 0.74-, and 0.80-fold of that in the control groups at 3 days after infection, respectively (Fig. 3A). The drug combination groups showed significantly higher LZM activity than that shown by the control group and the only infected, ENR and SHS groups throughout the test period after infection with VP_{AHPND} ($p < 0.05$; Fig. 3B). The ACP activity in all the treatment groups peaked at 1 day after the infection and then decreased gradually. After day 3, there was no significant difference between the control and only infected groups ($p > 0.05$) (Fig. 3C). The AKP activity in the treatment groups peaked at day 0.5. At day 5, there was no significant difference between the control and only infected groups ($p > 0.05$); however, the AKP activity in the drug treatment groups was higher than that in the aforementioned two groups ($p < 0.05$; Fig. 3D). Both the ACP and AKP activities in the drug combination groups were higher than those in the ENR and SHS groups, and increased with an increase in the dose in the drug combination groups.

3.5. Expression profiles of immune-related genes in hemocytes of the shrimp

The gene expression of ALF was up-regulated in the hemocytes during the experiment and reached a maximum at day 1. The ALF levels in the drug combination groups were significantly higher than those in the ENR and SHS groups throughout the experimental period after infection with VP_{AHPND} ($p < 0.05$; Fig. 4A). The catB expression level in the hemocytes was up-regulated at all time points ($p < 0.05$). However, there was no significant difference between the ENR and only infected group at 1, 3, and 5 day, respectively. ($p > 0.05$; Fig. 4B). Furthermore, the catB expression in the drug combination groups was significantly higher than that in the ENR and SHS groups during the experiment ($p < 0.05$). Crustin expression in all groups peaked at day 1, and the expression in the drug combination groups was significantly higher than that in the ENR and SHS groups ($p < 0.05$; Fig. 4C). Lec expression in the drug combination groups remained elevated at all time points after infection, while it elevated at day 0.5 and 1 and decreased at day 5 after infection in the ENR and SHS groups. Furthermore, Lec expression in the drug combination groups was higher than that in the ENR and SHS groups at all the time points ($p < 0.05$; Fig. 4D). LZM expression remained elevated throughout the experimental period in the drug combination groups. In the only infected, ENR and SHS groups, LZM expression enhanced from day 0.5–3. The LZM expression in the drug combination groups was significantly higher than that in the ENR and SHS groups at 1 day after infection ($p < 0.05$; Fig. 4E). TLR expression in the drug combination groups remained upregulated throughout the experimental period and were the highest at 0.5 and 5 days and were higher than that in the ENR and SHS groups ($p < 0.05$; Fig. 4F).

3.6. Histological analysis

As shown in Fig. 5, the epithelial cells of the hepatopancreatic tubules completely ruptured and disappeared in the only infected group. In the ENR and SHS groups, on the other hand, the cells were slightly intact. The hepatopancreatic structures in the drug combination groups were better than those in the ENR and SHS groups. The alignment and structural integrity of the hepatopancreatic tubules gradually improved with an increase in the drug combination dose, with the lumen becoming gradually clear and evident and the structural integrity of the

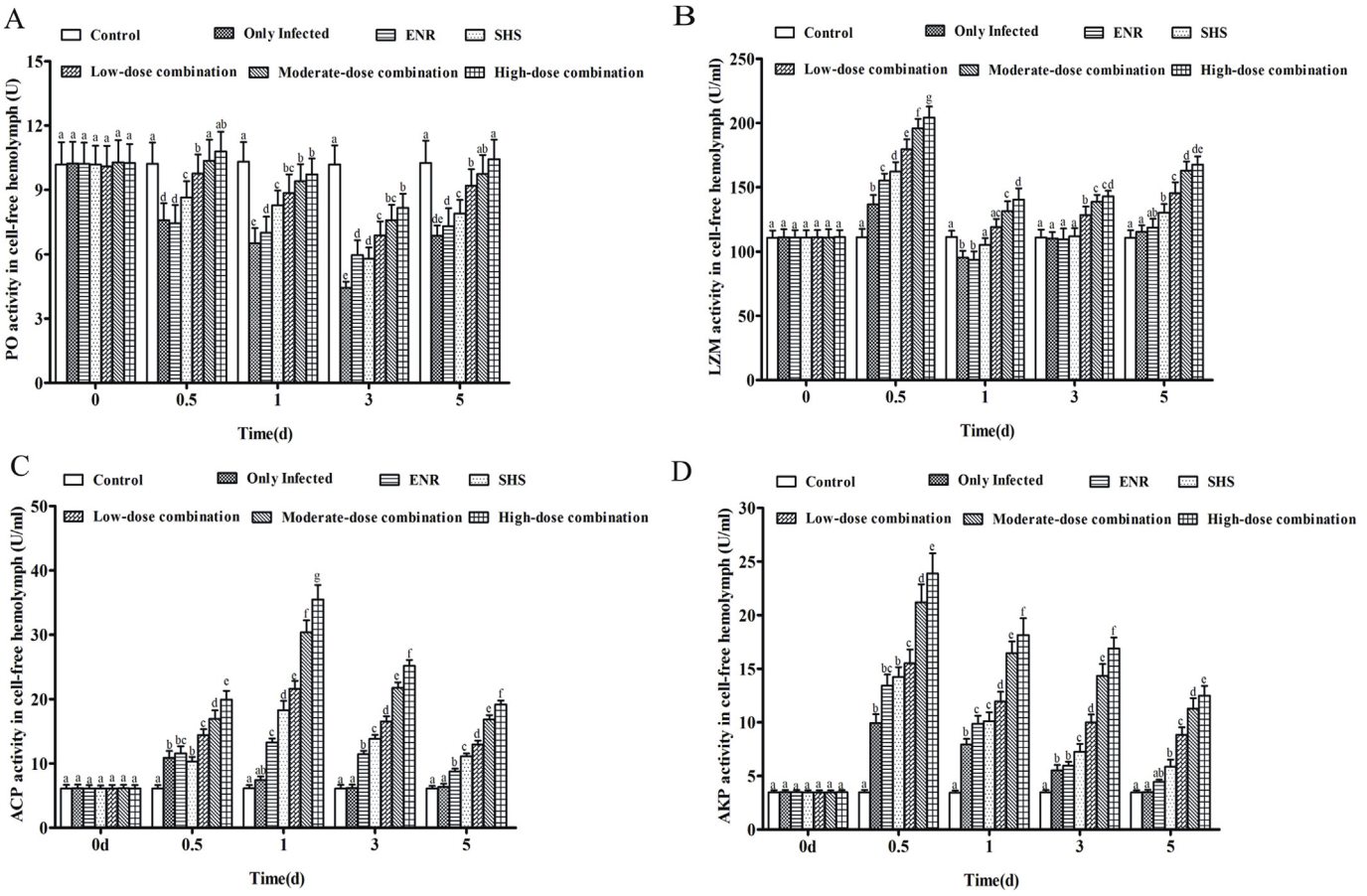

Fig. 3. The immune enzyme activities in cell-free hemolymph of shrimp after injection with VP_{AHPND}: phenoloxidase (PO) activity (A), lysozyme (LZM) activity (B), acid phosphatase (ACP) activity (C), and alkaline phosphatase (AKP) activity (D). Different letters indicate the significant differences between the different groups at the same time point ($p < 0.05$).

epithelial cells of the hepatopancreatic tubules increasing.

4. Discussion

AHPND can be caused by a specific set of virulent strains of *V. parahaemolyticus*, including VP_{AHPND}. It affects multiple shrimp species, especially *Penaeus monodon* and *L. vannamei*, not just in Asia, but also in Central America (Mexico) [33–35]. The disease is generally caused by *V. parahaemolyticus* harboring a 69-kb plasmid. VP_{AHPND} become virulent after acquiring the pVA1 plasmid, which encodes the deadly photorhabdus insect-related (Pir) binary toxins $pirA^{vp}$ and $pirB^{vp}$. VP_{AHPND} can multiply in the shrimp stomach and cause severe cellular damage by releasing virulent toxins into the shrimp hepatopancreas [36]. This disease has a high mortality rate (40–100%) accompanied by massive sloughing of hepatopancreatic epithelial cells [37].

At present, the most commonly used drugs for the treatment of bacterial diseases are chemotherapeutic drugs such as antibiotics and synthetic antibacterial drugs. However, long-term or large-scale use of these drugs can induce drug resistance, resulting in drug residues and other disadvantages. Traditional Chinese medicines can not only overcome the aforementioned shortcomings of antibiotics and synthetic antibacterial drugs but also can enhance animal immunity. Therefore, it is particularly important to scientifically and effectively use traditional Chinese medicines to reduce the use of chemotherapeutic drugs, for instance, antibiotics and synthetic antibacterial drugs.

Traditional Chinese medicines have attracted much attention in biomedical studies because of their broad spectrum of therapeutic activity and relatively low toxicity. With the increasing emphasis on food safety, a large number of Chinese herbal medicines have been developed as feed additives or medicines to reduce the use of chemotherapeutic drugs in animal production. SHS contains RR, SR, CP, and FI. The major constituents of RR are polyphenolic anthranoids, including sennoside A, sennoside B, emodin, aloe-emodin, chrysophanol, and rhein, which have been reported to show beneficial effects, such as anti-inflammatory and antioxidation activities [38,39]. The major constituents of SR are baicalin, baicalein, and wogonin, which have been reported to show anti-inflammatory [40], antitumor [41], immunomodulatory [26], and antioxidation [42] activities and hepatoprotective effects [27]. There are many bioactive components (e.g., obakunone, phellodendrine, and sterols) in CP, which mainly display anti-inflammatory and antibacterial activities [43]. The chemical compositions of FI vary, but include alkaloids [44], organic acids, glycosides, and flavonoids [45]. Most published reports have suggested that FI has anti-inflammatory and anti-oxidant activities, and strengthens immunity [46]. Dietary SHS supplementation is also effective to increase the daily weight gain and decreasing mortality of ducks [47]. In the present study, the effect of a combination of SHS and ENR, one of the most commonly used antibacterial drug in aquaculture, was evaluated and compared with the effects of SHS and ENR used alone.

Studies have shown that SHS has good anti-inflammatory effect and can effectively enhance the immunity of animals which improved the resistance to pathogenic bacterial infection [26]. The present study showed that oral administration of ENR or SHS effectively reduced the cumulative mortality rate of *L. vannamei* against VP_{AHPND} infection. The cumulative mortality rate showed a more significant reduction when ENR and SHS were used in combination. One possible explanation for this combined effect is that SHS enhanced the non-specific immunity

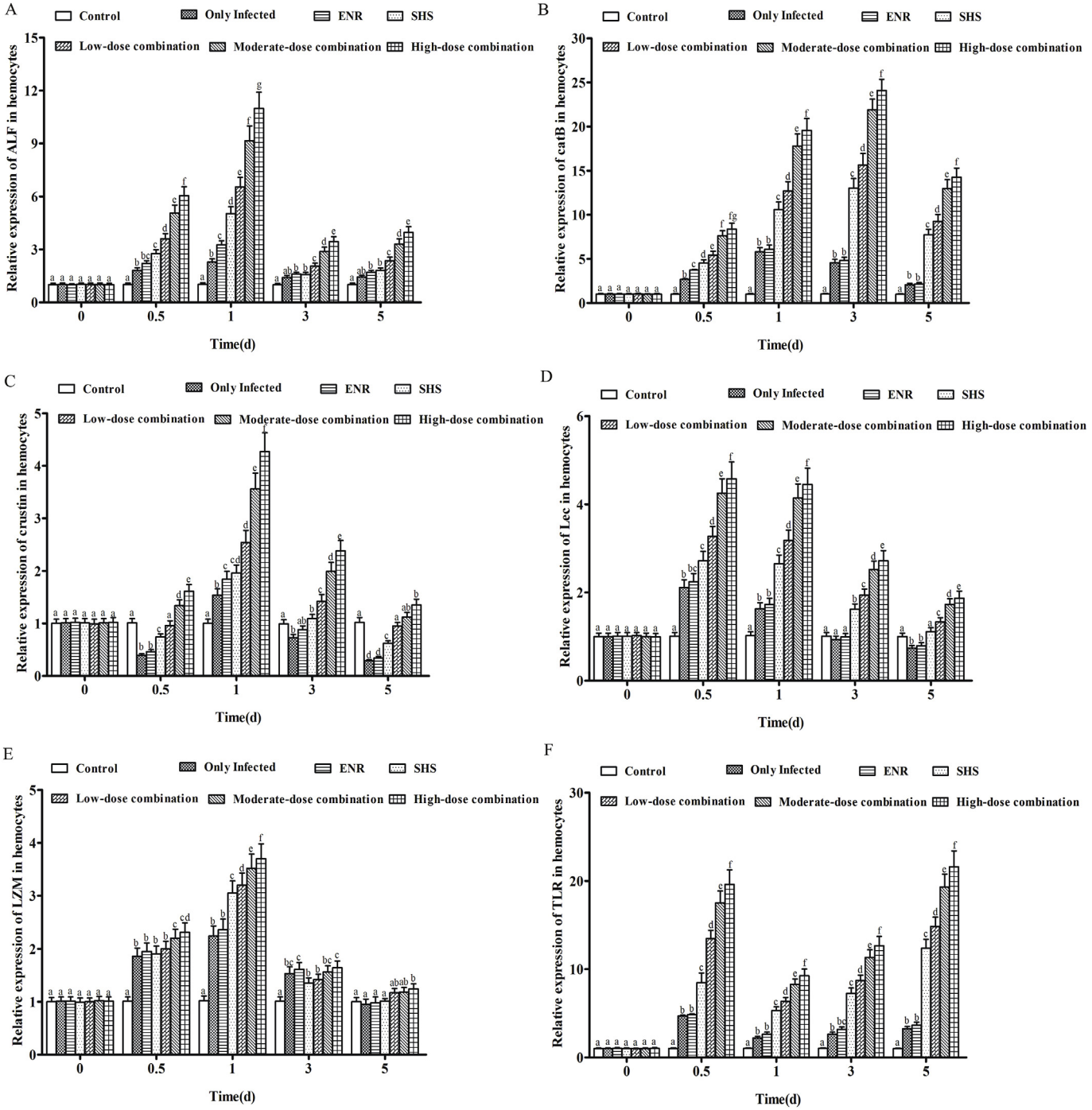

Fig. 4. Expression profiles of immune-related genes in hemocytes of shrimp after infection with VP_{AHPND}: anti-lipopolysaccharide factor (ALF) (A), cathepsin B (catB) (B), crustin (C), lectin (Lec) (D), lysozyme (LZM) (E), and Toll-like receptor (TLR) (F). Different letters indicate the significant differences between the different groups at the same time point ($p < 0.05$).

and improved various immune parameters in shrimp. The synergy between ENR and SHS thus increased the protection rate.

Shrimp lack typical adaptive immunity; therefore, they rely on the innate immune system to defend themselves against invading microbes by recognizing and clearing them through humoral and cellular immune responses [48,49]. In the present study, the effect of ENR, SHS, and the combination of ENR and SHS on the bacterial clearance in the hepatopancreas after the VP_{AHPND} infection was studied. The VP_{AHPND} clearance in the ENR, SHS, and drug combination groups improved compared with that in the only infected group. The VP_{AHPND} clearance of the drug combination group was better than that of the ENR and SHS groups. This indicated that compared with the use of ENR or SHS alone, dietary ENR with SHS can improve disease resistance against *V. parahaemolyticus*, which may be related to the antibacterial and anti-inflammatory effects of SHS and the immunological activity of SHS in shrimp.

L. vannamei, like other crustaceans, do not have specific immunity, instead they rely on innate immune mechanisms that include both cellular and humoral responses for defence against pathogens [50]. The cellular responses are mediated by haemocytes including phagocytosis, nodule formation, encapsulation, cytotoxicity, cell adhesion and hemolymph clotting mechanism, while humoral responses involve the

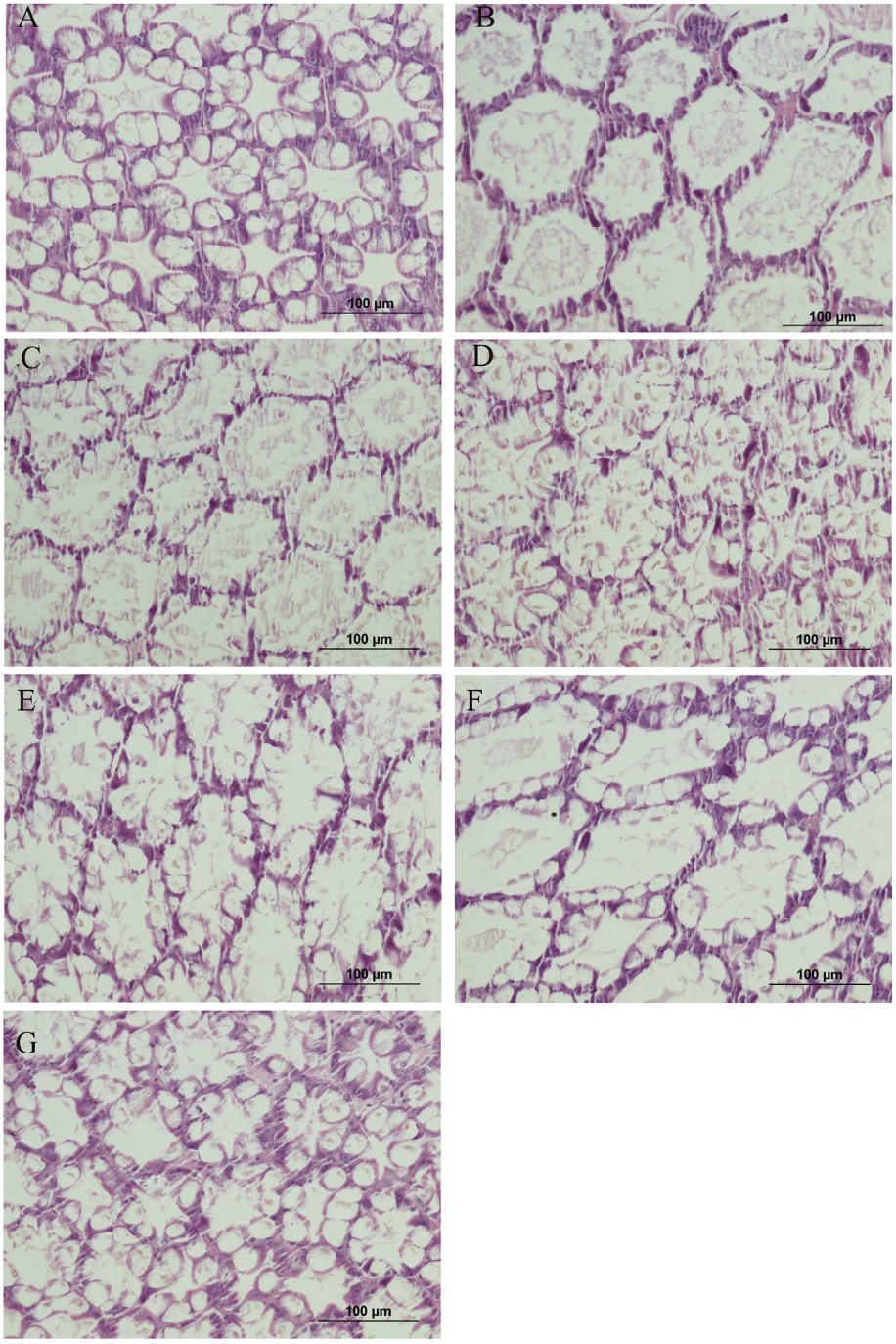

Fig. 5. Histological sections of the hepatopancreases obtained from shrimp in the control group (A), only infected group (B), ENR group (C), SHS group (D), low-dose combination group (E), moderate-dose combination group (F), and high-dose combination group (G). Scale bars, 100 μm.

prophenoloxidase-activating cascade and immune-related proteins such as lysozymes, lectins and antimicrobial peptides. The haemocytes which involve in cellular reactions and supply of factors related to humoral immunity play a crucial part in immune defence system [51]. Therefore, the THC can be used to reflect shrimp immunity ability [52]. The THC has been reported to decrease in shrimps exposed to infectious pathogens or environmental stress, which, in turn, may increase the risk of secondary infection [53]. In this study, THC gradually increased after drug administration and it was significantly higher in the drug combination groups than the ENR and SHS group. This may be due to the drugs increased the immunity of the shrimp and leads to an increase in THCs, and the drug combination ware more helpful in improving the immunity of the shrimp compared to ENR or SHS alone. This finding indicates that SHS might promote disease resistance in shrimp through proliferation of hemocytes and enhancement of their phagocytic activity. As the disease resistance improved, THC also changed accordingly.

Numerous studies have demonstrated that HEM may participate in the shrimp defense responses to pathogens, such as the PO activity and the antimicrobial and antiviral activities. It plays an important role in the disease immunity of shrimp [54]. Song et al. reported that HEM concentration decreases by about 33.3% after pathogenic infection [55], which was similar to the trend in the HEM levels after VP_{AHPND} infection in our study. The HEM content decreased significantly at day 0.5 and then increased. This was because after VP_{AHPND} infected shrimp, HEM participated in the infection resistance by converting to

the form of PO or degrading into antibacterial fragments [56]. After drug administration, the bacteria in the shrimp decreased and the immunity of shrimp increased, as a result the content of HEM increased. Moreover, in the present study, similar to the findings for THCs, the higher HEM concentration in the drug combination groups in comparison with that in the monotherapy groups (ENR and SHS groups) indicated that HEM concentration also increases with an increase in shrimp resistance to the pathogen.

The antibacterial activity in crustaceans is mainly attributable to antibacterial peptides found in abundance in the hemolymph. Shrimp can resist foreign pathogens by improving their antibacterial activity [57]. During the VP_{AHPND} infection in the present study, the infected shrimp showed greater antibacterial activity than that shown by the shrimp in the control group. Furthermore, antibacterial activity increased significantly in the early stage and decreased slightly in the late stage of the experiment, although it was always significantly higher than day 0. This might because of the VP_{AHPND} infection lead to stress reaction of shrimp which induced immune response, and the use of drugs further enhanced the immune response [58]. As the bacteria decreased in the late stage, the body's stress reaction was weakened and the immune response was slightly decreased. And notably, the antibacterial activity in the drug combination groups was higher than that in the ENR and SHS groups throughout the experiment.

Activated PO catalyses the stepwise oxidation of phenols to quinones which can inactivate viral pathogens [59]. Many studies have found that the activity of PO has a positive correlation with disease resistance of shrimp. Lysozyme participates in breaking down the polysaccharide wall of bacteria thus has been widely accepted as a crucial humoral immune factor [60,61]. ACP and AKP are important for the regulation of many phosphorylation and dephosphorylation processes [62]. Furthermore, ACP is an important component of phagocytic lysosomes, and in the phagocytosis and encapsulation of hemocytes, phagocytic lysosomes play a bactericidal action with the release of ACP [63]. As an important component of lysosomal enzymes, AKP plays a role in invertebrate immune responses [64]. Therefore, the various enzymes in hemolymph including PO, ACP, AKP and LZM are generally selected as the indicator to evaluate the immune state and disease resistance in shrimps [62,65,66]. It was reported the activity levels of immune enzymes such as PO, LZM, ACP, and AKP increased in the cell-free hemolymph of shrimp following VP_{AHPND} infection. Li et al. found that in *L. vannamei* that received *Vibrio alginolyticus* injection, PO activity levels decreased from 12 h and recovered at 120 h after injection [67], which was similar to the trend in the PO activity levels after VP_{AHPND} infection in our study. This might due to the inactive proPO is converted to active PO in haemocytes. After VP_{AHPND} infection, the content of PO in shrimp decreased with the decrease of THCs. After drug administration, the THCs gradually increased with the drug action, and the content of PO also increased. Chen et al. reported that the LZM activity of *P. trituberculatus* increased after *V. alginolyticus* injection [68]. In contrast, Feng et al. identified the ACP and AKP activity levels as indices for resistance against white spot syndrome virus (WSSV) in *E. carinicauda*. After shrimps were infected with VP_{AHPND}, the activity of LZM, AKP and ACP increased in the early stage and then decreased slightly, although they were always significantly higher than day 0. The reason should be the same as for the change in antibacterial activity in shrimp. These data also suggest that the immune enzymes in shrimp play roles in controlling systemic bacterial infections [69]. Interestingly, even the low-dose combination ($10\ mg{\cdot}kg^{-1}$ ENR + $250\ mg\ {\cdot}kg^{-1}$ SHS) group showed higher PO, LZM, ACP, and AKP activity levels than those shown by the ENR ($20\ mg{\cdot}kg^{-1}$) and SHS ($500\ mg{\cdot}kg^{-1}$) groups, indicating the use of ENR can be reduce when the same therapeutic effect is achieved compared with ENR used alone.

Activation of the innate immune response involves recognition of pathogens by pattern recognition receptors (PRRs) [70]. Lec is an important PRR and plays crucial roles in resisting pathogens [71]. Lec can interact particularly with carbohydrate moieties on the cell surface of non-self-molecules and thus initiate the innate immune responses for instance facilitating an opsonic effect to enhance phagocytosis, stimulating of prophenoloxidase activation system, and assisting in cell-cell interaction [72–75]. In this study, the expression level of Lec was up-regulated in hemocytes after VP_{AHPND} infection, and reached the highest value on day 0.5 and day 1. This is because of VP_{AHPND} invasion leads to bacterial agglutination and enhancement of adhesion and phagocytic activity of blood cells by Lec, thereby inducing up-regulation of Lec expression in the hemocytes [76]. The high level of Lec gradually shrinks as the bacteria in the body decreased. Thus, the high Lec expression level in the hemocytes in the drug combination groups may indicate higher levels of resistance. TLR is involved in PRRs and plays an essential role in recognizing pathogens in innate immunity [77–80]. TLR is a transmembranous glycoprotein (type-I protein) with a transmembrane domain, an extracellular N-terminus with leucine-rich repeats (LRRs) that mediate the recognition of pathogen-associated molecular patterns (PAMPs), and an intracellular C-terminus with the Toll/interleukin-1 receptor (TIR) domains that are required for downstream signal transduction [81,82]. TLR shows evolutionarily conserved regions in both vertebrates and invertebrates [83]. In our study, the TLR expression was also up-regulated after VP_{AHPND} infection. It is indicated that the expression level of TLR is closely related to the ability of shrimp to resist bacterial infection. Another interesting observation was that TLR expression was higher in the drug combination groups than in the ENR or SHS alone groups. Antimicrobial peptides (AMPs) are one of the key elements in innate immune system in crustaceans [84]. The AMPs have broad spectra of antimicrobial activity, an ability to kill or neutralize Gram-negative and Gram-positive bacteria, fungi, parasites and viruses [85]. Therefore, the AMPs are crucial for them to fight the pathogenic invasion. ALF, LZM and crustin are the most important AMPs reported in shrimp [86–88]. Lv et al. reported that silencing of ALF1 caused a hepatopancreatic lesion and finally led to the death of *E. carinicauda* [89]. In this study, the expression level of ALF, crustin and LZM were up-regulated in hemocytes after VP_{AHPND} infection, and reached the highest value on day 1. The reason should be similar to the change of antibacterial activity in shrimp. And similarly, higher expression levels of ALF, crustin, and LZM in hemocytes were observed in the drug combination groups than in the ENR and SHS groups. CatB, which acts as both endopeptidase and peptidyl-dipeptidase, is a unique member of the cathepsin superfamily, and it has been implicated in cancer progression and antigen processing through antigen-presenting cells [90,91]. As we known, cathepsins are essential enzymes in metabolism, and more and more research are focused on their ability in immune responses. The expression of cat B in hemocytes and hepatopancreas of shrimp infected by VP_{AHPND} was higher than that of the unscreened common shrimps, indicating that cat B can reflect the disease resistance of shrimp [28]. In *Fenneropenaeus chinensis*, catB expression in the gill, hepatopancreas, and muscle up-regulated after challenged with WSSV, suggesting that it may have a role in resisting WSSV infection [92]. In the present study, up-regulation of the catB transcripts in hemocytes was observed in response to VP_{AHPND} infection, and reached the highest value on day 3. This may also because of the VP_{AHPND} infection lead to stress reaction of shrimp that induced immune response, and drug administration further enhanced the immune response [59]. As the bacteria decreased later, the immune response was slightly decreased. It is worth noting that the catB expression was higher in the drug combination groups than in the ENR and SHS groups. Overall, the higher expression levels of immune-related genes in the drug combination groups suggest that the shrimp fed with diets supplemented with combination drugs showed more potent anti-infection activities than those shown by the shrimp fed with diets supplemented with ENR and SHS alone.

The hepatopancreas is the target tissue of VP_{AHPND}, as affected shrimp showed an abnormal hepatopancreas (shrunken, small, and discolored or black in coloration) and histopathological lesions on gross examination. Histopathological examination of the hepatopancreas

infected by VP_{AHPND} revealed the following characteristics. The tissue showed diffuse necrosis with rupture of the hepatopancreatic duct and only tissue fragments left. The epithelial cells of the hepatopancreatic tubule were disintegrated and the nucleus was deformed into a long or irregular shape and condensed or disappeared [93]. In this study, the effect of ENR, SHS, and the combination of ENR and SHS on the histological structure of the hepatopancreas after the VP_{AHPND} infection was studied. The histological structure in the ENR, SHS, and drug combination groups improved compared to that in the only infected group. The tissue structure of the drug combination group was better than that of the ENR and SHS groups. This indicates that compared with the ENR or SHS used alone, the combination use of the two drugs can improve the disease resistance of shrimp against *V. parahaemolyticus*, which may be related to the SHS-mediated hepatoprotective effects and the improvement in shrimp immunity.

In conclusion, the survival and drug protection performance during the 5 days after injection of VP_{AHPND} in the ENR + SHS combination groups were significant better than those in the ENR and SHS groups. The higher bacterial clearance, THCs, HEM concentrations, antibacterial activity, activity levels of four immune enzymes (PO, LZM, ACP, and AKP in cell-free hemolymph), and expression of six immune-related genes (ALF, catB, crustin, Lec, LZM, and TLR in hemocytes) in the drug combination groups compared to those in the ENR and SHS groups, suggest that the shrimp showed better disease-resistant capability when ENR and SHS were used in combination. In the histopathology experiment, increased anti-infective effect against VP_{AHPND} was observed when ENR and SHS were combined than when they were used alone. The above findings demonstrate that when used in combination, ENR and SHS could enhance the antibacterial efficacy compare to the drug used alone, which could in turn reduce the amount of antibiotics used and the bacterial drug resistance, moreover, to decreasing adverse reactions and antibiotic residues.

Conflicts of interest

Authors declare there is not conflict of interests.

Acknowledgments

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