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Evaluation of combination effects of *Astragalus* polysaccharides and florfenicol against acute hepatopancreatic necrosis disease-causing strain of *Vibrio parahaemolyticus* in *Litopenaeus vannamei*

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ABSTRACT

The effects of oral administration of *Astragalus* polysaccharides (APS) and florfenicol (FFC), 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 APS (200 mg·kg^{-1}) or FFC (15 mg·kg^{-1}), or diets containing low-dose (7.5 mg·kg^{-1} FFC + 100 mg·kg^{-1} APS), medium-dose (15 mg·kg^{-1} FFC + 200 mg·kg^{-1} APS), and high-dose (30 mg·kg^{-1} FFC + 400 mg·kg^{-1} APS) drug combinations for 5 days. The cumulative shrimp mortality over 5 days after injection of VP_{AHPND} in the APS + FFC combination groups was significantly lower than that in the APS or FFC alone groups ($p < 0.05$). Immune parameters, including the total hemocyte counts (THCs), hemocyanin (HEM) concentration, antibacterial activity, activity levels of lysozyme (LZM), and levels of 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 and hepatopancreas were determined in the shrimp. The values for these immune parameters in the drug combination groups were higher than those in the APS or FFC group ($p < 0.05$). Finally, in the histological examinations, the histological structural alignment and integrity of the hepatopancreatic tubules in the drug combination groups was better than that in the APS and FFC groups. Under the experimental conditions, dietary APS and FFC had a synergistic effect on immunity and disease resistance among shrimp after VP_{AHPND} infection.

1. Introduction

The white shrimp, *Litopenaeus vannamei*, is a commercially important shrimp species, and the main farmed species in China [1]. However, with the development of intensive culture and the deterioration of the ecological environment, the occurrence and spread of various infectious diseases, such as white spot syndrome (WSS) and acute hepatopancreatic necrosis disease (AHPND), have emerged as the most serious threats for shrimp aquaculture worldwide today, causing massive mortality and marked production loss [2–4]. AHPND is known to be caused by a unique strain of bacterium *Vibrio parahaemolyticus* carrying a toxin-producing plasmid [5]. AHPND-causing *V. parahaemolyticus* (VP_{AHPND}) has been shown to colonize and infect the stomach in the first stage of the infection, followed by the release of *Photorhabdus* insect-related (pir)A- and pirB-like toxins into the hepatopancreas. The toxins released result in severe cellular damage and hepatopancreas necrosis [6]. Thus, AHPND can cause mass mortalities (up to 100%) in *L. vannamei*.

For a long time, antibiotics and synthetic drugs have been widely used to prevent and control diseases. 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 [7,8]. Increasing public awareness of the side effects caused by overexposure to antibiotics and synthetic drugs has led to a search for ecofriendly and safe medicines. Therefore, medicinal herbs have attracted a lot of attention, because their active principles such as polysaccharides, flavonoids, alkaloids, phenolics, and essential oils, which have benefits such as appetite stimulation, growth promotion,

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immunoregulation and antimicrobial effects [9,10]. Furthermore, they are rich in resources and easy to prepare, which contributes to their application [10,11]. The combinations of antibiotics 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 [12]. Thus, to achieve better preventive and treatment effects, many researchers have focused on the synergistic effects of traditional Chinese medicines and antibiotics.

Florfenicol (FFC) is a third-generation broad-spectrum antibacterial analog of chloramphenicol; it is a veterinary drug. The mechanism of action of FFC is based on its ability to inhibit protein synthesis by binding the 50S ribosomal subunit of susceptible pathogens [13]. Compared with chloramphenicol and thiamphenicol, FFC is widely used in the prevention and control of aquatic diseases because of its broad-spectrum and strong antibacterial activity and mild side effects. It shows antibacterial activity against most aquaculture animal pathogens and it has been approved for use in various aquatic animals in Europe as well as Norway, Canada, China, and Korea; it has also been approved for use in El Ben [14]. *Astragalus membranaceus* polysaccharide (APS) is one of the major active substances isolated from the root of *A. membranaceus*, a traditional Chinese herbal medicine that has been commonly used as an immune booster for thousands of years in China [15]. There is evidence to indicate the importance of APS in the modulation of immune functions in both humans and in experimental animals [16–18]. APS has shown immune-stimulatory activity in a range of aquatic animals including the common carp (*Cyprinus carpio* L.) [19], Nile tilapia (*Oreochromis niloticus*) [20], Chinese mitten crab (*Eriocheir sinensis*) [21], and sea cucumber (*Apostichopus japonicus*) [22]. Dietary APS supplementation significantly promoted phagocytosis, respiratory burst, and the activity of various enzymes, including lysozyme phosphatase (LZM), acid phosphatase (ACP), alkaline phosphatase (AKP), prophenoloxidase-activating enzyme system (proPO system), and amylase, in aquatic animals. Both FFC and APS play important roles in antibacterial efficacy in aquatic animals. However, whether the two agents can act synergistically effects and whether combination with APS can reduce the use of FFC remain unknown.

Therefore, the present study was undertaken to evaluate the effects of the combination of FFC and APS on the survival rate, immunological parameters, and hepatopancreas histology of *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 FFC (purity ≥ 98%) used in the preparation of the medicated feed was purchased from Debon Pharmaceutical Company (Qingdao, China). APS, which is a polysaccharide complex extracted from the sliced roots of *A. membranaceus*, and the molecular weights were 20000–60000 Da was commercial product obtained from Beijing Centre Biology Co. Ltd., China.

Various drug-containing (APS and FFC) 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 $g{\cdot}kg^{-1}$ crude protein and 73 $g{\cdot}kg^{-1}$ crude fat, which are sufficient to support the optimal growth of *L. vannamei*. APS and FFC 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 $mg{\cdot}kg^{-1}$), only infected (0 $mg{\cdot}kg^{-1}$), APS (200 $mg{\cdot}kg^{-1}$) [23], FFC (15 $mg{\cdot}kg^{-1}$) [24], low-dose combination (7.5 $mg{\cdot}kg^{-1}$ FFC + 100 $mg{\cdot}kg^{-1}$ APS), moderate-dose combination (15 $mg{\cdot}kg^{-1}$ FFC + 200 $mg{\cdot}kg^{-1}$ APS), and high-dose combination (30 $mg{\cdot}kg^{-1}$ FFC + 400 $mg{\cdot}kg^{-1}$ APS) 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 ($cfu{\cdot}ml^{-1}$) was performed by bacterial plate count on thiosulfate-citrate-bile salts-sucrose (TCBS) agar.

2.3. Shrimp and culture conditions

White shrimp (*L. vannamei*; body length, 9.71 ± 0.28 cm and body weight, 9.02 ± 0.19 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 and sampling

Approximately 1260 healthy mature shrimp were divided into three batches. In the first batch, 350 shrimp were divided into seven groups (control, only infected, APS, FFC, 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 $cfu{\cdot}ml^{-1}$ (the 24 h half lethal concentration) VP_{AHPND}. 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 experiments were performed in triplicate. The cumulative mortality and protection ratio (RPS) at 0, 0.5, 1, 3, and 5 days after infection were recorded. The second batch of 105 shrimp was treated in the same way as the first batch, and after 5 days of administration, shrimp in the seven groups were carefully dissected and the hepatopancreas were immediately fixed in Davidson's solution for 24 h for histological analysis. The third batch of 105 shrimp was used to investigate changes in immune parameters in *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. 500 μl hemolymph of each shrimp was drawn directly from the cardiocoelom using sterile syringes containing of 500 μl anticoagulant solution. 300 μl of anticoagulant hemolymph was used for total hemocyte counts and hemocyanin concentration determination. The other 700 μl of anticoagulant hemolymph was centrifuged at 3000 r/min for 10 min at 4 °C to separate the supernatant fluid and hemocytes. The supernatant fluid was stored at −20 °C for antibacterial activity and immune enzyme activities measure; while the hemocytes dissolved Trizol Reagent was stored at −80 °C for RNA extraction. The hepatopancreas of each shrimp was also collected and grinded with Trizol Reagent and then frozen at −80 °C for RNA extraction.

Table 1
Formulations and approximate chemical compositions of the experimental diets.

	Groups						
	Control	Only infected	APS	FFC	Low-dose combination	Moderate-dose combination	High-dose combination
Ingredients							
Fish meala (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	179.8	179.99	179.89	179.79	179.57
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
APS (mg/kg)	0	0	200	0	100	200	400
FFC (mg/kg)	0	0	0	15	7.5	15	30
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.

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 immune parameters

2.6.1. Total hemocyte counts

Total hemocyte counts (THCs) were determined using a hemocytometer with a light microscope; 200 μl of anticoagulant hemolymph was placed on the hemocytometer and the hemocytes were counted (expressed as cells ml^{-1} hemolymph). The anticoagulant consists of the following ingredients: 1.588 g sodium citrate, 3.92 g sodium chloride, 4.56 g glucose, 0.66 g EDTA-2Na, 200 ml ddH_2O.

2.6.2. 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 [25].

2.6.3. Determination of antibacterial activity

Antibacterial activity was determined as described by Hultmark et al. [26]. The bacterial colonies cultured on the solid medium were washed with a sterile potassium phosphate buffer (0.1 M, pH = 7.4), and then the bacterial solution was centrifuged, resuspended, and finally formulated to a certain concentration of the bacterial suspension ($O.D._{570\ nm}$ = 0.3). 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: $U_a = \sqrt{(A0 - A)/A}$.

2.6.4. 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 [27]. 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 the kits from Jiancheng Bioengineering Institute (Nanjing, China) according to manufacturer's protocols.

2.6.5. 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 Primer5 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

Table 2
Primers used for qPCR in the experiment.

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

method ($2^{-\triangle\triangle}$CT) was used to calculate the fold change in the expression levels of the target genes.

2.7. Histological observation

The hepatopancreas in Davidson's solution for 24 h were 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).

2.8. 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. Result

3.1. Survival and drug protection ratio

Shrimp from each generation 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 during the 5-day feeding period were determined. The control group showed no obvious disease during the experiment. The shrimp fed with diets supplemented with APS and FFC alone or in combination, tended to have better survival than that of the only infected group ($p < 0.05$; Fig. 1). Survival was better and protection ratio was greater in the APS + FFC combination groups than those in the APS and FFC groups ($p < 0.05$). Furthermore, the results improved with an increase in the dose of the combined treatment.

3.2. 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. 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, APS, and FFC groups. In drug combination groups, the THCs were significantly higher than those in only infected, APS, and FFC 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

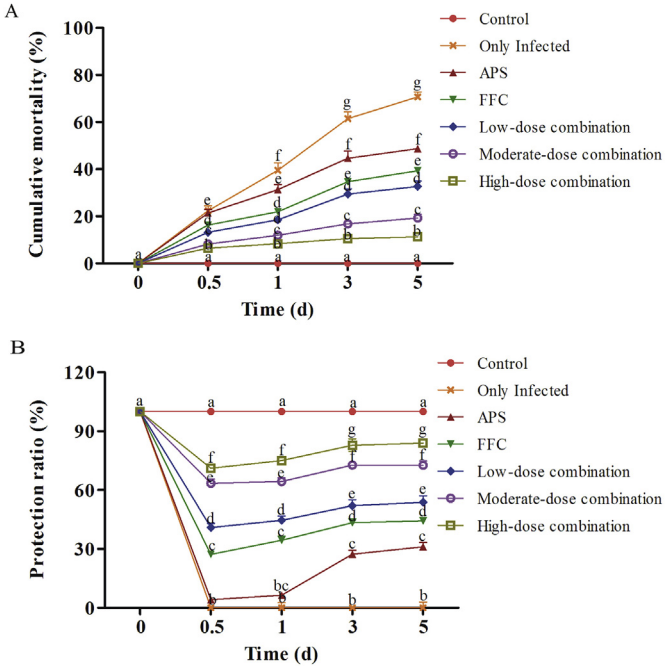

Fig. 1. Cumulative mortality (A) and protection rates (B) of *L. vannamei* in different groups after infection with VP_{AHPND}. Different letters indicate the significant differences between the different groups at the same time point ($p < 0.05$).

concentrations in the combination groups were higher than those in the only infection, APS, and FFC 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 and 1 days in the drug combination groups and at 0.5 days in the only infection, APS, and FFC groups. The antibacterial activities in the combination groups were higher than those in the APS and FFC groups ($p < 0.05$) (Fig. 2C).

3.3. 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, APS, FFC, low-dose combination, moderate-dose combination, and high-dose combination groups was approximately 0.34-, 0.73-, 0.54-, 0.71-, 0.78-, and 0.84-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, APS, and FFC 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 1. 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 APS and FFC groups, and increased with an increase in the dose in the drug combination groups.

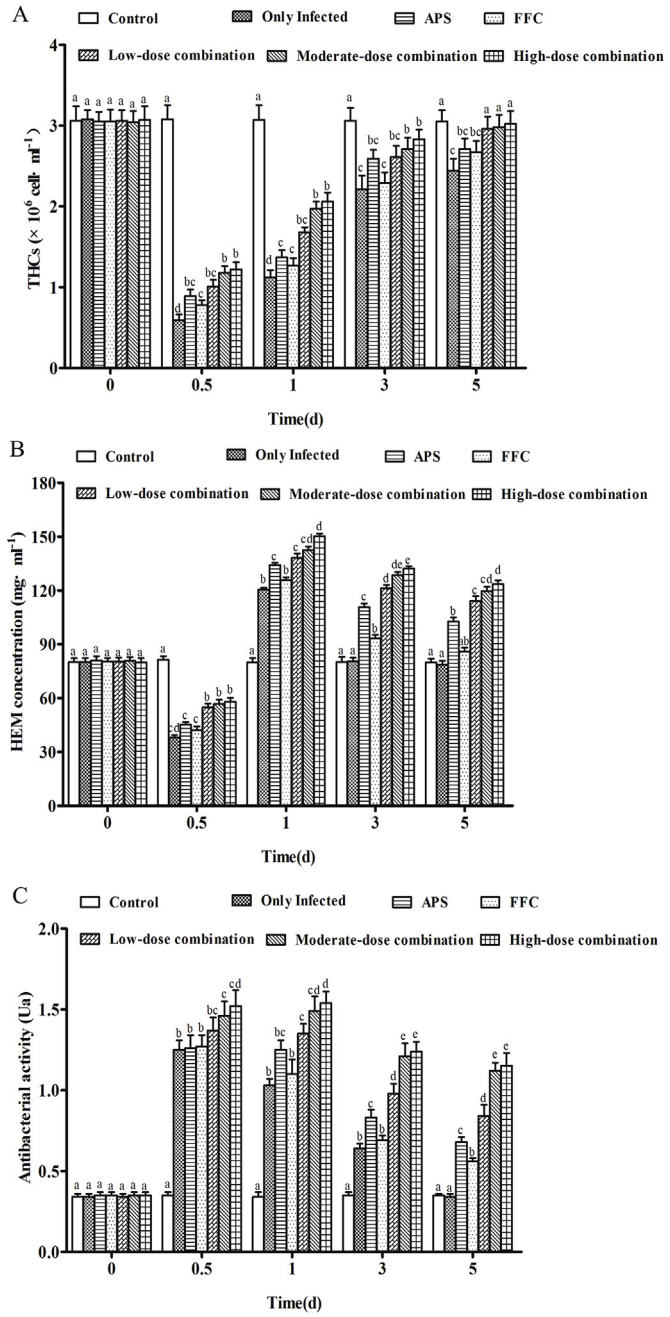

Fig. 2. The THCs (A), HEM concentration (B), and antibacterial activity (C) of shrimp after injection with VP_{AHPND}. Different letters indicate the significant differences between the different groups at the same time point ($p < 0.05$).

3.4. Expression profiles of immune-related genes in hemocytes and the hepatopancreas of the shrimp

The gene expression of ALF was up-regulated in the hemocytes and hepatopancreas 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 APS and FFC groups throughout the experimental period after infection with VP_{AHPND} ($p < 0.05$; Fig. 4A and Fig. 4B).

The catB expression level in the hemocytes was up-regulated in the APS and drug combination groups at all time points ($p < 0.05$). However, there was no significant difference between the control and only infected group or FFC group at 0.5 days ($p > 0.05$; Fig. 4C). Furthermore, catB expression in the hepatopancreas in the APS and drug combination groups elevated from day 0.5–5 ($p < 0.05$), while there was no significant difference in the expression between the control and only infected group or FFC group from day 1–5 ($p > 0.05$; Fig. 4D). The catB expression in the drug combination groups was significantly higher than that in the APS and FFC groups during the experiment ($p < 0.05$).

Crustin expression in the hemocytes in all groups peaked at day 1, and the expression in the drug combination groups was significantly higher than that in the APS and FFC groups ($p < 0.05$; Fig. 4E). Further, crustin expression in the hepatopancreas in the drug combination groups remained up-regulated throughout the experimental period, while it was only significantly high at day 1 in the APS and FFC groups ($p < 0.05$) (Fig. 4F).

Lec expression in the hemocytes 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 days 3 and 5 after infection in the APS and FFC groups. Furthermore, Lec expression in the drug combination groups was higher than that in the APS and FFC groups at all the time points ($p < 0.05$; Fig. 4G). Lec expression in the hepatopancreas in all the groups peaked at day 1 and then reduced sharply. Additionally, Lec expression in the drug combination groups was higher than that in the APS and FFC groups at days 0.5 and 1 ($p < 0.05$; Fig. 4H).

LZM expression remained elevated throughout the experimental period in the only infected, FFC, and the drug combination groups. In the APS group, LZM expression enhanced from day 0.5–3. The LZM expression in the drug combination groups was significantly higher than that in the APS and FFC groups at 0.5 and 1 days after infection ($p < 0.05$; Fig. 4I). The LZM expression in the hepatopancreas in the drug combination groups remained up-regulated throughout the experimental period, and was significantly higher than that in the APS and FFC groups ($p < 0.05$). LZM expression levels were only up-regulated at 0.5 and 1 days in the APS and FFC groups (Fig. 4J).

TLR expression in the hemocytes in the drug combination groups remained up-regulated throughout the experimental period and were the highest at 0.5 and 5 days and were higher than that in the APS and FFC groups ($p < 0.05$; Fig. 4K). Further, TLR expression in the hepatopancreas remained up-regulated after infection in all the treatment groups throughout the experimental period, and was higher in the drug combination groups than in the APS and FFC groups ($p < 0.05$) (Fig. 4L).

3.5. 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 APS and FFC groups, on the other hand, the cells were slightly intact. The hepatopancreatic structures in the drug combination groups were better than those in the APS and FFC 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 epithelial cells of the hepatopancreatic tubules increasing.

4. Discussion

AHPND is an emerging (since 2010) bacterial disease that affects multiple shrimp species, especially *Penaeus monodon* and *L. vannamei*, not just in Asia but also in Central America (Mexico) [28–30]. The disease is generally caused by *V. parahaemolyticus* harboring a 69-kb plasmid. VP_{AHPND} can multiply in the shrimp stomach and cause severe cellular damage by releasing virulent toxins into the shrimp hepatopancreas [31]. AHPND is characterized by severe atrophy of the shrimp hepatopancreas, along with sloughing of the epithelial cells of the hepatopancreatic tubules in the early stage followed by necrosis of the cells and massive hemocytic infiltration in the later stages of infection, eventually leading to a high mortality rate in shrimp [32].

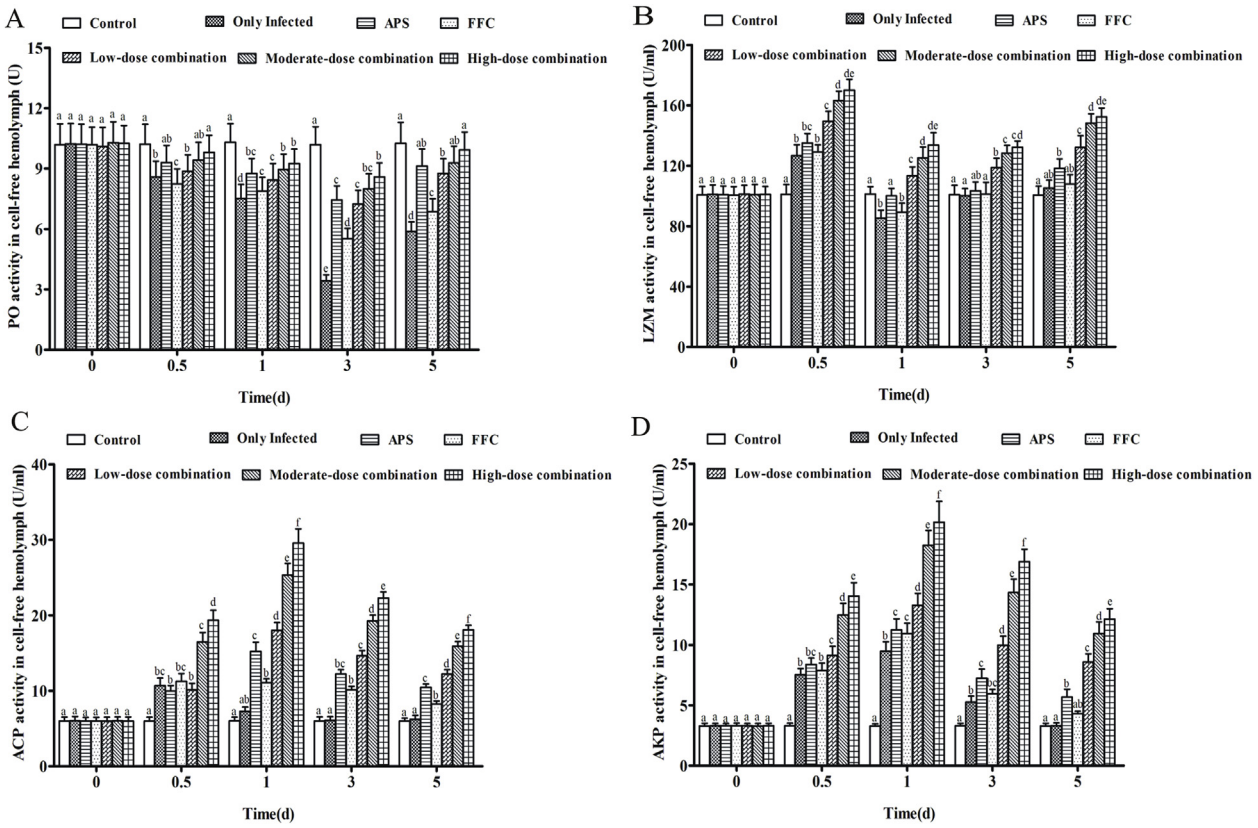

Fig. 3. The immune enzyme activities in cell-free hemolymph of shrimp after injection with VP_{AHPND}: PO (A), LZM (B), ACP (C), and AKP (D). Different letters indicate the significant differences between the different groups at the same time point ($p < 0.05$).

At present, the most commonly used drugs for the treatment of bacterial diseases are 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 extract effective active ingredients from traditional Chinese medicines to reduce the use of antibiotics and synthetic antibacterial drugs.

Polysaccharides isolated from mushrooms, algae, and many higher plants have attracted much attention in biomedical studies because of their broad spectrum of therapeutic activity and relatively low toxicity [33,34]. With the increasing emphasis on food safety, a large number of natural immunostimulants, including APS, have been developed as feed additives or medicines to reduce the use of chemotherapeutic drugs in animal production. As a natural polysaccharide, APS is a major active component in the Chinese herbal medicine *A. membranaceus*, and it has been proven to show excellent immune-enhancing effects in mammals, poultry, and fish by activating their specific and non-specific immune systems [20,35–37]. Dietary supplementation of APS has been reported to improve growth performance, immune organ indices, and immune activity in husbandry animals such as broilers, pig, rabbits, and cattle [38]. Dietary APS supplementation has also been found to be effective in increasing the daily weight gain and decreasing the mortality in crucian carp [39]. In this study, the effect of a combination of APS and FFC, the most commonly used antibacterial drug in aquaculture, was evaluated and compared with the effects of APS and FFC used alone.

Some studies have reported that oligosaccharides effectively enhanced the immunity of animals and thus improved the resistance to pathogenic bacterial infection [40–43]. The present study showed that oral administration of APS or FFC effectively reduced the cumulative mortality rate of *L. vannamei* against VP_{AHPND} infection. The cumulative mortality rate showed a more significant reduction when APS and FFC were used in combination. One possible explanation for this combined effect is that APS enhanced the non-specific immunity and improved various immune parameters in shrimp while FFC treated the infection. The synergy between APS and FFC thus increased the protection rate.

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 [44]. The present study showed that the combination of APS and FFC had a favorable effect on THC. This finding indicates that APS 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. Similar results have been found in giant tiger shrimp and Chinese shrimp fed with a diet supplemented with polysaccharides from *Durio zibethinus* and *Sargassum fusiforme*, respectively [45,46].

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 [47]. Song et al. reported that HEM concentration decreases by about 33.3% after pathogenic infection [48]. 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 (APS and FFC 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 [49]. 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, and notably, the antibacterial activity in

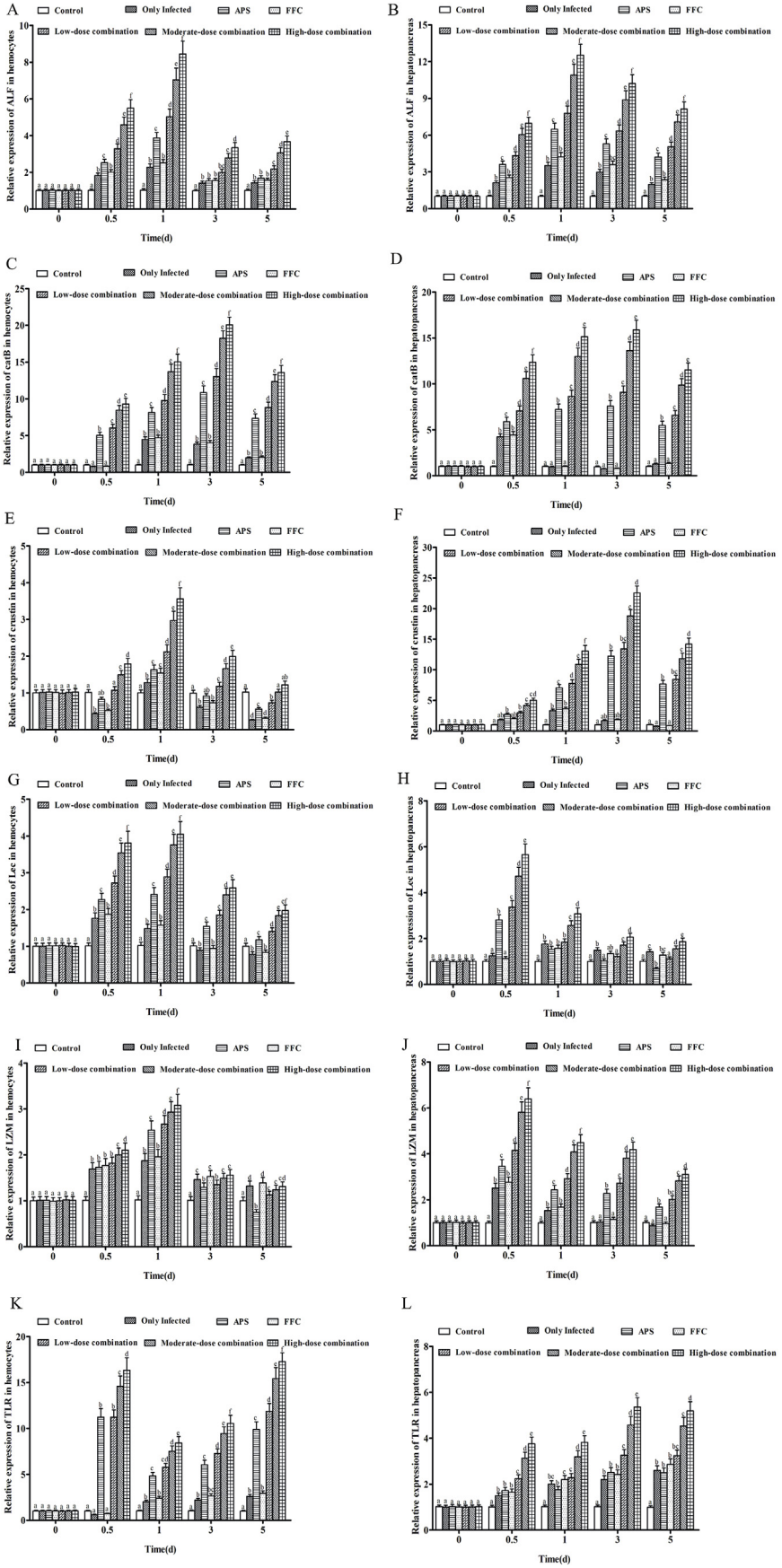

Fig. 4. Expression profiles of immune-related genes in hemocytes and the hepatopancreas of shrimp after infection with VP_{AHPND}: ALF (A), catB (C), crustin (E), Lec (G), LZM (I), and TLR (K) in hemocytes and ALF (B), catB (D), crustin (F), Lec (H), LZM (J), and TLR (L) in the hepatopancreas. Different letters indicate the significant differences between the different groups at the same time point ($p < 0.05$).

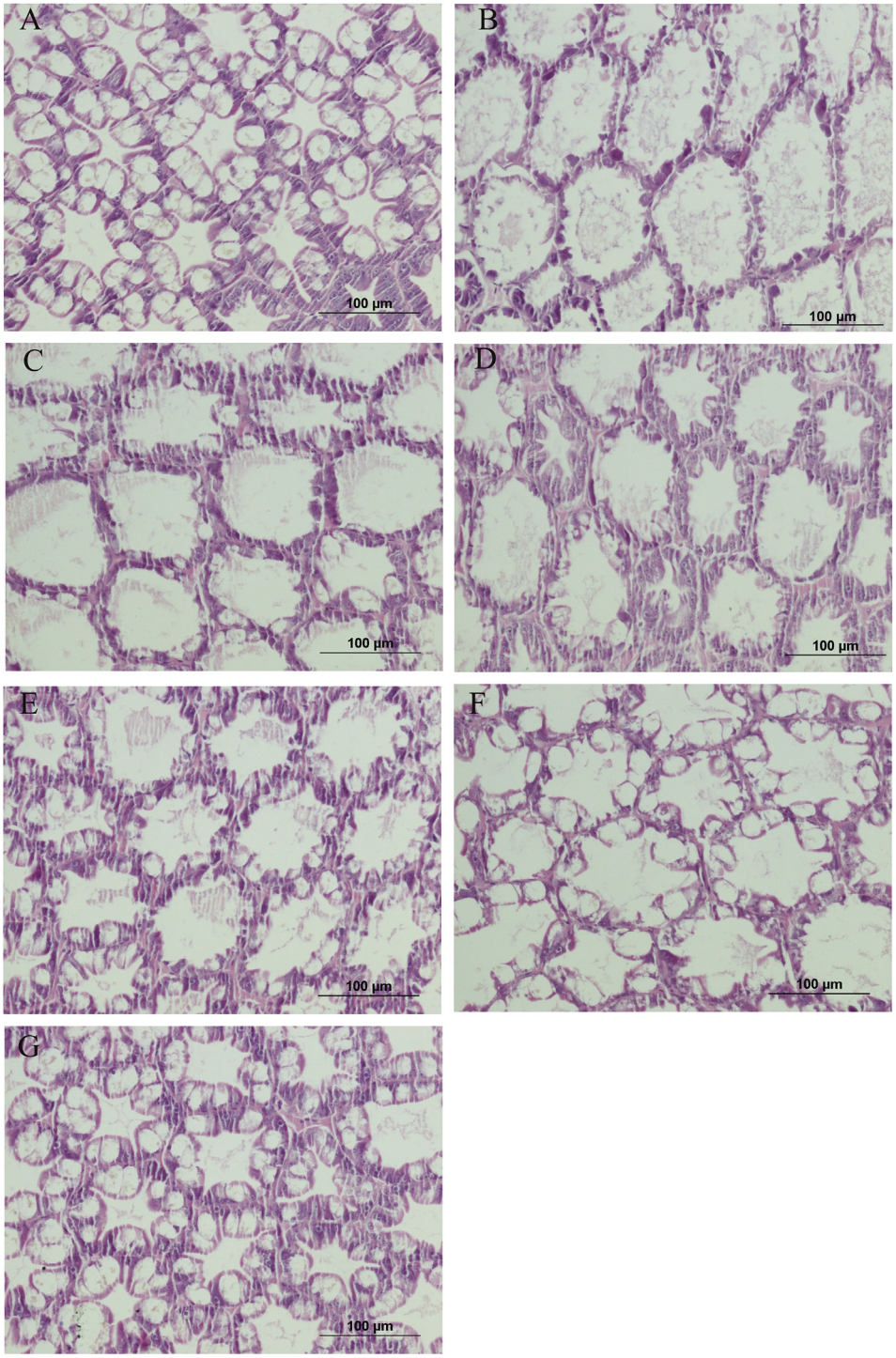

Fig. 5. Histological sections of the hepatopancreases obtained from shrimp in the control group (A), only infected group (B), APS group (C), FFC group (D), low-dose combination group (E), moderate-dose combination group (F), and high-dose combination group (G). Scale bars, 100 μm.

the drug combination groups was higher than that in the APS and FFC groups throughout the experiment.

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 [50], which was similar to the trend in the PO activity levels after VP_{AHPND} infection in our study. Chen et al. reported that the LZM activity of *P. trituberculatus* increased after *V. alginolyticus* injection [51]. 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*. These data suggest that the immune enzymes in shrimp play roles in controlling systemic bacterial infections [52]. Interestingly, even the low-dose combination (7.5 mg kg^{-1} FFC + 100 mg kg^{-1} APS) group showed higher PO, LZM, ACP, and AKP activity levels than those shown by the APS (200 mg kg^{-1}) and FFC (15 mg kg^{-1}) groups, indicating APS and FFC have a good synergistic effect.

Activation of the innate immune response involves recognition of pathogens by pattern recognition receptors (PRRs) [53]. Lec is an

important PRR and plays crucial roles in resisting pathogens [54]. In this study, the expression level of Lec was up-regulated in hemocytes and the hepatopancreas after VP_{AHPND} infection. 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 hepatopancreas and hemocytes [55]. Thus, the high Lec expression level in the hepatopancreas and hemocytes in the drug combination groups may indicate higher levels of resistance.

The Toll signal pathway, which serves as a major innate defense pathway, is involved in recognition of and defense against microbial infections in invertebrates and includes TLR, ALF, and crustin. TLR is involved in PRRs and plays an essential role in recognizing pathogens in innate immunity [56–59]. 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 [60,61]. TLR shows evolutionarily conserved regions in both vertebrates and invertebrates [62]. In our study, the TLR expression was also up-regulated after VP_{AHPND} infection. Another interesting observation was that TLR expression was higher in the drug combination groups than in the APS or FFC alone groups. ALF, crustin, and LZM are important AMPs, and several studies have provided evidence of the importance of AMPs in fighting pathogens in shrimp [63–65]. Lv et al. reported that silencing of ALF1 caused a hepatopancreatic lesion and finally led to the death of *E. carinicauda* [66]. In the present study, higher expression levels of ALF, crustin, and LZM in hemocytes and the hepatopancreas were observed in the drug combination groups than in the APS and FFC 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 [67,68]. 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 [69]. In the present study, up-regulation of the catB transcripts in hemocytes and hepatopancreas was observed in response to VP_{AHPND} infection. It is worth noting that the catB expression was higher in the drug combination groups than in the APS and FFC 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 APS and FFC 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 [68]. 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 [69,70]. In this study, the effect of APS, FFC, and the combination of APS and FFC on the histological structure of the hepatopancreas after the VP_{AHPND} infection was studied. The histological structure in the APS, FFC, 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 APS and FFC groups. This indicates that APS and FFC have synergistic effects against *V. parahaemolyticus*, which may be related to the APS-mediated improvement in shrimp immunity.

In conclusion, the survival and drug protection performance during the 5 days after injection of VP_{AHPND} in the APS + FFC combination groups were significant better than those in the APS and FFC groups. The higher 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 and the hepatopancreas) in the drug combination groups compared to those in the APS and FFC groups, suggest that the shrimp showed better disease-resistant capability when APS and FFC were used in combination. In the histopathology experiment, increased antimicrobial activity against VP_{AHPND} was observed when APS and FFC were combined than when they were used alone. The above findings demonstrate that APS and FFC have synergistic effects in combating VP_{AHPND} infection. The two drugs when used in combination could complement each other and enhance antibacterial efficacy, which could in turn the bacterial drug resistance in addition to decreasing adverse reactions, reduce the amount of antibiotics used and antibiotic residues.

Conflicts of interest

Authors declare there is not conflict of interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.11.065.

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