Fish and Shellfish Immunology 103 (2020) 88–94

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Dietary *Hizikia fusiforme* enhance survival of white spot syndrome virus infected crayfish *Procambarus clarkii*

Xinyue Jiang[a], Wenxin Jin[a], Fei Zhu[b,*]

[a] Jixian Honors College, Zhejiang Agriculture and Forestry University, Hangzhou, 311300, China
[b] Key Laboratory of Applied Technology on Green-Eco-Healthy Animal Husbandry of Zhejiang Province, College of Animal Science and Technology, College of Veterinary Medicine, Zhejiang Agriculture and Forestry University, Hangzhou, 311300, China

ARTICLE INFO

Keywords:
Hizikia fusiforme
White spot syndrome virus
Innate immunity
Procambarus clarkii

ABSTRACT

The sea vegetable *Hizikia fusiforme* is not only a good source of dietary fiber but also enhances immunity. In this study, we investigated the effects of *H. fusiforme* on innate immunity in invertebrates, using white spot syndrome virus (WSSV) challenge in the crayfish, *Procambarus clarkii*. Supplementation with *H. fusiforme* significantly reduced mortality caused by WSSV infection and also reduced copy numbers of the WSSV protein VP28. Quantitative reverse transcription-polymerase chain reaction showed that supplementation of feed with *H. fusiforme* increased the expression of immune-related genes, including NF-κB and crustin 1. Further analysis showed that supplementation with *H. fusiforme* also affected three immune parameters, total hemocyte count, and phenoloxidase and superoxide dismutase activity. *H. fusiforme* treatment significantly increased hemocyte apoptosis rates in both WSSV-infected and uninfected crayfish. *H. fusiforme* thus regulates the innate immunity of crayfish, and both delays and reduces mortality after WSSV challenge. Our study demonstrates the potential for the commercial use of *H. fusiforme*, either therapeutically or prophylactically, to regulate the innate immunity and protect crayfish against WSSV infection.

1. Introduction

The freshwater crayfish, *Procambarus clarkii*, is native to northern Mexico and the southern and southeastern United States [1]. This species has now been introduced into many areas of China and has become commercially important in freshwater aquaculture [2]. With the rapid development of crayfish pond culture and a comprehensive rice shrimp breeding industry, white spot syndrome (WSS) has become increasingly prevalent in crayfish. WSS is devastating to the shrimp industry and has dramatically reduced shrimp production worldwide [3,4]. Mortality rates are typically very high, and cumulative mortality can reach 100% within 3–10 days from the onset of gross visible signs of disease [3,4]. White spot syndrome virus (WSSV), a rod-shaped virus belonging to the genus *Whispovirus* of the *Nimaviridae* family, has a wide geographical distribution and host range [5,6]. All major cultivated species of penaeid shrimp can be naturally infected by WSSV [7–9], and both natural and experimental infections of other crustaceans have also been reported [10,11]. WSSV begins replication in host cells soon after infection and outbreaks of WSS cause extremely high mortality rates, leading to large economic losses [5]. The clinical symptoms of WSS include loss of appetite, lethargy, and the appearance of white spots on the exoskeleton [12,13]. Because of its economic importance, there is an urgent need to develop safe new treatments to reduce the mortality rate following WSSV infection.

Hizikia fusiforme is a sea vegetable that can be eaten fresh, but is mostly dried and rehydrated before use [14]. *H. fusiforme* and its extract show strong anti-tumor, anti-oxidant, anti-inflammatory, anti-melanogenesis, anti-diabetic and immunomodulatory activity [15–24]. Since dietary *H. fusiforme* has been shown to enhance growth and immunity of juvenile olive flounder, *Paralichthys olivaceus* [25], we decided to investigate whether it would inhibit replication of WSSV and reduce mortality in crayfish. To determine whether *H. fusiforme* is an effective antiviral additive, we measured innate immunity and immunological indicators before and after treatment, as well as copy numbers of the viral protein VP28 and mortality and apoptosis after WSSV infection. We found that *H. fusiforme* regulates innate immunity, inhibits viral replication and reduces mortality in crayfish challenged with WSSV.

* Corresponding author.
E-mail address: zhufei@zafu.edu.cn (F. Zhu).

https://doi.org/10.1016/j.fsi.2020.04.050
Received 24 February 2020; Received in revised form 17 April 2020; Accepted 22 April 2020
Available online 26 April 2020

2. Materials and methods

2.1. Crayfish, H. fusiforme and pathogens preparation

Healthy crayfish (approximately 15 g and 10 cm each) were purchased from a crayfish breeding company in Anhui, China. In order to adapt the crayfish to the new environment, the crayfish were bred for a week before the experiment, they were kept in tanks with fresh water and fed with commercial pellet feed at 5% of body weight per day, the water temperature were set at 25 ˚C. Hemolymph and gill tissues from cultured crayfish were subjected at random to PCR detection with WSSV-specific primers to ensure that the crayfish were WSSV-free. *Hizikia fusiforme* powder used in this study was purchased from Baiyuheng Biotechnology (Guangzhou, China). The commercial pellet feed was purchased from South Ranch (Henan, China). The preparation method of *H. fusiforme* additive feed is as follows: firstly, commercial pellet feed is crushed by disintegrator; secondly, crushed pellet feed is mixed with *H. fusiforme* in a certain proportion, added super-pure water to knead into 'dough', which is formed in dough press; finally, that is dried in an oven (40 ˚C). Oral use, *H. fusiforme* was added to commercial pellet feed for crayfish at a dose of 50, 60 and 70 mg/kg, stored at room temperature and kept dry.

WSSV used in this study originated from infected crayfish and was reserved at −80 ˚C as described previously [26]. The crayfish were randomly selected for WSSV challenge from the tanks, which were infected with WSSV. The minced meat of WSSV-infected crayfish used in the challenge experiments was prepared according to the previous study [27].

2.2. H. fusiforme treatment and WSSV challenge

Crayfish were randomly transferred into separate tanks as different groups. In the *H. fusiforme* treatment experiment, crayfish were fed with *H. fusiforme* additive feed every 24 h as the *H. fusiforme* group. Crayfish fed with commercial pellet feed as the control group. To determine the influence of *H. fusiforme* on the innate immunity of crayfish, the hemolymph of each group was collected after feeding to analyze gene expression and immune parameters. To determine the role of *H. fusiforme* in the WSSV infection process, crayfish in each group were fed with minced meat of WSSV-infected crayfish after 72 h of *H. fusiforme* treatment. Crayfish fed with commercial pellet feed alone were defined as the control group, and those fed with 50, 60 or 70 mg/kg *H. fusiforme* additive feed alone were defined as the 50 mg/kg *H. fusiforme*, 60 mg/kg *H. fusiforme*, or 70 mg/kg *H. fusiforme* groups, respectively. The results of pre-experiment about the dose of *H. fusiforme* showed that the mortality of crayfish fed with 30, 40, 50, 60 or 70 mg/kg *H. fusiforme* was 100%, 100%, 70%, 50% and 80% after WSSV challenge. After 72 h of *H. fusiforme* treatment, commercial pellet feed + minced meat, 50 mg/kg *H. fusiforme* additive feed + minced meat, 60 mg/kg *H. fusiforme* additive feed + minced meat, 70 mg/kg *H. fusiforme* additive feed + minced meat were challenged with minced meat of WSSV-infected crayfish. Crayfish fed with commercial pellet feed and minced meat was defined as the WSSV group. Crayfish fed with 50, 60 or 70 mg/kg *H. fusiforme* additive feed and minced meat were defined as the WSSV +40 mg/kg *H. fusiforme*, WSSV +50 mg/kg *H. fusiforme*, or the WSSV +60 mg/kg *H. fusiforme* groups, respectively. Based on the requirements of different analysis, crayfish samples were collected at different times after *H. fusiforme* or WSSV treatment. Experiments described above were all repeated three times. The mortality of experimental crayfish was recorded and analyzed according to the previous study [27].

2.3. WSSV replication analysis

The detection of WSSV copies in hemocytes of crayfish was performed according to the previous study [27].

Table 1
Real-time quantitative PCR primer sequences of immune signal pathways related genes in the hemocytes of *Procambarus clarkii*.

Primer Name	Primer Seqeunce (5′ to 3′)
β-Actin-F	ACCACTGCCGCCTCATCCTC
β-Actin-R	CGGAACCTCTCGTTGCCAATGG
Toll-like receptor-F	TTGCGTAGTGACTTGTGGAGC
Toll-like receptor-R	CTACTGTAACGCAGGCGATGG
NF-kappa B–F	TAGTGCGTGATGATGGGTCTT
NF-kappa B-R	GCTGATTATGGAGGCAGAAAA
crustin 1-F	CCACAGATGGCAATCGGAGTC
crustin 1-R	AGGGAACGAACGCTGGAAAGT
C-type-lectin-F	ACTTTGCTAACGCCAATCCAC
C-type-lectin-R	CTACGCTGTCATCGACGAACC

2.4. Expression analysis by real-time quantitative PCR

Crayfish hemocytes were collected from each group 24 h after feeding with 60 mg/kg *H. fusiforme*. For detect the expression of immune-related genes, the real-time quantitative PCR was performed following the previous study [28]. The gene expression level was calculated with the $2^{-\Delta\Delta CT}$ method [29], the amplification cycle of β-actin was used as an internal control to calculate the relative expression level. Expression levels of genes of the control group were used as index 1.

Five innate immune-related genes, Toll-like receptor (KP259728.1), proPO (MH156427.1), NF-kappa B (KF662471.1), crustin 1 (GQ301201.1), and C-type-lectin (KC857544.1), were selected to detect the influence of *H. fusiforme* treatment on the innate immune system. The design and synthesis of the RT-qPCR primers were entrusted to the Generay company (Shanghai, China). The primer sequences are listed in Table 1.

2.5. Total hemocyte count, phenoloxidase activity, and superoxide dismutase activity

Three immune parameters, total hemocyte count (THC), phenoloxidase (PO) activity and superoxide dismutase (SOD) activity were performed to analyze the innate immunity of the experimental crayfish. At 24 and 48 h after WSSV challenge, crayfish hemolymph was collected from each group and combined at a ratio of 1:1 with an anticoagulant (20 mM EDTA, pH 5.6), and the hemolymph mixtures were kept on ice constantly. The hemolymph (100 μL) was withdrawn from the ventral sinus of each crab into a 1 mL syringe containing 100 μL of 0.45 M NaCl (10% Methanal) in and transferred to a microfuge tube immediately. The hemocyte count was performed using a hemocytometer and defined as number of cells mL^{-1}, and the data presented as the total hemocyte count [30]. The PO activity of the experimental crayfish was quantified in the hemolymph mixture based on the formation of dopa chrome from L-3, 4-dihydroxyphenylalanine (L-DOPA), as described previously [31]. The SOD activity of the experimental crayfish was quantified in hemocytes isolated from the hemolymph mixture, according to the method described by Beauchamp and Fridovich [32]. The data was presented as measurements.

2.6. Apoptosis analysis

An apoptosis assay was conducted using Annexin V (Invitrogen, USA) according to an optimized method based on the manufacturer's protocol. The following manipulation steps were according to the previous study [27]. The apoptosis rate of the control group and the 60 mg/kg *H. fusiforme* group was measured after feeding the corresponding feed for 72 h. After feeding commercial pellet feed and *H. fusiforme* additive feed for 72 h, the apoptosis rate of WSSV group and WSSV +60 mg/kg *H. fusiforme* group was detected at 24 h after WSSV challenge. The data were presented as means ± standard deviation (SD) derived from three independent experiments.

2.7. Statistical analysis

Every experiment was performed three times as biological repeats to exclude variation from weather, individual difference, and anthropogenic operation. Quantitative data were expressed as mean ± standard deviation (SD). The data from three independent experiments were analyzed by one-way analysis of variance (ANOVA) to calculate the mean and standard deviation of the triplicate assays. All statistics were measured using SPSS software version 19 (IBM, USA). The differences between the different treatments were analyzed by multiple *t*-test method. Statistical significance was defined as *P < 0.05, **P < 0.01.

3. Results

3.1. Effects of H. fusiforme on survival rate of WSSV-challenged crayfish

Crayfish survival rates and viral copy numbers were used to demonstrate the protective effect of *H. fusiforme* in the WSSV challenge experiment. Crayfish survival was recorded at 12 h post infection. In uninfected animals, the survival rates in the untreated group and the group treated with 60 mg/kg *H. fusiforme* group were 90% and 100%, respectively, showing that this dose of *H. fusiforme* has no effect on the mortality of the crayfish. Crayfish challenged with WSSV were all dead at 168 h post infection but survival rates were significantly improved ($p < 0.01$) by treatment with 50, 60 or 70 mg/kg *H. fusiforme*. Survival rates were highest in the group treated with 60 mg/kg *H. fusiforme*; 50% of the crayfish in this group were alive at 168 h post infection (Fig. 1). Further studies were carried using 60 mg/kg *H. fusiforme* since this dose gave the highest survival rate in the WSSV challenge experiment.

3.2. Effects of H. fusiforme on WSSV copy numbers

WSSV copy numbers were measured at 0, 24, 48, 72 and 96 h post WSSV challenge. Viral copy numbers in both untreated crayfish and crayfish treated with 60 mg/kg *H. fusiforme* reached 1×10^5 at 24 h post infection, and then gradually increased from 24 to 96 h. Copy numbers were significantly lower in the treated group at 24, 72 and 96 h post infection. We concluded that *H. fusiforme* has some inhibitory effect on viral replication but that the reduction in crayfish mortality may not be directly due to inhibition of WSSV (Fig. 2).

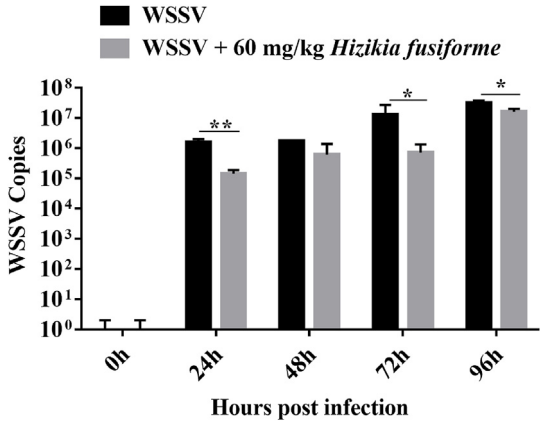

Fig. 2. WSSV copy numbers were measured 0, 24, 48, 72 and 96 h after WSSV challenge. The experiment was repeated three times as biological replicates. Values are presented as means, with standard deviations shown as error bars. Asterisks indicatesignificant differences between the untreated group and the group treated with60 mg/kg *H. fusiforme* group. *p < 0.05, **p < 0.01, analyzed by multiple *t*-test.

3.3. Effects of H. fusiforme on expression of immune pathway genes

Analysis of five immune-related genes by real-time reverse transcription-polymerase chain reaction (RT-PCR) showed that expression of crustin 1 and NF-κB was significantly up-regulated ($p < 0.05$) following treatment with 60 mg/kg *H. fusiforme*, whereas expression of C-type lectin was significantly down-regulated ($p < 0.01$) (Fig. 3). There was no significant change in the expression of proPO or Toll-like receptor. These results suggest that *H. fusiforme* may induce certain immune pathways in crayfish.

3.4. Effects of H. fusiforme on immune parameters

Total hemocyte count (THC), together with phenoloxidase (PO) and superoxide dismutase (SOD) activities, was measured at 24 and 48 h after administration of commercial feed (control group), commercial

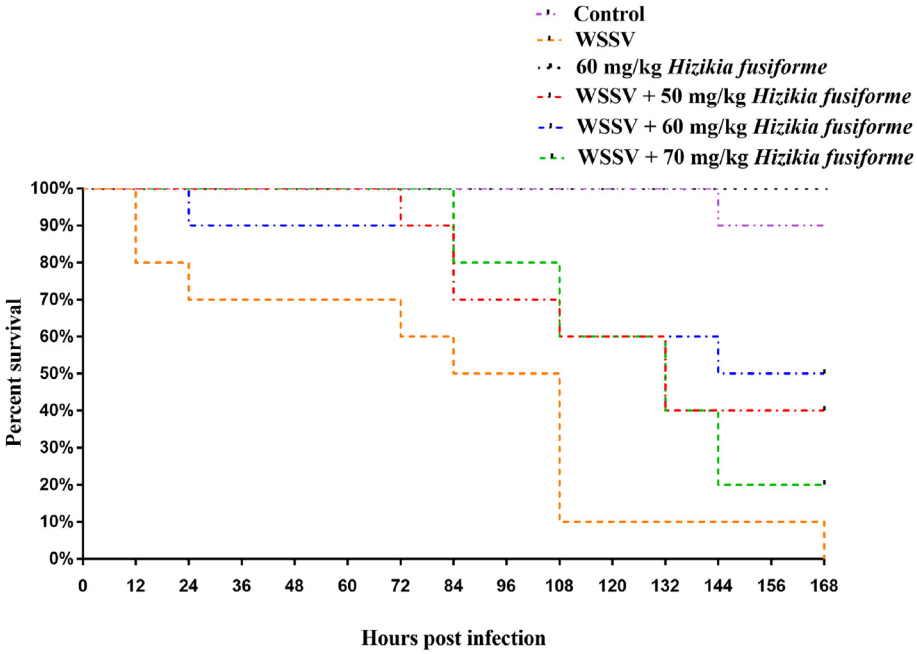

Fig. 1. Survival rates of WSSV-challenged crayfish with/without *Hizikia fusiforme* treatment. Crayfish were fed with commercial feed (control) or feed containing minced meat from WSSV-infected crayfish. *H. fusiforme* (50, 60 or 70 mg/kg) was added to the feed as shown above. Each group contained at least 15 individuals. The survival rate in each group was recorded every 12 h for 168 h.

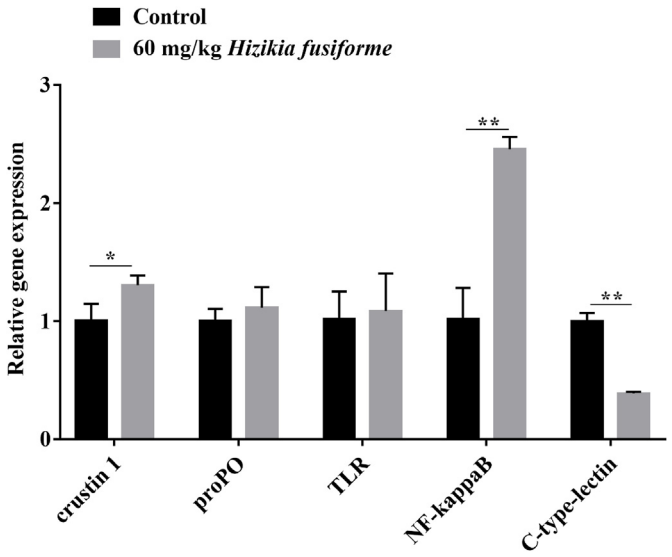

Fig. 3. RT-PCR analysis of expression of immune genes in crayfish. Expression of immune-related genes (Toll-like receptors (TLR), proPO, NF-κB, crustin 1 and C-type lectin) in crayfish hemocytes was measured 24 h after treatment with 60 mg/kg *H. fusiforme*. The mRNA expression was normalized to the β-actin transcript level. Data are presented as means ± standard deviations of three separate individuals. Asterisks indicate significant differences between the 60 mg/kg *H. fusiforme* group and the control group. *P < 0.05, **P < 0.01), analyzed by multiple *t*-test.

feed + 60 mg/kg *H. fusiforme*, WSSV-infected feed or WSSV-infected feed +60 mg/kg *H. fusiforme*. THC in the 60 mg/kg *H. fusiforme* group was significantly increased ($p < 0.01$) at 24 h, but significantly reduced ($p < 0.05$) at 48 h, compared with the control group (Fig. 4a). THC was significantly higher ($p < 0.05$), both at 24 and 48 h, in the WSSV-infected group compared with the control group (Fig. 4b). From 24 to 48 h after infection, THC in the WSSV +60 mg/kg *H. fusiforme* group increased from 1.08×10^6/mL to 1.53×10^6/mL and THC in the WSSV group increased from 8.3×10^5/mL to 1.9×10^6/mL.

There was no significant difference in PO activity between the control group and the 60 mg/kg *H. fusiforme* group at 24 h after treatment whereas PO activity in the treated group was significantly higher ($p < 0.01$) than in the control group at 48 h after treatment (Fig. 4c). PO activity was increased after WSSV challenge, indicating that WSSV infection temporarily enhanced PO activity in hemolymph. PO activity was significantly reduced ($p < 0.01$) in the WSSV + 60 mg/kg *H. fusiforme* group at 48 h after infection (Fig. 4d), showing that 60 mg/kg *H. fusiforme* blocked the effect of WSSV on PO activity and restored activity to a level similar to that of the control group.

The control group and the 60 mg/kg *H. fusiforme* group had similar SOD activity at 24 h after treatment. The 60 mg/kg *H. fusiforme* group, however, showed significantly lower ($p < 0.01$) SOD activity than the control group 48 h after treatment (Fig. 4e). SOD activity was significantly reduced ($p < 0.01$) both 24 and 48 h after WSSV challenge (Fig. 4f). In the WSSV challenge group treated with 60 mg/kg *H. fusiforme*, SOD activity remained at a similar level to that in the control group, but was significantly higher ($p < 0.01$) than in the WSSV challenge group, indicating that 60 mg/kg *H. fusiforme* may block the effect of WSSV on SOD activity.

3.5. Effects of H. fusiforme on apoptosis of hemocytes

Rates of hemocyte apoptosis in the presence and absence of 60 mg/kg *H. fusiforme* were measured using flow cytometry. The apoptosis rate in the 60 mg/kg *H. fusiforme* group was significantly higher ($p < 0.01$) than in the control group (Fig. 5a), indicating that *H. fusiforme* induces apoptosis of crayfish hemocytes. Hemocytes from treated crayfish are very fragile and we tried very hard to minimize damage and apoptosis caused by centrifugation and the liquid impact associated with pipetting and subsequent re-suspension. In the WSSV-infected group, the apoptosis rate increased to an average of 59.7%. In the WSSV + *H. fusiforme* group, the apoptosis rate was further increased to 69.9%, which was significantly higher than in the WSSV group ($p < 0.01$) (Fig. 5b).

4. Discussion

All animals have gradually evolved a series of mechanisms to protect themselves from viral attack. In crustaceans, innate immunity plays an important role in defense against viral infection and apoptosis is an important component of this response [33]. In the present study, we showed that *H. fusiforme* can directly regulate the innate immunity of crayfish.

We first investigated whether *H. fusiforme* can protect crayfish from infection with WSSV, which is the main pathogen for most crustaceans [5]. We found that the mortality rate of crayfish treated with *H. fusiforme* was significantly lower than that of untreated crayfish after WSSV infection, and that the best dose of *H. fusiforme* was 60 mg/kg. Treatment with *H. fusiforme* also significantly reduced WSSV copy numbers, although it is unclear whether this is caused by direct inhibition of viral replication or by an effect on the innate immunity of the crayfish. We next investigated the effect of adding 60 mg/kg *H. fusiforme* as a feed supplement for 24 h on expression levels of four important immune genes in crayfish. Expression levels of NF-κB and crustin 1 were significantly up-regulated, whereas expression of C-type lectin was significantly down-regulated. During viral infection, a series of signaling pathways can be triggered in the host, leading to apoptosis of infected cells [34,35]. NF-κB is involved in the transcriptional regulation of a variety of apoptosis-related genes and can either inhibit or promote apoptosis [36]. Polysaccharides from extracts of *H. fusiforme* have been shown to regulate NF-κB signaling pathways in human dermal fibroblasts [37]. After WSSV infection in crayfish, treatment with 60 mg/kg *H. fusiforme* significantly increased total hemocyte counts and hemocyte apoptosis rates, which may be associated with upregulation of NF-κB. *H. fusiforme* thus enhances apoptosis and also reduces mortality in WSSV-infected crayfish, suggesting that inhibition of WSSV replication may be due to regulation of apoptosis. Crustin plays an important role in innate immunity and could potentially be used as an antibacterial agents in shrimps [38]. Knockdown of shrimp crustin using RNAi has been shown to inhibit WSSV replication and to reduce mortality, WSSV copy numbers and expression of WSSV immediate early genes (IE1, IE2, DNA polymerase, VP28) [39]. We found that expression levels of crustin 1 in crayfish were significantly increased by treatment with *H. fusiforme*. C-type lectin plays a central role in the host immune response to pathogens and has been shown to have antiviral and antibacterial activity in shrimps [40,41]; many other members of the lectin family have also been shown to be involved in the response to viral infection [42,43]. The C-type lectin LvCTL1 in *Litopenaeus vannamei* has been shown to protect against WSSV infection, possibly by interaction of LvCTL1 with several viral proteins [43]. We found, however, that treatment of crayfish with *H. fusiforme* led to significantly reduced expression of C-type lectin. This is in agreement with a study that showed that C-type lectin acts with calreticulin to facilitate WSSV infection in shrimp [44]. The down-regulation of C-type lectin expression caused by treatment with *H. fusiforme* thus seems to be beneficial for the immune response of crayfish to viral infection.

To investigate the effect of *H. fusiforme* on the innate immunity of crayfish, we compared the immunological activity of crayfish fed *H. fusiforme* before and after WSSV challenge. Hemocyte, an important tissue for the innate immunity of crustaceans, is involved in cellular immunity and humoral immunity, and can directly recognize and engulf foreign pathogens [45]. Treatment with *H. fusiforme* increased total

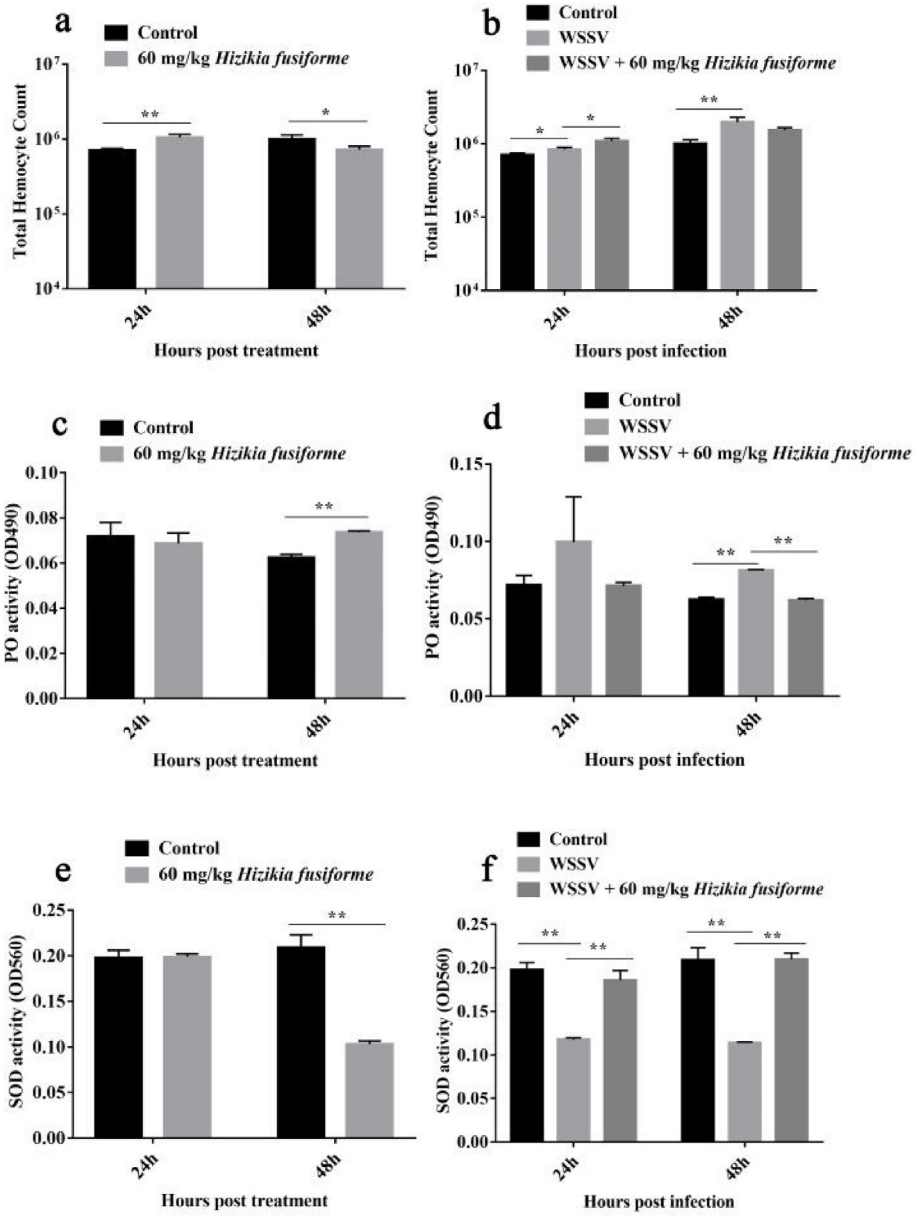

Fig. 4. Effects of WSSV challenge and 60 mg/kg *H. fusiforme* on immune parameters. Crayfish received commercial feed (control), commercial feed + 60 mg/kg *H. fusiforme*, WSSV-infected feed, or WSSV-infected feed + 60 mg/kg *H. fusiforme*. Three immune-related parameters, total hemocyte count (THC), phenol oxidase (PO) activity and superoxide dismutase (SOD) activity, were compared before and after WSSV challenge. In the WSSV-infected feed +60 mg/kg *H. fusiforme* group, the crayfish were challenged with WSSV-infected feed 72 h after receiving commercial feed +60 mg/kg *H. fusiforme*. Effects of 60 mg/kg *H. fusiforme* on (a) THC, (c) PO activity and (e) SOD activity in healthy crayfish. Effects of 60 mg/kg *H. fusiforme* on (b) THC, (d) PO activity and (f) SOD activity in WSSV-infected crayfish. Each value at each time point represents date for at least three individual crayfish. Data are presented as mean values with standard deviations. Asterisks indicate a significant difference between two groups when analyzed by a multiple *t*-test. *P < 0.05, **P < 0.01.

hemocyte counts in crayfish after WSSV challenge. Treatment with *H. fusiforme* also increased PO activity showing that treatment with *H. fusiforme* promotes the proPO pathway. However, the PO activity of *H. fusiforme* treatment was reduced by WSSV infection and showed no difference with that of the control. PO, the terminal enzyme of the proPO pathway, converts tyrosine to dihydroxyphenylalanine and then to dopaquinone and melanin [46]. PO activity is very important for the innate immune defense of crustacean against invading pathogens [47]. In the WSSV challenge experiments, SOD activity, which is very important for the stability and health of crayfish, was significantly higher in crayfish treated with 60 mg/kg *H. fusiforme* group than in untreated animals. Although reactive oxygen species (ROS) released during the innate immune response can destroy bacteria and viruses, large amounts of ROS can also nonspecifically attack host proteins, lipids and DNA, leading to cell damage [48]. To avoid ROS self-injury, SOD is activated as part of the antioxidant defense to remove superoxide anions and maintain homeostasis during the immune response [49]. Our results indicate that *H. fusiforme* reduces crayfish mortality mainly by enhancing innate immunity, rather than by a direct inhibitory effect on WSSV replication.

In conclusion, we have shown that supplementation with H. fusiforme promotes innate immunity of crayfish, especially cellular immunity following WSSV infection, by activating several innate immune pathways. Treatment with *H. fusiforme* also inhibited WSSV replication and reduced mortality in infected crayfish.

CRediT authorship contribution statement

Xinyue Jiang: Investigation, Data curation, Writing - original draft, Validation, Software. **Wenxin Jin:** Investigation, Data curation, Validation, Software. **Fei Zhu:** Conceptualization, Methodology, Investigation, Writing - review & editing.

Acknowledgments

This work was financially supported by Basic Public Welfare Research Project of Zhejiang Province (LY20C190001).

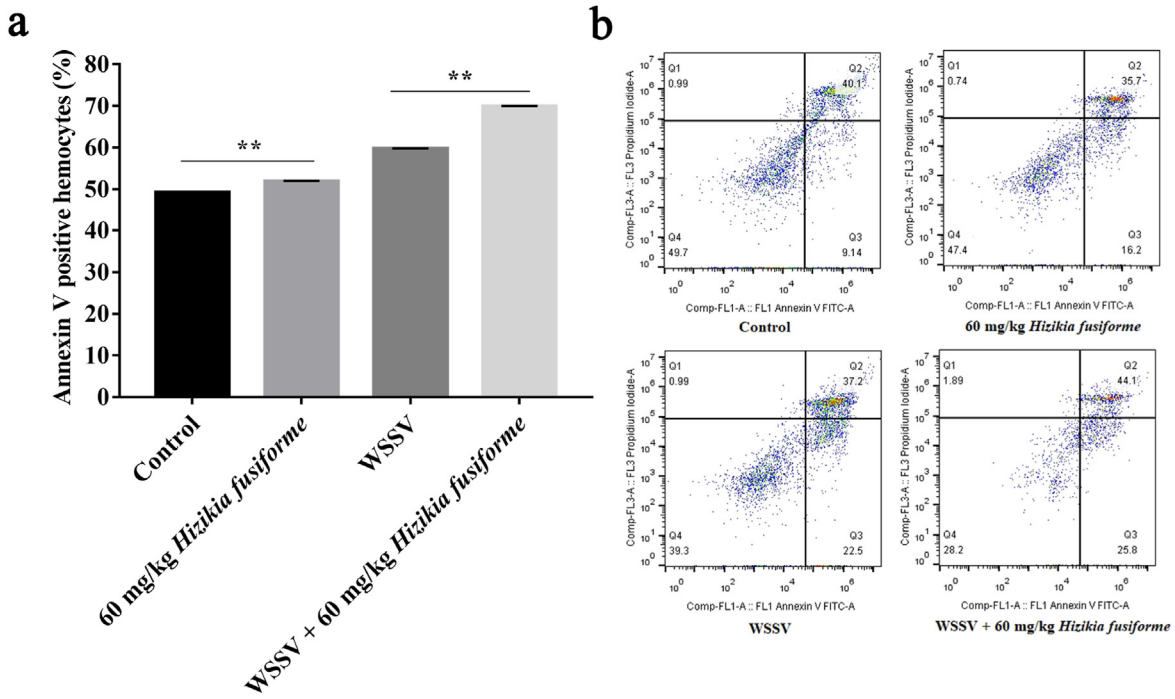

Fig. 5. Apoptosis of hemocytes in crayfish treated with *H. fusiforme*, analyzed by flow cytometry. Crayfish were treated with commercial feed or 60 mg/kg *H. fusiforme*, followed by challenge with WSSV. At 24 h post challenge, hemocyte samples from the different treatment groups were stained with Annexin-V FITC and propidium iodide (PI) for detection by flow cytometry. Fluorescent 1-Annexin V (FL1-A5) indicates apoptotic cells and fluorescent 3-PI (FL3-PI) indicates dead or damaged cells. Determination of the threshold was based on the empty control, negative control, and positive control. The percentage of Annexin V-positive cells indicates the apoptosis rate of the hemocytes. (a) Bar chart showing apoptosis rate of control group, 60 mg/kg *H. fusiforme* group, WSSV challenge group and WSSV + 60 mg/kg *H. fusiforme* group. (b) Scatter plots of one of the challenged groups, each column represents the mean value of three isolated repeats. Q1 represents cell fragments caused by centrifugation and resuspension, Q2 represents late stage apoptosis, Q3 represents early stage apoptosis and Q4 represents normal cells. The apoptosis rate of the sample was determined by the total fluorescence intensity of Q2 and Q3. Asterisks indicate a significant difference between two groups when analyzed by a multiple *t*-test. *P < 0.05, **P < 0.01.

References

[1] D.J. Harris, K.A. Crandall, Intragenomic variation within ITS1 and ITS2 of freshwater crayfishes (Decapoda: Cambaridae): implications for phylogenetic and microsatellite studies, Mol. Biol. Evol. 17 (2000) 284–291.

[2] D.M. Holdich, A review of astaciculture: freshwater crayfish farming, Aquat. Living Resour. 6 (1993) 307–317.

[3] H.Y. Chou, C.Y. Huang, C.H. Wang, et al., Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan, Dis. Aquat. Org. 23 (1995) 165–173.

[4] C. Wongteerasupaya, J.E. Vickers, S. Sriurairatana, et al., A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*, Dis. Aquat. Org. 21 (1995) 69–77.

[5] C.M. Escobedo-Bonilla, V. Alday-Sanz, M. Wille, et al., A review on the morphology, molecular characterization, morphogenesis and pathogenesis of white spot syndrome virus, J. Fish. Dis. 31 (2008) 1–18.

[6] J.H. Leu, F. Yang, X. Zhang, et al., Whispovirus, Curr. Top. Microbiol. Immunol. 328 (2009) 197–227.

[7] K. Inouye, S. Miwa, N. Oseko, et al., Mass mortalities of cultured kuruma shrimp *Penaeus japonicus* in Japan in 1993: electron microscopic evidence of the causative virus, Fish Pathol. 29 (1994) 149–158.

[8] C.V. Mohan, K.M. Shankar, S. Kulkarni, P.M. Sudha, Histopathology of cultured shrimp showing gross signs of yellow head and white spot syndrome during 1994 India epizootics, Dis. Aquat. Org. 34 (1998) 9–12.

[9] Y.G. Wang, M.D. Hassan, M. Shariff, S.M. Zamri, X. Chen, Histopathology and cytopathology of white spot syndrome virus (WSSV) in cultured *Penaeus monodon* from peninsular Malasia with emphasis on pathogenesis and the mechanism of white spot formation, Dis. Aquat. Org. 39 (1999) 1–11.

[10] P.S. Chang, H.C. Chen, Y.C. Wang, Detection of white spot syndrome associated baculovirus in experimentally infected wild shrimp, crab and lobsters by in situ hybridization, Aquaculture 164 (1998) 233–242.

[11] Q. Wang, L.M. Nunan, D.V. Lightner, Identification of genomic variations among geographic isolates of white spot syndrome virus using restriction analysis and southern blot hybridization, Dis. Aquat. Org. 43 (2000) 175–181.

[12] B. Verbruggen, L. Bickley, R. van Aerle, et al., Molecular mechanisms of white spot syndrome virus infection and perspectives on treatments, Viruses 8 (2016) 1–30.

[13] J. Huang, X.L. Song, J. Yu, C.H. Yang, Baculoviral hypodermal and hematopoietic necrosis-study on the pathogen and pathology of the explosive epidemic disease of shrimp, Mar. Fish. Res. 16 (1995) 1–10.

[14] M.L. Wells, P. Potin, J.S. Craigie, et al., Algae as nutritional and functional food sources: revisiting our understanding, J. Appl. Phycol. 29 (2017) 949–982.

[15] K. Jung, E. Ha, Y. Uhm, et al., Suppressive effect by *Hizikia fusiforme* on the production of tumor necrosis factor in BV2 murine microglial cells, Neurol. Res. 29 (2007) S88–S92.

[16] Y.L. Dai, Y.F. Jiang, H.G. Lee, et al., Characterization and screening of anti-tumor activity of fucoidan from acid-processed hijiki (*Hizikia fusiforme*), Int. J. Biol. Macromol. 139 (2019) 170–180.

[17] L. Wang, J.Y. Oh, H.S. Kim, et al., Protective effect of polysaccharides from cellulclast-assisted extract of *Hizikia fusiforme* against hydrogen peroxide-induced oxidative stress in vitro in vero cells and in vivo in zebrafish, Int. J. Biol. Macromol. 112 (2018) 483–489.

[18] L. Wang, J.Y. Oh, T.U. Jayawardena, et al., Anti-inflammatory and anti-melanogenesis activities of sulfated polysaccharides isolated from *Hizikia fusiforme*: short communication, Int. J. Biol. Macromol. 142 (2020) 545–550.

[19] S.Y. Kang, E. Kim, I. Kang, et al., Anti-diabetic effects and anti-inflammatory effects of *Laminaria japonica* and *Hizikia fusiforme* in skeletal muscle: in vitro and in vivo model, Nutrients 10 (2018) pii: E491.

[20] S.C. Jeong, Y.T. Jeong, S.M. Lee, et al., Immune-modulating activities of polysaccharides extracted from brown algae *Hizikia fusiforme*, Biosci. Biotechnol. Biochem. 79 (2015) 1362–1365.

[21] S.Y. Park, E. Hwang, Y.K. Shin, et al., Immunostimulatory effect of enzyme-modified *Hizikia fusiformein* a mouse model in vitro and ex vivo, Mar. Biotechnol. 19 (2017) 65–75.

[22] Y.D. Yoon, E.S. Lee, J.P. Park, et al., Immunostimulatory effect by aqueous extract of *Hizikia fusiforme* in RAW 264.7 macrophage and whole spleen cells, Biotechnol. Bioproc. Eng. 16 (2011) 1099–1105.

[23] S.O. Kim, Y.H. Choi, The ethyl alcohol extract of *Hizikia fusiforme* inhibits matrix metalloproteinase activity and regulates tight junction related protein expression in Hep3B human hepatocarcinoma cells, J. Med. Food 13 (2010) 31–38.

[24] J.H. Park, J.W. Choi, M.K. Lee, et al., Protective effects of *Hizikia fusiforme* and *Chlorella sp.* extracts against lead acetate-induced hepatotoxicity in rats, Fish. Aquat. Sci. 22 (2019) 2.

[25] Y.H. Choi, K.W. Kim, H.S. Han, et al., Dietary *Hizikia fusiformis* glycoprotein-induced IGF-I and IGFBP-3 associated to somatic growth, polyunsaturated fatty acid metabolism, and immunity in juvenile olive flounder *Paralichthys olivaceus*, Comp. Biochem. Physiol. Mol. Integr. Physiol. 167 (2014) 1–6.

[26] F. Zhu, X. Zhang, The Wnt signaling pathway is involved in the regulation of phagocytosis of virus in *Drosophila*, Sci. Rep. 3 (2013) 2069.

[27] X.Y. Qian, F. Zhu, Hesperetin protects crayfish *Procambarus clarkii* against white spot syndrome virus infection, Fish Shellfish Immunol. 93 (2019) 116–123.
[28] Z. Wang, B.Z. Sun, F. Zhu, Epigallocatechin-3-gallate inhibit replication of white spot syndrome virus in *Scylla paramamosain*, Fish Shellfish Immunol. 67 (2017) 612–619.
[29] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (2011) 402–408.
[30] K. Wongprasert, T. Rudtanatip, J. Praiboon, Immunostimulatory activity of sulfated galactans isolated from the red seaweed *Gracilaria fisheri* and development of resistance against white spot syndrome virus (WSSV) in shrimp, Fish Shellfish Immunol. 36 (2014) 52–60.
[31] Z. Zhao, C. Jiang, X. Zhang, Effects of immunostimulants targeting Ran GTPase on phagocytosis against virus infection in shrimp, Fish Shellfish Immunol. 31 (2011) 1013–1018.
[32] C.O. Beauchamp, I. Fridovich, Isozymes of superoxide dismutase from wheat germ, Biochim. Biophys. Acta 317 (1973) 50–64.
[33] S. Akira, S. Uematsu, O. Takeuchi, Pathogen recognition and innate immunity, Cell 4 (2006) 783–801.
[34] L. Deng, T. Adachi, K. Kitayama, Y. Bungyoku, S. Kitazawa, S. Ishido, et al., Hepatitis C virus infection induces apoptosis through a Bax-triggered, mitochondrion-mediated, caspase 3-dependent pathway, J. Virol. 21 (2008) 10375–10385.
[35] L. Wang, B. Zhi, W. Wu, X. Zhang, Requirement for shrimp caspase in apoptosis against virus infection, Dev. Comp. Immunol. 6 (2008) 706–715.
[36] J. Dutta, Y. Fan, N. Gupta, G. Fan, C. Gélinas, Current insights into the regulation of programmed cell death by NF-kappaB, Oncogene 25 (2006) 6800–6816.
[37] L. Wang, W. Lee, J.Y. Oh, et al., Protective effect of sulfated polysaccharides from celluclast-assisted extract of *Hizikia fusiforme* against ultraviolet B-induced skin damage by regulating NF-κB, AP-1, and MAPKs signaling pathways in vitro in human dermal fibroblasts, Mar. Drugs 16 (2018) pii: E239.
[38] M. Li, C. Ma, P. Zhu, et al., A new crustin is involved in the innate immune response of shrimp *Litopenaeus vannamei*, Fish Shellfish Immunol. 94 (2019) 398–406.
[39] B. Sun, Z. Wang, F. Zhu, The crustin-like peptide plays opposite role in shrimp immune response to *Vibrio alginolyticus* and white spot syndrome virus (WSSV) infection, Fish Shellfish Immunol. 66 (2017) 487–496.
[40] X.W. Wang, J.X. Wang, Diversity and multiple functions of lectins in shrimp immunity, Dev. Comp. Immunol. 39 (2013) 27–38.
[41] M. Li, C. Li, C. Ma, et al., Identification of a C-type lectin with antiviral and antibacterial activity from pacific white shrimp *Litopenaeus vannamei*, Dev. Comp. Immunol. 46 (2014) 231–240.
[42] D.D. Chen, X.L. Meng, J.P. Xu, et al., PcLT, a novel C-type lectin from *Procambarus clarkii*, is involved in the innate defense against *Vibrio alginolyticus* and WSSV, Dev. Comp. Immunol. 39 (2013) 255–264.
[43] Z.Y. Zhao, Z.X. Yin, X.P. Xu, et al., A novel C-type lectin from the shrimp *Litopenaeus vannamei* possesses anti-white spot syndrome virus activity, J. Virol. 83 (2009) 347.
[44] X.W. Wang, Y.H. Xu, J.D. Xu, et al., Collaboration between a soluble C-type lectin and calreticulin facilitates white spot syndrome virus infection in shrimp, J. Immunol. 193 (2014) 2106–2117.
[45] L. Cerenius, P. Jiravanichpaisal, H.P. Liu, I. Söderhill, Crustacean immunity, Adv. Exp. Med. Biol. 708 (2010) 239–259.
[46] K. Sritunyalucksana, K. Söderhäll, The proPO and clotting system in crustaceans, Aquaculture 191 (2000) 53–69.
[47] A. Tassanakajon, K. Somboonwiwat, P. Supungul, S. Tang, Discovery of immune molecules and their crucial functions in shrimp immunity, Fish Shellfish Immunol. 34 (2013) 954–967.
[48] P.T. Schumacker, Reactive oxygen species in cancer: a dance with the devil, Canc. Cell 27 (2015) 156–157.
[49] A.I. Campa-Córdova, N.Y. Hernández-Saavedra, R.D. Philippis, F. Ascencio, Generation of superoxide anion and SOD activity in haemocytes and muscle of American white shrimp (*Litopenaeus vannamei*) as a response to beta-glucan and sulphated polysaccharide, Fish Shellfish Immunol. 12 (2002) 353–366.