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Dietary *Bacillus amyloliquefaciens* enhance survival of white spot syndrome virus infected crayfish

Yongyong Lai[a,1], Ming Luo[b,1], Fei Zhu[a,*]

[a] 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
[b] Baiju Avenue 12, Meilan District, Haikou, Hainan Academy of Ocean and Fisheries Sciences, China

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ABSTRACT

Bacillus amyloliquefaciens, which is closely related to *Bacillus subtilis*, produces a series of metabolites that can inhibit the growth of fungi and bacteria. Here, we investigated the effect of *B. amyloliquefaciens* used as a probiotic on the innate immunity of the crayfish *Procambarus clarkii* when challenged with white spot syndrome virus (WSSV). Dietary *B. amyloliquefaciens* supplement significantly reduced the mortality of WSSV-challenged crayfish and reduced copy numbers of WSSV. The quantitative reverse transcription-polymerase chain reaction results showed that *B. amyloliquefaciens* supplement increased the expression of several immune-related genes, including Toll-like receptor, NF-κB and C-type-lectin. Further analysis showed that *B. amyloliquefaciens* supplement also had an effect on three immune parameters, including total hemocyte count, phenoloxidase activity and superoxide dismutase activity. In both infected and uninfected crayfish, *B. amyloliquefaciens* supplement significantly decreased hemocyte apoptosis. Our results showed that *B. amyloliquefaciens* can regulate innate immunity of crayfish and reduce the mortality following WSSV challenge. This study provides a novel insight into the potential for therapeutic or prophylactic intervention with *B. amyloliquefaciens* to regulate crayfish immunity and protect against WSSV infection, and also provides a theoretical basis for the use of probiotics as aquatic feed additives.

1. Introduction

The farming of freshwater crayfish (*Procambarus clarkii*) is mainly carried out in the southern states of the USA, and in Australia and Europe. This species has now been introduced into many areas of China and has become important economically in freshwater aquaculture [1]. With the rapid development of crayfish pond culture and a comprehensive rice shrimp breeding industry, white spot disease (WSD) has become increasingly prevalent in crayfish. White spot syndrome virus (WSSV) has a wide range of hosts, including all cultured prawns, and most wild shrimps and crabs [2–4]. Mortality exceeds 80% and death often occurs within one week. This epizootic disease has resulted in the lowest levels of shrimp production since the industry began [5]. The clinical symptoms of WSD include loss of appetite, lethargy, and the appearance of white spots on the exoskeleton [6]. During WSSV infection, the virus relies on host cell metabolism to complete its life cycle and, in doing so, disrupts the normal metabolism of the host cells [7]. Since WSSV was discovered, some progress has been made in preventing infection. For example, DNA [8] and recombinant protein [9] vaccines have been used to control WSSV infection. These vaccines cannot, however, easily be used in crayfish culture, and effective and convenient drugs are needed to cure WSSV-infected crayfish.

Dietary administration of functional (biologically active) feed additives has recently been suggested as an environmentally friendly approach to enhance the immune response and promote the growth of fish [10,11]. Microbes play a critical role in aquaculture, both at the hatchery and grow-out level, because water quality and disease control are directly affected by microbial activity [12]. Probiotics, which are live microbial feed supplements that benefit the host by improving its intestinal microbial balance [12], are widely prescribed to prevent antibiotics-associated dysbiosis and similar adverse effects [13]. *Bacillus amyloliquefaciens*, which is closely related to *Bacillus subtilis,* produces a series of metabolites that inhibit fungal and bacterial activity and is thus used as a probiotic. Antimicrobial proteins isolated and purified from *B. amyloliquefaciens* can increase the membrane permeability of pathogenic fungi, reduce the activity of human

* Corresponding author.
E-mail address: zhufei@zafu.edu.cn (F. Zhu).
[1] Same contributions to this work.

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immunodeficiency virus type 1 (HIV-1) reverse transcriptase, and inhibit the growth of liver, breast and colon cancer cells [14]. A bioactive peptide produced by *B. amyloliquefaciens* has also been shown to have an inhibitory effect on *Staphylococcus aureus* and *Listeria monocytogenes* [15]. *B. amyloliquefaciens* are active microorganisms that are beneficial to the host and are commonly used as immunomodulators [16]. When used as a feed additive in hybrid sturgeon, *B. amyloliquefaciens* improved both immunity and growth performance [17]. As an aquatic feed additive, *B. amyloliquefaciens* has been shown to be effective against some viruses and pathogens, and also to improve the immune activity of aquatic animals.

Here, we investigate whether using *B. amyloliquefaciens* as a feed additive could reduce mortality in crayfish exposed to WSSV, either by improving the immunity of the crayfish or by inhibiting replication of WSSV.

2. Materials and methods

2.1. Preparation of crayfish, Bacillus amyloliquefaciens and pathogens

Healthy crayfish (approximately 15 g in weight and 10 cm long) were purchased from a crayfish breeding center in Hangzhou, China. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Zhejiang **Agriculture and Forestry** University (Zhejiang, China). In order to allow the crayfish to adapt to their new environment, they were housed in tanks of freshwater for one week before the experiment. The water and air temperatures were maintained at 25 °C and the crayfish were fed with commercial pellet feed at a rate of 5% of body weight per day. The body weight of randomly selected individuals was recorded to calculate the average crayfish weight. Randomly selected hemolymph and gill tissues were subjected to PCR analysis, using WSSV-specific primers, to ensure that the crayfish were free from WSSV. The alive *B. amyloliquefaciens* (4×10^7 CFU/g) was provided by Zhejiang Science and Technology University, China, and stored at −80 °C. The commercial pellet feed used in this study was purchased from South Ranch (Henan, China). To prepare feed containing *B. amyloliquefaciens*, commercial pellet feed was first crushed using a disintegrator mill. The crushed pellets were mixed with sufficient amounts of *B. amyloliquefaciens* to provide doses of 4, 5 and 6 g/kg and enough super-pure water was added. The dough was kneaded, formed into pellets using a dough press, and the pellets were then dried in an oven at 40 °C. Once dry, the pellets were stored at room temperature and kept dry. The additive-containing pellets were fed to the crayfish at *B. amyloliquefaciens* doses rates of 4, 5and 6 g/kg [18].

WSSV (GenBank accession no. AF332093.1) used in this study originated from infected crayfish and was stored at −80 °C, as described previously [19]. Then PCR detection was conducted to collect the positive crayfish. A muscle sample (5 g) from WSSV-positive crayfish was placed in a beaker together with 200 mL TNE Buffer (50 mM Tris; 400 mM NaCl; 5 mM EDTA, pH7.5) containing 1 mM serine protease inhibitor (phenylmethylsulphonylfluoride, Solarbio, China). The mixture was homogenized at 3000 rpm for 15 min using a high speed disperser and the homogenate was then centrifuged at 6000 g for 10 min using a flop-mounted ultrahigh speed freezing centrifuge. The supernatant was removed and centrifuged three more times under the same conditions. The virus was present in the supernatant which basically removes muscle tissue. The final supernatant was passed through a 300-mesh cell sieve, centrifuged at 6000 g for 30 min and then passed through a 450 nm filter membrane to prepare the viral stock.

Ten crayfish were randomly selected from the tanks and infected with WSSV. The prepared WSSV extract was diluted with sterilized PBS to a density of 1×10^5 WSSV copies/mL and each crayfish was injected with 100 μL of the diluted solution. Approximately four or five days after infection, the crayfish started to display a variety of clinical symptoms, including lethargy, reduced food consumption, reduced preening activities, loosening of the cuticle and discoloration of the hepatopancreas. Before the crayfish died, partial appendages were removed from each crayfish for virus testing and infected crayfish were stored in an ultra-low temperature freezer. For virus challenge experiments, we removed WSSV-infected crayfish from the ultra-low temperature freezer, thawed them and minced the muscles to small pieces about the size of peas. The small pieces of muscle contain WSSV were fed to healthy crayfish in the WSSV challenge experiments.

2.2. B. amyloliquefaciens treatment experiment and WSSV challenge

Bacillus amyloliquefaciens was provided by Zhejiang Science and Technology University, China, and stored in 20% glycerol. The mixture bacterial liquid was inoculated into 5.0 mL LB medium (5 g/L peptone,10 g/L yeast extract,10 g/L NaCl in 1.0 L sterile water), inoculation amount of 1%, 37 °C, 220 rpm culture 12 h, transferred to 1.0 L LB medium, inoculated for 12 h under the same condition. After fermentation,the products was sent to spray drying (input temperature of 170 °C, output temperature of 85 °C, evaporation capacity 2 L/h) with corn starch as an auxiliary material (Wproducts:Wcorninlet = 1:10), to harvest *Bacillus amyloliquefaciens* preparation (4×10^7 CFU/g). Crayfish were randomly divided into different groups and transferred to separate tanks. In the treatment experiment, crayfish in the control group were fed commercial pellets and crayfish in the treatment group were fed with pellets supplemented with *B. amyloliquefaciens* every 24 h. To determine the effect of *B. amyloliquefaciens* on crayfish innate immune signaling pathways, hemolymph was collected from each group 24 h post feeding to analyze gene expression. In WSSV challenge experiment, the crayfish were given feed supplemented with *B. amyloliquefaciens* for 72 h and then fed with the minced meat of WSSV-infected crayfish. The crayfish were then divided into eight groups and fed as follows: commercial pellet feed, *B. amyloliquefaciens* supplemented feed (4 g/kg), *B. amyloliquefaciens* supplemented feed (5 g/kg), *B. amyloliquefaciens* supplemented feed (6 g/kg), commercial pellet feed + infected minced meat, *B. amyloliquefaciens* supplemented feed (4 g/kg) + infected minced meat, *B. amyloliquefaciens* supplemented feed (5 g/kg) + infected minced meat, *B. amyloliquefaciens* supplemented feed (6 g/kg) + infected minced meat. The crayfish were then placed back into their separate tanks for further research. Crayfish fed with commercial pellets were defined as the control group. Crayfish fed with 4, 5 or 6 g/kg B. *amyloliquefaciens* supplemented feed were defined as the 4 g/kg B. *amyloliquefaciens*, 5 g/kg B. *amyloliquefaciens* and 6 g/kg B. *amyloliquefaciens* groups, respectively. Crayfish fed with commercial pellets and infected minced meat was defined as the WSSV group. Crayfish fed with 4, 5 or 6 g/kg B. *amyloliquefaciens* supplemented feed and infected minced meat were defined as the WSSV + 4 g/kg B. *amyloliquefaciens*, WSSV + 5 g/kg B. *amyloliquefaciens* and WSSV + 6 g/kg B. *amyloliquefaciens* groups, respectively. Based on the requirements of different analyses, crayfish samples were collected at different times after treatment with *B. amyloliquefaciens* or WSSV.

To analyze the mortality after WSSV challenge, five groups of crayfish were kept in the tanks for 168 h, and the populations were counted every 12 h. Dead crayfish were removed immediately, and the water in the tank was replaced with clean water every day. To ensure statistical accuracy in the analysis of mortality, each group contained 15 individuals. The mortality data were arranged and analyzed using Microsoft GraphPad 5.0. For the analysis of WSSV copy numbers, three crayfish (as a technical repeat) from the control and WSSV-challenge groups were collected 24, 48, 72 and 96 h post-challenge. For the analysis of immune parameters, at least three crayfish (as a technical repeat) from each group were collected 24 and 48 h post-challenge with WSSV. The experiments described above were all repeated three times.

2.3. Quantitative analysis of WSSV

The detection of WSSV copies was based on previous studies

[20,21]. Briefly, DNA was extracted from a mixture of hemocytes collected from three randomly selected crayfish in each group, and TaqMan real-time quantitative PCR was performed. WSSV protein VP28-specific primers (5′-TTGGTTTCATGCCCGAGATT-3′ and 5′-CCTT GGTCAGCCCCTTG-3′) and TaqMan fluorogenic probe (5′-FAM-TGCT GCCGTCTCCAA-TAMRA-3′) were applied. Thermal cycling was performed on an iCycle IQ5 real-time PCR detection system (Bio-RAD, USA).

2.4. Expression analysis by real-time quantitative PCR

Three crayfish were selected from each group for mixed hemocytes collection at 24 h after feeding. Total RNA was extracted from the hemocytes using an RNApure Tissue & Cell kit (CWBIO, China) according to the manufacturer's protocol. In total, 200 ng RNA was applied to cDNA reverse transcription using a ReverTra Ace qPCR RT Master Mix with a gDNA remover kit (Toyobo, Japan). The cDNA of each group was applied to SYBR Green real-time quantitative PCR immediately. A two-step RT-qPCR method was performed using a Bio-Rad Two-Color Real-Time PCR Detection System. The gene expression level was calculated with the $2-\Delta\Delta CT$ method [22], 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 pathway-related genes, Toll-like receptor (KP259728.1), NF-kappa B (KF662471.1), crustin 1 (GQ301201.1), and C-type-lectin (KC857544.1), prophenoloxidase (proPO) (EF595973.1), were selected to detect the potential influence of *B. amyloliquefaciens* treatment on the innate immune system. The design and synthesis of the RT-qPCR primers were entrusted to the Generay Shanghai Company. The primer sequences are listed in Table 1.

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

For confirm the effect of *B. amyloliquefaciens* on the innate immunity of crayfish, the immune parameters included THC, PO and SOD activity were test. THC was determined as described previously. To determine total hemocyte count, hemolymph (100 μL) was withdrawn from the ventral sinus of individual crayfish into a 1 mL syringe containing 100 μL of 10% methanal in 0.45 M NaCl and transferred to a microfuge tube. 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 [23]. PO activity was quantified in the hemolymph mixture based on the formation of dopa chrome from the substrate L-3, 4-dihydroxyphenylalanine (L-DOPA), as described previously [24]. SOD activity was quantified in hemocytes isolated from 300 μL of the hemolymph mixture, according to the improved method described by Beauchamp and Fridovich [25]. The hemocytes were mixed from three crayfish randomly selected from each group. Data were presented as measurements.

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′–3′)
â-Actin-F	ACCACTGCCGCCTCATCCTC
â-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
ProPO-F	CCATAGGACGTTTGTCAGGGA
ProPO-R	GAGGTGGATCAGCCAGCAGTA

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. It first verified that whether *B. amyloliquefaciens* affects crayfish cell apoptosis. The apoptosis rate of the control group and the 5 g/kg B. *amyloliquefaciens* group was measured after feeding the commercial pellet feed and *B. amyloliquefaciens* additive feed for 24 h. Secondly, the apoptosis of the crayfish infected with WSSV after *B. amyloliquefaciens* treatment was confirmed. After feeding commercial pellet feed and *B. amyloliquefaciens* additive feed for 72 h, the apoptosis rate of WSSV group and WSSV + 5 g/kg B. *amyloliquefaciens* group was detected after WSSV challenge for 24 h. At 24 h post WSSV challenge, the hemolymph was drawn using 2 mL syringe with 20 mM of EDTA at a ratio of 1:1 and kept on ice. Samples were centrifuged at $800 \times g$ for 5 min at 4 °C to collect hemocytes. After washing with sterilized PBS, hemocytes were counted and adjusted to a density of 1–5 $\times$ 10^6 cells/mL. Hemocytes were re-suspended in ice-cooled 1 $\times$ binding buffer with Annexin-V FITC and propidium iodide (PI), and incubated at room temperature for 15 min to stain. After staining, the samples were centrifuged to remove residual dye and applied to flow cytometry immediately to avoid cell death. The empty control, negative control, and positive control for threshold values were prepared simultaneously with experimental samples. The data were presented as means ± standard deviation (SD) derived from three independent experiments. The hemocytes were mixed from three crayfish randomly selected from each group.

2.7. Statistical analysis

Quantitative data were expressed as mean ± standard deviation (SD). Data from three independent experiments were analyzed by one-way analysis of variance to calculate the means and standard deviations of the triplicate assays. Statistical differences were estimated using one-way ANOVA followed by least-significant differences (LSD) and Duncan's multiple range test. The differences between the different treatments were analyzed by multiple *t*-test method. All statistics were measured using SPSS software version 19 (IBM, USA). A probability level of 0.01 was used to indicate statistical significance ($P < 0.01$). All graphs were made using Microsoft GraphPad 5.0.

3. Results

3.1. Effects of B. amyloliquefaciens on survival of WSSV-challenged crayfish

Crayfish mortality was measured to determine the effects of *B. amyloliquefaciens* on survival of WSSV-challenged crayfish. There were no deaths in the control group or in the 4 g/kg B. *amyloliquefaciens*, 5 g/kg B. *amyloliquefaciens* and 6 g/kg B. *amyloliquefaciens* groups (data not shown). In the WSSV challenge experiments, crayfish began to die at 24 h after challenge and all crayfish in the WSSV group had died at 156 h after challenge. The mortality in the WSSV + 4 g/kg B. *amyloliquefaciens* and WSSV + 6 g/kg B. *amyloliquefaciens* groups were higher than in the WSSV + 5 g/kg B. *amyloliquefaciens* group. Crayfish in the 5 g/kg B. *amyloliquefaciens* + WSSV group did not die from 132 h to 168 h after challenge, and the eventual survival rate was above 40% (Fig. 1). A follow-up study was conducted with a new 5 g/kg B. *amyloliquefaciens* group based on the results of crayfish mortality.

3.2. Effects of B. amyloliquefaciens on the WSSV copy number

The sampling time for the WSSV copy number experiment was determined by the crayfish mortality, which began at 24 h after challenge.

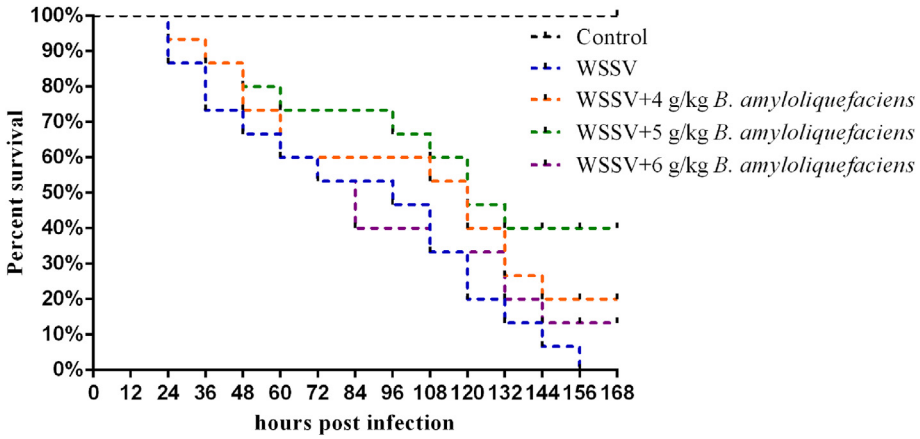

Fig. 1. Survival of WSSV-infected crayfish with/without *B. amyloliquefaciens* supplement. Crayfish were treated with commercial feed (control group), minced meat from WSSV-infected crayfish, or minced meat from WSSV-infected crayfish + *B. amyloliquefaciens*. Each group contained at least 15 individuals. The mortality of crayfish in each group was recorded over 168 h.

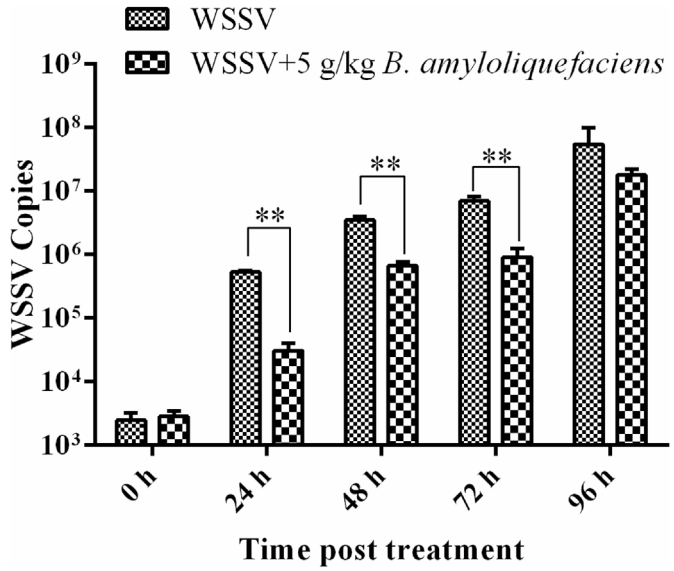

Fig. 2. WSSV copy numbers in hemocytes of crayfish detected 0, 24, 48, 72 and 96 h after WSSV challenge. The experiment was repeated three times. Data are presented as mean value with standard deviations. Asterisks indicate the significance of the difference between the WSSV group and WSSV + *B. amyloliquefaciens* group (*P < 0.05, **P < 0.01), analyzed by a multiple *t*-test.

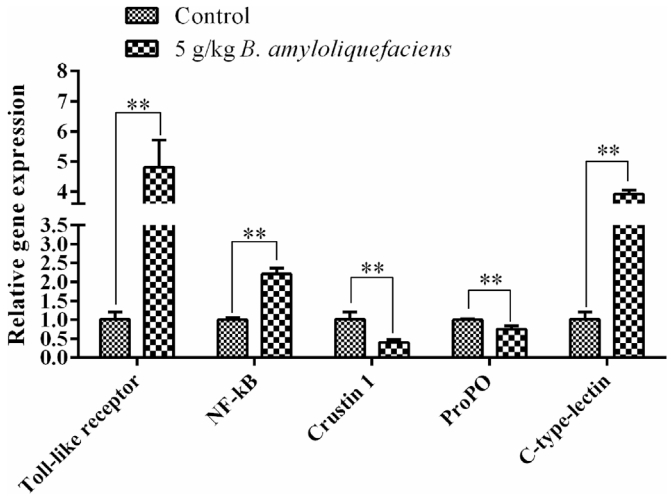

Fig. 3. Analysis of immune gene expression by RT-PCR. Expression of five immune-related genes (Toll-like receptor, NF-κB, crustin1, prophenoloxidase and C-type-lectin) was measured in hemocytes 24 h after treatment with 5 g/kg B. *amyloliquefaciens*. The mRNA expression was normalized to the β-actin transcript level. Data are shown as mean ± standard deviation of three separate individuals. Asterisks indicate a significant difference between the 5 g/kg B. *amyloliquefaciens* group and the control group (*P < 0.05, **P < 0.01), analyzed by a multiple *t*-test.

The sampling times were thus set at 0 h, 24 h, 48 h, 72 h and 96 h after challenge. The WSSV copy number of the WSSV group was significantly higher (P < 0.01) than that of WSSV + 5 g/kg B. *amyloliquefaciens* group at 24 h, 48 h and 72 h (Fig. 2). The WSSV copy number of WSSV + 5 g/kg B. *amyloliquefaciens* group at 96 h was significantly higher (P < 0.01) than at 72 h after challenge, which indicated that the protect effect of *B. amyloliquefaciens* against WSSV infection had been reduced at 96 h after challenge. Although *B. amyloliquefaciens* did show some inhibition of viral replication, it is uncertain whether the reduction in crayfish mortality was due to direct inhibition of WSSV replication.

3.3. Effects of B. amyloliquefaciens on expression of immune-related genes

Analysis of five immune-related genes by real-time reverse transcription polymerase chain reaction (RT-PCR) showed that expression of Toll-like receptor, NF-κB and C-type-lectin was significantly up-regulated (P < 0.01) following treatment with 5 g/kg B. *amyloliquefaciens*, whereas expression of prophenoloxidase and crustin 1 was significantly down-regulated (P < 0.01) (Fig. 3). These results suggested that *B. amyloliquefaciens* might induce certain immune pathways in crayfish.

3.4. Effects of B. amyloliquefaciens on THC, PO activity and SOD activity

There was no significant difference in total hemocyte count (THC) between crayfish in the 5 g/kg B. *amyloliquefaciens* group and the control group at 24 and 48 h after treatment (P = 0.252 at 24 h; P = 0.069 at 48 h) (Fig. 4a). Following challenge with WSSV, THC of WSSV + 5 g/kg B. *amyloliquefaciens* group was significantly higher than that of WSSV group at 48 h after treatment (P = 0.047) (Fig. 4b).

Phenoloxidase (PO) activity in the 5 g/kg B. *amyloliquefaciens* group was significantly lower than in the control group at 48 h (P = 0.002) after treatment (Fig. 5a). In the WSSV challenge experiment, PO activity in WSSV group was significantly higher than in the control group at 24 and 48 h after treatment (P = 0.027 at 24 h; P = 0.0168 at 48 h). When *B. amyloliquefaciens* was fed before WSSV challenge experiment, PO activity in WSSV + 5 g/kg B. *amyloliquefaciens* group was significantly lower than in WSSV group (P = 0.0003 at 24 h; P = 0.0058 at 48 h) (Fig. 5b).

Superoxide dismutase (SOD) activity in the 5 g/kg B. *amyloliquefaciens* group was significantly lower than in the control group at 24 and 48 h after treatment (P = 0.0045 at 24 h; P = 0.0004 at 48 h) (Fig. 6a). In the WSSV challenge experiment, SOD activity in WSSV group was

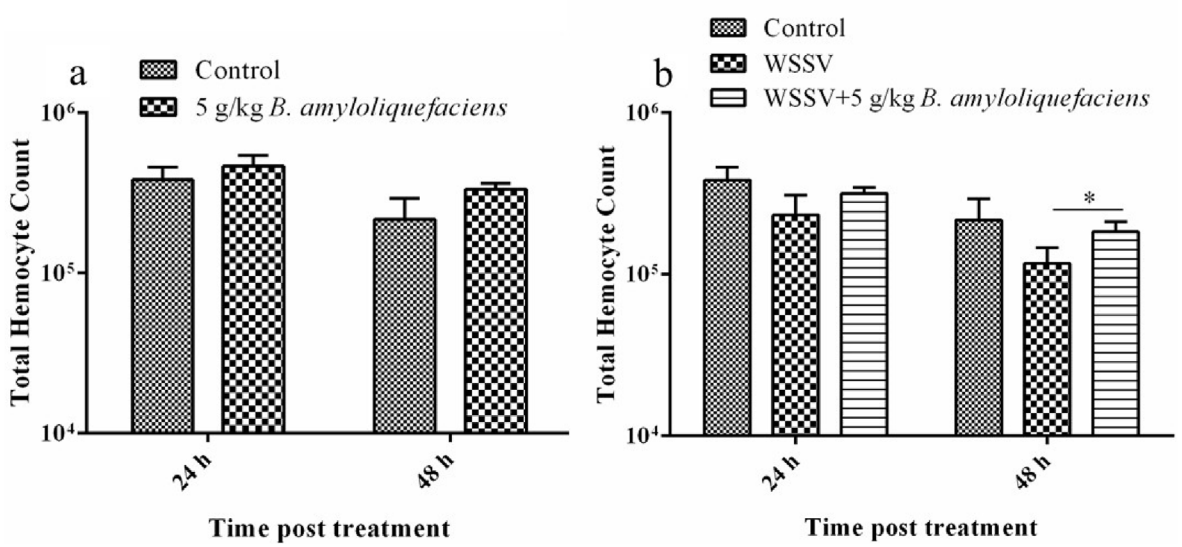

Fig. 4. Effects of 5 g/kg B. *amyloliquefaciens* on total hemocyte count (THC). Crayfish were fed commercial feed (control), 5 g/kg B. *amyloliquefaciens*, WSSV-infected meat, or WSSV-infected meat + 5 g/kg B. *amyloliquefaciens* and THC was measured after 24 and 48 h. (a) Effects of *B. amyloliquefaciens* supplement on healthy crayfish. (b) Effects of *B. amyloliquefaciens* supplement on WSSV-infected crayfish. Each treatment at each time point represents at least three individual crayfish. Data are presented as mean values with standard deviations. Significant differences are indicated by asterisks (*P < 0.05, **P < 0.01), analyzed by a multiple *t*-test.

significantly lower than in the control group at 24 and 48 h after treatment (P = 0.0004 at 24 h; P = 0.001 at 48 h). When *B. amyloliquefaciens* was fed before WSSV challenge experiment, SOD activity in WSSV + 5 g/kg B. *amyloliquefaciens* group was significantly higher than in WSSV group (P = 0.0021 at 24 h; P = 0.013 at 48 h) (Fig. 6b).

3.5. Effects of B. amyloliquefaciens on the apoptosis of hemocytes

Rates of hemocyte apoptosis were measured using flow cytometry. The apoptosis rate in the control group was approximately 34.4% and that in the 5 g/kg B. *amyloliquefaciens* group was 31.4%, which is significantly lower (P = 0.0132) (Fig. 7a). In the WSSV group, the apoptosis rate increased to 70.35%, which is significantly higher than in the control group (P < 0.01) (Fig. 7a). When *B. amyloliquefaciens* was fed before WSSV challenge experiment, the apoptosis rate in WSSV + 5 g/kg B. *amyloliquefaciens* group increased to 61.82%, which is significantly lower than in the WSSV group (P < 0.01) (Fig. 7a). In these experiments, the treated crayfish hemocytes were very fragile and we attempted to minimize the effects of centrifugation and the liquid impact associated with pipetting and subsequent re-suspension. Our results show that *B. amyloliquefaciens* can reduce the apoptosis rate to some extent.

4. Discussion

Because invertebrates, including crayfish, are generally believed to lack adaptive immunity, the innate immune system provides the main defense against invading pathogens [26–28]. Probiotics are potential feed additives in aquaculture and, since antimicrobial proteins have been isolated from *B. amyloliquefaciens* [29], which could potentially be used as a probiotic. *B. amyloliquefaciens* has been shown to improve the immune status and disease resistance of *Nile tilapia* [30]. For these

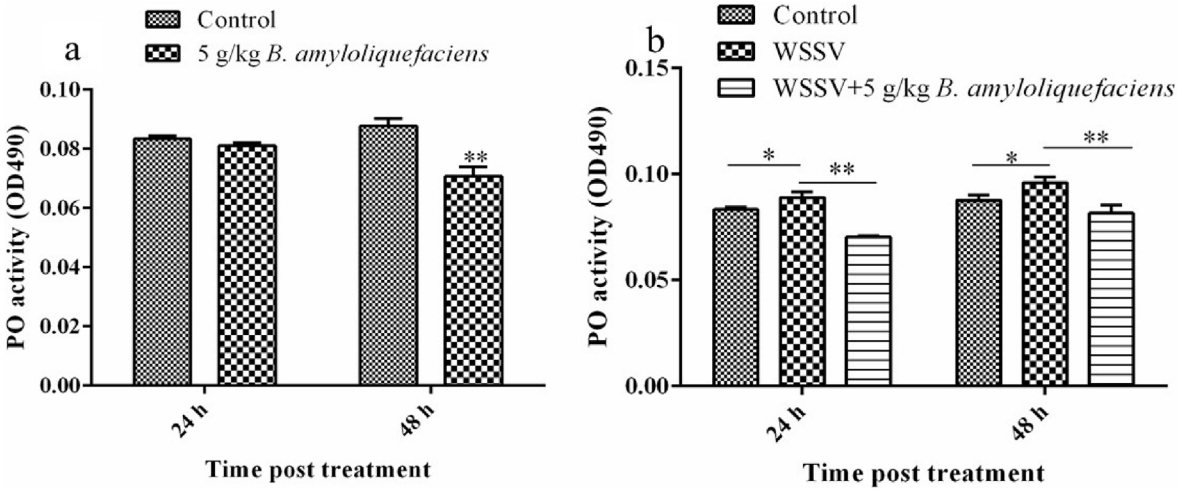

Fig. 5. Effects of 5 g/kg B. *amyloliquefaciens* on phenoloxidase (PO) activity. Crayfish were fed commercial feed (control), 5 g/kg B. *amyloliquefaciens*, WSSV-infected meat or WSSV-infected meat + 5 g/kg B. *amyloliquefaciens*. (a) Effects of *B. amyloliquefaciens* supplement on PO activity in healthy crayfish. (b) Effects of *B. amyloliquefaciens* supplement on PO activity in WSSV-infected crayfish. Each treatment at each time point represents at least three individual crayfish. Data are represented as mean values with standard deviations. Significant differences are indicated by asterisks (*P < 0.05, **P < 0.01), analyzed by a multiple *t*-test.

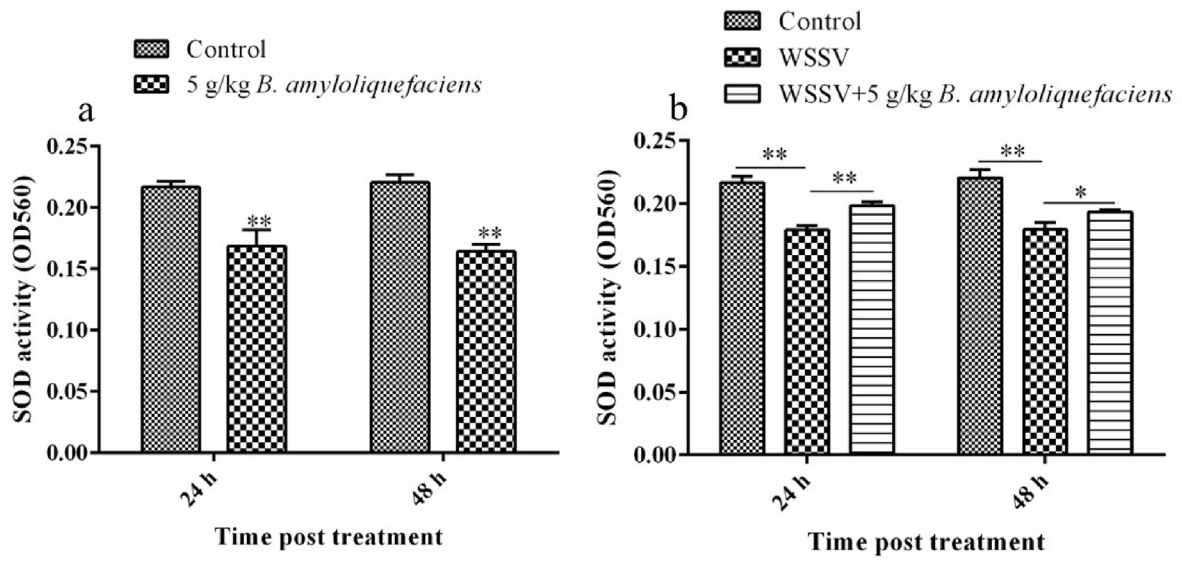

Fig. 6. Effects of 5 g/kg B. *amyloliquefaciens* on superoxide dismutase (SOD) activity. Crayfish were fed with commercial feed (control), 5 g/kg B. *amyloliquefaciens*, WSSV-infected meat or WSSV-infected meat + 5 g/kg B. *amyloliquefaciens*. (a) Effects of *B. amyloliquefaciens* supplement on SOD activity in healthy crayfish. (b) Effects of *B. amyloliquefaciens* supplement on SOD activity in WSSV-infected crayfish. Each treatment at each time point represents at least three individual crayfish. Data are presented as mean values with standard deviations. Significant differences are indicated by asterisks (*P < 0.05, **P < 0.01), analyzed by a multiple *t*-test.

reasons, we investigated whether *B. amyloliquefaciens*, as a feed additive, can protect crayfish from WSSV infection, which is the main pathogen for most crustaceans. We found that the mortality of crayfish treated with *B. amyloliquefaciens* was significantly lower than that of untreated crayfish after WSSV challenge, and that the best dose of *B. amyloliquefaciens* was 5 g/kg. Treatment with *B. amyloliquefaciens* also significantly reduced WSSV copy numbers, although it is uncertain whether this is a direct effect on viral replication. We then investigated the effect of feeding 5 g/kg B. *amyloliquefaciens* for 24 h on expression levels of five important immune genes in crayfish. Expression levels of proPO and crustin 1 were significantly down-regulated after fed with *B. amyloliquefaciens*, whereas expression levels of Toll-like receptor, NF-κB

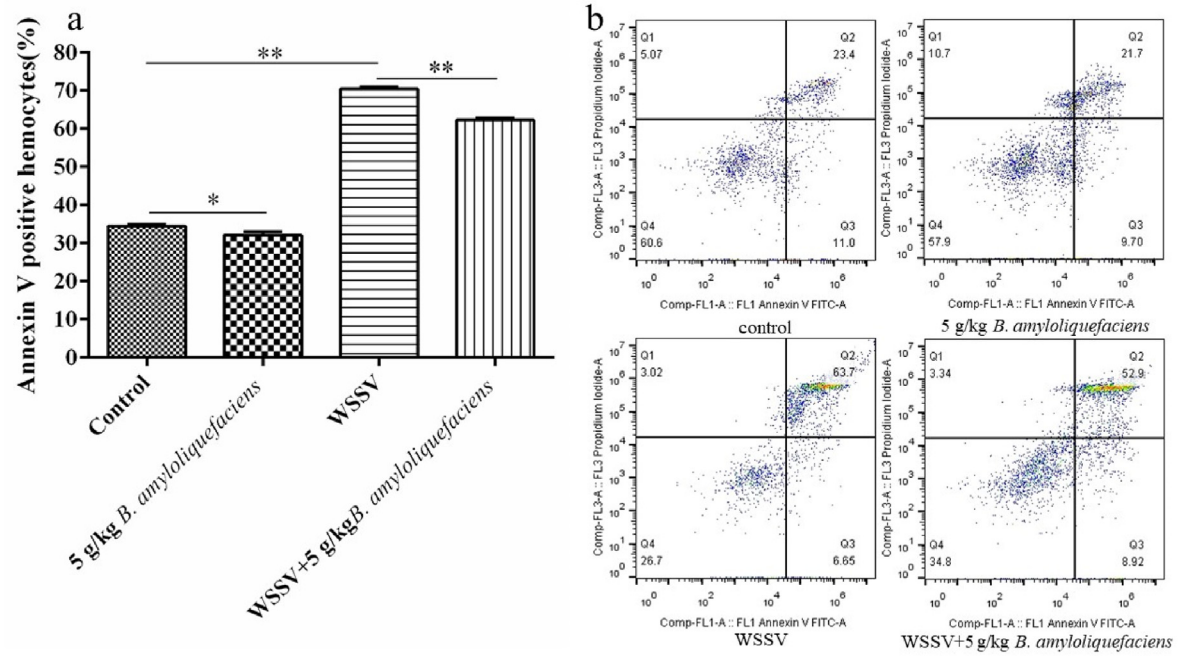

Fig. 7. Apoptosis of hemocytes in *B. amyloliquefaciens* treated crayfish, analyzed by flow cytometry. Crayfish were treated with commercial feed or 5 g/kg B. *amyloliquefaciens*, followed by challenge with WSSV. At 24 h post challenge, hemocyte samples from the different groups were stained with Annexin-V 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, the negative control and the positive control. The percentage of Annexin V-positive cells indicates the apoptosis rate of the hemocytes. (a) Bar chart showing apoptosis rate. Each column represents the mean value of three isolated repeats. (b) Scatter plots for one of the challenged groups, Q1 represents cell fragments caused by centrifugation and re-suspension, 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 fluorescent intensity of Q2 and Q3 (*P < 0.05, **P < 0.01).

and C-type-lectin were significantly up-regulated. The innate humoral immune response is mainly mediated by three immune signaling pathways: the Toll pathway, the IMD pathway and the JAK/STAT pathway [31,32]. In shrimp, the Toll and IMD pathways are two distinct NF-κB signaling pathways, which regulate the expression of antimicrobial peptide genes to counter invading microbes [33]. Toll like receptors, a family of pattern recognition receptors, participates in antiviral response against white spot syndrome virus via induction of anti-lipopolysaccharide factor in red claw crayfish *Cherax quadricarinatus* [34]. In shrimp, the NF-κB was found to be activated by WSSV to facilitate the replication of WSSV [35,36]. C-type lectin showed antiviral and antibacterial activity in pacific white shrimp *Litopenaeus vannamei* [37]. RNAi of crustin has been shown to inhibit replication of WSSV and reduce mortality of shrimp following WSSV challenge [38], The mortality, WSSV copy number and expressions of WSSV immediate early genes (IE1, IE2, DNA polymerase, VP28) were both decreased in crustin-dsRNA-treated shrimps, indicating that WSSV may take advantage of crustin to benefit its replication. Dietary supplementation of *Bacillus* PC465 also enhanced the transcription of C-type lectin but decreased the transcription of crustin in hemocytes of *Litopenaeus vannamei*, and provides protection against WSSV infection [39]. After WSSV challenge, expressions of proPO in crayfish hemocytes decreased at 12 h, then had respectively recovered and increased at 24 h, and at 48–72 h transcript levels were finally down-regulated [40]. WSSV inhibit shrimp melanization reaction, which activated by the prophenoloxidase activating (proPO) system, through viral protein WSSV453 interact with shrimp proPO-activating enzyme [41]. Our results show that *B. amyloliquefaciens* enhances disease resistance by altering the expression of important innate immune genes, thereby reducing mortality following WSSV infection.

We also investigated the effect of *B. amyloliquefaciens* on three immune parameters, THC, PO and SOD. Hemocytes, which are involved in cellular and humoral immunity and can directly recognize and engulf foreign pathogens [42], play a very important role in the innate immunity of crustaceans. Here, we found that the THC of the WSSV + 5 g/kg B. *amyloliquefaciens* group was significantly higher than that of WSSV group, indicating that *B. amyloliquefaciens* can enhance cellular immunity of crayfish. When *B. amyloliquefaciens* was used in WSSV challenge experiments, SOD activity, which is an important indicator of immunological activity in crustaceans [43], was significantly higher in the treated group than in the untreated group. The PO activity, on the other hand, was significantly lower in *B. amyloliquefaciens* treated group than in the untreated group. We think that *B. amyloliquefaciens* reduces the expression of the proPO gene, thus reducing PO activity compared with the control group. Judging by these innate immune parameters, the use of *B. amyloliquefaciens* as a feed additive is beneficial. Our results suggest that *B. amyloliquefaciens* may not have a direct effect on WSSV replication, but may instead have an indirect protective effect by enhancing the innate immune response.

Apoptosis and phagocytosis are also important components of immune response to foreign pathogens [44]. Apoptosis is a process of cell death caused by pre-existing death procedures triggered by internal and external factors, and it is also called programmed cell death [45]. We found that hemocyte apoptosis was significantly increased after WSSV challenge, and in both WSSV-challenged and unchallenged crayfish, apoptosis was significantly reduced by treatment with *B. amyloliquefaciens*. This explains why the total number of hemocytes in crayfish fed with *B. amyloliquefaciens* was higher than in the control group. Reducing apoptosis of hemocytes may be an important way in which *B. amyloliquefaciens* reduces the mortality of WSSV-challenged crayfish.

In conclusion, we have shown that *B. amyloliquefaciens* supplement may protect crayfish against WSSV infection and improve the innate immunity of crayfish, and we have preliminarily identified several innate immune pathways involved. *B. amyloliquefaciens* supplement effectively reduced copy numbers of WSSV and reduced the mortality of WSSV-challenged crayfish. The usage of *B. amyloliquefaciens* as a feed additive in crayfish farming would improve immune activity of crayfish, reduce the mortality caused by WSSV infection, and increase the yield of crayfish.

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CRediT authorship contribution statement

Yongyong Lai: Investigation, Data curation, Writing - original draft, Validation, Software. **Ming Luo:** Investigation, Data curation, Validation, Software. **Fei Zhu:** Conceptualization, Methodology, Investigation, Writing - review & editing.

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