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Hepatopancreatic antioxidant enzyme activities and disease resistance of Pacific white shrimp (*Litopenaeus vannamei*) fed diet supplemented with garlic (*Allium sativum*) extract

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Abstract

Garlic extract is herbal medicine that stimulate immune systems and disease resistance in many aquatic animals. This study evaluated garlic extract on antioxidant enzyme activity in the hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*) fed on diets supplemented with or without extracts of garlic (*Allium sativum*). Shrimp (8.36±0.17 g) were fed a garlic extract concentration of 2% or 4% for 30 d and then were challenged with *Vibrio parahaemolyticus* to study growth performance, cumulative mortality, oxidative enzyme activities and hepatopancreatic histopathology. The results showed that shrimp fed a 2% or 4% garlic-extract diet had higher ($p < 0.05$) survival rates (62% and 60%, respectively) than the control (25%). The hepatopancreas of shrimp was dissected out and analyzed for free-radical-scavenging enzymes, superoxide dismutase (SOD) and catalase (CAT) activity. The garlic-fed shrimp had higher expression of SOD activity compared to the control group ($p < 0.05$; 21.10, 21.97 and 19.27 units/mL, respective to the 2%, 4% and control group) but the CAT of garlic-extract-fed shrimp had lower ($p < 0.05$) activity than the control (3.63, 3.64 and 4.76 relative units/mg protein, respective to the 2%, 4% and control group). Histopathological study after challenge revealed atrophy of hepatopancreas cells compared to the control, whereas the hepatopancreas of garlic-fed shrimp was normal with clearly presented B-cells and R-cells. It was concluded that oxidative stress in the hepatopancreas played a major role in the pathogenesis and progression of hepatopancreatic diseases. Moreover, the survival rate of shrimp could be increased by enhancing the oxidative scavenging capacity through diet supplementation using garlic extract.

Introduction

Marine shrimp production is important for Thailand economy. Unfortunately, the production is corrupted by outbreak of several severe pathogens (Chanratchakool and Phillips. 2002: FAO, 2016); for examples, white-spot syndrome virus (WSSV), yellow-head virus (YHV), hepatopancreatic parvovirus (HPV), monodon baculovirus (MBV), Taura syndrome virus (TSV), infectious hypodermal and hematopoeitic virus (IHHNV) and acute hepatopancreatic necrosis disease (AHPND) (Flegel, 2006; Thitamadee et al., 2016).

Several strategies to reduce the impact of viral diseases have been developed and evaluated (Lakshmi et al., 2013). Treatments of viral infections in shrimp have proved more challenging, primarily because viruses are relatively tiny and reproduce inside cells and shrimp do not have the same immune system as vertebrates (Rouse and Sehrawat, 2010). Nevertheless, shrimp do have innate immunity to defend against viral infection (Chen et al., 2016). Thus, the enhancement of shrimp

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innate immunity of both humoral (anticoagulant protein, agglutinins, phenoloxidase enzymes, antimicrobial peptides, free radicals) and cellular (phagocytosis, encapsulation, nodules formation) components has become central to research (Jiravanichpaisal et al., 2006).

Following chemical restriction regulation in shrimp culture, farmers have used a combination of herbal plants and probiotics as an alternative medicine (Citarasu, 2010). Medicinal plant extracts have been used in controlling viral and bacterial diseases; for example, using guava (*Psidium guajava*) extracts on fish infectious haematopoietic necrosis virus (IHNV) and fish infectious pancreatic necrosis virus (IPNV) (Direkbusarakom et al., 1996) and luminous bacteria from Black tiger shrimp (*Penaues monodon*) (Direksabusarakom et al., 1996; Direkbusarakom, 2004).

Some plant extracts have been shown to possess antiviral activity; for example, the extracts of palomaría (*Callophyllum inophyllum*), purple basil (*Ocimum basilicum*) gooseberry (*Phyllanthus acidus*) and seed-under-leaf *Phyllanthus* spp., phayayo (*Clinacanthus nutans*) against YHV in *Penaues monodon* (Direksabusarakom et al. 1996; Janwitayanuchit et al., 2003), extract of *Sargassum weighti* (seaweed) against WSSV in *Penaeus indicus* and freshwater crab, *Paratelphusa hydrodomous* (Balasubramanian et al., 2006) and extracts of Bermuda grass (*Cynodon dactylon*) and yellow mangrove (*Ceriopstagal*) against WSSV in *Penaeus monodon* (Balasubramanian et al., 2008).

Garlic (*Allium sativum*) is a herbal plant that has displayed extensive efficacy against antimicrobial (Kumar and Berwal, 1998), anti-fungal and antioxidant properties in humans and animals (Lee and Gao, 2012). Studies carried out on the chemical composition of garlic showed that an organosulfur compound named allicin (diallyl dithiosulfinate) was an important constituent of the plant (McRae, 2005). Evidently, garlic and its active compounds were reported to have wide-spectrum pharmacological effects with low toxicity (Mikaili et al., 2013).

In Nile tilapia (*Oreochromis niloticus*) fed an allicin diet, the activity of antioxidant enzymes—such as—glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) of the fish increased significantly compared to the control group which finally led to therapeutic properties to protect Nile tilapia from subacute deltamethrin toxicity (Abdel-Daim et al., 2015). Although the efficiency of garlic regarding its bactericidal and anti-protozoal effects was discovered, its effect on anti-viral activity has not been well studied and requires further investigation (Weber et al., 1992). A few studies have reported roles of garlic on shrimp immunity enhancement; for example, garlic properties on cellular immune responses (phagocytic activity, superoxide anion activity, phenoloxidase activity) in Kuruma shrimp (*Marsupenaeus japonicus*) (Tanekhy and Fall, 2015) and the effect of garlic on monocytes and enhanced phagocytic activity of *Penaeus monodon* post larvae (Malar and Charles, 2013). Therefore, shrimp immunoenhancement by garlic supplementation is very appealing and deserves thorough investigation for its possible application at the farm scale.

In shrimp farms, the most effective preventive measures are limiting the spread of pathogens and rapid diagnosis of viral contamination, since there is currently no adequate treatment for viral infections (Seibert and Pinto, 2012). Basically, clinical signs, immune parameters and histopathological analyses are the first line of analysis to evaluate animal health before shrimp mortality occurs (Lightner et al., 2012).

The hepatopancreas or digestive gland of shrimp has similar responsibilities as that of the liver and pancreas in mammals being the metabolically active site for food absorption, transport, secretion of digestive enzymes and the storage of lipids, glycogen and minerals (Gibson and Barker, 1979; Díaz et al., 2010), as well as for the accumulation and biotransformation (detoxification) of various organic and inorganic toxic substances (Lee et al., 1976).

The hepatopancreas, in addition to haemolymphs (Sung et al., 2000; Chen and Sung, 2005), also plays an important role in synthesizing immune factors in crustaceans (Jiravanichpaisal et al., 2006; Ji et al., 2009; Cerenius et al., 2010). although reporting of the immune function of shrimp hepatopancreas has been rare (Du et al., 2013).

The purpose of this research was to investigate the effect of garlic extract as a dietary supplement for juvenile Pacific white shrimp, *Litopenaeus vannamei*. The studied parameters after experimental challenge with *Vibrio parahaemolyticus* covered growth performance, disease resistance, hepatopancreatic antioxidative enzymes and histopathological changes. Finally, the possibility of using garlic in shrimp feed for immune enhancement and health monitoring of the hepatopancreatic condition were investigated for farmer application.

Materials and Methods

Garlic extraction

Thai garlic (*Allium sativum*) and Chinese garlic (*Allium chinese*) were obtained from a local market near Kasetsart University, Bangkok, Thailand. Ethanol extract of garlic was prepared and the effects of the extract on *Vibrio parahaemolyticus* were evaluated.

Crude garlic extract was prepared by grinding fresh garlic in a domestic blender and then soaking in 95% (weight per volume) ethanol at a ratio of 2:1 at room temperature for 3 d. The mixture was filtered and the extracted garlic solution was evaporated to dryness under pressure in a rotary evaporator (Buchi Rotavapor, R-124) at 40°C. The ethanol extract of garlic thus obtained was used for assessment of antibacterial and hepatoprotective properties (Alfars, 2007).

Antibacterial activity test

The degree of antibacterial property of Thai and Chinese garlic extract was conducted by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using the broth macro dilution method (Clinical and Laboratory Standard Institute, 2008, document M27-A2).

Preparation of bacteria

V. parahaemolyticus (VP1.2) isolated from AHPND-infected *Litopenaeus vannamei* from Chantaburi province, Thailand was used for the bacterial challenge test. The bacteria from glycerol stocks were re-streaked on a Tryptic soy agar (TSA) plate containing 1.5% NaCl prior to the culture of a single colony in 5 mL TSB containing 1.5% NaCl at 30°C.

The bacterial inoculates were subsequently transferred to 150 mL TSB with 1.5% NaCl with vigorous shaking at 30°C until an optical density at 660 nm (OD600) of 0.6–0.8 was obtained. Then, the bacterial suspension was centrifuged at 3,500 revolutions per minute (rpm) for 10 min at 30°C and re-suspended in 1.5% NaCl to obtain a bacterial density of approximately 1×10^4 colony forming units (CFU)/mL (Joshi et al., 2014).

Determination of minimal inhibitory concentration and minimal bactericidal concentration

The MIC value was the minimum inhibitory concentration of garlic extract of testing bacteria compared with the control negative (no garlic extract) and control positive (no bacteria). MIC was determined using the microtiter broth dilution susceptibility test (Forbes et al., 1998). Concentrations of garlic extract were prepared at 2%, 4%, 6%, 8%, 10%, 20%, 30%, 40% and 50% of TSB suspended in 1.5% NaCl, *V. parahaemolyticus* and incubated at 35°C with continuous shaking (100 rpm) for 8 hr. The growth of bacteria was used to determine the MIC values.

The MBC value was the minimum bactericidal concentration of garlic extract with no growth of bacteria on thiosulfate-citrate-bile salts-sucrose agar namely, the lowest concentration that could kill the bacteria. The MBC was defined as the lowest concentration that demonstrated a pre-determined reduction (such as 99.9%) in colony forming units per milliliter compared to the MIC value. Determination of MBC was made by the inoculation of 10 µL from each tube (from the MIC test) onto TCBS agar plates. The plates were incubated at 35°C and examined for bacterial growth after 8 hr incubation.

A minimum of two concentrations of garlic extract from either the Thai or Chinese extract were determined according to the result of the antibacterial activity test. These concentrations were chosen not only for their degree of antibacterial effectiveness, but also for feed palatability by the shrimp.

Garlic extract feeding trial

Animal preparation

Juvenile Pacific white shrimp with an initial mean weight of 8.36±0.17 g was obtained from Suchart farm, Chonburi province, Thailand and stocked in the laboratory of the Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand. The experimental shrimp were acclimatized in 1 t concrete tanks provided with constantly aerated seawater (30 parts per trillion; ppt) for 7 d prior to the experiments. The shrimp were fed twice daily with commercial shrimp feed pellets. The un-eaten feed was siphoned out each day to maintain good water quality. The water temperatures during the experimental periods was kept in the range 27–28°C, with pH 7.9–8.1, salinity 30 ppt and dissolved oxygen (DO) 5.63–6.72 mg/L.

Garlic extract diet preparation

According to the higher antibacterial activity of Thai garlic than the Chinese garlic as shown by the MBC determined in the previous section, only the Thai garlic was used for further experiments. Experimental diets were prepared by mixing commercial shrimp diet with different levels of garlic extract (0%, 2%, 4% garlic extracts). The garlic-mix diets were then coated with 2% squid oil and air-dried before being kept in plastic bags at 4°C until further use. Experimental diets were given to shrimp twice daily at 0900 hours and 1700 hours.

Experimental design

The shrimp were randomly divided into two treatment groups (T1, T2) and one control with three replicates (100 shrimp each/1 t tank). Control shrimp were fed with commercial shrimp diet coated with squid oil. Treatment shrimp were fed with the garlic extract diet at 2% (T1) or 4% (T2) crude garlic extract. The feeding experiment was undertaken for 30 d.

At the end of the culture experiment, the growth performance of shrimp was determined based on the daily weight gain, survival rate and food conversion ratio (FCR). Subsequently, shrimp from each treatment were randomly subjected for the challenge test with *V. parahaemolyticus*.

Challenge test

The *V. parahaemolyticus* cultures in TSB at OD600 = 0.6–0.8 were collected using centrifugation at 3,500 rpm for 10 min and re-suspended prior to dilution with TSB containing 1.5% NaCl to a concentration of 1×10^6 CFU/mL.

After culturing the experimental shrimp for 30 d, the bacteria challenge test was performed by injecting *V. parahaemolyticus* isolates into the third abdominal segment of each shrimp at a concentration of 0.1 mL/shrimp with three replicates of 20 shrimp/250 L.

The hepatopancreas of moribund shrimp was homogenized with PBS (pH 7.4) for oxidative enzyme analysis and fixed with Davidson's fixative (Bell and Lightner, 1988) and further processed for histopathological analysis.

Oxidative enzyme measurement

The hepatopancreas of experimental shrimp (15 shrimps per replicate) after challenge testing were dissected out and homogenized in 50 mM ice-cold phosphate buffer (pH 7.4). The shrimp hepatopancreas homogenate was centrifuged at 20,000×*g* for 30 min at 4°C to obtain a supernatant for enzyme analysis.

Enzymatic antioxidants including superoxide dismutase (SOD) and catalase (CAT) in the shrimp hepatopancreas homogenate were evaluated. The SOD was assayed using an SOD kit (Sigma) based on the reaction of WST-1(2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with the superoxide anion. The rate of the reduction with a superoxide anion is linearly related to the xanthine oxidase activity and is inhibited by SOD so that subsequently, the inhibition activity of SOD can be determined by measuring the decrease in the color development at 440 nm (Peskin and Winterbourn, 2000).

Catalase activities were determined by measuring the decrease in the hydrogen peroxide concentration at 240 nm by the method described by *Dautremepuits* et al. (2003). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH = 7), 10.6 mM H_2O_2 and shrimp hepatopancreas supernatant. The absorbance was recorded at 240 nm and expressed as relative units per milligram of soluble protein (U/mg protein).

The protein concentration of shrimp hepatopancreas homogenate was measured using the Bradford method with bovine serum albumin as a protein standard (Bradford, 1976).

Shrimp hepatopancreas histopathological examination

The hepatopancreas of each experimental shrimp after challenge testing was dissected out and fixed in Davidson's fixative. The procedure for tissue processing followed Bell and Lightner (1988) and comprised dehydration in an alcohol series and embedding in paraffin wax. Then, each sample was cut and sectioned at 3 µm thickness using a rotary microtome (Weswox, MT1090/1090A). The thin sections of the hepatopancreas were stained using haematoxylin and eosin (H & E) for observation under a light microscope.

Each tissue section was evaluated for the presence of F-cells, R-cells and B-cells (hepatopancreas cells) using a 100× oil-immersion objective (Olympus CX41) and sequential random counting of hepatopancreas cells in three adjacent microscope fields. Numerical results were expressed as numbers of F-cells, R-cells and B-cells for each treatment (Franceschini-Vicentini et al., 2009)

Statistical analysis

A completely randomized design was used throughout the study. Data were expressed as mean ± SD. Data were analyzed using analysis of variance. Then, wherever the F value was significant, comparison of means was carried out using Duncan's multiple range tests (Steel and Torrie, 1980) and significance was tested at $p < 0.05$.

Results and Discussion

Antibacterial activity test

In this experiment, the MIC of garlic extract against *V. parahaemolyticus* was not detectable due to color interference from the garlic suspension. Therefore, only the MBC was measured and used in the diet application trial. This test clearly indicated that the Thai garlic extract had a higher level of antibacterial activity than the Chinese garlic extract with MBC values against *V. parahaemolyticus* of 2% and 4%, respectively (Table 2).

Table 1 Minimum bactericidal concentration of Thai garlic and Chinese garlic extract

Concentration (%)	Number of colonies	
	Thai garlic	Chinese garlic
2	0.0 ± 0.0[a]	49.3 ± 4.9[a]
4	0.0 ± 0.0[a]	44.0 ± 7.0[a]
6	0.0 ± 0.0[a]	43.6 ± 6.2[a]
8	0.0 ± 0.0[a]	0.0 ± 0.0[b]
10	0.0 ± 0.0[a]	0.0 ± 0.0[b]
20	0.0 ± 0.0[a]	0.0 ± 0.0[b]
30	0.0 ± 0.0[a]	0.0 ± 0.0[b]
40	0.0 ± 0.0[a]	0.0 ± 0.0[b]
50	0.0 ± 0.0[a]	0.0 ± 0.0[b]

Means within the same row with different lowercase superscripts are significantly ($p < 0.05$) different.

Beato et al. (2011) reported that Thai garlic had a greater bactericidal activity than Chinese garlic due to the higher amount of phenol compounds and allyl sulfide in Thai garlic. Based on the results in the current study, 2% and 4% of Thai garlic extract (coated with 2% squid oil) were used in the diet for the feeding trial. These two concentrations were pretested and the result showed that they had no effect on feed palatability by the shrimp.

The antimicrobial activities of garlic have been known for many years, with its active element identified as allicin or diallyl thiosulfinate (2-propenyl-2-propenethiol sulfonate) (Rahman, 2003). The bactericidal property of garlic in most studies has been shown to be dose dependent (Indu et al., 2006).

The bactericidal effects of a garlic-mixed diet on aquatic animals have been shown in numerous studies including on 20 serogroups of *E. coli*, 8 serotypes of *Salmonella* and *Aeromonas hydrophila* except *Listeria monocytogenes* (Indu et al., 2006); *Bacillus subtilis*, *Salmonella enteritidis* (Al-Turki et al., 2007.); *Edwardsiella tarda* (Chakravarty et al., 2017); *Pseudomonas fluorescens* (Diab et al, 2010); *Myxococcus piscicola*, (Shubha and Shyam, 2014); *Vibrio anguillarum* (Labrador et al., 2016); and *E. tarda* (Rahman, 2003).

In humans, pure allicin displays antibacterial activity against a wide range of Gram-negative and Gram-positive bacteria. Some research has revealed that the bactericidal effects of garlic applied only to Gram-negative bacteria; however, other research has produced different outcomes. For example, Wei et al. (2008) found that garlic extract was more effective at controlling Gram-negative bacteria including *E. tarda* than *Streptococcus agalactiae* and *Staphylococcus aureus*, whereas Nurtjahyani and Hadra (2016) reported that 50% garlic extract produced the highest clear zone only on *Penaeus monodon*, a Gram-positive bacterium.

Growth performance and survival rate

After 30 d of the feeding trial, there were no significant differences in shrimp average final weight, weight gain, FCR and survival rate among the treatment means (Table 2). This indicated that the garlic extract diet did not affect the growth performance of *L. vannamei* which was in agreement with experiments on *Labeo rohita* (Sahu et al., 2007).

Nonetheless, most other reports on the effect of garlic on aquatic fauna growth performance have produced positive results, including a garlic supplement diet enhancing growth and body composition of Benni fish (*Mesopotamichthys sharpeyi*) (Maniat et al., 2014), white leg shrimp (*L. vannamei)* (Labrador et al., 2016), , rainbow trout (*Oncorhynchus mykiss*) (Breyer et al., 2015; Gabor et al., 2012), *Macrobrachium rosenbergii* (Poongodi et al., 2012), juvenile sterlet sturgeons (*Acipenser ruthenus*) (Lee and Gao, 2012) and African catfish (*Clarias gariepinus*) (Eirna-liza et al., 2016).

Garlic has been used widely as a growth promoter for fish (Reuter et al., 1996). Fish fed a garlic-mix diet had higher weight gain because the allicin compound in the garlic enhanced gastrointestinal motility which resulted in increasing feed absorption and digestion by the fish (Diab et al., 2002). Allicin also improved the proteolytic activity of bacteria in the digestive tract (Khalil et al., 2001). This led to better utilization of energy and finally resulted in an increased protein content in the tested animals (Kamruzzaman et al., 2011).

Table 2 Growth performance and survival rate (mean ± SD) of Pacific white shrimp fed with diet containing 0%, 2% or 4% Thai garlic extract for 30 d, with no significant ($p \geq 0.05$) differences among means

Garlic extract	Initial weight (g)	Final weight (g)	ADG (g/shrimp/d)	FCR	Survival rate (%)
0%	8.42 ± 0.02	21.13 ± 1.56	0.42 ± 0.05	1.17 ± 0.02	65.00 ± 7.81
2%	8.19 ± 0.05	20.44 ± 0.52	0.41 ± 0.02	1.12 ± 0.06	72.33 ± 2.52
4%	8.46 ± 0.14	20.50 ± 1.43	0.40 ± 0.05	1.16 ± 0.05	74.33 ± 7.02

ADG = average daily gain; FCR = feed conversion ratio

However, garlic as a feed additive is not suitable for all fish species because of numerous factors involving, for example, garlic variation (sources, preparation techniques, active ingredients dosages), animal variation (species, age, sex), and feed management variation (diet composition, feeding program, length of application. environmental conditions) (Shalaby et al., 2006).

Some research found that garlic did not affect growth performance of livestock (Horton et al., 1991; Freitas et al., 2001; Bampidis et al., 2005) whereas it suppressed weight gain in fish (Platel and Srinivasan, 2004; Mesalhy et al., 2008). This was because the unpleasant odor and taste of garlic led to lower diet palatability and feed intake (Diab et al., 2002).

The optimal dosage of garlic mix in diet was crucial. High concentration of alkyl sulfide from the garlic interfered with normal metabolism resulting in slow growth and even death of Manila clam (*Ruditapes philippinarum*) (Yang et al., 2010) and red bellied pacu (*Colossoma barchypomum*) (Xiang and Liu, 2002). To benefit from growth enhancement by including the garlic, the feeding period to fish was also important as too short an experimental period introduced insufficient allicin into the body system (Ndong and Fall, 2006).

Challenge test

The results revealed that after challenging with *V. parahaemolyticus*, the control shrimp (no garlic diet) were lethargic at 8 hr and started to die at 12 hr post-challenge, whereas garlic-fed shrimp were slower to display any incidence of disease (12 hr for 2% garlic fed shrimp and 20 hr for 4% garlic fed shrimp). After 7 d of bacterial challenge, there was no further shrimp mortality recorded in any tank. Fig 1 shows that the shrimp mortality rate in the control group was the highest followed by the mortality rate from dietary supplementation with 2% and 4% garlic (75 ± 0.00, 38.33 ± 7.60 and 40 ± 5.00%, respectively) which were significantly different. However, at the end of the trial, the mortality rate of shrimp fed the diets supplemented with 2% or 4% garlic were not significantly different.

The effects of garlic are due to the presence of numerous organosulfur compounds, including allicin (Augusti and Mathew, 1974). Ankri et al. (1997) described that allicin strongly inhibited the cysteine protease and alcohol dehydrogenase enzymes produced by bacteria. Moreover, lectin in garlic could prevent bacterial infection and increase phagocytosis activity leading to immune activation in the infected animals (Nya and Austin, 2011)

The optimal concentration of garlic application was also an important criterion. The prophylactic effect of dietary garlic application even incorporated at a low level (0.5%) could enhance the hematological parameters and immunity of *Clarias gariepinus* fingerlings (Thanikachalam et al., 2010). However, regarding the Pacific white shrimp in the current study, incorporation of 2% garlic in the feed exhibited lower mortality percentage than 4% garlic in feed (Fig 1). The higher mortality rate resulting from incorporation at the 4% level of garlic than for the 2% garlic level might have been due to the excess alkyl sulfide in the garlic extract. Lee and Gao (2012) reported that garlic extract or allicin at higher than 2% supplementation did not improve fish growth and was harmful to fish health because excessive alkyl sulfide in the fish intestines would interfere with normal metabolism and suppress mitosis, which would eventually result in slow growth and even mortality of the fish.

Garlic has a bactericidal property and can constrain the growth of protozoa as well as gregarine infection in cultured shrimp; however, the most virulent diseases of shrimp culture in Thailand are mainly from virus infection rather than from bacteria (Flegel, 2006). The effect of garlic on viral disease infection has not been well studied and requires further investigation (Tanekhy and Fall, 2015). On the other hand, according to Thai farmers who survived outbreaks of AHPND, supplementation of assorted herbal drugs including garlic can sustain shrimp health, although there is no clear evidence of viricidal activity from garlic and the compounds responsible have not been identified (Weber et al., 1992). The benefit of using garlic in shrimp culture might be mainly on enhancing the immune defense mechanism of shrimp such as phagocytosis activity (Pope et al., 2011), the phagocytosis index (Ndong and Fall, 2006), superoxide anions (Kim et al., 2001), total white blood cell count (Iranloye, 2002) and respiratory bursts and lysozyme activity (Ndong and Fall, 2006).

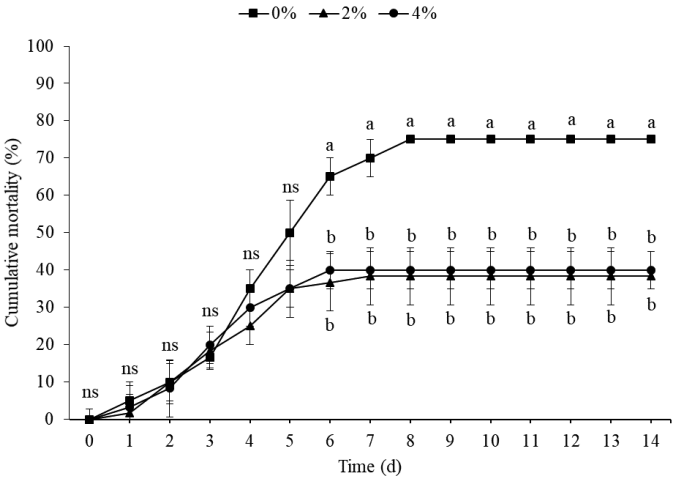

Fig. 1 Average cumulative mortality of *Litopenaeus vannamei* after challenge with *Vibrio parahaemolyticus* for 14 d, where error bars indicate ± SD ns denotes non-significant difference ($p > 0.05$) of means at each time point; different lowercase letters at each time point denote significant difference ($p < 0.05$) among means.

Antioxidant enzyme

The results showed the effectiveness of a garlic-supplemented diet in SOD synthesis activation as the shrimp fed the garlic supplement diets had higher mean SOD levels than the control shrimp (21.21 ± 1.80, 23.0 ± 91.92 and 22.97 ± 1.77 U/mL for the control, 2% and 4% garlic-supplemented diets, respectively). After challenge testing, shrimp fed the garlic-supplemented diets had higher survival rates than the control shrimp, and the SOD levels were significantly different between the control and the 4% garlic group, as shown in Table 3.

On the contrary, after challenge, shrimp fed the garlic-supplemented diets had significantly lower CAT than the control shrimp (4.76 ± 0.72 U/mg protein in the control, 3.64±0.43 and 3.65±0.23 U/mg protein in the 2% and 4% garlic supplement, respectively), as shown in Table 3.

Upon bacterial infection, host cells will generate reactive oxygen species (ROS) as signaling molecules for homeostasis and the uncontrolled production of ROS damages lipids, proteins and DNA which lead to oxidative stress (Cui et al., 2012) and loss of cell function and programmed cell death (Pallepati and Averill-Bates, 2012). To prevent cell damage from excess ROS, shrimp have an antioxidant defense system (SOD) that can catalyze the dismutation of superoxide into oxygen and hydrogen peroxide (H_2O_2) and then there may be further reduction by CAT to H_2O and oxygen (Fridovich, 1997).

In the current study, the SOD and CAT activity in hepatopancreas was determined in shrimp from all feeding groups before and after *V. parahaemolyticus* challenge. The results showed that before bacterial challenge the SOD and CAT activity in hepatopancreas of garlic-fed shrimp did not increase ($p \geq 0.05$) (Table 3).

Interestingly, the CAT activity in the control shrimp after challenge was significantly higher than for the garlic-fed shrimp. The control shrimp died suddenly because of the overwhelming amount of ROS which meant there was no enough time for CAT activity to adjust to the basal levels. The current results with *L. vannamei* were in agreement with previous studies involving numerous marine shrimp species that showed a direct relationship of SOD activity in the hepatopancreas with bacterial challenge (Ankri et al., 1997; Ji et al., 2009; Duan et al., 2013).

Increased antioxidant activity of SOD in cells is associated with a rapid detoxifying response and reflects the necessary roles of SOD and CAT in removing excessive reactive oxygen species from cells (Moreno et al., 2005). As the pathogen infection proceeded, the pathogens would continuously replicate in hosts, resulting in the infection progressively producing more pathogens that in turn would synthesize higher local levels of ROS (Duan et al., 2013).

Histopathological examination

The shrimp hepatopancreas is the central metabolic organ responsible for various anabolic and catabolic functions such as the synthesis and secretion of digestive enzymes (Ceccaldi, 1989), absorption (Díaz et al., 2010) and the storage of reserve material for detoxification and nutrient uptake (Icely and Nott, 1980).

In the current study, all shrimp before challenge testing had hepatopancreases that were normal in appearance, but after challenge, the control shrimp (fed diet without garlic) had significantly more severe pathological hepatopancreas conditions compared to the insubstantial signs of deterioration in the hepatopancreas samples of garlic-fed shrimp. These observations supported previous study that histopathological changes in the shrimp hepatopancreas represent a highly sensitive tool to evaluate bacterial infection (Aly and El-Attar, 2001).

The main hepatopancreatic alterations from bacterial infection were reductions in the number of F-cells, R-cells and B-cells, indicating the sensitivity of these cells to bacterial contamination. Other symptoms were cellular inflammation, tissue necrosis, dissolution of the well-organized liver structure, shrinkage of hepatocytes, slough hepatopancreatic tubule cells, degeneration of tubule lumen, necrotic cells, nodule formation, aggregated transformed microvilli (ATM), lack of F-cells, R-cells and B-cells and epithelial cells in the hepatopancreatic tubules and decreases in the numbers of B-cells and R-cells (Díaz et al., 2010; Joshi et al., 2014).

The degeneration of the E-cells, F-cells and R-cells, and the collapse of the epithelial tubules in the hepatopancreas were reported to be main signs of destruction of hepatopancreatic tissue caused by AHPND (Manan et al., 2015) which were similar to the current histopathological results identified in the shrimp exposed to bacterial challenge. AHPND infection suppresses the mitotic activity of E-cells (Lightner et al., 2012) followed by enlargement of vacuoles in E-cells (Joshi et al., 2014). Finally, massive intratubular hemocytic aggregation would occur, indicating secondary bacterial infections (Lightner et al., 2012; Tran et al., 2013).

Before challenge, the control shrimp had fewer F-cells but more B-cells than the garlic-fed shrimp 4while the numbers of R-cells in all treatments were not significantly different as shown in Table 4.

After challenge with *V. parahaemolyticus*, the numbers of F-cells, R-cells and B-cells of garlic-fed shrimp were significantly higher than those for the control shrimp (F-cells: 2.66±2.51 cells, 15.67±3.0 cells and 18.33±13.65 cells; R-cells: 47.33±36.7 cells, 439.67±88.2 cells and 471.33±41.1 cells; B-cells: 19.33±7.07 cells, 94.00±9.64 cells and 89.33±12.5 cells for control, 2% and 4% garlic-supplemented diets, respectively), as shown in Table 4. The alterations of these functional cells might reflect the condition of the hepatopancreas and overall shrimp health. F-cells are known to be responsible for protein synthesis (Sousa and Petriella, 2007) and mineral storage (Melba et al., 2013). The number of R-cells has been reported to decrease during the detoxicification process (Sousa and Petriella, 2007). The increased number of B-cells in the current study might have been due to the elevated level of SOD during the infective stage after challenge (Li et al., 2008). Overall, the pathological changes in the hepatopancreas caused by *V. parahaemolyticus* had a direct impact on the functional cells of this organ.

Table 3 Oxidative enzyme activity (mean ± SD) of Pacific white shrimp before challenge test (BC) and after challenge test (AC) with *Vibrio parahaemolyticus*

Group	Superoxide dismutase (U/mL)		Catalase (U/mg protein)	
	BC	AC	BC	AC
0%	21.21 ± 1.80[a]	19.27 ± 2.89[a]	3.75 ± 0.35[a]	4.76 ± 0.72[a]
2%	23.09 ± 1.92[a]	21.10 ± 3.36[ab]	3.42 ± 0.28[a]	3.64 ± 0.43[b]
4%	22.97 ± 1.77[a]	21.97 ± 1.84[b]	3.52 ± 0.26[a]	3.65 ± 0.23[b]

U = relative units; Means within the same column with different lowercase superscripts are significantly ($p < 0.05$) different.

Table 4 Number of hepatopancreatic cells of Pacific white shrimp before challenge test (BC) and after challenge test (AC) with *Vibrio parahaemolyticus* (mean ± SD)

Treatment	F-cells		R-cells		B-cells	
	BC	AC	BC	AC	BC	AC
0%	16.00 ± 5.20[a]	2.66 ± 2.51[a]	964.00 ± 57.10	47.33 ± 36.70[a]	45.00 ± 10.80[a]	19.33 ± 7.07[a]
2%	24.67 ± 11.10[b]	15.67 ± 3.00[b]	1,109.67 ± 79.40	439.67 ± 88.20[b]	36.67 ± 7.02[a]	94.00 ± 9.64[b]
4%	23.33 ± 6.02[b]	18.33 ± 13.65[b]	1,134.67 ± 75.00	471.33 ± 41.10[b]	15.00 ± 6.50[b]	89.33 ± 12.50[b]

Means within the same column with different lowercase superscripts are significantly ($p < 0.05$) different.

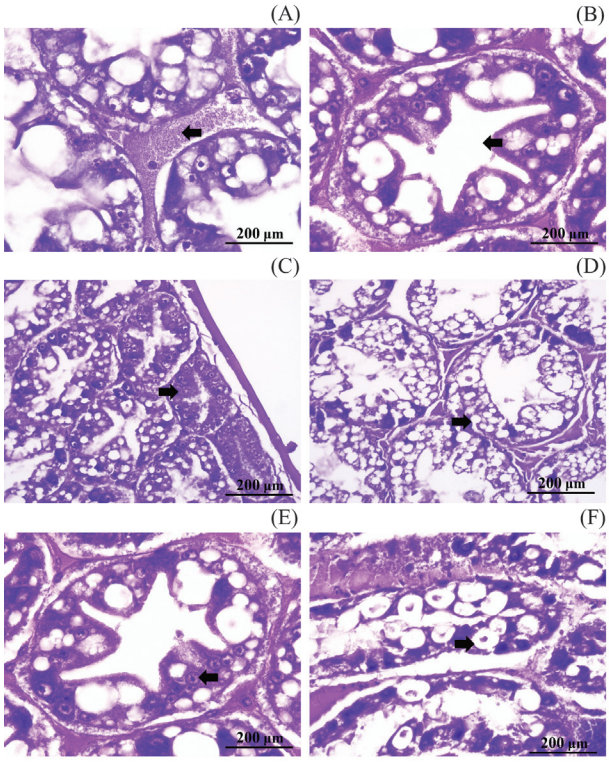

Fig. 2 Normal hepatopancreatic cells consisting of: (A) connective tissue (arrow); (B) tubule lumen (arrow); (C) E-cell (arrow); (D) R-cell (arrow); (E) F-cell (arrow); (F) B-cell (arrow) (hematoxylin and eosin staining, scale bar = 200 µm)

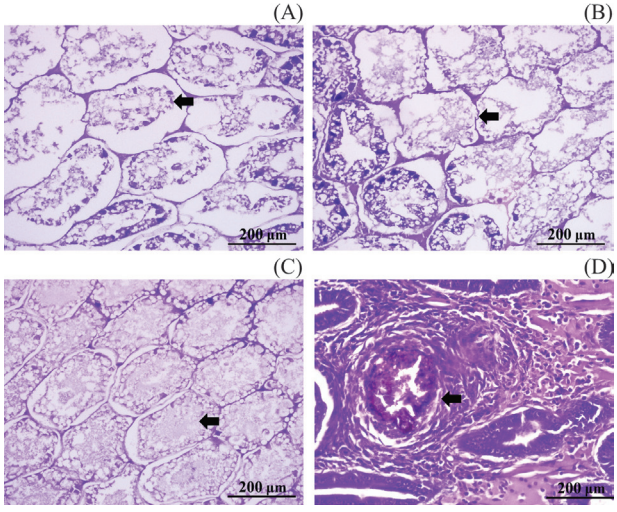

Fig. 3 Hepatopancreatic cells after challenge testing in the control group: (A) inclusion bodies within the cell (arrow); (B) hepatopancreatic ducts sloughing epithelial cells (arrow); (C) lack of R-cells, F-cells and B-cells (arrow); (D) nodule formation in the control (arrow) (hematoxylin and eosin staining, scale bar = 200 µm)

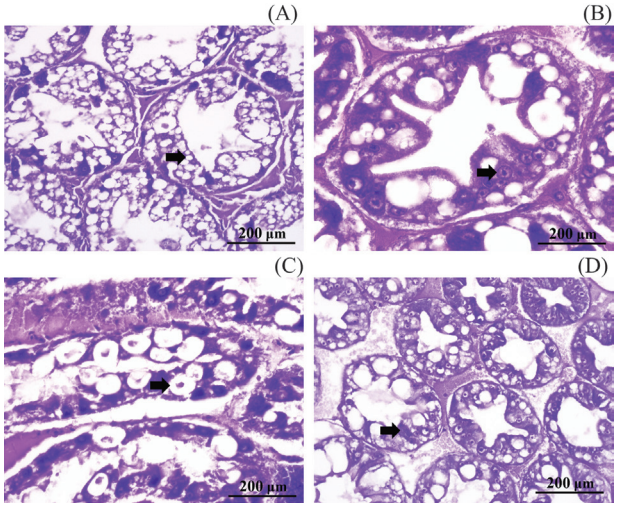

Fig. 4 Hepatopancreatic cells in shrimp with diet supplemented with 2% garlic extract: (A) R-cells (arrow); (B) F-cells (arrow); (C) B-cells (arrow); (D) pyknotic cells in hepatopancreatic cells after challenge testing (arrow) (hematoxylin and eosin staining, scale bar = 200 µm)

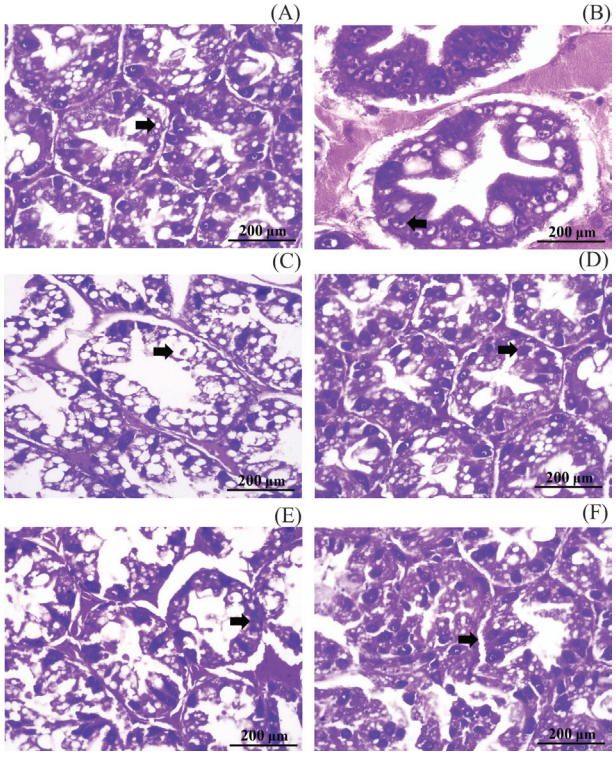

Fig. 5 Hepatopancreatic cells (arrows) in shrimp with diet supplemented with 4% garlic extract: (A) R-cells (arrow); (B) F-cells (arrow); (C) B-cells (arrow); (D) pyknotic cell (arrow); (E) aggregated transformed microvilli cells (arrow); (F) sloughing of epithelial cell after challenge testing (arrow) (hematoxylin and eosin staining, scale bar = 200 µm)

Infected shrimp with a degenerated hepatopancreas lost the ability to synthesize and secrete digestive enzymes (Lightner et al., 2012; Tran et al., 2013). The defense system caused famine and later loss of shrimp life (Manan et al., 2015). Preliminary MBC assay showed the higher antibiotic efficiency of Thai garlic over Chinese garlic. Therefore, Thai garlic was selected to be used in the diet. After feeding garlic supplemented diet to shrimp, it was shown that shrimp fed garlic supplement diet had higher SOD activity. Due to this enhanced immunity, the garlic fed shrimp had higher survival rate than control. The experimented shrimp also displayed lesser damage of hepatocytes compared to control shrimp. It can be concluded from this experiment that Thai garlic extract had potential as immunostimulant and bacteriocide for Pacific white shrimp.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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