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Immune responses and gene expression in white shrimp, *Litopenaeus vannamei,* induced by *Lactobacillus plantarum*

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Abstract

The total haemocyte counts, phenoloxidase (PO) activity, respiratory bursts, superoxide dismutase (SOD) activity, and phagocytic activity and clearance efficiency to *Vibrio alginolyticus*, as well as prophenoloxidase (proPO), lipopolysaccharide- and β-1,3-glucan-binding protein (LGBP), serine protein (SP), and peroxinectin (PE) mRNA transcription of *L. vannamei*, and its susceptibility to *V. alginolyticus* when the shrimp were fed diets containing *Lactobacillus plantarum* at 0 (control), 10^7, and 10^{10} cfu (kg diet) $^{-1}$ for 48 and 168 h were evaluated. The results indicated that PO activity, SOD activity, clearance efficiency to *V. alginolyticus*, proPO and PE mRNA transcription, and the survival rate after challenge with *V. alginolyticus* all significantly increased, but the total haemocyte counts significantly decreased in shrimp fed a diet containing *Lac. plantarum* at 10^{10} cfu (kg diet)$^{-1}$ for 168 h. However, no significant differences in phagocytosis, LGBP, or SP mRNA expression of shrimp were observed among the different treatments. It was concluded that administration of *Lac. plantarum* in the diet at 10^{10} cfu (kg diet)$^{-1}$ induced immune modulation and enhanced the immune ability of *L. vannamei*, and increased its resistance to *V. alginolyticus* infection.

Keywords: *Litopenaeus vannamei*; *Lactobacillus plantarum*; Probiotics; Phenoloxidase activity; Respiratory bursts; Superoxide dismutase; Haemocyte count; Phagocytic activity; Clearance efficiency; Challenge; Immune gene transcription

1. Introduction

White shrimp, *Litopenaeus vannamei,* which is distributed along the Pacific coast of Central and South America, has been introduced to the Eastern hemisphere, and has become the primary species currently being cultured in Southeast Asian countries. During the past two decades, shrimp culture around the world has suffered problems linked to deteriorating pond environments due to development of intensification, subsequently resulting in stress-induced

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disease incidence. Many shrimp farms have been particularly affected by epidemics of viruses and vibriosis. Therefore, the health of shrimp and enhancement of their immunity are of primary concern.

In decapod crustaceans, circulating haemocytes are generally classified into three types, hyaline, semi-granular, and large granular cells [1]. Haemocytes are involved not only in coagulation but also in the production of melanin via the prophenoloxidase (proPO) system, which plays an important defence role [2,3]. Both semi-granular and granular cells carry out functions of the proPO system, which has a role in recognition and defence [2]. Conversion of proPO to phenoloxidase (PO) occurs through prophenoloxidase-activating enzyme (ppA), a serine protease [4]. PO is the terminal enzyme in the proPO system, and ppA is activated by several microbial polysaccharides, including β-1,3-glucan and lipopolysaccharide (LPS) from fungal cell walls through the non-self recognition system [5]. Specific recognition proteins, including β-1,3-glucan-binding protein, LPS-binding protein, peptidoglycan-binding protein, and LPS- and β-1,3-glucan-binding protein (LGBP), which recognise and respond to intruders, have been reported in several crustacean species including penaeid shrimp [6–8].

In addition, peroxinectin (PE), an associated protein of the proPO system, is synthesised and stored in secretory granules of semi-granular cells and granular cells in an inactive form, is released in response to stimulus, and is activated outside the cells to mediate haemocyte attachment and spread [9,10]. PE has multiple functions of cell adhesion [9], opsonification [11], degranulation [12], peroxidase activity [13], and encapsulation enhancement [14]. The biological activity of PE is generated concomitantly with activation of the proPO system [13]. PE of *L. vannamei* has been cloned and characteristic by Liu et al. [15].

Several reactive oxygen species are produced during phagocytosis. Beginning this process, the membrane-bound enzyme complex, NADPH oxidase, assembles after binding the cell to a foreign particle, and reduces molecular oxygen to the superoxide anion (O_2^-), subsequently leading to the production of hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hydroxyl radicals ($OH^\bullet$), and numerous other reactive compounds [16]. The superoxide anion is the first product released from respiratory bursts, and plays an important role in microbicidal activity [17]. Although reactive oxygen intermediates (ROIs) play an especially important role in host defence, host cells can be damaged by the overexpression of ROIs. Most cells have also acquired the relevant protective mechanisms to maintain the lowest possible levels of ROIs inside the cell, including superoxide dismutase (SOD), catalase, and glutathione peroxidase.

Probiotics are defined as live microbial or cultured product feed supplements, which beneficially affect the host by producing inhibitory compounds, competing for chemicals and adhesion sites, modulating and stimulating the immune function, and improving the microbial balance [18–20]. They have been used in aquaculture as a means of disease control, supplementing or even in some cases replacing the use of antimicrobial compounds. A wide range of microalgae (*Tetraselmis*), yeasts (*Debaryomyces*, *Phaffia,* and *Saccharomyces*), and Gram-positive (*Bacillus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Micrococcus*, *Streptococcus*, and *Weissella*) and Gram-negative bacteria (*Aeromonas*, *Alteromonas*, *Photorbodobacterium*, *Pseudomonas,* and *Vibrio*) has been applied as probiotics in fish and shellfish. Applying probiotics to aquaculture ponds and feed to improve aquatic animal growth, survival, health, and disease prevention has been reviewed [20–22].

Administration of probiotics, such as *Thalassobacter ultilis* PM-4 and *Bacillus* sp., to elevate the survival rate in commercial shrimp hatcheries and farms has been evaluated [23,24] and reviewed [20]. Probiotics being used to stimulate the immune response of fish has also been considered [25]. However, the effect of probiotics on the immune response and gene expression of shrimp has not been clarified so far. *Lactobacillus plantarum* is an important species in the fermentation of various plant products [26], is known to produce antimicrobial substances, like plantaricin, that are active against certain pathogens, and is used as a probiotic [27].

The purpose of this study was to examine the immune parameters including total haemocyte counts, PO activity, respiratory bursts, SOD activity, phagocytic activity and clearance efficiency to *Vibrio alginolyticus*; the immune gene expression including proPO, LGBP, a serine protein, and PE mRNA transcription of *L. vannamei*; and its susceptibility to *V. alginolyticus* when the shrimp were fed diets containing *Lac. plantarum*.

2. Materials and methods

2.1. Preparation of the Lac. plantarum mixture

Lactobacillus plantarum 7-40 (NTU102) isolated from home-made Korean-style cabbage pickles were used in this study. Bacteria were cultured in a sterilised 2-L flask with de Man, Rogosa, and Sharpe (MRS) broth (Merck,

Darmstadt, Germany) for 24 h at 37 °C, and then centrifuged at 15,400 × *g* for 5 min at 4 °C. The pellet was collected and mixed with skim milk at a ratio of 1:4, and then freeze-dried. The bacterial mixture was stored at 4 °C until used. The viability of the bacterial mixture was determined by plate counting on MRS agar.

2.2. Preparation of feed

Three diets containing different levels of bacterial mixture (*Lac. plantarum*) were prepared as described in Table 1. The composition of the diets was prepared based on a diet used in a previous study [28]. The basal diet contained 0.1% skim milk. Proximate analysis of the basal diet was 40.7% crude protein, 7.4% crude lipid, 13.9% ash, and 9.0% moisture. A bacterial mixture (containing 1.01×10^{10} cfu g^{-1} *Lac. plantarum*) was added to the test diets at levels of 0.001 and 1.0 g (kg diet)$^{-1}$, resulting in 10^7 and 10^{10} cfu (kg diet)$^{-1}$, respectively, with corresponding decreases in the amount of skim milk. The ingredients were ground in a Hammer mill so as to pass through a 60-mesh screen. Experimental diets were prepared by mixing the dry ingredients with fish oil and then adding water until a stiff dough resulted. Each diet was then passed through a mincer with a die, and the resulting spaghetti-like strings were dried in a drying cabinet using an air blower at 38 °C until the moisture levels were at around 10%. After drying, the finished pellets were stored at −4 °C until being used.

2.3. Experimental design

White shrimp, *L. vannamei,* obtained from recirculating seawater (20–23‰) ponds at the Department of Aquaculture, National Pingtung University of Science and Technology, were acclimated to room temperature (26 ± 0.5 °C) and a salinity of 20‰ in an indoor cement pond (6 × 2 × 1.5 m) with recirculating water for 2 weeks before experimentation. During the acclimation period, shrimp were fed twice daily with the control diet, and 50% of the water was exchanged weekly to maintain water quality. Only shrimp in the intermolt stage (stage C) were used for the subsequent tests. The molt stage was determined by examination of the uropoda in which partial retraction of the epidermis could be distinguished [29]. Four studies were conducted. For the susceptibility experiment, the test and control groups were comprised of ten shrimp each in triplicate, and were conducted on shrimp following 48 and 168 h of feeding of *Lac. plantarum*-containing and control diets. For the study of immune parameters and immune gene assays, tests and controls were carried out on eight replicate test groups consisting of one shrimp each in 20-L PVC tanks containing 10 L of aerated water. In all tests, shrimp were fed the test diet twice daily. No significant difference in weight was observed among the treatments. During the experiments, the water temperature was maintained at 26 ± 0.5 °C, the pH at 7.8–8.2, and the salinity at 20‰.

Table 1
Composition of the basal diet (g kg^{-1}) for *Litopenaeus vannamei*

Ingredients	*Lactobacillus plantarum* in diet (cfu kg^{-1})		
	Control	10^7	10^{10}
Fish meal	430	430	430
Soybean meal	50	50	50
Yeast meal	25	25	25
Shrimp shell meal	70	70	70
Wheat flour	353	353	353
Cellulose	1	1	1
Skim milk	1	0.999	0
Probiotic	0	0.001	1
Gluten	25	25	25
Fish oil	20	20	20
Mineral mixture[a]	21	21	21
Vitamin mixture[b]	4	4	4

[a] Mineral mix (Shinta Feed Company, Pingtung, Taiwan).
[b] Vitamin mix (Shinta Feed Company, Pingtung, Taiwan).

2.4. Susceptibility of shrimp to V. alginolyticus

A known pathogenic strain, *V. alginolyticus* (CH003), isolated from diseased *L. vannamei*, which displayed symptoms of anorexia, inactivity, poor growth, and necrotic musculature, was used for the study. The pathogen was cultured on tryptic soy agar (TSA supplemented with 1.5% NaCl, Difco) for 24 h at 28 °C before being transferred to 10 ml tryptic soy broth (TSB supplemented with 1.5% NaCl, Difco), where it remained for 24 h at 28 °C as the stock culture for the tests. The broth cultures were centrifuged at 7155 × *g* for 15 min at 4 °C. The supernatant fluids were removed, and the bacterial pellets were re-suspended in a saline solution at 3×10^7 and 1×10^7 as stock bacterial suspensions for the susceptibility study, and at 1×10^{10} cfu ml^{-1} for the phagocytic activity and clearance efficiency studies.

Challenge trials were carried out in shrimp following 48 and 168 h of being fed *Lac. plantarum*-containing and control diets by injecting them with 20 μl of a bacterial suspension (at 3×10^7 or 1×10^7 cfu ml^{-1}) resulting in 6×10^6 and 2×10^6 cfu shrimp^{-1} into the ventral sinus of the cephalothorax, respectively. Shrimp fed the control diet and then saline (20 μl) served as the unchallenged control. Experimental shrimp (10 shrimp in each aquarium) were kept in 60-l glass aquaria containing 40 l of water at 20‰ and 26 ± 0.5 °C. There were four treatments for each test. Each treatment used 30 shrimp. Twenty percent of the water was renewed daily, and the experiment lasted 168 h. Shrimp were fed twice daily with the test diets after challenge.

2.5. Immune parameters of the shrimp

The immune parameters of the shrimp following feeding with different levels of *Lac. plantarum*-containing diets were determined at the beginning and after 24, 72, and 168 h of feeding. Haemolymph (100 μl) was withdrawn from the ventral sinus of each shrimp into a 1-ml sterile syringe (25-gauge) containing 0.9 ml anticoagulant solution (30 mM trisodium citrate, 0.34 M sodium chloride, and 10 mM EDTA, at a pH of 7.55 and with the osmolality adjusted with glucose to 780 mOsm kg^{-1}). They were divided into two parts. A drop of the anticoagulant-haemolymph mixture (100 μl) was placed on a haemocytometer to measure the THC using an inverted phase-contrast microscope (Leica DMIL, Leica Microsystems, Wetzlar, Germany). The remainder of the haemolymph mixture was used for subsequent tests.

PO activity of haemocytes was measured spectrophotometrically by recording the formation of dopachrome produced following the procedures of Hernãndez-López et al. [30]. The L-3, 4-dihydroxyphenylalanine and trypsin were served as a substrate and an elicitor, respectively. The details of the measurement were described previously [31]. The optical density of the shrimp's PO activity was expressed as dopachrome formation in 50 μl of haemolymph.

Respiratory bursts of haemocytes were quantified using the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion (O_2^-) formation as described previously [17]. The optical density at 630 nm was measured using a microplate reader (Model VERSAmax, Molecular Devices, Sunnyvale, CA, USA). Respiratory bursts were expressed as NBT-reduction per 10 μl of haemolymph.

SOD activity of haemocytes was measured by its ability to inhibit superoxide radical-dependent reactions using the Ransod kit (Randox, Crumlin, UK) based on the methods described in the instructions. Briefly, the reaction mixture (1.7 ml) contained 0.05 mM xanthine and 0.025 mM 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) dissolved in 50 mM CAPS (pH 10.2) and 0.94 mM EDTA. In the presence of xanthine oxidase (80 U L^{-1}, 250 μl), superoxide and uric acid were produced from xanthine. The superoxide radical then reacted with INT to produce a red formazan dye. The optical density was measured at 505 nm and 37 °C, and the rate of the reaction was estimated from the absorbance readings 0.5 and 3 min after adding xanthine oxidase. A reference standard (SOD) was supplied with the Ransod kit. The unit of SOD was calculated according to the ratio of xanthine inhibition of the SOD standard. Specific activity was expressed as SOD units (mg protein)$^{-1}$.

The total protein was quantified as described by Bradford [32] using a Bio-Rad Protein Assay Kit (no. 500-0006, Bio-Rad Laboratories, USA) with bovine serum albumin as a standard.

2.6. Phagocytic activity and clearance efficiency of shrimp to V. alginolyticus

For the phagocytic activity and clearance efficiency tests, 20 μl of a bacterial suspension (1×10^{10} cfu ml^{-1}) resulting in 2×10^8 cfu shrimp^{-1} was injected into the ventral sinus. After injection, the shrimp were kept for 3 h in

separate tanks containing 40 L of seawater (20‰) at 26 ± 1 °C. Then, 200 μl of haemolymph was collected from the ventral sinus and mixed with 200 μl of sterile anticoagulant. This mixture was divided into two equal sub-samples: one to measure phagocytic activity and the other to measure clearance efficiency.

The methods for the measurements of phagocytic activity and clearance efficiency were described previously [31]. Two hundred haemocytes were counted. Phagocytic activity, defined as the phagocytic rate (PR), was expressed as:

$$\mathrm{PR} = [(\text{phagocytic hemocytes})/(\text{total hemocytes})] \times 100$$

The clearance efficiency, defined as percentage inhibition (PI) of *V. alginolyticus,* was calculated as:

$$\mathrm{PI} = 100 - [(\text{cfu in the test group})/(\text{cfu in the control group})] \times 100$$

2.7. Quantification of shrimp immune genes

The immune genes of shrimp following feeding on different levels of *Lac. plantarum*-containing diets were determined by quantitative real-time RT–PCR at the beginning and after 24, 72, and 168 h of feeding. Haemocytes of shrimp were collected as described above. Total RNA of haemocytes was extracted and purified using the guanidinium thiocyanate method [33]. The RNA was adjusted to the same concentration with DEPC-water and accurately quantified with a spectrophotometer. First-strand cDNA synthesis by reverse transcription (RT) was accomplished using SuperScript II RNase H^- reverse transcriptase (Promega Corp., Madison, WI, USA) to transcribe poly $(A)^+$ RNA with oligo-$d(T)_{18}$ as the primer. Reaction conditions recommended by the manufacturer were followed.

For quantification of the shrimp immune genes, the specific primer pairs of prophenoloxidase (proPO), peroxinectin (PE), lipopolysaccharide- and β-1,3-glucan-binding protein (LGBP), a serine protein (SP), and β-actin were designed as follows: proPO forward primer, 5′-GCCTTGGCAACGCTTTCA-3′ and reverse primer, CGCGCATCA GTTCAGTTTGT-3′ [34]; LGBP forward primer, 5′-CATGTCCAACTTCGCTTTCAGA-3′ and reverse primer, 5′-ATCACCGCGTGGCATCTT-3′ [8]; PE forward primer, 5′-TGGACCTCGCGGGAGAT-3′ and reverse primer, 5′-GACCGATAGCCACCATGCTT-3′ [15]; SP forward primer, 5′-CGTCGTTAGGTTAAGTGCGTTCT-3′ and reverse primer, 5′-TTTCAGCGCATTAAGACGTGTT-3′ [35]; and β-actin forward primer, 5′-GAGCAACACGG AGTTCGTTGT-3′ and reverse primer, 5′-CATCACCAACTGGGACGACATGGA-3′ (GenBank accession no.: AF300705).

The SYBR green I real-time RT–PCR assay was carried out in an ABI PRISM™ 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The amplifications were performed in a 96-well plate in a 25-μl reaction volume containing 12.5 μl of 2× SYBR Green Master Mix (PE Applied Biosystems), 2.5 μl (each) of the forward and reverse primers (10 μM), 1 μl of template (1 μg cDNA), and 9 μl of DEPC-water. The thermal profile for the SYBR green real-time RT–PCR was 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. In a 96-well plate, each sample was analysed in duplicate. DEPC-water replaced the template as the negative control.

Data analysis of the RT–PCR was performed with SDS software version 2.0 (Perkin–Elmer Applied Biosystems). Relative quantification of gene expression was performed according to the manufacturer's instructions. Briefly, the threshold PCR cycle (Ct) was defined as the cycle number at which a statistically significant increase in the fluorescence of SYBR green against the internal passive dye, ROX (ΔR_n), was first detected. The copy number of the target gene and Ct values were inversely related; thus, a sample containing a higher number of copies of the target gene had a lower Ct value than that of a sample with a lower number of copies of the same target. Differences in the Ct values of immune genes and the corresponding internal control β-actin gene, called ΔCt, were calculated to normalise any differences in the amount of total RNA added to the cDNA reaction mixture and the efficiency of the reverse-transcription reaction. The value of ΔCt in the treated sample was subtracted from the value of ΔCt of the control sample. The difference was expressed as the ΔΔCt value that allowed measurement of the change in the expression of immune genes in the treated sample relative to the control sample. A 3.3-fold change in the Ct value is considered to be equivalent to a 10-fold change in expression.

2.8. Statistical analysis

A multiple comparison (Tukey's) test was conducted to examine the significant differences among treatments using the SAS computer software (SAS Institute, Cary, NC, USA). Before analysis, percent data were normalised using arcsine transformation before analysis. It was required that $p < 0.05$ to achieve statistical significance.

3. Results

3.1. Challenge tests

White shrimp, *L. vannamei,* following feeding with *Lac. plantarum*-containing and control diets after 48 h and 168 h were challenged with *V. alginolyticus*. All unchallenged control shrimp survived in the two challenge trials. The cumulative mortality of shrimp following feeding with different *Lac. plantarum*-containing diets for 48 h, and then challenge with *V. alginolyticus* did not significantly differ in any time interval. With the same treatments after 168 h and then challenge with *V. alginolyticus*, the cumulative mortality of shrimp fed 10^{10} cfu (kg diet)$^{-1}$ of the *Lac. plantarum*-containing diet was significantly lower than that of shrimp fed the control diet after 144 h. After 168 h of challenge, the cumulative mortalities of shrimp fed the 0, 10^7, and 10^{10} cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diets were 43.3%, 33.3%, and 23.3%, respectively (Table 2).

3.2. Immune parameters of shrimp

The total haemocyte count (THC) of shrimp fed the diet containing *Lac. plantarum* at 10^{10} cfu (kg diet)$^{-1}$ after 48 h was significantly lower than that of shrimp fed the control diet. After 168 h of feeding, the THCs of shrimp fed the diets containing *Lac. plantarum* at 10^7 and 10^{10} cfu (kg diet)$^{-1}$ were significantly lower than that of shrimp fed the control diet. However, no significant differences in the THCs were detected between shrimp fed the diets containing *Lac. plantarum* at 10^7 and 10^{10} cfu (kg diet)$^{-1}$. The relative THC (compared to the THC of shrimp fed the control diet) of shrimp fed the 10^7 and 10^{10} cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diets decreased to 77.8% and 72.4%, respectively (Fig. 1).

The phenoloxidase activity of shrimp receiving the diet containing 10^{10} cfu (kg diet) $^{-1}$ *Lac. plantarum* was significantly lower than that of shrimp fed the control diet at 48 h. In contrast, those of shrimp fed the 10^7 and 10^{10} cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diets were significantly higher than that of shrimp fed the control diet after 168 h. The relative phenoloxidase activity levels (compared to the activity of shrimp fed the control diet) of shrimp fed the 10^7 and 10^{10} cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diets increased to 156.5% and 137.0%, respectively (Fig. 2).

The respiratory bursts of shrimps fed the 10^7 cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diet were significantly higher than those of shrimp fed the 10^{10} cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diet and the control diet after 48 and 168 h. However, no significant differences in respiratory bursts of shrimp were observed between shrimp fed the 10^{10} cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diet and the control diet from 48 to 168 h. The relative respiratory bursts (compared to the activity of shrimp fed the control diet) of shrimp following 48 and 168 h of feeding the 10^7 cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diets increased to 216.2% and 174.1%, respectively (Fig. 3).

Table 2
Cumulative mortality (mean ± S.E.) of *Litopenaeus vannamei* challenged with *Vibrio alginolyticus*, when the shrimp were fed different levels of *Lactobacillus plantarum* (0, 10^7, 10^{10} cfu kg^{-1}) containing diets after 168 h

Bacteria (cfu shrimp^{-1})	*Lactobacillus plantarum* in diet (cfu kg^{-1})	No. of Shrimp	Cumulative mortality (%) after time elapsed					
			6	12	24	48	144	168
Saline	0	30	0	0	0	0	0	0
2×10^5	0	30	3.3 ± 3.3^a	20.0 ± 5.8^a	30.0 ± 0^a	33.3 ± 3.3^a	43.3 ± 3.3^a	43.3 ± 3.3^a
2×10^5	10^7	30	13.3 ± 3.3^a	30.0 ± 0^a	30.0 ± 0^a	30.0 ± 0^a	33.3 ± 3.3^{ab}	33.3 ± 3.3^{ab}
2×10^5	10^{10}	30	3.3 ± 3.3^a	20.0 ± 5.8^a	26.7 ± 6.7^a	26.7 ± 6.7^a	23.3 ± 3.3^b	23.3 ± 3.3^b

Data in the same column with different letters significantly differ ($P < 0.05$) among different treatments. Values are mean ± S.E.

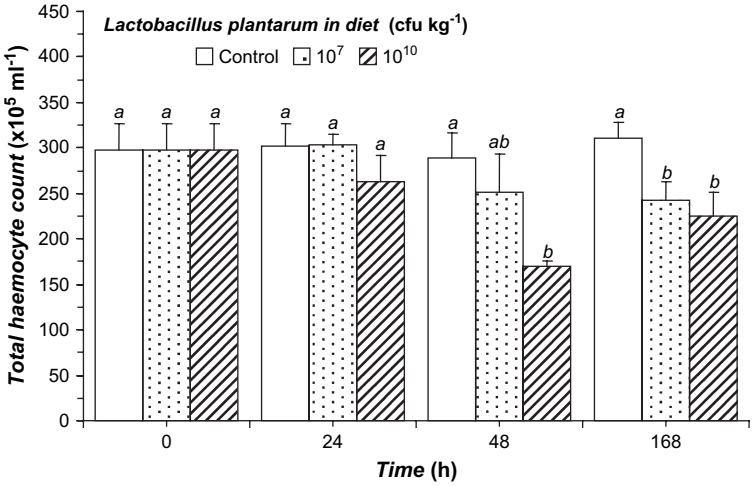

Fig. 1. Total haemocyte counts of *Litopenaeus vannamei* fed the control diet and *Lactobacillus plantarum*-containing diets at 10^7 and 10^{10} cfu (kg diet)$^{-1}$, respectively. Each bar represents the mean value from eight determinations with the standard error. Data (mean ± S.E.) with different letters significantly differ ($p < 0.05$) among treatments.

The superoxide dismutase activity in haemocytes of shrimp fed the 10^{10} cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diet was significantly higher than those of shrimp fed the 10^7 cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diet and the control diet from 24 to 168 h. The relative superoxide dismutase activity (compared to the activity of shrimp fed the control diet) of shrimp following 24, 48, and 168 h of feeding the 10^{10} cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diets increased to 420.9%, 244.3%, and 496.6%, respectively (Fig. 4).

No significant differences in phagocytic activity of shrimp were observed among the three treatments from 24 to 168 h.

The clearance efficiency, an overall antibacterial indicator, of shrimp was significantly enhanced when shrimp were fed the 10^7 and 10^{10} cfu (kg diet) $^{-1}$ *Lac. plantarum*-containing diets from 48 to 168 h. The clearance efficiencies of shrimp following 48 h of feeding the 10^7 and 10^{10} cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diets increased by 79.8% and 74.4%, respectively, and following 168 h of feeding the 10^7 and 10^{10} cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diets, they increased by 82.4% and 66.6%, respectively (Fig. 5).

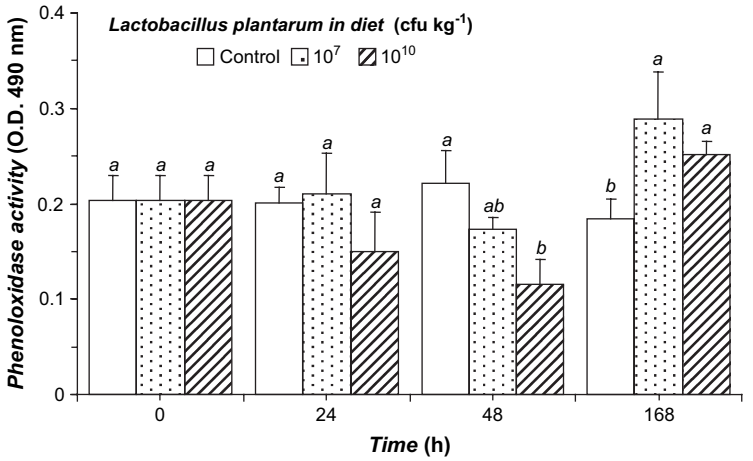

Fig. 2. Phenoloxidase activity of *Litopenaeus vannamei* fed the control diet and *Lactobacillus plantarum*-containing diets at 10^7 and 10^{10} cfu (kg diet)$^{-1}$, respectively. See Fig. 1 for statistical information.

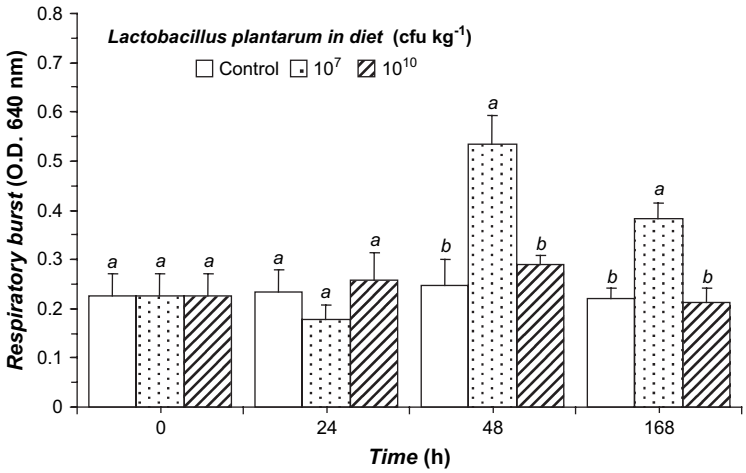

Fig. 3. Respiratory bursts of *Litopenaeus vannamei* fed the control diet and *Lactobacillus plantarum* containing diets at 10^7 and 10^{10} cfu (kg diet)$^{-1}$, respectively. See Fig. 1 for statistical information.

3.3. Expression of immune genes

mRNA expressions of immune genes of shrimps fed the 0, 10^7, and 10^{10} cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diets were measured using real-time RT–PCR at the beginning and after 48 and 168 h. No significant differences in LGBP mRNA transcriptions of shrimp were observed among the three treatments from 24 to 168 h.

The proPO mRNA expression levels of shrimp which had been fed the 10^7 and 10^{10} cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diets after 168 h were significantly higher than that of shrimp fed the control diet. The proPO gene expression levels of shrimp fed the 10^7 and 10^{10} cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diets were -2.0 and -1.5, respectively, relative to shrimp fed the control diet after 168 h. Shrimp fed the 10^7 and 10^{10} cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diets had approximately 6.1- and 4.4-fold higher expression levels of proPO mRNA, respectively, than did shrimp fed the control diet after 168 h (Fig. 6A,B).

No significant differences in serine protease mRNA transcription levels of shrimp were observed among the three treatments from 24 to 168 h.

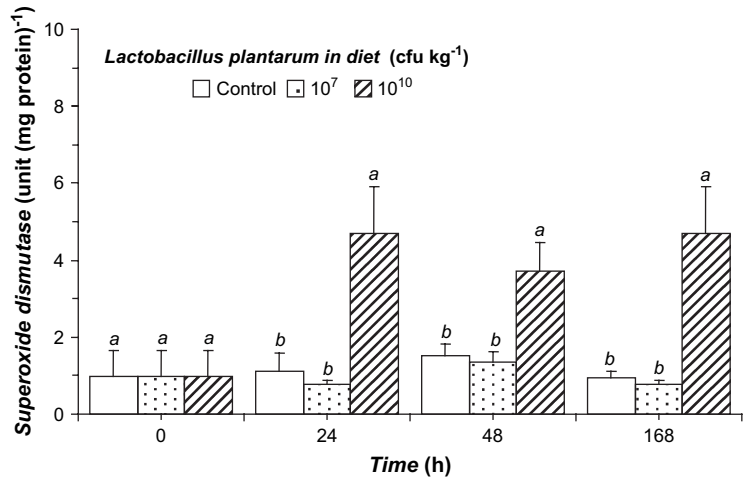

Fig. 4. Superoxide dismutase of *Litopenaeus vannamei* fed the control diet and *Lactobacillus plantarum*-containing diets at 10^7 and 10^{10} cfu (kg diet)$^{-1}$, respectively. See Fig. 1 for statistical information.

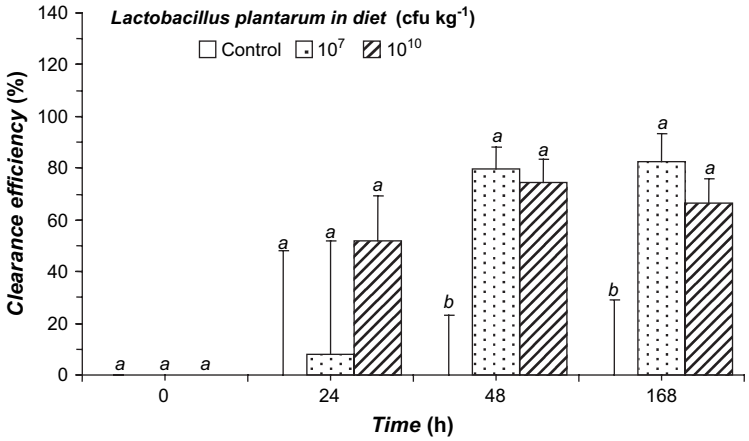

Fig. 5. Clearance efficiency of *Litopenaeus vannamei* fed the control diet and *Lactobacillus plantarum*-containing diets at 10^7 and 10^{10} cfu (kg diet)$^{-1}$, respectively. See Fig. 1 for statistical information.

The PE mRNA transcription levels of shrimp fed the 10^7 and 10^{10} cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diets were significantly higher than that of shrimp fed the control diet after 168 h. The PE gene expression levels of shrimp fed the 10^7 and 10^{10} cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diets were −1.4 and −1.2, respectively, relative to shrimp fed the control diet after 168 h. Shrimp fed the 10^7 and 10^{10} cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diets had approximately 4.3- and 3.7-fold higher expression levels of PE mRNA, respectively, than did shrimp fed the control diet after 168 h (Fig. 7A,B).

4. Discussion

In the present study, white shrimp receiving *Lac. plantarum*-containing diets exhibited enhanced immune modulation resulting in increased resistance against a pathogen, similar to that reported with the administration of probiotics in several vertebrate [25,36–42].

Chang and Liu [43] used *Enterococcus faecium* SF 68 from commercial products to reduce edwardsiellosis in European eel, *Anguilla anguilla,* resulting in reduced mortality of eels and suppressed growth of *E. tarda in vitro*. The human probiotic, *Lactobacillus rhamnosus* ATCC (American Type Culture Collection, Rockville, MD, USA) 53101, was administered at a dose of 10^9 and 10^{12} cells g^{-1} of feed to rainbow trout, *Oncorhynchus mykiss,* for 51 days, and reduced mortalities from 52.6% to 18.9% (at 10^9 cells g^{-1} of feed) and to 46.3% (at 10^{12} cells g^{-1} of feed) following challenge with *Aeromonas salmonicida* [44]. Maeda and Liao [23] reported that the soil bacterial strain, PM-4, promoted the growth of *Penaeus monodon* nauplii. This strain also showed an *in vitro* inhibitory effect against a *V. anguillarum* strain. When added to tanks inoculated with diatoms and rotifers, the strain resulted in 57% survival of the larvae after 13 days, while without the bacterium all the larvae had died after 5 days [45]. After being fed for 100 days with *Bacillus* strain S11-supplemented feed, *P. monodon* postlarvae were challenged with a pathogenic *V. harveyi* strain, D331, by immersion of the shrimp. After 10 days, all groups treated with the S11 *Bacillus* strain showed 100% survival, whereas the control group had only 26% survival [24]. In the present study, white shrimp, *L. vannamei,* following feeding with *Lac. plantarum*-containing diets for 168 h, were challenged with *V. alginolyticus* and showed reduced mortalities of from 43.3% to 33.3% (at 10^7 cfu kg^{-1} of feed) and to 23.3% (at 10^{10} cfu kg^{-1} of feed) indicating that *L. plantarum* 7-40 (NTU102) has the potential as a probiotic to improve white shrimp, *L. vannamei,* resistance against *V. alginolyticus* infection.

It is well known that the life cycle, food intake, disease outbreak, pollutants, and environmental stress affect the circulating haemocyte count of crustaceans, both in quantity and quality [46–48]. An increase in the THC provides enhanced immune capability during periods of stress [49] leading to disease resistance in crustaceans [50]. There is a direct relationship between the bacterial concentration in the water and in haemolymph [51]. In the present study, the THCs of shrimp fed diets containing *Lac. plantarum* at 10^7 and 10^{10} cfu (kg diet)$^{-1}$ were significantly lower at 168 h and at 48–168 h, respectively, than that of shrimp fed the control diet. These facts suggest that after a certain period,

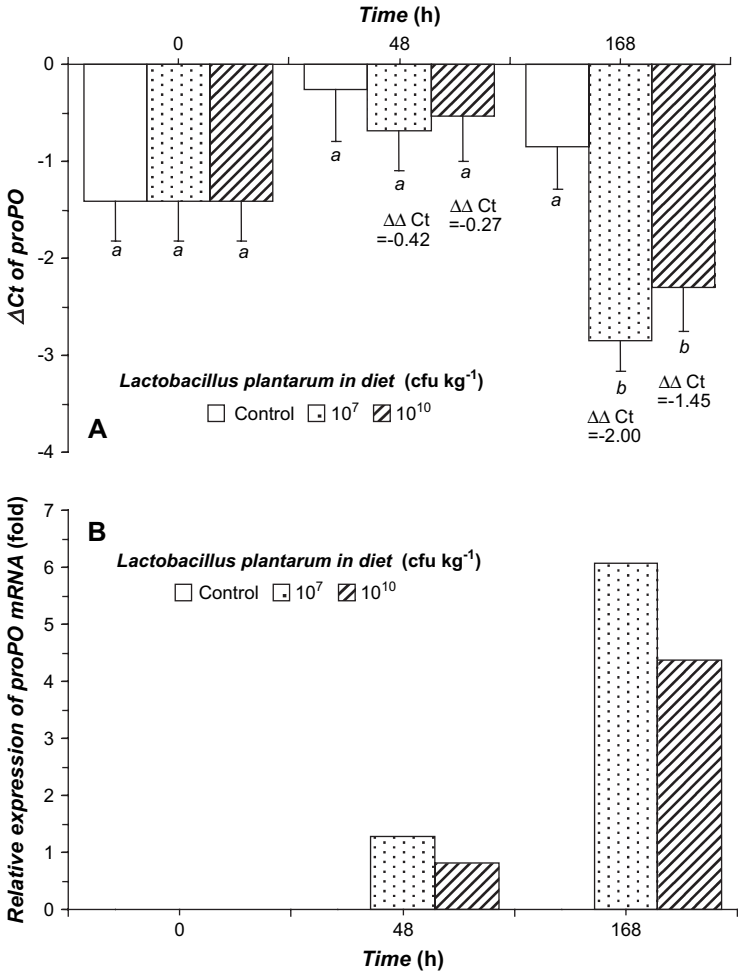

Fig. 6. Measurement of prophenoloxidase (proPO) mRNA expression in *Litopenaeus vannamei* fed the control diet and fed the *Lactobacillus plantarum*-containing diets at 10^7 and 10^{10} cfu (kg diet)$^{-1}$, respectively, by SYBR green RT–PCR. Each bar represents the ΔCt of proPO (the Ct value of the proPO gene minus the Ct value of the β-actin gene) as measured by SYBR green RT–PCR. Numbers above the bars indicate the ΔΔCt value (the ΔCt value of proPO in each treatment shrimp minus the ΔCt value of proPO in control shrimp) (A). A Ct value change (ΔΔCt) of 3.3 is equivalent to a 10-fold difference between the control and treatments (B). Bars in the same treatment time interval with different letters significantly differ ($p < 0.05$) among shrimp with different treatments.

shrimp fed the *Lac. plantarum*-containing diets may have increased bacterial levels in the digestive tract or the test water may have induced more haemocytes to attach to the epidermis to offer better protection. Unfortunately, bacterial levels in the digestive tract or test water were not determined in this study.

In the present study, the THCs of shrimp fed the diets containing *Lac. plantarum* at 10^7 and 10^{10} cfu (kg diet)$^{-1}$ were significantly lower at 168 h and 48–168 h, respectively, than those of shrimp fed the control diet. The phenoloxidase (PO) activity of shrimp fed diets containing *Lac. plantarum* at 10^7 and 10^{10} cfu (kg diet)$^{-1}$ were lower at 48 h, but significantly higher at 168 h than those of shrimp fed the control diet. In addition, the mRNA transcription of proPO in shrimp fed the 10^7 and 10^{10} cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diets after 168 h were significantly higher than that of shrimp fed the control diet. However, the cumulative mortality of shrimp following feeding on different levels of *Lac. plantarum*-containing diets for 48 h and then challenge with *V. alginolyticus* did not significantly differ at any time interval. These facts suggest that the PO activity of shrimp decreases as a consequence of the decrease in the circulating haemocyte count after 48 h of feeding. After168 h of feeding, upregulation of proPO mRNA by the shrimp resulted in increased PO activity which enhanced the resistance against the pathogen, *V. alginolyticus*.

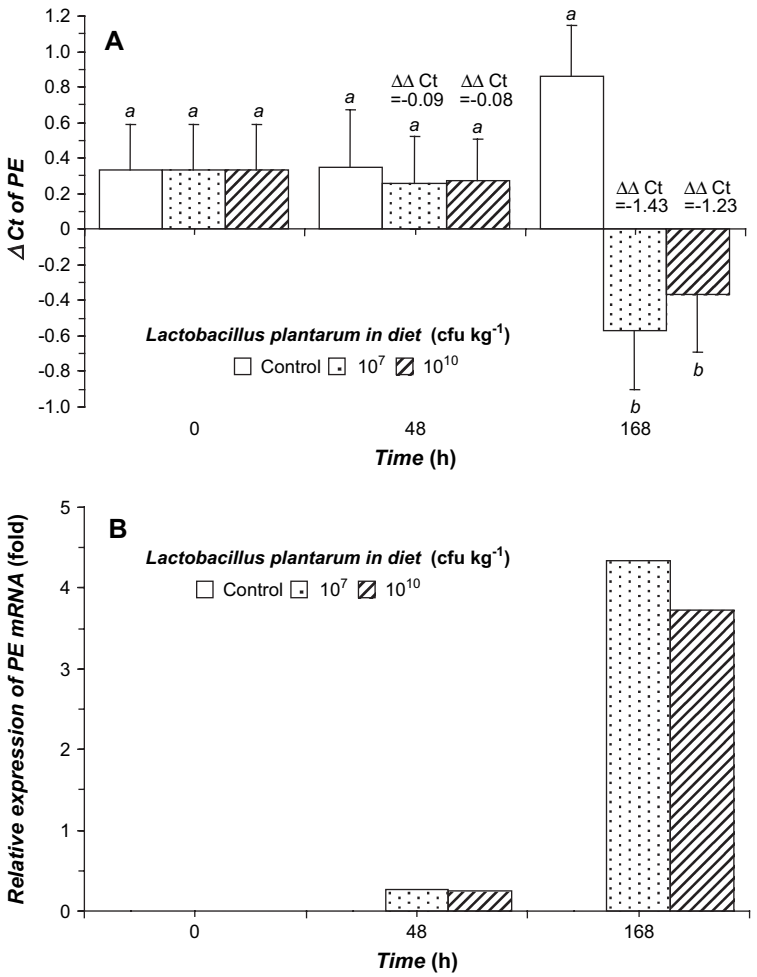

Fig. 7. Measurement of peroxinectin (PE) mRNA expression in *Litopenaeus vannamei* fed the control diet and fed the *Lactobacillus plantarum*-containing diets at 10^7 and 10^{10} cfu (kg diet)$^{-1}$, respectively, by SYBR green RT–PCR. Each bar represents the ΔCt of PE (the Ct value of the PE gene minus the Ct value of the β-actin gene) as measured by SYBR green RT–PCR. Numbers above the bars indicate the ΔΔCt value (the ΔCt value of PE in each treatment shrimp minus the ΔCt value of PE in control shrimp) (A). A Ct value change (ΔΔCt) of 3.3 is equivalent to a 10-fold difference between the control and treatment groups (B). See Fig. 6 for statistical information.

Respiratory bursts in haemocytes have been widely used to evaluate the defence ability against pathogens in shrimp [28,52]. They are produced by phagocytes in order to attack invasive pathogens during phagocytosis. Rainbow trout fed *Lac. rhamnosus* demonstrated a significant increase in respiratory burst activity of blood cells [40]. Also, the phagocytic activity of head kidney leucocytes increased in rainbow trout fed the same species [41]. A similar response was reported in gilthead seabream, *Sparus aurata*, fed *Lac. delbrueckii* [42], and rainbow trout, *Oncorhynchus mykiss,* fed *Carnobacterium maltaromaticum* B26 and *C. divergens* B33 at a dose $> 10^7$ cells (g diet)$^{-1}$ [25].

In the present study, the phagocytic activities of shrimp insignificantly differed among different treatments. Respiratory bursts of haemocytes increased in shrimp following feeding of the 10^7 cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diet from 48 to 168 h, and showed an insignificant change in shrimp following feeding with the 10^{10} cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diet for 168 h. The superoxide dismutase (SOD) activity and peroxinectin (a multifunctional protein containing biological activity of peroxidase) mRNA transcription level of shrimp following feeding with the 10^{10} cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diet significantly increased from 24 to 168 h and at 168 h, respectively. These facts suggest that the difference in respiratory bursts of shrimp administered different levels of *Lac. plantarum*-contained diets was a consequence of increases in the activity of SOD, which catalyses the superoxide

anion to hydrogen peroxide inducing an increase in PE gene transcription. Therefore, *Lac. plantarum* administration can enhance the situation with shrimp antioxidation.

The recognition protein, lipopolysaccharide and β-1, 3-glucan binding (LGBP) protein [8,53], a serine protease (SP) [4], and the cell adhesive protein, peroxinectin (PE) [13,15,54], are immune molecules relative to the proPO system. In this study, only PE of shrimp fed the *Lac. plantarum*-containing diet significantly increased after 168 h, suggesting that an increase in PE can increase the biological activities of cell adhesion [9], opsonin [11], degranulation [12], peroxidase [13], and encapsulation [14] of shrimp.

Phagocytosis is an important cellular defence mechanism, whereas clearance efficiency is an important humoral defence mechanism in crustaceans [55]. There was significantly higher serum lysozyme activity in rainbow trout fed *Lac. rhamnosus* [41], and fed *C. divergens* B33 [25]. In the present study, the clearance efficiency significantly increased in *L. vannamei* following feeding of the 10^7 and 10^{10} cfu (kg diet)$^{-1}$ *Lac. plantarum*-containing diets from 48 to 168 h, suggesting that *Lac. plantarum* was beneficial to shrimp in terms of enhancing the humoral immune response which may compensate for the decrease in PO activity after 48 h of feeding. Further research is necessary to determine the humoral immune parameters such as antimicrobial peptides of shrimp receiving *Lac. plantarum*.

In order to get information about the mechanism on the immune system of shrimp by probiotic stimulation, more experiments are needed to confirm the immune response of shrimp fed with dead or lysed *La. Plantarum* contained diets. Additional information about molecular characteristics of the lysed *La. Plantarum* surface, and compared with those from other probiotics, could also be useful to understand the chemical nature of the stimulus.

In conclusion, the *Lactobacillus plantarum* culture was beneficial for white shrimp in terms of resisting challenge by *Vibrio alginolyticus* and enhancing cellular and humoral immune responses, such as PO activity, SOD activity, and clearance efficiency against *V. alginolyticus*, and proPO and PE mRNA transcription levels.

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