Fish and Shellfish Immunology 88 (2019) 142–149

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Changes in the intestine barrier function of *Litopenaeus vannamei* in response to pH stress

Yafei Duan, Yun Wang, Qingsong Liu, Jiasong Zhang*, Dalin Xiong

Key Laboratory of South China Sea Fishery Resources Exploitation & Utilization, Ministry of Agriculture and Rural Affairs, Key Laboratory of Fishery Ecology and Environment, Guangdong Province, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou, 510300, PR China

ARTICLE INFO

Keywords:
Shrimp
pH stress
Intestine barrier
Immune
Microbiota

ABSTRACT

pH of water environment affects the survival of aquatic animals. Intestine barrier function influences the health of animals, which is related to its mucosa structure, immune components, and microbial communities. In this study, we investigated the histological structure, digestive and metabolic capacity, immune responses, and microbial composition in the intestine of *Litopenaeus vanmei* under three different conditions: control (pH 8.3), low pH stress (pH 6.9), and high pH stress (pH 9.7) for 72 h. The results showed both low and high pH stress disrupted the intestine morphological structure, and induced variations in the activities of digestive (AMS, LPS, Tryp, and Pep) and metabolic (HK, PK, CCO, and LDH) enzymes. Low and high pH stress also increased oxidative stress (MDA, LPO, PC, and $\cdot O_2^-$ generation), and decreased the antioxidant enzyme activities (T-AOC, SOD, and GST); shrimp enhanced CAT activity and *HSP70*, *Trx*, *MT* and *Fer* gene transcripts as defense mechanism. Additionally, Immune confusion was also found in the shrimp intestine in response to low and high pH stress, including the antibacterial ability (T-NOS, PO, *proPO*, *ALF*, and *Lys*), pathogen recognition (*TLR* and *Lec*), apoptosis (*Casp*, *IAP* and *p53*), and mucus homeostasis (*Muc-1*, *Muc-2*, *Muc*-5AC, *Muc-5B*, and *Muc-19*). pH exposure also decreased the diversity of the intestine bacterial, disturbed the composition of microbiota, and decreased the microbial metabolite SCFA contents. Our results indicated that acute pH stress can impair the intestine barrier function of white shrimp, probably via destroying mucosa structure, confusing digestion and metabolism, inducing oxidative stress, disordering immunity, and disrupting the microbial composition.

1. Introduction

The intestine of shrimp is an important organ for nutrient and immunity. The intestine barrier function serves as a front-line defense to diseases in shrimp, which is associated with its structural integrity, immune proteins, and a stable microbiota [1]. Shrimp rely on its innate immune responses to defense against stresses, including antibacterial, antioxidant, pathogen pattern recognition, and apoptosis, etc. The shrimp intestine also harbors abundant microbiota, and the function and stability of which is important to the host health [2]. Intestine microbiota is a complex microbial ecosystem that exist in a symbiotic relationship with the host, contributes to multiple physiological processes of the host [3]. Healthy intestine microbiota can produce beneficial metabolites, and protect the host from colonization of pathogenic microbes; conversely, unbalanced microbiota (dysbiosis) may produce harmful metabolites, leading to alteration of immunity and increasing the susceptibility to diseases [4,5]. If the structural integrity of the intestine is destroyed by various stresses, the opportunistic pathogen may invade and disorder the immunity of the host. Thus, intestine barrier function affects the overall health of shrimp.

Shrimp habitats are a pathogen-rich water environment; thus, its intestine may be affected by the water environment [1]. The pH is an important indicator of water environment affecting the life of shrimp. In mariculture system, pH levels fluctuate from 6.6 to 10.2 because of the consumption and release of carbon dioxide [6]. The pond sediments frequently induce the decreasing of pH [7]; conversely, organic wastes accumulation and algal bloom may cause red tide, resulting in the water pH rise to 9.0 [8,9]. Acidification water environment can reduce the blood pH value of aquatic animals, weaken its oxygen carrying capacity, and resulting in animal physiological hypoxia despite the high dissolved oxygen in water [10,11]. Alkaline water environment may corrode the animal's gill tissue, causing it to lose its absorb ability and die in large numbers [12,13]. Additionally, pH variation could cause a stress response in shrimp, leading to high mortality, oxidative stress, immune suppression, and pathogen susceptibility [10,11,14–16]. Till now, a clear understanding of the effects of pH stress on the intestine

* Corresponding author. South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, 231 Xingangxi Road, Guangzhou, 510300, PR China.
E-mail address: jiasongzhang@hotmail.com (J. Zhang).

https://doi.org/10.1016/j.fsi.2019.02.047
Received 11 December 2018; Received in revised form 21 February 2019; Accepted 22 February 2019
Available online 23 February 2019
1050-4648/

barrier function of shrimp is still lacking.

The Pacific white shrimp, *Litopenaeus vannamei*, is one of the most important marine species in the world. Shrimp cultivation has been suffering serious problems caused by polluted environment and diseases. Disease is the result of complex interaction of the host, its environment, and pathogens; of these, environmental stress is a main inducement factor [14]. In this study, we investigated the changes of the intestine barrier fuction of *L. vannamei* when exposed to acute low pH (pH 6.9) and high pH (pH 9.7) stress (compared to pH 8.3; control). Of that, intestine histological morphology, digestive and metabolism capacity, oxidative stress and immune responses, and microbiota composition were detected after pH stress 72 h.

2. Materials and methods

2.1. Experimental animals and pH exposure

Healthy juvenile *L. vannamei* were randomly selected from a local farming pond in Shenzhen, China, the average body weight was 3.2 ± 0.3 g. The shrimp were acclimated for 7 days before the stress experiment. The rearing water of the shrimp was sand-filtered seawater, and aerated with two stones. The parameters of water quality was pH 8.3, salinity 30‰, temperature 30 ± 0.5 °C, dissolved oxygen 6.5 ± 0.5 mg L^{-1}. The shrimp were fed daily with commercially feed at 5% of body weight. Changed one-third of water per day.

After acclimation, the shrimp were divided into three groups: the control group (CG), the low pH stress group (LG), and the high pH stress group (HG). Per group contained three replicate tanks. Shrimp were housed at 50 shrimp per tank and the tanks contained 500 L of filtered, aerated seawater. pH values of the CG, LG, and HG were set at 8.3, 6.9, and 9.7 by adding 1.0 mol/L HCl or 1.0 mol/L NaOH solution to the regular seawater. The stability of the water pH was continuously maintained using a portable multiparameter meter (YSI, USA). The pH stress experiment lasted for 72 h. At 72 h, intestine of the shrimp of each tank were sampled individually, snap-frozen in liquid nitrogen for biochemical, gene-expression, and microbiota analysis.

2.2. Intestine morphology

The intestine from three shrimp per tank were extracted at 72 h of pH stress, and fixed in Davidson solution (330 mL 95% ethanol, 220 mL formalin, 115 mL acetic acid, and 335 mL H_2O) for 24 h. The samples were placed in flush water for 8 h, and the embedded lumps were put into 70% ethanol overnight. Then, the samples were dehydrated with different concentrations of ethanol (80%, 90%, and 100%), followed by acetone and xylene transparent, paraffin embedding. The intestine sections was sliced to 4 μm, stained with hematoxylin and eosin (HE), and examined with a microscopy (Olympus, Tokyo, Japan).

2.3. Biochemical assays

The intestine from three shrimp per tank were broken to 10% tissue homogenate with 0.9% saline solution, then centrifuged (3500 rpm, 4 °C, 10 min). The supernatant was immediately detected for biochemical parameters using a microplate reader (Bio-Rad, USA). Amylase (AMS), lipase (Lip), trypsin (Tryp), pepsin (Pep), hexokinase (HK), pyruvate kinase (PK), cytochrome *c* oxidase (CCO), lactate dehydrogenase (LDH), malondialdehyde (MDA), lipid peroxidation (LPO), protein carbonyl (PC), $\cdot O_2^-$ generation capacity, total antioxidant capacity (T-AOC), superoxide dismutase (SOD), catalase (CAT), glutathione *S*-transferase (GST), phenoloxidase (PO), and total nitric oxide synthase (T-NOS) were measured using related commercial assay kits (Nanjing Jiancheng Institute, China) according to the manufacturer's protocol. The total protein concentration in tissue homogenates was measured using a Coomassie brilliant blue protein assay kit (Jiancheng, Ltd., Nanjing, China) according to the manufacturer's protocol. The assays were all run in three replicate samples.

One unit of AMS activity was defined as the amount of enzyme per mg tissue protein every 30 min hydrolyzes 10 mg starch at 37 °C. One unit of LPS activity was defined as the amount of enzyme per g tissue protein every minute catalyzes 1 mmol substrate at 37 °C. One unit of Tryp activity was defined as the amount of enzyme per mg tissue protein every minute make the absorbance change 0.003 at 37 °C under the pH 8.0 assay condition. One unit of Pep activity was defined as the amounts of enzyme per mg tissue protein every minute generate 1 mg amino acid by hydrolyzing protein at 37 °C. One unit of HK activity was defined as the amount of enzyme per g tissue protein producing 1 mmol/L NADH every minute at 37 °C and pH 6.0. One unit of PK activity was defined as the amount of enzyme per g tissue protein making 1 μmoL phosphoenolpyruvate into pyruvic acid every minute at 37 °C and pH 6.0. CCO activity was calculated from the linear rate of absorbance decrease at 550 nm. One unit of LDH activity was defined as per g tissue protein reacted with a substrate at 37 °C to produce 1 μmol pyruvic acid within 15 min. MDA and LPO content was measured spectrophotometrically with thiobarbituric acid (TBA) method and the product were read spectrophotometrically at 586 nm and 532 nm respectively. PC content was measured spectrophotometrically at 370 nm, and expressed as nmol/mgprot. $\cdot O_2^-$ generation capacity was measured spectrophotometrically using xanthine/xanthine oxidase as the $\cdot O_2^-$ generator and the product were read spectrophotometrically at 550 nm. One unit of T-AOC was defined as the amount of enzyme per mg tissue protein inducing an absorbance increase of 0.01 every minute at 37 °C. One unit of SOD activity was defined as the amount of SOD required per mg of tissue protein to inhibit the rate of xanthine reduction by 50% in 1 mL of reaction solution. One unit of CAT activity was defined as the amount of enzyme per *mg* of tissue protein needed to reduce 1 μmol of H_2O_2 in 1 s under the assay conditions. GST was determined by monitoring the conjugation of reduced glutathione with 1-chloro-2, 4-dinitrobenzene (CDNB) at 412 nm. One unit of GST activity was defined as the amount of enzyme per mg tissue protein every minute deduction of enzymatic reaction reduce 1 μmol/L GSH at 37 °C. T-NOS activity was measured by the catalysing reaction of L-arginine and O_2 at 530 nm. PO activity was measured using L-3,4-dihydroxyphenylalanine (L-DOPA) as a substrate. One unit of PO activity was defined as a linear increase in absorbance of 0.001 per mg tissue protein every minute.

2.4. Gene expression analysis

Total RNA from the intestine of three shrimp per tank was extracted using TRIzol Reagent (Invitrogen, USA), then the genome DNA was eliminated using RQ1 RNase-Free DNase (Promega, USA). Total RNA (8 μg) was reverse transcribed to cDNA using PrimeScript™ RT Reagent Kit (Takara, China) according to the manufacturer's protocol. Real-time RT-qPCR was conducted in a LightCycler480 System using a SYBR® Premix Ex Taq™ II Kit (TaKaRa, Japan). *β-actin* gene of *L. vannamei* was chosen as an internal control, and the specific primer sequences of the target genes were designed using Primer Premier 5.0 software and listed in Table 1. The RT-qPCR was carried out in a total volume of 20 μL, containing 10 μL SYBR Premix Ex Taq II (Tli RNaseH Plus) (2 ×), 2 μL of the 1:7 diluted cDNA, 0.8 μL each of 10 μmol/L forward and reverse primers, and 6.4 μL DEPC-treated water. The PCR program was 95 °C for 30 s, then 40 cycles of 95 °C for 5 s and 60 °C for 30 s, followed by 1 cycle of 95 °C for 5 s, 60 °C for 1 min and 95 °C for 15 s. DEPC-treated water for the replacement of template was used as negative control. To estimate transcript copy numbers for each sample, RT-qPCR data from three replicate samples were analyzed with the LightCycler480 system SDS Software. The relative gene expression level is shown as the fold-change in expression, relative to the *β-actin* gene, calculated by the $2^{-\triangle\triangle CT}$ comparative C_T method.

Table 1
Primer sequence used in this study.

Primer name	Sequence (5′-3′)	GenBank accession number
proPO-F	CAATGACCAGCAGCGTCTTC	AY723296
proPO-R	CACGGAAGGAGGCGTATCAT	
ALF-F	TTACTTCAATGGCAGGATGTGG	KJ000049
ALF-R	GTCCTCCGTGATGAGATTACTCTG	
Lys-F	GTTCCGATCTGATGTCCGATG	AY170126
Lys-R	AAGCCACCCAGGCAGAATAG	
HSP70-F	CAACGATTCTCAGCGTCAGG	AY645906
HSP70-R	ACCTTCTTGTCGAGGCCGTA	
Trx-F	TTCCTGAAGGTGGATGTGGA	EU499301
Trx-R	AGTTGGCACCAGACAAGCTG	
MT-F	CTGATCCATGCTGTAACGAG	KJ701600
MT-R	CATCTTGTTGCACACTCCTC	
Fer-F	ACCTTCAAGCGAACCTCTGG	AY955373
Fer-R	GGTAGTTCTGGCGGACTTGG	
TLR-F	CCTTGCTGACTGGGCATTTGG	KM272333
TLR-R	TGCATTGCTGGCTCCGAGACTTA	
Lec-F	GCAGCAACCTGATAATGCACA	DQ871245
Lec-R	TCGGATGGTGGCTTCACATA	
Casp-F	CGAAGTCAAAGCCAGAAACA	EU421939
Casp-R	ACTGCTACTTCCCTGGTGAC	
IAP-F	CAACACCTGCCTCAGGACAA	GQ293142
IAP-R	CTTCCATTGCCTCCTCGTCT	
p53-F	AAGACACCGAACGATGGAAG	KC422442
p53-R	TGGGGGACTCGTTCTTTATG	
Muc-1-F	GGCTCGGAAGTTGGCGATGATG	Duan et al. [1]
Muc-1-R	CGATGGCTCAATGGCGAAGAGG	
Muc-2-F	TGCCAGCCACGTCCTCCTTG	Duan et al. [1]
Muc-2-R	CCGCAGCCGAGGCAGTCC	
Muc-5AC-F	AGCAGGACTTCAACGACTACAACAG	Duan et al. [1]
Muc-5AC-R	GCGCGACGCCGATGATGG	
Muc-5B-F	CTTGACGCATACGCTCAGGTTCC	Duan et al. [1]
Muc-5B-R	TCCGCCGCCTTCATCCTCTG	
Muc-19-F	GAAGAGGAGGAAGAGGACGAGGAG	Duan et al. [1]
Muc-19-R	GGACCACCAGGCACAAGAACATC	
β-actin-F	GCCCTGTTCCAGCCCTCATT	AF300705
β-actin-R	ACGGATGTCCACGTCGCACT	

2.5. Intestine microbiota analysis

Intestine microbial DNA of three shrimp per tank was extracted using a PowerSoil™ DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's protocol. The purity DNA quality was examined with 1.0% agarose electrophoresis. The V4 region of the bacterial 16S rRNA gene was amplified using the barcoded fusion primers 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACNVGGGTWTCTAAT). The PCR reactions was 20 μL, containing the FastPfu polymerase 0.4 μL, dNTPs (2.5 mM) 2 μL, 5 × FastPfu buffer 4 μL, each primer (5 μM) 0.8 μL, and template DNA 10 ng. The PCR reaction programs were 1 cycle of 95 °C for 5 min, 27 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, followed by 72 °C for 10 min. The PCR products was purified by a PCR purification kit (Qiagen), then was sequenced with an Illumina HiSeq platform. The raw sequences were processed using the BIPES pipeline. Chimeric sequences were determined by undetected chimera (UCHIME) [17]. The operational taxonomy units (OTUs) were defined with a threshold of 97% identity by UPARSE [18]. Taxonomies were assigned with uclust for each OTU, and alpha and beta diversity analyses were determined for each library using quantitative insights into microbial ecology (QIIME).

2.6. Statistical analysis

The value of each variable was expressed as the mean ± SE. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Duncan multiple range tests (SPSS Ver 17.0). Significance was set at $P < 0.05$.

3. Results

3.1. Intestine histological structure

The intestine mucosa of the CG showed good morphology (Fig. 1A). After shrimp exposed to low and high pH stress, there were obvious pathological damage in the intestine mucosa of the LG and HG, such as epithelial cells were exfoliated from the basement membrane, and out of shape; lots of them had cavitation bubble (Fig. 1B and C).

3.2. Digestive and metabolism capacity of shrimp to low and high pH stress

After pH stress, digestive enzymes including AMS, LPS, and Tryp activity were decreased in the LG and HG, while Pep activity was only decreased in the LG (Fig. 2A–D). The activities of energy metabolism enzymes HK and PK were decreased in the LG and HG (Fig. 2E and F). Respiratory metabolism enzymes including CCO activity was decreased in the LG and HG, while LDH was increased in the two groups (Fig. 2G and H).

3.3. Oxidative stress responses of shrimp to low and high pH stress

After pH stress, the contents of MDA, LPO and PC, and the capacity for $\cdot O_2^-$ generation were all increased in the LG and HG (Fig. 3A–D). In response to oxidative stress, antioxidant enzymes including T-AOC, SOD, and GST activity were all decreased in the LG and HG, while CAT activity was increased (Fig. 3E–H). The relative expressions of antioxidant related genes including heat shock protein 70 (*HSP70*), thioredoxin (*Trx*) and metallothionein (*MT*) were all increased in the LG and HG; ferritin (*Fer*) was increased in the LG, while decreased in the HG (Fig. 3I–L).

3.4. Immune responses of shrimp to low and high pH stress

After pH stress, immune enzymes including T-NOS and PO activity

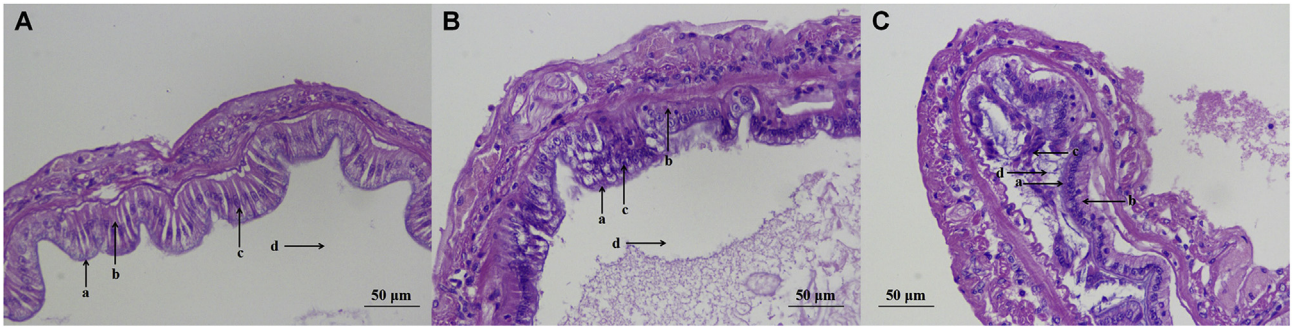

Fig. 1. Intestine tissue of *L. vannamei* stained with HE exposed to low and high pH stress 72 h. **(A)** The control group (CG), × 400; **(B)** the low pH group (LG), × 400; **(C)** the high pH group (HG), × 400. The letters in the Figure indicate that: **(a)** brush border, **(b)** epithelium, **(c)** nuclei, **(d)** lumen.

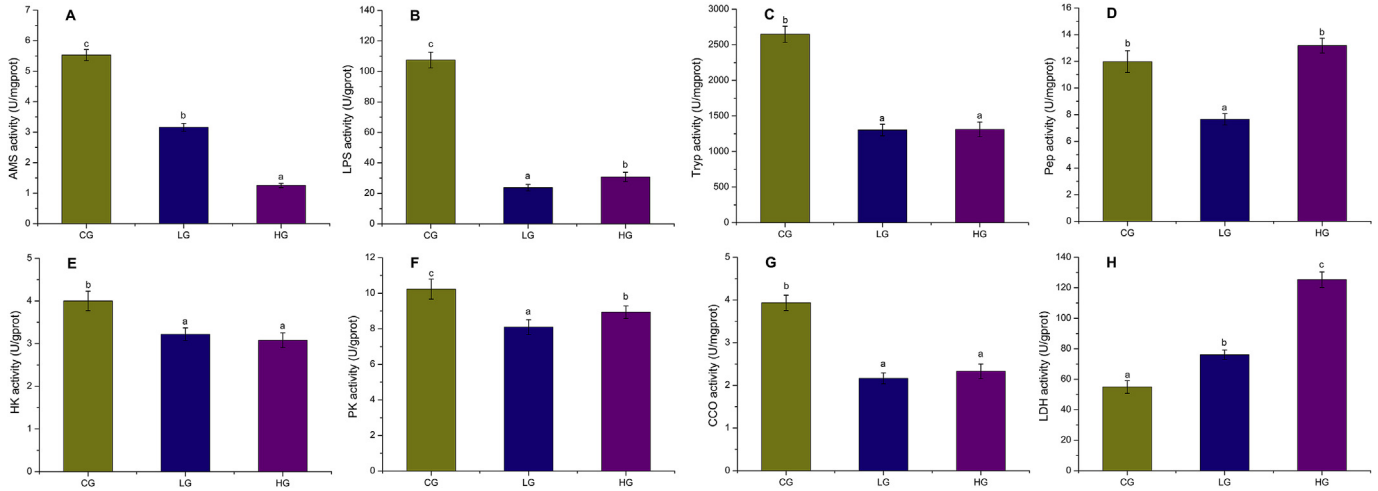

Fig. 2. Intestine digestive and metabolism capacity of *L. vannamei* exposed to low and high pH stress 72 h. **(A)** AMS activity; **(B)** LPS activity; (**C**) Tryp activity; (**D**) Pep activity; **(E)** HK activity; **(F)** PK activity; **(G)** CCO activity; (**H**) LDH activity. Vertical bars represent the mean ± SE ($n = 3$). The different letters (a, b, c) indicate significant differences ($P < 0.05$) among groups.

were both decreased in the LG and HG (Fig. 4A and B). The relative expressions of antibacterial-related genes including prophenoloxidase (*proPO*) were decreased in the LG and HG, while anti-lipopolysaccharide factor 1 (*ALF*) were increased in the two groups; lysozyme (Lys) was only decreased in the HG (Fig. 4C–E). The relative expressions of pathogen pattern recognition genes including toll like receptor 4 (*TLR*) were decreased in the LG and HG, while C-type lectin (*Lec*) were increased in the two groups (Fig. 4F and G). The relative expressions of apoptosis genes including caspase-3 (*Casp*), inhibitor of apoptosis protein (*IAP*), and p53 were all decreased in the LG and HG (Fig. 4H–J). The relative expressions of mucin (*Muc*) genes including *Muc-1*, *Muc-5AC*, and *Muc-5B* were all decreased in the LG and HG, while *Muc-2* and *Muc-19* were both increased in the two groups (Fig. 4K–O).

3.5. Intestine microbiota responses of shrimp to low and high pH stress

Illumina sequencing of intestine microbial produced 496,732 high-quality sequences, with an average of 55,192 sequences per sample. The coverage for each group exceeded 99%. Rarefaction curves analysis of the observed species per sample was sufficient (Fig. 5A). A total of 389 OTUs were shared by the three groups with Venn diagram analysis, and the number of unique OTUs in the LG and HG were lower than the

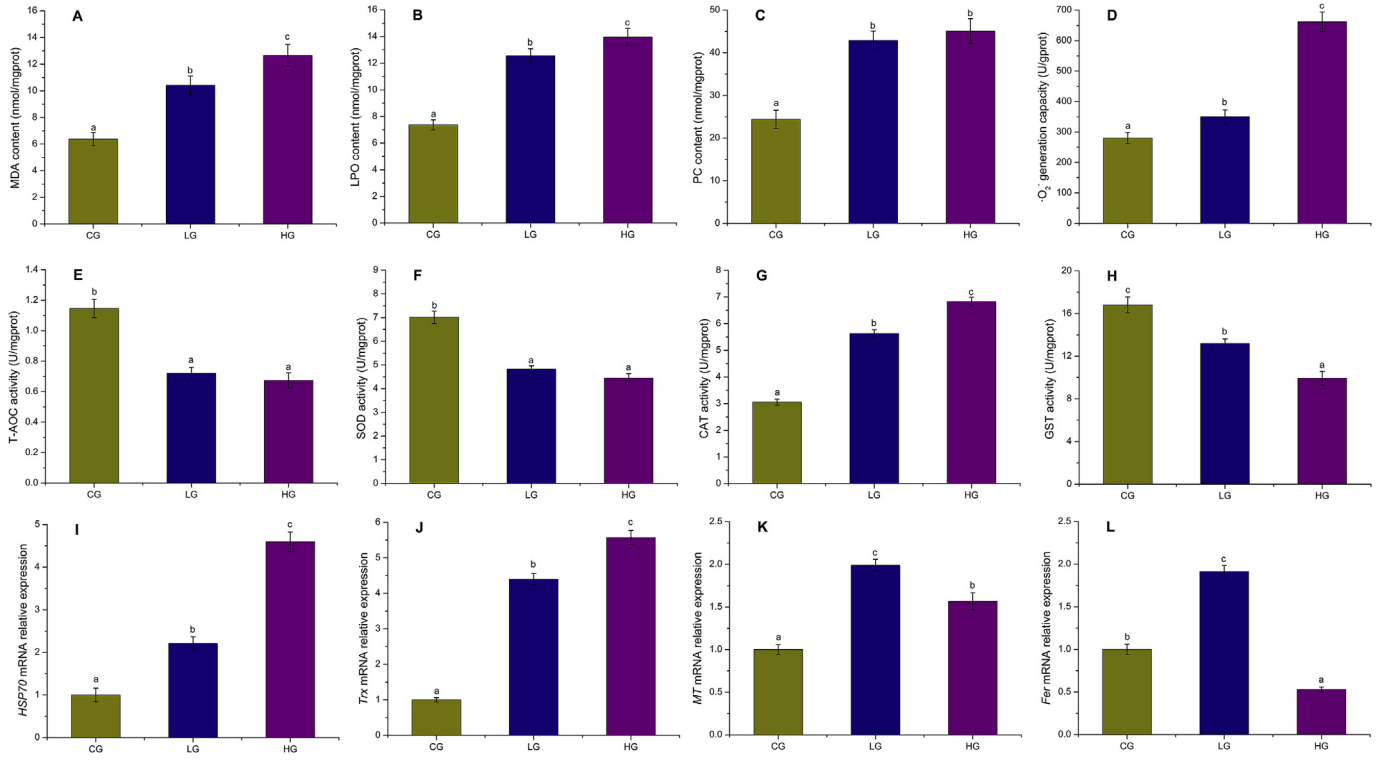

Fig. 3. Intestine oxidative stress responses of *L. vannamei* exposed to low and high pH stress 72 h. **(A)** MDA content; **(B)** LPO content; (**C**) PC content; (**D**) $\cdot O_2^-$ generation capacity; **(E)** T-AOC activity; **(F)** SOD activity; **(G)** CAT activity; (**H**) GST activity; (**I**) *HSP70* mRNA expression; (**J**) *Trx* mRNA expression; **(K)** *MT* mRNA expression; (**L**) *Fer* mRNA expression. Vertical bars represent the mean ± SE ($n = 3$). The different letters (a, b, c) indicate significant differences ($P < 0.05$) among groups.

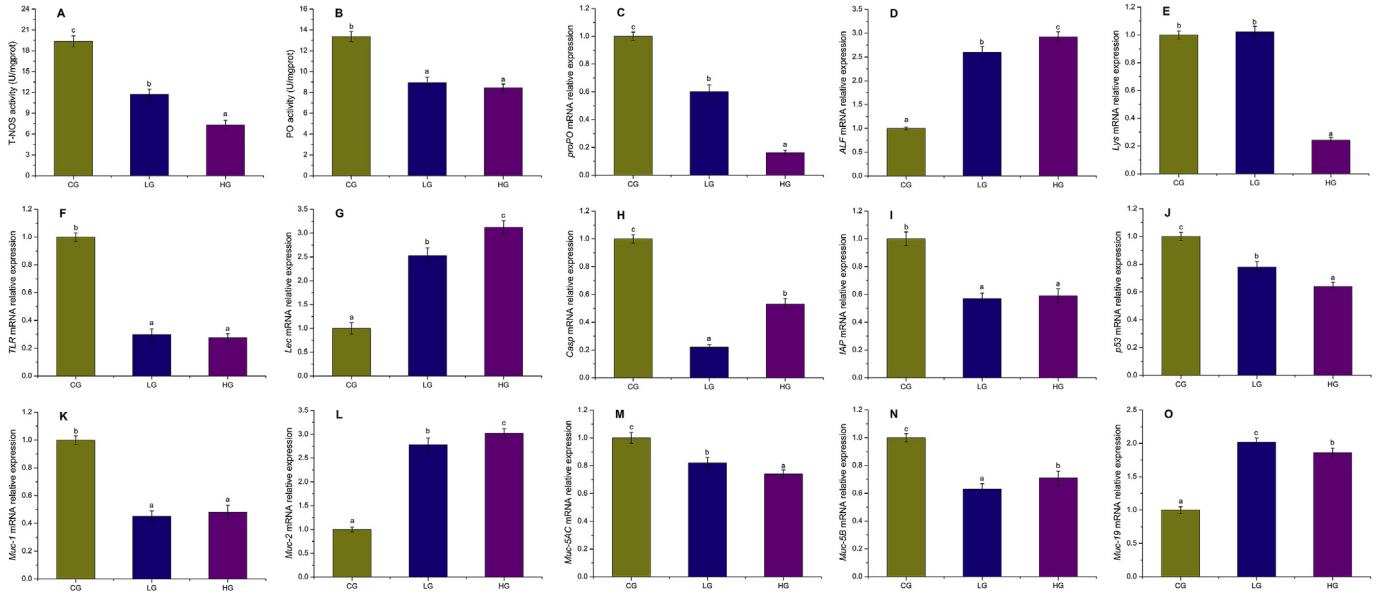

Fig. 4. Intestine immune responses of *L. vannamei* exposed to low and high pH stress 72 h. **(A)** T-NOS activity; **(B)** PO activity; (**C**) proPO mRNA expression; (**D**) *ALF* mRNA expression; **(E)** *Lys* mRNA expression; **(F)** *TLR* mRNA expression; **(G)** *Lec* mRNA expression; (**H**) *Casp* mRNA expression; (**I**) *IAP* mRNA expression; (**J**) *p53* mRNA expression; (**K**) *Muc*-1 mRNA expression; (**L**) *Muc*-2 mRNA expression; (**M**) *Muc*-5AC mRNA expression; (**N**) *Muc-5B* mRNA expression; (**O**) *Muc*-19 mRNA expression. Vertical bars represent the mean ± SE ($n = 3$). The different letters (a, b, c) indicate significant differences ($P < 0.05$) among groups.

CG (Fig. 5B). The alpha diversity analysis showed that the Chao1, ACE, Shannon, and Simpson indexes of the LG and HG were all lower than those of the CG; there was no significant difference between LG and HG (Table 2). Non-metric Multi-Dimensional Scaling (NMDS) based on Bray-Curtis distance was performed to detect the relationships of the microbial in different samples, and confirmed that the intestine microbial in the three groups were separated (Fig. 5C).

A total of 33 different bacterial phylum were identified. Proteobacteria and Bacteroidetes were two of the dominant phyla in the three clustered groups. Compared with the CG, the relative abundance of Proteobacteria, Verrucomicrobia, Actinobacteria, and Firmicutes were decreased in the LG and HG, while that of Bacteroidetes was increased (Fig. 5C). At the class level, Flavobacteriia, Gammaproteobacteria, and Alphaproteobacteria were the primary intestine bacterial

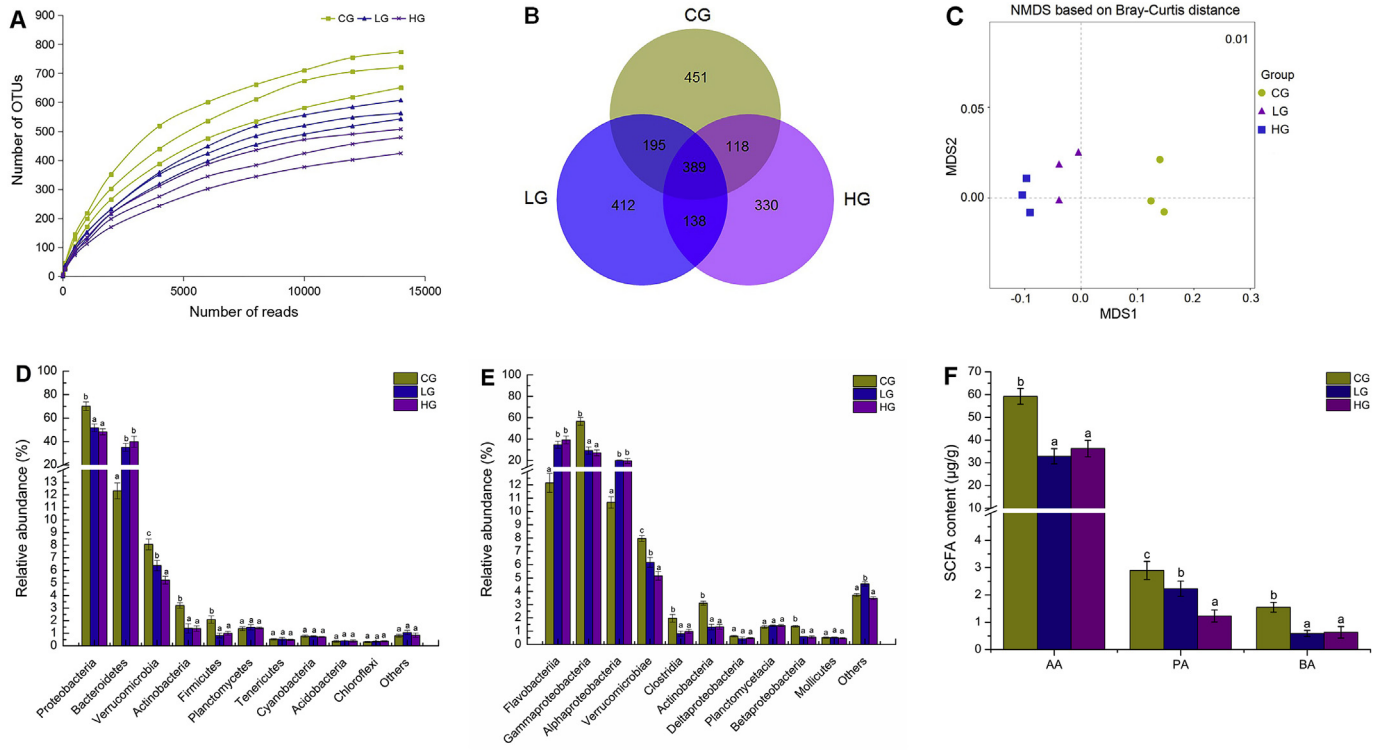

Fig. 5. Intestine microbiota responses of *L. vannamei* exposed to low and high pH stress 72 h. **(A)** Rarefaction curves; **(B)** Venn diagram; (**C**) NMDS based on Bray-Curtis distance; (**D**) Relative abundance of intestine microbial at the phylum level; **(E)** Relative abundance of intestine microbial at the class level; **(F)** SCFA content. Vertical bars represent the mean ± SE ($n = 3$). The different letters (a, b, c) indicate significant differences ($P < 0.05$) among groups.

Table 2
Alpha diversity of intestinal microbial after *L. vannamei* exposed to low and high pH stress 72 h.

Group	Observed	Chao1	ACE	Shannon	Simpson
CG	672 ± 39	817 ± 36[a]	845 ± 37[a]	3.36 ± 0.15[a]	0.82 ± 0.05[a]
LG	638 ± 24	613 ± 42[b]	713 ± 25[b]	3.02 ± 0.18[b]	0.76 ± 0.03[b]
HG	646 ± 47	633 ± 31[b]	698 ± 34[b]	2.89 ± 0.13[b]	0.72 ± 0.06[b]

ACE, abundance-based coverage estimator. Values represent the mean ± SE ($n = 3$). The different letters (a, b, c) indicate significant differences ($P < 0.05$) among groups.

in all of the groups. Compared with the CG, the relative abundance of Flavobacteriia and Alphaproteobacteria were increased in the LG and HG, while that of Gammaproteobacteria, Verrucomicrobia, Clostridia, Actinobacteria, and Betaproteobacteria were decreased (Fig. 5D). Gas chromatography was performed to detect the contents of intestine microbial metabolites short-chain fatty acids (SCFA) including acetic acid (AA), propionic acid (PA) and butyric acid (BA), showed that these were all decreased in the LG and HG (Fig. 5E).

4. Discussion

The nutrient uptake ability of shrimp intestine mainly depend on its intestine epithelium and microvilli, and the digestive function of intestine mainly rely on its digestive enzymes [19]. The structural integrity of intestine mucosa benefits the intestine health of shrimp. It was reported that environmental stress could damage the intestine mucosa of the shrimp [19,20]. In this study, after low and high pH stress, the intestine mucosa epithelial cells were damaged obviously, and the activities of AMS, LPS, and Tryp were decreased in the LG and HG, the activity of Pep was decreased in the LG, which indicated that low and high pH stress destroyed the structural integrity of intestine, and disordered its digestion function. Glycolysis is an important energy metabolic pathway in organisms, which can provide energy to organisms [21]. Of that, HK and PK are two important rate-limiting enzymes [22]. Additionally, organisms can also obtain energy respiratory metabolism; CCO and LDH are two marker enzymes of aerobic and anaerobic respiration respectively [23,24]. In this study, the activities of HK, PK, and CCO were decreased in the LG and HG, while the activity of LDH was increased in the two groups. Hence forecasts low and pH stress might disorder the energy and respiratory metabolic homeostasis in the intestine of shrimp.

The increasing of oxidative stress is one of the toxic effect of environmental pollutants in shrimp [25]. The excessive of ROS is damage to organism. LPO and PC are considered to be consequences of oxidation of lipid and protein by ROS; MDA is the end product of LPO that is recognized as an indicator of oxidative damage [19]. It was reported that pH stress could influence the oxygen affinity and consumption of *Palaemontes kadiakensis* [26]. ROS production in haemolymph of *L. vannamei* was also increased [16,27]. Similarly, in this study, the contents of MDA, LPO and PC, and the capacity of $\cdot O_2^-$ generation were increased in the intestine of *L. vannamei* in response to low and high pH stress, indicating that oxidative stress occurred in the shrimp intestine. To counteract oxidative stress damage, organisms have evolved antioxidant defense system, including antioxidant enzymes and proteins. SOD, CAT, and GST are important antioxidant enzymes to defense oxidative stress, and the total status of antioxidant enzymes can be reflected with T-AOC activity [19]. As important antioxidant proteins, HSPs can increase endogenous peroxidase activity to catalyze the conversion of ROS [28]; Trx acts as a substrate of peroxiredoxin and function in cell redox homeostasis [29]; Fer orchestrates the cellular defense against stress and inflammation by regulating the cellular availability of transition iron in Fenton reaction [30]; MT plays an antioxidant role as an effective free radical scavenger [31]. In this study, significant deduction decreases of T-AOC, SOD and GST activity, and induction of CAT activity and *HSP70*, *Trx* and *MT* gene transcripts were observed in the intestine of *L. vannamei* in response to low and high pH stress, while *Fer* gene transcripts was induced in the LG, but deduced in the HG. These mechanisms indicated that pH stress might break the balance of redox status in the shrimp intestine, probably via increasing the oxidative stress parameters, and confusing the antioxidant enzyme activities and gene transcripts.

Intestine immune function of shrimp plays a vital role in resisting pathogens infection and environmental stress. As antibacterial molecules, T-NOS, ALF and Lys are important components of the non-specific immune system against pathogenic microbes [32–34]; proPO plays its immune function by increasing PO activity [35]. In this study, after pH stress, a significant decrease in the T-NOS and PO activity and *proPO* gene expression in the LG and HG, while *Lys* gene expression was only decreased in the HG, *ALF* gene expression was increased in the LG and HG, indicating pH stress might influence the antibacterial function of shrimp. With exception to the immune system, an early pivotal event in the immune process is to recognize the pathogen. *TLR* and *Lec* initiate the immune response of organism by facilitating pathogen recognition associated molecule patterns [36,37]. In this study, after pH stress, the transcript levels of *TLR* was decreased, while that of *Lec* was increased, indicating that exposure of pH stress disturbed the intestine pathogen pattern recognition process. Apoptosis is a normal physiological process for eliminating harmful cells and preventing excessive cell proliferation [38]. Of that, *Casp* and *p53* play important roles in mediating apoptosis [39,40]; contrarily, *IAP* has a strong inhibitory effect on apoptosis [41]. In this study, *Casp*, *IAP* and *p53* gene transcripts were down-regulated in the shrimp intestine in response to pH stress, suggesting that apoptosis program was failed to remove unnecessary or deleterious cells to resist stress. Mucus is present at the interface between the epithelial surfaces of the intestine and its internal environment, representing the principal immune response of the intestine mucosa [42]. Mucs are the major organic components of the intestine mucus layer that contribute to the mucosal barrier against enteric pathogens [43]. In this study, the expressions of *Muc-2* and *Muc-19* gene were up-regulated, while the expressions of *Muc-1*, *Muc*-5AC, and *Muc-5B* were down-regulated, indicating that pH stress influenced the intestine mucus homeostasis of the shrimp. In general, pH stress might disorder the immune functions in the shrimp intestine, including the antibacterial ability, pathogen recognition, apoptosis, and mucus homeostasis.

The intestine microbial plays important roles in the intestine barrier function of the host. Intestine microbiota includes various opportunistic pathogens. When the integrity of intestine mucosa is broken by stress, pathogens can seize the opportunity to penetrate the barrier of the intestine, enter the body and disorder the immunity of the host [1]. Previous studies have shown that water environmental mutations and exposure to environmental pollutants caused significant changes in the intestine microbial of aquatic animals [1,20,44–46], thus induced the inflammation and immune disorders of the host [47]. Bacterial diversity functions in important ecological ability, which was decreased in the diseased shrimp, and closely associated with the severity of shrimp disease [48]. In this study, after shrimp exposure to low and high pH stress, the diversity of intestine bacterial was decreased; the dominant bacterial phylum Proteobacteria abundance was decreased, while the Bacteroidetes abundance was increased; the Firmicutes abundance was also decreased. Proteobacteria and Bacteroidetes are both intestine dominant bacterial of shrimp [1,49–51]. Firmicutes bacteria provide a good index regarding the state of the intestine, including producing SCFA and prebiotics [52]. SCFA, including AA, PA, and BA, are beneficial metabolites of the intestine bacterial, which can provide nutrition for the intestine mucosa, and benefit the regulation of the intestine micro-ecological environment of the host [53]. In this study, SCFA contents in the intestine of shrimp were also decreased, indicating that pH stress influenced the production of beneficial metabolites of the intestine bacterial. Together, these phenomena revealed that low and high pH stress could impair the functions and stability of

intestine microbial of the shrimp, by decreasing the bacterial diversity, disrupting the composition of intestine microbial, and reducing the bacterial metabolite SCFA contents. These might compromise the intestine immunity of the host.

In conclusion, acute low and high pH stress damaged the morphologic structure, affected the levels of genes expression and enzymes activity related to digestive and metabolism, oxidative stress response, and immune function, and caused biomolecule damage in the intestine of *L. vannamei*. Acute exposure to pH stress resulted in a significant changes in the composition and metabolites content of intestine microbiota. Our results suggest that pH stress impaired the intestine barrier function of the shrimp. Further studies need to explore the correlation of the intestine microbiota with the shrimp immune and metabolism in response to pH stress.

Acknowledgments

This study was supported by the earmarked fund for the Central Public-interest Scientific Institution Basal Research Fund, South China Sea Fisheries Research Institute, CAFS (2019TS18, 2017YB12), Guangdong Provincial Special Fund for Marine Fisheries Technology (A201701B09), Guangdong Natural Science Foundation (2017A030313147), and Central Public-interest Scientific Institution Basal Research Fund, CAFS (2019CY0103). The authors are grateful to the laboratory staff for technical assistance.

References

[1] Y.F. Duan, Q.S. Liu, J.S. Zhang, Y. Wang, D.L. Xiong, Impairment of the intestine barrier function in *Litopenaeus vannamei* exposed to ammonia and nitrite stress, Fish Shellfish Immunol. 78 (2018) 279–288.
[2] K. Brown, D. DeCoffe, E. Molcan, D.L. Gibson, Diet-induced dysbiosis of the intestinal microbiota and the effects on immunity and disease, Nutrients 4 (2012) 1095–1119.
[3] M. Levy, E. Blacher, E. Elinav, Microbiome, metabolites and host immunity, Curr. Opin. Microbiol. 35 (2017) 8–15.
[4] E.Y. Hsiao, S.W. McBride, S. Hsien, G. Sharon, E.R. Hyde, T. McCue, et al., Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders, Cell 155 (2013) 1451–1463.
[5] R.A. Koeth, Z. Wang, B.S. Levison, J.A. Buffa, E. Org, B.T. Sheehy, et al., Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis, Nat. Med. 19 (2013) 576–585.
[6] C.E. Boyd, Water Quality in Ponds for Aquaculture, (1990).
[7] H. Lemonnier, E. Bernard, E. Boglio, C. Goarant, J.C. Cochard, Influence of sediment characteristics on shrimp physiology: pH as principal effect, Aquaculture 240 (2004) 297–312.
[8] A.L. Wang, W.N. Wang, The study of the relationship between red tide and environment factors in shrimp farming, Acta Oceanol. Sin. (Chin.) 17 (1995) 128–133.
[9] W.N. Wang, A.L. Wang, L. Chen, Y. Liu, R.Y. Sun, Effects of pH on survival, phosphorus concentration, adenylate energy charge and Na+-K+ ATPase activities of Penaeus chinensis Osbeck juveniles, Aquat. Toxicol. 60 (2002) 75–83.
[10] S.Y. Han, B.J. Wang, M. Liu, M.Q. Wang, K.Y. Jiang, X.W. Liu, L. Wang, Adaptation of the white shrimp *Litopenaeus vannamei* to gradual changes to a low-pH environment, Ecotoxicol. Environ. Saf. 149 (2018) 203–210.
[11] S.Y. Han, M.Q. Wang, B.J. Wang, M. Liu, K.Y. Jiang, L. Wang, A comparative study on oxidative stress response in the hepatopancreas and midgut of the white shrimp *Litopenaeus vannamei* under gradual changes to low or high pH environment, Fish Shellfish Immunol. 76 (2018) 27–34.
[12] Z. Qian, X. Liu, L. Wang, X. Wang, Y. Li, J. Xiang, P. Wang, Gene expression profiles of four heat shock proteins in response to different acute stresses in shrimp, *Litopenaeus vannamei*, Comp. Biochem. Physiol., C 156 (2012) 211–220.
[13] J. Zhou, W.N. Wang, G.Z. Ma, A.L. Wang, W.Y. He, P. Wang, et al., Gene expression of ferritin in tissue of the Pacific white shrimp, *Litopenaeus vannamei* after exposure to pH stress, Aquaculture 275 (2008) 356–360.
[14] Y.Y. Chen, J.C. Chen, K.C. Tseng, Y.C. Lin, C.L. Huang, Activation of immunity, immune response, antioxidant ability, and resistance against *Vibrio alginolyticus* in white shrimp *Litopenaeus vannamei* decrease under long-term culture at low pH, Fish Shellfish Immunol. 46 (2015) 192–199.
[15] C.C. Li, J.C. Chen, The immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio alginolyticus* under low and high pH stress, Fish Shellfish Immunol. 25 (2008) 701–709.
[16] W.N. Wang, J. Zhou, P. Wang, T.T. Tian, Y. Zheng, Y. Liu, et al., Oxidative stress, DNA damage and antioxidant enzyme gene expression in the Pacific white shrimp, *Litopenaeus vannamei* when exposed to acute pH stress, Comp. Biochem. Physiol., C 150 (2009) 428–435.
[17] R.C. Edgar, B.J. Haas, J.C. Clemente, C. Quince, R. Knight, UCHIME improves sensitivity and speed of chimera detection, Bioinformatics 27 (2011) 2194–2200.
[18] R.C. Edgar, UPARSE: highly accurate OTU sequences from microbial amplicon reads, Nat. Methods 10 (2013) 996–998.
[19] Y.F. Duan, Y. Wang, J.S. Zhang, Q.S. Liu, X. Ding, Morphologic, digestive enzymes and immunological responses of intestine from *Litopenaeus vannamei* after lipopolysaccharide injection, J. Invertebr. Pathol. 153 (2018) 186–194.
[20] Y.T. Suo, E.C. Li, T.Y. Li, Y.Y. Jia, J.G. Qin, Z.M. Gu, et al., Response of gut health and microbiota to sulfide exposure in Pacific white shrimp *Litopenaeus vannamei*, Fish Shellfish Immunol. 63 (2017) 87–96.
[21] Z. Wang, X. Ren, B. Gao, X. Yu, X. Zhang, J. Zhang, P. Liu, Comparison of carbohydrate metabolism key enzymes in different generations of growth-selected *Portunus trituberculatus* families, Aquaculture 477 (2017) 6–14.
[22] A.H. Romano, T. Conway, Evolution of carbohydrate metabolic pathways, 14th Forum in Microbiology, 1996, pp. 448–455.
[23] J. Kan, H.M. Wang, C.H. Jin, H.Y. Xie, Changes of reactive oxygen species and related enzymes in mitochondria respiratory metabolism during the ripening of peach fruit, Agric. Sci. China 9 (1) (2010) 138–146.
[24] K. Vijayavel, M.P. Balasubramanian, Changes in oxygen consumption and respiratory enzymes as stress indicators in an estuarine edible crab *Scylla serrata* exposed to naphthalene, Chemosphere 63 (9) (2005) 1523–1531.
[25] V.I. Lushchak, Environmentally induced oxidative stress in aquatic animals, Aquat. Toxicol. 101 (2011) 13–30.
[26] D.L. McCulloch, Metabolic response of the grass shrimp *Palaemontes kadiakensis*, Aquat. Toxicol. 17 (1990) 263–274.
[27] W.N. Wang, B.S. Li, J.J. Liu, L. Shi, M.J. Alam, S.J. Su, et al., The respiratory burst activity and expression of catalase in white shrimp, *Litopenaeus vannamei*, during long-term exposure to pH stress, Ecotoxicology 21 (2012) 1609–1616.
[28] J.X. Shi, M.J. Fu, C. Zhao, F.L. Zhou, Q.B. Yang, L.H. Qiu, Characterization and function analysis of Hsp60 and Hsp10 under different acute stresses in black tiger shrimp, *Penaeus monodon*, Cell Stress Chaperones 21 (2016) 295–312.
[29] Q. Ren, R.R. Zhang, X.F. Zhao, J.X. Wang, A thioredoxin response to the WSSV challenge on the Chinese white shrimp, *Fenneropenaeus chinensis*, Comp. Biochem. Physiol., C 151 (2010) 92–98.
[30] F.M. Torti, S.V. Torti, Regulation of ferritin genes and proteins, Blood 99 (2002) 3505–3516.
[31] D.P. Steenvoorden, G.M. van Henegouwen, The use of endogenous antioxidants to improve photoprotection, J. Photochem. Photobiol., B 41 (1997) 1–10.
[32] E. de la Vega, N.A. O'Leary, J.E. Shockey, J. Robalino, C. Payne, C.L. Browdy, et al., Anti-lipopolysaccharide factor in *Litopenaeus vannamei* (LvALF): a broad spectrum antimicrobial peptide essential for shrimp immunity against bacterial and fungal infection, Mol. Immunol. 45 (7) (2008) 1916–1925.
[33] S. Saurabh, P.K. Sahoo, Lysozyme: an important defence molecule of fish innate immune system, Aquacult. Res. 39 (2008) 223–239.
[34] C.L. Yao, P.F. Ji, Z.Y. Wang, F.H. Li, J.H. Xiang, Molecular cloning and expression of NOS in shrimp, *Litopenaeus vannamei*, Fish Shellfish Immunol. 28 (2010) 453–460.
[35] C. Lai, W. Cheng, C. Kuo, Molecular cloning and characterisation of prophenoloxidase from hemocytes of the white shrimp, *Litopenaeus vannamei*, Fish Shellfish Immunol. 18 (2005) 417–430.
[36] M. Li, C.Z. Li, C.X. Ma, H.Y. Li, H.L. Zuo, S.P. Weng, et al., Identification of a C-type lectin with antiviral and antibacterial activity from pacific white shrimp *Litopenaeus vannamei*, Dev. Comp. Immunol. 46 (2014) 231–240.
[37] Q.Q. Ge, J.P. Liang, J.T. Li, J. Li, Y.F. Duan, F.Z. Zhao, et al., Molecular cloning and expression analysis of Relish gene from the ridgetail white prawn *Exopalaemon carinicauda*, Fish. Sci. 81 (2015) 699–711.
[38] N.N. Danial, S.J. Korsmeyer, Cell death: critical control points, Cell 116 (2004) 205–219.
[39] Y. Wang, J. Li, P. Liu, J.T. Li, Z. Zhang, Z.Q. Chang, et al., The responsive expression of a caspase gene in Chinese shrimp Fenneropenaeus chinensis against pH stress, Aquacult. Res. 42 (2011) 1214–1230.
[40] W.T. Dai, M.J. Fu, C. Zhao, Z.H. Ma, F.L. Zhou, Q.B. Yang, L.H. Qiu, Characterization, expression analysis and RNAi of p53 gene in *Penaeus monodon*, Mol. Biol. Rep. 43 (6) (2016) 549–561.
[41] J.H. Leu, Y.C. Chen, L.L. Chen, K.Y. Chen, H.T. Huang, J.M. Ho, et al., *Litopenaeus vannamei* inhibitor of apoptosis protein 1 (LvIAP1) is essential for shrimp survival, Dev. Comp. Immunol. 38 (2012) 78–87.
[42] M.E. Johansson, G.C. Hansson, Immunological aspects of intestinal mucus and mucins, Nat. Rev. Immunol. 16 (10) (2016) 639–649.
[43] M. Derrien, M.W. Passel, J.H. Bovenkamp, R. Schipper, W.M. Vos, et al., Mucin bacterial interactions in the human oral cavity and digestive tract, Gut Microb. 1 (4) (2010) 254–268.
[44] W. Rungrassamee, A. Klanchui, S. Maibunkaew, N. Karoonuthaisiri, Bacterial dynamics in intestines of the black tiger shrimp and the Pacific white shrimp during *Vibrio harveyi* exposure, J. Invertebr. Pathol. 133 (2016) 12–19.
[45] M.L. Zhang, Y.H. Sun, Y.K. Liu, F. Qiao, L.Q. Chen, W.T. Liu, et al., Response of gut microbiota to salinity change in two euryhaline aquatic animals with reverse salinity preference, Aquaculture 454 (2016) 72–80.
[46] L. Zhou, S.M. Limbu, M. Shen, W. Zhai, F. Qiao, A. He, et al., Environmental concentrations of antibiotics impair zebrafish gut health, Environ. Pollut. 235 (2018) 245–254.
[47] D.N. Frank, A.L. St Amand, R.A. Feldman, E.C. Boedeker, N. Harpaz, N.R. Pace, Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases, Proc. Natl. Acad. Sci. U. S. A 104 (2007) 13780–13785.
[48] J.B. Xiong, K. Wang, J.F. Wu, L.L. Qiuqian, K.J. Yang, Y.X. Qian, D.M. Zhang, Changes in intestinal bacterial communities are closely associated with shrimp disease severity, Appl. Microbiol. Biotechnol. 99 (2015) 6911–6919.
[49] Y.F. Duan, Y. Wang, H.B. Dong, X. Ding, Q.S. Liu, H. Li, et al., Changes in the

intestine microbial, digestive, and immune-related genes of *Litopenaeus vannamei* in response to dietary probiotic *Clostridium butyricum* supplementation, Front. Microbiol. 9 (2018) 2191.
[50] D.M. Zhang, X. Wang, J.B. Xiong, J.L. Zhu, Y.N. Wang, Q.F. Zhao, et al., Bacterioplankton assemblages as biological indicators of shrimp health status, Ecol. Indicat. 38 (2014) 218–224.
[51] Z.B. Huang, X.Y. Li, L.P. Wang, Z.Z. Shao, Changes in the intestinal bacterial community during the growth of white shrimp, *Litopenaeus vannamei*, Aquacult. Res. 47 (6) (2016) 1737–1746.
[52] R. Barczynska, K. Slizewska, M. Litwin, M. Szalecki, A. Zarski, J. Kapusniak, The effect of dietary fibre preparations from potato starch on the growth and activity of bacterial strains belonging to the phyla Firmicutes, Bacteroidetes, and Actinobacteria, J. Funct. Foods 19 (2015) 661–668.
[53] A. Koh, F. De Vadder, P. Kovatcheva-Datchary, F. Backhed, From dietary fiber to host physiology: short-chain fatty acids as key bacterial metabolites, Cell 165 (2016) 1332–1345.