Fish and Shellfish Immunology 88 (2019) 335–343

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Transcriptomic and microbiota response on *Litopenaeus vannamei* intestine subjected to acute sulfide exposure

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ARTICLE INFO

Keywords:
Shrimp
Sulfide
Intestine microbiota
Immune response
RNA-seq

ABSTRACT

Harmful effects of water pollutants are myriad. Sulfide from water bodies affects the aquatic animals. Intestine barrier function serves as the front-line of animals defense. Our previous study confirmed the toxic effect of sulfide on intestine immune response of *Litopenaeus vannamei*, but the underlying mechanisms remained elusive. Therefore, in this study, we investigated the transcriptomic and microbiota responses of the *L. vannamei* intestine subjected to acute sulfide exposure. Sulfide decreased bacterial richness and altered the intestine microbiota composition. Specifically, sulfide increased the abundances of Bacteroidetes and Actinobacteria, but decreased the abundance of Proteobacteria. At the genus level, sulfide increased typical cellulolytic characteristics bacteria, such as *Formosa*, *Sphingomonas*, and *Demequina*. RNA-seq analysis identified differential expression of 1799 genes (701 up-regulated and 1098 down-regulated) were grouped into 267 pathways. The most enriched pathway 'amoebiasis' was related to the intestine mucus homeostasis. A number of immune-related genes associated with antimicrobial, antioxidant, pathogen attachment and recognition, and apoptosis processes in contrasting accessions; they were correlated with the abundance of intestine bacterial at the phylum level. This study provides an insight into the mechanisms associated with molecular and microbiota response and processes involved in adaptation strategies towards sulfide stress.

1. Introduction

The Pacific white shrimp, *Litopenaeus vannamei,* is a species important to the global economy. Shrimp aquaculture suffers economic losses due to diseases [1,2], which result from complex interactions among the host, environment, and pathogen, and environmental stress is often an inducing factor. *L. vannamei* often occurs in pond benthic zones, and therefore, it is frequently affected by environmental pollutants, such as sulfide [3]. Sulfide is generated from the anaerobic decomposition of organic wastes and accumulates in the bottom layers and sediments of aquatic systems [4]. Sulfide is an important constituent of hydrogen sulfide (H_2S), bisulfide anion (HS^-) and sulfide anion (S^{2-}), and H_2S plays a decisive role in sulfide stress [5]. H_2S can inhibit the electron transport chain of cytochrome oxidase and make the glutathione inactivation through combining with the thiol of glutathione, thus affected the biological oxidation process, blocked the cell respiration and added to the hypoxia in vivo [6,7]. Additionally, chronic exposure of shrimp to sulfide can decrease health and survival through functional changes in gluconeogenesis, protein synthesis and energy metabolism [4]. It was reported that the 50% lethal concentration (LC_{50}) of sulfide greatly varies among aquatic species, including mollusks (1.4–50.0 mg/L), fish (1.38–22.4 mg/L) and crustacean (0.2–52.0 mg/L) [4]. In crustaceans, the 96 h LC_{50} of sulfide is high, such as *L. vannamei* (4.25 mg/L), *Macrobrachium rosenbergii* (4.2 mg/L), *Eriocheir sinensis* (3.09 mg/L), and *Eohaustorus estuaries* (3.32 mg/L) [4,8–10]. Although sulfide concentration in natural water environment is typically low, low dose exposure can cause the immune depression and increase the pathogen susceptibility of the shrimp [5,11–13]. Therefore, better understanding of toxicological effects of sulfide will be important for the health of the shrimp.

The intestine of shrimp is an important organ for nutrient. The shrimp intestine possess a large surface area that provides a barrier to the variety of pathogen infection and stresses; thus, intestine immune function serves as the first line of the host defense, and the damages of the organ clearly effects the shrimp health [14]. The intestine immune function is associated with its structural integrity, microbiota composition, and immune compounds. The functional activity and stability of the microbiota is important for shrimp health, acting via functions

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https://doi.org/10.1016/j.fsi.2019.02.021
Received 28 November 2018; Received in revised form 9 February 2019; Accepted 13 February 2019
Available online 14 February 2019

related to immunity and pathogen resistance [15–17]. Additionally, shrimp mainly rely on innate immune actors for protecting them against stress. Because the shrimp habitats in the pathogen rich water environment; thus, the intestine is continuously exposed to foreign substances, and may be affected by the water quality. It was reported that ammonia and nitrite exposure could disrupt the mucosal tissue, change the microbiota composition, and confuse the immune status in the intestines of shrimp [14].

In previous studies, we found that acute sulfide exposure disrupted structural integrity, and induced an immune response in the *L. vannamei* intestine [18]. However, a clear understanding of the intestine immune function of *L. vannamei* in response to sulfide toxicity is lacking. To characterize the immune actors and their associated microbiota variation in shrimp intestine immune function, here we investigated transcriptomic and microbiota response on *L. vannamei* intestine subjected to acute sulfide exposure. This new information affords a greater understanding of the intestine immune functions of *L. vannamei* in response to sulfide toxicity, and the foundation for further developing of biomarkers.

2. Materials and methods

2.1. Shrimp culture conditions and sulfide exposure

Healthy juvenile *L. vannamei*, with an average weight of 5.4 ± 0.3 g, were randomly collected from a local hatchery and reared in a semi-intensive culture pond at Shenzhen Base, South China Sea Fisheries Research Institute of Chinese Academy of Fishery Sciences (Shenzhen, China). The shrimp were acclimated for 7 days prior to the experiment, and were fed a formulated pellet feed (Haida Feed, Jieyang, China) daily at a ratio of 5% of their body weight. The rearing water was multistep sand-filtered, well aerated seawater collected from the nearby ocean, and aerated with two stones. The parameters of water quality were salinity 30, pH 8.3, dissolved oxygen 6.0 ± 0.2 mg/L, and temperature 30 ± 0.5 °C. One-third of the water was changed daily.

After acclimation, the experimental shrimp were divided into two groups—the control group (CG) and the sulfide stress group (SG)—with three replicate tanks per group. The tanks used in the experimental stage were the same as those used in the acclimation stage, and the shrimp were not moved to new tanks. Shrimp were housed at a density of 50 shrimp per tank (300 L, 0.5 m^2 round bottom area), and the tanks contained 300 L of filtered, aerated seawater. The sulfide concentration of the CG was 0 mg/L (normal seawater as control). Based on the 96 h LC_{50} of sulfide (4.3 mg/L) for *L. vannamei* reported by Li et al. [4], the sulfide concentration of the SG group in the present study was set to 5.0 mg/L by adding sodium sulfide ($Na_2S{\cdot}9H_2O$) to regular seawater. The sulfide level was measured by the methylene blue spectrophotometric method, and the water was renewed every 4 h by adding Na_2S solution to maintain the concentration of sulfide at 5.0 ± 0.2 mg/L. Shrimp were fed twice daily (08:00 and 18:00) with a commercial diet at a ratio of 5% of the average shrimp body weight.

The sulfide exposure experiment lasted for 72 h. At 72 h, whole intestine tissue (without faeces) of six shrimp from each tank was collected, mixed, and snap frozen in liquid nitrogen for intestine transcriptome analysis. Additionally, whole intestine tissue (including feces) of another six shrimp from each tank was collected and mixed, then frozen at −80 °C for intestine microbiota analysis.

2.2. Intestine microbiota analysis

Microbial DNA was extracted using the DNeasy PowerSoil Kit (QIAGEN) and analyzed by using 0.8% agarose gel electrophoresis. DNA concentration and quality were checked using a NanoDrop spectrophotometer. Amplification of the bacterial 16S rDNA gene V4 region was performed using the barcoded fusion primers 515F (GTGYCAGCMGCCGCGGTAA) [19] and 806R (GGACTACNVGGGTWTCTAAT) [20]. PCR was performed using the KOD-Plus-Neo DNA polymerase kit (TOYOBO, Shanghai) with a Gene Amp® PCR System 9700 (Applied Biosystems, USA). The PCR fragments were subjected to electrophoresis in 2.0% agarose gels, and the target band was purified using a PCR purification kit (Omega Bio-Tek, USA). The amplicons were pooled at equimolar concentrations and sequenced on an Illumina HiSeq platform.

Raw sequences were processed using the BIPES pipeline. Chimeric sequences were determined by undetected chimera (UCHIME) [21]. Operational taxonomy units (OTUs) were defined with a threshold of 97% identity by UPARSE [22]. Taxonomies were assigned with uclust for each OTU, and alpha and beta diversity analyses were performed for each library using quantitative insights into microbiota ecology (QIIME). A heatmap was constructed using the heatmap 2 function of the R g-plots package based on the top 50 genera in the samples.

2.3. Intestine transcriptome analysis

2.3.1. RNA extraction, library construction and sequencing

Total RNA was extracted from the intestines of shrimp in each group using Trizol Reagent (Invitrogen, USA) according to the manufacturer's instructions, and contaminant DNA was removed using RQ1 RNase-Free DNase (Promega, USA). Sequencing libraries were constructed using the NEBNext Ultra™ RNA Library Prep Kit for Illumina (NE, USA) according to the manufacturer's instructions. The library products were sequenced on an Illumina HiSeq X Ten system.

2.3.2. Transcriptome assembly and functional annotation

Raw reads were processed to screen clean reads by removing adaptor sequences, low-quality sequences, and reads with more than 5% poly-N. Additionally, the Q20, Q30 and GC contents of the clean reads were calculated. After filtering clean reads, transcriptome de novo assembly was performed with Trinity software [23]. The identities of unigenes were evaluated with the Basic Local Alignment Search Tool (BLAST) program against the non-redundant (Nr) protein, nucleotide (Nt), SwissProt, Cluster of Orthologous Groups (COG), Gene Ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. Sequence homology was accepted at an *E*-value < 1e-5.

2.3.3. Identification of differentially expressed genes (DEGs)

DEGs analysis of CG vs SG was performed using the DESeq R package [24], and the threshold was set as a *q*-value < 0.01. GO analysis of DEGs was performed with the GOseq R package [25], and GO terms were enriched by DEGs with a corrected *P*-value < 0.05. Pathway analysis of the DEGs was downloaded from KEGG, and statistical enrichment was checked using KOBAS software (v2.0) [26].

2.3.4. Validation of RNA-seq profiles by qPCR

To validate the differentially expressed genes identified by RNA-seq, 28 genes with different expression patterns and differential involvement in immune responses were selected for qPCR confirmation using the same RNA samples analyzed for the transcriptome profiling. The *β-actin* gene of *L. vannamei* was used as an internal control to verify the successful reverse transcription and to calibrate the cDNA template. The gene-specific qPCR primers designed with Primer Premier 5.0 software (Table S1), the efficiency was evaluated with amplification plot and melt curve. The qPCR method was previously described by Duan et al. [18].

2.4. Statistical analysis

In the analysis of the relative abundance of intestine bacterial at the phyla and class level, 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 $P < 0.05$. Pearson correlation

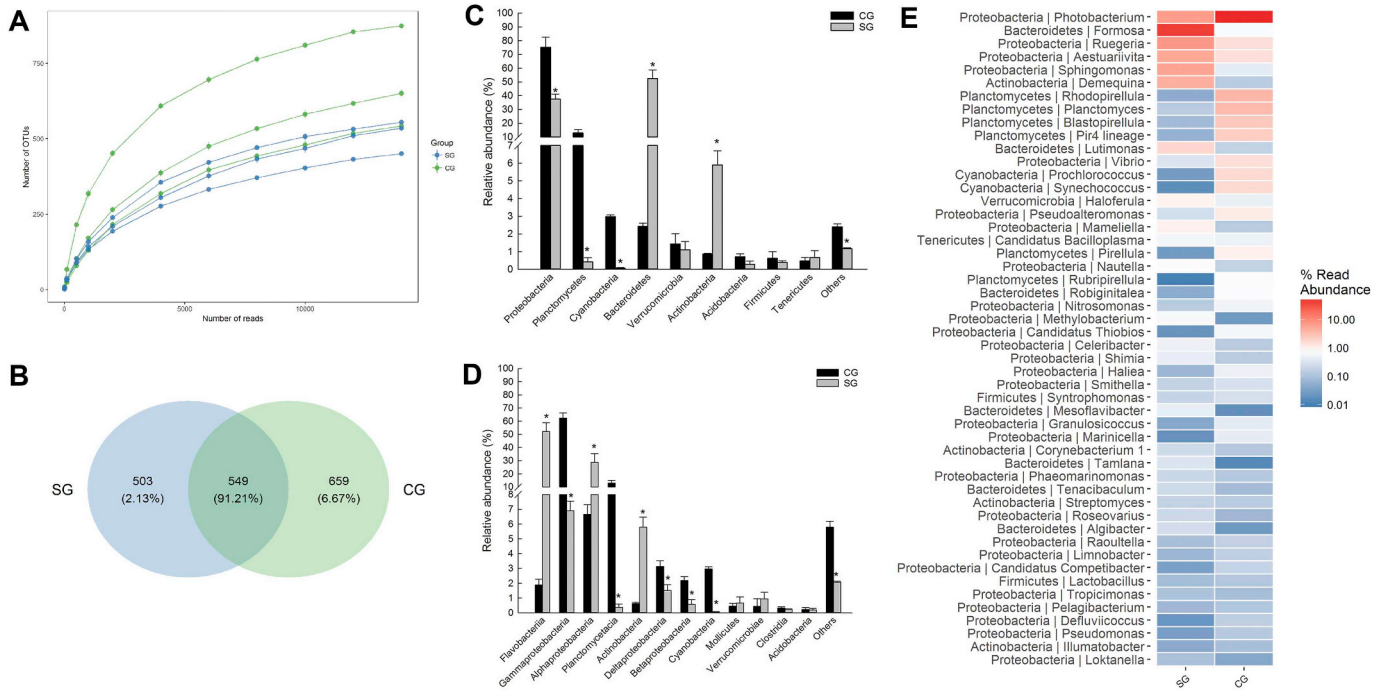

Fig. 1. Alteration of intestine microbial structure of *L. vannamei* by sulfide stress. **(A)** Rarefaction curves. **(B)** Venn diagram. **(C)** Relative abundance of intestine microbial at phylum level. **(D)** Relative abundance of intestine microbial at class level. **(E)** Heat map analysis of intestine microbial on the top 50 genera. Bars show the mean ± SE ($N = 3$). * indicate significant differences ($P < 0.05$) between groups. The read abundance indicates the relative abundance of species of each row after standardization in heatmap. The redder color show the higher abundance of the genera, and the black color was the lower abundance.

analysis was used to reveal the relationships between phylum abundance and immune indices.

3. Results

3.1. Intestine microbiota analysis

3.1.1. Richness and diversity

A total of 2,320,275 sequences were obtained from the intestine microbiota of *L. vannamei* by 16S rDNA gene Illumina sequencing, with an average of 38,644 sequences per sample obtained after optimization and quality control. The mean sequence length was 316 bp. The rarefaction per sample was sufficient (Fig. 1A). A kind of 549 OTUs were shared between the two groups, and the percent of the sequence which constituted OTUs was 91.21% (Fig. 1B). Intestine bacterial richness and diversity indices were evaluated from the OTUs. The bacterial richness analysis showed that Chao1 and ACE index were decreased in the SG, when compared with the CG. Bacterial diversity was estimated by the Shannon and Simpson index, while no significant difference in two indices between the SG and CG (Table 1).

3.1.2. Changes in the intestine bacterial composition

A total of 37 different bacterial phylum were identified. Of the dominant phyla, the abundances of Bacteroidetes and Actinobacteria in the SG were increased, while the abundances of Proteobacteria, Planctomycetes, and Cyanobacteria were decreased (Fig. 1C). At the class level, the abundances of Flavobacteriia, Alphaproteobacteria, and Actinobacteria in the SG were increased, while the abundances of Gammaproteobacteria, Planctomycetacia, Deltaproteobacteria, Betaproteobacteria, and Cyanobacteria were decreased (Fig. 1D). Differences were also observed at the genus level: certain genera, such as *Formosa*, *Ruegeria*, *Sphingomonas*, and *Demequina*, were more dominant in the SG. However, some genera, such as *Photobacterium*, *Rhodopirellula*, and *Planctomyces*, were reduced in the SG (Fig. 1E).

The linear discriminant analysis (LDA) effect size (Lefse) package was used to determine the differential abundances of microbiota taxa between the two groups. LDA scores of Lefse showed that 14 taxa were increased and 10 taxa were decreased in the SG, and there were many groups only enriched at genus level, including *Formosa*, *Ruegeria*, and *Demequina* (Fig. 2A). There were 16 bacterial taxa distinguishing SG from CG with LDA value; 3 class, 3 orders, and 3 families were enriched in SG, including Bacteroidetes (from class to family levels), Actinobacteria (from class to family levels), and Alphaproteobacteria (class level). While 3 class, 3 orders, and 3 families were enriched in CG, including Planctomycetacia (from class to family levels), Cyanobacteria (from class to family levels), and Gammaproteobacteria (class level) (Fig. 2B). Furthermore, a principal coordinates analysis (PCoA)of weighted and unweighted UniFrac distances analysis was conducted to analyze the functional content similarity of all samples, and the results indicated that the SG samples were clearly separated from the CG samples (Fig. 2C and D).

Table 1
Richness and diversity indices of intestine microbiota after *L. vannamei* exposed to sulfide stress 72 h.

Group	OTUs	Chao1	ACE	Shannon	Simpson	ECS (%)
CG	1405 ± 169	1190.27 ± 53[a]	1204 ± 19[a]	3.25 ± 0.33	0.67 ± 0.06	99.51 ± 0.15
SG	1247 ± 62	875 ± 39[b]	954 ± 17[b]	3.09 ± 0.19	0.81 ± 0.03	99.36 ± 0.11

ACE: abundance-based coverage estimator. ECS: Good's estimated sample coverage. Vertical bars represent the mean ± SE ($N = 3$). Data indicated with different letters were significantly different ($P < 0.05$) between groups.

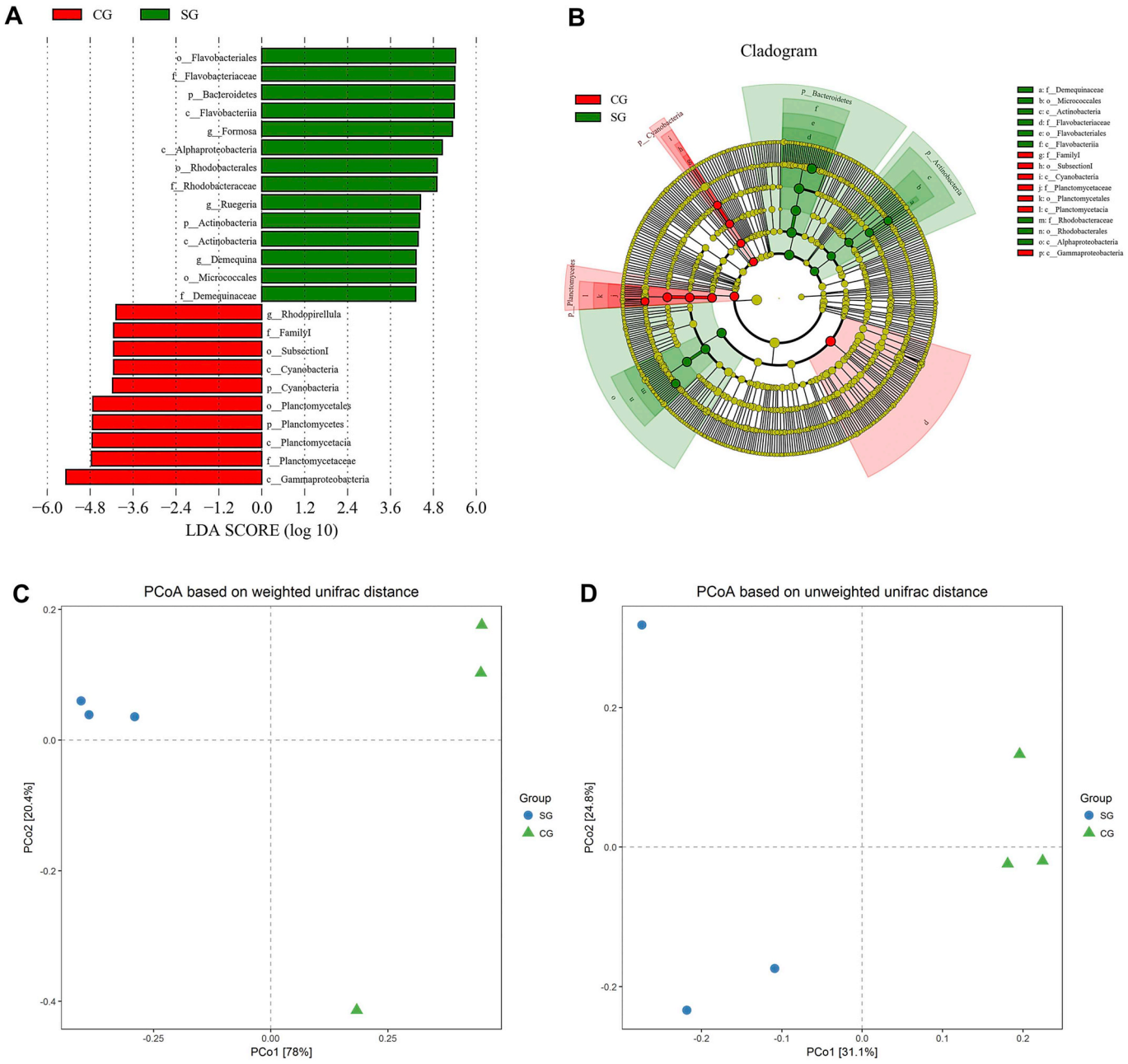

Fig. 2. Inter-group variation and principal coordinates analysis (PCoA) in the relative abundance of the intestine microbial communities between CG and SG. **(A)** LDA score of Lefse-PICRUSt. The length of column represent the effect size of bacterial lineages. **(B)** Lefse cladogram. The bacterial groups from family to class level are listed from center to outside. Each circle's diameter is proportional to the bacterial taxa's abundance. Green: bacterial taxa enriched in SG; red: bacterial taxa enriched in CG; yellow: no significant differences. Only the taxa that linear discriminate analysis (LDA) value above 2.5 are shown. **(C)** PCoA Plots based on weighted UniFrac metrics. **(D)** PCoA Plots based on unweighted UniFrac metric. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. Intestine transcriptome analysis

3.2.1. Sequencing, de novo assembly, and gene annotation

Illumina RNA-seq was performed on six intestine samples from *L. vannamei*, and the person correlation coefficient between samples showed that the SG samples were clearly separated from the CG samples (Fig. S1A). The total number of raw reads for all six samples sequenced was 299,911,286, which were submitted to the Sequence Read Archive (SRA) (accession: SRP133473). Total clean reads and clean bases for all samples were 296,558,418 and 44,483,762,700, respectively. Total clean reads ratio was 98.57%–99.12%. Trinity software generated approximately 53,271 transcripts. The lengths ranged from 200 bp to 28,723 bp, with a mean length of 1,212 bp and an N50 of 2,361 (Fig. S1B). The number of unigenes greater than 3,000 bp was 5,427 (Fig. S1C). A total of 32,503 unigenes were annotated by alignment with public databases, and 8,546 unigenes were co-owned (Fig. S2). The distribution of the unigenes classified by gene function is presented in Fig. S3.

3.2.2. Functional classification of DEGs

A total of 1799 DEGs in the intestines were identified between the CG and SG, including 701 up-regulated genes and 1098 down-regulated genes (Fig. 3). The difference between these two groups was clear. DEGs were further annotated with the GO database. According to a

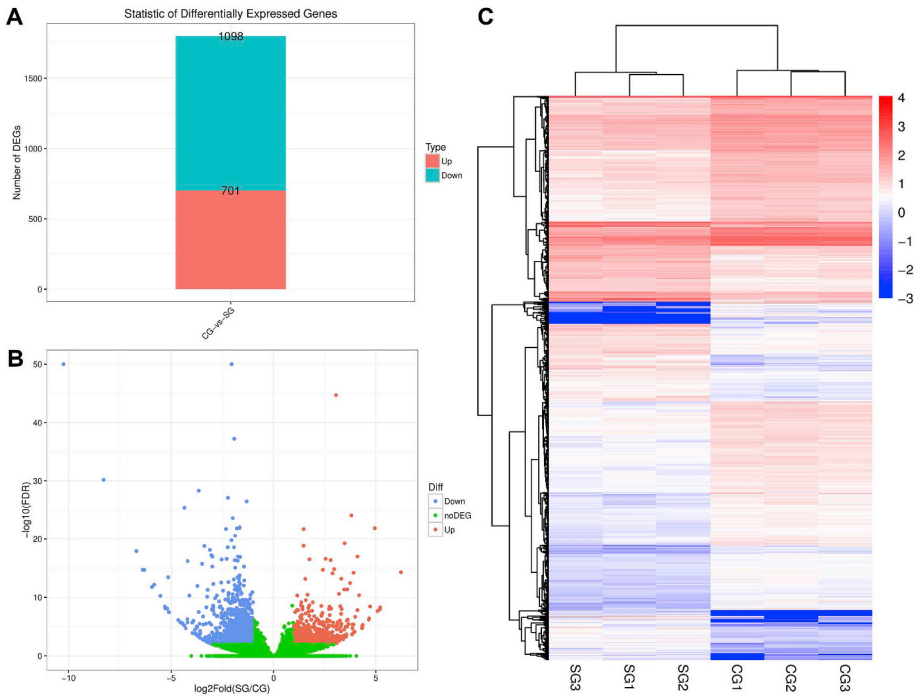

Fig. 3. Analysis of all DEGs in response to sulfide stress in intestine of *L. vannamei*. **(A)** Statistic of DEGs. **(B)** Cluster analysis of DEGs. **(C)** Distribution of DEGs.

GOTerm analysis, 'oxidation-reduction process' and 'protein phosphorylation' were the most well-represented terms among biological processes, 'integral component of membrane' and 'membrane' represented a large proportion of cellular components, and 'protein binding' was the most enriched term among molecular functions (Fig. 4A).

The DEGs were also grouped into 267 pathways by KEGG analysis, and the summary of pathway enrichment analysis results was shown in Table S2. The most enriched pathway was 'amoebiasis', followed by 'focal adhesion', '*Vibrio cholerae* infection', and 'endocytosis' (Fig. 4B and C). In the most enriched pathway "amoebiasis", certain DEGs were involved in the homeostasis of the intestine mucus, such as mucins (MUC), collagen (COL), and laminin (Fig. S4).

3.2.3. Identify and validation of immune-related DEGs

According to the Nr database, many DEGs were also related to the immune defence system and clustered into 6 categories: 'mucus immune', 'antimicrobial', 'antioxidant', 'pathogen attachment and recognition', 'apoptosis', and 'other immune-related genes' (Table S3). These immune-related DEGs showed different expression trends. For example, antimicrobial genes, such as anti-lipopolysaccharide factor (*ALF*) and prophenoloxidase (*proPO*), serine proteinase (*serP*), serine proteinase inhibitor (*serPI*), alpha 2 macroglobulin (*α2M*), and kazal type protease inhibitor (*kPI*)was up-regulated. Antioxidant enzymes, such as copper/zinc superoxide dismutase (*Mn/Zn-SOD*), glutathione synthetase (*GSS*), glutathione S-transferase (*GST*), and sulfhydryl oxidase (*SOX1*), were all down-regulated, while heat shock proteins (*HSP21*, *HSP70*, and *HSP90*) were all up-regulated. Pathogen attachment and recognition genes, such as peroxinectin (*Pxt*), lectin (*Lec*), focal adhesion kinase (*FDK*), fibrinogen (*FGL*), clottable protein (*CP*), transglutaminase (*TGase*), and integrin alpha 5 (*ITGA5*), were up-regulated, while integrin alpha-IIb (*ITGA2b*), melanization interactin protein (*MIP*), and toll-like receptor (*TLR*) were down-regulated. Apoptosis pathway, such as *p53*, caspase (*Casp*), and inhibitor of apoptosis protein (*IAP*); the expression of these genes were down-regulated. Other immune genes, such as acid phosphatase and chitinase, were down-regulated.

To verify the immune-related DEG expression profiles identified by RNA-seq, 28 genes involved in intestine immune responses were randomly selected for qPCR confirmation. The up-regulation or down-regulation trends of the genes between CG and SG was generally consistent between qPCR and RNA-seq study (Fig. 5A). Thus, the DEGs identified by RNA-seq analysis were accurate.

3.3. Correlations between intestine bacterial and immune-related DEGs

The correlations between intestine bacterial at the phylum level and immune-related DEGs were shown in Fig. 5B. *Muc-2*, *Muc*-5AC, *COL1A3*, *COL1A17*, *Laminin-β1*, *ALF*, *serP*, *HSP21*, *HSP90*, *Lec*, and *FDK* were positively correlated with the abundance of Bacteroidetes and Actinobacteria. *Muc-19*, *proPO*, *HSP70*, and *Chit* were positively correlated with the abundance of Bacteroidetes. *Muc-5B*, *Laminin-γ1*, *Mn/Zn-SOD*, *TLR*, and *Ras* were positively correlated with the abundance of Proteobacteria. *p53* was positively correlated with the abundance of Planctomycetes. *Muc-1*, *COL1A10*, *COL5A4*, and *ACP* were negatively correlated with the abundance of Bacteroidetes and Actinobacteria. *COL1A4*, *Casp*, and *IAP* were negatively correlated with the abundance of Bacteroidetes.

4. Discussion

4.1. Intestine microbiota in response to sulfide exposure

Animal intestines possess a large surface area that acts as a immune barrier to inflammation and pathogen infection. The intestine barrier of animals is associated with structural integrity, proteins involved in immunity, and a stable microbiota. If the structural integrity of the intestine is damaged, pathogens can enter the intestine epithelium and disrupt host immunity [27]. It was reported that *Vibrio* and chronic sulfide exposure altered the intestine microbiota structure of shrimp [3,16]. In the present study, after shrimp exposure to sulfide, the intestine bacterial richness was decreased, and microbiota composition was altered. The dominant bacterial phylum changed from Proteobacteria to Bacteroidetes, and Actinobacteria increased. Similar results was also found in the intestine of shrimp after exposure to ammonia and nitrite [14]. Proteobacteria are a group of commensal bacteria and

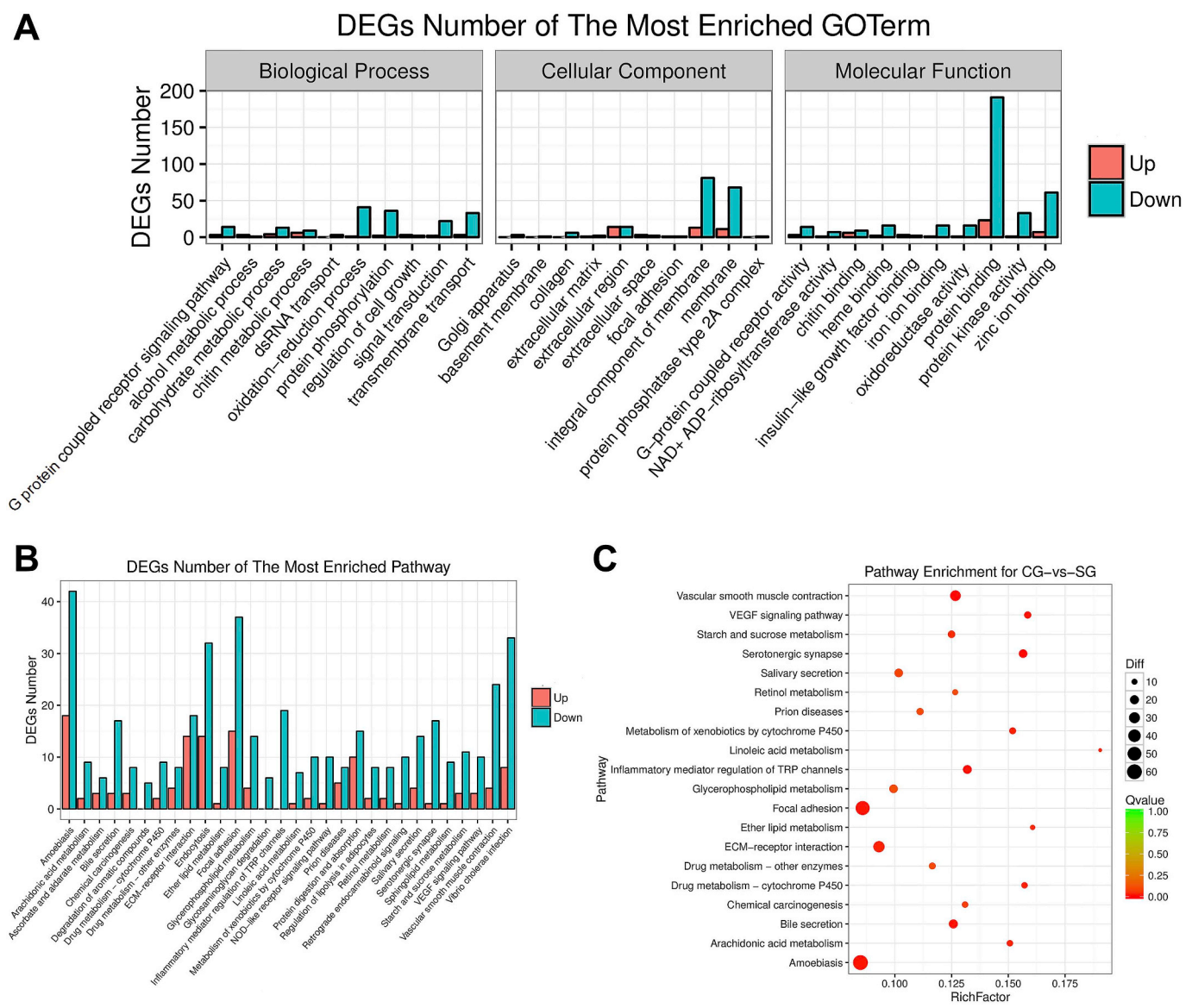

Fig. 4. The most enriched GOTerm and KEGG pathways of all DEGs in response to sulfide stress in intestine of *L. vannamei*. **(A)** GO classification. **(B)** The 30 most enriched pathways. **(C)** Pathway enrichment of DEGs.

opportunistic pathogens, which is a microbial signature of dysbiosis in intestine microbiota [28]. Bacteroidetes, especially the class Flavobacteriia, are known degraders of polymeric organic matter [29]. Actinobacteria are considered excellent elaborators of pharmaceutical products such as antibiotics, antimicrobial agents, and industrial enzymes [30]. Hence forecasts sulfide might disrupt the intestine microbiota balance, cause the unhealthy status of the intestine microecosystem, and different types of bacterial have differently responses to stress.

At the genus level, after sulfide stress, the dominate genera *Photobacterium* belonging to the phylum Proteobacteria changed to those genera belonging to Bacteroidetes and Actinobacteria, such as *Formosa* and *Demequina*; opportunistic pathogens including *Sphingomonas* was increased. The role of *Formosa* was not previously reported in other studies, and more work is needed to determine the effects of this bacteria. *Demequina* is isolated from marine organisms and can produce α-amylase, which is involved in starch degradation to glucose and dextrine [31]. In the present study, the abundance of *Demequina* in response to sulfide suggested intestine beneficial bacterial might provide additional nutrition for protecting the host. *Photobacterium* is a marine bacterium of the family Vibrionaceae, and some species are symbiotic or pathogenic for their hosts, including fish and crustaceans [32]. *Photobacterium* abundance was previously shown to decrease in the intestines of *Oreochromis niloticus* and *L. vannamei* after salinity stress [33]. Similarly, in the present study, the abundance of *Photobacterium* was also decreased after sulfide stress, which indicated that *Photobacterium* is a symbiotic bacterial in the intestine of shrimp, and non pathogenic in our experimental conditions. *Sphingomonas* is a strictly aerobic, non-spore-forming Gram-negative bacillus, ubiquitous bacterium, thought to be an opportunistic pathogen and can cause infection in humans [34]. In the present study, the abundance of *Sphingomonas* was increased in response to sulfide, which indicated that sulfide stress might increase the risk of opportunistic pathogen infection to shrimp. Thus, the dominance of these genera suggested a select community categorized by typical cellulolytic characteristics, and sulfide may increase the chances for opportunistic or pathogenic bacteria to adhere to or penetrate the damaged intestine epithelium of shrimp, thus compromising the intestine immunity of the host.

4.2. Intestine transcriptomic in response to sulfide exposure

The intestine mucosa is a major guardian against pathogen invasion

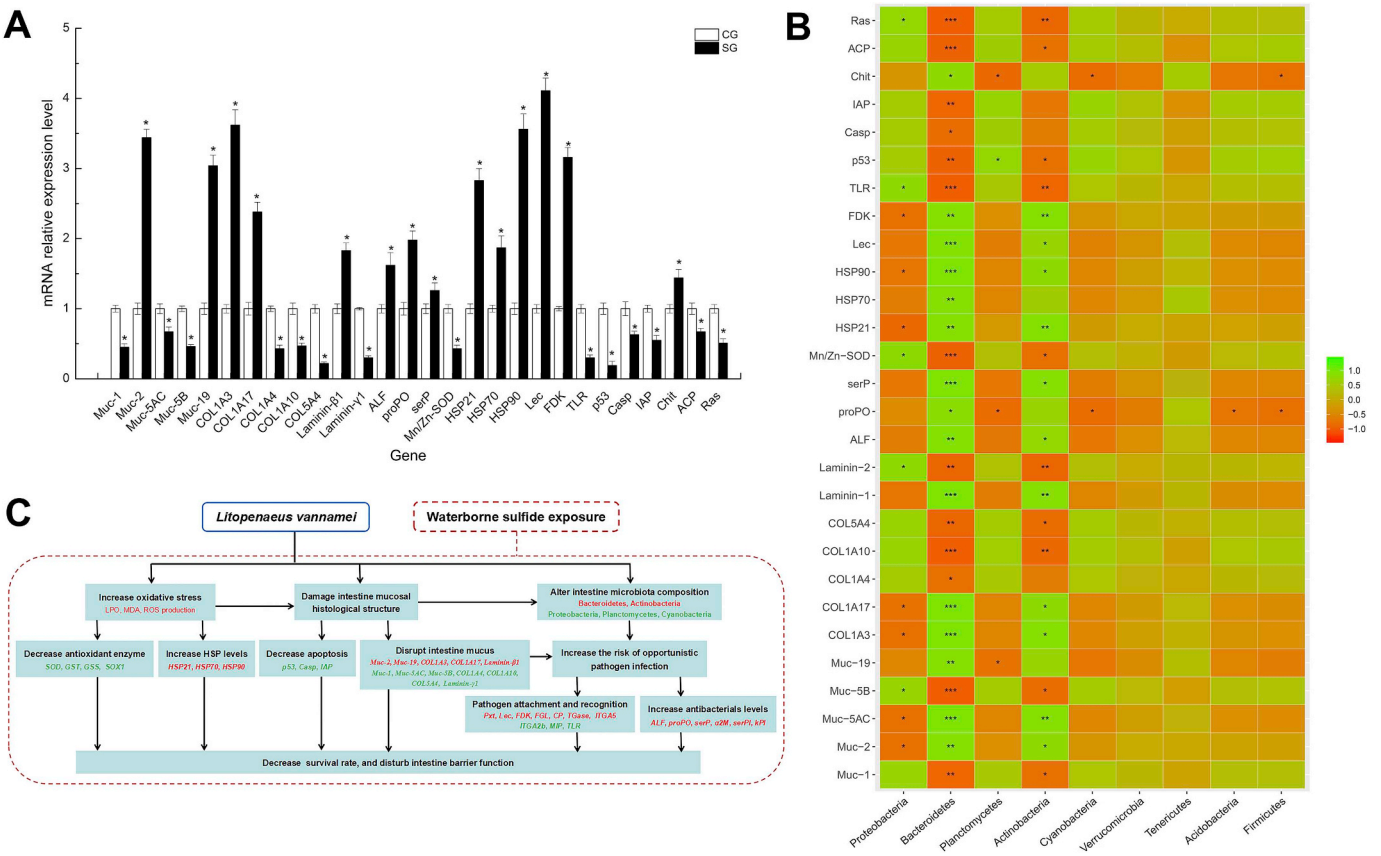

Fig. 5. qPCR verification of immune-related DEGs, significant correlation between intestine bacterial and immune-related DEGs, and potential mechanism of intestine barrier function in the contrasting accessions towards sulfide stress. **(A)** qPCR verification. Bars represent the mean ± SE ($N = 3$). * indicate significant differences ($P < 0.05$) between groups. **(B)** Significant correlation between intestine bacterial at the phylum level and immune-related DEGs. Correlation coefficient is represented by different colors (green: positive correlation; red: negative correlation). * represent significantly negative or positive correlations (*$P < 0.05$; **$P < 0.01$; ***$P < 0.0011$). **(C)** A schematic model representing the potential mechanism of intestine barrier function in the contrasting accessions towards sulfide stress. Bacterial, genes, and enzymes mentioned in Red and Green colour signifies increased and decreased, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and stress, and its barrier function is related to the expression of tight junctions, mucus components and immune molecules. Tight junctions contribute to the structural integrity of the intestine epithelium [35]. Mucins are the major components of mucus secreted by intestine goblet cells to resist infection [27]. When the intestine mucus barrier is disrupted, bacteria invade the intestine epithelium and colonize the organism. For example, *V. cholera* uses mucins as a nutrient source [36]. In the present study, the RNA-seq results showed that most DEGs were down-regulated. To further explore the intestine response in detail, DEGs were categorized using KEGG pathway analysis, which revealed that intestine mucus barrier pathways, such as 'amoebiasis', 'tight junction', and '*V. cholerae* infection', were more strongly activated by sulfide. Thus, sulfide influenced the immune function homeostasis of the intestine mucosa in shrimp.

In the most enriched pathway, 'amoebiasis', several DEGs, such as mucin, collagen (COL), and laminin, play a protective role on intestine epithelium of shrimp in response to sulfide. Of these, COL and laminin are also the proteins involved in 'focal adhesion' signaling. Here, 'focal adhesion' was the second highest enriched DEG pathway. Cells utilize focal adhesion to facilitate and regulate mechanical coupling with the extracellular matrix (ECM) in physiological processes [37]. Focal adhesion are complex organelles which span the actin cytoskeleton and the ECM [38]. Mucins are a group of large glycoproteins that play important roles in bacterial attachment and invasion [39–41]. *V. vulnificus* increases host susceptibility by modifying the expression of mucin-2 in intestine epithelial cells [42]. In the present study, the expression of *Muc-2* and *Muc-19* was up-regulated, while *Muc-1*, *Muc-5A*, and *Muc-5B* were down-regulated, indicating that sulfide induced the immune components of the intestine mucus. COLs are the most abundant of glycoproteins and are components of the ECM. The balance between collagen synthesis and breakdown is considered to be among the most important factors determining disease recurrence [43]. In the present study, the expression of *COL1A3* and *COL1A17* was up-regulated, while the expression of *COL1A4*, *COL1A10* and *COL5A4* was down-regulated, indicating that sulfide influenced the ECM of the intestine. Laminins are proteins of the ECM that play critical roles the formation and stability of the basement membranes of intestine epithelium [44]. In the present study, the expression of laminin-β1 was up-regulated, while laminin-γ1 was down-regulated; these changes might influence the stability of the basement membrane of intestine epithelial cells. In summary, an external sulfide stimuli sensed by the host as stressor might promote the modulation of these genes precisely to reinforce/confer additional protection to the mucosal barrier.

The oxidation-reduction process is responsible for maintaining cellular homeostasis, and phosphorylation processes constitute a category of post-translational modifications [45]. Antioxidant enzymes and HSPs participate in antioxidant functions in response to environmental stress [18,46]. In the present study, DEGs were enriched for the oxidation-reduction process and phosphorylation processes according to GO annotation. Additionally, antioxidant enzymes (*Cu/Zn-SOD*, glutathione synthetase, *GST*, and sulfhydryl oxidase 1) were all down-regulated, while HSP genes (*HSP21*, *HSP70*, and *HSP90*) were all up-regulated. These results indicated that the oxidation-reduction process was activated in the shrimp intestines in order to counteract the oxidative

stress; antioxidant defense mechanisms mounted on the host because its high allostatic load provoked by the sulfide exposure.

Sulfide increases the risk of pathogen infection in aquatic species [11–13]. Successful bacterial infection always starts with adhesion to the mucosal surfaces of the host [27]. Pathogen pattern recognition proteins (PRPs) can recognize the pathogen associated molecule patterns that released through infection [47]. In the present study, a number of genes involved in host-pathogen attachment and recognition were significantly altered, including lectin, fibrinogen, clottable protein, integrin, peroxinectin, and toll-like receptor, suggesting that the intestine immune system of shrimp undergoes pathogen attack induced by sulfide. Antimicrobial peptides and the prophenoloxidase (proPO) system contribute to the antimicrobial capabilities of shrimp [48,49]. In the present study, *ALFs*, and *proPO* system related genes were identified among DEGs, and all were up-regulated. This suggested that these antimicrobial molecules might involve in the intestine immune response to sulfide stress.

Apoptosis is a genetically programmed cellular suicide process that eliminates unwanted or diseased cells, and it can be induced by a wide range of stimuli [50]. Caspases and p53 are the main executors of the apoptosis program, which can mediate apoptosis and resist cellular stress [51,52]. As anti-apoptosis regulators, IAP can inhibit the activity of caspases, and play important roles in regulating the progression of apoptosis [53]. In the present study, p53, caspase-3, caspase-4, and IAP gene were down-regulated in the shrimp intestine after sulfide stress. This suggested that apoptosis program was activated to remove unnecessary or deleterious cells to resist sulfide stress. Additionally, several genes in the other immune gene groups were also significantly changed, which also have been reported to be involved in immune responses in crustaceans. For example, acid phosphatase, a multifunctional phosphoric monoester hydrolase that is sensitive to environmental changes in shrimp [54]. Ras family proteins are involved in the immune response to pathogen infection [55]. Chitinase is produced by shrimp and may help to control pathogens that contain chitin [56]. In the present study, acid phosphatase and ras was down-regulated, while chitinase was up-regulated, suggesting that sulfide influenced the intestine immunity of shrimp, and immune system utilized different response mechanism to defence the stress.

4.3. Increase of intestine immune response and changes of bacterial populations

Proteobacteria and Bacteroidetes are normally dominant in the intestines of shrimp at all growth stages [57,58]. The pearson correlation analysis showed that the immune genes in the shrimp intestine were correlated with the abundance of Proteobacteria and Bacteroidetes. Of these 28 immune genes, the up-regulated expression of *Muc-2*, *Muc-19*, *COL1A3*, *COL1A17*, *Laminin-β1*, *ALF*, *serP*, *proPO*, *HSP21*, *HSP70*, *HSP90*, *Lec*, *FDK*, and *Chit* were positively correlated with the abundance of Bacteroidetes, which indicated that these Bacteroidetes bacterial might contribute to the immune response of the host, including mucus, antimicrobial, HSPs, and PRPs genes. The down-regulated expression of *Mn/Zn-SOD* were positively correlated with the abundance of Proteobacteria, which indicated that these Proteobacteria bacterial might contribute to the decreasing of antioxidant enzymes.

5. Conclusions

This study provides data on the mechanisms associated with molecular and microbiota response and processes on *L. vannamei* intestine involved in adaptation strategies towards sulfide stress. Sulfide decreased bacterial richness and changed the composition of the microbial in the shrimp intestine. RNA-seq analysis led to the identification of 1799 specific genes responsible for sulfide tolerance in contrasting accessions. Immune-related genes identified through transcriptomic data include those associated with mucus homeostasis, antimicrobial, antioxidant, pathogen attachment and recognition, and apoptosis, which might be a contributing factor towards tolerance or sensitivity to sulfide stress. On the basis of this study, a potential mechanism of intestine barrier function in the contrasting accessions towards sulfide stress has been speculated and presented in Fig. 5C. Further studies will be focus on the relevance of intestine microbiota, metabolites and shrimp immunity in adaptation strategies towards sulfide stress.

Acknowledgements

This research was supported by the Central Public-interest Scientific Institution Basal Research Fund, South China Sea Fisheries Research Institute, CAFS (2019TS18, 2017YB12), Guangdong Provincial Key Laboratory of Fishery Ecology and Environment (LFE-2016-12), Guangdong Natural Science Foundation (2017A030313147), Guangdong Provincial Special Fund for Marine Fisheries Technology (A201701B09), Central Public-interest Scientific Institution Basal Research Fund, CAFS (2019CY0103). The authors are grateful to the laboratory staff for technical assistance, especially the RNA-seq analysis supported by Sui Ma Yang Biotechnology Co. Ltd. The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.02.021.

References

[1] T.W. Flegel, Historic emergence, impact and current status of shrimp pathogens in Asia, J. Invertebr. Pathol. 110 (2012) 166–173.
[2] J. Joshi, J. Srisala, V.H. Truong, I.T. Chen, B. Nuangsaeng, O. Suthienkul, et al., Variation in *Vibrio parahaemolyticus* isolates from a single Thai shrimp farm experiencing an outbreak of acute hepatopancreatic necrosis disease (AHPND), Aquaculture 428 (2014) 297–302.
[3] 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.
[4] T.Y. Li, E.C. Li, Y.T. Suo, Z.X. Xu, Y.Y. Jia, J.G. Qin, L.Q. Chen, Z.M. Gu, Energy metabolism and metabolomics response of Pacific white shrimp *Litopenaeus vannamei* to sulfide toxicity, Aquat. Toxicol. 183 (2017) 28–37.
[5] B. Vismann, Sulfide species and total sulfide toxicity in the shrimp *Crangon crangon*, J. Exp. Mar. Biol. Ecol. 204 (1996) 41–54.
[6] E. Affonso, V. Polez, C. Correa, A. Mazon, M. Araujo, G. Moraes, et al., Blood parameters and metabolites in the teleost fish *Colossoma macropomum* exposed to sulfide or hypoxia, Comp. Biochem. Physiol., C 133 (2002) 375–382.
[7] M. Konishi, T.O. Watsuji, S. Nakagawa, Y. Hatada, K. Takai, T. Toyofuku, Effects of hydrogen sulfide on bacterial communities on the surface of galatheid crab, *Shinkaia crosnieri*, and in a bacterial mat cultured in rearing tanks, Microb. Environ. 28 (2013) 25–32.
[8] R. Reiffenstein, W.C. Hulbert, S.H. Roth, Toxicology of hydrogen sulfide, Annu. Rev. Pharmacol. Toxicol. 32 (1992) 109–134.
[9] L.A. Gorodezky, J.J. Childress, Effects of sulfide exposure history and hemolymph thiosulfate on oxygen-consumption rates and regulation in the hydrothermal vent crab *Bythograea thermydron*, Mar. Biol. 120 (1994) 123–131.
[10] M.J. Kravitz, J.O. Lamberson, S.P. Ferraro, R.C. Swartz, B.L. Boese, D.T. Specht, Avoidance response of the estuarine amphipod *Eohaustorius estuarius* to polycyclic aromatic hydrocarbon-contaminated, field-collected sediments, Environ. Toxicol. Chem. 18 (6) (1999) 1232–1235.
[11] L. Hagerman, B. Vismann, Anaerobic metabolism in the shrimp *Crangon crangon* exposed to hypoxia, anoxia and hydrogen sulfide, Mar. Biol. 123 (1995) 235–240.
[12] S.W. Hsu, J.C. Chen, The immune response of white shrimp *Penaeus vannamei* and its susceptibility to *Vibrio alginolyticus* under sulfide stress, Aquaculture 271 (2007) 61–69.
[13] S.Y. Cheng, S.W. Hsu, J.C. Chen, Effect of sulfide on the immune response and susceptibility to *Vibrio alginolyticus* in the kuruma shrimp *Marsupenaeus japonicus*, Fish. Shellfish Immun 22 (2007) 16–26.
[14] 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.
[15] J.P. Jacobs, J. Braun, Immune and genetic gardening of the intestinal microbiome, FEBS Lett. 588 (22) (2014) 4102–4111.
[16] 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.
[17] M.L. Zhang, Y.H. Sun, K. Chen, N. Yu, Z.G. Zhou, L.Q. Chen, Z.Y. Du, E.C. Li, Characterization of the intestinal microbiota in Pacific white shrimp, *Litopenaeus*

vannamei, fed diets with different lipid sources, Aquaculture 434 (2014) 449–455.
[18] Y.F. Duan, H.B. Dong, Y. Wang, H. Li, Q.S. Liu, Y. Zhang, J.S. Zhang, Intestine oxidative stress and immune response to sulfide stress in Pacific white shrimp *Litopenaeus vannamei*, Fish Shellfish Immunol. 63 (2017) 201–207.
[19] A. Apprill, S. McNally, R. Parsons, L. Weber, Minor revision to V4 region SSU rRNA 351 806R gene primer greatly increases detection of SAR11 bacterioplankton, Aquat. Microb. Ecol. 75 (2015) 129–137.
[20] A.E. Parada, D.M. Needham, J.A. Fuhrman, Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples, Environ. Microbiol. 18 (5) (2016) 1403–1414.
[21] 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.
[22] R.C. Edgar, UPARSE: highly accurate OTU sequences from microbial amplicon reads, Nat. Methods 10 (2013) 996–998.
[23] M.G. Grabherr, B.J. Haas, M. Yassour, J.Z. Levin, D.A. Thompson, I. Amit, X. Adiconis, L. Fan, R. Raychowdhury, Q. Zeng, Full-length transcriptome assembly from RNA-Seq data without a reference genome, Nat. Biotechnol. 29 (7) (2011) 644–652.
[24] L. Wang, Z. Feng, X. Wang, X. Wang, X. Zhang, DEGseq: an R package for identifying differentially expressed genes from RNA-seq data, Bioinformatics 26 (1) (2010) 136–138.
[25] M.D. Young, M.J. Wakefield, G.K. Smyth, A. Oshlack, Gene ontology analysis for RNA-seq: accounting for selection bias, Genome Biol. 11 (2010) R14.
[26] X.Z. Mao, T. Cai, J.G. Olyarchuk, L.P. Wei, Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary, Bioinformatics 21 (2005) 3787–3793.
[27] C. Gao, Q. Fu, B. Su, S. Zhou, F. Liu, L. Song, M. Zhang, Y. Ren, X. Dong, F. Tan, C. Li, Transcriptomic profiling revealed the signatures of intestinal barrier alteration and pathogen entry in turbot (*Scophthalmus maximus*) following *Vibrio anguillarum* challenge, Dev. Comp. Immunol. 65 (2016) 159–168.
[28] N.R. Shin, T.W. Whon, J.W. Bae, Proteobacteria: microbial signature of dysbiosis in gut microbiota, Trends Biotechnol. 33 (9) (2015) 496–503.
[29] J.W. Liu, C.X. Xue, H. Sun, Y.F. Zheng, Z. Meng, X.H. Zhang, Carbohydrate catabolic capability of a Flavobacteriia bacterium isolated from hadal water, Syst. Appl. Microbiol. (2019), https://doi.org/10.1016/j.syapm.2019.01.002.
[30] Zothanpuia A.K. Passari, V.V. Leo, P. Chandra, B. Kumar, C. Nayak, et al., Bioprospection of actinobacteria derived from freshwater sediments for their potential to produce antimicrobial compounds, Microb. Cell Factories 17 (2018) 68.
[31] H.W. Wei, L.H. Wang, M. Hassana, B. Xie, Succession of the functional microbial communities and the metabolic functions in maize straw composting process, Bioresour. Technol. 256 (2018) 333–341.
[32] A.M. Labella, M.D. Castro, M. Manchado, T. Lucena, D.R. Arahal, J.J. Borrego, *Photobacterium malacitanum* sp. nov., and *Photobacterium andalusiense* sp. nov., two new bacteria isolated from diseased farmed fish in Southern Spain, Syst. Appl. Microbiol. 41 (5) (2018) 444–451.
[33] M.L. Zhang, Y.H. Sun, Y.K. Liu, F. Qiao, L.Q. Chen, W.T. Liu, Z.Y. Du, E.C. Li, Response of gut microbiota to salinity change in two euryhaline aquatic animals with reverse salinity preference, Aquaculture 454 (2016) 72–80.
[34] S. Walayat, A. Malik, N. Hussain, T. Lynch, *Sphingomonas paucimobilis* presenting as acute phlebitis: a case report, IDCases 11 (2017) 6–8.
[35] M. Zuhl, S. Schneider, K. Lanphere, C. Conn, K. Dokladny, P. Moseley, Exercise regulation of intestinal tight junction proteins, Br. J. Sports Med. 48 (12) (2014) 980–986.
[36] M. Mondal, D. Nag, H. Koley, D.R. Saha, N.S. Chatterjee, The *Vibrio cholerae* extracellular chitinase ChiA2 is important for survival and pathogenesis in the host intestine, PLoS One 9 (2014) e103119.
[37] P.W. Oakes, M.L. Gardel, Stressing the limits of focal adhesion mechanosensitivity, Curr. Opin. Cell Biol. 30 (2014) 68–73.
[38] M.A. Schwartz, Integrins and extracellular matrix in mechanotransduction, Cold Spring Harb. Persp. Biol. (2010) 2 a005066.
[39] M. Derrien, M.W. van Passel, J.H. van de Bovenkamp, R.G. Schipper, W.M. de Vos, J. Dekker, Mucin-bacterial interactions in the human oral cavity and digestive tract, Gut Microb. 1 (4) (2010) 254–268.
[40] S. Ogata, K. Shimizu, S. Tominaga, K. Nakanishi, Immunohistochemical study of mucins in human intestinal spirochetosis, Hum. Pathol. 62 (2017) 126–133.
[41] E. Peatman, C. Li, B.C. Peterson, D.L. Straus, B.D. Farmer, B.H. Beck, Basal polarization of the mucosal compartment in Flavobacterium columnare susceptible and resistant channel catfish (*Ictalurus punctatus*), Mol. Immunol. 56 (2013) 317–327.
[42] S.J. Lee, Y.H. Jung, S.Y. Oh, K.K. Jang, H.S. Lee, S.H. Choi, H.J. Han, *Vibrio vulnificus* VvpE inhibits mucin 2 expression by hypermethylation via lipid raftmediated ROS signaling in intestinal epithelial cells, Cell Death Dis. 6 (2015) e1787.
[43] W. Szczęsny, J. Szczepanek, A. Tretyn, S. Dąbrowiecki, J. Szmytkowski, J. Polak, An analysis of the expression of collagen I and III genes in the fascia of obese patients, J. Surg. Res. 195 (2) (2015) 475–480.
[44] J. Pickering, V.T. Cunliffe, F. Van Eeden, A.G. Borycki, Hedgehog signalling acts upstream of Laminin alpha1 transcription in the zebrafish paraxial mesoderm, Matrix Biol. 62 (2017) 58–74.
[45] V.I. Lushchak, Environmentally induced oxidative stress in aquatic animals, Aquat. Toxicol. 101 (2011) 13–30.
[46] 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.
[47] Y.Y. Chen, J.C. Chen, Y.H. Kuo, Y.C. Lin, Y.H. Chang, H.Y. Gong, et al., Lipopolysaccharide and β-1,3-glucan-binding protein (LGBP) bind to seaweed polysaccharides and activate the prophenoloxidase system in white shrimp *Litopenaeus vannamei*, Dev. Comp. Immunol. 55 (2016) 144–151.
[48] E. de la Vega, N.A. O'Leary, J.E. Shockey, J. Robalino, C. Payne, C.L. Browdy, G.W. Warr, 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.
[49] N. Khorattanakulchai, P. Amparyup, A. Tassanakajon, Binding of PmClipSP2 to microbial cell wall components and activation of the proPO-activating system in the black tiger shrimp *Penaeus monodon*, Dev. Comp. Immunol. 77 (2017) 38–45.
[50] N.N. Danial, S.J. Korsmeyer, Cell death: critical control points, Cell 116 (2004) 205–219.
[51] 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.
[52] 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.
[53] 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.
[54] C.H. Yang, J. Kong, Q.Y. Wang, Q.H. Liu, Y. Tian, K. Luo, Heterosis of haemolymph analytes of two geographic populations in Chinese shrimp *Fenneropenaeus chinensis*, Fish, Shellfish Immun 23 (2007) 62–70.
[55] M.X. Ning, Y.J. Xiu, M. Yuan, J.X. Bi, M. Liu, P.P. Wei, et al., Identification and function analysis of ras-related nuclear protein from *Macrobrachium rosenbergii* involved in *Spiroplasma eriocheiris* infection, Fish Shellfish Immunol. 70 (2017) 583–592.
[56] Y.F. Duan, P. Liu, J.T. Li, J. Li, Y. Wang, P. Chen, The responsive expression of a chitinase gene in the ridgetail white prawn *Exopalaemon carinicauda* against *Vibrio anguillarum* and WSSV challenge, Cell Stress Chaperones 19 (2014) 549–558.
[57] 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.
[58] 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.