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Individual and combined effects of ammonia-N and sulfide on the immune function and intestinal microbiota of Pacific white shrimp *Litopenaeus vannamei*

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ABSTRACT

In this study, we explored the individual and combined effects of ammonia-N and sulfide stress (1 mg/L sulfide and 15 mg/L ammonia-N) on the oxidation resistance, immune response and intestinal health of *Litopenaeus vannamei* during 72 h exposure. The total antioxidant capacity (T-AOC), malonaldehyde (MDA) and nitric oxide (NO) content, superoxide dismutase (SOD) and catalase activity (CAT), the immune-relative gene (*caspase-3*, *hsp70* and *IMD*) expression in hepatopancreas and intestine of *L.vannamei* and the intestinal microbiota were measured. The result showed that MDA and NO contents in hepatopancreas of *L. vannamei* in all treatment groups increased and remain were at high levels at the end of the stress exposure. The *L. vannamei* employ antioxidant defense system by increasing the activities of T-AOC, SOD and CAT enzymes in hepatopancereas and intestine to reduce oxidant damage. More severe damages with combined ammonia-N and sulfide stress to antioxidant systems were observed. The gene expression results also demonstrated that antioxidant capacity of *L. vannamei* was severely impaired and the apoptosis cell was initiated under the ammonia-N and sulfide stress. In addition, the environmental stress also reshaped the intestinal microbial community structure of *L. vannamei* that a number of original genera decreased, such as *Cellvibrio, Vibrio* and *Rheinheimera*; some new genera increased or appeared, such as *Photobacterium* in all treatment groups, *Arcobacter* and *Fusibacter* in sulfide stress group. Therefore, the health of *L. vannamei* was severely impacted when exposed to the stress of ammonia nitrogen and sulfide and these two factors can have weak synergic effects.

1. Introduction

The Pacific white shrimp (*Litopenaeus vannamei*) is an economically valuable species, which is the most popular aquaculture products all over the world, particularly in Southeast Asia [1]. The production of *L. vannamei* has increased rapidly due to intensive cultivation [2]. However, the high densities of aquatic organisms and overfeeding activities during the modern aquaculture process often severely deteriorated environments of aquaculture systems [3,4] and caused harsh environmental conditions and the accumulation of hazardous compounds, such as ammonia nitrogen and sulfide [4–6]. Ammonia-N is the main product of protein catabolism in aquatic systems, while sulfide is widely distributed at the interface between water and sediment, despite its low natural concentration [7]. In natural aquatic environments, appropriate concentrations of ammonia-N and sulfide do not stress aquatic plants and animals [8,9]. However, both ammonia-N and sulfide accumulate over time in shrimp ponds, due to the decomposition of organic wastes [10,11]. At high concentrations, ammonia-N may seep into animal tissues, affecting the ammonia metabolism and leading to ammonia poisoning; high concentrations of ammonia-N in the blood destroy the

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excretory system and the osmotic balance [12]. Both ammonia-N and sulfide strongly suppress the immune system and negatively affect physiological activities in shrimp due to the excessive production or accumulation of reactive oxygen species (ROS) [9,13]. When concentrations of ammonia-N and sulfide exceed specific tolerance levels, the susceptibility of shrimp to pathogenic bacterium increases, leading to disequilibrium and death [13,14].

Although previous studies have typically focused on the effects of a single stressor, shrimp are usually exposed to a variety of environmental stressors simultaneously [15]. Results of separate studies have indicated that both excessive ammonia-N and excessive sulfide in aquaculture systems have adverse impacts on shrimp growth [16], antioxidant systems [17,18], and immune responses [18,19]. However, the combined effects of these compounds on *L. vannamei* might be different since stressors may have additive, synergistic, or antagonistic effects [20]. Therefore, it is of significant interest to quantify the response of *L. vannamei* to the combination of ammonia-N and sulfide.

Environmental stress generally triggers an overproduction of ROS, which may cause cellular damage due to the formation of lipid peroxide [21]. As one of the end products of lipid peroxidation, the concentration of malonic dialdehyde (MDA) is most frequently used to monitor lipid peroxidation [14]. Organisms employ antioxidant defense systems to remove excess ROS, protect cells, and maintain homeostasis [22]. Therefore, antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT), are often induced by environmental stress. New biotechnological techniques also can provide rich genetic resources for studies of the interactions between environmental stress and aquatic animals [22–26]. The libraries of differentially expressed genes of crustacean had been established in previous and genes with obvious changes in expression in response to environmental stress also have identified [11,24–27]. Environmental stressors, such as cytotoxins, pollutants, and other contaminants, may induce apoptosis and further cell death [28,29]. Oxidative stress is a regulatory response used by organisms in response to environmental stress, and the toxic effects induced by environmental stress factors may be mediated by the regulation and induction of apoptosis and redox signaling [20,30,31]. Therefore, the expression of several immunomodulatory- and apoptosis-related genes provide helpful information for the underlying mechanisms of shrimp to resist environmental stress.

In addition to food digestion and nutrient absorption, the gut and its symbiotic bacteria are also vital to the maintenance of shrimp immunity [7,32]. Acute exposure to environmental stressors, such as sulfide, may detrimentally affect the composition of the gut microbiotas of aquatic animals by increasing the abundance of pathogenic bacteria [33]. An increased abundance of pathogenic bacteria might can cause further physiological and metabolic disorders, possibly resulting in death [3]. It has been suggested that the impact of environmental stressors might be reduced by regulating intestinal microorganisms.

In this study, we evaluated the effects of ammonia-N and sulfide on the oxidation resistance and immune response of juvenile white shrimp (*L. vannamei*). We also quantified the expression levels of three immune-related genes (*caspase-3*, *hsp70*, and *IMD*) in the hepatopancreas and intestine of *L. vannamei* and investigated the composition of the intestinal microbiota composition after exposure to ammonia-N and sulfide. Thus, we characterized the individual and the combined effects of these two environmental stressors. Our results would provide a basis for the improvement of the aquatic environment and are useful for the exploration of the regulatory gene mechanisms underlying stress tolerance.

2. Materials and methods

2.1. Experimental animals

Healthy juvenile white shrimp (*L. vannamei*), weighing 7.71 ± 0.48 g, were obtained from a local shrimp pond (Pinggang Farm, Yangjiang, Guangdong Province, China). Shrimp were cultured in artificial aerated seawater (salinity 5‰; pH 7.90 ± 0.11) for one week before experimentation. Shrimp were fed feed pellets daily, at 5% of total shrimp body weight. During the experiment, water temperature was controlled at 27.9 ± 0.6 °C, salinity at 5‰, pH at 7.90 ± 0.11, and dissolved oxygen at 5.85 ± 0.50 mg/L.

2.2. Ammonia and sulfide exposure

After the one-week acclimation, shrimps were divided into 12 plastic buckets (160 L each) representing four groups (the control group: CK, the ammonia-N stress group: N group, the sulfide stress group: S group, and the co-stress group: NS group) with three replicate buckets per group. We allocated 30 shrimp to each bucket. Buckets were filled with 120 L artificial aerated water. According to previous research on the 96-h lethal concentration of ammonia-N and sulfide for *L. vannamei* at 5‰ salinity [34,35], the stress factor concentrations were set for the three experimental groups: 15 mg/L ammonia for N group, 1 mg/L sulfide for S group, 15 mg/L ammonia + 1 mg/L sulfide for NS group. Before experimentation, a stock solution of 1000 mg/L sulfide was prepared by dissolving 7.49 g of $Na_2S{\cdot}9H_2O$ in 1 L of distilled water, and a stock solution of 10,000 mg/L ammonia was prepared by dissolving 29.72 g of NH_4Cl in 1 L of distilled water. We then mixed 120 ml of the sulfide solution into each sulfide and co-stress bucket (for a final concentration of 1 mg/L sulfide per bucket), and 180 ml of the ammonia solution to each ammonia and co-stress bucket (for a final concentration of 15 mg/L ammonia per bucket). The concentrations of sulfide and ammonia in each bucket were measured with a spectrophotometer UV-6000 (Metash, Shanghai, China) every 4 h during the experiment. Appropriate concentrations were maintained via supplementation with the stock solutions.

2.3. Sampling

Three individuals from each bucket were sampled on the ice after 0, 6, 12, 24, 48, and 72 h of stress exposure. Three individuals were collected from each bucket at each time point and were mixed as a replicate. The hepatopancreas and intestine of each shrimp individuals was dissected and placed in a 2-ml sterilized internal thread cryogenic vial (Corning, New York, USA). At 72 h of stress exposure, the intestines from three individuals of each bucket were mixed as a replicate and placed in sterilized cryogenic vials, which were rapidly frozen in liquid nitrogen. Three replicates were sampled for each treatment group.

2.4. Biochemical analysis

Each hepatopancreas was accurately weighted and mechanically homogenized in sterile 0.85% saline at 1:9 (w:v) saline to hepatopancreas ratio. We measured the biochemical parameters of the hepatopancreases, including total antioxidant capacity (T-AOC), malondialdehyde (MDA) concentration, nitric oxide (NO) concentration, superoxide dismutase (SOD) activity level, and catalase (CAT) activity level, using commercial test kits (Jiancheng, Ltd, Nanjing, China), following the manufacturer's instructions. Results were read on a microplate reader (Bio-RAD, California, USA) and a spectrophotometer UV-6000 (Metash, Shanghai, China).

2.5. Immune-associated gene expression

2.5.1. Total RNA extraction and reverse transcription PCR

The total RNAs of the hepatopancreases and hemocytes of three shrimp per bucket were extracted with TRIzol reagent (Invitrogen, California, USA). We placed 0.5 g of hepatopancreas tissue in a mortar with liquid nitrogen, and ground the tissue into a powder. The powdered tissue was equally divided among six 1.5 ml sterilized centrifuge tubes containing 1.0 ml TRIzol reagent. The tubes were then stored at

−80 °C or used for the extraction of total RNA, following the manufacturer's instructions. The integrity of each total RNA sample was determined using 1.0% agarose electrophoresis. Total RNA yield and purity were quantified at 260 nm and 280 nm, respectively. The OD260/OD280 ratios of all of the samples were between 1.8 and 2.0. Total RNA was reverse transcribed using the PrimeScript RT reagent Kit (Takara, Dalian, China) for real-time quantitative PCR. First strand cDNA was generated in a 20 μl reaction volume, containing 2 mg total RNA, 1 × RT buffer, 1 mM dNTP, 0.2 mM Oligo (dT), 10 U RNase inhibitor, and 50 U Multi-Scribe Reverse Transcriptase (Applied Biosystems, California, USA). The reaction was performed at 37 °C for 2 h.

2.5.2. Quantitative real-time PCR analysis of gene expression

Gene expression was using quantitative real-time PCR (qPCR) using SYBR Fast qPCR Mix (Takara, Dalian, China) and a LightCycler 480 Real-Time PCR System (Roche, Basel, Switzerland). The qPCR conditions were 95 °C for 3 min, follows by 35 cycles of 95 °C for 10 s and 60 °C for 30 s. Relative gene expression was calculated as $2^{-\Delta\Delta Ct}$, where Ct was the threshold cycle [36]. The expression levels of the housekeeping gene *β-actin* were unaltered across all of the groups; *β-actin* was thus used as the internal reference gene (see Table 1).

2.6. Intestinal microbiota analysis

2.6.1. DNA extraction and PCR amplification

Microbial DNA was extracted from each intestinal sample using an E.Z.N.A. stool DNA Kit (Omega Biotek, Norcross, GA, US), following the manufacturer's protocols. The V3–V4 region of the eukaryotic ribosomal 16S rDNA gene was amplified using PCR (95 °C for 2 min; 30 cycles of 98 °C for 10 s, 62 °C for 30 s, and 68 °C for 30 s; and a final extension at 68 °C for 10 min) and primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 806R (5′-GGACTACHVGGGTATCTAAT-3′). The barcode was an eight-base sequence unique to each sample. PCR reactions were performed in triplicate in 50 μL reaction volumes, each containing 5 μL of 10 × KOD Buffer, 5 μL of 2.5 mM dNTPs, 1.5 μL of each primer (5 μM), 1 μL of KOD Polymerase, and 100 ng of template DNA.

2.6.2. Illumina Hiseq2500 sequencing

Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions and quantified using QuantiFluor-ST (Promega, Wisconsin, USA). Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 250) by using a Hiseq2500 PE250 (Illumina, California, USA) according to the standard protocols. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database and processed (e.g., tag splicing and chimera removal) to obtain effective tags for operational taxonomic units (OTU) clustering.

2.6.3. Bioinformatics analysis

Raw data containing adapters or low quality reads would affect the following assembly and analysis. Thus, to get high quality clean reads, raw reads were further filtered according to the following rules:

1) Removing reads containing more than 10% of unknown nucleotides (N);
2) Removing reads containing less than 80% of bases with quality (Q-value) > 20.

Paired end clean reads were merged as raw tags using FLASH [37] (v 1.2.11) with a minimum overlap of 10bp and mismatch error rates of 2%. Noisy sequences of raw tags were filtered by QIIME [38] (V1.9.1) pipeline under specific filtering conditions to obtain the high-quality clean tags. The effective tags for all of the samples were clustered into OTUs, using a 97% identity threshold, with UPARSE (usearch v9.2.64) [39]. The tag sequence with highest abundance was selected as reprehensive sequence within each cluster. Between groups Venn analysis was performed in R to identify unique and common OTUs.

Chao1, Simpson and all other alpha diversity index were calculation in QIIME. OTU rarefaction curve and Rank abundance curves was plotted in QIIME. Statistics of between group Alpha index comparison was calculated by a Welch's *t*-test and a Wilcoxon-rank test in R. Alpha index comparing among groups was computed by a Tukey's HSD test and a Kruskal-WallisH test in R.

The representative sequences were classified into organisms by a naive Bayesian model using RDP classifier (Version 2.2) based on SILVA Database [40]. Biomarker features in each group were screened by Metastats and LEfSe software.

2.7. Statistical analysis

Data were expressed as means ± standard deviation. Data were analyzed by one-way analysis of variance (ANOVA) followed by multiple-comparison Tukey tests to identify significant differences among groups. Data were tested for normality and were log-transformed to meat the assumption when required. $P < 0.05$ was considered as statistic significance. All of the statistical analyses were performed in SPSS for Windows v22.0 (IBM, New York, USA).

3. Results

3.1. Oxidative stress parameters

T-AOC activity levels in the shrimp hepatopancreas increased sharply after exposure to ammonia-N, sulfide, and both combined. However, T-AOC activity levels decreased across all of the treatment groups after 24 h of exposure; T-AOC activity levels in the ammonia-N group (N) and the co-stress group (NS) were lower than those of the control group at 72 h (Fig. 1E). The T-AOC activity levels in N and NS groups peaked at 12 h, and at 6 h in the S group. These levels were significantly higher than those of all other groups ($P < 0.05$). SOD activity levels in all of the treatment groups (N, S, and NS) peaked at 6 h ($P < 0.05$). SOD activity levels in the NS and N groups were significantly less than those of the control group at 72 h ($P < 0.05$). CAT activity levels in the hepatopancreas samples across all of the treatment groups followed a similar pattern to T-AOC activity levels (Fig. 1A and B). MDA and NO concentrations in the hepatopancreases of all of the treatment groups were significantly higher than the control at all of the time points except NO in NS group at 12 h ($P < 0.05$) (Fig. 1C and D).

T-AOC, SOD, and CAT activity levels in the shrimp intestine were significantly higher than those in the control group after 6–24 h of

Table 1
Immune-related genes of white shrimp *Litopenaeus vannamei* and their PCR primers used for RT-PCR in this study.

Gene	Forward sequence (5′ to 3′)	Reverse sequence (5′ to 3′)
β-actin	CATCACCAACTGGGACGACATGGA	GAGCAACACGGAGTTCGTTGT
caspase 3	CGAAGTCAAAGCCAGAAACA	ACTGCTACTTCCCTGGTGAC
hsp70	CTCCTGCGTGGGTGTGTT	GCGGCGTCACCAATCAGA
IMD	TGGGTCCGTGTCCAGTGAT	ACAAACAACCACACACAAGCAG

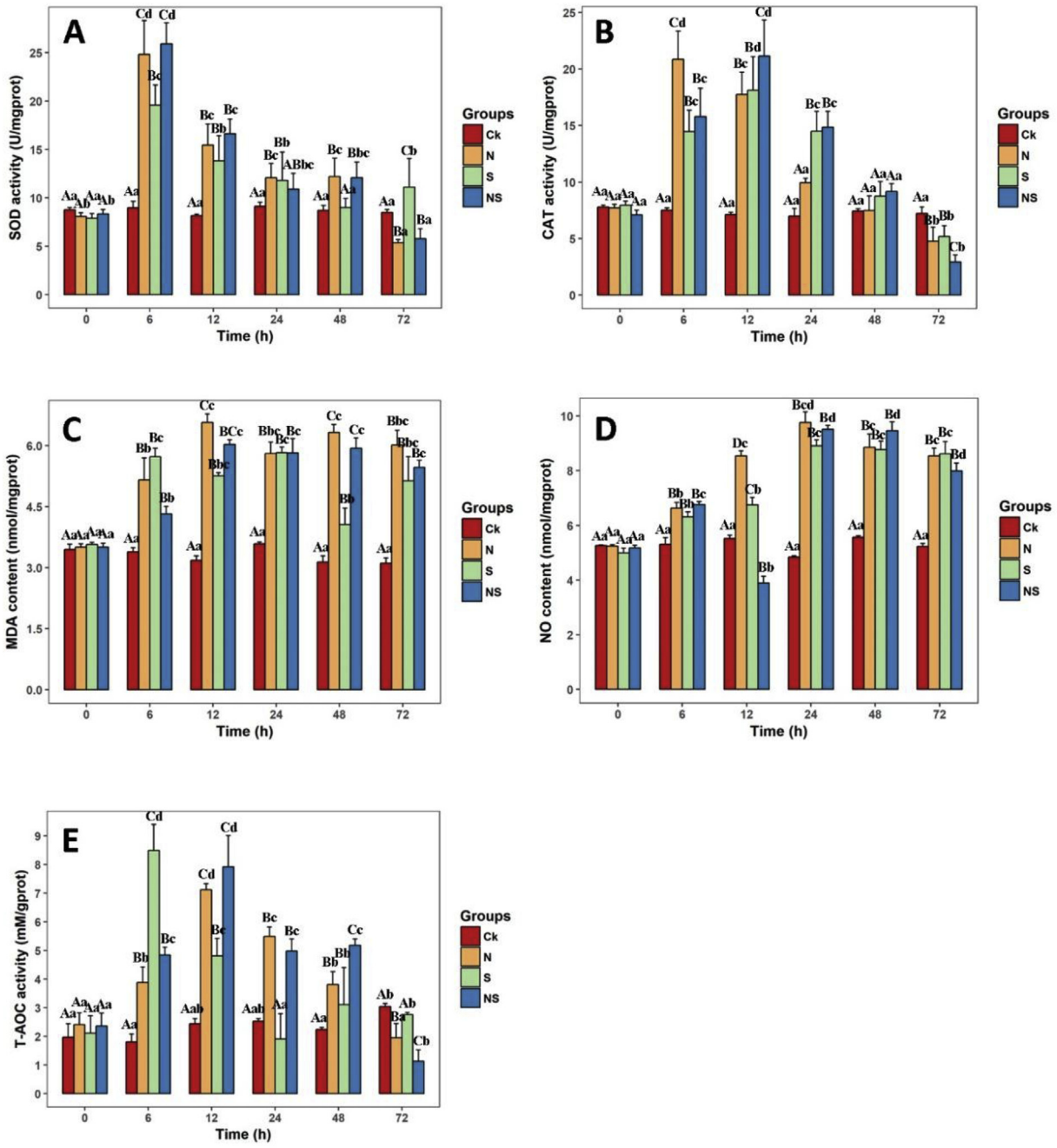

Fig. 1. superoxide dismutase activity (A), catalase activity (B), Malondialdehyde concentration (C), nitric oxide concentration (D) and total antioxidant capacity (E) in the hepatopancreas of *Litopenaeus vannamei* over 72 h of ammonia or/and sulfide exposure. Error bars indicate the SD of the mean of three replicates. Uppercase letters indicate significant differences among groups at the same time points, and lowercase letters indicate significant differences among time points ($P < 0.05$).

exposure ($P < 0.05$) (Fig. 2E). The levels of T-AOC and CAT activity in the intestines from the N and NS groups decreased at 48 h; at 48 h, no significant differences were found in T-AOC and CAT activity levels between the NS group and the control group (P < 0.05). Indeed, T-AOC and CAT activity levels were lower in the N group than in the control group at 48 h ($P < 0.05$). No significant differences in SOD and CAT activity levels in the intestine were identified between any treatment group and the control at 72 h ($P > 0.05$) (Fig. 2A and B). The S group had the highest level of T-AOC activity, while the NS group had the lowest; no significant difference in T-AOC activity level was found between the N group and the control group.

MDA concentrations in the intestine samples from all of the treatment groups were greater than the MDA concentrations in the intestine samples from the control group after 12 h of exposure ($P < 0.05$) (Fig. 2C). Only the NS group had a higher MDA concentration than the control at 6 h. The NO concentrations in the N and NS groups were significantly higher than those of the control group at all of the time points, while the NO concentration in the S group was only significantly higher than the control at 12 h and 24 h ($P < 0.05$). The NO concentrations in S group were not significantly different from the control at 6 h, 48 h, or 72 h ($P > 0.05$) (Fig. 2D). The S group has significantly lower levels of NO than did the other two treatment group throughout the experiment ($P < 0.05$).

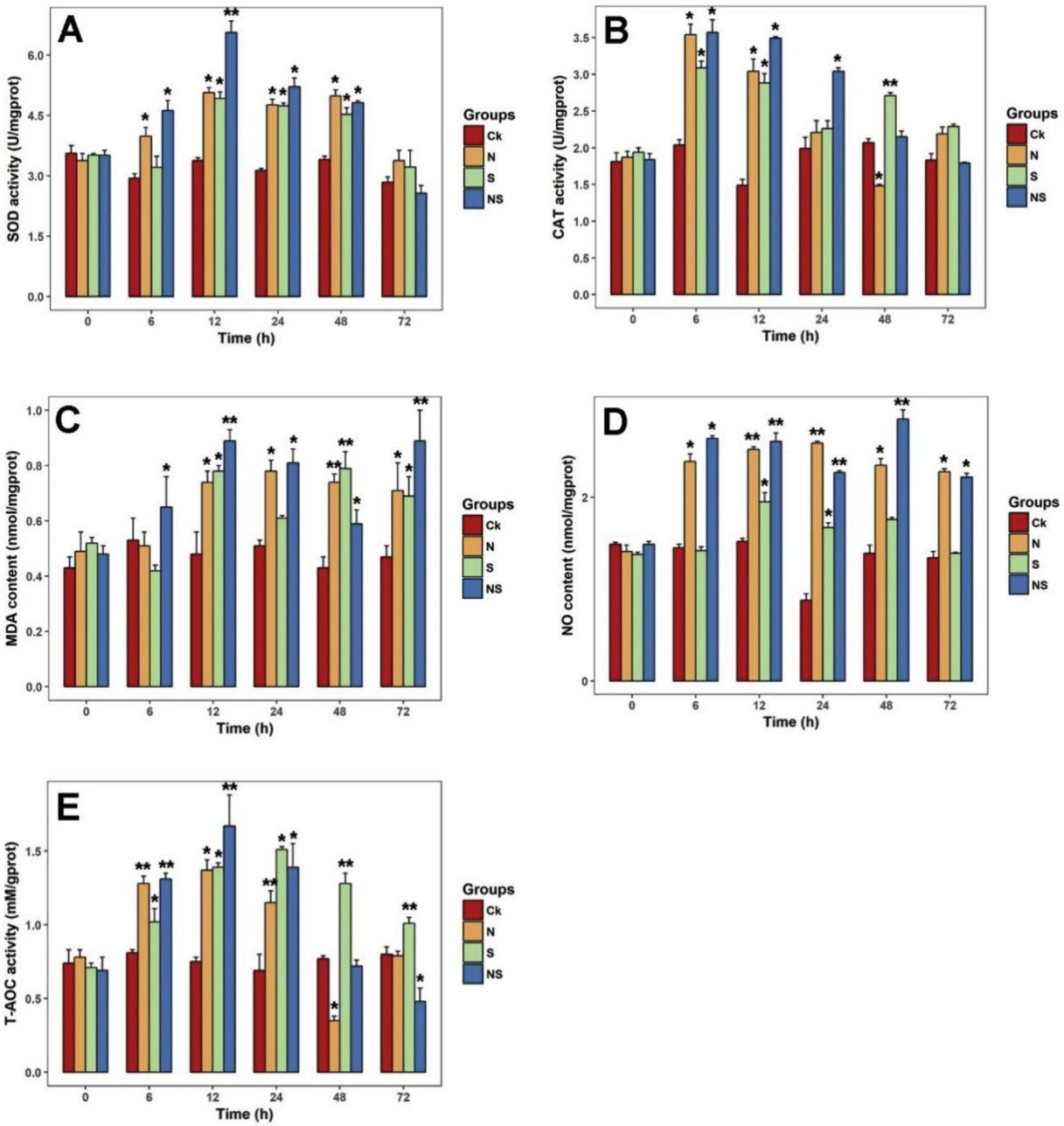

Fig. 2. superoxide dismutase activity (A), catalase activity (B), Malondialdehyde concentration (C), nitric oxide concentration (D) and total antioxidant capacity (E) in the intestine of *Litopenaeus vannamei* over 72 h of ammonia or/and sulfide stress. Error bars indicate the SD of the mean of three replicates. Asterisks indicate significant differences among groups at the same time point ($P < 0.05$). Control group, Ck; ammonia group, N; sulfide group, S; co-stress group, NS.

3.2. Immune-related gene expression

The relative mRNA expression levels of *caspase-3* and *hsp*70 in the hepatopancreas samples from all of the treatment groups increased significantly over the 72 h of exposure, except for the expression of *caspase-3* in the N group at 12 h, and the expression of *hsp70* at 48 h and 72 h in the N group ($P < 0.05$) (Fig. 3A and B). The relative gene expression of *caspase-3* gene was highest at 6 h and 12 h, and then decreased ($P < 0.05$) (Fig. 3A). The relative gene expression levels of *IMD* did not differ significantly in the treatment groups as compared to the control group from 0 to 12 h of exposure ($P > 0.05$). However, *IMD* gene expression levels increased sharply in the treatment groups at 24 h ($P < 0.05$), and remained significantly higher than levels in the control group at 72 h ($P < 0.05$) (Fig. 3C).

The relative mRNA expression levels of *caspase-3* in the intestines of the treatment groups were significantly greater than those of the control at all of the time points ($P < 0.05$), except for the S group at 6 h and the N group at 24 h (Fig. 3D). The relative gene expression of *hsp*70 in the intestines of N and NS group increased sharply, peaking 6–12 h after exposure, while the relative gene expression of *hsp70* in S group peaked at 12 h ($P < 0.05$). The gene expression levels of *hsp70* in the intestines from all treatment groups decreased after 24 h of exposure. Indeed, the *hsp70* gene expression levels in the S and NS groups were significantly lower than in the control group ($P < 0.05$, Fig. 3E). The relative gene expression levels of *IMD* in the intestines of all of the treatment groups increased sharply at 12 h as compared to the control group ($P < 0.05$), peaking after 24–72 h of exposure ($P < 0.05$, Fig. 3F).

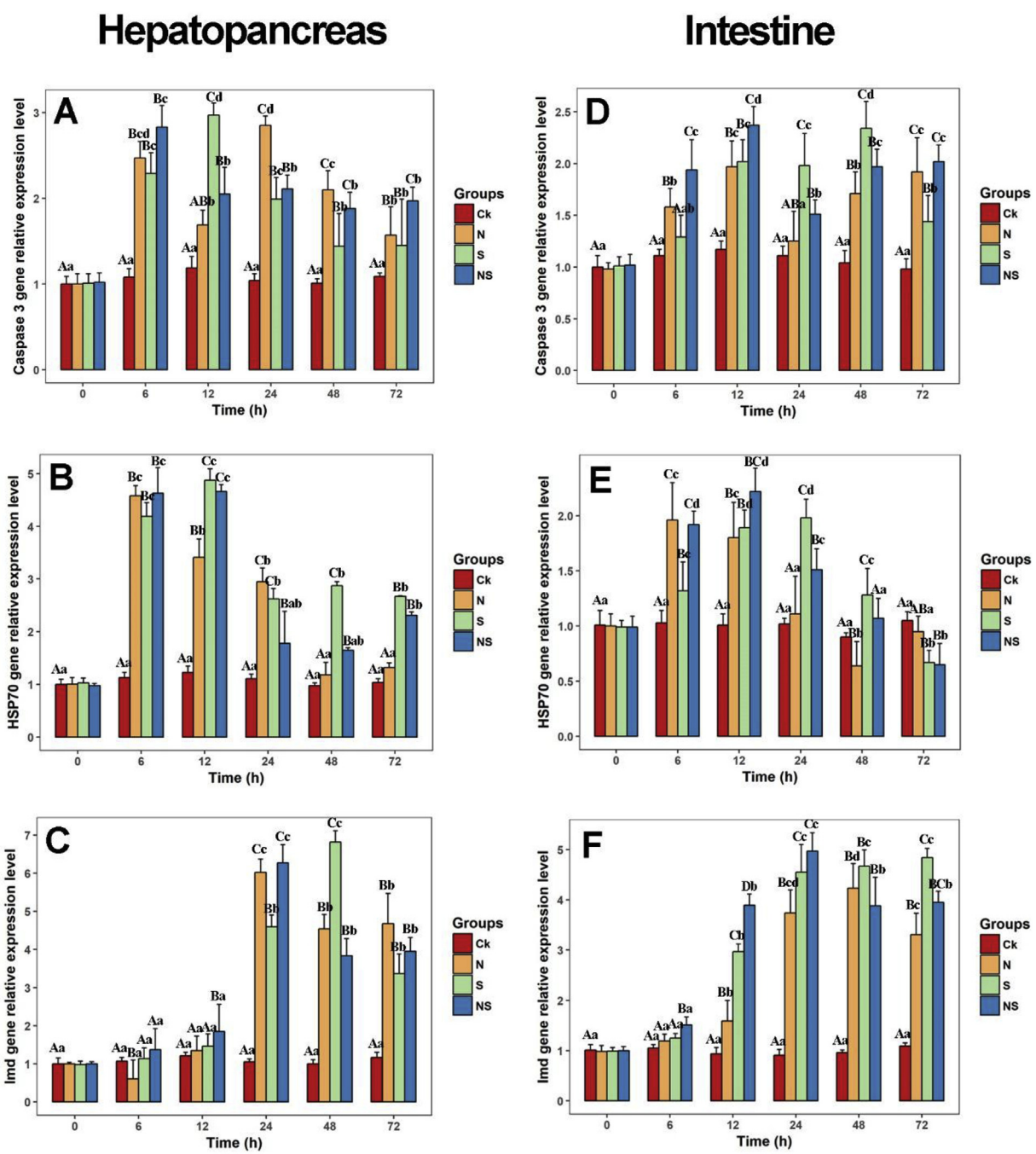

Fig. 3. Relative gene expression of *caspase-3*, *hsp70*, and *IMD* in the hepatopancreas (A–C) and intestine (D–F) of *Litopenaeus vannamei* over 72 h of ammonia or/and sulfide exposure. β-actin was selected as the internal reference gene. Error bars indicate the SD of the mean of three replicates. Uppercase letters indicate significant differences among groups at the same time points, and lowercase letters indicate significant differences among time points ($P < 0.05$). Control group, Ck; ammonia group, N; sulfide group, S; co-stress group, NS.

3.3. Intestinal microbiota analysis

3.3.1. Richness and diversity analysis

We obtained 1,099,092 Tags sequences from the *L. vannamei* intestinal bacteria (Fig. 4). After data processing, a total of 1092395 effective Tags sequences were obtained, with an average length of 462bp, for the subsequent Operational Taxonomic Units clustering. 0.61% of invalid Tags were removed in the process. UPARSE software was used to cluster the effective Tags sequences of all samples, and the sequences were clustered into OTUs with 97% Identity, resulting in a total of 4494 OTUs.

Compared with the control group, ammonia stress significantly decreased bacterial richness, as estimated by the Chao1 and Ace indices ($P < 0.05$), while sulfide exposure and the two compounds together significantly increased bacterial richness ($P < 0.05$) (Table 2). No significant differences in the richness estimators were identified between the S and NS groups ($P > 0.05$). The NS group had the highest diversity estimate of all of the groups ($P < 0.05$). The N and S groups both had higher diversity estimates than the control group ($P < 0.05$), with the exception of the Shannon Weiner index, which was not significantly different between the S group and the control group. The Shannon-Wiener index of the N group was higher than that of the S group, but no significant differences in the Simpson index were identified between the two groups ($P > 0.05$).

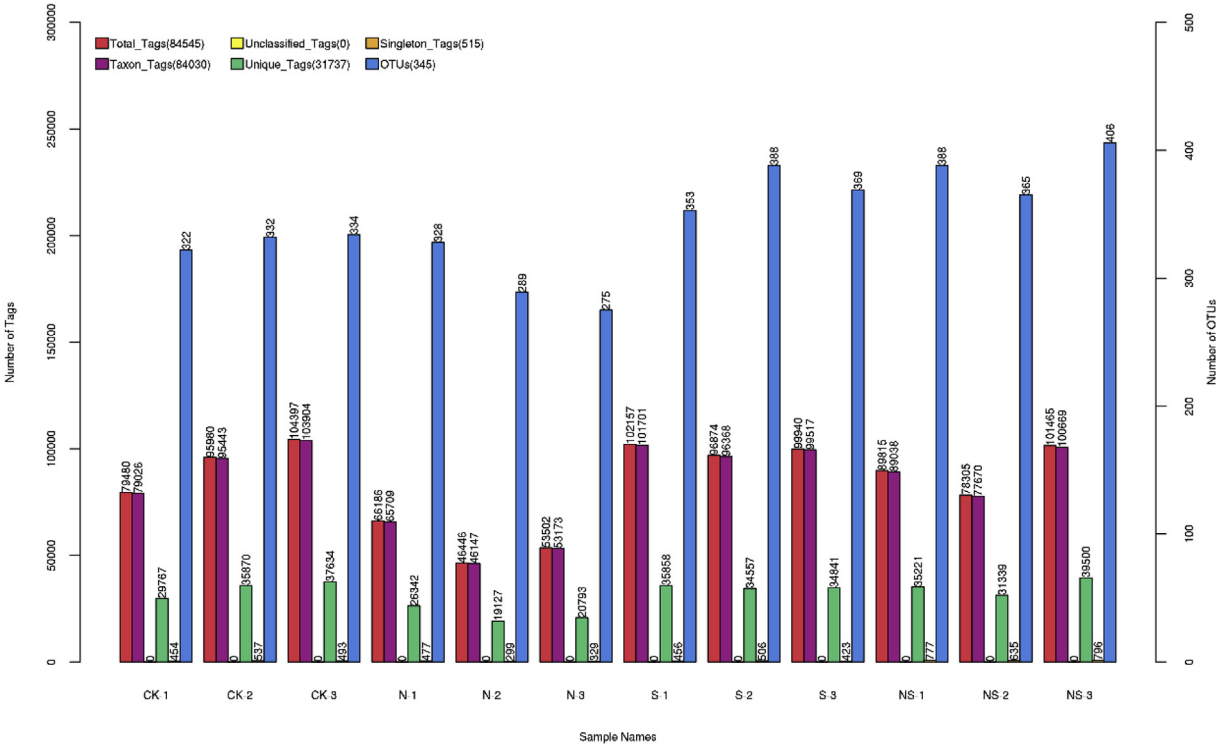

Fig. 4. Statistics chart of OTUs and Tags for different samples in the four groups of *Litopenaeus vannamei*. (control, CK; ammonia group, N; sulfide group, S; co-stress group, NS).

Table 2
Intestinal bacterial diversity and richness, based on the diversity index (means ± standard deviation) and the estimated number of operation taxonomic units (OTUs) in different groups.

	Control (CK)	Ammonia (N)	Sulfide (S)	Co-stress (NS)
Observed species	327	295	363	386
Richness estimators				
Chao1	458.91 ± 56.01^{b}	405.38 ± 25.97^{a}	505.92 ± 14.39^{c}	528.87 ± 18.50^{c}
Ace	459.41 ± 39.81^{b}	398.76 ± 20.86^{a}	504.42 ± 16.08^{c}	506.06 ± 8.31^{c}
Diversity estimators				
Shannon	3.132 ± 0.016^{a}	3.589 ± 0.075^{b}	3.133 ± 0.019^{a}	4.136 ± 0.008^{c}
Simpson	0.712 ± 0.007^{a}	0.787 ± 0.003^{b}	0.791 ± 0.001^{b}	0.866 ± 0.002^{c}

Lowercase letters indicate significant differences among groups ($P < 0.05$).

We identified 881 OTUs and 121 OTUs were identified in all of the samples, 97 were identified in the samples of control group only; 38 were identified in the samples of N group only; 62 were identified in the samples of S group only; and 108 OTUs in the samples of NS group only. We identified 36 OTUs appeared at the same time between the samples of N and NS groups, and 20 OTUs appeared at the same time between the samples of S and NS groups (Fig. 5).

Vibrio had the highest abundance across all of the OTUs (Fig. 7). However, the abundances of other bacterial taxa differed among the groups. In the N group *Cetobacterium*, *Photobacterium,* and *Acinetobacter* were most abundant; in the S group *Fusibacter*, *Arcobacter,* and *Pseudomonas* were most abundant; and in the NS group *Spongiimonas*, *Flavobacterium,* and *Brevundimonas* were most abundant.

3.3.2. Bacterial community analysis

Our heatmap analysis of *L. vannamei* intestinal bacterial abundance at the genus level indicated that *Aeromonas*, *Cetobacterium,* and *Photobacterium* were more abundant in the N and NS groups than in the control group ($P < 0.05$). In the S and NS groups, *Fusibacter*, *Oceanobacter,* and *Arcobacter* were more abundant than in the control group ($P < 0.05$) (Figs. 6 and 7). However, *Vibrio*, *Rheinheimera,* and *Cellvibrio* were more abundant in the control group than in any treatment group ($P < 0.05$).

At the phylum level, the abundances of *Fusobacteria, Chlamydiae,* and *Planctomycetes* were greater in the N group than in the control ($P < 0.05$), while *Saccharibacteria*, *Gracilibacteria,* and *Firmicutes* were more abundant in the S group than in the control ($P < 0.05$). Finally, *Tenericutes* was more abundant in the NS group than in the control ($P < 0.05$).

4. Discussion

Treatment of shrimp with ammonia-N has been shown to regulate the physiological metabolism and immune system, increasing the risk of pathogenic infection [15–17,41]. Excessive ammonia nitrogen may affect crucial neuroendocrine substances, and cause an immune response via the cAMP- (cyclic adenosine monophosphate), CaM- (calmodulin), and cGMP- (cyclic guanosine monophosphate) dependent pathways [22,42]. Similar results were found in this study that the expression levels of hepatopancreatic and intestinal immune-related genes were significantly increased after ammonia-N exposure. As for sulfide, it may inhibit cytochrome C oxidase, affecting aerobic respiration and damaging the colonic mucosal epithelial cells [43]. Sulfide may also damage gut structure and alter the gut microbiota, reducing the gut health of *L.*

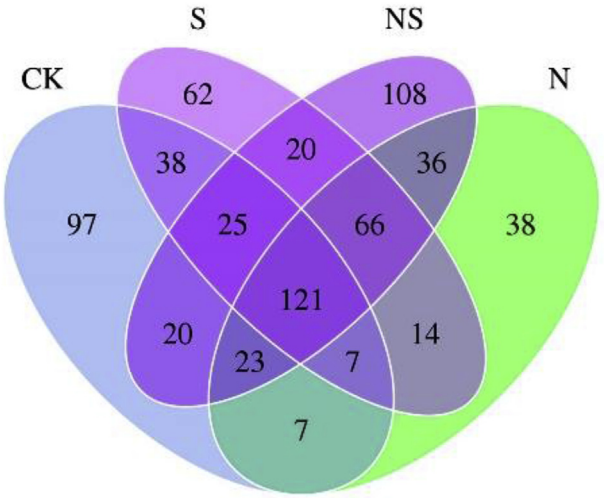

Fig. 5. Distribution of the operation taxonomic units (OTUs) shared by the four groups of *Litopenaeus vannamei* (control, CK; ammonia group, N; sulfide group, S; co-stress group, NS) as shown with a Venn diagram. Numbers indicate number of OTUs within each subset. Colors indicate different subsets with each group; numbers represent number of OTUs. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

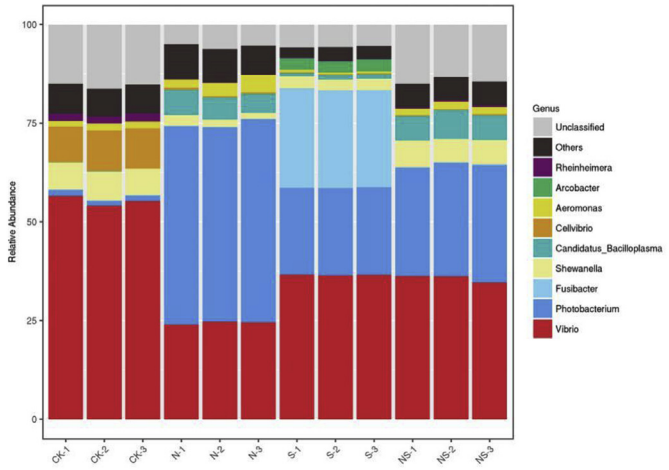

Fig. 7. Stack graph of the top 10 most abundant bacterial genera (> 2% of all bacteria) in the *Litopenaeus vannamei* intestinal samples across all of the groups.

vannamei [7]. Our results confirmed that both ammonia-N and sulfide exposure severely damaged the immune functions of the hepatopancreas and intestine in *L. vannamei*, while the exposure to the two compounds together increased the severity of the damage.

4.1. The antioxidant capacity of L. vannamei under ammonia nitrogen and sulfide stress

Suboptimal environmental conditions induce toxic oxidative stress in aquatic organisms; in response, affected organisms employ antioxidant defense systems to reduce oxidant damage [29]. In this study, remarkable oxidative stress reactions were observed in shrimp exposed to ammonia-N, sulfide, and combined stressors. MDA and NO concentrations in the *L. vannamei* hepatopancreas increased gradually in all treatment groups during the early stages of the experiment. This indicated that ROS were overproduced, inducing severe membranous lipid peroxidation. MDA and NO concentrations remained high throughout exposure, suggesting that cells might have been severely damaged and that the antioxidant system was unable to remove the excess ROS.

As ROS increased, the *L. vannamei* can activate the oxidative stress system [14]. Thus, the activity levels of the T-AOC, SOD and CAT enzymes in the *L. vannamei* hepatopancreas and intestine were rapidly increased in response to stress. However, the enzymatic activity levels in the hepatopancreases of stressed *L. vannamei* were significantly lower than those in the control group after 72 h exposure. This indicated that exposure to excessive ammonia and sulfide had already damaged the antioxidant system of *L. vannamei*. These results were consistent with our observations of the MDA and NO concentrations.

The MDA concentrations in the intestines of the NS group were slightly higher than the control at 6 h, 12 h, and 72 h. However, the NO

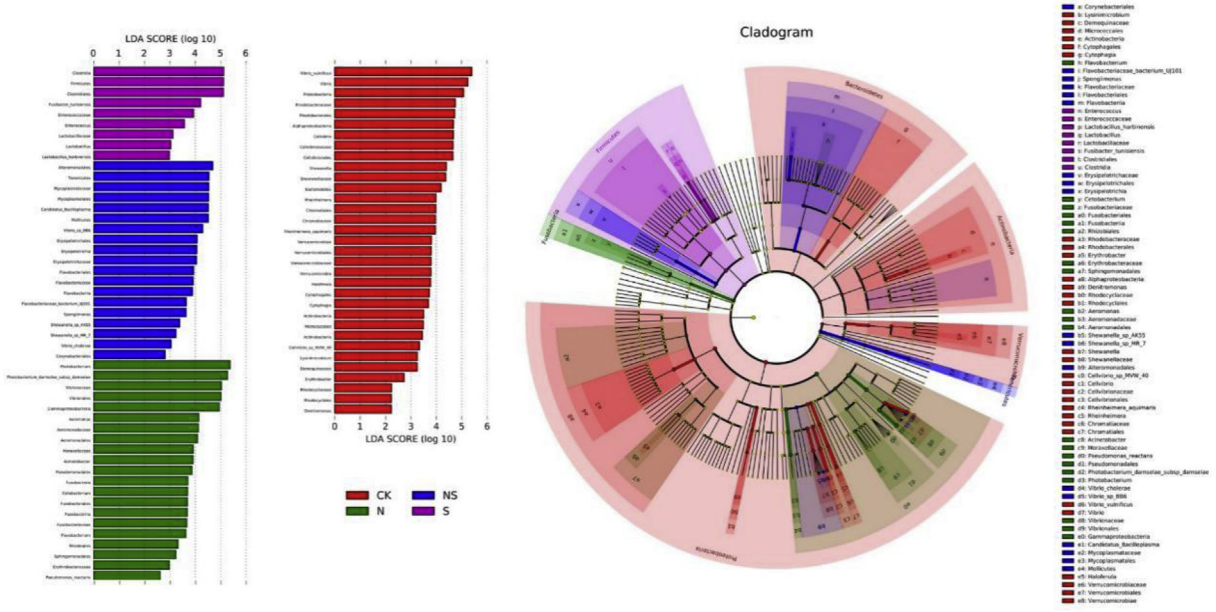

Fig. 6. Cladogram of the genera of intestinal microbiota recovered by LEfSe[1] analysis. The length of each histogram bar corresponds to the influence of the represented species (i.e., the LDA[2] Score). The circles, from inner to outer, represent bacterial classifications from phylum to species. The size of the circles at different levels of classification represents the relative abundance of the taxa within that clade. (LEfSe[1]: LDA Effect Size; LDA[2]: Linear Discriminant Analysis).

concentrations in the hepatopancreases of the NS group were significantly lower than those of the other groups at 12 h, indicating that these two stress factors might have an antagonistic effect on ROS at initial exposure. Lower T-AOC activity levels were observed in the hepatopancreases and intestines of the NS, as compared to either the N or the S group at 72 h. Thus, the immune defense system of *L. vannamei* exposed to combined stressors might be more severely affected as exposure duration increases. This suggested that ammonia-N and sulfide had a weak synergic effect on *L. vannamei* immune function after 72 h of exposure, while further experiments were needed to confirm this.

4.2. Gene expression in L. vannamei in response to ammonia nitrogen and sulfide exposure

The activation of *caspase-3* demonstrates that cell apoptosis has entered an irreversible stage [44]. We found that, apoptosis in *L. vannamei* began immediately in response to severe environmental stress [45,46]. In consistent with our biochemical results, the upregulation of *caspase-3* suggested that the antioxidant capacity of *L. vannamei* was severely impaired. *Caspase-3* gene expression in the hepatopancreas peaked prior to *caspase-3* gene expression in the intestine in all groups, indicating that apoptosis began in the hepatopancreas before the intestine. In addition, *caspase-3* gene expression in the hepatopancreas of *L. vannamei* peaked earlier in the co-stress groups than sulfide or ammonia-N stress groups. This indicated the synergistic effects of sulfide stress and ammonia-N stress.

As one of the most well-conserved heat shock proteins, *hsp70* protects cells from damage in almost all living organisms [47]. This protein regulates a series of antioxidant reactions, inhibiting apoptosis downstream of cytochrome *c* release and upstream of Caspase-3 activation [48–50]. Here, *hsp70* gene expression in the hepatopancreas and intestine first increased and then decreased as the duration of ammonia nitrogen and sulfide exposure increased. At the early stages of exposure, *hsp70* was upregulated by the stress factors, consistent with the intense cellular antioxidant reaction and the synthesis of antioxidant enzymes for the removal of ROS. Owing to the destruction of antioxidant systems, excessive ROS was not able to be removed, leading to a downregulation of *hsp70* expression after 72 h.

IMD pathways are one of the major signaling pathways that are essential for inducing immune related genes during invasion of pathogens [51–53]. In the *L. vannamei* hepatopancreas and intestine across all of the treatments, *IMD* gene expression was sharply upregulated at 12 h and 24 h. This possibly due to a bacterial infection, considering the change of gut microbiota *of L. vannamei* (Fig. 7). However, no significant difference between single stress and co-stress groups were observed in this study. Therefore, the two environmental stress factors ammonia-N and sulfide not only caused oxidative stress in shrimp, but also increased the vulnerability of the shrimp to pathogenic attack.

4.3. The gut microbiota of L. vannamei in response to ammonia nitrogen and sulfide exposure

Exposure to ammonia-N and sulfide greatly affected the intestinal microbial community structures of *L. vannamei*. The richness of the microbial community increased after exposure to sulfide and both compounds. The N and NS groups had higher diversity indices, while the S group had a higher Simpson index (Table 2).

Many bacterial species disappeared from the intestine of *L. vannamei* during the exposure to environmental stress, and many species were introduced. The intestinal microbiota directly affects the growth, feeding, and immune responses of shrimp [54]. Thus changes in microbiotal community structures might strongly negatively affect the digestive system of *L. vannamei*.

At the genus level, the abundance of *Fusibacter* in the S group and the abundance of *Photobacterium* in all of the treatment groups were increased compared to the control. *Fusibacter* can use a truncated sox complex, along with reverse-acting sulfate-reducing systems, to conserve energy in haloalkaliphilic sulfate-reducing bioreactors [55]. Thus, the increased abundance of *Fusibacter* might indicate an adaptation of the *L. vannamei* microbiota to ambient sulfide exposure. Previous studies showed that two pathogenic *Photobacterium* strains isolated from *Exopalaemon carinicauda* could lead to mortality in both *E. carnicauda* and *L. vannamei* [56]. *Photobacterium* was the second most abundant family across all treatment groups, and was particularly abundant in the N group. Thus, the *L. vannamei* exposed to ammonia nitrogen might be more easily infected. However, the effects of sulfide exposure on *Photobacterium* infections in crustaceans were poorly understood and further researches in this area are needed.

Vibrio is a ubiquitous genus of bacteria, and some of which are beneficial to shrimp survival and growth and could be used as probiotic strains [57]. *Vibrio* abundance was reduced in the guts of *L. vannamei* exposed to sulfide and ammonia-N, as compared to unexposed controls. This finding suggested a direct relationship between the sulfide and ammonia-N exposure and the susceptibility of crustaceans to *Vibrio*. Large *Vibrio* populations may benefit shrimp gut health, as these bacteria secrete mucus and promote colonization by probiotics [58,59]. The stress of ammonia nitrogen and sulfide exposure might destroy these beneficial strains of *Vibrio*, adversely affecting *L. vannamei* health.

5. Conclusion

In conclusion, this study indicates that acute exposure to sub-lethal ammonia-N and sulfide could lead to the over production of ROS. The MDA and NO contents in hepatopancreas of *L. vannamei* in all treatment groups increased and remain were at high levels at the end of the stress exposure. The *L. vannamei* employ antioxidant defense system by increasing the activities of T-AOC, SOD and CAT enzymes in hepatopancereas and intestine to reduce oxidant damage. However, the excessive ammonia-N and sulfide resulted in the damage to the antioxidant system of *L. vannamei*. The *caspase-3*, *hsp70* and *IMD* gene expression results also demonstrated that antioxidant capacity of *L. vannamei* was severely impaired and the apoptosis was initiated under the ammonia-N and sulfide stress. In addition, the environmental stress also reshaped the intestinal microbial community structure of *L. vannamei* that the abundance of pathogenic *Photobacterium* was increased, while some beneficial *Vibrio* were decreased. Therefore, the health of *L. vannamei* was severely impacted when exposed to the stress of ammonia nitrogen and sulfide and these two factors can have synergic effects in some ways. Our results could provide a reliable experimental basis for further study of ammonia-N and sulfide stress and the healthy industry of *L. vannamei*.

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