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RESEARCH REPORT

Effects of the glutathione administration via dietary on intestinal microbiota of white shrimp, *Penaeus vannamei*, under cyclic hypoxia conditions

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Abstract

Environmental stress can impair the survival, growth performance, and intestinal environment of shrimp. The objective of this study was to investigate the effect of glutathione (GSH) on the survival, growth performance, intestinal oxidation, microbiota, and histology of shrimp under hypoxia. Four treatments were used: (1) normoxia, (2) cyclic serious/medium hypoxia (CSMH), (3) CSMH and 75 mg kg^{-1} GSH, and (4) CSMH and 150 mg kg^{-1} GSH. White shrimp (*Penaeus vannamei*) in groups 3 and 4 were fed a commercial diet supplemented with 75 and 150 mg kg^{-1} GSH for a 28 day period, respectively, and they were cultured under CSMH (0.8 – 3.5 mg L^{-1} dissolved oxygen) for the last 14 days of the experiment. *P. vannamei* supplemented with 75 mg kg^{-1} GSH showed significantly improved survival and growth performance under CSMH compared with the CSMH condition alone. The dose of 75 mg kg^{-1} GSH completely eliminated overproduction of reactive oxygen species and malondialdehyde to suppress serious histopathological lesions and improve bacterial diversity and the relative abundance of beneficial bacteria, such as Rhodobacteraceae, thereby preventing pathogen (e.g., *Vibrio*) invasion in the intestine of shrimp under CSMH. However, the dose of 150 mg kg^{-1} GSH was excessive, as it led to serious impairment of survival and growth. These results indicate that 75 mg kg^{-1} GSH has the potential to control shrimp mortality and growth inhibition under CSMH in the shrimp farm setting.

Key Words: *Penaeus vannamei*; glutathione; intestinal microbiota; reactive oxygen species; histology

Introduction

During the course of normal growth of aquatic invertebrates, the intestine and its symbiotic bacteria play a key role in digestion, nutrient supply, and immune function and serve as a barrier between the external environment and internal structures to defend against invading pathogens (Harris, 1993; Garcia-Garcia *et al*., 2013). However, variations in the environment that inevitably occur in aquaculture systems, such as pollution and changes in controlled conditions, may increase the risk of bacterial infection, leading to disease outbreaks (Le and Haffner, 2000; Zhou *et al*., 2009). Previous investigations of shrimp species such as *Homarus gammarus* (Kristensen, 2015), *Neocaridina denticulata* (Cheung *et al*., 2015), and *Penaeus monodon* (Rungrassamee *et al*., 2013) suggested that intestinal microbiota structures could be influenced by environmental, physiological, and

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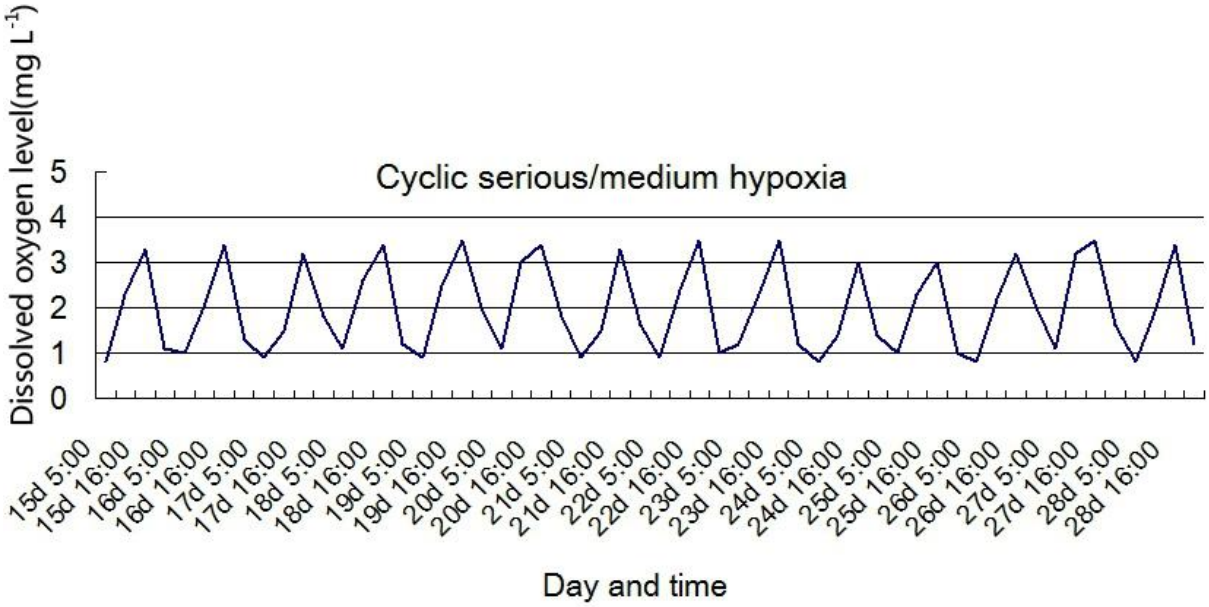

Fig. 1 Monitoring of DO levels during the 28-day experimental period. Experiments were set in order to obtain the lowest DO in the early morning and the highest DO in the afternoon.

nutritional factors. Xiong *et al*. (2015) reported that the dynamic changes of the bacterial community in the intestine of shrimp were closely related to the severity of shrimp disease. These findings suggested that the intestinal microbiota in shrimp interact with environmental stressors, and microbiota homeostasis in the intestine is beneficial for maintaining health and growth of shrimp under complex conditions (Gómez and Balcázar, 2008; Cardona *et al*., 2016).

As an environmental stressor, hypoxia refers to a state of dissolved oxygen (DO) deficiency, and it is observed frequently in mariculture ponds (Diaz and Rosenberg, 2008). Hypoxia is especially critical in rearing ponds that do not use aerators, where shrimp can be exposed to hypoxia as DO levels drop from 3 mg L^{-1} to less than 1 mg L^{-1} due to respiration of organis-ms and decomposition of accumulated organic matter, which can lead to death of the shrimp (Cheng *et al*., 2003; Chantal *et al*., 2008). *Penaeus vannamei* is one of the most important farmed shrimp species in the world (Zeng *et al*., 2013). In a previous study, we created an experimental environment of cyclic serious/medium hypoxia (CSMH, DO 0.8 – 3.5 mg L^{-1}) that simulated the practical shrimp culture conditions, and we found that CSMH led to death, growth impairment, and histopathological lesions in the intestine of *P. vannamei* (Han *et al*., 2018).

When aquatic organisms encounter environmental stress, they are likely to produce elevated levels of reactive oxygen species (ROS), and a series of cellular antioxidant defense mechanism is initiated to control excessive ROS in order to maintain homeostasis (Franzellitti, 2005; Sheikhzadeh *et al*., 2012). The antioxidant defense system consists of enzymatic antioxidants and non-enzymatic small molecules. The multiple enzyme system includes superoxide dismutase, catalase, and glutathione peroxidase, whereas the non-enzymatic system includes glutathione (GSH), vitamins A, C, and E, and ceruloplasmin (Trasviña-Arenas *et al*., 2013). Among these non-enzymatic compounds, the tripeptide GSH is very important for cellular defense against ROS. As a carrier of an active thiol group in the form of a cysteine residue in living organisms, GSH can react directly with ROS in non-enzymatic reactions (Cooper *et al*., 2011).

In this study we investigated (1) survival and growth performance, (2) intestinal microbiota, (3) intestinal ROS and malondialdehyde (MDA) content, and (4) intestinal histology of *P. vannamei* fed with GSH under CSMH with DO ranging from 0.8 to 3.5 mg L^{-1}. The goal of this study was to determine if adding GSH to the shrimp diet is an effective strategy for controlling shrimp death and growth inhibition by protecting the intestinal environment under CSMH.

Materials and Methods

Experimental shrimp

For this study, 1,200 healthy *Penaeus. vannamei* postlarvae of similar size (mean weight 0.26 ± 0.01 g) were obtained from the Ruizi Seafood Development Co. Ltd. (Qingdao, China). Shrimp were placed in 12 cylindrical tanks (640 L) equipped with net covers (N = 100 per tank). Every 640 L cylindrical tank contained 500 L of aerated seawater (DO 6.4 – 7.5 mg L^{-1}). The initial seawater was unfiltered water directly from the ocean with pH 8.0 – 8.4, salinity 30–31 ‰, total ammonia 0.022 – 0.038 mg L^{-1}, nitrite 0.015 – 0.032 mg L^{-1}, and nitrate 0.120 – 0.205 mg L^{-1} at 28 – 32 °C. Shrimp were acclimated for 2 weeks under a natural photoperiod (12 h light: 12 h dark). The shrimp were fed three times daily with a commercial diet (41.52 % crude protein, 7.42 % lipid, and 12.03 % crude ash, supplied by Yantai Dale Feed Co. Ltd, Shandong,

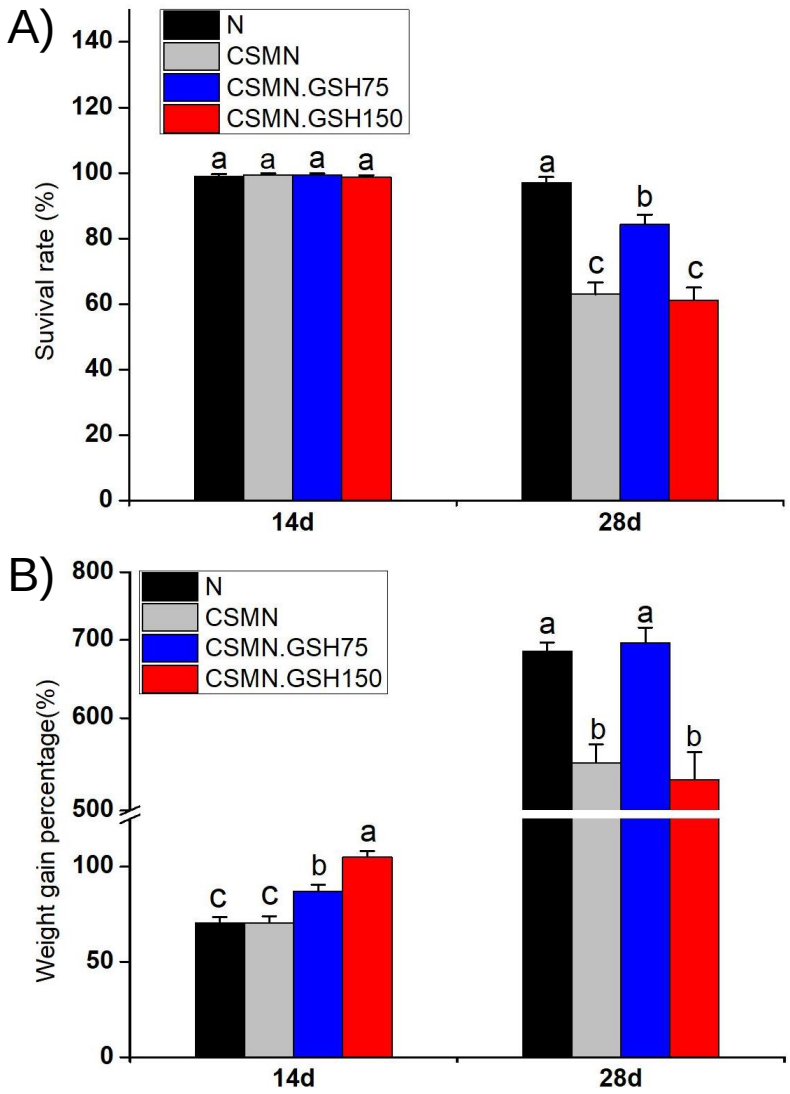

Fig. 2 Survival rate (A) and weight gain percentage (B) of shrimp during the 28-day experimental period. Each bar represents the mean value from three replicates with standard error. Values in the same time period with different letters differ significantly ($p < 0.05$)

China) at 07:00, 11:00, and 17:00; this represented a daily feeding rate that was 10 % of the total weight of shrimp. The unconsumed feed and feces were removed with a siphon tube, and 35 % seawater was replaced once daily. Unfiltered seawater was prepared in three 1000 L cylindrical tanks to use for daily water changes.

Experimental design for GSH test and CSMH challenge

Following acclimation, the 12 cylindrical tanks were randomly divided into four groups. Each group had three cylindrical tanks constituting three replicates: (1) normoxia (N, DO 6.4 – 7.5 mg L^{-1}), (2) CSMH (DO 0.8 – 3.5 mg L^{-1}), (3) CSMH and 75 mg kg^{-1} GSH added to the diet (CSMH.GSH75, DO 0.8 – 3.5 mg L^{-1} + 75 mg kg^{-1} dietary GSH), and (4) CSMH and 150 mg kg^{-1} GSH added to the diet (CSMH.GSH150, DO 0.8 – 3.5 mg L^{-1} + 150 mg kg^{-1} dietary GSH). The experiment lasted for 28-day. The photoperiod, water exchange, and waste disposal were exactly as described for the acclimation period.

Each day, the N and CSMH groups were fed with the commercial diet, whereas the CSMH.GSH75 and CSMH.GSH150 groups were fed the commercial diet supplemented with 75 mg kg^{-1} GSH and 150 mg kg^{-1} GSH, respectively. The GSH test diets were prepared by thoroughly mixing the commercial diet with an aqueous solution of 75 mg kg^{-1} GSH and 150 mg kg^{-1} GSH, respectively, and then thoroughly covering them with egg white. Egg white inhibits the leaching of GSH in seawater, ensuring the accuracy of the experiment. The commercial diet for the N and CSMH groups was also covered with egg white. These diets were air dried, sealed in plastic bags, and stored frozen at −20 °C until used.

Table 1 Sampling depth and diversity indices in the shrimp intestines

Item	N.1	N.2	N.3	CSMH.1	CSMH.2	CSMH.3	CSMH. GSH75.1	CSMH. GSH75.2	CSMH. GSH75.3	CSMH. GSH150.1	CSMH. GSH150.2	CSMH. GSH150.3
Sampling depth												
No. of sequence	40156	38963	39954	44328	41718	39736	41908	41849	41937	43886	41394	41371
OTUs (97 %)	2887	3180	2580	2513	3584	3839	3363	3642	3981	3120	2605	2185
Phylum	648	713	573	574	803	872	780	830	921	722	589	495
Class	647	711	571	570	801	870	775	829	916	718	587	492
Order	635	696	563	549	792	857	759	818	899	691	571	496
Family	611	669	543	501	764	809	686	779	835	633	545	431
Genus	231	258	216	222	292	283	248	266	280	266	230	209
Diversity indices												
Chao1	649.21	714.00	574.00	676.15	811.32	875.00	782.25	832.56	925.00	862.39	614.16	530.96
Ace	653.51	714.00	574.38	676.62	832.92	875.74	786.47	844.95	925.74	844.96	633.19	542.71
Simpson	0.92	0.95	0.92	0.90	0.93	0.92	0.94	0.91	0.93	0.96	0.95	0.91
Shannon	5.18	5.91	4.98	4.72	5.45	5.71	5.67	5.56	5.88	6.02	5.55	4.90

During days 0 – 14 of the experiment, the water in each tank was aerated enough to generate DO 6.4 – 7.5 mg L^{-1} automatically. The daily DO concentration was characterized by the lowest DO in the early morning and the highest DO in the afternoon and by exposure to DO 6.4 – 7.0 mg L^{-1} and 7.0 – 7.5 mg L^{-1} for about 16 and 8 h, respectively, over every 24 h cycle. During days 14 – 28 of the experiment, the water in the N group tanks was still aerated enough to generate DO 6.4 – 7.5 mg L^{-1} automatically. However, the water in the tanks of the other three groups was not aerated enough to generate DO 0.8 – 3.5 mg L^{-1} automatically. Thus, the DO concentration in the three CSMH groups was characterized by the lowest DO in the early morning and the highest DO in the afternoon and by exposure to DO 0.8–2.0 mg L^{-1} and 2.0 – 3.5 mg L^{-1} for about 16 h and 8 h, respectively, over every 24 h cycle. The DO levels were monitored four times a day during the experimental period using a YSI model 55 DO meter (YSI Incorporated, Yellow Springs, OH, USA) (Fig. 1).

Measurement of survival and growth and sampling procedure

The number of dead shrimp in each tank was recorded every 24 h during the experimental period. Shrimp were considered dead when they failed to move even when gently stimulated with a glass pipette. Dead shrimp were removed to prevent fouling. Ten shrimp from each tank were randomly selected on days 14 and 28 during the experimental period, and each shrimp was weighed. Survival and growth were evaluated in terms of survival rate (SR) and weight gain percentage (WGP) based on the following standard formulae: SR (%) = 100(cumulative dead shrimp number)/(initial shrimp number); WGP (%) = 100(final weight–initial weight)/initial weight. Twelve other shrimp were randomly selected and removed from each tank on day 28, and the intestine of each shrimp was collected using sterilized scissors and forceps. Nine intestines were immediately ground in liquid nitrogen; three of them were stored at −80 °C until use in the ROS production and MDA content assay, and six were saved in dry ice and sent to Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China) for DNA extraction and Illumina sequencing. The remaining three intestines were immediately fixed in 10% formaldehyde for histological analysis.

Sequence Analysis

Polymerase chain reaction (PCR) products were generated with the forward primer 338F (5'-ACTCCTACGGGAGGCAGCA-3') and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'), which amplified the V3−V4 regions of the bacterial 16S rRNA gene. PCR amplicons were purified with Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN, USA) and quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). The products of pair-end 2 × 300 bp sequencing were sequenced with the Illumina Miseq platform (Illumina, San Diego, CA, USA). The short reads that overlapped were assembled with FLASH (Edgar, 2013), and the poor-quality assembled reads were filtered with Quantitative Insights Into Microbial Ecology (QIIME) (Wang *et al.*, 2007). In QIIME, poor-quality sequences were defined as sequences with a length of less than 150 bp, average

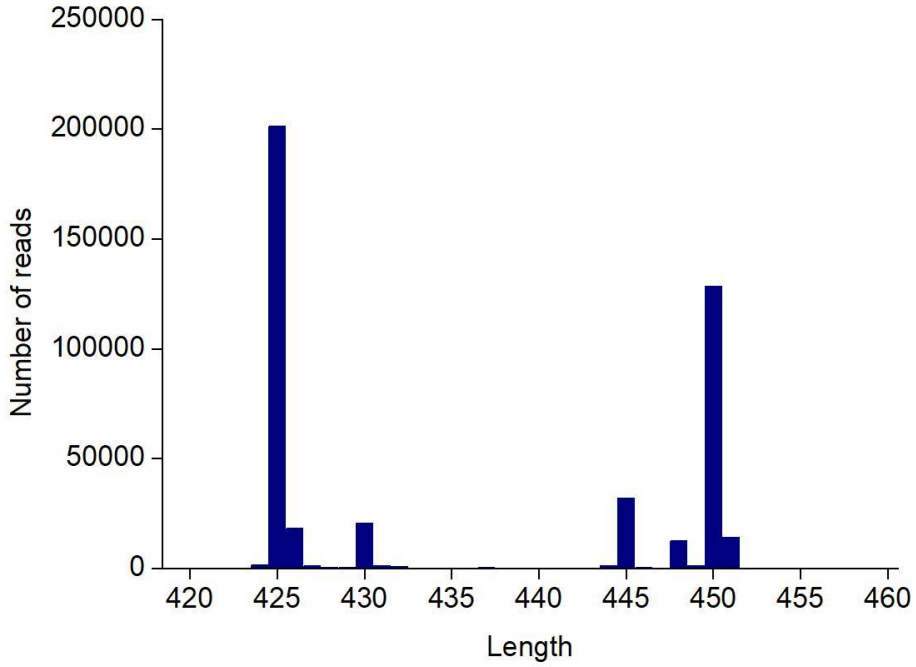

Fig. 3 Length distribution of high quality sequences in the shrimp intestines after the 28-day experiment

Phred scores of less than 20, and containing ambiguous bases and mononucleotide repeats of more than 8 bp. After chimera detection, the remaining high-quality sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity by UCLUST (Edgar, 2010). OTU taxonomic classification ranging from phylum to species levels was conducted using the Greengenes Database (Desantis *et al*., 2006). OTU-level alpha diversity indices, such as the Chao biodiversity index, ACE index, Shannon index, and Simpson index, were calculated for each sample using the OTU table in QIIME. A beta diversity analysis was used to compare the microbial community compositions in the different samples, and results were visualized via nonmetric multidimensional scaling (NMDS) (Ramette, 2007). The taxonomy compositions and abundances were visualized using MEGAN (Huson *et al*., 2011) and GraPhlAn (Asnicar *et al*., 2015).

ROS and MDA content in the intestines

Intestines of three shrimp from each tank were mixed at a 1:5 ratio (w/v) with chilled Tris-hydrochloric acid buffer solution (pH 7.6, 10 mmol L^{-1}) and homogenized under ice-chilled conditions. The homogenate was centrifuged at 10,000 *g* for 10 min at 4 °C, and the supernatant was then collected for sample analysis. ROS content in the intestine was measured using the 2′,7′-dichlorofluorescein diacetate (DCFH-DA) method (Guo *et al*., 2017). The supernatant was incubated with DCFH-DA for 30 min in darkness and then diluted with Modified Alsevier's Solution to a final concentration of 1×10^6 cells mL^{-1}, followed by flow cytometry analysis (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). ROS production was expressed as mean fluorescence of DCF. MDA content was evaluated using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. MDA content was expressed as the relative concentration per milligram of soluble protein (nmol per mg protein). Both ROS production and MDA content were analyzed with three replicates of each sample.

Measurement of intestinal histology

The fixed intestines were dehydrated in an ascending alcohol series (50 % to 95 % concentration). Dehydrated tissues were embedded in paraffin, sectioned into 5 μm thick sections, and stained with hematoxylin and eosin (HE). Sections were then examined with a light microscope (Casado *et al*., 2001).

Statistical analysis

Data for different bacterial community composition in the shrimp intestines are all presented as mean ± standard error (SE). Statistical analysis was performed using SPSS (version 17.0) (IBM, Armonk, NY, USA). One-way analysis of variance and the least significant difference test were used to analyze the differences among the different treatment groups. $p < 0.05$ was considered to be statistically significant. All images were generated with Origin 8.6 software (OriginLab, Northampton, MA, USA).

Results

Survival and growth of shrimp

After the first 14 days of the experiment, all shrimp in the four groups had survived (Fig. 2A). The average WGP values of N, CSMH, CSMH.GSH75,

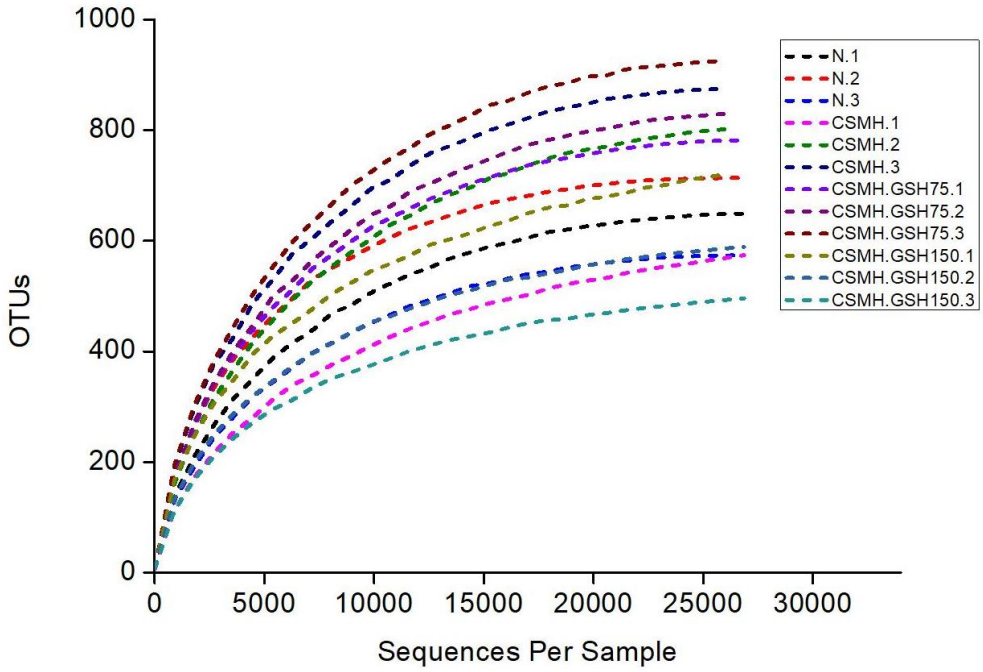

Fig. 4 Rarefaction curves of the bacterial 16S rDNA gene sequences in the shrimp intestines after the 28-day experiment

and CSMH.GSH150 shrimp were 71.26 %, 71.54 %, 83.62 %, and 106.34 %, respectively. The WGP values of N and CSMH shrimp did not differ significantly, but those of CSMH.GSH75 and CSMH.GSH150 shrimp were significantly higher (p < 0.05) than the values for the N and CSMH shrimp. Additionally, the WGP of CSMH.GSH150 shrimp was significantly higher (p < 0.05) than that of CSMH.GSH75 shrimp (Fig. 2B).

At the end of the 28-day experiment, the average SR values of N, CSMH, CSMH.GSH75, and CSMH.GSH150 shrimp were 96.97 %, 63.03 %, 84.24 %, and 61.21 %, respectively. The SR values of the CSMH and CSMH.GSH150 shrimp did not differ significantly, but the values of the N and CSMH.GSH75 shrimp were significantly higher (p < 0.05) compared with those of CSMH and CSMH.GSH150 shrimp. (Fig. 2A). The average WGP values of N, CSMH, CSMH.GSH75, and CSMH.GSH150 shrimp were 684.23 %, 549.23 %, 695.77 %, and 531.54 %, respectively. The WGP values of the N and CSMH.GSH75 shrimp did not differ significantly, and the WGP values of the CSMH and CSMH.GSH150 shrimp did not differ significantly. However, the WGP values of the N and CSMH.GSH75 shrimp were significantly higher (p < 0.05) than those of the CSMH and CSMH.GSH150 shrimp (Fig. 2B).

Overview of 16S rDNA high-throughput sequencing analysis

High-throughput sequencing of the 16S rDNA gene amplicons was performed to determine bacterial diversity in intestines of N, CSMH, CSMH.GSH75, and CSMH.GSH150 shrimp after the 28-day experiment (Table 1). A total of 497,200 reads were obtained from 12 samples with an average read length of 438 bases (Fig. 3). In the rarefaction curves, the number of OTUs almost reached the plateau phase with the increasing read number at 20,000 (Fig. 4), which suggested sufficient sampling depth in all samples. The average number of OTUs was highest in the CSMH.GSH75 group (3662) and lowest in the CSMH.GSH150 group (2637) (Fig. 5).

Relationships among the bacterial communities in the four groups

We used NMDS analysis to investigate the similarity of community structure among different samples. The results of the N group and CSMH.GSH75 groups both showed good repeatability, and they had different bacterial communities. However, the CSMH group and CSMH.GSH150 group had similar bacterial communities (Fig. 6). Heat map was constructed at the genus level based on the clustering analysis of bacterial communities. The result was consistent with the NMDS findings to some extent (Fig. 7).

Bacterial community composition

The composition and abundance of bacterial communities in different samples were investigated according to five classification levels (Table 2). Figure 8A shows the top 20 bacteria at the phylum level. Proteobacteria were the predominant microflora in the four groups, accounting for more than 71 % of the bacterial communities, followed by Actinobacteria and Bacteroidetes. The relative abundances of Bacteroidetes and Verrucomicrobia

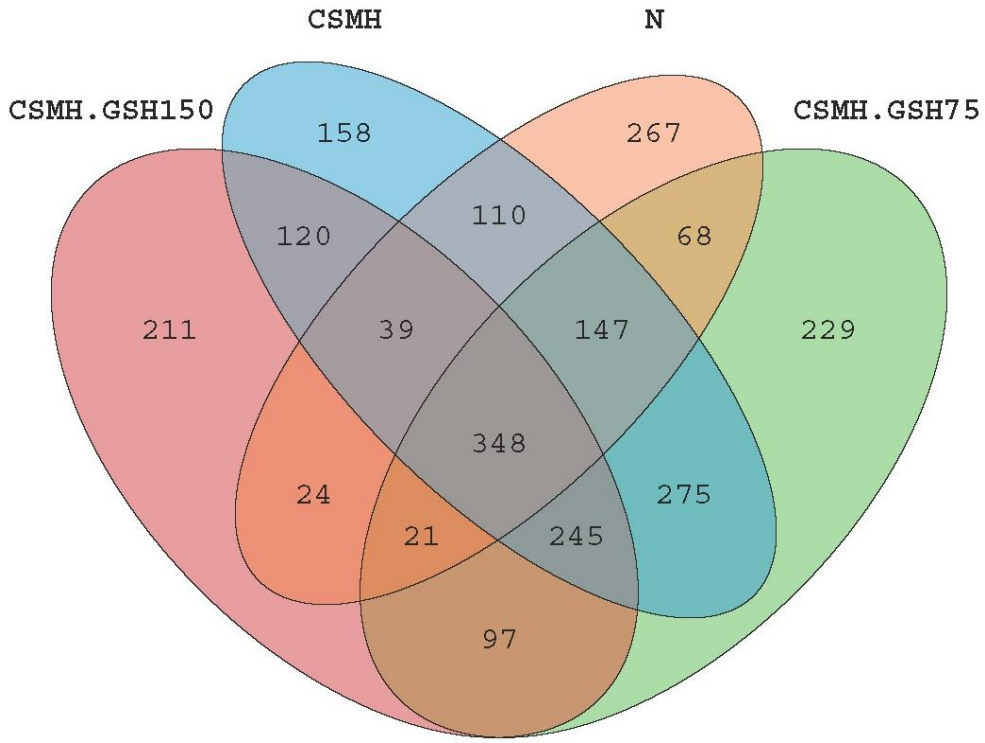

Fig. 5 Venn diagram showing the unique and shared operational taxonomic units (OTUs) in the shrimp intestines after the 28-day experiment

were significantly higher in the N group than in the other three groups (p < 0.05). The relative abundance of Cyanobacteria was highest in the CSMH.GSH150 group, and it was significantly higher than that in the N group (p < 0.05).

At the class level, the relative abundance of Gammaproteobacteria was significantly lower in the CSMH.GSH75 group than in the other three groups (p < 0.05). The relative abundance of Verrucomicrobiae was significantly higher in the N group than in the other three groups (p < 0.05).

In Figure 8B, it is shown that the top 20 bacteria at the order level. The relative abundance of Rhodobacterales was significantly higher in the CSMH.GSH75 group than in the other three groups (p < 0.05). The relative abundance of Vibrionales was highest in the CSMH group, and it was significantly higher than that in the CSMH.GSH75 group (p < 0.05). The relative abundance of Verrucomicrobiales was significantly higher in the N group than in the other three groups (p < 0.05).

At the family level (Fig. 8C), the relative abundance of Rhodobacteraceae was significantly higher in the CSMH.GSH75 group than in the other three groups (p < 0.05). The relative abundances of *Rhodobacteraceae* in the N and CSMH groups were significantly higher than that in the CSMH.GSH150 group (p < 0.05). The relative abundance of Verrucomicrobiaceae was significantly higher in the N group than in the other three groups (p < 0.05). The relative abundance of Vibrionaceae was highest in the CSMH group, and it was significantly higher than that in the N group (p < 0.05). The relative abundance of Streptococcaceae was significantly higher in the CSMH.GSH150 group than in the other three groups (p < 0.05).

At the genus level, the relative abundance of *Streptococcus* was significantly higher in the CSMH.GSH150 group than in the other three groups (p < 0. 05).

ROS production and MDA content of the intestines

At the end of the 28-day experiment, ROS production and MDA content in the intestines of CSMH and CSMH.GSH150 shrimp were significantly higher (p < 0.05) than values in the N and CSMH.GSH75 shrimp. Additionally, ROS production and MDA content in the intestines of CSMH.GSH150 shrimp were significantly lower (p < 0.05) compared with values in the CSMH shrimp. (Fig. 9A, B).

Histology assays of the intestines

After the 28-day experiment, the intestinal villi and the lamina propria of N shrimp were arranged regularly. Compared with N shrimp, intestinal villi were exfoliated completely and the lamina propria was cleaved in the CSMH shrimp. The intestinal villi length appeared shorter and the intestinal villi connection appeared to be twisted in the CSMH.GSH75 shrimp, whereas intestinal villi were exfoliated and the lamina propria was scattered in the CSMH.GSH150 shrimp (Fig. 10).

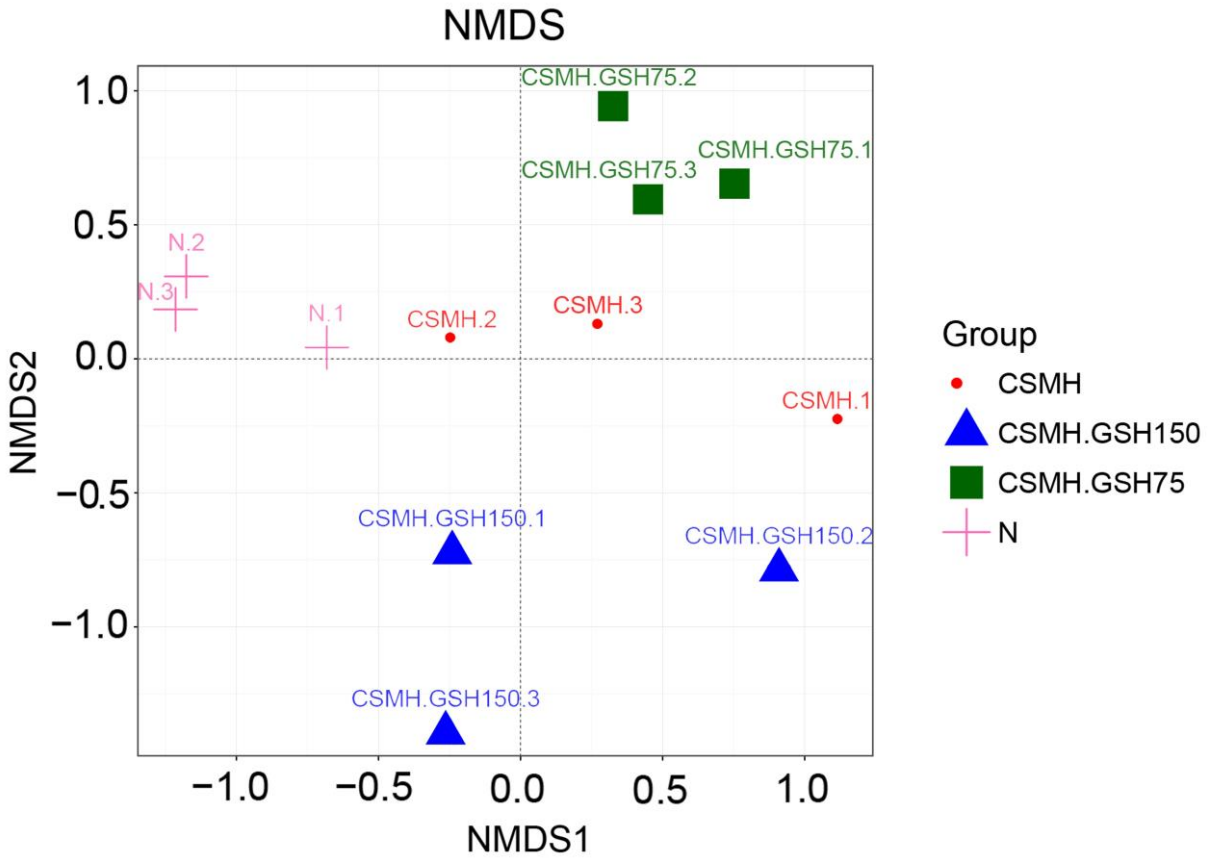

Fig. 6 Non-metric multidimensional scaling (NMDS) showing bacterial community differences in the shrimp intestines after the 28-day experiment

Discussion

Intestinal bacteria in shrimp play important roles in maintaining the health of the host, such as promoting digestion and inhibiting growth of pathogenic bacteria (Ringø *et al*., 2003; Round and Mazmanian, 2009; Clemente *et al*., 2012). Previous research indicated that alteration of intestinal microbiota of *P. vannamei* under high or low salinity was likely attributed to pathogenic bacteria, leading to poorer growth (Zhang *et al*., 2016). Decreased bacterial diversity and anti-stress bacteria in the intestine of *P. vannamei* also promoted pathogenic bacteria invasion under exposure to increasing concentrations of sulfide, leading to slower growth (Suo *et al*., 2017). In the present study, we found that CSMH induced impairment of survival and growth performance of *P. vannamei* and that the addition of 75 mg kg^{-1} GSH to the diet significantly improved survival and growth of shrimp under CSMH. Thus, we speculated that both CSMH and GSH addition affected bacterial diversity and microbiota structures in the intestine of the shrimp.

Proteobacteria, Bacteroidetes, and Actinobacteria were predominately distributed in the intestine of *P. vannamei* under different environmental conditions, such as normal (Huang *et al*., 2016), hyposaline, hypersaline (Zhang *et al*., 2016), and high-concentration sulfides (Suo *et al*., 2017). Similarly, these phyla accounted for the majority of intestinal bacteria in the four groups tested in the present study. Bacteroidetes may play a specialized role in biopolymer degradation and uptake of micromolecular organics (Kirchman, 2002; Williams *et al*., 2013). However, CSMH significantly reduced the relative abundance of Bacteroidetes in the intestine of *P. vannamei* in our study, as was also reported for *P. vannamei* and Nile tilapia (*Oreochromis niloticus*) facing hyposaline or hypersaline stress (Zhang *et al*., 2016). Cardman *et al*. (2014) reported that Verrucomicrobiaceae were capable of degrading polysaccharides in aquatic animals, but we found that CSMH significantly reduced the relative abundance of Verrucomicrobiaceae at the phylum, class, order, and family level in *P. vannamei*. The diversity indices in the present study indicated that CSMH could reduce bacterial diversity in the intestine of *P. vannamei*. Thus, we surmised that CSMH reduced bacterial diversity and the beneficial bacteria community, which might have suppressed digestion and uptake in the intestine of shrimp, thereby impairing survival and growth performance.

Prebiotics, probiotics, and synbiotics are reported to improve health status of fish and shellfish through modulation of the gastrointestinal

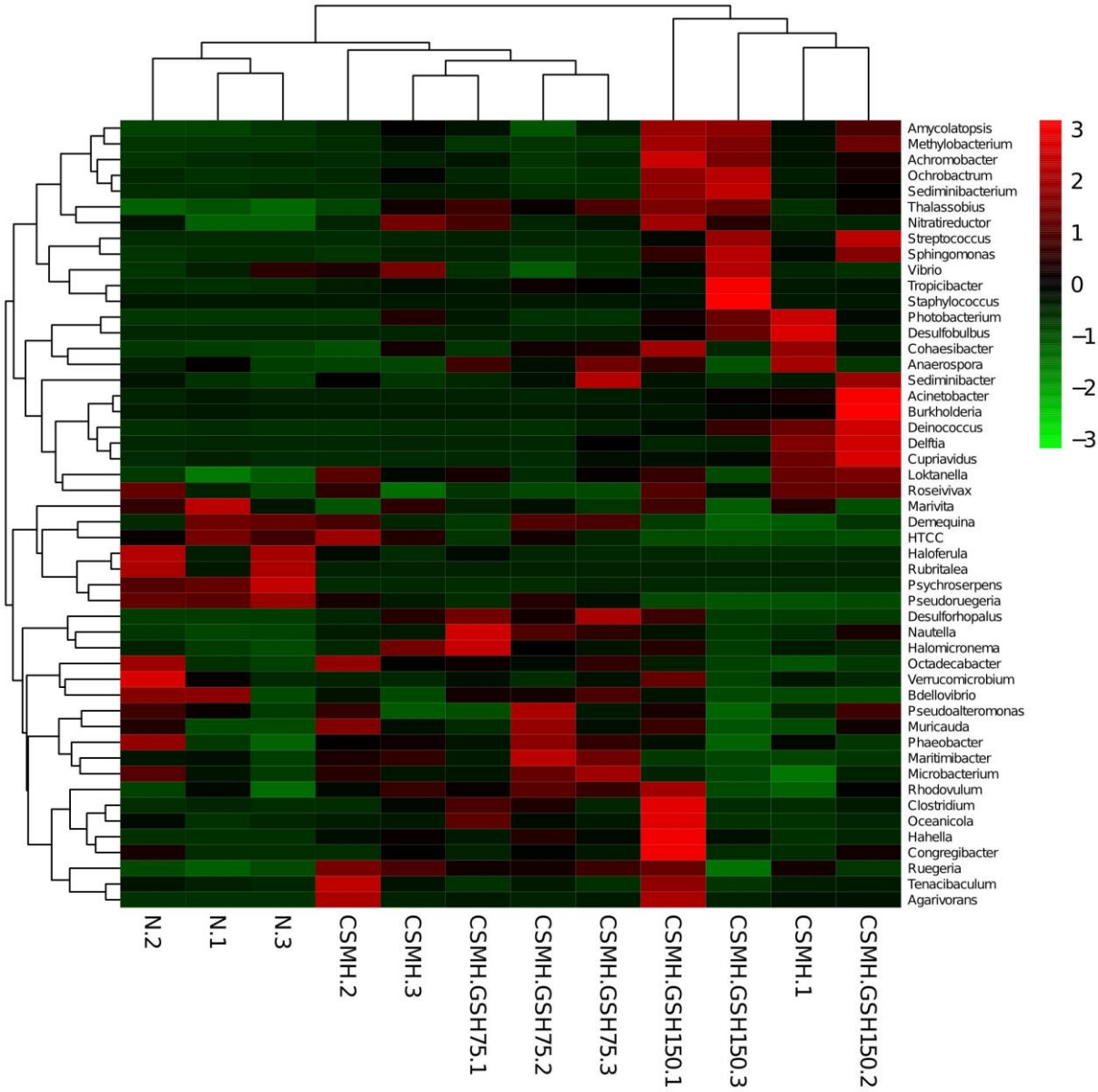

Fig. 7 Heat map showing bacterial community differences in the shrimp intestines after the 28-day experiment

tract microbiome, thus reducing the incidence of diseases in aquaculture (Merrifield *et al*., 2014; Hoseinifar *et al*., 2016; Sha *et al*., 2016). In the present study, the diversity indices indicated that dietary GSH addition could improve bacterial diversity in the intestine of *P. vannamei* under CSMH. In particular, CSMH.GSH75 shrimp had the highest bacterial diversity. Cardona *et al*. (2016) reported that the Rhodobacteraceae could have limited the survival of pathogenic bacteria in *Litopenaeus stylirostris* reared with biofloc technology. This finding supports the premise that addition of 75 mg kg^{-1} GSH to the diet could significantly improve the relative abundance of Rhodobacteraceae at the order and family levels in the intestine of *P. vannamei* under CSMH. Thus, we propose that adding 75 mg kg^{-1} GSH to the diet improved bacterial diversity and the beneficial bacteria community, which might have helped the shrimp resist pathogenic bacteria in the intestine under CSMH, thereby enhancing survival and growth performance.

Generally, bacteria in the class Gammaproteobacteria are more adaptable to oligotrophic marine environments, and previous studies reported that the relative abundance of members of this class in *P. vannamei* was higher in the diseased samples than that in the healthy samples (Bowman and McCuaig, 2003; Zheng *et al*., 2016). In the present study, CSMH significantly increased the relative abundance of Gammaproteobacteria in the intestine of *P. vannamei*,

Table 2 Bacterial community composition in the shrimp intestines from different groups

Classification	N	CSMH	CSMH.GSH75	CSMH.GSH150
Phylum				
Proteobacteria	71.57 ± 4.80^{b}	81.17 ± 3.56^{a}	76.27 ± 6.08^{ab}	75.27 ± 4.23^{ab}
Actinobacteria	4.73 ± 0.90^{b}	9.47 ± 5.75^{ab}	16.23 ± 6.23^{a}	4.03 ± 2.31^{b}
Bacteroidetes	15.97 ± 2.74^{a}	5.30 ± 3.67^{b}	2.70 ± 1.70^{b}	7.70 ± 3.21^{b}
Tenericutes	1.8 ± 1.15^{b}	1.83 ± 0.65^{b}	1.23 ± 0.58^{b}	6.7 ± 1.95^{a}
Firmicutes	0.03 ± 0.06^{b}	1.07 ± 1.19^{b}	1.8 ± 1.67^{b}	4.43 ± 1.68^{a}
Verrucomicrobia	5.33 ± 4.11^{a}	0.43 ± 0.49^{b}	0.57 ± 0.46^{b}	0.23 ± 0.06^{b}
Cyanobacteria	0.07 ± 0.06^{b}	0.17 ± 0.15^{ab}	0.3 ± 0.26^{ab}	0.57 ± 0.31^{a}
Class				
Alphaproteobacteria	44.60 ± 4.08^{b}	45.37 ± 3.91^{b}	56.00 ± 7.33^{a}	37.13 ± 2.30^{b}
Gammaproteobacteria	26.8 ± 0.82^{a}	30.57 ± 3.70^{a}	15.63 ± 2.21^{b}	30.30 ± 8.34^{a}
Flavobacteria	15.77 ± 2.727^{a}	4.837 ± 3.70^{b}	1.93 ± 1.21^{b}	6.93 ± 2.87^{b}
Mollicutes	1.8 ± 1.15^{b}	1.83 ± 0.65^{b}	1.23 ± 0.58^{b}	6.70 ± 1.95^{a}
Verrucomicrobiae	5.33 ± 4.11^{a}	0.40 ± 0.52^{b}	0.53 ± 0.49^{b}	0.23 ± 0.06^{b}
Bacilli	0 ± 0^{b}	0.2 ± 0.26^{b}	0.17 ± 0.12^{b}	3.77 ± 2.57^{a}
Order				
Rhodobacterales	44.13 ± 4.23^{b}	44.1 ± 4.57^{b}	55.3 ± 7.56^{a}	31.1 ± 3.52^{c}
Vibrionales	16.57 ± 2.37^{ab}	25.63 ± 4.01^{a}	13.23 ± 1.06^{b}	24.43 ± 11.18^{ab}
Flavobacteriales	15.77 ± 2.72^{a}	4.83 ± 3.70^{b}	1.93 ± 1.21^{b}	6.93 ± 2.87^{b}
Alteromonadales	10.03 ± 3.07^{a}	4.2 ± 4.92^{b}	2.1 ± 1.13^{b}	2 ± 1.39^{b}
Verrucomicrobiales	5.33 ± 4.11^{a}	0.4 ± 0.52^{b}	0.53 ± 0.49^{b}	0.23 ± 0.06^{b}
Rhizobiales	0.27 ± 0.12^{b}	0.83 ± 0.40^{b}	0.47 ± 0.15^{b}	2.93 ± 1.07^{a}
Family				
Rhodobacteraceae	44.13 ± 4.23^{b}	44.1 ± 4.57^{b}	55.3 ± 7.56^{a}	31.1 ± 3.52^{c}
Flavobacteriaceae	15.33 ± 3.02^{a}	4.23 ± 4.03^{b}	1.77 ± 1.20^{b}	3.3 ± 4.19^{b}
Vibrionaceae	1.2 ± 0.35^{b}	7 ± 4.36^{a}	1.93 ± 0.35^{ab}	6 ± 3.29^{ab}
Verrucomicrobiaceae	5.33 ± 4.11^{a}	0.4 ± 0.52^{b}	0.53 ± 0.49^{b}	0.23 ± 0.06^{b}
Streptococcaceae	0 ± 0^{b}	0.2 ± 0.26^{b}	0.17 ± 0.12^{b}	3.33 ± 2.31^{a}
Genus				
Psychroserpens	7.93 ± 2.76^{a}	0.23 ± 0.06^{b}	0.2 ± 0.26^{b}	0.1 ± 0.17^{b}
Nautella	0.43 ± 0.15^{b}	1.1 ± 0.17^{b}	4.17 ± 1.80^{a}	1.43 ± 0.85^{b}
Haloferula	4.07 ± 3.09^{a}	0.37 ± 0.55^{b}	0.50 ± 0.44^{b}	0.13 ± 0.12^{b}
Streptococcus	0 ± 0^{b}	0.20 ± 0.26^{b}	0.17 ± 0.12^{b}	3.33 ± 2.31^{a}

but the addition of 75 mg kg^{-1} GSH to the diet significantly suppressed the relative abundance of this group under CSMH. Numerous studies have reported that *Vibrio*, which is a major pathogenic bacteria affecting shrimp, caused stressed shrimp to be susceptible to infectious diseases (Vandenberghe *et al*., 1999; Austin and Zhang, 2006). In our study, CSMH significantly increased the relative abundance of Vibrionaceae in the intestine of *P. vannamei*, but adding 75 mg kg^{-1} GSH to the diet significantly suppressed the relative abundance of Vibrionales under CSMH. Therefore, we suggest that the CSMH environment might have been oligotrophic, which created a suitable environment for opportunistic pathogenic bacteria, which in turn impaired survival and growth performance of shrimp. However, adding 75 mg kg^{-1} GSH to the diet improved the beneficial bacteria

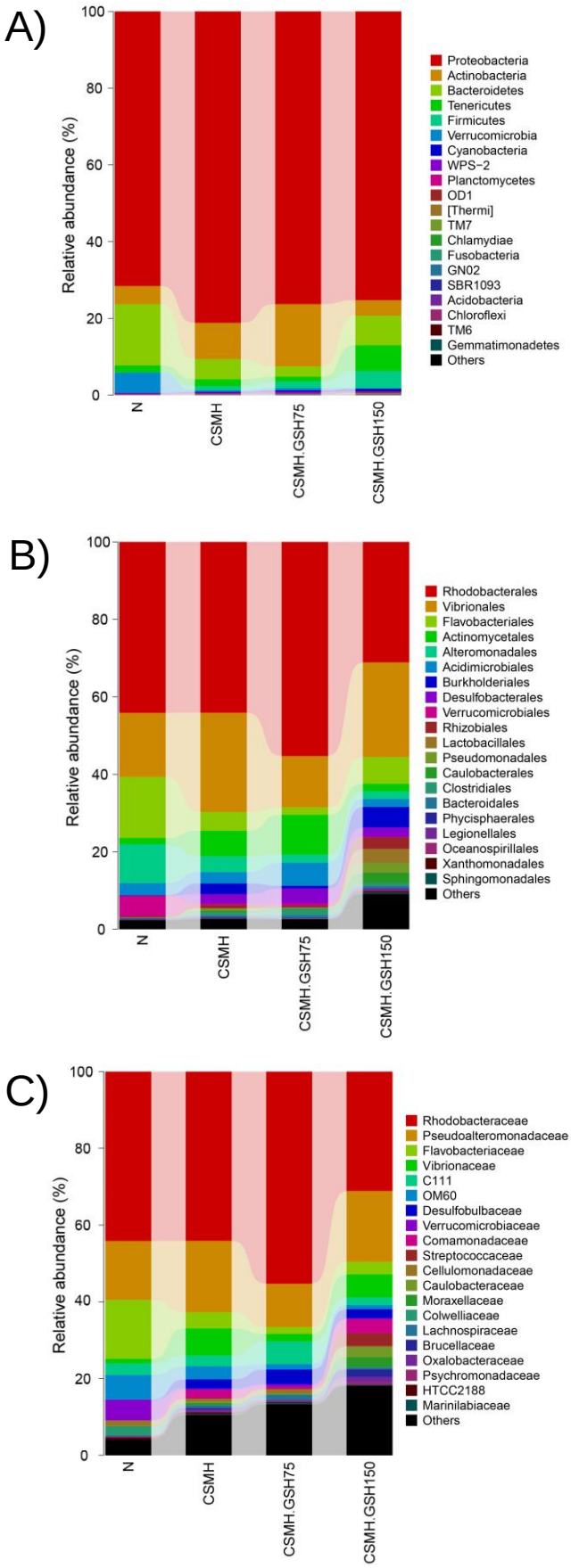

Fig. 8 Bacterial community compositions at phylum (A), order (B), and family (C) levels in the shrimp intestines after the 28-day experiment

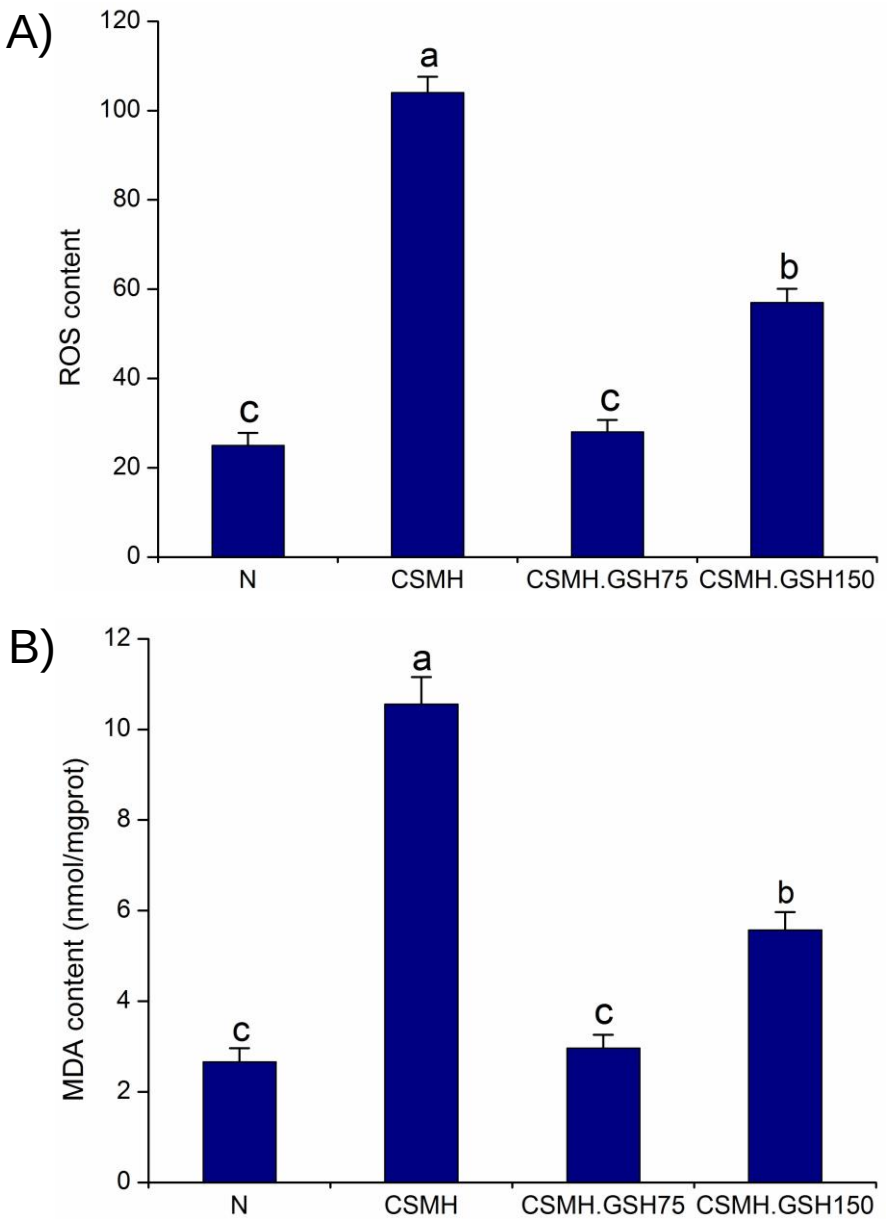

Fig. 9 ROS content (A) and MDA content (B) in the shrimp intestines after the 28-day experiment. Each bar represents the mean value from three replicates with standard error. Values with different letters differ significantly ($p < 0.05$)

community, which prevented pathogen invasion in the intestine of shrimp under CSMH and enhanced survival and growth performance.

ROS, including superoxide anion, hydroxyl radical, and hydrogen peroxide, can damage important biomolecules, such as DNA, proteins, and lipids (Halliwell and Gutteridge, 1999). As the main component of lipid peroxides, MDA has strong biotoxicity and can damage tissue structure and function (Freeman and Crapo, 1982). At the tissue and cellular levels, environmental stresses are likely to produce elevated levels of ROS (Lushchak, 2011). In the present study, CSMH induced overproduction of ROS and MDA in the intestine of *P. vannamei*, leading to serious histopathological lesions. Han *et al*. (2018) found similar results when shrimp were exposed to low or high pH as environmental stressors. Additionally, Qi *et al*. (2017) reported that histopathological lesions in the intestine of *P. vannamei* could promote pathogen invasion due to impaired function of the intestinal barrier. In our study, CSMH reduced the beneficial bacteria community and improved that of pathogenic bacteria in the intestine of *P. vannamei*. Thus, we propose that excessive ROS was the main reason why CSMH promoted pathogen invasion. However, addition of 75 mg kg^{-1} GSH to the diet completely eliminated excessive ROS and MDA to suppress

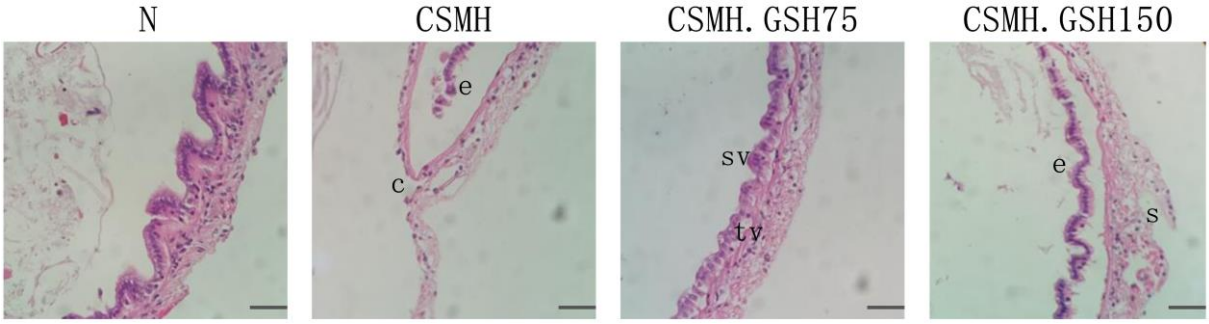

Fig. 10 Photomicrographs of the shrimp intestines after the 28-day experiment. The pathologies observed were: dilatation (d); vacuoles (v); shorter intestinal villi (sv); twisting intestinal villi (tv); exfoliation (e); cleavage (c); scatter(s). Scale bar = 100 μm.

serious histopathological lesions, preventing pathogen invasion under CSMH.

Although GSH plays an important role in scavenging ROS, too much GSH can be a problem when it combines with a variety of compounds, such as aldehydes and alkenyl halide, as these toxic metabolites covalently combine with DNA and produce ROS or tumors (Monks *et al*., 1990; Thomas *et al*., 1998). In the present study, 150 mg kg^{-1} GSH, compared with 75 mg kg^{-1} GSH, induced significant overproduction of ROS and MDA in the intestine of *P. vannamei* under CSMH, leading to serious histopathological lesions. Jia *et al*. (2016) reported that a low abundance of Cyanobacteria was beneficial to aquatic animal growth, but other studies showed that a high abundance of Cyanobacteria produced hepatotoxic microcystins and cytotoxic lipopeptides, which can cause cell necrosis (Howard, 2012; Kang 2012). In our study, 150 mg kg^{-1} GSH significantly increased the relative abundance of Cyanobacteria in the intestine of *P. vannamei* under CSMH compared with normoxia. *Streptococci* are the cause of an emerging disease in penaeid shrimp in the Americas and in regions of east Africa (Hasson *et al*., 2009; Lightner *et al*., 2009). No *Streptococci* were detected in the intestine of *P. vannamei* under normoxia, but the addition of 150 mg kg^{-1} GSH to the diet significantly increased the relative abundance of *Streptococci* at the family and genus level under CSMH. Thus, 150 mg kg^{-1} GSH was excessive supplementation for *P. vannamei* under CSMH, as it induced excessive ROS to promote pathogen invasion, leading to serious survival and growth impairment.

Conclusions

This study was the first investigation of the effect of GSH on the survival, growth performance, intestinal microbiota, oxidation, and histology of *P. vannamei* under CSMH. Dietary *P. vannamei* supplementation with 75 mg kg^{-1} GSH completely eliminated overproduction of ROS and MDA to suppress serious histopathological lesions and improved bacterial diversity and the relative abundance of beneficial bacteria community such as Rhodobacteraceae, which prevented pathogen (e.g., *Vibrio*) invasion in the intestine of shrimp under CSMH and enhanced survival and growth performance. However, supplementation with 150 mg kg^{-1} GSH under CSMH was excessive and led to serious impairment of survival and growth. Therefore, adding 75 mg kg^{-1} GSH to the diet could improve the health status of shrimp experiencing CSMH by protecting the intestinal environment. This supplementation dose could be used to control shrimp mortality and growth inhibition under CSMH in the shrimp farm setting.

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References

Asnicar F, Weingart G, Tickle TL, Huttenhower C, Segata N. Compact graphical representation of phylogenetic data and metadata with GraPhlAn. Peer J. 3: e1029, 2015.

Austin B, Zhang X. *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates. Lett. Appl. Microbiol. 43: 119, 2006.

Bowman JP, McCuaig RD. Biodiversity, community structural shifts, and biogeography of prokaryotes within antarctic continental shelf sediment. Appl. Environ. Microb. 69: 2463-2483, 2003.

Cardona E, Gueguen Y, Magré K, Lorgeoux B, Piquemal D, Pierrat F, *et al*. Bacterial community characterization of water and intestine of the shrimp *Litopenaeus stylirostris* in a biofloc system. BMC Microbial. 16: 157, 2016.

Cardman Z. Arnosti C, Durbin A, Ziervogel K, Cox C, Steen AD, *et al*. *Verrucomicrobia* are candidates for polysaccharide-degrading bacterioplankton in an arctic fjord of Svalbard. Appl. Environ. Microb. 80: 3749-3756, 2014.

Casado JM, Theumer M, Masih DT, Chulze S, Rubinstein HR. Experimental subchronic mycotoxicoses in mice: individual and combined effects of dietary exposure to fumonisins and aflatoxin b1. Food Chem. Toxicol. 39: 579-86, 2001.

Cheng W, Liu CH, Kuo CM. Effects of dissolved oxygen on hemolymph parameters of freshwater giant prawn, *macrobrachium rosenbergii* (de man). Aquaculture 220: 843-856, 2003.

Cheung MK, Yip HY, Nong W, Law PTW, Chu KH, Kwan HS, *et al*. Rapid change of microbiota diversity in the gut but not the hepatopancreas during gonadal development of the new shrimp model *Neocaridina denticulate*. Mar. biotechnol. 17: 811-819, 2015.

Chantal M, Etienne Z, Cyrille G, Hugues L. Combined effect of exposure to ammonia and hypoxia on the blue shrimp *Litopenaeus stylirostris* survival and physiological response in relation to molt stage. Aquaculture 274: 398-407, 2008.

Clemente JC. Ursell LK, Parfrey LW, Knight R. The impact of the gut microbiota on human health: an integrative view. Cell 148: 1258-1270, 2012.

Cooper AJL, Pinto JT, Callery PS. Reversible and irreversible protein glutathionylation: biological and clinical aspects. Expert Opin. Drug Metab. Toxicol. 7: 891-910, 2011.

Diaz RJ. Rosenberg R. Spreading dead zones and consequences for marine ecosystems. Science 321: 926-929, 2008.

Desantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, *et al*. Greengenes, a chimera-checked 16s rrna gene database and workbench compatible with arb. Appl. Environ. Microb. 72: 5069-5072, 2006.

Edgar RC. Uparse: highly accurate otu sequences from microbial amplicon reads. Nat. Methods 10: 996, 2013.

Edgar RC. Search and clustering orders of magnitude faster than blast. Bioinformatics 26: 2460, 2010.

Franzellitti SFE. Differential hsp70 gene expression in the *mediterranean mussel* exposed to various stressors. Biochem. Biophys. Res. Commun. 336: 1157-1163, 2005.

Freeman BA. Crapo JD. Biology of disease: free radicals and tissue injury. Lab. Investig. 47: 412-426, 1982.

Garcia-Garcia E, Galindo-Villegas J. Mulero V. Mucosal immunity in the gut: the non-vertebrate perspective. Dev. Comp. Immunol. 40: 278, 2013.

Gómez GD, Balcázar JL. A review on the interactions between gut microbiota and innate immunity of fish. Fems Immunol. Med. Mic. 52: 145, 2008.

Guo H, Li K, Wang W, Wang C, Shen Y. Effects of copper on hemocyte apoptosis, ROS production, and gene expression in white shrimp *Litopenaeus vannamei*. Biol. Trace Elem. Res. 179: 318-326, 2017.

Harris JM. The presence, nature, and role of gut microflora in aquatic invertebrates: A synthesis. Microb. Ecol. 25: 195-231, 1993.

Halliwell B. Gutteridge JMC. Antioxidant defenses. In Free Radicals in Biology and Medicine. Oxford Univ. Press, New York, 1999.

Han SY, Wang BJ, Liu M, Wang MQ, Jiang KY, Liu XW, *et al*. Adaptation of the white shrimp *Litopenaeus vannamei* to gradual changes to a low-pH environment. Ecotox. Environ. Safe. 149: 203-210, 2018.

Han SY, Wang MQ, Liu M, Wang BJ, Jiang KY, Wang L. Comparative sensitivity of the hepatopancreas and midgut in the white shrimp *Litopenaeus vannamei*, to oxidative stress under cyclic serious/medium hypoxia. Aquaculture 49: 44-52, 2018.

Hasson KW, Wyld EM, Fan Y, Lingsweiller SW, Weaver SJ, Cheng J, *et al*. *Streptococcosis* in farmed *Litopenaeus vannamei*: a new emerging bacterial disease of penaeid shrimp. Dis. Aquat. Organ. 86: 93-106, 2009.

Hoseinifar SH, Ringø E, Shenavar Masouleh A, Esteban MÁ. Probiotic, prebiotic and synbiotic supplements in sturgeon aquaculture: a review. Rev. Aquacult. 8: 89-102, 2016.

Huson DH, Mitra S, Ruscheweyh HJ, Weber N, Schuster SC. Integrative analysis of environmental sequences using MEGAN4, Genome Res. 21: 1552-1560, 2011.

Huang Z, Li X, Wang L, Shao Z. Changes in the intestinal bacterial community during the growth of white shrimp, *Litopenaeus vannamei*. Aquac. Res. 47: 1737-1746, 2016.

Howard A. Cyanobacteria (Blue-green Algae). Encyclopedia of Lakes and Reservoirs 174-175, 2012.

Jia J, Chen Q, Lauridsen TL. A systematic investigation into the environmental fate of microcystins and the potential risk: study in lake Taihu. Toxins 8: 170, 2016.

Kristensen E. Temporal development of the gut microbiota in European lobster (*Homarus gammarus*) juveniles exposed to two different water treatment systems (Master's thesis, NTNU), 2015.

Kirchman DL. The ecology of Cytophaga–Flavobacteria in aquatic environments. Fems Microbial. Ecol. 39: 91-100, 2002.

Kang HS. Minutissamides E-L, antiproliferative cyclic lipodecapeptides from the cultured freshwater cyanobacterium cf. *Anabaena* sp, Bioorgan. Med. Chem. 20: 6134, 2012.

Le MG, Haffner P. Environmental factors affecting immune responses in crustacea. Aquaculture 191: 121-131, 2000.

Lightner DV, Redman RM, Pantoja CR, Navarro SA, Tang-Nelson KFJ, Noble BL, *et al*. Emerging non-viral infectious and noninfectious diseases of farmed penaeid shrimp and other decapods. The rising tide: proceedings of the special session on sustainable shrimp farming. 46-52, 2009.

Lushchak VI. Environmentally induced oxidative stress in aquatic Animals. Aquat. Toxicol. 101: 13-30, 2011.

Merrifield DL, Balcázar JL, Daniels C, Zhou Z, Carnevali O, Sun YZ, *et al*. Indigenous lactic acid bacteria in fish and crustaceans. Aquacult. Nutr. 128-168, 2014.

Monks TJ, Anders MW, Dekant W, Stevens JL, Lau SS, Bladeren PJV. Glutathione conjugate mediated toxicities. Toxicol. Appl. Pharm. 106:1-19, 1990.

Qi C, Wang L, Liu M, Jiang K, Wang M, Zhao W, *et al*. Transcriptomic and morphological analyses of *Litopenaeus vannamei* intestinal barrier in response to *Vibrio paraheamolyticus* infection reveals immune response signatures and structural disruption. Fish Shellfish Immun. 70: 437-450, 2017.

Ramette A. Multivariate analyses in microbial ecology. Fems Microbial. Ecol. 62: 142-160, 2007.

Ringø E, Olsen RE, Mayhew TM, Myklebust R. Electron microscopy of the intestinal microflora of fish. Aquaculture 227: 395-415, 2003.

Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. Nat. Rev. Immunol. 9: 313, 2009.

Rungrassamee W, Klanchui A, Chaiyapechara S, Maibunkaew S, Tangphatsornruang S, Jiravanichpaisal P, *et al*. Bacterial population in intestines of the black tiger shrimp (*Penaeus monodon*) under different growth stages. PloS one 8: e60802, 2013.

Sha Y, Liu M, Wang B, Jiang K, Qi C, Wang L, Bacterial population in intestines of *Litopenaeus vannamei* fed different probiotics or probiotic supernatant, J. Microbiol. Biotechnol. 26: 1736-1745, 2016.

Sheikhzadeh N, Tayefi-Nasrabadi H, Oushani AK, Enferadi MH. Effects of *Haematococcus pluvialis* supplementation on antioxidant system and metabolism in rainbow trout (*Oncorhynchus mykiss*). Fish Physiol. Biochem. 38: 413-419, 2012.

Suo Y, Li E, Li T, Jia Y, Qin JG, Gu Z, *et al*. Response of gut health and microbiota to sulfide exposure in pacific white shrimp *Litopenaeus vannamei*. Fish Shellfish Immun. 63: 87-96, 2017.

Trasviña-Arenas CH, Garcia-Triana A, Peregrino-Uriarte AB, Yepiz-Plascencia G. White shrimp *Litopenaeus vannamei* catalase: gene structure, expression and activity under hypoxia and reoxygenation. Comp. Biochem. Physiol. B 164 44-52, 2013.

Thomas S, Lowe JE, Hadjivassiliou V, Knowles RG, Green IC, Green MHL. Use of the comet assay to investigate the role of superoxide in glutathione-induced DNA damage. Biochem. Bioph. Res. Co. 243: 241-245, 1998.

Vandenberghe J, Verdonck L, Robles-Arozarena R, Rivera G, Bolland A, Balladares M, *et al*. Vibrios associated with *litopenaeus vannamei* larvae, postlarvae, broodstock, and hatchery probionts. Appl. Environ. Microb. 65: 2592, 1999.

Wang Q, Garrity GM, Tiedje JM, Cole JR. Naïve Bayesian classifier for rapid assignment of RNA sequences into the new bacterial taxonomy. Appl. Environ. Microb. 73: 5261, 2007.

Williams TJ, Wilkins D, Long E, Evans F, DeMaere MZ, Raftery MJ, *et al*. The role of planktonic Flavobacteria in processing algal organic matter in coastal East Antarctica revealed using metagenomics and metaproteomics, Environ. Microbiol. 15: 1302-1317, 2013.

Xiong J, Wang K, Wu J, Qiuqian L, Yang K, Qian Y, *et al*. Changes in intestinal bacterial communities are closely associated with shrimp disease severity, Appl. Microbial. Biot. 99: 6911-6919, 2015.

Zeng D, Chen X, Xie D, Zhao Y, Yang C, Li Y, *et al*. Transcriptome analysis of Pacific white shrimp (*Litopenaeus vannamei*) hepatopancreas in response to Taura syndrome Virus (TSV) experimental infection, PloS one 8 e57515, 2013.

Zhang M, Sun Y, Liu Y, Qiao F, Chen L, Liu WT, *et al*. Response of gut microbiota to salinity change in two euryhaline aquatic animals with reverse salinity preference. Aquaculture 454: 72-80, 2016.

Zheng Y, Yu M, Liu Y, Su Y, Xu T, Yu M, *et al*. Comparison of cultivable bacterial communities associated with pacific white shrimp (*Litopenaeus vannamei*) larvae at different health statuses and growth stages. Aquaculture 451: 163-169, 2016.

Zhou J, Wang WN, Wang AL, He WY, Zhou QT, Yuan L, *et al*. Glutathione s-transferase in the white shrimp *Litopenaeus vannamei*: characterization and regulation under pH stress. Comp. Biochem. Physiol. C 150: 224, 2009.