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Beneficial effects of dietary β-glucan on growth and health status of Pacific white shrimp *Litopenaeus vannamei* at low salinity

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ABSTRACT

An 8-week trial was conducted to evaluate the effect of dietary β-glucan supplement (0, 0.01%, 0.02%, or 0.04%) on growth and health of Pacific white shrimp *Litopenaeus vannamei* at low salinity of 3 practical salinity unit (psu). The *L. vannamei* fed 0.02% and 0.04% β-glucan gained more weight and showed higher activities of protease, amylase, superoxide dismutase, and glutathione peroxidase in the intestine than in the control (0% β-glucan). The *L. vannamei* fed 0.04% β-glucan had a higher condition factor than those fed the control diet. Amylase activity in the hepatopancreas of *L. vannamei* fed 0.02% β-glucan was higher than those fed the control diet. Dietary β-glucan supplement increased the mRNA expressions of Toll-like receptor, myostatin, immune deficiency or heat shock protein 70, but decreased the mRNA expressions of tumor necrosis factor-α and C-type lectin 3 in both hepatopancreas and intestine. The response of intestine microbiota in *L. vannamei* fed 0.04% β-glucan was further compared to the control. The 0.04% β-glucan supplement reduced richness and diversity of the intestinal microbial community as indicated by the low values of Chao1 estimator, ACE estimator, Simpson index and Shannon diversity index. Abundances of *Bacillus*, *Chitinibacter*, *Geobacillus* and *Vibrio* in the intestine increased, while *Flavobacterium*, *Microbacterium* and *Mycobacterium* decreased significantly in *L. vannamei* fed 0.04% β-glucan compared to the control. This study indicates that dietary β-glucan supplement at 0.02%–0.04% can significantly improve digestibility, antioxidant capacity and immunity in *L. vannamei*, and thus improve growth performance and survival at low salinity. These beneficial effects of β-glucan probably are related to the dominance of probiotics over potential pathogens in the intestine.

1. Introduction

The Pacific white shrimp *Litopenaeus vannamei* is a euryhaline species and can tolerate a wide range of salinity from 0.5 to 50 practical salinity unit (psu) [1,2]. Low salinity cultivation (< 5 psμ) of *L. vannamei* has been used in many countries in the world. Previous research has shown that low salinity can cause metabolic disorder, and reduce growth, survival, and immune defense ability, which seriously affect the economic benefits and sustainable development of inland farming of *L. vannamei* at low salinity [3,4]. Therefore, the method toward overcoming poor growth performance of white shrimp at low salinity has become a research focus.

Aquaculture feed not only provides energy and nutrients to animals, but also improves performance by adding active substances to regulate physiological metabolism. Nutritional regulation through diet ingredients is an effective method to reduce negative effects of variable environmental factors on organisms [1,5,6]. β-Glucans are the major compositions of various nutritional diets, which is ubiquitous in fungi, yeast, oats, and seaweed [7]. It possesses various health-promoting properties such as anticancer, antioxidant, anti-inflammatory and immune-modulating effects, and is widely used in pharmaceutics, nutrition, health protection and animal feed [8,9]. Existing research indicates that β-glucan can improve growth performance and enhance immune function in a variety of aquatic animals, including Nile tilapia, zebrafish and *Pagrus major* [10–12]. During a 30-day feeding trial, *L. vannamei* fed 0.2% β-glucan showed higher survival and respiratory burst values than those fed the basal diet, suggesting that dietary supplementation of β-glucan has beneficial effects in improving shrimp

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performance at low salinity (5 ppt) [13]. Pompano fish *Trachinotus ovatus* fed 0.05%–0.20% β-glucan can tolerate low salinity stress while *L. vannamei* fed 0.035% β-glucan can increase haemolymph glucose and osmolarity significantly [14,15]. These results suggested that β-glucan can relieve the low-salinity stress in *L. vannamei.* Meanwhile, the intestinal microbiota also plays a critical role in the maintenance of gut homeostasis and host health. But the proportion of bacteria regarded as opportunists increased while those regarded as beneficial bacteria decreased when facing hyposaline stress in *L. vannamei* [16]. These alterations of intestinal microbiota may cause adverse effects on the host. Recent research has indicated that β-glucan supplementation can change gut microbiota significantly [17]. Much of the research of β-glucan on gut microbiota were focused on mammals, such as human and rats, which indicated that β-glucan can enhanced the growth of probiotic strains, such as *Lactobacilli* and *Bifidobacteria* [18,19]. And the gut microbiota helps to digest the nondigestible β-glucans into short-chain fatty acids (SCFA) with biological activity [20]. SCFA is beneficial to reduce the intestinal pH, and thereby help in competitive exclusion of pathogens [21]. In another study, the levels of SCFA were significantly increased in the rats fed oats [22]. In aquatic animals, dietary β-glucan supplementation could alter the network interactions among different microbial functional groups by changing the microbial community composition and topological roles of the OTUs in the ecological network [23]. Furthermore, *Scophthalmus maximus* fed dietary with β-glucan obtained a faster maturation of the larval compared to the control [24].

The hepatopancreas and intestine are the major components of the digestive system in shrimp. The hepatopancreas is the most important detoxification organ, and the intestine is an efficient immune organ [25]. The occurrence of a variety of diseases is associated with the health status of the hepatopancreas and intestines. Previous studies have shown that *L. vannamei* fed β-glucan could improve the digestive enzyme of hepatopancreas and intestinal villi in the distal part [14,26]. And *L. vannamei* fed dietary β-1, 3-glucan could significantly increase the activities of superoxide dismutase (SOD), lysozyme and respiratory burst (RB) in the hepatopancreas [27]. However, as these studies were done under normal salinity range in a farming condition, there is a need to test the effect dietary β-glucan on *L. vannamei* at low salinity.

In this study, the effects of β-glucan on the growth performance, proximate composition, digestive enzymes activity, antioxidant capacity, immunity and intestinal microbiota of *L. vannamei* were investigated at 3 psμ. Results indicate that proper dietary β-glucan (0.02%–0.04%) can significantly improve digestibility, antioxidant capacity and immunity. These beneficial effects can provide a reference for β-glucan supplement in aquatic animal cultivation at low salinity or other environmental stress.

2. Materials and methods

2.1. Experimental diets

According to the existing research, the *L. vannamei* diet was formulated to adapt to the low salinity environment [28]. Soybean meal and fish meal were used as protein, fish oil, soybean oil, cholesterol and lecithin as lipid, wheat starch as carbohydrate. The ratio of carbohydrate to sugar was 35:20. β-glucan was purchased from Swiss abac Research and Development Co., Ltd. and extracted from yeast cell walls. β-glucan was proved to be (1, 3) - (1, 6) - β-glucan with 88% purity. Ingredient and proximate composition of experimental diets are given in Table 1. β-glucan was added to a basal diet at four concentrations (0, 0.01%, 0.02% and 0.04% dry diets). Raw materials were crushed by a pulverizer and sieved through an 80-mesh and then mixed evenly before being extruded into 2-mm-diameter pellets in a double helix plodder (F-26, SCUT, industrial factory, Guangdong, China). The wet pellets were dried by a fan at room temperature until moisture was less than 10% and then stored at −20 °C until use.

Table 1
Ingredient formulation (g/kg dry basis) and proximate composition (%) of the four experimental diets fed to *L. vannamei*.

Ingredients	Diets (g kg $^{-1}$)			
	Control	0.01%	0.02%	0.04%
Fish meal	370	370	370	370
Soybean meal	280	280	280	280
Wheat starch	200	200	200	200
Fish oil	22.8	22.8	22.8	22.8
Soybean oil	25	25	25	25
Lecithin	10	10	10	10
Cholesterol	5	5	5	5
Vitamin Premix[a]	20	20	20	20
Mineral Premix[b]	5	5	5	5
Vitamin C	1	1	1	1
Sodium Carboxymethyl cellulose	30	30	30	30
Cellulose	11.2	11.1	11.0	10.8
Calcium carbonate	20	20	20	20
β-glucan[c]	0	0.1	0.2	0.4
Total	1000	1000	1000	1000
Analyzed proximate composition				
Crude protein	353	349	351	355
Crude lipid	88.3	87.4	88.9	87.9
Ash	118.1	117.5	119.4	118.7
Moisture	9.2	8.9	8.7	9.0

[a] Vitamin premix, diluted in cellulose, provided the following vitamins (g kg^{-1} premix): thiamin HCl 0.5; riboflavin 3.0; pyridoxine HCl 1.0; DL-calcium pantothenate 5.0; nicotinic acid 5.0; biotin 0.05; folic acid 0.18; vitamin B12 0.002; choline chloride 100.0; inositol 5.0; menadione 2.0; vitamin A acetate (20 000 IU g^{-1}) 5.0; vitamin D3 (400 000 IU g^{-1}) 0.002; DL-alpha-tocopheryl acetate (250 IU g^{-1}) 8.0; α-cellulose 865.266.

[b] Trace mineral premix provided the following minerals (g 100 g^{-1} premix): cobalt chloride 0.004; cupric sulphate pentahydrate 0.250; ferrous sulphate 4.0; magnesium sulphate heptahydrate 28.398; manganous sulphate monohydrate 0.650; potassium iodide 0.067; sodium selenite 0.010; zinc sulphate heptahydrate 13.193; sodium dihydrogen phosphate 15; filler 38.428.

[c] β-glucan was extracted from yeast cell wall and purchased from Swiss abac Research and Development Co., Ltd.

2.2. Experimental design, management procedure

Shrimps were obtained from a shrimp hatchery center in Danzhou, Hainan, China. *L. vannamei* were acclimated for two weeks prior to the experiment. During the acclimation period, the salinity was reduced to 3 psμ at a daily rate of ~2 psμ by adding freshwater.

After acclimation, 420 shrimps (0.020 ± 0.005 g) were randomly assigned into four groups: 0% (control), 0.01%, 0.02%, 0.04% of β-glucan in three replicates in 12 tanks (110 × 80 × 40 cm) with 35 shrimps each. Seawater was added to fresh water to adjust the salinity to 3 psμ. After thorough aeration, 1/3 of the tank water was exchanged per day. During the whole experiment, culture water was maintained at 24.5–30.0 °C, pH 7.5–8.0, dissolved oxygen ≥7 mg/L and total ammonia content < 0.05 mg/L. Shrimps were fed three times daily at 7:00, 12:00 and 20:00 h. Feeding ration was adjusted according to the response of feeding on the previous day.

2.3. Sampling

At the end of 8 weeks of the feeding trial, shrimps in each tank were counted to calculate survival. After 24-h fasting, the shrimps in each tank were weighed and then they were randomly measured for body length and body weight to determine weight gain (WG) and condition factor (CF). Growth indicators were calculated based on the following formulae:

Weight gain (WG, %) = (final weight – initial weight) / initial weight × 100

Survival (%) = (final number of shrimp / initial number of shrimp) × 100

Feed conversion ratio (FCR) = total feed intake / total wet gain

Condition factor (CF, g cm^{-3}) = final weight / $length^3$ × 100

Five shrimps from each tank were preserved at −20 °C to determine body proximate composition. The whole intestine of five shrimps from each tank were aseptically dissected and the intestinal contents from one tank were mixed as one sample for microbiota analysis. The other shrimps were anesthetized in ice bath and the hepatopancreas and intestines were dissected and stored at −80 °C for biochemical assays and gene expression analysis.

2.4. Body composition analysis and biochemical assays

The composition of whole shrimp was analyzed according to the standard method of AOAC (AOAC, 1995) [29]. The moisture was measured by drying at 105 °C to a constant weight (DZF-6050, Jinghong, Ltd., Shanghai, China) and the ash was measured by theb 550 °C muffle furnace burning method (PCD-E3000 Serials, Peaks, Japan). The crude protein and crude lipid were calculated by the Kjeldahl method (Kjeltec8100, FOSS, Sweden) and the Soxhlet extraction method respectively.

Activities of protease, amylase and lipase were analyzed by ELISA kits (Shanghai Hengyuan Biotechnology Co, Ltd). The phenol oxidase (POX) ELISA kit, malondialdehyde (MDA) assay kit (TBA method), total superoxide dismutase (T-SOD) assay kit (Hydroxylamine method), catalase (CAT) assay kit (visible light), glutathione peroxidase (GSH-PX) assay kit (colorimetric method) were purchased from Nanjing Jiancheng Bioengineering Institute. The test was carried out according to the instructions of assay kits.

2.5. RNA extraction, synthesis of cDNA, and real-time PCR analysis

Total RNA was extracted from the hepatopancreas and intestine of *L. vannamei* using Trizol reagent (RN0101, Aidlab, China). The optical density (260/280) of total RNA ranged between 1.80 and 2.00 by a Nano Drop 2000 spectrophotometer (Thermo, Wilmington, USA). The integrity of RNA was detected by 1.5% agarose electrophoresis. The total RNA was reversely transcribed to cDNA using the PrimeScript ™ RT reagent kit (RR047A, Takara, Japan) for real time-PCR (RT-PCR). RT-PCR was implemented using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Richmond, CA). β-actin was used as the housekeeping gene. The primers for β-actin, heat shock protein 70 (HSP70), myostatin (MSTN), tumor necrosis factor-α (TNF-α), C-type lectin 3 (CTL3), Toll and immune deficiency (IMD) gene were obtained from previous studies [30–33]. The reactions were carried out in a final volume of 10 μL including 5 μL of 2 × UltraSYBR mixture, 0.4 μL of 10 mM gene-specific forward and reverse primers, 1 μL of cDNA template, and 3.6 μL of RNase-free H_2O. The cycling parameters involved an activation of 95 °C for 10 min, 40 cycles (95 °C for 15 s, 60 °C for 1 min to anneal), and 95 °C for 5 s. Melting curve analysis was then created from 65 to 95 °C, and increased by 0.5 °C per 0.05 s. The mRNA relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

2.6. Illumina high-throughput sequencing of barcoded 16S rRNA genes

The gut microbiota of shrimp fed 0.04% β-glucan was compared to the control. Total bacterial community DNA was isolated with an E.Z.N.A.™ soil DNA kit according to the manufacturer's instructions. DNA yield was measured using a NanoDrop spectrophotometer (Thermo, Wilmington, USA). DNA quality was assessed by PCR amplification of the bacterial 16S rRNA genes. Bacterial DNA was used as the template for 16S rRNA gene V4–V5 region amplification [34]. Unique eight-base barcodes were added to each primer to distinguish the PCR products. The 2% agarose gel was used to detected PCR products to ascertain that the target bands of V4–V5 area are all amplified. In this study, each purified PCR product was then submitted to Illumina-based high-throughput sequencing.

2.7. Bioinformatics and statistical analysis

Raw Fastq file reads were analyzed and quality-filtered using the QIIME (version 1.8.0, http://qiime.org/). USEARCH (v5.2.236, http://www.drive5.com/usearch/) was used to check and remove the chimera sequence through the QIIME software. Operational taxonomic units (OTUs) were clustered with a threshold of 97% similarity using UPARSE via QIIME. Taxonomic richness estimators and community diversity were determined for each library in Mothur. Alpha diversity indexes, including Chao1, ACE, Shannon and Simpson, were used for evaluating community diversity. All these indices were estimated based on OUT abundance matrices.

All data were reported as the mean ± standard error (mean ± S.E). The variance in growth performance, enzyme activity and gene mRNA expression among groups were tested by one-way ANOVA using SPSS statistics 23 (IBM, Armonk, NY, USA) followed by Duncan's multiple rang test. Significant differences of alpha diversity indexes between the control and shrimp fed 0.04% dietary β-glucan were determined by using Student's t tests. $P < 0.05$ was considered to be statistically significant. Using GraPhlAn Construct the grade tree for the composition of the sample population at each classification level. The proportion of common and unique OTU for each group is presented by Venn Graph. The abundance of classification unit degree and the similarity of community composition between samples were shown by heatmap. The effect of classification model was tested by partial least squares discriminant analysis (PLS-DA). At the genus levels, the significant difference in species richness between the control and shrimp fed 0.04% dietary β-glucan was calculated by Metastats [35].

3. Results

3.1. Growth performance and body composition

Compared with the control, *L. vannamei* fed 0.02% or 0.04% β-glucan showed significantly higher WG and lower FCR ($P < 0.05$,

Table 2
Growth performance and morphological parameter of *L. vannamei* fed diets with different β-glucan at 3 psμ for 8 weeks.

	β-glucan (%)			
	0 (Control)	0.01	0.02	0.04
Weight gain (%)	11922.50 ± 398.57^{b}	10290.73 ± 393.98^{b}	16273.32 ± 1581.63^{a}	17647.14 ± 515.87^{a}
Survival (%)	91.43 ± 1.65	76.19 ± 5.04	83.81 ± 6.67	89.52 ± 4.15
Feed conversion ratio	2.00 ± 0.08^{a}	2.03 ± 0.05^{a}	1.75 ± 0.06^{b}	1.70 ± 0.06^{b}
Condition factor (%)	0.96 ± 0.04^{b}	1.01 ± 0.01^{ab}	0.99 ± 0.01^{ab}	1.03 ± 0.01^{a}

Values of weight gain, survival and feed conversion ratio are mean of three tanks. Values of condition factor are means of ten determinations of ten shrimp per tank and three tanks per treatment.Different superscript letters indicate significant differences between the same row ($P < 0.05$) for *L. vannamei*.

Table 3
Proximate composition of *L. vannamei* fed diets with different β-glucan at 3 psμ for 8 weeks.
Wet weight ratio (%).

Parameters	β-glucan (%)			
	0 (Control)	0.01	0.02	0.04
Ash	2.86 ± 0.19	2.91 ± 0.18	3.28 ± 0.13	3.07 ± 0.21
Crude protein	14.27 ± 0.14^{c}	14.41 ± 0.03^{bc}	14.71 ± 0.17^{ab}	14.92 ± 0.10^{a}
Crude lipid	2.73 ± 0.11	2.62 ± 0.14	2.75 ± 0.07	2.52 ± 0.13
Moisture	77.10 ± 0.56	76.84 ± 1.05	77.08 ± 0.52	77.66 ± 0.32

Values are means of two determinations of pooled samples of five shrimp per tank and three tanks per treatment. Different superscript letters indicate significant differences between the same row ($P < 0.05$) for *L. vannamei*.

Table 2). The CF of *L. vannamei* fed 0.04% dietary β-glucan was significantly higher than that in the control ($P < 0.05$). No significant difference was observed in survival among all the groups ($P > 0.05$, Table 2).

Whole body proximate crude protein contents increased with the increase of dietary β-glucan, and significantly higher values were found in *L. vannamei* fed 0.02% and 0.04% β-glucan than the control ($P < 0.05$, Table 3). No significant difference was found in crude lipid, moisture and ash contents among all treatments.

3.2. Digestive enzymes activity in hepatopancreas and intestine

Compared with the control, amylase activity in the hepatopancreas of *L. vannamei* fed 0.02% dietary β-glucan was significantly higher ($P < 0.05$). Protease and amylase activities in the intestine of *L. vannamei* fed 0.04% dietary β-glucan were significantly higher than in the control ($P < 0.05$, Table 4). *L. vannamei* fed 0.04% β-glucan showed significantly lower lipase activity than that of *L. vannamei* fed 0.02% β-glucan ($P < 0.05$), but there was no significant difference when compared to the control (Table 4).

3.3. Antioxidant capacity and immune related parameters in hepatopancreas

The MDA contents in hepatopancreas decreased while the CAT activities increased with the increase of dietary β-glucan (Table 5). SOD, GSH-PX and POX activities in the hepatopancreas of *L. vannamei* fed 0.02% and 0.04% β-glucan were higher than those in the control ($P < 0.05$) (Table 5).

3.4. mRNA expressions of immune related genes

In the hepatopancreas (Fig. 1-A), HSP70 and MSTN mRNA expressions were significantly higher in *L. vannamei* fed 0.02% β-glucan than those in the control ($P < 0.05$), and *L. vannamei* fed 0.04% dietary β-glucan obtained significantly higher Toll mRNA expression compared with the control ($P < 0.05$). Lower mRNA expressions of TNF-α and CTL3 were found in the hepatopancreas of *L. vannamei* fed 0.02% and 0.04% dietary β-glucan ($P < 0.05$). In the intestine (Fig. 1-B), *L. vannamei* fed 0.02% β-glucan showed significantly higher Toll, IMD, and MSTN mRNA expressions and significantly lower expressions of TNF-α, CTL3 compared to the control ($P < 0.05$). *L. vannamei* fed 0.04% β-glucan also showed lower mRNA expression of CTL3 ($P < 0.05$). There was no significant difference in IMD mRNA expression in the hepatopancreas and HSP70 in the intestine ($P > 0.05$).

3.5. Intestinal microbiota analysis

3.5.1. Intestinal microbiota richness and diversity analysis

In this study, the DNA fragments were sequenced by the Illumina Miseq platform. The total number of sequences was 314 429, with an average of 52 405 sequences per sample. The obtained sequences were divided to OTU with a threshold of 97% similarity.

Chao 1 and ACE analysis for bacterial richness varied from 954 to 1065 and 984 to 1111, respectively (Table 6). To estimate and compare the bacterial diversity between the control and *L. vannamei* fed 0.04% β-glucan, bacterial diversity was estimated by Shannon and Simpson index, which varied from 0.94 to 0.96 and 6.79 to 7.31 in two groups, respectively (Table 6). Compared with the control, *L. vannamei* fed 0.04% dietary β-glucan had lower Chao1, ACE, Simpson and Shannon index (Table 6).

With 97% sequence similarity as the OUT partition threshold, the control and *L. vannamei* fed 0.04% dietary β-glucan shared 870 OTUs, accounting for 47.34% and 50.84% of the total number of OTUs, respectively (Fig. 2). Compared with the control, 841 OTUs were different in *L. vannamei* fed 0.04% β-glucan.

3.5.2. Bacterial community analysis and comparison

As shown in Fig. 3, the points in the control were relatively closer, and one point in the *L. vannamei* fed 0.04% dietary β-glucan was farther than the other two points. There was no overlap between two groups, which was far apart, indicating that the classification model in this study was reliable.

In this study, the abundance of top 50 genera was clustered and the heatmap analysis showed that *Algoriphagus*, *Mycobacterium*, *Pseudoxanthomonas*, *Flavobacterium*, and *Haloferula* were more

Table 4
Activities of protease, amylase and lipase in hepatopancreas and intestines of *L. vannamei* fed diets with different β-glucan at 3 psμ for 8 weeks.

Organ	Parameters	β-glucan (%)			
		0 (Control)	0.01	0.02	0.04
Hepatopancreas	Protease (IU mg protein^{-1})	0.175 ± 0.022	0.188 ± 0.029	0.209 ± 0.037	0.198 ± 0.019
	Amylase (U mg protein^{-1})	0.402 ± 0.030^{b}	0.438 ± 0.040^{b}	0.554 ± 0.033^{a}	0.467 ± 0.038^{ab}
	Lipase (mU mg protein^{-1})	0.0066 ± 0.0005^{ab}	0.0068 ± 0.0007^{ab}	0.0070 ± 0.0002^{a}	0.0053 ± 0.0005^{b}
Intestine	Protease (IU mg protein^{-1})	0.151 ± 0.001^{b}	0.162 ± 0.007^{b}	0.186 ± 0.033^{ab}	0.245 ± 0.032^{a}
	Amylase (U mg protein^{-1})	0.408 ± 0.019^{b}	0.423 ± 0.039^{b}	0.454 ± 0.016^{ab}	0.530 ± 0.038^{a}
	Lipase (mU mg protein^{-1})	0.0065 ± 0.0008	0.0056 ± 0.0006	0.0057 ± 0.0004	0.0060 ± 0.0008

Values are means of two determinations of two shrimp per tank and three tanks per treatment. Different superscript letters indicate significant differences between the same row ($P < 0.05$) for *L. vannamei*.

Table 5
Effects of β-glucan supplementation on the activities of enzymes about antioxidant and immune in the hepatopancreas of *L. vannamei* fed diets with different β-glucan contents at 3 psμ for 8 weeks.

Parameters	β-glucan (%)			
	0 (Control)	0.01	0.02	0.04
MDA (nmol mg protein^{-1})	2.06 ± 0.49	2.03 ± 0.25	1.75 ± 0.21	1.50 ± 0.19
CAT (U mg protein^{-1})	4.75 ± 0.92	4.78 ± 1.08	5.74 ± 0.79	6.17 ± 1.21
SOD (U mg protein^{-1})	18.68 ± 0.94^{b}	18.75 ± 0.79^{b}	22.33 ± 0.92^{a}	23.81 ± 1.36^{a}
GSH-PX (U mg protein^{-1})	65.31 ± 3.22^{b}	67.04 ± 4.89^{b}	102.07 ± 15.26^{a}	97.71 ± 5.47^{a}
POX (ng mg protein^{-1})	2.24 ± 0.10^{b}	2.52 ± 0.16^{ab}	3.61 ± 0.55^{a}	3.53 ± 0.51^{a}

MDA: malondialdehyde; CAT: catalase; SOD: superoxide dismutase; GSH-PX: glutathione peroxidase; POX: phenol oxidase. Values are means of two determinations of two shrimp per tank and three tanks per treatment. Different superscript letters indicate significant differences between the same row ($P < 0.05$) for *L. vannamei*.

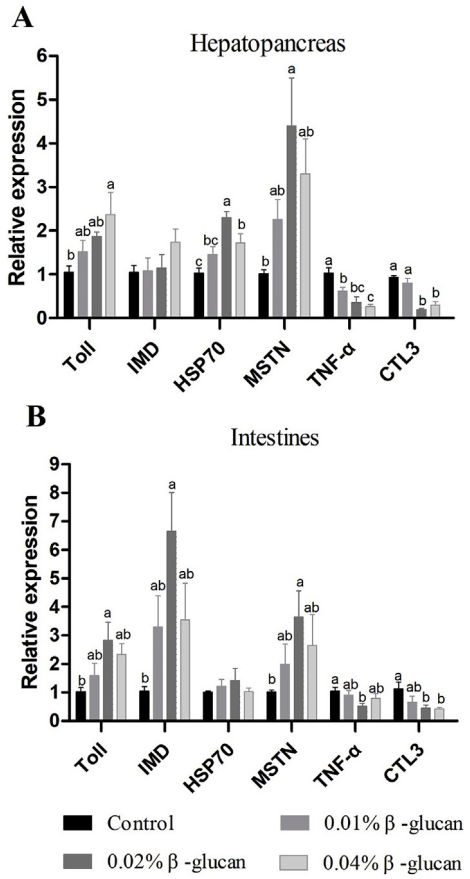

Fig. 1. Relative mRNA expressions of immune-related genes in hepatopancreas (A) and intestine (B) of *L. vannamei* fed diets with different β-glucan contents at 3 psμ for 8 weeks. Different superscript letters indicate significant differences in the same genes ($P < 0.05$) between different β-glucan supplements in *L.vannamei*.

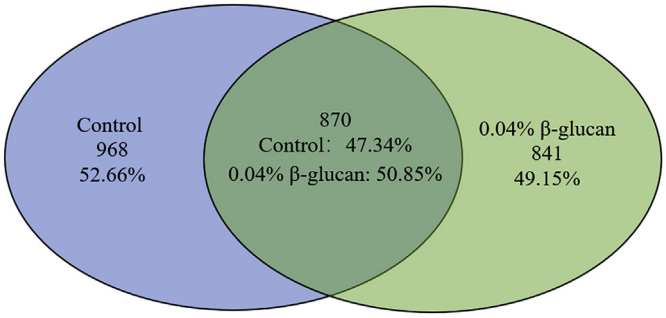

Fig. 2. Venn diagrams showing the distribution of OTUs of the control and *L.vannamei* fed 0.04% dietary β-glucan. Note: OTU numbers are generated from the subsets of each group. The percentage indicates the ratio of correlated OTUs in the total sequences of each group.

abundant in the control than those in *L. vannamei* fed 0.04% dietary β-glucan. The abundances of *Chitinibacter* and *Bacillus* were higher in *L. vannamei* fed β-glucan than control (Fig. 4). Compared with the control, the reduced bacteria of *L. vannamei* fed 0.04% dietary β-glucan were mainly *Proteobacteria*, and the increased bacteria were mainly *Firmicutes*.

In the intestine, 13 bacterial phyla were detected with the major of sequences belong to the *Proteobacteria* (varied from 38.9% to 72.6%), and five were with the abundance of > 1% of the total sequences (Fig. 5-A). For these five phyla, *Proteobacteria* was the most dominant member followed by *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia* and *Firmicutes*. No significant difference was found in the relative abundance of bacteria at the phylum level.

There were significant differences in the 16 bacteria at the genus level (Fig. 5-B). Among these genera, the abundances of *Algoriphagus*, *Flavobacterium*, *Haloferula*, *Microbacterium*, *Mycobacterium*, *Pseudoxanthomonas* and *Salinibacterium* were significantly decreased in *L. vannamei* fed 0.04% dietary β-glucan compared with those in the control ($P < 0.05$). The abundances of *Aminobacter*, *Bacillus*, *Brachybacterium*, *Brevundimonas*, *Chitinibacter*, *Geobacillus*, *Phenylobacterium*, *Sphingopyxis* and *Vibrio* increased significantly in *L. vannamei* fed β-glucan compared to the control ($P < 0.05$).

Table 6
Summary of Illumina high-throughput bacterial diversity richness (OTU), diversity index (Shannon & Simpson) and estimated OUT richness (Chao 1 & ACE) for intestinal bacterial diversity analysis of *L. vannamei* fed diets with different β-glucan at 3 psμ for 8 weeks.

	Richness estimate		Diversity estimators	
	Chao1	ACE	Simpson	Shannon
Control	1065.37 ± 316.80	1111.19 ± 355.73	0.96 ± 0.01	7.31 ± 0.36
0.04% β-glucan	954.42 ± 184.48	984.44 ± 219.53	0.94 ± 0.02	6.79 ± 0.19

Statistically significant differences between control and *L. vannamei* fed 0.04% β-glucan were determined by using Student's t tests. $P < 0.05$ was considered to be statistically significant difference. Values are means of three replicates per treatment.

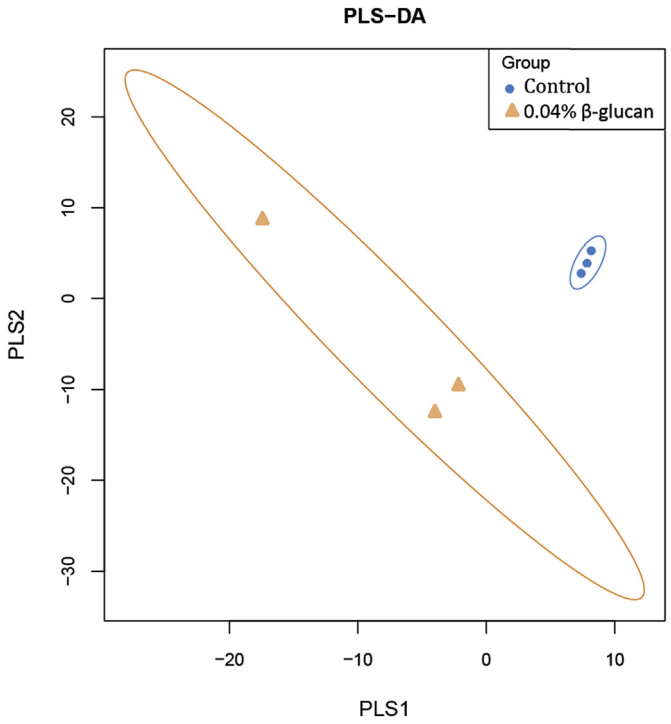

Fig. 3. PLS-DA discriminant analysis chart. Each point represents a sample, and the points of same color belong to the same group, and the points of the same group are marked with an ellipse. If the samples belonging to the same group are closer to each other, and the distance between the points of different groups is farther a part, the classification model will be more reliable. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

L. vannamei have showed strong adaptability to environmental salinity fluctuation, and its isotonic point concentration is about 718 mOsm/kg [36]. The optimal growth rate of *L. vannamei* occurs at environmental salinity ≥20 psμ [37]. More energy is needed to regulate osmotic pressure at low salinity [38,39]. Various prebiotics can promote shrimp growth. For instance, oligofructose can significantly increase weight gain and decrease food conversion ratio of *L. vannamei* at 13.5 psμ [40], and peptidoglycan can improve the growth of *Penaeus japonicus* [41]. In this study, dietaryβ-glucan supplement at 0.02% and 0.04% significantly improved growth performance of *L. vannamei* at low salinity. The growth promoting effects of β-glucan in aquatic animals also found in *Oreochromis niloticus*, *Acipenser persicus*, and *Paramisgurnus dabryanus* [10,42,43]. *L. vannamei* fed β-glucan (0.35 g kg^{-1}) exhibited higher growth performance and body protein content, and *L. vannamei* fed β-glucan (2 g kg^{-1}) regardless of continuous or discontinuous feeding showed significantly higher specific growth rate than those in the control group [14,44]. Although many studies have shown that β-glucan can promote the growth of *L. vannamei*, the related mechanism is rarely discussed. The change of growth performance is closely related to the change of digestive enzymes. Digestive enzyme activity directly reflects the degree of feed digestion and utilization, and a higher activity indicates that the utilization of feed is more adequate. In this study, dietary β-glucan significantly increased protease and amylase activities of *L. vannamei*, and the crude protein contents were also increased. It has been reported that prebiotics can increase the activity of digestive enzymes [45,46]. In this study, higher abundance of *Bacillus* and *Geobacillus* observed in the shrimp fed 0.04% dietary β-glucan, which may be an important reason of the increase of digestive enzyme activity in *L. vannamei* fed dietary β-glucan. *Bacillus* produces extracellular proteases, amylases and lipases in the gut of animals [47]. *Geobacillus* has a high-producing chitinase activity, which can convert chitin conversion to N-acetyl-D-glucosamine [48]. These findings indicate that β-glucan can induce digestive enzyme synthesis and improve the digestion and absorption capacity for diets to enhance the accumulation of nutrients, which may be closely related to the change of probiotics such as *Bacillus* and *Geobacillus*. The reduction of lipase activity may be related to the hypolipidemic effect of β-glucan [49].

Regulation of antioxidant enzymes in the animals must have contributed to the hardiness of animals [50]. Multiple stressors can cause oxidative damage to shrimp, such as nitrite and ammonia [51,52]. With the increase of dietary β-glucan, the content of MDA gradually decreased, which is consistent with the trend of change in CAT, SOD and GSH-PX. MDA is a metabolite of lipid peroxidation, which reflects the degree of lipid peroxidation in the body and is used as an evaluation index for cell damage. The antioxidant capacity of shrimp has positive correlation with salinity stress tolerance. Myo-inositol could improve the anti-oxidative capacity of *L.vannamei*, and the shrimp fed myo-inositol showed better ability of salinity stress tolerance [53]. Supplementation of *Lactobacillus plantarum* significantly improved the resistance of *L. vannamei* against the stress of acute low salinity, as indicated by higher transcript levels of ProPo, SOD and Lys gene [54]. In this study, *L. vannamei* fed 0.02% and 0.04% dietary β-glucan significantly increased the activities of SOD, GSH-PX in the hepatopancreas. These findings indicate that β-glucan can increase the activity of antioxidant enzymes to alleviate oxidative damage caused by low salinity stress.

Crustaceans lack specific immunoglobulin-mediated immunity, and their immunity depends mainly on a variety of cellular and humoral immune factors [55]. Main signaling pathways of nonspecific immunity in crustaceans are Toll, IMD and JAK/STAT [56]. In *L. vannamei,* the Toll pathway can be activated by gram-positive, gram-negative bacteria and white spot syndrome virus (WSSV), while the IMD pathway preferentially recognizes gram-negative bacteria [57,58]. JAK/STAT pathway showed important response to virus infection in *L. vannamei* [59]. Pattern recognition is the first step of innate immunity. Shrimp cells can recognize the invading microbes innately by the pattern recognition receptors (PRPs) with pathogen-associated molecular patterns (PAMPs) and then activate immune responses. Innate immune cells make cellular and humoral immune responses through intracellular signaling cascades [60]. The prophenoloxidase (proPO) system is important humoral responses. In crustaceans, β-1, 3-glucanase related protein (BGRP) is a typical pattern recognition receptor family. The member of BGRP possess different recognizing properties, such as lipopolysaccharide and β-1, 3-glucan binding protein (LGBP) and β-1, 3-glucan binding protein (BGBP). In decapod crustaceans, BGRP family is essential for proPO activation. β-1, 3-glucan can bind to LGBP and BGBP effectively and subsequently activate the immune signaling pathway and enhance the PO activity [61,62]. In this study, the results showed that β-glucan could effectively activate the immune system of *L. vannamei*. Shrimp fed 0.02% or 0.04% dietary β-glucan effectively increased the relative expression of Toll, IMD and HSP70 genes under low salinity. Previous research also indicated that β-glucan could improve the immune response of loach *Paramisgurnus dabryanus* [43], *Cyprinus carpio koi* [63] and *Pagrus major* [64]. β-Glucan can directly interact with TLR to induce the synthesis of cytokines, chemokines and the prophenoloxidase activating system (proPO-AS) [65]. The shrimp fed 0.02% β-glucan showed a higher level of expression in immune genes than those fed 0.04% β-glucan, which may be related to immune fatigue. Heat shock proteins (HSPs) are evolutionarily highly conserved cellular proteins in bacteria, animals and humans. Multiple stressors induce cells to produce HSPs such as toxins, hypoxia, and bacterial infections [66]. HSP70 plays an important role in aquatic animals to cope with environmental stress. In *L. vannamei*, LvHSP70 activates the immune signaling pathway to induce the production of immune proteins, thereby preventing infectious diseases [67]. In this study, significantly higher POX content was found in *L. vannamei* fed 0.02% and

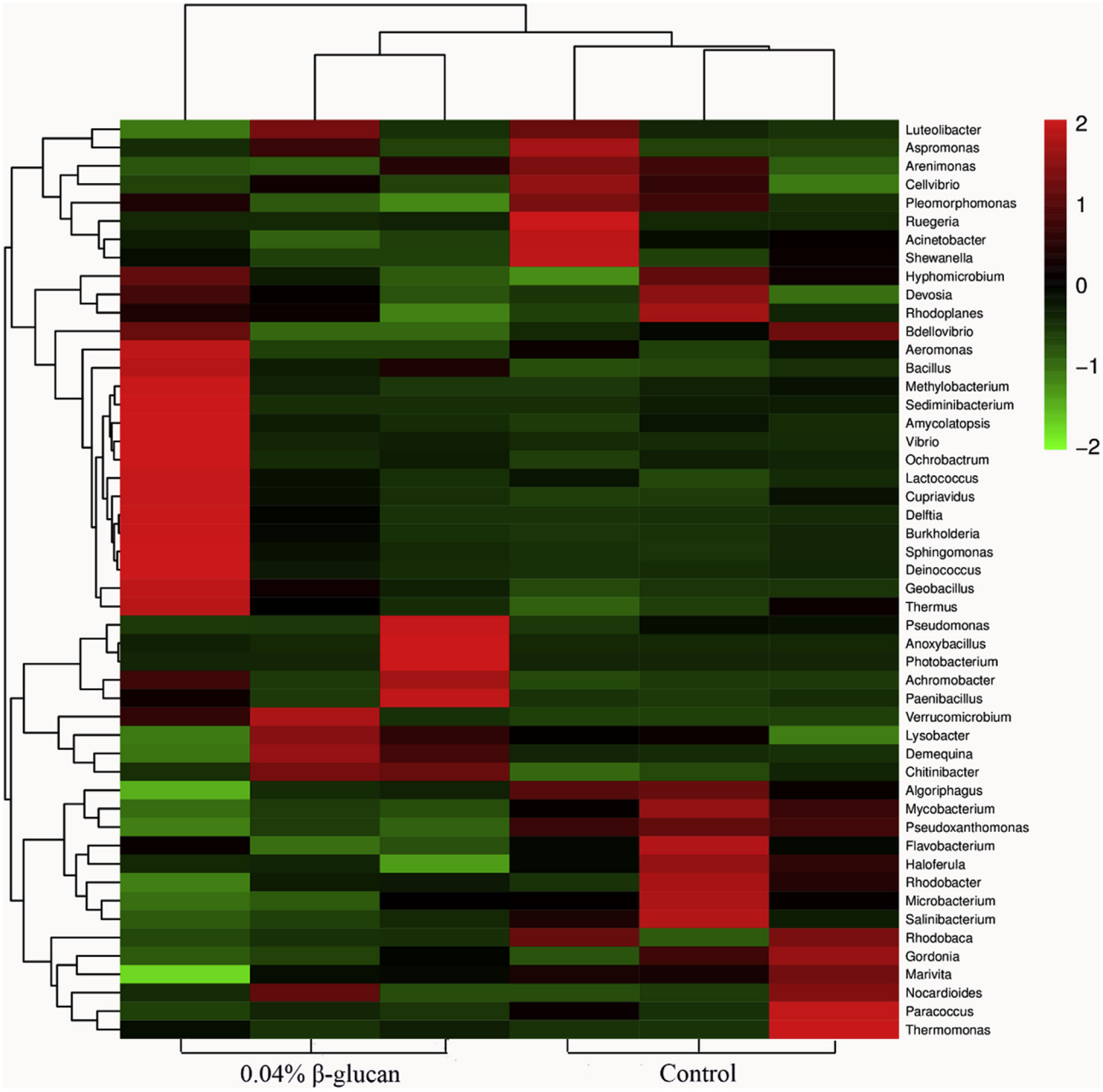

Fig. 4. Heatmap of the abundance of *L.vannamei* intestinal bacteria at the genus level in the control and *L. vannamei* fed 0.04% dietary β-glucan. In the figure, red represents the genera with higher abundance in the corresponding, and green represents the genera with lower abundance. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

0.04% dietary β-glucan, which is consistent with the change of gene expression. β-glucan induced immune gene up-regulation and further activated proPO-AS, thus increasing the POX content. POX is a non-specific immune factor producing melanin killing pathogens in crustaceans [68]. These results indicate that *L. vannamei* fed moderate β-glucan were in a positive immune response state, which is beneficial to resist various bacterial viruses in a low salinity environment.

In this study, *L. vannamei* fed 0.02% dietary β-glucan significantly increased the relative expression of MSTN mRNA, which is a positive signal for growth and cell differentiation in the hepatopancreas and intestine [69,70]. TNF-α is widely involved in immunity, inflammatory response, apoptosis, differentiation and other physiological processes [33,71]. The shrimp fed 0.02% dietary β-glucan significantly reduced the relative expression of TNF-α mRNA in the hepatopancreas and intestine, which is consistent with the findings of β-glucan in the *Carassius auratus gibelio* and rat models [72,73]. CTLs promote immune signaling to activate humoral immune responses and kill pathogens in the body of invertebrates [32]. In this study, shrimp fed 0.02% or 0.04% dietary β-glucan effectively reduced the relative expression of CTL3 mRNA. Variations in TNF-α and CTL3 mRNA may suggest that cytokines is an important pathway for β-glucan to function. β-glucan can reduce the inflammatory response, especially the intestinal inflammatory response to save energy, improve feed utilization efficiency, and promote growth [10,74].

The structure and composition of intestinal microbes affect immune response, nutrient absorption and energy balance in a host. The diet and host phylogeny are important factors influencing the composition of the intestinal microbiota [75]. Meanwhile, multiple environmental factors also can affect the diversity of intestinal microflora [76]. Comparisons between the compositions of intestinal microbiota at

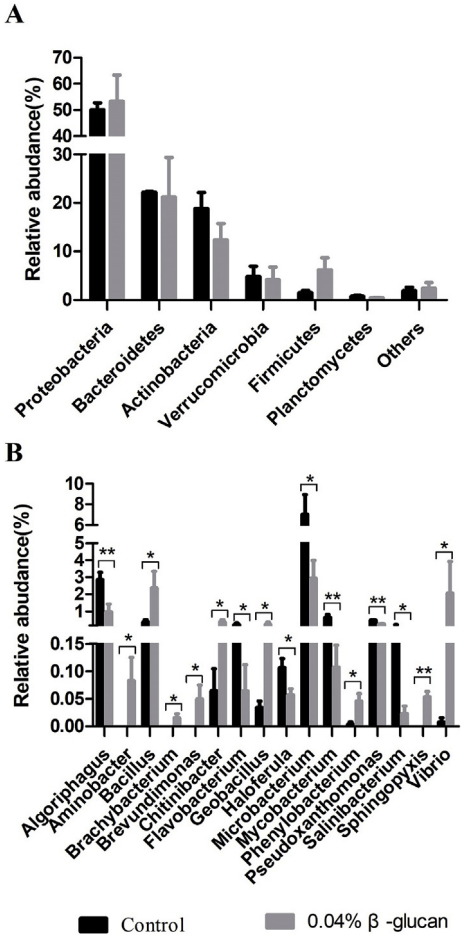

Fig. 5. Comparisons of the relative abundance of major bacteria in *L. vannamei* of the control and *L. vannamei* fed 0.04% dietary β-glucan at the phylum level (A) and genus level (B), respectively. The difference between groups was based on Metastats statistical algorithm. Asterisk (*) represents a significant difference ($P < 0.05$) between groups. Double asterisk (**) represents a significant difference ($P < 0.01$) between groups.

different salinities reveal that salinity also plays an important role in shaping the intestinal microbiota [16]. The results showed that the core bacteria in the intestine of *L. vannamei* were *Proteobacteria*, followed by *Actinobacteria*, *Bacteroidetes*, *Verrucomicrobia* and *Firmicutes,* which is consistent with previous studies of various aquatic animals [16,25]. At the phylum level, the addition of β-glucan did not significantly alter the abundance of major bacterial taxa.

Bacillus is a beneficial bacterium for nutrient absorption and immune response in the intestine of *L. vannamei* [77]. *Bacillus* not only can promote growth, but also can enhance immunity and improve disease resistance [78,79]. *Geobacillus* has thermophilic, facultative anaerobic properties and contains a variety of thermostable enzymes used to degrade cellulose and starch [80–82]. Chitinase is a key enzyme to digest chitin in crustaceans and is closely related to the growth and development of crustaceans, molt and immune function [83]. Under a short-term low salinity stress (e.g., 2 psμ for 24 h), the chitinase mRNA expression of *L. vannamei* was up-regulated, but the expression decreased after a 56-d exposure at low salinity (2 psμ), indicating that a long term stress at low salinity inhibits expression of chitinase [84]. Proteomics analysis reveals that a long-term stress at low salinity stress can decrease chitinase 3 [85]. The abundance of *Geobacillus* increased significantly in *L. vannamei* fed 0.04% dietary β-glucan, which is beneficial to increase the activity of chitinase and enhance the efficiency of chitin digestion and utilization.

Vibrio is a common bacterial flora in aquatic animals. However, environmental deterioration can cause an outbreak of vibriosis in shrimp farms, suggest they are opportunistic pathogens [86]. In this study, the abundance of *Vibrio* increased significantly in *L. vannamei* fed 0.04% β-glucan. However, it should be pointed out that the pathogenic effect of *Vibrio* is species-specific. For example, *Vibrio harveyi* and *Vibrio alginolyticus* showed strong pathogenicity [87–89], but *Vibrio azureus* has no pathogenicity [90]. *Vibrio alginolyticus* UTM 102 can be used as a probiotic to resist *Vibrio parahaemolyticus* [91]. It can be seen that the species and abundance of *Vibrio* have different effects on intestinal health. It is therefore worthwhile to study whether it is possible to enhance the resistance to pathogenic vibrio by regulating the abundance of non-pathogenic vibrio. *Flavobacterium* is a potential pathogen and often causes massive fish mortality in aquaculture [92,93], but its impact on prawn needs to be further studied. *Mycobacteriu* has a negative impact on shrimp as it was isolated from sick shrimp [94]. Although many other bacteria also showed significant changes in shrimp fed dietary 0.04% β-glucan relative to the control, the cause of these changes in this study is not clear.

5. Conclusions

The present study indicates that addition of dietary β-glucan at 0.02%–0.04% can improve growth, digestive enzymes activity, antioxidant capacity and immunity of *L. vannamei* under a long-term stress at low salinity. β-Glucan can shape the microbiota structure in the intestine of *L. vannamei*, particularly by significant increase of probiotics such as *Bacillus*. This study provides an experimental foundation for the application of β-glucan for shrimp farming at low salinity. Further research is necessary to understand the instability of β-glucan structure, and whether β-glucan can cause immune fatigue under low salinity.

Acknowledgement

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