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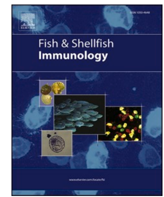
Fish and Shellfish Immunology 105 (2020) 104–116

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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



Full length article

Effects of Yu-Ping-Feng polysaccharides (YPS) on the immune response, intestinal microbiota, disease resistance and growth performance of *Litopenaeus vannamei*

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A R T I C L E I N F O

Keywords:
Litopenaeus vannamei
Yu-Ping-Feng polysaccharides
Immune response
Intestine microbiota
Prebiotics

A B S T R A C T

A 28-day feeding trial was conducted to investigate the effects of Yu-Ping-Feng polysaccharides (YPS) containing *Astragalus* polysaccharides (APS), *Atractylodes macrocephala* polysaccharides (AMP) and *Saposhnikoviae* polysaccharides (SPS) on the immune response, intestinal microbiota, disease resistance and growth performance of *Litopenaeus vannamei*. Seven hundred and twenty shrimp (3.04 ± 0.33 g) were fed the following diets: Control, YPS1 (0.13% APS + 0.0325% AMP + 0.0325% SPS), YPS2 (0.13% APS + 0.0325% AMP + 0.065% SPS) and YPS3 (0.13% APS + 0.0325% AMP+0.0975% SPS). After 14 and 28 days of feeding, the immune responses of hemocytes and intestine were measured. Intestinal microbiota and growth performance were measured after 28 days of feeding, after that, a 7-day challenge test against *Vibrio harveyi* was conducted. A significant ($P < 0.05$) increase of the total haemocyte count (THC), phagocytic activity, antibacterial activity and phenoloxidase (PO) activity was observed in shrimp fed YPS diets compared to the control. Also, dietary YPS supplementation particularly YPS3 group significantly increased the expressions of immune-related genes in the hemocytes and intestine. Regarding the intestinal microbiota, the microbial diversity and richness decreased and functional genes associated with short-chain fatty acids metabolism increased in YPS groups. After *Vibrio harveyi* challenge, the cumulative mortality in YPS groups was significantly lower than that of the control. Besides, dietary YPS had no significant effect on growth performance of shrimp ($P > 0.05$). The present results suggested that YPS could be considered as potential prebiotics for aquaculture farmed shrimp.

1. Introduction

The commercial production of farmed shrimp especially white shrimp (*Litopenaeus vannamei*) has been expanded steadily all over the world [1]. While the emergence and outbreak of shrimp diseases have become severer in recent years, causing serious economic losses to shrimp culture industry [2,3]. Previously, antibiotics and chemical drugs were used to prevent the disease infections of shrimp, but the long-term usage of these treatments seriously threatened the sustainability of shrimp culture and the health of ecosystem [4–7]. Meanwhile, plant extract due to its relative safety, environmental friendliness and economic feasibility has attracted more and more attention to cope with the disease infections of shrimp [8]. As one of the most important plant extracts, plant polysaccharides have been regarded as the natural alternative for chemical drugs as well as antibiotics and have widely applied as dietary immunostimulant in aquaculture practice [9,10].

Like other invertebrates, shrimp rely on the nonspecific immune system to fight against pathogens [11]. It has been proved that dietary intake of plant polysaccharides such as *Astragalus* polysaccharides (APS) or *Angelica sinensis* polysaccharides significantly improved the nonspecific immunity of *L. vannamei* [12,13]. Nevertheless, little information is available on the mechanism of plant polysaccharides regulating the immune system of shrimp. Besides, intestinal immune system is also an important part of shrimp immune system. Specifically, the intestine tract could defend against external microorganisms, pathogens and toxins from water environment or feeds [14,15]. And intestine epithelial cells could maintain intestinal immune homeostasis by regulating the expression of signaling pathways [16]. Whereas, there are few studies about effects of dietary plant polysaccharides on intestinal immune system of shrimp and the underlying mechanism has not been reported

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https://doi.org/10.1016/j.fsi.2020.07.003
Received 20 March 2020; Received in revised form 28 June 2020; Accepted 1 July 2020
Available online 3 July 2020
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as well.

Prebiotics are indigestible substances that allow specific changes in the composition and/or activity of gastrointestinal microbiota, which has a positive effect on the nutrition and health status of the host [17]. The most important value of prebiotics is their ability to activate the intestinal immune system, which has been reported in shrimp and fish, such as *L. vannamei* [18,19], *Oreochromis niloticus* [20], *Cyprinus carpio* [21] and *Sciaenops ocellatus* [22]. In addition to directly activating the immune system, prebiotics could be fermented by intestinal bacteria, thus producing different metabolites such as short-chain fatty acids (SCFAs) with the immunomodulatory and anti-inflammatory activities [23]. So far, the most studied prebiotics are usually some indigestible carbohydrates such as Fructooligosaccharide, Galactooligosaccharide, inulin and xylooligosaccharides [15]. In this sense, some indigestible plant polysaccharides derived from herbs might have the potential as prebiotics [24,25].

Yu-Ping-Feng polysaccharide (YPS) is a blend of three plant polysaccharides including APS, *Atractylodes macrocephala* polysaccharides (AMP) and *Saposhnikoviae* polysaccharide (SPS), which is derived from the traditional Chinese herbal formula Yu-Ping-Feng [26,27]. Modern pharmacological researches demonstrate that YPS can regulate humoral immunity and cellular immunity [28], and suppress inflammation [29]. Clinically, YPS is widely used to strengthen immunity and treat asthma, allergic rhinitis, severe acute respiratory syndrome or other human diseases [30–32]. Besides, as an indigestible plant polysaccharide, YPS is believed to have prebiotics potential in livestock which could improve the intestinal microbiota homeostasis and reduce the abundance of potential pathogens [27]. Nonetheless, whether and how YPS supplement affect the immune system of shrimp or whether YPS could be the potential prebiotics of shrimp are still unclear. Therefore, the present study addressed to investigate the effects of dietary YPS on immune response, intestinal microbiota, disease resistance and growth performance of *L. vannamei*.

2. Materials and methods

2.1. Preparation of plant polysaccharides

Each plant polysaccharide of YPS (APS, AMP and SPS) was prepared separately and then mixed into different YPS in a preferred ratio. The raw medicinal herbs *Radix Astragali*, *Rhizoma Atractylodis Macrocephalae* and *Radix Saposhnikoviae* were purchased from Beijing Tong Ren Tang medicinal materials Co., Ltd. (Qingdao, China). Three herbs were ground into powder and boiled twice with distilled water (1:12, w/v) for 2 h, respectively. The obtained filtrate was centrifuged at 6000×*g* for 10 min and the protein was removed through the Sevag's method. Subsequently, anhydrous ethanol was added to the decoction to obtain ethanol (80%, v/v), and the mixture was centrifuged at 4000 r/min for 15 min, followed by standing for 12 h. The precipitate was washed with anhydrous ethanol, acetone and ether in turn. After vacuum freezing, the APS, AMP and SPS powders were obtained. The carbohydrate contents of APS, AMP and SPS were 75.35%, 70.22% and 68.75% as measured via phenol-sulfuric acid method [33].

2.2. Preparation of experimental diets

Diet composition was shown in Table 1. According to the results of the preliminary experiment of primary shrimp hemocyte culture, the experimental group was: control (0%), YPS1 (0.13% APS + 0.0325% AMP + 0.0325% SPS), YPS2 (0.13% APS + 0.0325% AMP + 0.063% SPS) and YPS3 (0.13% APS + 0.0325% AMP+0.0975% SPS). APS, AMP and SPS were weighed, mixed and completely dissolved in sterile water in different combinations and doses to prepare the corresponding YPS, then sprayed slowly on the commercially basal pellet diet (Hengxing Group Co., Ltd., China) and the control diet was supplemented with the same volume of sterile water. All diets were coated with sodium alginate and placed at 25 °C for 30 min to fully absorb the YPS. Finally, all diets were air-dried for 12 h and stored at −20 °C until use.

Table 1
Experimental diets composition and nutrition level.

Ingredient (%)	Diets			
	Control	YPS1	YPS2	YPS3
Fish meal	29	29	29	29
Peanut meal	18	18	18	18
Soybean meal	16	16	16	16
Squid visceral meal	5	5	5	5
Wheat flour	25.3	25.3	25.3	25.3
Fish oil	2.5	2.5	2.5	2.5
Vitamin C ester (35%)	0.1	0.1	0.1	0.1
$Ca(H_2PO_4)_2$	1	1	1	1
Choline chloride (50%)	0.25	0.25	0.25	0.25
Soybean phospholipid	2	2	2	2
Cholesterol	0.1	0.1	0.1	0.1
Vitamin premix[a]	0.25	0.25	0.25	0.25
Mineral premix[b]	0.5	0.5	0.5	0.5
APS	0	1.3	1.3	1.3
AMP	0	0.325	0.325	0.325
SPS	0	0.325	0.65	0.975
Proximate analysis (%)				
Crude protein	45.6	45.1	45.8	45.8
Crude lipid	8.3	8.4	8.3	8.2
Ash	11.6	10.8	11.4	11.2
Fibre	3.8	3.6	3.8	3.4
Moisture	7.1	7.6	7.5	7.5

[a] Per kg Vitamin premix contains the following: V_A 4,000,000 IU, V_{B1} 5g, V_{B2} 15 g, V_{B6} 8g, V_{B12} 40 g, V_D 2,000,000 IU, V_E 30 g, V_K 10 g, calcium pantothenate 25 g, folic acid 2.5 g, biotin 0.08 g, nicotinic acid 30 g, inositol 150 g.

[b] Per kg Mineral premix contains the following: $MgSO_4 \cdot H_2O$ 12 g, KCl 90 g, Met-Cu 3 g, Met-Co 0.16 g, $FeSO_4 \cdot H_2O$ 1 g, $ZnSO_4 \cdot H_2O$ 10 g, $Ca(IO_3)_2$ 0.06 g, $NaSeO_3$ 0.0035 g.

2.3. Animals preparation and sampling

The experimental shrimp of *L. vannamei* were obtained from Shazikou, Qingdao, China. The shrimp (3.04 ± 0.33 g) were fed with control (without YPS) and acclimated for 7 days prior to the experiment. After acclimation, 720 shrimp were weighed and randomly assigned into 12 tanks (60 × 100 × 100 cm) with 60 shrimp each and each diet was randomly assigned to three tanks. During the whole experiment, one third of the water in each tank was replaced twice daily and culture water was maintained at 23–27.0 °C, salinity 33‰, pH 7.9–8.2, dissolved oxygen 5.6–6.2 mg/L and total ammonia content <0.05 mg/L. Shrimp were fed their respective diets twice daily (8:00 and 20:00) with 5% body weight per day for 4 weeks.

Fifteen individuals from each tank were sampled after 14 and 28 days of feeding. The midgut of ten shrimp from each tank were collected and mixed, then stored at −80 °C for the further microbiota analysis. Additionally, the midgut of the other five shrimp were cooled with liquid nitrogen, ground with mortar and pestle, and stored at −80 °C until the total RNA extraction. Hemolymph sampling (0.25 mL) was individually withdrawn from the ventral sinus according to procedures described by Sung et al. (1998) [34]. After collection, 4.5 mL of hemolymph sample was centrifuged at 800×*g* for 10 min at 4 °C. The supernatant (plasma) was store at −80 °C for measurements of immune response parameters. The pellet was suspended in 1 mL Trizol reagent (TransGen, China) and stored at −80 °C until the gene expression analysis.

2.4. Immune response parameters assay

After hemolymph was collected, 50 μL anticoagulant-hemolymph was mixed with 50 μL of 10% formaldehyde for 30 min at 4 °C. Total haemocyte counts (THC) were measured by Pan et al. (2010) [35].

Phagocytic activity was evaluated as described by Yue et al. (2010)

[36]. Phagocytic activity, defined as phagocytic rate (PR) was calculated as:

PR (%) = (number of phagocytic hemocytes/200 hemocytes) × 100%

Antibacterial activity of plasma was measured using *Vibrio harveyi* which was provided by Fisheries College of Ocean University of China according to the method of Hultmark et al. (1980) [37].

Phenoloxidase (PO) activity of plasma was determined spectrophotometrically according to the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) as Yeh and Chen (2009) described [38].

2.5. Gene expression assay

Total RNA from the hemocytes and intestine were extracted using RNAiso Plus reagent (TaKaRa, Dalian, China) and treated with RNase-free DNase following the protocol of the manufacturer (TaKaRa, Dalian, China). RNA quantity, purity and integrity were verified spectrophotometrically (A260/A280) and by electrophoresis on 1% agarose gels. The RNA sample was reverse transcribed using SMARTer™ PCR cDNA Synthesis Kit (Clontech, USA). The expressions of genes were detected by real-time quantitative PCR (qPCR), and the primers of immune-related genes, Toll, immune deficiency (Imd), Dorsal, Relish, penaeidins (Pen), crustins (Cru), anti-lipo-polysaccharide factor (ALF) and Tumour necrosis factor-α (TNF-α) were shown in Table 2. After verification of PCR efficiency to be 97.3–99.2%, the expression levels were compared with the relative Ct method [39].

2.6. Intestinal microbiota analysis

DNA of intestinal microbiota of all samples were extracted and assessed according to Zhang et al. (2019) [40]. Then, all samples were sent to Novogene Biological Information Technology Co. (Tianjin, China) for analysis. For the Illumina HiSeq platform sequencing, the hypervariable regions V4–V5 of the 16S rRNA gene were amplified using universal primers: forward primers were 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and reverse primers were 907R (5′-CCGTCAATTCCTTTGAGTTT-3′). All PCR reactions were performed on Phusion® High-Fidelity PCR Master Mix (New England Biolabs) and parameters have been described by Xiong et al. (2014) [41]. Sequencing library was generated by TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA). Library quality was assessed on the Qubit ® 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system and quantified by Q-PCR. Lastly, library was sequenced on IlluminaHiSeq2500 platform and 250 bp paired-end reads were formed. The sequences obtained in this paper were available in the GenBank with the accession number PRJNA601100.

Sequences sharing ≥97% similarity were appointed as the identical OTUs by UPARSE [42]. Taxonomic classification of OTU-representative sequences was executed with MOTHUR program via SILVA database with 80% confidence threshold [43]. Subsequently, OTUs abundances were normalized based on the least number of taxon tags to analyze the alpha diversity by QIIME (version 1.7.0) [44]. Venn diagrams and NMDS based on Bray-distance were performed. The functional prediction of the OTUs was inferred using Tax4Fun (v1.0) [45].

Table 2
Primer sequences used in this study.

Primer name	Primer sequences (5′–3′)	GenBank accession number	Product size (bp)
Toll-F	TCGACCATCCCTTTTACACC	DQ923424	88bp
Toll-R	TTGCCTGGAAGGTCTGATTC	DQ923424	
IMD-F	TCACATTGGCCCCGTTATCC	FJ592176	117bp
IMD-R	ATCTCGCGACTGCACTTCAA	FJ592176	
Dorsal-F	TTGCGACCACCAGACAAGAG	SRP132193	142bp
Dorsal-R	GCAAGGTAACGACTAATCTTCTCTG	SRP132193	
Relish-F	CTACATTCTGCCCTTGACTCTGG	FJ592176	152bp
Relish-R	GGCTGGCAAGTCGTTCTCG	FJ592176	
Pen-F	CACCCTTCGTGAGACCTTTG	Y14926	141bp
Pen-R	AATATCCCTTTCCCACGTGAC	Y14926	
Cru-F	ATTCTGTGCGGCCTCTTTAC	AY488496	185bp
Cru-R	ATCGGTCGTTCTTCAGATGG	AY488496	
ALF-F	GGTGTTCCTGGTGGCACTCT	GQ227486	114bp
ALF-R	AGCTCCGTCTCCTCGTTCCT	GQ227486	
TNF-a-F	CTCAGCCATCTCCTTCTTG	JN180639.1	114bp
TNF-a-R	TGTTCTCCTCGTTCTTCAC	JN180639.1	
β-actin-F	CCACGAGACCACCTACAAC	AF300705	142bp
β-actin-R	AGCGAGGGCAGTGATTTC	AF300705	

2.7. Challenge test

The challenge trials began after 4 weeks of growth trial. 15 shrimp from each replicate were captured and stocked into 150 L tank. *Vibrio harveyi* was provided by Fisheries College of Ocean University of China and cultured in Luria-Bertani (LB) broth overnight at 28 °C and 200 r/min. Then the bacterial culture was centrifuged at 5000×*g* for 15 min at 4 °C, the supernatant was removed and the bacterial pellet was resuspended in sterile saline solution with the final concentration of 5.0 × 10^7 CFU/mL for the bacterial inoculum. According to the results of the preliminary experiment, all shrimp were challenged with 50 μL V. *harveyi* (5.0 × 10^7 CFU/mL) by intra muscular (IM) injection. The cumulative mortality was recorded twice a day for 7 days in all group.

2.8. Survival and growth

Shrimp were weighed on 0 d and 28 d and the survival rate (%), weight gain (%) and specific growth rate (SGR) were calculated as follows:

$$\text{Weight gain (WG) (\%)} = 100 \times (W_f - W_i)/W_i$$

$$\text{Specific growth rate (SGR) (\% day}^{-1}) = 100 \times (\ln W_f - \ln W_i)/t$$

$$\text{Survival (\%)} = 100 \times N_t/N_0$$

Where W_f and W_i were final and initial shrimp body weights; t was the experimental duration in days; N_t was number of shrimp at the end of the trials and N_0 at the start.

2.9. Statistical analysis

All data were presented as the means ± standard deviation (SD). The significant difference between the control and the treatment groups at the same time point was determined by a one-way ANOVA followed by a multiple comparison of Tukey's test tests. The level of statistical significance was set at $P < 0.05$. The survival analysis was determined by the Kaplan-Meier survival analysis [46].Statistical calculations were performed using SPSS 24.0 software.

3. Results

3.1. Effects of dietary YPS on immune response parameters

Effects of dietary YPS on THC, phagocytic activity, antibacterial activity, and PO activity of shrimp were shown in Fig. 1. The THC in YPS groups were significantly increased after 28 days of feeding ($P < 0.05$). All shrimp fed diets containing YPS resulted in higher ($P < 0.05$) phagocytic capacity and antibacterial activity with the highest value in YPS3 group at the end of the experiment. Meanwhile, PO activity in YPS groups reached the peak at 14 d and then exhibited a downward trend, but was still significantly ($P < 0.05$) higher than the control group.

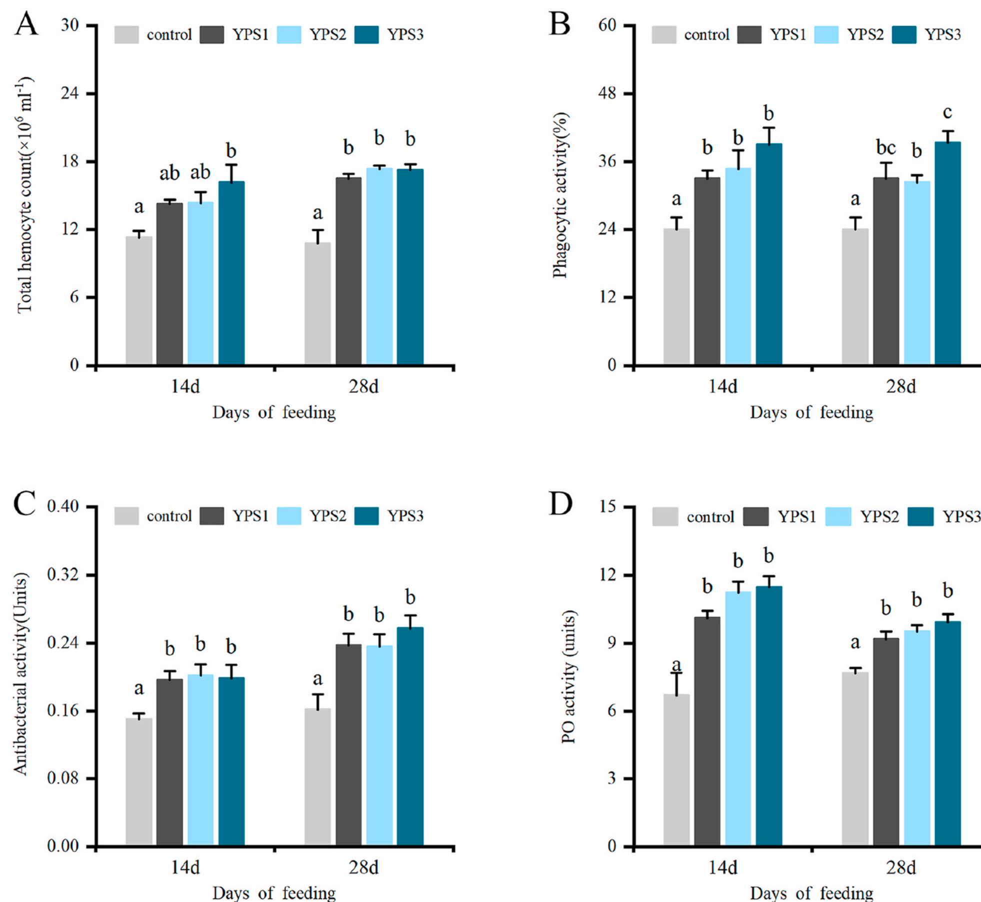


Fig. 1. Total haemocyte count (THC) (A) in hemolymph, phagocytic activity (B), antibacterial activity (C) and phenoloxidase (PO) activity (D) in plasma of *L. vannamei* fed YPS diets. Data are means ± SD. (n = 3). Bars with different letters represent significant differences ($P < 0.05$, Tukey's test) among the control and YPS groups.

3.2. Effects of dietary YPS on the expressions of genes related to immune response

Fig. 2 presented the effects of dietary YPS on the expression levels of immune-related genes in the hemocytes of shrimp. YPS3 group induced the highest expression levels of Toll, Imd and Dorsal genes after 28 days of feeding. The expression levels of Relish gene in YPS groups were significantly increased at 14 d and recovered to the control level at 28 d. As for antimicrobial peptides (AMPs), the expression levels of Pen gene in YPS groups were significantly up-regulated ($P < 0.05$) while Cru gene was significantly down-regulated at 28 d. During the whole experiment, the expression levels of ALF and TNF-α genes in YPS groups had no significant difference with control group ($P > 0.05$).

Fig. 3 represented the effects of dietary YPS on the expression level of immune-related genes in the intestine of shrimp. Dietary YPS significantly ($P < 0.05$) increased the expression levels of Toll and Imd genes compared to the control group, and YPS3 induced the highest expression level of Imd gene at 28 d. The expression levels of Dorsal and Relish genes in YPS groups were remarkably ($P < 0.05$) higher than those in control, while, there was no significant difference among all YPS groups ($P > 0.05$). The expression levels of Pen and Cru genes in YPS groups were significantly upregulated after 14 days of feeding and the expression level of ALF gene in YPS groups had no significant difference with control group ($P > 0.05$). In addition, the expression level of TNF-α gene was significantly down-regulated in YPS2 and YPS3 groups ($P < 0.05$).

3.3. Effects of dietary YPS on intestinal microbiota

3.3.1. Beta-diversity of intestinal microbiota

The high-quality sequences were clustered into 558 OTUs according to the 97% similarity. The microbial diversity and richness in all 12 samples were estimated (Table 3). By comparison with control group, Shannon indexes in YPS2 and YPS3 groups decreased significantly ($P <$ 0 0.05), and Simpson index reduced significantly in YPS2 group. While, as for microbial richness, the Chao1 and ACE indexes decreased significantly in all YPS groups. The rarefaction curve reached the asymptote when the sequencing depth was until 42112 reads (Fig. S1).

3.3.2. Composition of intestinal microbiota

16S rRNA gene sequencing in triplicate was used to explore bacterial community composition. Proteobacteria and Bacteroidetes were the most abundant phyla in all samples (Fig. 4A). Compared with the control group, the abundances of Proteobacteria, Planctomycetes, Chloroflexi and Chlamydiae decreased while the abundance of Bacteroidetes increased in all YPS groups. Furthermore, Verrucomicrobia, Actinobacteria, Tenericutes, Firmicutes and Kiritimatiellaeota were also the top ten phyla in the intestine. At the genus level, *Tenacibaculum* and *Pseudoalteromonas* were found to be the dominant genera in YPS groups, whereas *Ruegeria* was the dominant genus in control group (Fig. 4B). Compared to the control group, relative abundances of *Ruegeria*, *Formosa*, *Paracoccus* and *Owenweeksia*, belonging to the top ten genera, were changed in YPS groups.

As shown in Fig. 5A, 558 OTUs were distributed among all samples and 169 OTUs were co-owned by the four groups. The NMDS analysis (Fig. 5B) showed that the three YPS groups were classified into three

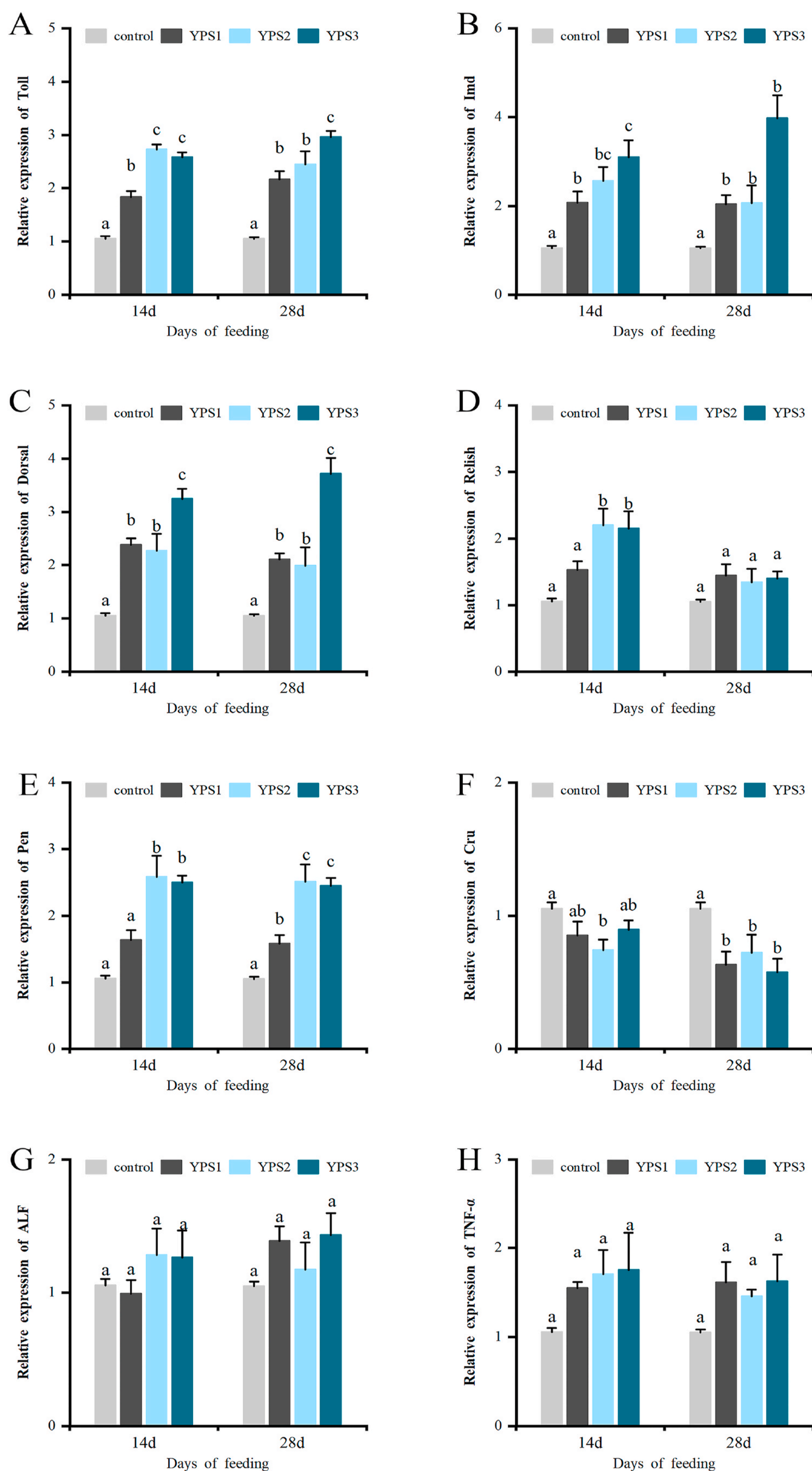


Fig. 2. The immune related genes expression in the hemocytes of *L. vannamei* fed YPS for 28 days (A: Toll; B: Imd; C: Dorsal; D: Relish; E: Pen; F: Cru; G: ALF; H: TNF-α). Data are means ± SD. (n = 3). Bars with different letters represent significant differences ($P < 0.05$, Tukey's test) among the control and YPS groups.

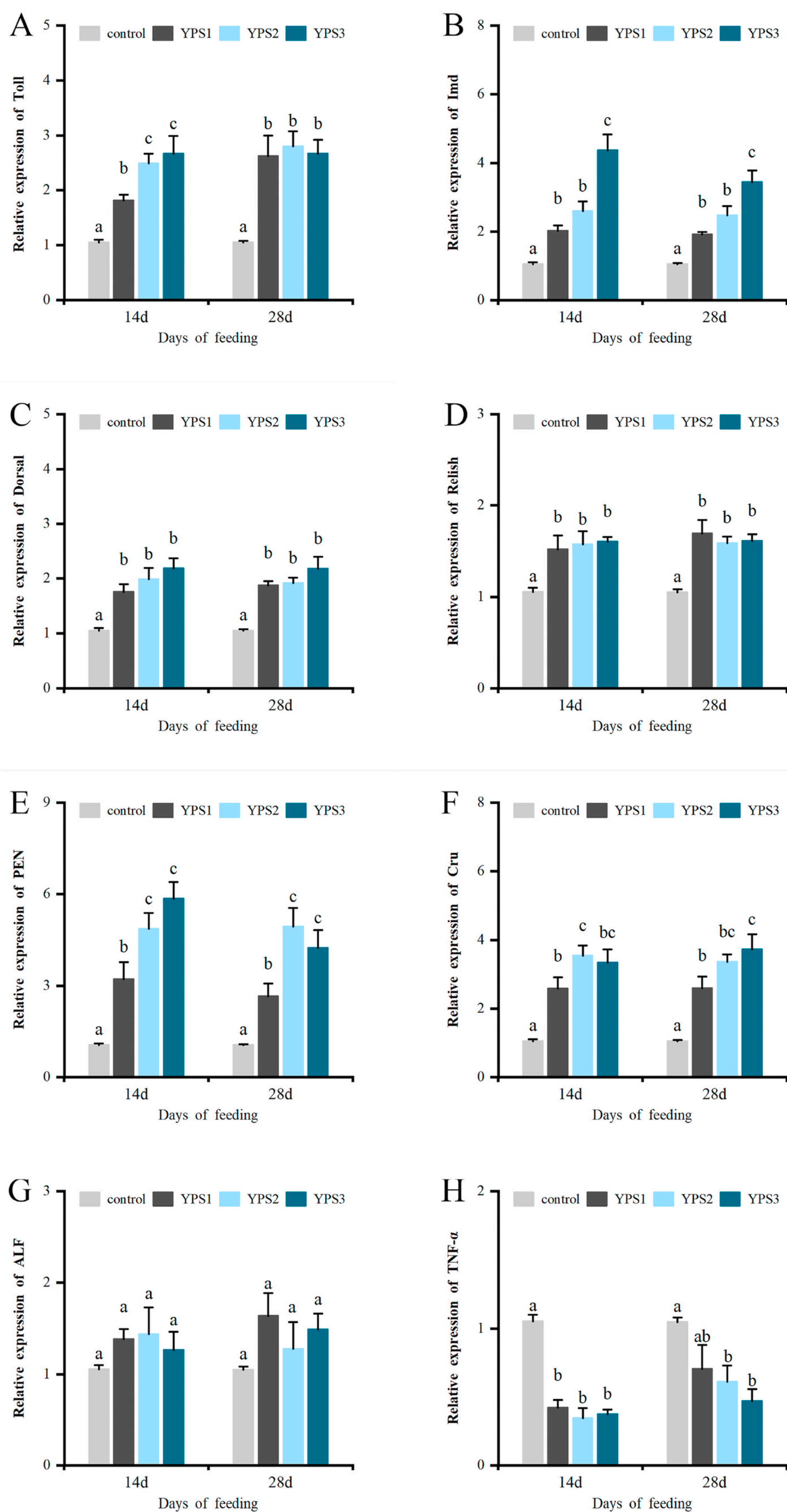


Fig. 3. The immune related genes expression in the intestine of *L. vannamei* fed YPS for 28 days (A: Toll; B: Imd; C: Dorsal; D: Relish; E: Pen; F: Cru; G: ALF; H: TNF-α). Data are means ± SD. (n = 3). Bars with different letters represent significant differences ($P < 0.05$, Tukey's test) among the control and YPS groups.

Table 3
Diversity indices of intestine microbiota used in this study.

Sample name	Observed species	Shannon	Simpson	Chao1	ACE	Good's coverage
Control	374.667 ± 23.029[a]	5.567 ± 0.331[a]	0.935 ± 0.016[a]	425.141 ± 64.704[a]	401.702 ± 22.087[a]	0.999 ± 0.001[a]
YPS1	256.667 ± 5.033[b]	5.004 ± 0.226[ab]	0.946 ± 0.013[a]	309.929 ± 19.580[b]	308.180 ± 12.942[b]	0.998 ± 0.001[a]
YPS2	211.667 ± 17.898[c]	4.209 ± 0.077[c]	0.887 ± 0.003[b]	262.521 ± 30.701[b]	266.585 ± 32.054[bc]	0.999 ± 0.001[a]
YPS3	195.667 ± 5.508[c]	4.475 ± 0.152[bc]	0.920 ± 0.015[a]	237.509 ± 9.744[b]	245.800 ± 9.743[c]	0.999 ± 0.000[a]

Data are presented as mean ± SD. Data indicated with different letters were significantly different ($P < 0.05$) among treatments.

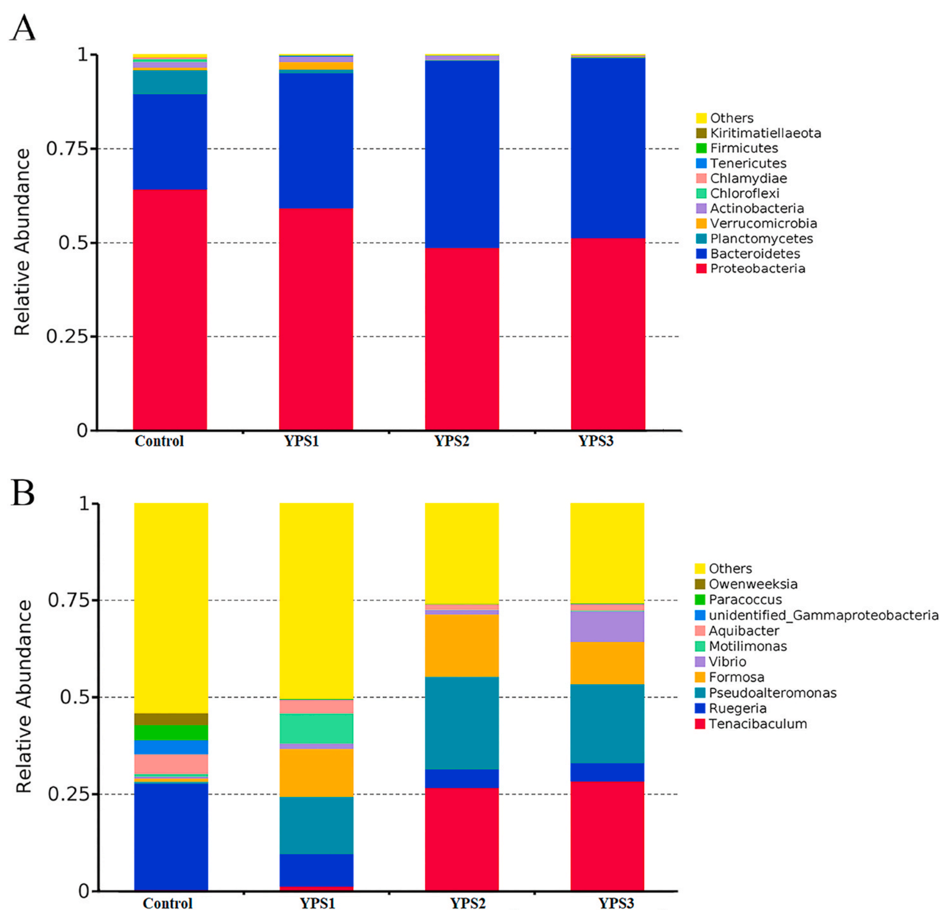


Fig. 4. Relative abundances of the dominant bacterial phyla (A) and genus (B) in *L. vannamei* from different YPS diets treatments.

categories and separated from the control group. Similarly, the UPGMA clustering analysis (Fig. 5C) demonstrated that YPS groups were clustered more closely.

3.3.3. *Function of intestinal microbiota*

The functional profiling of the intestinal microbiota of YPS and control groups was predicted using Tax4Fun (v1.0). Results showed that "Metabolism", "Genetic Information Processing", "Environment Information Processing" and "Cellular Processes" were the four dominant predicted functions in all samples (Fig. S2). At KEGG level 3, YPS groups showed increased abundance in the terms "Transporters", "ABC transporters" and "Quorum sensing" (Fig. 6). Furthermore, functional genes associated with SCFAs metabolism, such as propanoate metabolism and butanoate metabolisms, were significantly activated in YPS groups ($P < 0.05$). Specifically, dietary YPS significantly improved the ability of intestinal microbiota to convert pyruvate, acetate and succinate to butyrate as well as to convert glucose and succinate to propionate (Fig. 7).

3.4. *Cumulative mortality*

Cumulative mortality curves of *L. vannamei* fed diets containing YPS after *V. harveyi* challenge were shown in Fig. 8. The cumulative mortalities were 80%, 47%, 42% and 31% respectively in control, YPS1, YPS2 and YPS3 groups after *V. harveyi* challenge. Kaplan-Meier survival analysis revealed that the cumulative mortalities of *L. vannamei* fed diets containing YPS1 (log-rank X^2: 7.942, $P < 0.01$), YPS2 (log-rank X^2: 12.318, $P < 0.001$) and YPS3 (log-rank X^2: 20.901, $P < 0.0001$) were significantly lower than control group. There was no significant difference in cumulative mortalities of all YPS groups after *V. harveyi* challenge.

3.5. *Growth performance and survival*

Table 4 showed the effects of dietary YPS on growth performance and survival of *L. vannamei*. Both weight gain and specific growth rate did not show significant differences ($P > 0.05$) in YPS and control groups. Survival rates were above 90%, with no significant differences ($P > 0.05$) in YPS and control groups.

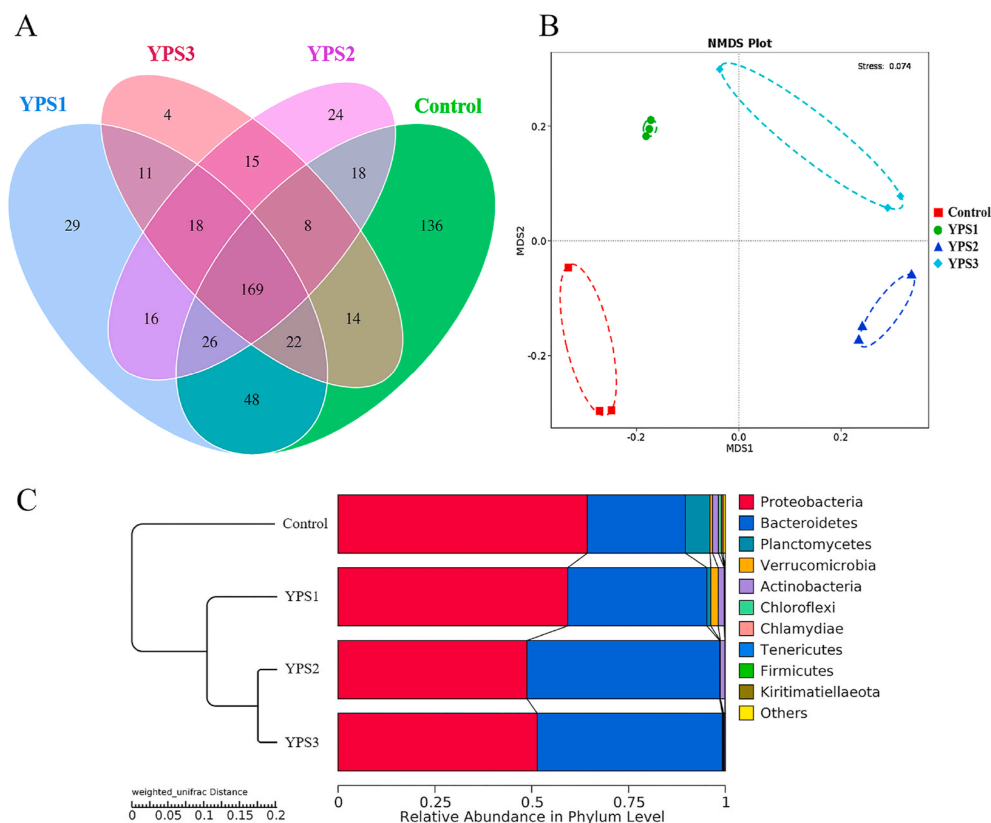


Fig. 5. Analysis of intestinal microbiota structure of *L. vannamei* from different YPS diets treatments (A: Venn diagram of shared and specific microbial communities at OTUs among four groups; B: Nonmetric Multidimensional scaling (NMDS) analysis among microbial communities of 12 samples; C: UPGMA clustering based on four groups from the gut samples).

4. Discussion

The herbs, such as the traditional Chinese medicines, Indian Ayurveda, Japanese Kampo medicines, and western countries' Phytomedicines, have a long history for the prevention and treatment of various diseases all over the world. Till now, different types of active components such as terpenoids, polysaccharides, flavonoids, saponins and alkaloids extracted from medicinal herbs have been identified. Among them, plant polysaccharide is widely studied due to its diverse biological activities, including immunostimulatory activity, antiviral activity, antioxidant activity, antitumor activity, radioprotection effect, hepatoprotection effect, and antifatigue effect [47]. And the mixture of three plant polysaccharides (APS, AMP and SPS), YPS, has been elucidated to enhance the immunity and disease resistance of human and livestock [27,32,48]. In present study, effects of dietary YPS on the immune response and intestinal microbiota of shrimp were investigated.

Hemocytes are the main actors of the cellular immune responses (recognition, phagocytosis, and melanization) [49] in shrimp. Meanwhile, the prophenoloxidase (proPO) system is a significant part of immune system of shrimp as well, and phenoloxidase (PO) is the terminal enzyme of proPO system, whose activity is positively correlated with disease resistance capability of shrimp [56]. Previous studies reported that dietary plant polysaccharides extracted from *Angelica sinensis* [13], *Panax ginseng* [50], *Astragalus* [12] and *Ulva rigida* [51] significantly improved the innate immunity parameters of *L. vannamei*, such as THC, phagocytic activity, and PO activity. Huang et al. (2006) showed *Sargassum fusiforme* polysaccharide could reduce the cumulative mortality of *Fenneropenaeus chinensis* injected with *V. harveyi* by increasing the PO activity [52]. Consistent with these previous studies, the results suggested that dietary YPS significantly increased THC of *L. vannamei* after 14 days of feeding. Evidence showed that YPS can regulate cell cycle progression into S and G2/M phase and promote mitosis [48], which may explain the increased THC in present results. To confirm this hypothesis, experiments such as primary cell cultures merit investigations in future studies. Meanwhile, the increase of phagocytic activity, PO activity and antibacterial activity in YPS groups further confirmed the beneficial effects of YPS on the immune system of shrimp. These results suggested YPS had the potential to serve as immunostimulant for shrimp farming. In this case, further work is required to explore the underlying mechanism by which YPS stimulate the immune response of shrimp.

Recently, Toll pathway and Imd pathway have been identified as the major signaling pathways in *L. vannamei* to participate in the process of immune responses [53]. NF-κB is a key regulator for immune and inflammatory responses, and Dorsal and Relish are two Rel/NF-κB transcription factors involved in the Toll and Imd pathways, respectively [54]. In this study, the expression levels of Toll and Imd genes in hemocytes and intestine of all YPS groups were significantly higher than those of control. One possible explanation was that YPS contained a variety of immune-stimulating oligosaccharides such as glucan and arabinogalactans [46–48], which could bind to pattern recognition proteins (PRPs) on the cell surface thus activating the immune system [26]. Meanwhile, the expression levels of Imd in hemocytes and intestine varied with the composition of YPS, while similar results were not observed in the expression level of Toll, which may be attributed to the fact that the Toll pathway was more sensitive than the Imd pathway under the stimulation of YPS. Previous studies suggested that the expression level of NF-κB in sea cucumber increased after dietary APS supplementation [55], and Wongprasert et al. (2015) reported that dietary sulfated galactans extracted from *Gracilaria fisheri* effectively activated the NF-κB signaling pathway in the hemocytes of *L. vannamei* [56]. In accordance with previous studies, YPS supplementation significantly increased the expression levels of Dorsal and Relish in present study. It was worth noting that the expression level of Relish in

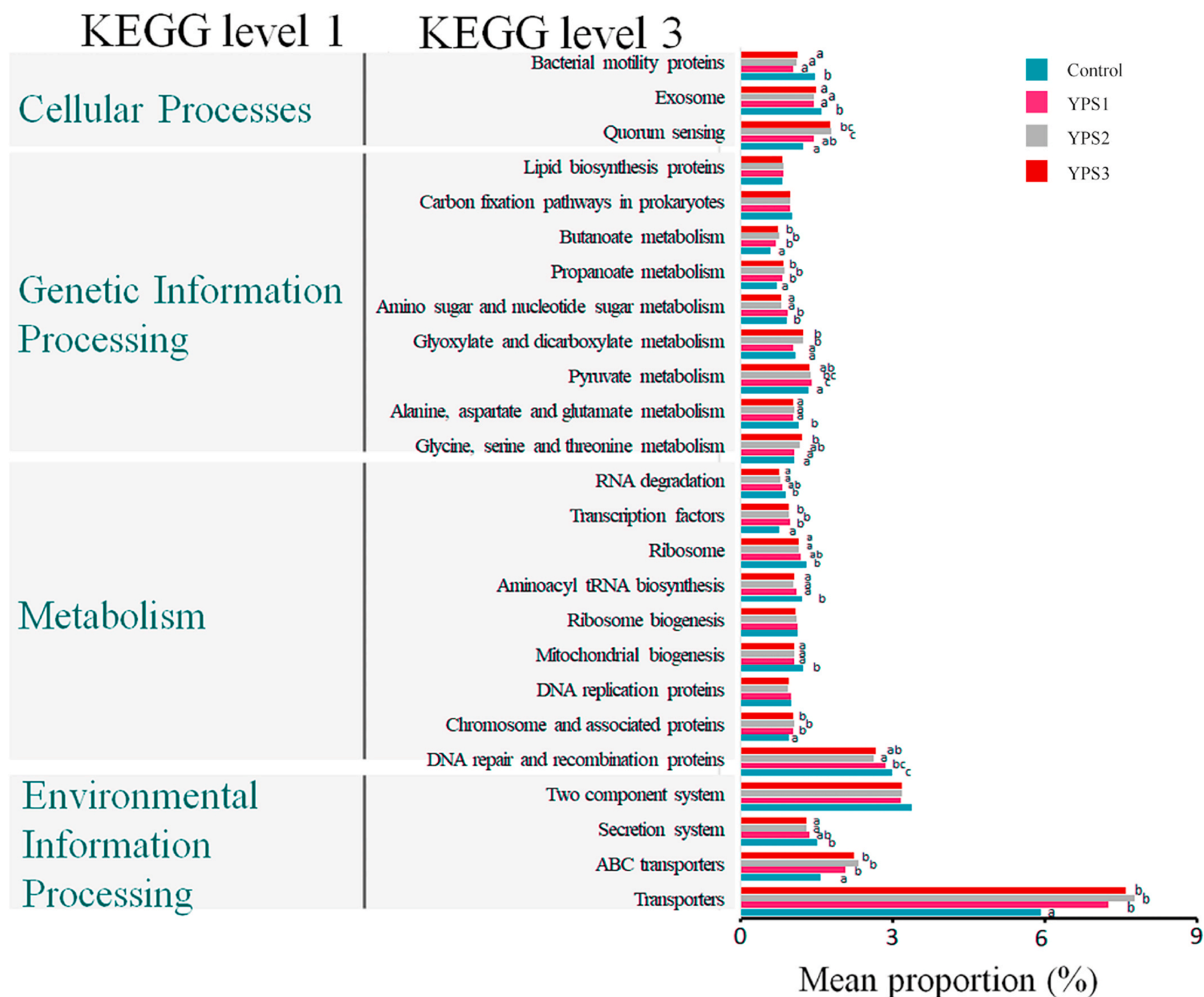
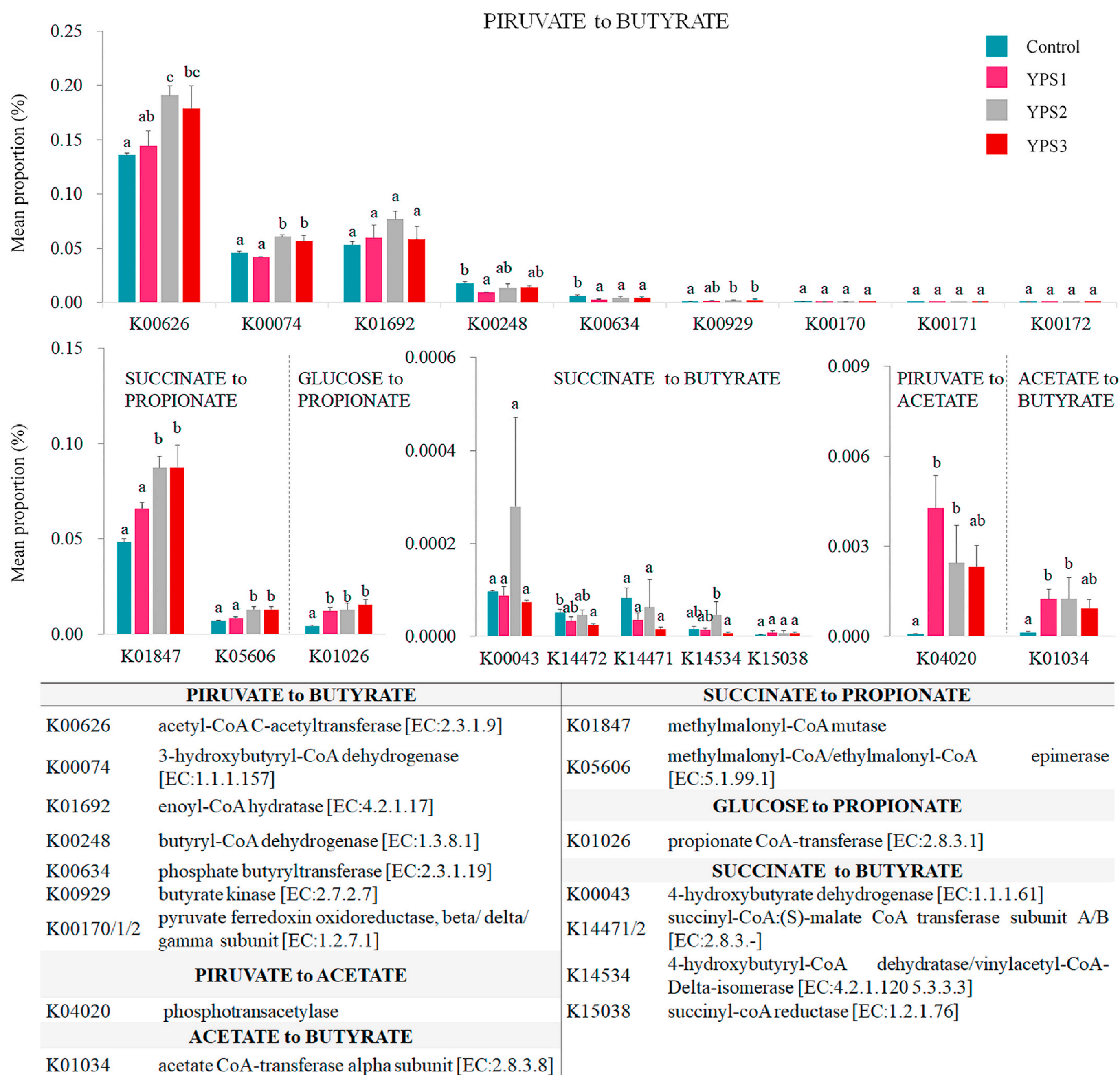


Fig. 6. Analysis of Predicted functions in KEGG level3 of intestinal microbiota in *L. vannamei* from different YPS diets treatments. Data indicated with different letters were significantly different ($P < 0.05$) among treatments.

hemocytes reached the peak at 14 d and recovered to the normal at 28 d, which may be related to the immune fatigue of hemocytes in shrimp. These results suggested that the feeding time or cycle should be taken into account when supplementing YPS in shrimp aquaculture practice.

As the important innate immune effectors, AMPs are regulated by NF-κB signaling pathways [54]. In the past two to three decades, many AMPs such as Pen, Cru and anti- ALF have been identified and characterized in shrimp [57]. And AMPs have been proved to play vital roles in the immune system of shrimp, while studies about effects of dietary plant polysaccharides on expression levels of AMPs in shrimp are rare. In the present study, YPS supplementation particularly YPS2 group significantly increased the expression level of Pen in both hemocytes and intestine, decreased the expression level of Cru only in hemocytes and did not significantly affect the expression level of ALF. There were two possible explanations for this phenomenon 1) Pen, Cru and ALF exerted different functions in immune response to YPS supplementation; 2) dietary YPS tended to increase the expressions of AMPs more easily in intestine than in hemocytes, possibly because the intestine had a lower immune response threshold than hemocytes when stimulated by YPS [58].The findings of these differences suggested that we should pay more attention to the intestinal immunity of shrimp in the future studies, and also provided significant inspiration for the development of shrimp feed supplement in the future work.

Intestinal microbiota play important roles in the immunity, digestion and nutrient absorption of the host [59,60]. Results of Illumina sequencing showed that microbial diversity and richness were decreased in YPS groups. On the one hand, enhanced intestinal immune response in present study, such as the increased expression of AMPs, may put more pressure to certain bacteria in the intestine and hinder their proliferation [61]. On the other hand, the production of SCFAs, ultimate fermentation products of plant polysaccharides might lead to the strong decline of pH in intestine, thus inhibiting the metabolism of specific pathogens [62], and this view was also supported by the Tax4fun analysis that functional genes associated with SCFAs metabolism were activated in all YPS groups. At the phylum level, dietary YPS supplementation decreased the abundance of Proteobacteria and Chlamydiae, and increased the abundance of Bacteroidetes in YPS groups. It has been proposed that the increased abundance of Proteobacteria may bring potential risks to shrimp, since more abundant Proteobacteria were observed in pathogen-infected [63] and slow-growing shrimp [64]. Chlamydiae were well-known intracellular pathogens that might be lethal to fishes, bivalves and crustaceans [65]. In addition, numerous



PIRUVATE to BUTYRATE		SUCCINATE to PROPIONATE	
K00626	acetyl-CoA C-acetyltransferase [EC:2.3.1.9]	K01847	methylmalonyl-CoA mutase
K00074	3-hydroxybutyryl-CoA dehydrogenase [EC:1.1.1.157]	K05606	methylmalonyl-CoA/ethylmalonyl-CoA epimerase [EC:5.1.99.1]
K01692	enoyl-CoA hydratase [EC:4.2.1.17]		**GLUCOSE to PROPIONATE**
K00248	butyryl-CoA dehydrogenase [EC:1.3.8.1]	K01026	propionate CoA-transferase [EC:2.8.3.1]
K00634	phosphate butyryltransferase [EC:2.3.1.19]		**SUCCINATE to BUTYRATE**
K00929	butyrate kinase [EC:2.7.2.7]	K00043	4-hydroxybutyrate dehydrogenase [EC:1.1.1.61]
K00170/1/2	pyruvate ferredoxin oxidoreductase, beta/ delta/ gamma subunit [EC:1.2.7.1]	K14471/2	succinyl-CoA:(S)-malate CoA transferase subunit A/B [EC:2.8.3.-]
	PIRUVATE to ACETATE	K14534	4-hydroxybutyryl-CoA dehydratase/vinylacetyl-CoA-Delta-isomerase [EC:4.2.1.120 5.3.3.3]
K04020	phosphotransacetylase	K15038	succinyl-coA reductase [EC:1.2.1.76]
	ACETATE to BUTYRATE		
K01034	acetate CoA-transferase alpha subunit [EC:2.8.3.8]		

Fig. 7. The relative abundance of candidate genes associated with short-chain fatty acid metabolism of intestinal microbiota. Data indicated with different letters were significantly different ($P < 0.05$) among treatments.

studies have shown that Bacteroidetes have the ability to produce SCFAs to regulate host immune homeostasis [66–68]. Thus, changes of the microbial abundance at the phylum level in YPS groups may be related to the stronger immune response of shrimp. At the genus level, the abundance of *Pseudoalteromonas* was greatly increased in YPS groups. It has been elucidated that by producing exopolysaccharides, *Pseudoalteromonas* can improve the health status of organisms [69], and can also be used as the biological agent to inhibit the proliferation of pathogens [70].Overall, these results probably suggested that dietary YPS could regulate intestinal community composition by increasing the abundance of potential probiotics and decreasing the abundance of opportunistic pathogens, thus exerting beneficial effects to shrimp [25]. Nevertheless, functions of many intestinal bacteria whose abundances changed after dietary YPS supplementation are still unclear in present study, and the widespread application of high-throughput sequencing technology in aquaculture would explain these results more comprehensively.

As the key bacterial metabolites and anti-inflammatory factors in intestine, SCFAs could be produced through the fermentation process by intestinal microbiota [23]. Hoseinifar et al. (2016) [71] reported that dietary sodium propionate improved mucosal and humoral immune responses of Caspian Sea white fish. And dietary intake of organic acids or poly-β-hydroxybutyrate could significantly change the composition of intestinal microbiota of *L. vannamei* [72,73]. The results showed that after dietary YPS supplementation, the abundance of functional genes associated with SCFAs metabolism in intestine were increased, perhaps implying that YPS could significantly improve the ability of intestinal microbiota to produce SCFAs. Increasing the production of SCFAs in

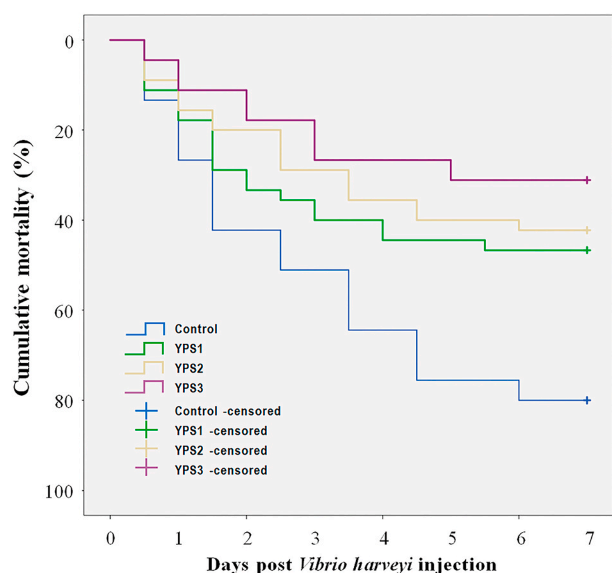


Fig. 8. The cumulative mortality curves of *L. vannamei* fed with YPS diets in pathogen challenge experiments. Differences in cumulative mortality levels between treatments were analyzed by Kaplan-Meier plot log-rank X^2 test.

intestine may be an another important way for YPS to exert biological activity [74], which may further explain the increased expression levels of immune-related genes in all YPS groups [75]. By combination with the changes in the composition of intestinal microbiota, Bacteroides may be related to the metabolism of SCFAs in this study [76], but the correlation analysis between species of Bacteroidetes and metabolism of SCFAs needs to be explored in the future work. Therefore, a topic that merits investigations in further studies is the global metabolome profiling of intestinal content after dietary YPS supplementation, in order to investigate the relationship between different metabolites and specific intestinal microbiota as well as evaluate the overall contribution of YPS to the physiological state of the shrimp.

The cumulative mortality after pathogen challenge can directly reflect the health status and immune response performance of shrimp. As a serious pathogen, *V. harveyi* can cause different kinds of severe diseases to shrimp [77]. It has been shown that dietary intake of *Sargassum fusiforme* polysaccharide extracts could improve the survival rate of *Fenneropenaeus chinensis* infected with *V. harveyi* [52]. And survival rate of *L. vannamei* fed probiotic-supplemented diet was increased after 10 h of challenge with *V. harveyi* [78]. In this study, the cumulative mortality of *L. vannamei* fed YPS after challenge with *V. harveyi* was significantly lower than control group, possibly due to the enhanced immune response caused by dietary YPS. On the other hand, the intestinal bacterial structure of shrimp was altered by YPS, which may enhance the ability of beneficial bacteria to compete for adhesive sites with *V. harveyi* and other pathogens [79]. In addition, a topic merits investigation in the future work is to determine the immune response of shrimp after the challenge with *V. harveyi*, so as to explore the beneficial effect of YPS on infected shrimp. Even so, this study still provides valuable information for our understanding towards the immune-promoting mechanism of YPS in shrimp.

Table 4
Effects of diets containing YPS on growth performance of *L. vannamei* after 28 days of feeding.

Index	Treatment			
	Control	YPS1	YPS2	YPS3
SR%	91.11 ± 3.42^a	92.78 ± 2.83^a	94.44 ± 0.79^a	95.00 ± 3.60^a
Initial weight/g	3.04 ± 0.33^a	3.04 ± 0.33^a	3.04 ± 0.33^a	3.04 ± 0.33^a
Final weight/g	5.04 ± 0.22^a	5.34 ± 0.27^a	5.50 ± 0.17^a	5.70 ± 0.45^a
WG%	77.94 ± 7.17^a	75.74 ± 8.74^a	81.01 ± 5.61^a	87.06 ± 14.97^a
SGR%/d	2.06 ± 0.12^a	2.01 ± 0.14^a	2.12 ± 0.09^a	2.24 ± 0.24^a

Data are presented as mean ± SD. Data indicated with different letters were significantly different ($P < 0.05$) among treatments.

YPS can promote the growth performance of mammals. And Sun et al. (2016) reported that increased growth performance was observed in rabbit after dietary YPS supplementation [27]. Furthermore, SCFAs can also serve as an energy source for the host [80,81]. In the present study, YPS3 exhibited the best growth performance, but no statistical differences in weight gain rate and SGR were observed between control and all YPS groups. Previous studies have demonstrated that dietary APS supplementation could increase the final weight and SGR in *Micropterus salmoides* [82] while no obvious growth promoting effects were exhibited in *L. vannamei* [12].Further, the growth rates of *Macrobrachium rosenbergii* were not significantly improved after 4 or 6 weeks of feeding with 0.2% anthraquinone extracts, but was significantly higher than that of control after 8 weeks of feeding [83]. Thus, the growth-promoting effects of YPS may be species-specific and time-dependent, and further study may needed to clarify these differences.

5. Conclusions

In conclusion, the present study revealed that dietary supplementation of YPS for 28 days significantly increased the immune response of *L. vannamei*. Moreover, composition and metabolism of intestinal microbiota significantly changed following YPS administration in diet while no significant effects on growth performance were observed. These results suggested that YPS could be a functional feed additive for shrimp and encourage further studies on the relationship between intestinal microbiota, bacterial metabolites, and host immune system.

Disclosure summary

The authors have nothing to disclose.

Declaration of competing interest

There were no conflicts of interest to declare.

CRediT authorship contribution statement

Chen Su: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Project administration. **Depeng Fan:** Resources, Methodology. **Luqing Pan:** Supervision, Conceptualization, Writing - review & editing. **Yusong Lu:** Investigation. **Yuxuan Wang:** Investigation. **Mengyu Zhang:** Investigation, Writing - review & editing, Formal analysis.

Acknowledgments

This study was supported by the Key Laboratory of Mariculture (KLM2017005), Ministry Education, Ocean University of China (OUC), and Bio-Form biotechnology (Guangdong) co. LTD (20160359). We thank all the staff at the Laboratory of Environmental Physiology of Aquatic Animal for helping with sampling.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2020.07.003.

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