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Effects of dietary mixed probiotics on growth, non-specific immunity, intestinal morphology and microbiota of juvenile pacific white shrimp, *Litopenaeus vannamei*

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ABSTRACT

This study was conducted to elucidate the effects of dietary mixed probiotics on growth, non-specific immunity, intestinal morphology and microbiota of juvenile pacific white shrimp, *Litopenaeus vannamei*. Juvenile shrimp (initial body weight 1.21 ± 0.01 g) were fed diets containing graded probiotics (F1: 0 mg/kg probiotics; F2: 1000 mg/kg probiotics; F3: 2000 mg/kg probiotics; F4: 4000 mg/kg compound probiotics; F5: 6000 mg/kg probiotics; F6: 8000 mg/kg probiotics) for 8 weeks. The result of this trial showed that the growth performance (SGR, WG, FBW) of shrimp fed diets containing probiotics (F2 ~ F6) were significantly higher than that of shrimp fed diet without supplemental probiotics (F1) ($P < 0.05$), and the highest values of the growth performance (SGR, WG, FBW) and lowest FCR were found in shrimp fed the diet containing 2000 mg/kg probiotics. Total antioxidant capacity of shrimp fed diet F2 and F3 were significantly higher than that of shrimp fed the basal diets ($P < 0.05$). Superoxide dismutase in F4 treatment was significantly higher than that of basal treatment ($P < 0.05$). Catalase of shrimp in all probiotics supplemented (F2 ~ F6) treatments were significantly higher than that of the control one (F1) ($P < 0.05$). Malondialdehyde in F5 groups was significantly lower than that of F1 groups ($P < 0.05$). Alkline phosphatase and acid phosphatase in F3 treatments were significantly higher than those of the basal one ($P < 0.05$). Lysozyme of shrimp fed F2 ~ F6 were significantly higher than that of shrimp fed F1 diet ($P < 0.05$). The lipase and amylase activities in 2000 mg/kg probiotics groups showed the highest activities and were significantly higher than that of control one ($P < 0.05$). Intestinal villi height in F3 ~ F6 treatments were significantly higher than that of control one ($P < 0.05$). Alpha diversity indices including observed species, chao1, ACE and shannon indices showed that F2 and F3 groups had higher microbial diversity in their intestines, both richness and evenness. PCA plot showed that there was a clear shift of F2 and F3 groups from the control groups in microbial community structure. The dominant phyla in pacific white shrimp are *proteobacteria*, *bacteroidetes* and *actinobacteria*, the dominant genus were *algoriphagus* and *vibrio*. As the probiotics increased, the *gemmatimonadetes, acidobacteria*, *deltaproteobacteria* and *xanthomonadales* firstly increased and then decreased, with the highest content in F2 group, which was no significant difference to F3 group ($P > 0.05$) while significantly higher than other groups ($P < 0.05$). In conclusion, the supplement of mixed species probiotics can promote growth performance, enhance the non-specific immunity, influence the microbiota of the pacific white shrimps and the recommended optimum dosage in diet of *Litopenaeus vannamei* was 2000 mg/kg.

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1. Introduction

Aquaculture is emerging as one of the most viable and promising enterprises for keeping pace with the surging need for animal protein, providing nutritional and food security to humans [1]. However, there is an increasing stress for fish with intensive cultivation, leading to infectious diseases and finally causing economic losses [2,3]. The main treatment methods in conventional way are antibiotics and chemotherapeutants, which are neither effective nor consumer or environment-friendly [4]. Consequently, probiotics, environmentally friendly alternatives, are widely used to take place of the conventional methods, which improve activity of gastrointestinal microbiota. Research showed that microbiota helps to digest complex dietary macronutrients and provides the host with nutrients, vitamins [5] and digestive enzymes [6], thus facilitating feed utilization and digestion. The probiotics can therefore enhance feed utilization and growth performance [1]. Bairagi et al. showed that the adding B. *subtilis* and B. *circulans* to the diet of rohu, *Labeo rohita* resulted in extracellular cellulolytic and amylolytic enzyme production, hence increased performance [7]. Probiotics also help to maintain intestinal microbial balance and development gut mucosa, improving digestion and absorption rate and thus improving production [8].

The most commonly proposed mode of action is that probiotics work by competitive exclusion whereby the live organism enters the digestive tract and interferes with the action of potential pathogens by the production of inhibitory molecules and direct competition for space or oxygen [9]. Probiotic is regarded as occupying and colonizing sites in the digestive tract particularly the gastro-intestinal mucosal epithelium [10], blocking adhesion receptors and the portals of entry for fish pathogens [11], therefore forming a barrier against pathogenic bacteria and to stimulate the host's immune system [12], with the reason that the probiotic showed higher adhesion to mucus hence reducing the pathogenic bacteria, e.g. V. *harveyi* [13].

Research showed that probiotics are also able to inhibit pathogens. *Carnobacterium* sp. has been shown to be antagonistic against *Fl. Psychrophilum* [14]. The strains of *P. damselae* subsp. *Piscicida* were inhibited by four potential probiotics obtained from gilthead sea bream [15]. Besides, probiotics are found to trigger the lysozyme level in teleosts either single or in combination [12], enhance natural complement activity of fish [16], improve respiratory burst activity [17] and secrete antimicrobial peptides [18].

The present study mainly focused on effects of mixed-species probiotics (1.8×10^8 CFU *Bacillus subtilis*,2×10^7 CFU *Bacillus licheniformis* and 3×10^7 CFU *lactobacillus* per kilogram) on growth performance, immunity and microbiota of juvenile shrimp, *Litopenaeus vannamei*.

2. Materials and methods

2.1. Diet preparation and dietary treatments

The formulation and proximate composition of the six experimental diets with or without probiotics supplemented (T1: control diet; T2: 1000 mg/kg; T3: 1000 mg/kg; T4: 1000 mg/kg; T5: 1000 mg/kg; T6: 1000 mg/kg) are presented in Table 1. The method of diet preparation was the same as described by Niu et al. [19]. All the dry ingredients for each diet were weighed, combined and thoroughly mixed in a Hobart-type mixer, and then oils were added and thoroughly mixed for 5 min. Deionized water (250 mL/kg dry ingredients mixture) was added and mixed for another 5 min. The wet dough was placed in a monoscrew extruder (Institute of Chemical Engineering, South China University of Technology, Guangzhou, China) and extruded through a 1.2-mm die. The diets were then ground using a mortar and pestle and graded through a series of different-sized metal sieves. The resulting pellets were dried at 25 °C with the aid of an air conditioner and an electrical fan. After drying, all diets were packed in bags and stored at −20 °C until used.

Table 1
Ingredients and proximate composition1 of the four experimental diets (%).

Ingredients (mg kg^{-1})	F1	F2	F3	F4	F5	F6
	0	1000	2000	4000	6000	8000
Fish meal	12	12	12	12	12	12
Soybean meal	26	26	26	26	26	26
Peanut meal	12	12	12	12	12	12
Peanut meal	23.23	23.13	23.03	22.83	22.63	22.43
Shrimp head meals	5	5	5	5	5	5
Beer yeast	4	4	4	4	4	4
Chicken powder	5	5	5	5	5	5
Soybean lecithin	1	1	1	1	1	1
Fish oil	1	1	1	1	1	1
Soybean oil	1	1	1	1	1	1
CaH_2PO_4	1	1	1	1	1	1
Vitamin premix[1]	1	1	1	1	1	1
Mineral premix[2]	1	1	1	1	1	1
Ascorbic Phosphate ester	0.1	0.1	0.1	0.1	0.1	0.1
lysine	0.16	0.16	0.16	0.16	0.16	0.16
DL-Methionine	0.3	0.3	0.3	0.3	0.3	0.3
Mixed probiotics	0	0.1	0.2	0.4	0.6	0.8
Y_2O_3	0.01	0.01	0.01	0.01	0.01	0.01
Alginic acid sodium	1	1	1	1	1	1
Proximate composition						
Crude protein	40.51	41.40	41.18	40.54	40.83	40.36
Crude lipid	5.57	5.78	5.98	5.86	5.77	6.32
ash	9.95	9.75	9.82	9.69	9.83	9.81
Moisture	10.50	10.58	12.17	11.17	11.97	11.77

Note:Vitamin premix (In 1 kg contains):vitaminA,250,000IU; vitaminC,7000 mg; folicacid,125 mg; biotin,10 mg; riboflavin, 750 mg; calcium pantothenate,1250 mg; pyridoxine HCL,400 mg; cyanocobalamin,1 mg; thiamin,250 mg; menadione,250 mg; α-tocopherol,2.5 g; myo-inositol,8000 mg; nicotinicacid,2000 mg; cholinechloride,8000 mg; vitamin 3,45,000IU; cellulose was used as a carrier.
Mineral premix (In 1 kg contains):$ZnSO_4{\cdot}7H2O$,0.04 g; KCL,5.3 g; KI,0.04 g; NaCl,2.6 g; $CuSO_4{\cdot}5H_2O$,0.02 g; $MnSO_4{\cdot}H_2O$,0.03 g; $CaCO_3$,37.9 g; $MgSO_4{\cdot}7H_2O$,3.5 g; $Ca(HPO4_2{\cdot}2H2O$,9.8 g; $CoSO_4{\cdot}7H_2O$,0.02 g; $FeSO_4{\cdot}7H_2O$,0.9 g; cellulose was used as a carrier.

2.2. Animal rearing and experimental procedures

The feeding trial was conducted at Sanya, Hainan province. Prior to the start of the trial, juvenile *Litopenaeus vannamei* were acclimated to a control diet for 2 weeks. At the beginning of the feeding trial, the shrimps were starved for 24 h, weighed, and then shrimps with similar size (initial body weight 1.21 ± 0.01 g) were randomly allotted to twenty-four tanks (1 m × 1 m × 1 m). Water exchange in each tank was adjusted to approximately 1.0 L/min with a flowing filtered water system. Each tank was covered by a plastic mesh lid to prevent the shrimp from jumping out. The shrimp were cultured outdoors, subjected to a natural photoperiod. During the experimental period, water quality parameters were monitored daily. All shrimp in each aquarium were initially fed 6% of their total body weight daily. The feeding frequency was three times per day at 07:00, 14:00 and 21:00 h and lasted for 56 d. During the feeding trial, the amount of the diet given was progressively changed and adjusted according to the appetite of shrimp by checking the bottom of the tank for excess feed remaining 2 h after feeding.

2.3. Sample collection

At the end of the feeding trial, shrimps were starved for 24 h and then weighed and counted the total number. Ten fish from each cage were randomly collected for sampling, four for analysis of whole-body composition and six were anesthetized to obtain hepatopancreas and gut samples. The samples were rapidly removed and frozen in the liquid nitrogen for analysis.

Table 2
Effects of different levels of dietary mixed probiotics on growth performance of white shrimp (*Litopenaeus vannamei*).

mg/kg	F1	F2	F3	F4	F5	F6
	0	1000	2000	4000	6000	8000
IBW(g)	1.20 ± 0.01	1.20 ± 0.01	1.21 ± 0.01	1.22 ± 0.01	1.20 ± 0.01	1.22 ± 0.01
FBW(g)	23.96 ± 0.28^{a}	27.10 ± 0.51^{bc}	27.74 ± 0.15^{c}	26.54 ± 0.30^{bc}	25.92 ± 0.56^{b}	26.80 ± 0.28^{bc}
WG (%)	1893 ± 23.69^{a}	2157 ± 24.02^{cd}	2204 ± 25.03^{d}	2080 ± 28.87^{bc}	2052 ± 36.83^{b}	2101 ± 18.36^{bc}
SGR	5.34 ± 0.02^{a}	5.56 ± 0.02^{bc}	5.60 ± 0.02^{c}	5.50 ± 0.02^{bc}	5.48 ± 0.03^{b}	5.52 ± 0.01^{bc}
FCR	1.24 ± 0.01^{c}	1.10 ± 0.02^{b}	1.04 ± 0.01^{a}	1.02 ± 0.02^{a}	1.03 ± 0.02^{a}	1.01 ± 0.04^{a}
SR (%)	97.5 ± 1.44	99.17 ± 0.83	97.5 ± 1.44	100 ± 0	98.33 ± 0.83	100 ± 0

2.4. Biochemical composition analysis

Chemical composition of diets and shrimps were determined by standard methods [20]. Moisture was determined by oven drying at 105 °C until a constant weight was obtained. Crude protein content (N × 6.25) was determined according to the Kjeldahl method after acid digestion using an Auto Kjeldahl System (1030- Autoanalyzer; Tecator, Höganäs, Sweden). Crude lipid was determined by the ether extraction method using a Soxtec extraction System HT (Soxtec System HT6, Tecator). Ash content was determined after samples were placed in a muffle furnace at 550 °C for 4 h.

2.5. Hepatopancreas antioxidant status and digestive enzymes analysis

Hepatopancreas and gut samples were homogenized in ice-cold phosphate buffer (1:10 dilution). The homogenate was then centrifuged for 20 min (4 °C, 3000 rpm) and aliquots of the supernatant were used to quantify hepatopancreas antioxidant status and digestive enzymes analysis. All indices were measured with commercial assay kits (Nanjing Jian cheng Bioengineering Institute Nanjing, China) in accordance with the instructions of the manufacturer.

2.6. Intestinal morphology

Samples fixed in Bouin solution were dehydrated in ethanol, equilibrated in xylene and embedded in paraffin according to the method described by Krogdahl et al. [21]. The paraffin blocks was sectioned (5 μm) in serial sagittal section using a Leica RM 2135 rotary microtome and stained with haematoxylin and eosin (H & E). The sections were examined using a light microscope with villi height measured. Photographs were taken with an Olympus digital camera attached to the microscope. 10 random villi from each segment were measured.

2.7. DNA extraction and 16S DNA gene sequencing

Total DNA of microbes in intestine was extracted directly with the E.Z.N.A. Stool DNA Kit (OMEGA,US) according to manufacturer's instructions. Amplification and sequencing of the V4 region of the bacterial 16S DNA gene was performed using barcoded fusion primers 515F (GTGCCAGCMGCCGCGGTAA) 806R (GGACTACHVGGGTWTCTAAT). 16S rRNA tag-encoded high-throughput sequencing was carried out in Illumina MiSeq platform at the Novogene (Beijing, China). Sequencing reads were assigned to each sample according to the individual unique barcode. Sequences were analyzed with QIIME software package (Quantitative Insights Into Microbial Ecology) and UPARSE pipeline [22]. The reads were first filtered and clustered into Operational Taxonomic Units (OTUs) at an identity threshold of 97%. For each OTU, a representative sequence was selected and used to assign taxonomic composition by using the RDP classifier [23]. Alpha and beta diversity analyses were also performed using QIIME.

2.8. Calculations and statistical analysis

The following variables were calculated:

Initial body weight (IBW, g) = initial body weight / initial number of fish;

Final body weight (FBW, g) = final body weight / final number of fish;

Weight gain rate (WG, %) = 100 × (final body weight − initial body weight) / initial body weight;

Specific growth rate (SGR, % day − 1) = 100 × (Ln final individual weight − Ln initial individual weight) / number of days; Feed conversion ratio (FCR) = dry diet fed /wet weight gain;

Survival rate (%) = 100 × (final number of fish) / (initial number of fish);

All data are presented as means ± S.E.M. and subjected to independent-sample *t*-test to test the effects of experimental diets using the software of the SPSS for windows (ver 16.0, U.A.S). Statistical significance was examined at $P < 0.05$ unless otherwise noted.

3. Results

3.1. Growth performance and body composition

Growth performance and feed utilization of juvenile *Litopenaeus vannamei* fed different levels of dietary complex probiotics are shown in Table 2. Results showed that FBW, WG and SGR were significantly influenced by different levels of mixed probiotics. In general, final body weight (FBW), weight gain (WG) and special growth rate (SGR) of shrimps fed mixed species probiotics diets were higher than that of the control group ($P < 0.05$). FBW, WG and SGR increased significantly ($P < 0.05$) with dietary mixed probiotics levels supplemented from 0 to 2000 mg/kg, and then decreased and leveled off. FCR were significant higher in control group than that in any other groups ($P < 0.05$).SR were no significant differences in any groups ($P > 0.05$).

The proximate compositions of whole body and muscle of white shrimp fed the diets containing grade mixed probiotics levels are shown in Table 3. Moisture decreased with mixed probiotics supplemented. The control group was significantly higher than F4, F5 and F6 in whole shrimp ($P < 0.05$). Crude lipid in control group was significantly higher than that of other groups except for F6 ($P < 0.05$) in whole shrimp. Ash in control groups both whole shrimp and muscle were significantly higher than supplemented groups ($P < 0.05$).

3.2. Hepatopancreas antioxidant status and immunity and gut digestive enzymes analysis

The antioxidant status and immunity of juvenile *Litopenaeus vannamei* are presented in Table 4. Results showed that T-AOC increased

Table 3
Effects of different levels of dietary mixed probiotics on whole shrimp and muscle composition of white shrimp (*Litopenaeus vannamei*).

Whole shrimp	F1	F2	F3	F4	F5	F6
	0	1000	2000	4000	6000	8000
Moisture	76.07 ± 0.51^{b}	75.11 ± 0.25^{ab}	75.17 ± 0.25^{ab}	74.71 ± 0.25^{a}	74.07 ± 0.55^{a}	74.32 ± 0.44^{a}
Crude protein	75.53 ± 0.13	76.45 ± 0.23	75.76 ± 0.06	75.97 ± 0.88	75.99 ± 0.39	76.14 ± 0.19
Crude Lipid	4.51 ± 0.22^{b}	3.98 ± 0.08^{a}	3.69 ± 0.04^{a}	3.53 ± 0.18^{a}	3.78 ± 0.12^{a}	4.43 ± 0.08^{b}
Ash	13.99 ± 0.38^{b}	13.14 ± 0.54^{ab}	13.25 ± 0.26^{ab}	12.60 ± 0.45^{ab}	12.23 ± 0.67^{a}	12.95 ± 0.50^{ab}
Muscle composition						
Moisture	74.92 ± 0.09	74.56 ± 0.47	74.60 ± 0.26	74.17 ± 0.53	74.23 ± 0.38	74.19 ± 0.16
Crude protein	88.02 ± 0.16	88.46 ± 0.87	88.09 ± 0.53	88.20 ± 0.26	87.50 ± 0.11	87.82 ± 0.17
Crude Lipid	2.43 ± 0.23	2.45 ± 0.07	2.05 ± 0.10	2.26 ± 0.17	2.10 ± 0.09	2.22 ± 0.05
Ash	6.41 ± 0.10^{b}	6.13 ± 0.04^{a}	6.22 ± 0.09^{ab}	6.17 ± 0.05^{a}	6.24 ± 0.08^{ab}	6.14 ± 0.03^{a}

significantly as mixed species probiotics supplemented from 0 to 2000 mg/kg ($P < 0.05$), with the highest in 2000 mg/kg mixed species probiotics supplemented group, while the 4000–8000 mg/kg groups were no significant differences compared to control groups ($P > 0.05$). With dietary complex probiotics supplemented, SOD increased at first and then decreased. The activities were significant higher in 4000 mg/kg groups than any other groups ($P < 0.05$). CAT increased and then leveled off as dietary mixed species probiotics supplemented, with the highest level in 2000 mg/kg, significantly higher than control group ($P < 0.05$). MDA showed the decreasing trend with dietary complex probiotics supplemented, and the lowest content showed in 6000 mg/kg groups, significant lower than any other groups ($P < 0.05$). With dietary mixed species probiotics added, the alkaline phosphatase and acid phosphatase increased at first and then decreased; the highest value showed in 2000 mg/kg, significant higher than that of any other treatments ($P < 0.05$). The LYZ showed the same trends while the highest value showed in 4000 mg/kg groups, significant higher than that of any other treatments ($P < 0.05$). As can be shown from Table 5, lipase and amylase were higher in shrimps fed mixed probiotics groups. The activities of lipase and amylase were significantly higher with 2000 mg/kg complex probiotics than that of control group ($P < 0.05$).

3.3. Intestinal morphology

As is shown in Fig. 1, height of intestinal villi increased at first and then decreased and leveled off. Shrimps fed diets containing 2000 mg/kg mixed probiotics owned the highest intestinal villi, significantly higher than F1, F2 and F5 groups ($P < 0.05$). All treatments except for 1000 mg/kg were significantly higher than the control one ($P < 0.05$) (see Fig. 2).

3.4. Microbiota

3.4.1. Richness and diversity

A total of 63437–81876 6S rRNA gene sequences were analyzed and assigned to 771 to 2219 OTUs. The observed species, Chao1, abundance-based coverage estimator (ACE) and Shannon indices obtained for all the samples in the 6 groups are reported in Table 6 to assess the alpha diversity of intestinal microbiota of the 6 groups of *Litopenaeus vannamei*. The non-parametric species-richness index Chao1 showed an Operational Taxonomic Units (OTUs) minimum count of 896 in F5 group and a maximum count of 2992 in F2 group, with no significant difference with F3 ($P > 0.05$), while signifcantly higher than other groups ($P < 0.05$). The relative high supplemented content of probiotics (4000, 6000 and 8000 mg/kg) had lower OUT than that of control groups with no significantly difference ($P > 0.05$). Observed species and ACE showed the same trend, with F2 significantly higher than other groups except for F3 ($P < 0.05$). With mixed-species probiotics supplemented, observed species increased at first and then decreased, observed species in F5 and F6 groups significantly lower than that in F1 groups ($P < 0.05$). Shannon index, which combines estimates of richness and evenness within the samples [24], ranged from 5.55 to 7.14 in F2 groups, significantly higher than other groups ($P < 0.05$). Phylogenesis was measured using PD whole tree estimator, which ranged from 65.08 to 129.19. All those indices suggest the F2 and F3 groups had higher microbial diversity in their intestines, whether measured in richness or evenness (see Table 7).

3.4.2. Beta diversity analysis

The differences of gut microbiota between treatments were analyzed by the beta diversity metric. Principal component analysis (PCA) and principal coordinates analysis (PCoA) based on unweighted-UniFrac distance for bacterial profiles were used in the present study to indicate the community change in different samples. The PCA two-dimensional plot showed that the gut microbiota of the basal diets were similar with the F4, F5 and F6, while different to F2 and F3 (Fig. 3). But unweighted (Fig. 4) UniFrac PCoA analysis showed that F2 and F3 were similar to F1, different with F5 and F6, which were confirmed by cluster analysis (Fig. 5). As is shown in Fig. 5, cluster analysis revealed that F2 and F3 were clustered into a clade, and then clustered with F1, indicating that the bacteria communities in F2 and F3 were quiet similar, different with that of shrimps fed the basal and high mixed species

Table 4
Effect of different levels of dietary mixed probiotics on hepatopancreas antioxidative indices of white shrimp (*Litopenaeus vannamei*).

	F1	F2	F3	F4	F5	F6
	0	1000	2000	4000	6000	8000
T-AOC,U/mgprot	2.02 ± 0.10^{a}	2.78 ± 0.16^{b}	2.95 ± 0.03^{b}	2.09 ± 0.15^{a}	2.35 ± 0.18^{a}	2.11 ± 0.10^{a}
SOD,U/mgprot	179.30 ± 12.9^{a}	200.19 ± 2.5^{ab}	201.60 ± 9.1^{ab}	294.14 ± 17.9^{c}	218.55 ± 5.2^{b}	187.27 ± 1.9^{ab}
CAT,U/mgprot	17.94 ± 1.52^{a}	26.83 ± 3.69^{b}	45.28 ± 0.35^{c}	43.63 ± 2.24^{c}	37.92 ± 2.83^{c}	39.56 ± 3.49^{c}
MDA, nmol/mgprot	1.70 ± 0.28^{c}	1.15 ± 0.14^{abc}	1.60 ± 0.08^{bc}	1.25 ± 0.03^{abc}	0.90 ± 0.12^{a}	1.31 ± 0.15^{abc}
ALP,KU/gprot	5.81 ± 0.36^{a}	7.20 ± 0.52^{ab}	9.20 ± 0.34^{b}	8.44 ± 1.27^{b}	8.16 ± 0.89^{b}	8.05 ± 0.11^{ab}
ACP,KU/gprot	12.90 ± 0.46^{a}	14.16 ± 0.78^{a}	18.24 ± 1.04^{b}	13.05 ± 0.86^{a}	13.07 ± 0.41^{a}	15.11 ± 0.45^{a}
LZM,U/mgprot	12.30 ± 1.38^{a}	15.87 ± 0.47^{ab}	16.67 ± 0.39^{b}	23.33 ± 1.61^{c}	20.26 ± 0.33^{bc}	17.87 ± 1.05^{bc}

T-AOC, total antioxidant capacity; SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde; ALP, Alkaline phosphatase; ACP, Acid phosphatase; LZM, lysozyme.

Table 5
Effect of different levels of dietary mixed probiotics on intestinal digestive enzymes of white shrimp (*Litopenaeus vannamei*).

	F1	F2	F3	F4	F5	F6
	0	1000	2000	4000	6000	8000
Lipase (U/gprot)	5.82 ± 0.38^{a}	8.50 ± 0.70^{ab}	9.47 ± 0.91^{b}	7.97 ± 0.94^{ab}	7.08 ± 0.45^{ab}	9.25 ± 0.49^{b}
Amylase (U/mgprot)	0.68 ± 0.03^{a}	0.81 ± 0.02^{ab}	0.83 ± 0.03^{b}	0.70 ± 0.06^{ab}	0.72 ± 0.03^{ab}	0.81 ± 0.03^{ab}

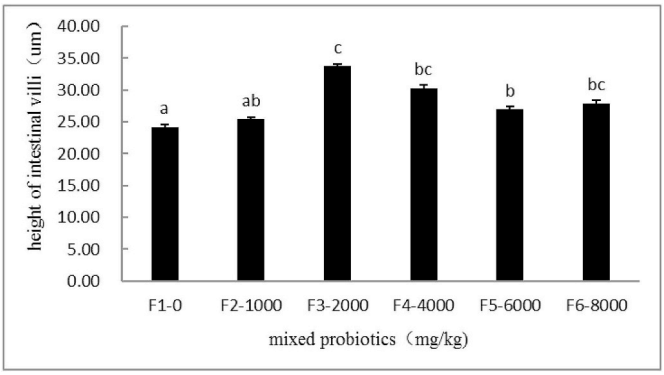

Fig. 1. Effect of different levels of dietary mixed probiotics on height of intestinal villi of pacific white shrimp (*Litopenaeus vannamei*).

probiotics diets. Besides, as the probiotics increased up to 4000 mg/kg, the communities differed from the F1 groups with diversity decreased. Venn diagram was constructed to identify the core and different OTUs existing in shrimp samples under different diets. In this regard, 793 OTUs were shared among all shrimp gut samples (Fig. 6). In contrast, 80 OTUs, 237 OTUs, 127 OTUs, 55 OTUs, 28 OTUs and 43 OTUs unique to basal, F2, F3, F4, F5 and F6 diets, respectively. This observation suggested that the exposure of shrimp to different graded probiotics led to the selection of unique microbial populations.

3.4.3. Gut microbiota composition of Litopenaeus vannamei and changes with probiotics supplemented

The dominant phyla in all groups are *Proteobacteria* (36.86 ± 1.27%), *Bacteroidetes* (29.80 ± 2.02%) and *Actinobacteria* (17.48 ± 2.20%). In general, the most abundant taxonomic groups for shrimp gut samples were *Cytophagia*, *Gammaproteobacteria* and *Alphaproteobacteria* at class level, *Cytophagales* and *Rhodobacterales* at order level and *Cyclobacteriaceae* and *Rhodobacteraceae* at family level *Algoriphagus* and *Vibrio* at genus level. The microbial community structure after the probiotic treatments were changed at different levels with different bacteria mainly in F2 and F3 groups. As the probiotics increased, the *Gemmatimonadetes*, *Acidobacteria*, *Deltaproteobacteria* and *Xanthomonadales* firstly increased and then decreased, with the highest content in F2 group, which was no significant difference to F3 group ($p > 0.05$) while significantly higher than other groups ($P < 0.05$). The control group was significantly higher than that of the supplementary groups in *Cyclobacteriaceae*, *Rhodobacteraceae* and *Algoriphagus* ($P < 0.05$). At the genus level, the microbial diversity increased, and the abundance of other bacteria was also increased at the expense of *Bacillus* in F3 groups compared with the control and high probiotics groups. With probiotics supplemented, *Bacillus* firstly increased and then decreased, with the highest content in F3 group, significantly higher than other groups ($P < 0.05$). In family level, *Bacillaceae* were first increased and then decreased with mixed probiotics supplemented, with the highest content in F3 group, higher than other groups except for F2 group ($P < 0.05$). Similar trend occurred in *Bacillales*.

4. Discussion

The current study demonstrates that supplementing mixed probiotics in feed improved growth performance. Similar findings have previously been documented in preliminary trials on European lobster (*Homarus gammarus* L.) [25], tiger shrimp *Penaeus monodon* [26], and *Penaeus vannamei* by photosynthetic bacteria and *Bacillus* sp. mixture [27]. Earlier study [28] showed that mixed probiotics induced the best growth performance of common carp compared with individual probiotics. The better growth performance may be due to the ability of probiotics to influence the digestion mediated via enzyme production [29]. Supplement of probiotics to shrimp resulted in an increasing activity of lipase and amylase in the shrimp's digestive tract in the present study. Similar study has been reported by Ziaei-Nejad et al. who observed higher digestive enzyme activity in Indian shrimp (*Fenneropenaeus indicus*) gut after fed bacillus probiotics [30]. Liu reported that bacillus enhanced protease of white shrimp, *Litopenaeus vannamei* [31]. Gram-positive bacteria do secrete a wide range of exoenzymes [32],

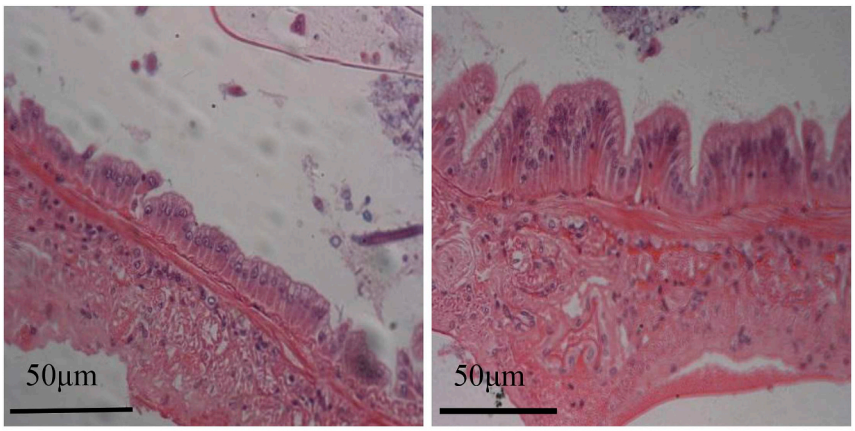

Fig. 2. Comparison of intestinal villi height between shrimps fed diets supplemented 0 mg/kg and 2000 mg/kg mixed probiotics (200 ×).

Table 6
Richness and diversity indices used in this study.

	F1	F2	F3	F4	F5	F6
Observed species	1402.33 ± 84.05cd	1903.67 ± 75.34e	1655.00 ± 67.68de	1180.00 ± 140.83bc	794.67 ± 84.36a	1026.00 ± 121.13 ab
Chao1	1653.24 ± 107.91a	2992.58 ± 873.43b	1908.33 ± 65.13 ab	1410.54 ± 152.01a	896.94 ± 94.59a	1206.94 ± 159.94a
ACE	1711.28 ± 114.55bc	2431.90 ± 256.52d	1974.31 ± 64.22cd	1491.37 ± 157.67bc	921.29 ± 93.34a	1296.08 ± 179.29 ab
Shannon	5.90 ± 0.21 ab	7.14 ± 0.11c	6.54 ± 0.13bc	5.56 ± 0.51a	5.62 ± 0.32 ab	5.55 ± 0.27a
PD whole tree	102.80 ± 4.71cd	129.19 ± 4.95e	116.01 ± 3.19de	86.76 ± 8.71bc	65.08 ± 4.61a	81.17 ± 7.03 ab

Table 7
Changes of various bacteria in shrimps fed with graded probiotics.

	F1	F2	F3	F4	F5	F6
Gemmatimonadetes	0.40 ± 0.07a	0.96 ± 0.20b	0.69 ± 0.09 ab	0.29 ± 0.06a	0.33 ± 0.17a	0.37 ± 0.14a
Acidobacteria	0.47 ± 0.06b	1.07 ± 0.05c	1.08 ± 0.10c	0.31 ± 0.13 ab	0.08 ± 0.04a	0.17 ± 0.07a
Deltaproteobacteria	0.77 ± 0.15 ab	1.48 ± 0.10c	1.13 ± 0.25bc	0.69 ± 0.21 ab	0.36 ± 0.14a	0.62 ± 0.12 ab
Cytophagales	24.21 ± 4.51b	11.55 ± 1.75 ab	14.01 ± 5.02 ab	14.58 ± 3.60 ab	12.90 ± 3.82 ab	10.73 ± 3.77a
Rhodobacterales	15.66 ± 1.67 ab	9.11 ± 2.19a	12.64 ± 2.74 ab	20.08 ± 3.57b	10.43 ± 2.78a	12.97 ± 3.14 ab
Xanthomonadales	1.77 ± 0.25a	3.96 ± 0.46b	2.69 ± 0.42 ab	1.62 ± 0.49a	1.82 ± 0.48a	2.15 ± 0.43a
Cyclobacteriaceae	19.11 ± 5.09b	1.82 ± 0.21a	5.03 ± 1.25a	8.03 ± 5.01a	5.87 ± 2.75a	5.23 ± 2.77a
Rhodobacteraceae	15.66 ± 1.67 ab	9.11 ± 2.19a	12.64 ± 2.74 ab	20.08 ± 3.57b	10.43 ± 2.78a	12.97 ± 3.14 ab
Algoriphagus	19.11 ± 5.09b	1.82 ± 0.21a	5.03 ± 1.25a	8.03 ± 5.01a	5.87 ± 2.75a	5.23 ± 2.77a
Bacillales	0.085 ± 0.016a	0.138 ± 0.017 ab	0.224 ± 0.058b	0.094 ± 0.003a	0.070 ± 0.010a	0.040 ± 0.029a
Bacillaceae	0.058 ± 0.016a	0.083 ± 0.015 ab	0.153 ± 0.050b	0.213 ± 0.141 ab	0.034 ± 0.016a	0.009 ± 0.005a
Bacillus	0.046 ± 0.005a	0.085 ± 0.009a	0.153 ± 0.050b	0.075 ± 0.003a	0.056 ± 0.011a	0.016 ± 0.003a

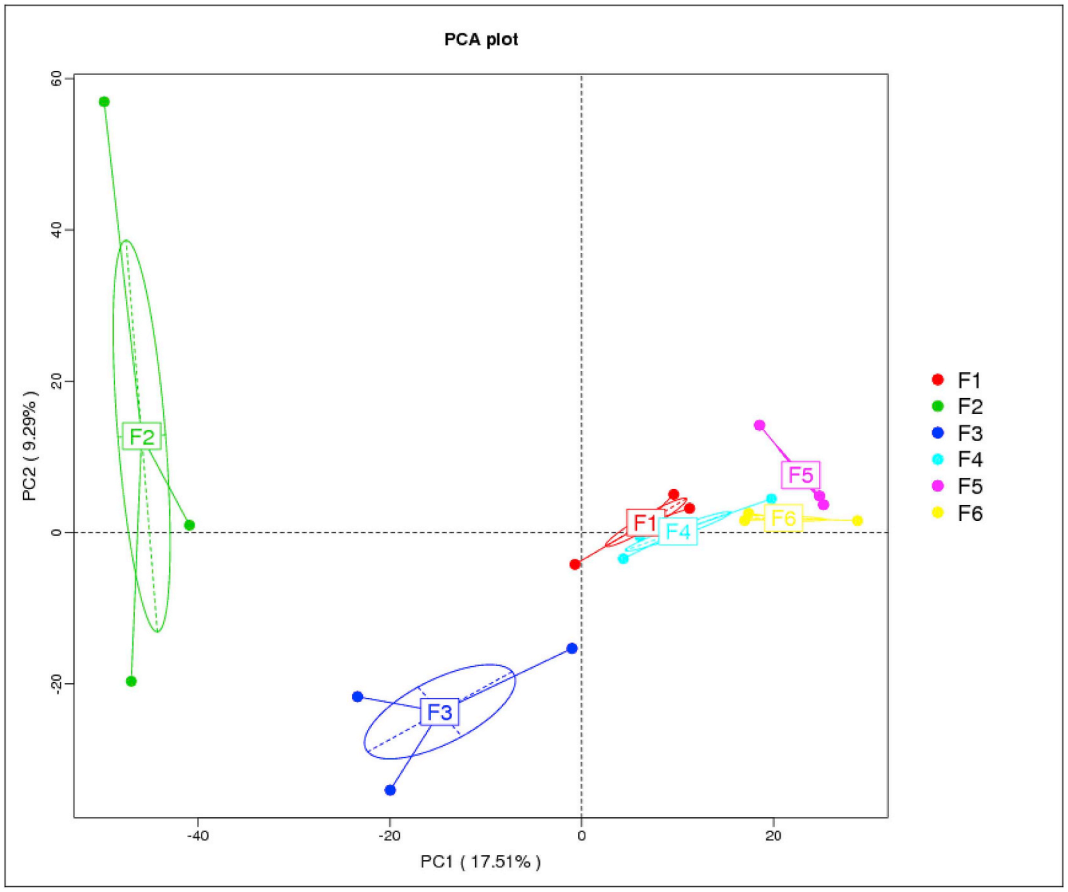

Fig. 3. Principal component analysis (PCA) plot.

and the presence of the probiotics may in some way stimulate endogenous enzymes produced by the shrimp [30]. The increases in activities of digestive enzymes in probiotic treatments may lead to enhanced digestion and increased absorption of food, which in turn contributed to the improved growth performance and feed utilization. Besides the increasing activities of enzymes, shrimp fed diets supplemented mixed-species probiotics had higher intestinal villi than control groups. The villi are important for digestion and absorption in intestine. To some extent, the villi heights reflect the function of the intestinal wall [33]. Study on broilers suggested that dietary inclusion of

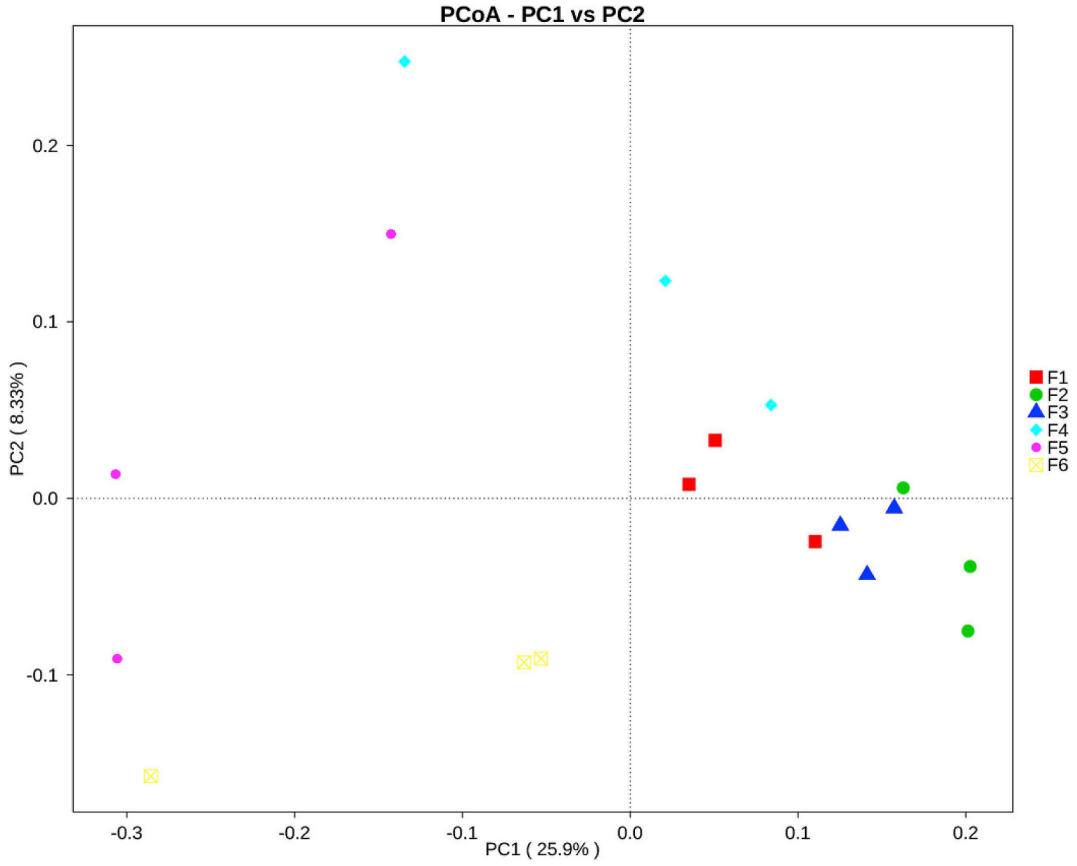

Fig. 4. Principal coordinate analysis (PCoA) plot based on the 16S rRNA sequencing genes from 18 samples. The scatter plot is of principal coordinate 1 (PC1) vs principal coordinate 2(PC2). The percentages are the percentage of variation explained by the components.

probiotics had increased the villi heights and led to better nutrient absorption and better growth performance [34]. Standen showed that probiotics on tilapia may improve the microvilli density and microvilli length, thus increasing the intestinal absorptive surface area and promoting growth performance [35].

In response to bacterial ingestion, invertebrate animals produce ROS and the express antimicrobial peptides [36]. ROS-dependent innate immunity effectively operates in the majority of infections in some certain tissues [37]. The excessive ROS can be removed by internal antioxidants and anti-oxidative systems, normally [38]. Total antioxidant capacity (T-AOC) is an overall indicator of the antioxidant status of an individual, representing the level of enzyme and non-enzyme original antioxidant of the body [39]. As it increases, the antioxidant defense against free radical reaction and reactive oxygen intermediates increases [40]. Results showed that 1000 mg/kg and 2000 mg/kg mixed-species probiotics had higher T-AOC than control groups, suggesting that probiotics can improve the antioxidant status. Tissue MDA content is widely used as biomarker for oxidative damage of lipids, which may result from generation of excess reactive oxygen species (ROS) [37]. SOD is considered to be molecular biomarkers for evaluating the oxidative stress status of aquatic organisms, due to its catalyzing the dismutation of superoxide anions to hydrogen peroxide

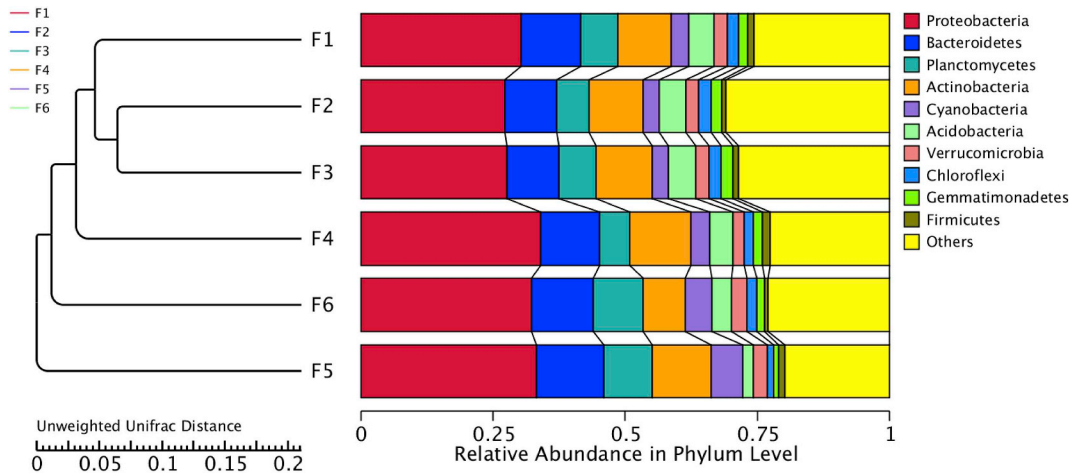

Fig. 5. UniFrac UPGMA cluster of microbial communities associated with graded supplemented mixed species probiotcs gut samples. The figure was constructed on the basis of Illumina sequencing data.

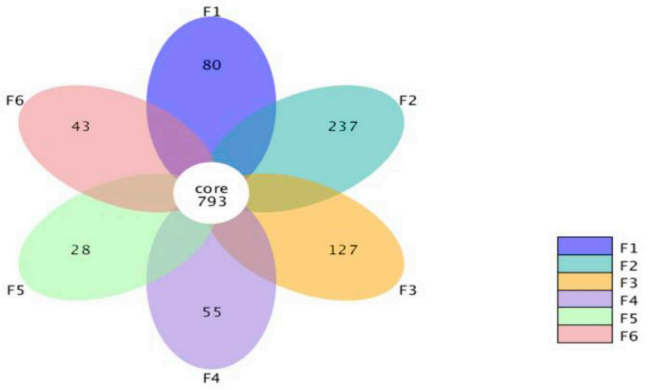

Fig. 6. Venn diagram of OUT in six treatments.

and molecular oxygen, forming the first line antioxidant enzymatic defense [41]. CAT is an important oxidoreductase enzyme that catalyzes the conversion of hydrogen peroxide molecules into water and oxygen during immune responses in different species [42]. MDA decreased with dietary mixed-species probiotics supplemented. SOD and CAT showed the similar trend, increasing at first and then decreasing and leveling off, suggesting that dietary mixed-species probiotics improved shrimp hepatopancreas antioxidant capacity.

ACP and ALP were composed of many kinds of phosphomonoesterases, which was very important to the crustacea immune system [43]. ACP was a sign of lysosome activity to digest the invading organisms [44] and ALP was a kind of phosphomonoesterase to detoxify during the normal living and phagolysis and to digest and absorb many nutriments [45]. The alkaline phosphatase and acid phosphatase in 2000 mg/kg were significant higher than control groups. Lysozyme is a component of the innate immune system in invertebrates, functioning as an antibacterial protein [46]. It hydrolyzes mucopolysaccharides, which are basic components of bacterial cell wall and kill pathogens [47]. Shrimp fed diets containing mixed-species probiotics showed higher LYZ activities in the present study. Similar researches were reported on rainbow trout [48] and Yoshitomi tilapia [49]. All indices considered, mixed-species probiotics boosted the shrimp immunity.

To assess the diversity between the mixed-species probiotics groups and control groups, α-diversity metrics were calculated to identify species richness and diversity within each sample. Among these α-diversity indices, chao1 and ACE were used to estimate the species richness of microbiome [50]; observed species is the amount of unique OTUs found in each sample; and shannon indices estimate the diversity of microbial communities. Species richness is simply prediction of species number, and it does not take into account the abundances of the species or their relative abundance distributions [51]. Species diversity takes into account both species richness and species evenness [52]. Chao1, observed species, ACE and shannon indices all showed a significant difference between 1000, 2000 mg/kg mixed-species probiotics groups and control groups, suggesting that the greater diversity of bacterial species in mixed-species probiotics groups than in control groups. With mixed-species probiotics supplemented, observed species increased at first and then decreased, observed species in F5 and F6 groups significantly lower than that in F1 groups. Observed species and ACE showed a similar trend with no significant difference, indicating that 1000/2000 mg/kg mixed-specices probiotics enhance shrimp gut microbiota richness and diversity, but high concentrations of probiotics may decrease the species richness of shrimp gut microbiota. In our data, the phylogenetic method PD whole tree revealed significant differences between different groups, indicating when taxonomy is taken into account those differences even out as the different OTUs may be mapped to the different taxonomic assignment [53]. All indices taken into account, 1000/2000 mg/kg mixed-species probiotics may improve both species richness and species evenness of shrimp microbiota, which means that there is more colonization resistance to a pathogen [54]. Our findings further indicated influences of mixed-species probioticcs on the intestinal bacterial composition of Pacific white shrimp. Principal component analysis (PCA) and principal co-ordinates analysis (PCoA) plots were used to further assess and visualize beta diversity. A clear shift of F2 and F3 groups from the control groups in microbial community structure was observed based on the PCA plot, suggesting that probiotics was a critical factor in shaping gut microbiota. The observation that the core microbial populations accounted for a significant fraction of total populations in shrimp guts under graded concentrations of probiotics indicated that the core populations could tolerate supplementation of probiotics. Among those phyla, *proteobacteria* and *bacteroidetes* were predominated in pacific white shrimp intestines and this was consistent with the previous findings [55–57]. *Proteobacteria* is widely dispersed in marine environments and might participate in the degradation of complex compounds [58]. However, a bloom of *proteobacteria* in the gut reflcts dysbiosis or an unstable gut microbial community structure, which may trigger inflammatory responses [59]. The 2000 mg/kg mixed-species probiotics decreased the content of *proteobacteria* as the results showed, indicated that probiotics may maintain the homeostasis of pacific white shrimp. *Actinobacteria* phylum, plays a pivotal role in the development and maintenance of gut homeostasis, modulating gut permeability, immune system and metabolism [60]. *Actinobacteria* increased with the mixed-species probiotic added, indicating that it may be beneficial to the growth and immunology of shrimp. The ability to balance the host gut microbiota with probiotics has been documented. The presence of the mixed probiotic significantly improved shrimp growth performance and survival rate in most treatments in this present study mainly by increasing the abundance of the *Bacillus* interacting with other bacteria. *Bacillus* has been proven to produce antimicrobial substances that are active against many microbes [61]. Research showed that the probiotic *Bacillus* supplementation caused the increase in the lipase and amylase activity regardless of its concentration [62].

Lactobacillus is a facultative anaerobic bacterium, which is difficult to maintain at desired viability during storage as they do not form spores to cope with adverse conditions, such as feed processing [63]. The application of non-viable beneficial microbes was due to certain practical applicability and functionality issues associated with viable microbes [64]. Many beneficial effect are involved from the application of live form of probiotics and non-viable form of probiotics [65].

There is increasing trends that mixed multiple probiotics were used because many bacteria have symbiotic relationships with each other, which needs further analysis.

5. Conclusion

Supplementation of mixed species probiotics can promote growth performance of juvenile pacific white shrimp, *Litopenaeus vannamei*, enhance the non-specific immunity, influence the microbiota by increasing the abundance of the *Bacillus*. The recommended optimum dosage in diet of *Litopenaeus vannamei* was 2000 mg/kg.

Authors' contributions

The authors thank the participants who gave their time to the trial. Jin Niu, Yong-Jian Liu and Li-Xia Tian designed the study. Jia-Jun Xie and Qiang-Qiang Liu carried out the rearing work and analyzed the results and wrote the paper with contributions from the other authors. There are no conflicts of interest.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in Sun Yat-sen University, Guangzhou, China, and conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85–23, revised 1985).

Availability of data and materials

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References

[1] C.D. B, D.K. Meena, B.K. Behera, P. Das, P.K. Das Mohapatra, A.P. Sharma, Probiotics in fish and shellfish culture: immunomodulatory and ecophysiological responses, Fish Physiol. Biochem. 40 (3) (2014) 921–971.
[2] J.W. Pridgeon, Major Bacterial Diseases in Aquaculture and Their Vaccine Development vol. 7, (2013) 48.
[3] M.J. Costello, The global economic cost of sea lice to the salmonid farming industry, J. Fish Dis. 32 (1) (2010) 115–118.
[4] V. Kiron, Fish immune system and its nutritional modulation for preventive health care, Anim. Feed Sci. Technol. 173 (1–2) (2012) 111–133.
[5] A. Koh, F. De Vadder, P. Kovatcheva-Datchary, F. Backhed, From dietary fiber to host physiology: short-chain fatty acids as key bacterial metabolites, Cell 165 (6) (2016) 1332–1345.
[6] A.K. Ray, K. Ghosh, E. Ringo, Enzyme-producing bacteria isolated from fish gut: a review, Aquacult. Nutr. 18 (5) (2012) 465–492.
[7] A. Bairagi, K. Sarkar Ghosh, S.K. Sen, A.K. Ray, Evaluation of the nutritive value of Leucaena leucocephala leaf meal, inoculated with fish intestinal bacteria Bacillus subtilis and Bacillus circulans in formulated diets for rohu, Labeo rohita (Hamilton) fingerlings, Aquacult. Res. 35 (5) (2015) 436–446.
[8] B. Mohan, R. Kadirvel, A. Natarajan, M. Bhaskaran, Effect of probiotic supplementation on growth, nitrogen utilisation and serum cholesterol in broilers, Br. Poult. Sci. 37 (2) (1996) 395–401.
[9] R. Fuller, Probiotics in man and animals, J. Appl. Bacteriol. 66 (5) (2010) 365–378.
[10] T.L. Korkea-Aho, J. Heikkinen, K.D. Thompson, W.A. Von, B. Austin, Pseudomonas sp. M174 inhibits the fish pathogen Flavobacterium psychrophilum, J. Appl. Microbiol. 111 (2) (2011) 266.
[11] S. Nikoskelainen, S. Salminen, G. Bylund, A.C. Ouwehand, Characterization of the properties of human- and dairy-derived probiotics for prevention of infectious diseases in fish, Appl. Environ. Microbiol. 67 (6) (2001) 2430.
[12] S.K. Nayak, Probiotics and immunity: a fish perspective, Fish Shellfish Immunol. 29 (1) (2010) 2–14.
[13] M. Chabrillón, R.M. Rico, S. Arijo, P. Díaz-Rosales, M.C. Balebona, M.A. Moriñigo, Interactions of microorganisms isolated from gilthead sea bream, Sparus aurata L., on Vibrio harveyi, a pathogen of farmed Senegalese sole, Solea senegalensis (Kaup), J. Fish Dis. 28 (9) (2005) 531–537.
[14] P.A.W. Robertson, C. O'Dowd, C. Burrells, P. Williams, B. Austin, Use of Carnobacterium sp. as a probiotic for Atlantic salmon (Salmo salar L.) and rainbow trout (Oncorhynchus mykiss , Walbaum), Aquaculture 185 (3–4) (2000) 235–243.
[15] M. Chabrillón, R.M. Rico, M.C. Balebona, M.A. Moriñigo, Adhesion to sole, Solea senegalensis Kaup, mucus of microorganisms isolated from farmed fish, and their interaction with Photobacterium damselae subsp. piscicida, J. Fish Dis. 28 (4) (2005) 229–237.
[16] A. Panigrahi, V. Kiron, S. Satoh, I. Hirono, T. Kobayashi, H. Sugita, J. Puangkaew, T. Aoki, Immune modulation and expression of cytokine genes in rainbow trout Oncorhynchus mykiss upon probiotic feeding, Dev. Comp. Immunol. 31 (4) (2007) 372–382.
[17] D.H. Kim, B. Austin, Cytokine expression in leucocytes and gut cells of rainbow trout, Oncorhynchus mykiss Walbaum, induced by probiotics, Vet. Immunol. Immunopathol. 114 (3–4) (2006) 297.
[18] J.M. Laparra, Y. Sanz, Interactions of gut microbiota with functional food components and nutraceuticals, Pharmacol. Res. 61 (3) (2010) 219–225.
[19] J. Niu, C.H. Li, Y.J. Liu, L.X. Tian, X. Chen, Z. Huang, H.Z. Lin, Dietary values of astaxanthin and canthaxanthin in Penaeus monodon in the presence and absence of cholesterol supplementation: effect on growth, nutrient digestibility and tissue carotenoid composition, Br. J. Nutr. 108 (1) (2012) 80–91.
[20] G.W. Latimer, Official Methods of Analysis of AOAC International, AOAC international, 2012.
[21] Å. Krogdahl, A. Bakke-McKellep, G. Baeverfjord, Effects of graded levels of standard soybean meal on intestinal structure, mucosal enzyme activities, and pancreatic response in Atlantic salmon (Salmo salar L.), Aquacult. Nutr. 9 (6) (2003) 361–371.
[22] J.G. Caporaso, C.L. Lauber, W.A. Walters, D. Berglyons, C.A. Lozupone, P.J. Turnbaugh, N. Fierer, R. Knight, Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample, Proc. Natl. Acad. Sci. U. S. A 108 (Supplement_1) (2011) 4516–4522.
[23] Q. Wang, G.M. Garrity, J.M. Tiedje, J.R. Cole, Naïve bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy, Appl. Environ. Microbiol. 73 (16) (2007) 5261.
[24] J. Mariani, C. Favero, A. Spinazzè, D.M. Cavallo, M. Carugno, V. Motta, M. Bonzini, A. Cattaneo, A.C. Pesatori, V. Bollati, Short-term particulate matter exposure influences nasal microbiota in a population of healthy subjects, Environ. Res. 162 (2018) 119–126.
[25] C.L. Daniels, D.L. Merrifield, D.P. Boothroyd, S.J. Davies, J.R. Factor, K.E. Arnold, Effect of dietary Bacillus spp. and mannan oligosaccharides (MOS) on European lobster (Homarus gammarus L.) larvae growth performance, gut morphology and gut microbiota, Aquaculture 304 (1) (2010) 49–57.
[26] S. Rengpipat, W. Phianphak, S. Piyatiratitivorakul, P. Menasveta, Effects of probiotic bacterium on black tiger shrimp Penaeus monodon survival and growth, Aquaculture 167 (3–4) (1998) 301–313.
[27] Y.B. Wang, Effect of probiotics on growth performance and digestive enzyme activity of the shrimp Penaeus vannamei, Aquaculture 269 (1) (2007) 259–264.
[28] Y. Wang, Z. Xu, Effect of probiotics for common carp (Cyprinus carpio) based on growth performance and digestive enzyme activities, Anim. Feed Sci. Technol. 127 (4) (2006) 283–292.
[29] H.A. Hong, H.D. Le, S.M. Cutting, The use of bacterial spore formers as probiotics, FEMS Microbiol. Rev. 29 (4) (2005) 813–835.
[30] S. Ziaeinejad, M.H. Rezaei, G.A. Takami, D.L. Lovett, A.R. Mirvaghefi, M. Shakouri, The effect of Bacillus spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp Fenneropenaeus indicus, Aquaculture 252 (2) (2006) 516–524.
[31] C.H. Liu, C.S. Chiu, P.L. Ho, S.W. Wang, Improvement in the growth performance of white shrimp, Litopenaeus vannamei, by a protease-producing probiotic, Bacillus subtilis E20, from natto, J. Appl. Microbiol. 107 (3) (2009) 1031–1041.
[32] D.J.W. Moriarty, Microbial biotechnology: a key ingredient for sustainable aquaculture, Infofish Int 4 (1996) 29–33.
[33] N.K. Emami, A. Samie, H.R. Rahmani, C.A. Ruiz-Feria, The effect of peppermint essential oil and fructooligosaccharides, as alternatives to virginiamycin, on growth performance, digestibility, gut morphology and immune response of male broilers, Anim. Feed Sci. Technol. 175 (1–2) (2012) 57–64.
[34] A.R. Al-Fataftah, A. Abdelqader, Effects of dietary Bacillus subtilis on heat-stressed broilers performance, intestinal morphology and microflora composition, Anim. Feed Sci. Technol. 198 (2014) 279–285.
[35] B.T. Standen, A. Rodiles, D.L. Peggs, S.J. Davies, G.A. Santos, D.L. Merrifield, Modulation of the intestinal microbiota and morphology of tilapia, Oreochromis niloticus, following the application of a multi-species probiotic, Appl. Microbiol. Biotechnol. 99 (20) (2015) 8403–8417.
[36] N. Buchon, N.A. Broderick, B. Lemaitre, Gut homeostasis in a microbial world: insights from Drosophila melanogaster, Nat. Rev. Microbiol. 11 (9) (2013) 615–626.
[37] J. Zhao, L. Feng, Y. Liu, W. Jiang, P. Wu, J. Jiang, Y. Zhang, X. Zhou, Effect of dietary isoleucine on the immunity, antioxidant status, tight junctions and microflora in the intestine of juvenile Jian carp (Cyprinus carpio var. Jian), Fish Shellfish Immunol. 41 (2) (2014) 663–673.
[38] Y.Y. Chen, P.C. Lee, Y.L. Wu, L.Y. Liu, In vivo effects of free form astaxanthin powder on anti-oxidation and lipid metabolism with high-cholesterol diet, PLoS One 10 (8) (2015) e0134733.
[39] N. Xiao, X.C. Wang, Y.F. Diao, R. Liu, K.L. Tian, Effect of initial fluid resuscitation on subsequent treatment in uncontrolled hemorrhagic shock in rats, Shock 21 (3) (2004) 276–280.
[40] Y.-H. Chien, C.-H. Pan, B. Hunter, The resistance to physical stresses by Penaeus monodon juveniles fed diets supplemented with astaxanthin, Aquaculture 216 (1) (2003) 177–191.
[41] A. Valavanidis, T. Vlahogianni, M. Dassenakis, M. Scoullos, Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants, Ecotoxicol. Environ. Saf. 64 (2) (2006) 178–189.
[42] H.T. Yang, M.C. Yang, J.J. Sun, F. Guo, J.F. Lan, X.W. Wang, X.F. Zhao, J.X. Wang, Catalase eliminates reactive oxygen species and influences the intestinal microbiota of shrimp, Fish Shellfish Immunol. 47 (1) (2015) 63–73.
[43] C. Yang, J. Kong, Q. Wang, Q. Liu, Y. Tian, K. Luo, Heterosis of haemolymph analytes of two geographic populations in Chinese shrimp Fenneropenaeus chinensis, Fish Shellfish Immunol. 23 (1) (2007) 62–70.
[44] S.Q. Liu, X.L. Jiang, H.J. Mou, H.M. Wang, H.S. Guan, Effects of Immunopolysaccharide on LSZ, ALP, ACP and POD Activities of Penaeus Chinensis

Serum, (1999).
[45] M. Zhang, L. Wang, Z.Y. Guo, B.J. Wang, Effect of lipopolysaccharide and Vibrio anguillarum on the activities of phosphatase,superoxide dismutase and the content of hemocyanin in the serum of Fenneropenaeus chinesis, Mar. Sci. 28 (7) (2004) 22–25.
[46] R.R. Sotelo-Mundo, M.A. Islas-Osuna, E. De-La-Re-Vega, J. Hernández-López, F. Vargas-Albores, G. Yepiz-Plascencia, cDNA cloning of the lysozyme of the white shrimp Penaeus vannamei, Fish Shellfish Immunol. 15 (4) (2003) 325–331.
[47] M. Samuel, T.J. Lam, Y.M. Sin, Effect of Laminaran [β(1,3)-D-Glucan] on the protective immunity of blue gourami, Trichogaster trichopterus against Aeromonas hydrophila, Fish Shellfish Immunol. 6 (6) (1996) 443–454.
[48] A. Newajfyzul, A.A. Adesiyun, A. Mutani, A. Ramsubhag, J. Brunt, B. Austin, Bacillus subtilis AB1 controls Aeromonas infection in rainbow trout (Oncorhynchus mykiss, Walbaum), J. Appl. Microbiol. 103 (5) (2007) 1699.
[49] L. Tang, K. Huang, J. Xie, D. Yu, L. Sun, Q. Huang, Y. Bi, 1-deoxynojirimycin from Bacillus subtilis improves antioxidant and antibacterial activities of juvenile Yoshitomi tilapia, Electron. J. Biotechnol. 30 (C) (2017).
[50] M. Hamady, R. Knight, Microbial community profiling for human microbiome projects: tools, techniques, and challenges, Genome Res. 19 (7) (2009) 1141–1152.
[51] K. Robert Colwell, III.1 Biodiversity: Concepts, Patterns, and Measurement : the Princeton Guide to Ecology, (2009).
[52] C.E. Moreno, P. Rodríguez, A consistent terminology for quantifying species diversity? Oecologia 163 (2) (2010) 279–282.
[53] C.E. Dehler, C.J. Secombes, S.A. Martin, Environmental and physiological factors shape the gut microbiota of Atlantic salmon parr (Salmo salarL.), Aquaculture 467 (2017) 149.
[54] D.J. Hodgson, P.B. Rainey, A. Buckling, Mechanisms linking diversity, productivity and invasibility in experimental bacterial communities, Proc. Biol. Sci. 269 (1506) (2002) 2277–2283.
[55] Z. Huang, X. Li, L. Wang, Z. Shao, Changes in the intestinal bacterial community during the growth of white shrimp, Litopenaeus vannamei, Aquacult. Res. 47 (6) (2016) 1737–1746.
[56] Y. Zheng, M. Yu, Y. Liu, Y. Su, T. Xu, M. Yu, X.H. Zhang, Comparison of cultivable bacterial communities associated with Pacific white shrimp (Litopenaeus vannamei) larvae at different health statuses and growth stages, Aquaculture 451 (2016) 163–169.
[57] W. Rungrassamee, A. Klanchui, S. Maibunkaew, N. Karoonuthaisiri, Bacterial dynamics in intestines of the black tiger shrimp and the Pacific white shrimp during Vibrio harveyi exposure, J. Invertebr. Pathol. 133 (2016) 12–19.
[58] M.T. Cottrell, L. Kirchman, Natural Assemblages of Marine Proteobacteria and Members of the Cytophaga Flavobacter Cluster Consuming Low- and High-Molecularweight Dissolved Organic, (2013), (2000).
[59] N.R. Shin, T.W. Whon, J.W. Bae, Proteobacteria : microbial signature of dysbiosis in gut microbiota, Trends Biotechnol. 33 (9) (2015) 496–503.
[60] C. Binda, L.R. Lopetuso, G. Rizzatti, G. Gibiino, V. Cennamo, A. Gasbarrini, Actinobacteria: a relevant minority for the maintenance of gut homeostasis, Dig. Liver Dis. 50 (5) (2018) 421–428.
[61] M. Kavitha, M. Raja, P. Perumal, Evaluation of probiotic potential of Bacillus spp. isolated from the digestive tract of freshwater fish Labeo calbasu (Hamilton, 1822), Aquac. Reports. 11 (2018) 59–69.
[62] A.I. Zaineldin, S. Hegazi, S. Koshio, M. Ishikawa, A. Bakr, A.M.S. El-Keredy, M.A.O. Dawood, S. Dossou, W. Wang, Z. Yukun, Bacillus Subtilis as Probiotic Candidate for Red Sea Bream: Growth Performance, Oxidative Status, and Immune Response Traits Fish Shellfish Immunol vol. 79, (2018), pp. 303–312.
[63] S.K. Nayak, Role of gastrointestinal microbiota in fish, Aquacult. Res. 41 (11) (2010) 1553–1573.
[64] G. Dash, R.P. Raman, K.P. Prasad, M. Makesh, M.A. Pradeep, S. Sen, Evaluation of paraprobiotic applicability of Lactobacillus plantarum in improving the immune response and disease protection in giant freshwater prawn, Macrobrachium rosenbergii (de Man, 1879), Fish Shellfish Immunol. 43 (1) (2015) 167–174.
[65] C. A, Adams the probiotic paradox: live and dead cells are biological response modifiers, Nutr. Res. Rev. 23 (1) (2010) 37–46.