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Dietary prebiotic inulin benefits on growth performance, antioxidant capacity, immune response and intestinal microbiota in Pacific white shrimp (*Litopenaeus vannamei*) at low salinity

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ABSTRACT

Dietary manipulation is a useful approach to improve production and health of farmed shrimp. However, no study has investigated the effect of prebiotic inulin on intestinal microbiota response and physiological status of shrimp at low salinity of 3 psu (practical salinity unit). The effects of dietary inulin additive at 0%, 0.1%, 0.2%, and 0.4% on growth performance, antioxidant capacity, immune response and intestinal microbiota of Pacific white shrimp (*Litopenaeus vannamei*) at a low salinity of 3 psu were evaluated after an 8-week feeding trial. Shrimp fed the 0.2% and 0.4% inulin diet significantly increased final weight, weight gain, specific growth rate compared to those fed the control diet. Body ash content tended to increases with the increasing level of inulin. Intestinal amylase and hepatopancreas superoxide dismutase (SOD) activity in 0.4% inulin group were higher than in control group. The acid phosphatase (ACP) and phenol oxidase (PO) activities of hepatopancreas were significantly increased in 0.2% and 0.4% inulin group, respectively, compared to control group. Shrimp fed the 0.2% and 0.4% inulin diet reduced hepatopancreas oxidative stress by increasing the catalase (CAT) activity and decreasing the malondialdehyde (MDA) content compared to those fed the control diet. Shrimp fed 0.4% inulin changed intestinal microbiota by increasing the relative abundance of *Firmicutes* phylum and *Bacillus* genus. PICRUSt analysis show that the KEGG pathway involved in aldosterone-regulated sodium reabsorption was significantly increased in shrimp fed 0.4% inulin. The shrimp fed 0.4% inulin exhibited more negative interspecies interactions than those fed the control diet. This study suggests that inulin can serve as a potential feed additive that helps shrimp to cope with low salinity stress.

1. Introduction

The Pacific white shrimp (*Litopenaeus vannamei*) is one of the most important shrimp species in global aquaculture. To further explore more natural resources for expansion of shrimp farming areas and to meet the increasing demand of seafood, *L. vannamei* farming in low-salinity water has been practiced in many regions (Li et al., 2017). Although white shrimp has been found at salinities range from < 1 (practical salinity unit) psu to over 40 psu (Castille and Lawrence, 1981), this does not mean that shrimp can achieve maximum growth, survival and immune response, and various adverse effects have been reported, including lower specific growth rate, food consumption and absorption efficiency (Wang et al., 2006), decreased final shrimp body weight (Decamp et al., 2003) and survival (Laramore et al., 2001; Li et al., 2007a), impaired immune response (Lamela et al., 2005; Lin et al., 2012; Xu et al., 2017), disturbed gut microbiota composition and construction (Zhang et al., 2016). and increased susceptibility to pathogens (Wang and Chen, 2005; Lin et al., 2012). Therefore, it is necessary to find a safe and effective method to solve these problems.

Dietary manipulation is a useful approach to alleviate negative effects by low salinity stress in shrimp farming (Chen et al., 2015; Romano and Zeng, 2012). Previous research has indicated that dietary supplementation with minerals (Huang et al., 2017), amino acids (Xie et al., 2014), cholesterol (Roy et al., 2006), carbohydrates (Wang et al., 2015), lipids (Chen et al., 2015), proteins (Li et al., 2011) and β-glucan (Li et al., 2019) could improve the ability of shrimp to cope with low

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salinity stress. However, no attempt has been made to investigate the effects of inulin on shrimp at low salinity. Prebiotic is a substrate that is selectively utilized by host microorganisms conferring a health benefit (Gibson et al., 2017). Prebiotics including inulin (Hoseinifar et al., 2015; Li et al., 2018b; Luna-González et al., 2012; Partida-Arangure et al., 2013), β-glucan (Boonanuntanasarn et al., 2016; Li et al., 2019), mannanoligosaccharides (MOS) (Li et al., 2018b; Sang et al., 2014; Zhang et al., 2012), fructooligosaccharides (FOS) (Zhou et al., 2007), isomaltooligosaccharides (IMO) (Li et al., 2009), xylooligosaccharides (XOS) (Wang et al., 2010) were studied in shrimp because of stimulating growth performance, food utilization, positive effects on gut morphology, gut microbiota, immune system, and disease resistance (Yousefian and Amiri, 2009). However, several studies reported that dietary inulin had no obvious benefit effects, even negative effects on aquatic animals (Cerezuela et al., 2013; Reza et al., 2009). Those contradictory results suggest that the effects of prebiotic inulin on aquatic animals is case-dependent. Although inulin are now widely used in aquaculture, information about the effects on shrimp is still scarce, especially on gut microbiota of shrimp. Considering the fact that the benefits effects of inulin are driven, in part at least, by influencing the host microbiota, culture-dependent and denaturing gradient gel electrophoresis (DGGE) techniques are used to reveal the effect of dietary inulin on gut microbiota (Hoseinifar et al., 2015; Li et al., 2007b). With the advance in high-throughput sequencing technologies, 16S rRNA gene amplicon sequencing can provide a promising approach to understand the role of inulin in regulating shrimp intestinal microbiota.

To the best of our knowledge, this was the first attempt to investigate the effects of dietary inulin on growth, antioxidant capacity, immune response and gut microbiota of L. *vannamei* that reared at low salinity of 3 psu. Results obtained from this study would provide a practical solution through dietary manipulation to improve growth performance and reduce environmental stress for inland white shrimp farming at low salinities.

2. Materials and methods

2.1. Prebiotic

Inulin (Orafti® GR, CAS: 9005-80-5) was purchased form Beneo-Orafti. Prebiotic inulin is a powder food ingredient that contains about 92% inulin. It is a standard form of chicory inulin. The average degree of polymerization of inulin is equal or > 10.

2.2. Experimental diets

The experimental diets were formulated based on the nutritional requirement of L. *vannamei*. A total of four experimental diets were formulated with different levels of inulin at 0% (control), 0.1%, 0.2%, and 0.4%. All the ingredients were ground into powder through a 0.28 mm mesh sieve and thoroughly mixed before being extruded into 2 mm diameter pellets in a double helix plodder (F-26, SCUT, industrial factory, Guangdong, China). The diets were air-dried at room temperature till the moisture content was below 10% and then stored at −20 °C until use. The ingredients and proximate composition of the experimental diets is shown in Table 1.

2.3. Shrimp rearing and feeding trial

L. vannamei postlarvae (~P10) were obtained from a shrimp hatchery center in Danzhou, Hainan, China, and were acclimated for two weeks prior to the experiment. During the acclimation period, the salinity was reduced to 3 psu at a daily rate of 2–3 psu by adding freshwater into seawater. Then, shrimp were randomly divided into four treatments namely 0% (control), 0.1%, 02%, and 0.4% of inulin in 12 tanks (110 × 80 × 40 cm, three tanks per treatment, 35 shrimps per tank). Shrimp were fed three times a day at 07:00, 12:00 and 20:00 until apparent satiation with accurate records of diet application per tank and water was exchanged by 1/3 of the tank volume each day. The water quality parameters maintained at 24.5–30 °C, pH 7.5–8.0, dissolved oxygen ≥7 mg/L, and total ammonia < 0.05 mg/L. Shrimp were maintained under a natural daylight cycle.

Table 1
Dietary formulations and proximate composition of the experimental diets.

	Experiment diets			
	Control	0.1% inulin	0.2% inulin	0.4% inulin
Ingredients (g / kg)				
Fish meal	370	370	370	370
Soybean meal	280	280	280	280
Wheat starch	200	200	200	200
Fish oil	22.8	22.8	22.8	22.8
Soybean oil	25	25	25	25
Lecithin	10	10	10	10
Cholesterol	5	5	5	5
Vitamin Premix [a]	20	20	20	20
Mineral Premix [a]	5	5	5	5
Vitamin C	1	1	1	1
Sodium carboxymethyl cellulose	30	30	30	30
Cellulose	11.2	10.2	9.2	7.2
Calcium carbonate	20	20	20	20
inulin	0	1	2	4
Proximate compositions (g / kg)				
Moisture	94.2	91.7	90.1	88.9
Crude protein	346	355	353	349
Crude lipid	87.5	88.1	88.3	88.7
Ash	115.3	109.4	111.7	114.2

[a] Same composition as reported by Li et al., 2019

2.4. Sample collection

At the end of eight weeks of the feeding trial, shrimp were starved for 24 h before sampling. The weight of hepatopancreas and the length of shrimp were also measured. Five shrimps from each tank were preserved at −20 °C to determine body proximate composition. The body surface of each shrimp was sterilized with 70% ethanol, the intestine and hepatopancreas samples were aseptically extracted and placed into a 1.5 mL sterile centrifuge tube. One part of intestine sample was used for intestinal microbial analysis, considering the inter-individual variation of intestinal microbiota, the intestines of five shrimps were pooled as one sample, with three samples in total per group. The rest intestine samples were used for determination of digestive enzymes activities. Hepatopancreas samples were for determination of antioxidant and immune enzymes activities. All samples were collected and immediately stored in liquid nitrogen before being transferred to the lab for preservation at −80 °C.

2.5. Growth performance

Shrimp in each tank were counted and weighted to calculated survival and final weight of shrimp, respectively. Ten shrimps from each tank were randomly selected to measure their body length and body weight. The hepatopancreas of each shrimp was extracted and weighed for computation of hepatosomatic index. Growth parameters were calculated as below:

$$\text{Weight gain (WG,\%)} = (\text{final weight} - \text{initial weight})/\text{initial weight} \times 100$$

$$\text{Survival (\%)} = \text{final number of shrimp}/\text{initial number of shrimp} \times 100$$

$$\text{Specific growth rate (SGR,\%day}^{-1}\text{)} = 100 \times (\text{Ln final weight} - \text{Ln initial weight})/\text{number of days}$$

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

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

Hepatosomatic index (HIS,%)
= hepatopancreas weight/final weight × 100

2.6. Chemical analysis of diet and shrimp composition

The proximate composition of the whole shrimp and experimental diets was analyzed following the standard procedures. Moisture content was estimated by drying at 105 °C to a constant weight. Crude protein was determined by the Kjeldahl method using an Auto Kjeldahl System (Kjeltec8100, FOSS, Sweden). Crude lipid was calculated by the Soxhlet extraction method. Ash content was determined by incinerating samples at 550 °C in a muffle furnace.

2.7. Biochemical assays

The entire intestinal of three shrimps mixed as one sample, the hepatopancreas of one shrimp as one sample, each group have six samples. All samples were weighed and homogenized with 9 vol (*v/w*) of 0.86% physiological saline. Then, the homogenate was centrifuged at 2500 ×*g* at 4 °C for 10 min and the supernatant was collected for biochemical assays according to the manufacturer's instruction. Activities of intestinal protease, amylase, and lipase were measured using an enzyme-linked immune sorbent assay (ELISA) kit (Shanghai Hengyuan Biotechnology Co, Ltd) (Li et al., 2019). Phenol oxidase (PO) activity in hepatopancreas was measured using an enzyme-linked immune sorbent assay (ELISA) kit (Nanjing Jiancheng Institute, China). The contents of malondialdehyde (MDA), total superoxide dismutase (T-SOD), catalase (CAT), acid phosphatase (ACP) in hepatopancreas were measured by using specific commercial assay kits (Nanjing Jiancheng Institute, China) (Zhang et al., 2008). Results were recorded on a microplate reader (Epoch, BioTek, USA).Total SOD activity was determined by using WST-1 method. One unit of SOD activity was defined as the amount of enzyme required for 1 mg tissue proteins in 1 mL of a reaction mixture SOD inhibition rates to 50% as monitored at 550 nm. CAT activity was determined by using ammonium molybdate colorimetric method. One unit of CAT activity was defined as 1 mg tissue proteins consumed 1 μmol H_2O_2 at 405 nm for 60 s. MDA content was determined by using TBA method, which was measured at 532 nm. ACP activity was determined by using colorimetric method. One unit of ACP activity was defined as 1 mg tissue proteins produced 1 mg phenol at 37 °C for 30 min as monitored at 550 nm. Activities of protease, amylase, lipase and phenol oxidase were measured using the enzyme-linked immunosorbent assay (ELISA) and the absorbance (OD) was measured at 450 nm. Total protein content was measured by using Coomassie brilliant blue method.

2.8. Intestinal microbiota analysis

The total intestinal bacterial DNA was isolated using a E.Z.N.A.® soil DNA kit (OMEGA, USA) according to the manufacturer's instruction. DNA quantity and quality were measured using a NanoDrop 2000 spectrophotometer (Thermo, USA), The V3-V4 region of the bacteria 16S ribosomal RNA gene was amplified by PCR using the forward primer 338F (5' ACTCCTACGGGAGGCAGCA 3′) and the reverse primer 806R (5' GGACTACHVGGGTWTCTAAT 3′). PCR reactions were performed in a 25 μL mixture containing 5 μL of 5 × reaction Buffer, 5 μL of 5 × GC buffer, 2 μL of 2.5 mM dNTPs, 1 μL of forward primer (10 μM), 1 μL of reverse primer (10 μM), 2 μL of DNA template, 8.75 μL of ddH_2O, 0.25 μL of Q5® high-fidelity DNA polymerase (NEB). The PCR conditions were as follows: initial denaturation at 98 °C for 2 min, followed by 26 cycles of denaturation at 98 °C for 15 s, annealing at 55 °C for 30 s; extension at 72 °C for 30 s, and final extension at 72 °C for 5 min. Purified PCR products were subjected to Illumina MiSeq PE300 platform (Shanghai Personal Biotechnology Co., Ltd., China), generating paired-end reads. The sequences obtained in this paper are available in SRA with the accession number SRP216804.

The Quantitative Insights Into Microbial Ecology (QIIME, v1.8.0) pipeline was employed to process the sequencing data, as previously described (Caporaso et al., 2010). Briefly, the low-quality sequences (length below 150 bp, average Phred scores < 20, mononucleotide repeats over 8 bp, and with ambiguous bases) were removed.

(Chen and Jiang, 2014; Gill et al., 2006). Averaged, rounded rarefied OTU table was generated by averaging 100 evenly resampled OTU subsets under the 90% of the minimum sequencing depth for further analysis. Operational taxonomic units (OTUs) were clustered based on a similarity cutoff of 97% using UPARSE (version 7.1 http://drive5.com/uparse/) (Edgar, 2013). The most abundant sequence in the OTU was selected as the representative sequence and then taxonomically assigned in the Greengenes database (release 13.8) using confidence threshold of 70% (DeSantis et al., 2006). Rarefaction curves were created in Mothur to determine whether sequencing depth was sufficient to cover the expected number of OTUs at the level of 97% sequence similarity. Alpha diversity indices (ACE, Chao1, Simpson, and Shannon) were calculated by QIIME. Partial least squares discriminant analysis (PLS-DA) was used to test the effect of classification model by R software. Venn diagram was constructed to identify the shared and unique OTUs. Linear discriminant analysis (LDA) effect size (LEfSe) was applied to detecting bacterial taxa that respond (i.e., increase or decrease in its relative abundance) to prebiotic inulin administration (Segata et al., 2011). The bioinformatics software package PICRUSt was used to predict the functional profiling of the intestinal microbiota and the predicted functional pathways were annotated by using the Kyoto Encyclopedia of Genes and Genomes (KEGG) at levels 1, 2 and 3 (Langille et al., 2013). The Spearman correlation coefficients between genera with abundance in the top 50 were calculated by using Mothur. Constructing interspecies interaction network with Rho > 0.6 and $P < .01$. The network was visualized by using Gephi software.

2.9. Statistical analysis

Results are presented as means ± standard error of means (SEM). One-way ANOVA followed by Duncan's multiple rang test was used to compare significant differences among the control, 0.1% inulin, 0.2% inulin, and 0.4% inulin treatments, Student's *t*-test was used to analyze differences between the control and 0.4% inulin treatments. All statistical analyses were conducted using SPSS statistics 23 (IBM, Armonk, NY, USA). A value of $P < .05$ was regarded as statistical significance.

3. Result

3.1. Growth performance and body composition

The growth performance of shrimp fed diets containing inulin is presented in Table 2. Shrimp fed 0.2% and 0.4% inulin diets had significantly higher final weight, weight gain and specific growth rate than shrimp fed control diet. Hepatosomatic index was significantly decreased in shrimp fed inulin diets compared to shrimp fed control diet. There were no significant effects of inulin supplementation on survival, feed conversion ratio and condition factor of shrimp among treatments. The whole body ash content of shrimp fed 0.4% inulin diet was significantly higher than those fed the control diets (Table 3). No significant differences were detected on moisture, crude protein and crude lipid content among all dietary treatments.

3.2. Digestive, immune enzymes activities and antioxidant capacity

Intestinal amylase activity was significantly higher in the 0.4%

Table 2
Growth performance of *Litopenaeus vannamei* fed experimental diets at 3 psu for 8 weeks.

	Experiment diets			
	Control	0.1% inulin	0.2% inulin	0.4% inulin
Initial weight (g)	0.0213 ± 0.00	0.0206 ± 0.00	0.0206 ± 0.00	0.0208 ± 0.00
Final weight (g)	2.55 ± 0.17^{a}	2.67 ± 0.24^{ab}	3.54 ± 0.05^{b}	3.63 ± 0.23^{b}
Weight gain (%)	11,879.49 ± 932.43^{a}	12,944.95 ± 1394.83^{ab}	17,129.83 ± 233.26^{b}	17,415.66 ± 1282.14^{b}
Survival (%)	88.57 ± 1.65	88.57 ± 1.65	87.62 ± 4.75	85.71 ± 4.36
Specific growth rate (% day^{-1})	8.53 ± 0.14^{a}	8.68 ± 0.19^{ab}	9.19 ± 0.24^{b}	9.22 ± 0.13^{b}
Feed conversion ration	1.86 ± 0.10	1.98 ± 0.11	1.76 ± 0.0	1.72 ± 0.07
Condition factor (%)	1.02 ± 0.02	1.07 ± 0.03	1.05 ± 0.02	1.05 ± 0.01
Hepatosomatic index (%)	6.01 ± 0.27^{a}	5.16 ± 0.21^{b}	5.23 ± 0.20^{b}	4.90 ± 0.17^{b}

Growth parameters (initial weight, final weight, weight gain, survival,specific growth rate and feed conversion ration) are expressed as mean ± SE (n = 3), condition factor and hepatosomatic index are expressed as mean ± SE (n = 30). Different letters in the same row represent significant difference (P < .05).

inulin group than in the control (Fig. 1A), while no significant difference was observed among control, 0.1% inulin, and 0.2% inulin groups. Moreover, shrimp fed the inulin diet did not show significant differences on intestinal protease and lipase activities (Fig. 1B, C). The effects of dietary inulin on immune enzymes were assessed. The ACP and PO enzyme activities of hepatopancreas in the 0.2% and 0.4% inulin groups were significantly higher than those in the control, respectively (Fig. 1G, H). The CAT activity of hepatopancreas was significantly increased in the 0.2% and 0.4% inulin groups compared to the control (Fig. 1F). By contrast, the MDA content of hepatopancreas exhibited an opposite trend (Fig. 1D). However, no significant differences were observed for CAT and MDA between the 0.1% inulin group and the control. The SOD activity of hepatopancreas was only significantly increased in the 0.4% inulin group compared to the control (Fig. 1E).

3.3. Microbiota composition and diversity

A total of 343,605 high-quality sequences were produced in this study, with an average of 57,268 sequences per sample. To minimize the difference of sequencing depth across samples, an averaged, rounded rarefied OTU table was generated by averaging 100 evenly resampled OTU subsets under the 90% of the minimum sequencing depth for further analysis. Before subsample, OTUs containing < 0.001% of total sequences across all samples were discarded. The reads of each sample after subsample are shown in Table 4. The most dominant phyla in all samples were *Proteobacteria* followed by *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia*, and *Firmicutes* (Fig. 2A). The relative abundances of *Firmicutes* was significantly increased in the 0.4% inulin group compared with control (Fig. 2B). According to the result of PLS-DA, the control group and 0.4% inulin group were clearly separated and formed two distinct clusters (Fig. 2C). The alpha diversity analysis showed that the ACE, Chao1, Simpson, and Shannon indexes tend to decrease with the supplementation of 0.2% inulin in the diet, but the difference was not significant (Table 4). The rarefaction curves were approaching the saturation plateau (Fig. 2D), which suggested that sufficient sampling depth was achieved for each sample. Venn diagram showed that 547 OTUs shared by all samples, 893 and 396 OTUs were only found in control and 0.4 inulin group, respectively (Fig. 2E). LEfSe analysis revealed that some beneficial bacteria such as *Bacillus* were significantly enriched in the 0.4% iunlin group (Fig. 2F).

3.4. Functional prediction of the intestinal microbiota

Bacterial gene functions were predicted from the 16S rRNA sequencing data by using PICRUSt. The prediction accuracy of PICRUSt can be assessed through calculating the value of the nearest sequenced taxon index (NSTI), for each sample the NSTI vaule range from 0.07 to 0.08 (average NSTI = 0.08). Comparing the 0.4% inulin group and the control group, KEGG pathways with significant difference were divided into five categories at KEGG level 1: “Metabolism”, “Genetic Information Processing”, “Cellular Processing”, “Organismal Systems” (Table 5) and “Human Diseases” (data not shown). Notably, the KEGG pathway related to aldosterone-regulated sodium reabsorption was significantly increased in the 0.4% inulin group compared to the control (Table 5).

3.5. Intestine microbiota interspecies interaction

To evaluate the effect of dietary inulin on interspecies interaction of the intestine microbiota, the interspecies interaction network was established. Shrimp fed 0.4% inulin had higher ratio of negative association as evidenced by more negative links than that in the control (Fig. 3B). The network was more connected in the 0.4% inulun group as evidenced by more links than that in the control (Fig. 3A, B).

4. Discussion

It was reported that L. *vannamei* reared at low salinity of 3 psu had lower weight gain and survival (Li et al., 2007a). In the present study, the final weight, weight gain and specific growth rate of shrimp in 0.2% inulin and 0.4% inulin groups was significantly higher than in the control group after the 8-week feeding trial. The improved growth performance is possibly related to nutrient enhancement and availability as evidenced by the increased amylase activity. Our results indicate that inulin supplementation can have a positive effect on shrimp growth at low salinity of 3 psu. Inulin has been demonstrated to

Table 3
Whole body composition of *Litopenaeus vannamei* fed experimental diets at 3 psu for 8 weeks.

	Experiment diets			
	Control	0.1% inulin	0.2% inulin	0.4% inulin
Moisture	77.57 ± 0.13	77.79 ± 0.22	77.97 ± 0.12	77.83 ± 0.20
Crude protein	14.48 ± 0.15	14.34 ± 0.18	14.31 ± 0.17	14.76 ± 0.16
Crude lipid	2.84 ± 0.04	2.77 ± 0.10	2.79 ± 0.07	2.78 ± 0.09
Ash	2.96 ± 0.07^{a}	3.03 ± 0.07^{ab}	3.10 ± 0.11^{ab}	3.35 ± 0.16^{b}

All data are expressed as mean ± SE (n = 3). Different letters in the same row represent significant difference (P < .05).

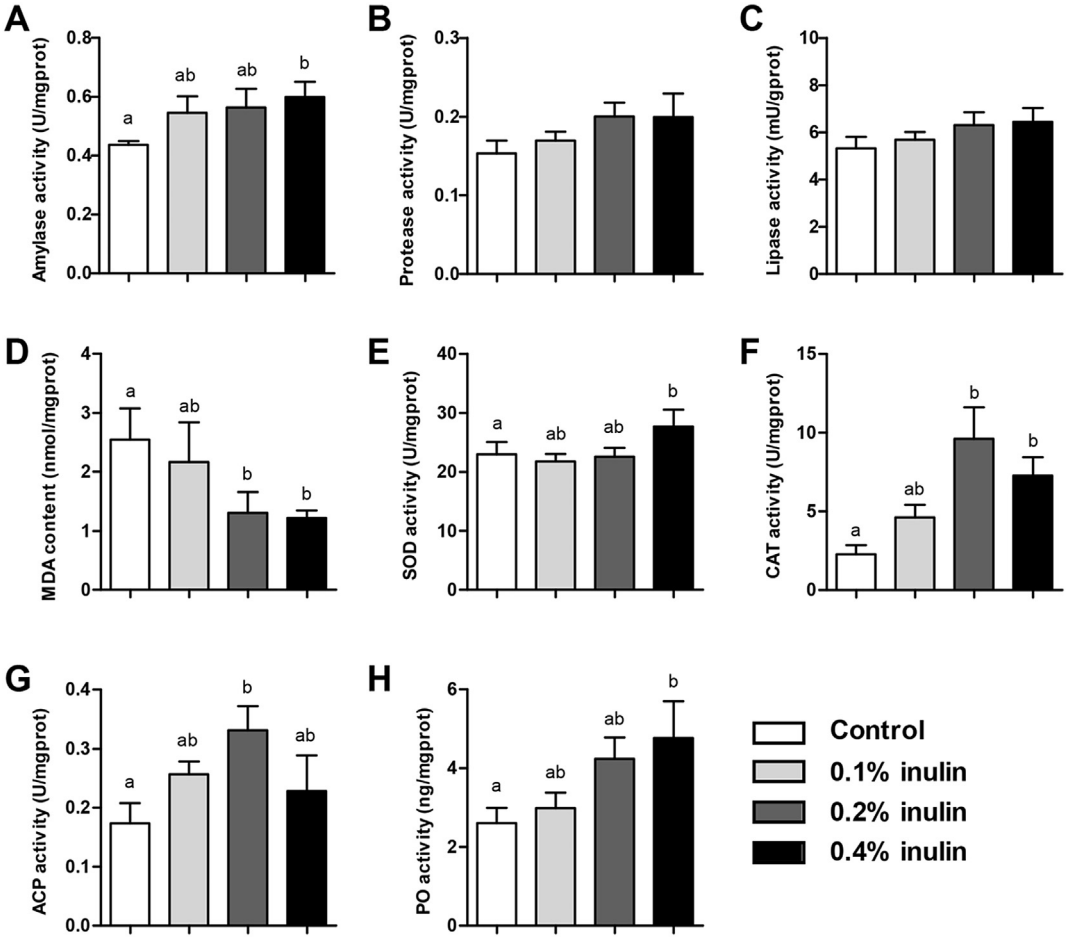


Fig. 1. Effects of dietary inulin on digestive, immune enzymes activities and antioxidant capacity in Pacific white shrimp at low salinity of 3 psu. (A) Amylase activity in intestine, (B) Protease activity in intestine, (C) Lipase activity in intestine, (D) Malondialdehyde (MDA) content in hepatopancreas, (E) Total superoxide dismutase (SOD) activity in hepatopancreas, (F) Catalase (CAT) activity in hepatopancreas, (G) Acid phosphatase (ACP) activity in hepatopancreas, (H) Phenol oxidase (PO) activity in hepatopancreas. All data are expressed as mean ± SE ($n = 6$). *$P < .05$.

enhanced specific growth rate in Pacific white shrimp when provided at 10 g/kg diet for 28 days (Li et al., 2018b). However, in a study on Pacific white shrimp for 60 days, 0.4 and 8 g/kg of dietary inulin had no significant effect on specific growth rate (Partida-Arangure et al., 2013). These inconsistent results possibly due to the difference in developmental stage, property of inulin, the dosage, the duration of application and the culture conditions.

The ash content of the whole shrimp was significantly increased by 0.4% dietary inulin supplementation, indicating that inulin-enriched diet can promote mineral element deposition. A research carried out on broiler chickens has indicated that inulin can increase the content of ash and calcium in the tibiae (Ortiz et al., 2009). Likewise, dietary supplementation with inulin can significantly increase the concentration of several minerals in the blood of Nile tilapia, including magnesium, calcium, and iron (Tiengtam et al., 2015). Prebiotics like inulin can be fermented by specific intestinal microbiota to produce short-chain fatty acids, which can decrease the pH of intestinal (Nabizadeh, 2012; Wu et al., 2019) and low pH would enhance mineral absorption and bioavailability in rats (Azorín-Ortuño et al., 2009; Lobo et al., 2009) and human (Holloway et al., 2007).

Low salinity can induce oxidant status and generate excess reactive oxygen species (ROS) to damage cells and tissues in shrimp (Liu et al., 2007; Li et al., 2017). Antioxidant enzymes such as SOD and CAT play an important role in removing excess ROS. MDA as a terminal product of lipid peroxidation is often used as indicator of oxidative damage. Previous studies on humans, piglets and mice have demonstrated that inulin could alleviate negative effects of oxidative stress induced by lipopolysaccharide (Barszcz et al., 2018; Pasqualetti et al., 2014), high-fat-diet (Zhu et al., 2019) and deoxynivalenol (Abdel-Wahhab et al., 2018). Our result showed that shrimp fed the 0.2 and 0.4% inulin diet significantly increased CAT activity and reduced MDA content, while SOD activity was significantly increased in shrimp fed 0.4% inulin. These findings indicate that inulin supplementation can inhibit lipid peroxidation and enhance anti-oxidative ability of shrimp at low salinity of 3 psu.

Shrimp reared at low salinity of 3 psu are likely to be susceptible to

Table 4
Alpha diversity of intestinal microbiota in Pacific white shrimp (*Litopenaeus vannamei*) at low salinity of 3 psu.

	Reads	ACE	Chao1	Shannon	Simpson	Coverage
Control	31,428 ± 32	798 ± 182	772 ± 163	5.05 ± 0.06	0.91 ± 0.01	99.6% ± 0.2%
0.4% Inulin	31,454 ± 23	568 ± 71	556 ± 65	4.45 ± 0.23	0.84 ± 0.04	99.7% ± 0.1%

All data are expressed as mean ± SE (n = 3).

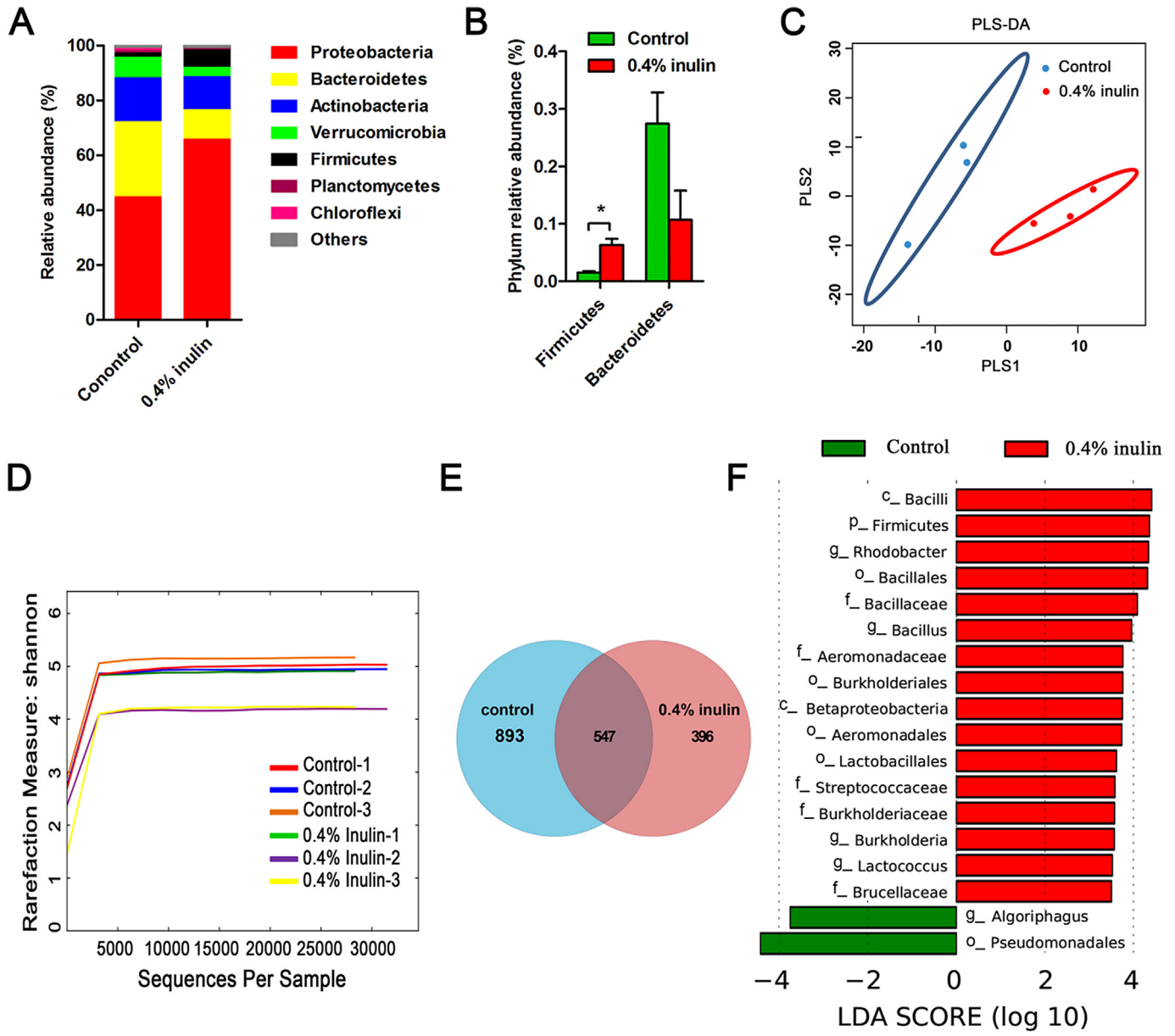


Fig. 2. Differences in intestinal microbiota of composition and diversity between the 0.4% inulin and the control groups in Pacific white shrimp at low salinity of 3 psu. (A) Microbiota composition at the phylum level with relative abundance in the top seven. (B) Differences in relative abundance of *Firmicutes* among two groups. (C) Partial least squares discrimination analysis (PLS-DA). Each point represents a sample, and the points of same color belong to the same group, and the points of the same group are marked with an ellipse. If the samples belonging to the same group are close to each other, and the distance between the points of different groups is farther, the classification model will be more reliable. (D) Rarefaction analysis of shrimp intestines. (E) Venn diagram showed the numbers of shared and unique OTUs. (F) Bacterial taxa differentially displayed in two groups identified by LEfSe using LDA score threshold of > 3.5. All data are expressed as mean ± SE (n = 3). $^*P < .05$.

pathogen infection due to weakened immunity such as low immune-related proteins expression (Xu et al., 2017). In the present study, the application of dietary inulin at 0.2 and 0.4% enhance non-specific immune response as evidenced by the increased ACP and PO enzyme activities. Similarly, dietary supplementation with 2.5 g/kg inulin significantly increases the PO activity and improved the host's defence against white spot syndrome virus (WSSV) infection in Pacific white shrimp (Luna-González et al., 2012). A study showed that L. *vannamei* fed with 5 mg/g dietary inulin for 28 days significantly elevated immune-related gene expression of toll-like receptor1, 2 and 3 (TLR1, 2, 3), signal transducer and activator of transcription (STAT), crustin, anti-lipopolysaccharide factor (ALF) as well as prophenoloxidase (proPO) (Partida-Arangure et al., 2013). It has been suggested that prebiotic enhance the immunity in host by stimulating the growth of benefit bacterial such as *Lactic acid* and *Bacillus* (Nie et al., 2017; Song et al., 2014). Our results support this view as *Bacillus* genus significantly increased in shrimp fed 0.4% inulin. *Bacillus* not only can promote growth, but also can enhance immunity (Wang et al., 2019; Zokaeifar et al., 2014). Our results suggest that shrimp fed dietary inulin can be an immunostimulant to enhance immune response at low salinity of 3 psu.

Although inulin have been reported to exert a significant impact on α-diversity of intestinal microbiota (Zhang et al., 2017), the result of present study is at odds with this claim as no significant difference in α-diversity indices (ACE, Chao 1, Simpson, and Shannon) was detected in shrimp among all treatments. Although bacterial diversity plays an important role in maintaining the gut ecological function, its beneficial effect depend on the presence or enrichment of certain beneficial

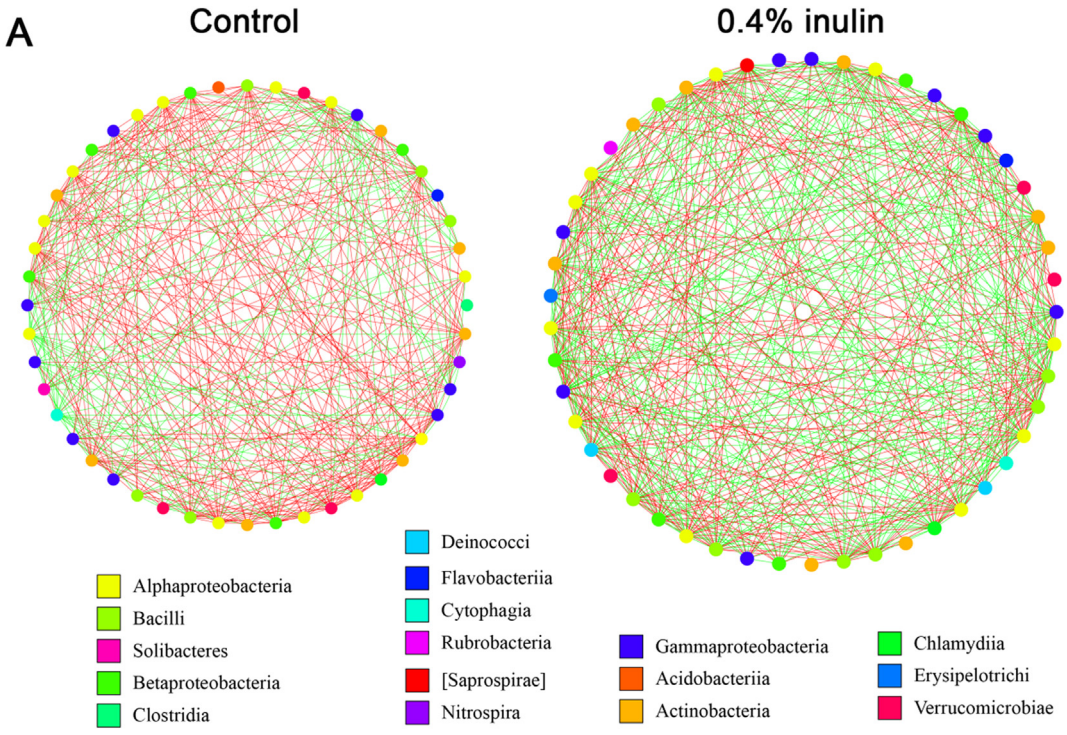
Table 5
Relative abundance of predicted microbial-mediated function with significant difference in Pacific white shrimp at low salinity of 3 psu are listed (n = 3).

KEGG level	KEGG pathway	Control (%)	0.4% inulin (%)	*P*-value
1	Metabolism			
3	Biosynthesis of ansamycins	0.060	0.053	0.048
3	Dioxin degradation	0.031	0.043	0.029
3	Flavonoid biosynthesis	0.024	0.017	0.040
3	Taurine and hypotaurine metabolism	0.145	0.155	0.021
1	Genetic Information Processing			
3	Homologous recombination	0.677	0.659	0.030
1	Cellular Processes			
3	Cell cycle - *Caulobacter*	0.418	0.404	0.031
1	Organismal Systems			
3	Aldosterone-regulated sodium reabsorption	8.68E-06	5.42E-05	0.006

genera/species in the community rather than diversity itself (Siriyappagouder et al., 2018). *Proteobacteria* are the most prevalent members reported to be present in L. *vannamei*, while *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* are reported as the most dominant phyla after *Proteobacteria* and the relative abundances of these bacteria change in the L. *vannamei* intestine depending on the environment and on diet composition (Cornejo-Granados et al., 2017; Li et al., 2018a), In the present study, our result indicated that dietary inulin influenced intestinal bacterial composition by increasing the relative abundance of *Firmicutes* phylum. The PLS-DA results show a clear separation of microbial samples among the experimental groups, indicating dietary inulin has a strong effect on the overall structure of intestinal microbiota in L. *vannamei* at low salinity of 3 psu.

The PICRUSt analysis showed that there was significant difference between the control and 0.4% inulin group in the aldosterone-regulated sodium reabsorption pathway, which is a downstream target pathway of the renin-angiotensin system (Shao et al., 2015). The renin-angiotensin-aldosterone system plays a primary role in hormonal osmoregulation of amphibians and fish (Uchiyama et al., 2014; Wong et al., 2006). In this regard, we speculate that inulin supplementation participates in osmoregulation by chancing the microbial-mediated function. The significantly increased pathway related to aldosterone-regulated sodium reabsorption in shrimp fed 0.4% inulin is possibly associated with adaptation to the low salinity seawater of 3 psu. However, limited information is available about the effect of dietary inulin on osmoregulatory ability, and more studies are needed.

Intestinal microbiota contains a diverse of species interacting with each other to form a complex ecological network through various types of interactions such as cooperation, competition and predation (Deng et al., 2012).The cooperative and competitive interaction through positive and negative links, respectively, could be interpreted by



B

	Positive links	Negative links	Negative association (%)
Control	252	123	32.8
0.4% inulin	243	248	50.5

Fig. 3. The ecological network analysis of the intestinal microbiota in Pacific white shrimp at low salinity of 3 psu. (A) Interspecies interaction network of intestinal microbiota for shrimp fed the 0.4% inulin and control diets. Each node represents a bacterial OUT (genus). Node colors indicate OTUs affiliated to different major classes. The blue edge indicates negative interaction, whereas the red edge indicates positive interaction between two individual nodes. (B) Interspecies interaction types and the ratio of negative interaction in the ecological network. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ecological interactions (Faust and Raes, 2012). The high ratio of negative link in shrimp fed 0.4% inulin indicates that species are involved more in interactions such as amensalism, competition or predation. It proves that a cooperative network of microbes is often unstable, while a higher proportion of competitive interaction improve microbiota stability (Coyte et al., 2015; Xiong et al., 2018).

In conclusion, the present study suggests that inulin can serve as a potential feed additive that helps shrimp to cope with low salinity stress. However, further studies are needed to determine the optimal dietary level of inulin for shrimp farming at low salinity of 3 psu.

Declaration of Competing Interest

None.

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