



Full length article

Dietary fructooligosaccharide can mitigate the negative effects of immunity on Chinese mitten crab fed a high level of plant protein diet

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ABSTRACT

An 8-week feeding trial was carried out under controlled condition to evaluate the effect of dietary fructooligosaccharide (FOS) on growth performance, whole body composition, antioxidant status and immunity of crabs fed high levels of plant protein diets. Thus, six experimental diets were formulated (designated as F₀P₅₀, F₀P₆₀, F₀P₇₀, F_{0.2}P₅₀, F_{0.2}P₆₀ and F_{0.2}P₇₀), which contain two FOS levels (0 or 0.2%) and three plant protein levels (50, 60, or 70%) according to a 2 × 3 factorial design. The results showed that weight gain increased significantly as dietary plant protein level decreased from 70% to 50%. At 50% plant protein level, the addition of 0.2% FOS can significantly elevate weight gain (WG) ($P < 0.05$). The highest value in survival rate was observed in crabs fed F_{0.2}P₅₀ and F_{0.2}P₆₀ diet. Crabs fed F_{0.2}P₅₀ diet showed significantly higher crude protein content ($P < 0.05$) compared with those in other groups, but there were no significant differences in the contents of moisture, crude lipid and ash among all groups ($P > 0.05$). Catalase (CAT) activity in crabs fed F_{0.2}P₅₀ increased significantly ($P < 0.05$) compared with crabs fed F₀P₆₀, F₀P₇₀, F_{0.2}P₆₀ and F_{0.2}P₇₀, but malondialdehyde (MDA) concentrations decreased significantly ($P < 0.05$). Meanwhile, nitric oxide (NO) concentration, acid phosphatase (ACP) and alkaline phosphatase (AKP) activities of crabs fed 0.2% FOS diets increased significantly ($P < 0.05$) compared with crabs fed 0% FOS diets. The expressions of prophenoloxidase (*propo*) was significantly ($P < 0.05$) affected only by dietary plant protein levels with the highest values observed in 50% plant protein diet, whereas the opposite was true for Myeloid differentiation factor 88 (*myd88*). The mRNA expressions of mitochondrial manganese superoxide dismutase (*mtmnsod*), lipopolysaccharide-induced TNF- α factor (*litaf*) and toll like receptors (*tlrs*) were significantly affected ($P < 0.05$) by both FOS and plant protein levels. The cytosolic manganese superoxide dismutase (*cytmnsod*) mRNA expressions in F_{0.2}P₅₀ and F_{0.2}P₆₀ groups were significantly higher than those in F₀P₇₀ and F_{0.2}P₇₀ groups. The results in this study indicated that supplementation with 0.2% FOS can enhance growth performance in crabs fed lower plant protein diets and as well improve immunity in those fed with higher plant protein diets.

1. Introduction

Chinese mitten crab (*Eriocheir sinensis*) is an economically important species in China and its farming may also be extended to other Asian countries. The worldwide fast-growing crab culture has resulted in an increase demand of pelletized feed year by year. And the crab is an omnivorous animal in natural environment, while the farmed crabs can be trained to eat artificial feeds. Most of the feed formulas of Chinese mitten crab contain fishmeal as the main protein sources. Feed has accounted for a large proportion of the total production cost [1]. Such large portion of cost is mainly due to high inclusion of expensive

protein sources, especially fishmeal (FM).

Fishmeal has been the protein source of choice in aquafeed for many reasons, including its high protein content, excellent amino acid profile and high nutrient digestibility [2]. Nevertheless, the current use of fishmeal in the diets of fish and crustaceans is largely viewed as both an uneconomical and unsustainable practice [3]. Thus, the aquafeed industry had to search for alternative protein sources less expensive to reduce their dependence on FM [4,5]. Plant feedstuffs (PF) are nowadays the more available alternatives to FM, and can overcome the problems associated with the by-products of fish, such as organic and inorganic contaminants, shortage of supply and net effect of demand-

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and-supply economics [6]. However, the negative effects on plant feedstuffs, such as anti-nutritional factors, imbalance of amino acids and less palatability, and species differences should be the major factors for this wide variation in the replacement levels [7]. An alternative approach to reduce adverse factors of plant protein may be the inclusion of diet additives that can improve growth performance in aquatic and, possibly, compensate performance loss in low FM diets [8].

A range of diet supplements, including prebiotics and probiotics were commercially available for aquatic animals. Prebiotics appear as an alternative that similarly allow to improve fish health status by modulating the host gut microbiota. Prebiotics has shown promise as eco-friendly additives to improve growth performance, feed utilization, immunological status and disease resistance in aquaculture [9–12]. Fructooligosaccharides (FOS), mannanoligosaccharides (MOX), galactooligosaccharides (GOX), xylooligosaccharide (XOS) and other related carbohydrates have all received considerable attention due to their health benefits to fish [12]. For instance, FOS supplementation positively affected growth performance, survival and feed utilization of the blunt snout bream (*Megalobrama amblycephala*) [13]. Soleimani et al. reported that 2% and 3% FOS can be considered as a beneficial dietary supplement for improving the immune response, stress resistance and digestive enzyme activities of Caspian roach (*Rutilus rutilus*) fry [14]. Li et al. reported that FOS supplementation influenced potentially on growth performance, feed utilization, intestinal microflora and immunity of Pacific white shrimp (*Litopenaeus vannamei*) [15]. In addition, Guerreiro studied that plant feedstuffs supplemented with FOS can decrease the activity of superoxide dismutase (SOD), but scFOS had little effect of oxidative status on European sea bass (*Dicentrarchus labrax*) [6]. However, up to date, there was no report concerning plant feedstuffs supplemented with FOS on growth, immunity and disease resistant in crabs and therefore, further studies were required.

Thus, this study was particularly relevant when FM was highly replaced by PF and to evaluate the effects of dietary FOS on the growth performance, whole body composition, antioxidant status and immune of crabs. The present work will elucidate the potential of prebiotics to mitigate some of the negative effects on crab's health as feeding PF-based diets, which will contribute to result in functional diets with both health and environmental benefits.

2. Materials and methods

2.1. Experimental diets

The FOS product was obtained from by Meiji Holdings Co., Ltd., Japan and characterized as having a typical composition of 95% 1-3fructose. A 2 (FOS level) × 3 (Plant protein level) factorial design with three replicates per dietary treatment was adopted in this study. So, six experimental diets (designated as F₀P₅₀, F₀P₆₀, F₀P₇₀, F_{0.2}P₅₀, F_{0.2}P₆₀ and F_{0.2}P₇₀), which contained 0 or 0.2% dietary FOS combined with plant protein at 50, 60, or 70%. The diets were prepared by sieving the protein sources, and all dry ingredients were thoroughly mixed, followed by the addition of the lipid sources, and thoroughly mixing again. Distilled water was then added at 30% of the ingredient weight and after mixing for 15 min, the resulting mash was then pelleted through a 2.5 mm diameter die and extruded through a single-screw meat grinder extruder. Extruded diets were dried in the ventilation room for 24 h, ground to appropriate size for crabs, and then stored in sealed plastic bags at –20 °C until used. Proximate analyses of the experimental diets (Table 1) were determined according to the method of AOAC [16]. Crude protein content was determined by Kjeldahl method using an Auto Kjeldahl System (Kjeltec™ 2300, Foss, Sweden). Crude lipid was analyzed by Soxtec system, moisture content by a dry oven (D-63450, Heraeus, Hanau, Germany) drying at 105 °C for 24 h and ash by a furnace muffler (550 °C for 4 h). Crabs were hand-fed twice daily at 08:00 a.m. and 18:00 p.m. for 8 weeks.

Table 1

Ingredients and proximal composition of experimental diets.

	Experimental diets					
	F ₀ P ₅₀	F ₀ P ₆₀	F ₀ P ₇₀	F _{0.2} P ₅₀	F _{0.2} P ₆₀	F _{0.2} P ₇₀
Ingredients (% dry matter)						
Fish meal	27.58	22.8	17.55	27.58	22.8	17.55
Soybean meal	22.31	26.33	30.45	22.31	26.33	30.45
Peanut meal	14.55	17.17	19.89	14.55	17.17	19.89
Rapeseed meal	4.85	5.72	6.68	4.85	5.72	6.68
Wheat flour	19	16.27	13.72	19	16.27	13.72
Soybean oil	1.46	1.46	1.46	1.46	1.46	1.46
a-Cellulose	0.2	0.2	0.2	0	0	0
FOS	0	0	0	0.2	0.2	0.2
Fish oil	0.97	0.97	0.97	0.97	0.97	0.97
Ca(H ₂ PO ₄) ₂	2.16	2.16	2.16	2.16	2.16	2.16
Attapulgite clay	0.97	0.97	0.97	0.97	0.97	0.97
Zeolite powder	0.97	0.97	0.97	0.97	0.97	0.97
Blood powder	1.94	1.94	1.94	1.94	1.94	1.94
Premix ^a	1.00	1.00	1.00	1.00	1.00	1.00
Mixture ^b	2.04	2.04	2.04	2.04	2.04	2.04
Proximate analysis (% dry weight)						
Dry matter	90.28	89.35	91.54	90.82	89.49	89.23
Crude protein	40.86	40.70	39.95	39.96	40.23	39.63
Crude lipids	5.24	5.35	5.35	5.45	5.51	5.46
Ash Starch	13.68	13.83	13.94	13.83	13.89	13.85

Note: ^a Premix supplied the following minerals (g/kg) and vitamins (IU or mg/kg): CuSO₄·5H₂O, 2 g; FeSO₄·7H₂O, 25 g; ZnSO₄·7H₂O, 22 g; MnSO₄·4H₂O, 7 g; Na₂SeO₃, 0.04 g; KI, 0.026 g; CoCl₂·6H₂O, 0.1 g; Vitamin A, 900,000 IU; Vitamin D, 200,000 IU; Vitamin E, 4500 mg; Vitamin K₃, 220 mg; Vitamin B₁, 320 mg; Vitamin B₂, 1090 mg; Vitamin B₅, 2000 mg; Vitamin B₆, 500 mg; Vitamin B₁₂, 1.6 mg; Vitamin C, 10,000 mg; Pantothenate, 1000 mg; Folic acid, 165 mg; Choline, 60,000 mg; Biotin, 100 mg; Myoinositol 15,000 mg. ^b Mixture includes the following ingredients (%): choline chloride 4.75%; antioxidants 1.72%; mildew-proof agent 2.35%; salt 22.06%; Lvkyanguan 59.30% and biostimep 9.51%.

2.2. Crab and experimental design

Chinese mitten crab were collected from a local farm in Pukou, Jiangsu province, China. Crabs were fed a commercial diet (Haipurui Feed Co., Ltd., Jiangsu) twice a day and acclimated to experimental conditions for 2 weeks. A total of 288 crabs (average initial weight: 30.85 ± 1.02 g) were randomly distributed into 18 cement pools (2.0 × 2.0 × 0.5 m, L: W: H) at a density of 16 crabs per pool. During the experimental period, water temperature, pH and dissolved oxygen were monitored daily and recorded as 24 ± 2 °C, 8.5–8.6 and 5 mg/L respectively.

2.3. Sample collection

Crabs were anaesthetized on ice for 10 min. Because of low temperature can reduce the crab vigor. After that, six crabs were sampled in each pool. Hemolymph was collected using syringes from each crab's second last pair of walking legs, mixing 1:1 with precooling anticoagulant solution (100 mmol L⁻¹ glucose, 26 mmol L⁻¹ citrate, 30 mmol L⁻¹ citric acid, 450 mmol L⁻¹ NaCl, 10 mmol L⁻¹ EDTA, pH = 7.2) [17] and then immediately centrifuged at 9000 rpm and 4 °C for 20 min. After that, hepatopancreas was dissected aseptically and frozen immediately in liquid nitrogen and stored at –80 °C for subsequent analysis.

2.4. Growth performance

At the end of feeding trial, all crabs were deprived of food for 24 h before weighing and sampling, and then the following parameters were measured:

$$\text{Weight gain (WG)} = 100 \times (W_2 - W_1) / W_1$$

Table 2
Nucleotide sequences of the primers used to assay gene expression by RT-qPCR.

Target gene	Forward (5'-3')	Reverse (5'-3')
<i>propo</i>	CCATCCCTTCCTGCTTACCA	CTCCATCACAACCCTAACGACTT
<i>mtmsod</i>	AAGGTTCTGGTTGGGGCT	AACATTCTGTACTGCAG
<i>cytmsod</i>	AAGAAGGACTTTGGTTCC	CGTCCAGACCAAGCAGCG
<i>litaf</i>	CAGGAGTAGTGTGGGATTTGC	AGTTGTTGGAGCAGCACCTTG
<i>tlrs</i>	CTCCTTACCTGCCCTAACTGCT	CTCCAGITTTGTAITGCTGTGCGAAA
<i>myd88</i>	GCAACAGGTGGACTTTGAGGAGTG	CACGGACAACCAGCAGTAAACC
β -actin	GCATCCACGAGACCACCTTACA	CTCCTGCTTGTGATCCACATC

Survival rate (SR) (%) = $N_t \times 100 / N_0$

Specific growth rate (SGR, % / d) = $100 \times (\ln W_2 - \ln W_1) / T$

Where W_1 is the initial weight, W_2 is the final weight and T is the number of days in the feeding period. Where N_t and N_0 were the final and initial number of crabs.

2.5. Analysis of antioxidant capability

The activities of total catalase (CAT) and glutathione peroxidase (GPX) were all determined following the procedures described by Bjarte et al. [18]. The CAT activities in hepatopancreas and hemocytes were determined according to a method recording the decrease in H_2O_2 concentration at 240 nm. Samples were incubated in ethanol (1%, v/v) for 30 min on ice before the enzyme assay to decompose and prevent the formation of inactive complex of CAT with H_2O_2 . 2 mL sample was diluted in phosphate buffer and then added 1 mL of a freshly prepared substrate solution (30 mM H_2O_2). The absorbance was monitored in two replicates for 30 s at intervals of 5 s using a Shimadzu UV-240 spectrophotometer at a reaction temperature of 20 °C. The GPX activity of the compounds was determined using H_2O_2 as the substrate in the presence of GSH.

Hepatopancreas malondialdehyde (MDA) content was estimated using the thiobarbituric acid test [19]. This method is based on the reaction between malondialdehyde and other aldehyde, a by-product of reactive oxygen species (ROS), which react with thiobarbituric acid (TBA) at low pH and high temperature forming a complex with maximum light absorption at 534 nm. One mL of thiobarbituric acid (TBA) was added to 200 μ L of plasma, mixed well in the test tube and boiled in 95 °C boiling water bath for 30 min. The absorbance of sample against blank and standard against distilled water were read at 534 nm.

2.6. Measurement of hemocytes immune parameters

Plasma acid phosphatase (ACP) activity was measured according to a disodium phenyl phosphate method [20]. 1 mL of samples supernatant was mixed with 4 mL of modified universal buffer (pH 6.5). Further 1 mL of 0.025 mM disodium p-nitrophenyl phosphate (tetrahydrate) was mixed with the culture supernatant and incubated at 37 °C for 1 h. After incubation for 1 h, 4 mL of 0.5 M NaOH and 1 mL of 0.5 M $CaCl_2$ were added to stopped the reaction. The concentration of p-nitrophenol was measured by measuring the absorbance at 420 nm using a Shimadzu UV-240 spectrophotometer and the values were extrapolated base on standard curve determined by serially diluted solutions of p-nitrophenol. One unit (U) of phosphatase activity was defined as the amount of enzyme required to release 1 μ mol of p-nitrophenol/mL/min from di-Na p-nitrophenyl phosphate (tetrahydrate) under the assay condition.

Alkaline phosphatase (AKP) activity was carried out based on the procedures given by Rausch and Moore [21]. AKP was determined in 0.62 M 2-amino-2-methyl-1-propanol buffer (pH = 10.2) with 15.2 mM p-nitrophenylphosphate and 0.1 mM $MgCl_2$ at 37 °C for 30 min. The reaction was stopped with 1.25 M NaOH and the absorbance values were read in a Beckman DU recording spectrophotometer at 410 nm.

Plasma nitric oxide (NO) levels were determined by the nitrate reductase assay using a commercial kit (ref. no. A012) produced by Jiancheng Bioengineering Institute (Nanjing, China).

2.7. Analysis of gene expression in tissues

2.7.1. Total RNA extraction and reverse transcription

Total RNA was extracted from hepatopancreas of using RNAiso Plus (Takara Co. Ltd. Dalian, China) according to the manufacturer's instructions. The concentration and purity of total RNA were measured by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) using the absorbance value of A260/A280. Only the RNA samples with A260/A280 ratio between 1.8 and 2.0 were used for the subsequent analysis. The reverse transcription-PCR reaction of PrimeScript™ RT Master Mix (Takara Co. Ltd. Dalian, China) was as follows: 37 °C for 15 min, 85 °C for 5 s, and 4 °C thereafter. The cDNA was kept at -20 °C for real-time quantitative PCR (RT-qPCR).

2.7.2. Real-time quantitative PCR analysis

RT-qPCR was performed using a SYBR Premix Ex Taq II kit (TaKaRa, Co. Ltd. Dalian, China) on a real-time PCR detection system (ABI USA-7500). β -actin was adopted as the internal standard gene in arthropods. It has been demonstrated to be reliable internal reference gene in Chinese mitten crab. RT-qPCR primers for prophenoloxidase (*propo*) (EF493829.1), cytosolic manganese superoxide dismutase (*cytmsod*), mitochondrial manganese superoxide dismutase (*mtmsod*) [22], toll like receptors (*tlrs*) (JX295852.1), myeloid differentiation factor 88 (*myd88*) (KM433864.1) and lipopolysaccharide-induced TNF- α factor (*litaf*) (KF892539.1) and β -actin [23] were presented in Table 2. The RT-qPCR reactions were carried out in a final volume of 20 μ L, containing 10 μ L 2 \times SYBR Premix Ex Taq™, 0.4 μ L (10 μ M) of each primer, 0.4 μ L ROX, 6.8 μ L DEPC water and 2 μ L of cDNA template. The reactions were initially denatured at 95 °C for 10 min and then 40 cycles at 95 °C for 15 s, followed by annealing at 60 °C for 34 s. To assess the specificity of each amplicon, the melt curve analysis of 5 s per step from 65 to 95 °C was performed at the end of each PCR thermal profile. Ct values were obtained and the relative mRNA levels were calculated by the $2^{-\Delta\Delta Ct}$ method as described [24], using β -actin as the endogenous control to normalize the mRNA expression of the target genes.

2.8. Statistics

The data were analyzed by analysis of variance with the quadratic in the SPSS version 22.0 software (SPSS Inc., Chicago, IL, USA). The normality of data was performed by Shapiro–Wilk test and then they were subjected to one and two-way analysis of variance (ANOVA) to test the effect of dietary FOS and plant protein levels on crabs. The hypothesis of equal mean responses among treatment groups was tested using Duncan procedure. Differences were considered significant at $P < 0.05$. The data are presented as mean \pm SEM.

Table 3

Growth performance of crabs fed diets with two levels of dietary FOS and three levels of plant protein.

Diets	Initial weight (g)	Final weight (g)	WG (%)	Survival rate (%)	SGR (%)
F ₀ P ₅₀	29.71 ± 0.51	56.84 ± 0.60 ^{ab}	91.43 ± 1.59 ^b	81.25 ± 3.60 ^{ab}	1.16 ± 0.03
F ₀ P ₆₀	29.73 ± 0.18	54.59 ± 0.84 ^a	83.61 ± 1.71 ^{ab}	81.25 ± 0.25 ^{ab}	1.08 ± 0.02
F ₀ P ₇₀	30.26 ± 0.72	54.19 ± 0.67 ^a	79.24 ± 3.25 ^a	83.33 ± 5.51 ^{ab}	1.04 ± 0.03
F _{0.2} P ₅₀	30.78 ± 0.79	59.19 ± 0.51 ^b	97.6 ± 1.67 ^c	87.50 ± 3.60 ^b	1.16 ± 0.02
F _{0.2} P ₆₀	31.03 ± 1.14	55.24 ± 2.34 ^{ab}	82.60 ± 1.76 ^a	87.50 ± 6.25 ^b	1.03 ± 0.06
F _{0.2} P ₇₀	30.96 ± 0.61	55.81 ± 1.31 ^{ab}	80.48 ± 2.67 ^a	70.83 ± 2.08 ^a	1.05 ± 0.07
Two-way ANOVA					
FL	ns	ns	*	ns	ns
PL	ns	*	***	ns	*
FL × PL	ns	ns	ns	*	ns

Means in the same column with different superscripts are significantly different ($P < 0.05$). *, $P < 0.05$; ***, $P < 0.001$; ns, $P > 0.05$.

3. Result

3.1. Growth performance

Growth performances were shown in Table 3. At the same dietary FOS levels, crabs fed with 50% plant protein obtained a significantly higher WG compared to those fed with 70% plant protein diets. Crabs fed F_{0.2}P₅₀ diet had higher final weight compared to those in the F₀P₆₀ and F₀P₇₀ groups, and at 50% plant protein level, the inclusion of 0.2% FOS can dramatically elevate WG compared with no supplementation of FOS ($P < 0.05$). Among all the groups supplemented with 0.2% FOS, the inclusion of 50% and 60% plant protein significantly increased survival rate, compared to 70% plant protein group. At FOS levels, no significant ($P > 0.05$) differences were found in SGR of crab in all groups. In addition, a significant interaction ($P < 0.05$) between FOS levels and plant protein levels was observed in survival rate.

3.2. Body composition

As can be seen in Table 4, compared with F₀P₆₀, F₀P₇₀, F_{0.2}P₆₀ and F_{0.2}P₇₀ groups, the crude protein content of crabs was significantly higher in F_{0.2}P₅₀ ($P < 0.05$). However, the moisture, crude lipid and ash contents were observed no significant differences ($P > 0.05$) among all groups. In addition, a significant interaction ($P < 0.05$) between FOS levels and plant protein levels was observed in crude protein content.

3.3. Antioxidant enzyme activity

The results of antioxidant enzyme activities and MDA concentrations were shown in Table 5. There was no significant ($P < 0.05$) interaction between dietary plant protein and FOS levels on MDA concentrations, CAT and GPX activities. The highest CAT activity was observed in F_{0.2}P₅₀ group, which was significantly higher than those in

Table 4

Whole-body composition of crabs fed diets with two levels of dietary FOS and three levels of plant protein.

Diets	Crude protein (%)	Moisture (%)	Crude lipid (%)	Ash (%)
F ₀ P ₅₀	13.98 ± 0.09 ^{bc}	66.86 ± 0.24	4.97 ± 0.08	10.05 ± 0.01
F ₀ P ₆₀	13.18 ± 0.26 ^a	64.55 ± 0.73	5.01 ± 0.17	10.18 ± 0.16
F ₀ P ₇₀	13.74 ± 0.11 ^{ab}	63.04 ± 0.19	5.37 ± 0.23	10.82 ± 0.24
F _{0.2} P ₅₀	14.56 ± 0.35 ^c	64.67 ± 0.36	5.00 ± 0.17	9.95 ± 0.07
F _{0.2} P ₆₀	13.22 ± 0.50 ^a	66.45 ± 0.60	4.60 ± 0.03	10.64 ± 0.08
F _{0.2} P ₇₀	13.73 ± 0.64 ^{ab}	66.25 ± 1.83	4.68 ± 0.34	10.48 ± 0.43
Two-way ANOVA				
FL	ns	ns	ns	ns
PL	*	ns	ns	ns
FL × PL	*	ns	ns	ns

Means in the same column with different superscripts are significantly different ($P < 0.05$). *, $P < 0.05$; ns, $P > 0.05$.

Table 5

Hemocyte antioxidant status of crabs fed diets with two levels of dietary FOS and three levels of plant protein.

Diets	MDA (nmol/ml)	CAT (u/ml)	GPX (U/L)
F ₀ P ₅₀	5.78 ± 0.41 ^b	3.28 ± 0.21 ^{bc}	928.18 ± 9.06
F ₀ P ₆₀	5.98 ± 0.18 ^b	2.88 ± 0.15 ^{ab}	930.91 ± 6.99
F ₀ P ₇₀	6.08 ± 0.42 ^b	2.44 ± 0.08 ^a	907.27 ± 11.15
F _{0.2} P ₅₀	4.32 ± 0.25 ^a	3.52 ± 0.23 ^c	917.27 ± 6.36
F _{0.2} P ₆₀	5.49 ± 0.33 ^b	2.78 ± 0.20 ^{ab}	923.64 ± 7.67
F _{0.2} P ₇₀	5.69 ± 0.25 ^b	2.86 ± 0.29 ^{ab}	909.09 ± 7.92
Two-way ANOVA			
FL	**	ns	ns
PL	*	*	*
FL × PL	ns	ns	ns

Means in the same column with different superscripts are significantly different ($P < 0.05$). *, $P < 0.05$; **, $P < 0.01$; ns, $P > 0.05$.

F₀P₆₀, F₀P₇₀, F_{0.2}P₆₀ and F_{0.2}P₇₀. Among all the groups, the observed lowest MDA concentration was in F_{0.2}P₅₀ group, and this value was significantly higher than those in other groups including F₀P₅₀ group. No significant ($P > 0.05$) differences were found in GPX activity of crab in all groups.

3.4. Immune parameters

The results of hemocytes immune parameters are shown in Fig. 1. In the groups with same levels of plant protein (50% and 60%), 0.2% FOS addition in diets significantly increased ACP and AKP activities. But, 0.2% FOS addition in diets could not change ACP and AKP activities in 70% plant protein groups. In addition, the NO contents of crabs fed the low-plant protein diets were significantly higher than those of crabs fed the high-plant protein diets at the same dietary FOS levels. And at the same dietary plant protein levels, 0.2% FOS inclusion in diets significantly increased NO content. Furthermore, AKP activity and NO concentration were significantly ($P < 0.05$) affected by the interaction between dietary FOS levels and plant protein levels.

3.5. The relative mRNA quantification of antioxidant and immune genes

The mRNA levels of antioxidant genes were presented in Fig. 2. A, B and C. With increasing dietary plant protein level from 50% to 70%, the mRNA expressions of *propo*, *mtmsod* and *cytmsod* decreased significantly ($P < 0.05$) at the same dietary FOS levels. 0.2% FOS supplementation led to a remarkable increase of *propo* expression in crab fed F_{0.2}P₅₀ diet compared with F₀P₅₀ group. But, in the groups with same levels of high plant protein (60% and 70%), 0.2% FOS addition in diets can't affect *propo* expression. At 70% plant protein levels, *mtmsod* expression of crabs fed with 0.2% FOS supplemented diet was significantly ($P < 0.05$) higher than that in the group without FOS inclusion. Among all the groups supplemented with 0.2% FOS, the inclusion of 50% and 60% plant protein significantly increased *cytmsod*

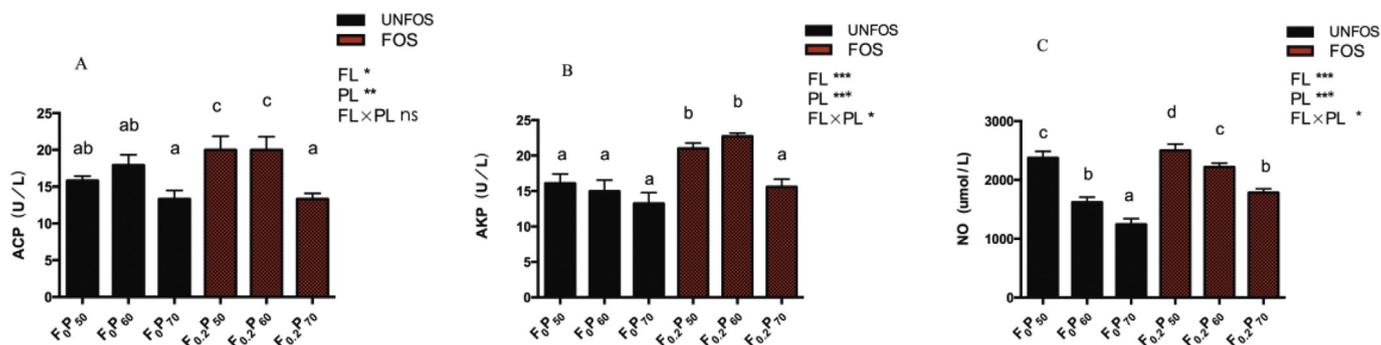


Fig. 1. Effects of crabs fed diets with two levels of dietary FOS and three levels of plant protein on immune parameters (ACP (A), AKP (B) and NO (C)). Each datum represents the mean of three replicates. Bars assigned with different superscripts are significantly different ($P < 0.05$). UNFOS, un-supplementation fructooligosaccharides; FOS, fructooligosaccharides; FL, FOS level; PL, plant protein level; FL \times PL, interaction FOS levels and plant protein levels.

expression, but not significantly difference were found between F_{0,2}P₅₀ and F_{0,2}P₆₀ groups ($P > 0.05$). Furthermore, a significant interaction between dietary FOS levels and plant protein levels was observed in the expressions of *mtmsod*.

The mRNA levels of immunity-related genes were presented in Fig. 2. D, E and F. The expression of *litaf* was significantly lower in crabs fed F_{0,2}P₅₀ diet than crabs in F₀P₅₀, F₀P₇₀ and F_{0,2}P₇₀ groups. At 50% plant protein levels, 0.2% FOS addition in diets can significantly down-regulate *litaf* expression ($P < 0.05$). The expression of *tlrs* in hepatopancreas was significantly enhanced with dietary plant protein level increasing from 50% to 70% at the same FOS addition levels ($P < 0.05$). And in the groups with 50% plant protein, 0.2% FOS addition in diets significantly decreased *tlrs* expression ($P < 0.05$). In 0% FOS diets groups, *myd88* expression was not significantly ($P > 0.05$) affected by different levels of plant protein. But, among all the groups supplemented with 0.2% FOS, the mRNA expression of *myd88* significantly decreased in crabs fed 50% or 60% plant protein diet compared to 70% plant protein diet ($P < 0.05$). In 60% plant protein groups, 0.2% FOS addition in diets significantly decreased *myd88* expression ($P < 0.05$). In addition, a significant interaction ($P < 0.05$) between FOS levels and plant protein levels was observed in the relative expression of *litaf*.

4. Discussion

In recent years, replacing fishmeal by plant protein is becoming a somewhat well-established strategy which avoids overexploitation of natural fish resources and marine ecosystems [25]. In general, crustacea cannot effectively utilize high levels of plant proteins. Bulbul et al. reported that 50% fishmeal can be successfully reduced by the blend of SBM and canola meal (CM), the combination provided good performances of shrimp, but growth performance of kuruma shrimp significantly decreased, when 60% of FM was replaced with plant protein mixture [26]. This is similar to our results, which showed a negative effect on crab's growth at high plant protein levels (60% and 70%) whether FOS was supplemented or not. Some of these detrimental effects may be linked to reduced utilization of plant proteins and associated with low nutrients digestibility, poor palatability, imbalanced amino acids and/or the presence of anti-nutritional factors (ANF) [27–29]. However, the present study demonstrated that at 50% plant protein levels, dietary supplement FOS group showed a higher WG. It indicated that under a certain range of plant protein level, FOS supplementation could improve the digestion, absorption and metabolism of various nutrients in aquatic animals [14,30]. Chen et al. reported that dietary FOS significantly stimulated the growth of the prawns [31]. Inês et al. also reported that dietary scFOS and XOS supplementation can have positive effect on gut morphology of European sea bass (*Dicentrarchus labrax*) fed PF-based diets [32], thereafter which may improve final body weight.

In the present study, little differences were observed in moisture, crude lipid and ash content of crab fed with different levels of dietary plant protein. However, the content of crude protein decreases as the dietary plant protein level increases. Catacutan et al. [33] also found similar results on the mangrove red snapper (*Lutjanus argentimaculatus*), which may be related with the process of protein digestion, absorption and deposition. The F_{0,2}P₅₀ group displayed the uppermost crude protein values that could explain the good growth performance registered in the group. However, other studies reported that there is no effect of dietary plant protein level on whole body composition, such as juvenile black tiger shrimp [34] and kuruma shrimp [26]. It may be related to feed ingredients, the proportion of alternative fishmeal and the culture environment. In addition, our results showed there was no significant difference in dietary FOS levels on crude protein, moisture, crude lipid and ash contents. This was consistent with what Fuchs et al. reported that whole body composition of turbot remained unaffected by additive inclusions in both, high and low FM diets as in earlier studies comparing FM- or PF-based diets supplemented with some additives [8].

Regarding to antioxidants, animal cells produce reactive oxygen species (ROS) under normal physiological conditions, and meanwhile, the body has designed several antioxidant defense mechanisms to cope with it. GPX and CAT are two main antioxidant enzymes of the antioxidant defense system. In addition, the concentration of MDA is well correlated with the activity of GPX and usually determined to evaluate whether the body is in oxidative status [35]. In order to detect the effect of plant protein levels on crabs, antioxidant status of crabs administered by different plant protein levels was studied. The results showed that among all the groups supplemented with 0.2% FOS, the highest CAT activity and the lowest MDA concentration were observed in crabs fed with 50% plant protein supplemented diets, which is consistent with our previous study [36]. It indicated that dietary FOS could stimulate antioxidant system, and thus involve in immune responses. But, there are no significant differences on GPX activity in all groups. Similar phenomenon was reported that high inclusion (over 60%) of plant protein reduced serum antioxidant activities but elevated MDA content in juvenile starry flounder (*Platichthys stellatus*) [37]. Lin et al. [38] also studied that the antioxidant capability in liver significantly decreased in tilapia when fed diets containing over 50% plant protein. This deteriorated hemocyte antioxidant status was associated with malnutrition and indicative of potential damage from free radicals.

In the present study, different levels of FOS and plant protein in diets can significantly affect the innate immunity of crabs. This was supported by the fact that in 0.2% FOS addition groups, crabs fed diets with the inclusion of 50% and 60% plant protein obtained significantly higher ACP and AKP activities as well as NO contents, compared with crabs fed 70% plant protein diets. A similar result was reported that replacement of 60% fishmeal with plant protein mixture had significant decrease on the activities of ACP and AKP in Pacific White Shrimp

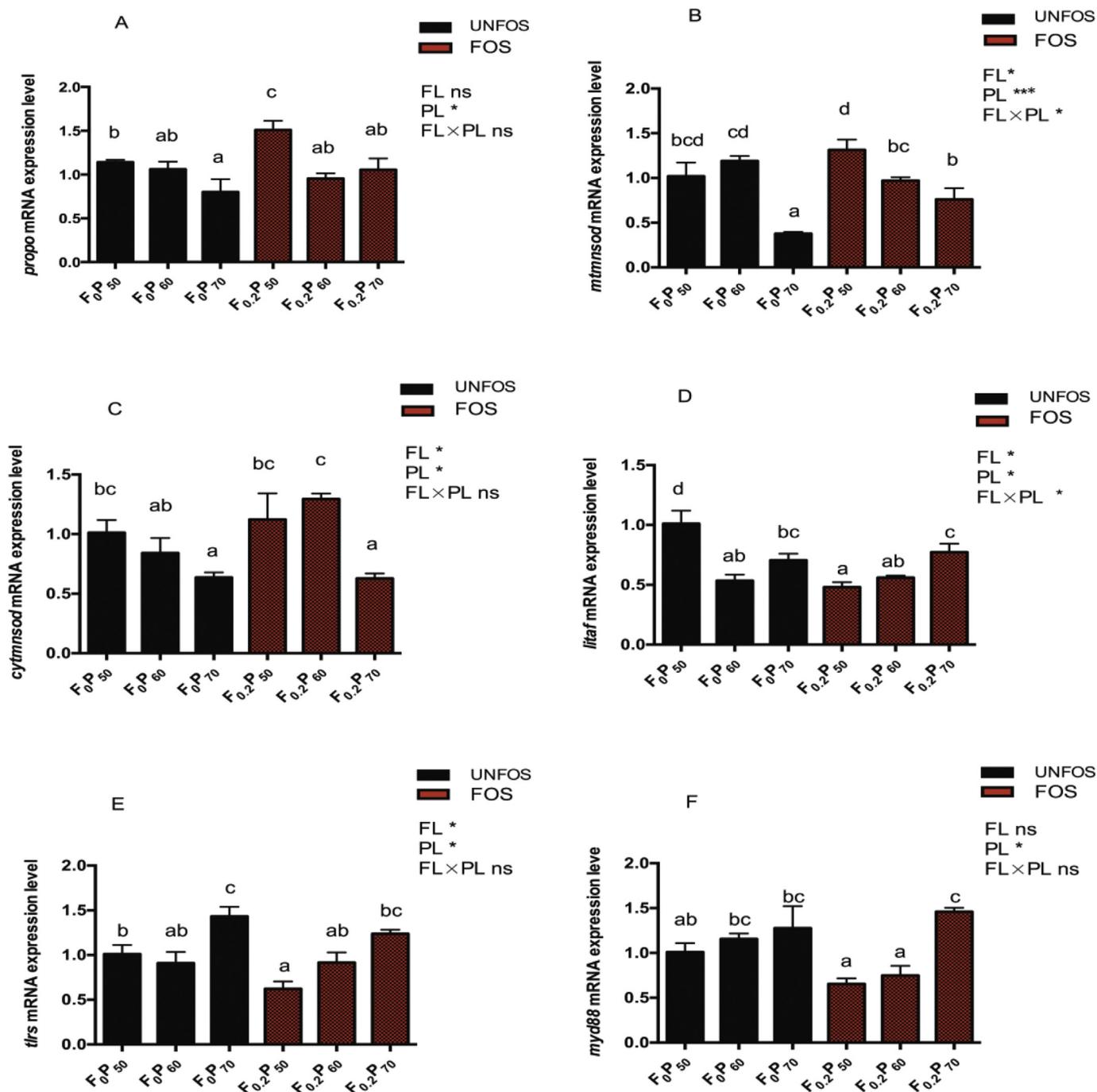


Fig. 2. Effects of crabs fed diets with two levels of dietary FOS and three levels of plant protein on *propo* (A), *mtmnsod* (B), *cytmnsod* (C), *litaf* (D), *tlrs* (E) and *myd88* (F) genes in hepatopancreas. Each data represents the mean of three replicates. Bars assigned with different superscripts are significantly different ($P < 0.05$). UNFOS, un-supplementation fructooligosaccharides; FOS, fructooligosaccharides; FL, FOS level; PL, plant protein level; FL \times PL, interaction FOS levels and plant protein levels.

(*Litopenaeus vannamei*) [39]. Zhang et al. also reported that replacement of fishmeal by soybean meal attenuated activities of the AKP in large yellow croaker [40]. The possible reason might be that most plant protein sources contain ANF and restrict growth and protein utilization and affect the food intake in aquatic animals [41], which may lead to nutrient deficiency [42] and lead to a severe decrease in the nonspecific immune function [43]. In addition, the application of dietary FOS had beneficial effects on the nonspecific immune function of crabs. The present study also demonstrated that at the same dietary plant protein levels (50% and 60%), crabs fed 0.2% FOS groups had significant higher immune parameters (including ACP and AKP activities as well as

NO contents). The possible reason is that when FM is highly replaced by plant feedstuffs, the combination of prebiotics with PP would result in functional diets with both health and environmental benefits [25]. It may be that FOS could delay the occurrence of immunity fatigue by activating different pathways of immune system [44]. Hoseinifar et al. studied that the observed elevation of respiratory burst activity is probably caused by the stimulation of carp immune response due to FOS supplementation [45]. Guerreiro et al. also reported that dietary FOS supplementation with PF-based diets can improve fish gut histomorphology and enhance immune status [46]. According to our previous studies, Bifidobacteriales and Bacteroides relative abundance

significantly increased in crabs fed diets with the inclusion of 0.2% FOS [36]. Besides, these oligosaccharides can be selectively used by bifidobacterium to reproduce probiotic bacteria and restrain the adherence and colonization of pathogenic microorganism [47]. Furthermore, the fermentation of FOS including acetate, propionate and butyrate, as well as lactic acid, carbon dioxide and hydrogen also plays a crucial role in health as a bifidogenic agent, modulating the immune system [48,49].

In invertebrates, hepatopancreas is regarded as the main innate immune organ where antioxidant defenses and multiple oxidative reactions occur with high metabolic activity [50]. The important antioxidant enzymes, PROPO and SOD were also more abundant in hepatopancreas. SOD can clear the internal ROS to avoid the occurrence of fatty acid oxidation and relieve toxic effects of ROS, and subsequently protect organism from oxidative damage [51]. As one of the SOD metal cofactors, MNSOD have two types, CYTMNSOD and MTMNSOD [52]. In the present study, the mRNA expressions of *propo*, *mtmnsod* and *cytmnsod* in hepatopancreas of crabs fed non-FOS diet were significantly decreased with dietary plant protein level increasing to 70%, which suggested anti-oxidative and immune ability of crabs were damaged in high plant protein level. The possible reason was that plant protein can produce the negative effects including anti-nutritional factors and toxic factors, such as trypsin inhibitor, aflatoxin and phytic acid. Low plant protein levels (50% or 60%) may activate the PROPO activating system, thus involve in cell adhesion, cellular responses to inflammatory cytokines and environmental stresses [53]. In addition, in the groups with 50% plant protein, 0.2% FOS addition in diets significantly increased in expression of *propo*. In the groups with 70% plant protein, 0.2% FOS addition in diets significantly increased in expression of *mtmnsod*. And in the groups with 60% plant protein, 0.2% FOS addition in diets significantly increased in expression of *cytmnsod*. It may be that CYTMNSOD and MTMNSOD reduce the superoxide anions generated during phagocytosis and convert them to hydrogen peroxide in supplementation 0.2% FOS groups [54]. In addition, the CYTMNSOD functions as the essential scavenger of ROS, and it also could be involved in many important physiological processes of crabs, including innate immunity [55]. Meanwhile, TLRS, MYD88 and LITAF [56,57] are important components of NF- κ B pathway to activate the expression of multiple genes involved in immune responses [58]. Accumulating evidences have proved that LITAF is an important transcription factor in downstream of TLR signaling pathway, which is closely associated with transcriptional regulation of TNF- α and other cytokines dependent on MYD88 [56,59]. In the present study, crabs fed un-supplemented FOS groups, the mRNA expression of *litaf* was significantly decreased with dietary plant protein level increasing to 70%, but the expression of *tlrs* was opposite. And no significant differences were found in the expression of *myd88* of crabs in all un-supplemented FOS groups. High plant protein feed can cause the body immune damage through the invasion of pathogens. Dechamma et al. studied that *tlrs* expressed to a maximum level in lymphoid organ was observed at 24 h post *V. harveyi* infection [60]. Differentially modulated *myd88* mRNA expression in shrimp in response to viral infections, such as white spot syndrome virus (WSSV), suggested the involvement of *myd88* in antiviral immune responses in invertebrates [61,62]. However, in our results, 0.2% FOS addition in diets can significantly decreased *litaf* and *tlrs* expressions in 50% plant protein levels groups and decreased *myd88* expression in 60% plant protein levels groups. It might be possible that the effects of both FOS and plant protein levels on these gene was associated with their effects on the translation process and/or post-translational process of these antioxidant immune genes. However, further investigation is still needed to elucidate this.

5. Conclusion

The present study indicated that the supplementation with 0.2% FOS can enhance growth performance in crabs fed lower plant protein diets and as well improve immunity in those fed with higher plant

protein diets.

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