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Short communication

Effect of dietary date seed meal as an alternative carbohydrate source on immune-related gene expression of Pacific white shrimp *Penaeus vannamei*

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ABSTRACT

Current study aimed to examine the effect of dietary date seed meal (DSM) as an alternative carbohydrate ingredient on immune-related genes expression of Pacific white shrimp (*Penaeus vannamei*). A total number of 750 shrimp (4.0 ± 0.1 g) were randomly assigned into five experimental treatments, each with three replicates. Experimental diets contained 0, 50, 100, 150 and 230 g/kg DSM substituted for both wheat and rice flour. Shrimp were fed at a rate of 5% body weight three times daily and the mRNA expression of α2-macroglobulin (α2-M), lipopolysaccharide and beta-1,3-glucan-binding protein (LGBP), integrin β, and peroxinectin (PX) was studied on five shrimp per treatment after 8 weeks feeding trial. The results showed that replacement of DSM up to 100 g/kg in the diet of *P. vannamei* caused no significant changes in the expression of α2-M and integrin β, however both of these genes showed lower expression in shrimp fed diets containing 150 and 230 g/kg DSM compared to control group. Moreover, no significant differences were observed in the expression of PX and LGBP between shrimp fed diets containing different amounts of DSM and control group. These findings demonstrated that as an inexpensive source of carbohydrate, DSM could be substituted up to 100 g/kg for wheat and rice flour in the diet of farmed shrimp without suppressive effects on the immune status.

1. Introduction

The high cost of aquatic feed has led to a wide research for the use of inexpensive ingredients. Agriculture creates wastes and by-products that could be hazardous to the environment and therefore researchers have made efforts to use or recycle them. One approach for recycling these agricultural wastes is to use them in aquafeed industry [1–3]. Among the agricultural by-products, date seed meal (DSM) is considered as a promising source of inexpensive carbohydrate [2]. DSM is a waste by-product of date that is used for various purposes such as confectionery and prepare date syrup [4]. DSM consists an excellent nutritional quality due to high amounts of minerals (i.e. selenium, magnesium, potassium, calcium and phosphorus), vitamins, lipids and protein. Moreover, DSM is rich in antioxidants and phenolic compounds. Total polyphenol content of DSM is higher than in the edible date flesh [5–8]. It is known that DSM consists numerous favorable properties such as antiviral, antibacterial, and antifungal activity [9–11]. Moreover, DSM has been shown to improve the function and integrity of the immune system [12]. For example, polyphenolic compounds of DSM may affect the immune function of shrimp. It is well known that Polyphenolic compounds can modulate the immune system influencing both activation and differentiation of multiple cell types involved in the immune response [13]. Therefore, phenolic compounds of DSM may increase the immune responses of shrimp and their resistance against pathogens.

DSM is usually used as complementary feed ingredient for animals including cattle, sheep, camel and poultry [4,14]. It has been also incorporated to the diet of some farmed fish species e.g. Nile tilapia (*Oreochromis niloticus*) [15,16], Afrsican catfish (*Clarias gariepinus*) [17], and common Carp (*Cyprinus carpio*) [18]. However, studies exploring the inclusion of DSM in aquatic organisms are mostly limited to evaluating the growth performance [17,19,20]. Our pervious study showed that DSM meal could be incorporated up to 230 g/kg in the diet of farmed shrimp, *P. vannamei* with no adverse effects on growth performance, fatty acid composition and hemolymph biochemical parameters (unpublished results). For evaluation of the suitability of a novel source of food in the diet of farmed animals, its physiological function

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Table 1
The ingredient and chemical composition of the experimental diets with 0 (Control), 50, 100, 150 and 230 g/kg of date seed meal (DSM).

Ingredients (g/kg)	Diet				
	Control	50	100	150	230
Fish meal	329.20	329.20	329.20	329.20	329.20
Soybean meal	200.00	200.00	200.00	200.00	200.00
Meat powder	96.00	96.00	96.00	96.00	96.00
Date seed meal	00.00	50.00	100.00	150.00	230.00
Rice flour	90.00	70.00	50.00	30.00	00.00
Wheat flour	184.80	154.80	124.80	94.80	44.80
Fish oil	40.00	40.00	40.00	40.00	40.00
Soybean oil	20.00	20.00	20.00	20.00	20.00
Vitamin mixture[a]	10.00	10.00	10.00	10.00	10.00
Mineral mixture[b]	10.00	10.00	10.00	10.00	10.00
Filler (cellulose)	20.00	20.00	20.00	20.00	20.00
Chemical composition (g/kg)					
Crud protein	385.00	398.80	385.80	389.70	397.80
Crud lipid	208.60	186.50	212.70	204.90	219.10
Carbohydrate	188.30	195.60	161.30	158.80	127.00
Fiber	29.80	30.40	32.10	31.60	32.90
Ash	87.20	87.70	94.90	98.70	114.40
NFE	259.70	266.20	242.40	243.50	202.80
Dry matter (%)	89.88	89.90	88.68	88.36	89.13
Moisture (%)	10.12	10.10	11.32	11.64	10.87
Energy (kcal kg^{-1})[c]	4170.60	4056.10	4102.70	4038.10	3999.10

[a] Supplied (IU or mg/kg diet): vitamin A, 1800 IU; vitamin D3, 1200 IU; vitamin E, 120 mg; vitamin B_{12}, 24 mg; Riboflavin, 15 mg; Niacin, 90 mg; D-pantothenic acid, 27 mg; Menadione, 3 mg; Folic acid, 4.8 mg; Pyridoxine, 9 mg; Thiamine, 9 mg; D-biotin, 0.48 mg; Choline chloride 360 mg; Cobalamin 24 mg; Ascorbic acid 156 mg; Nicotinic acid 90 mg; Inositol 72; antioxidant 15 mg.

[b] Supplied (mg/kg diet): Zn, 18 mg; I, 0.6 mg; Mg, 7.8 mg; Co, 0.15 mg; Se, 0.15 mg; CU, 1.8 mg; Fe, 12 mg.

[c] Energy was calculated as 4, 4 and 9 kcaL/kg of protein, carbohydrate and lipids, respectively (Calculated from physiological fuel values).

should also be taken into account. For example, understanding the impacts of nutrition on immunity and health status of aquaculture species is a major focus in recent years [21].

Given that DSM is a very rich source of bioactive compounds, it could be considered as a strong candidate for functional feed additive and nutraceutical. Phenolic compounds of DSM may affect the expression of immune related genes and increase the immune responses of shrimp and their resistance against pathogens. In this regard this study aimed to evaluate the expression of a panel of immune-related genes including α2-macroglobulin (α2-M), lipopolysaccharide and beta-1,3-glucan-binding protein (LGBP), integrin β, and peroxinectin (PX) in white shrimp *P. vannamei* fed diet containing different amounts of DSM.

2. Material and methods

2.1. Diet preparation

A date by-product company, Isfahan, Iran, provided DSM. Five diets were prepared by replacing wheat flour and rice flour with different levels of DSM including 0 (Control diet), 50, 100, 150 and 230 g/kg of diet (Table 1). All trial diets were formulated on iso-nitrogenous and iso-energetic. The ingredients of the experimental diets were mixed and pellets were made by an extruder (diameter of pellet was 2 mm), then diets were dried and preserved as described in Pakravan et al. [22,23].

2.2. Experimental shrimp and feeding

P. vannamei were provided from a shrimp culture farm in North Tiab, Hormozgan Iran, and transferred to Kolahi Shrimp development and Training center located at Hormozgan province, Iran. Briefly, after one week of acclimation to laboratory condition, shrimp with weight of 4.0 ± 0.1 g were distributed into five experimental treatments as described above with three replicates. Fifty shrimp were randomly placed in each circular fibreglass tank (300 L volumes with about 200 L water). Shrimp were fed with experimental diets at a rate of 5% body weight per day three times a day at 8:00, 14:00 and 20:00 h during a period of eight weeks. Daily water replacements was about 50%. Total length and weight of shrimp were measured every 2 weeks, and the amounts of daily feeds were readjusted by defining the shrimp total weight in each replicate. During the experimental period, temperature, DO, salinity and pH were about 32 ± 2 °C, 8 mg L^{-1}, 36.5 g L^{-1}, and 8.1 ± 0.1, respectively. At the end of the feeding trial, the hepatopancreas of three shrimp from each tank (nine shrimp from each treatment fed with different diets) were dissected from each shrimp and immediately deep-frozen in liquid nitrogen, and stored in a −80 °C freezer until RNA extraction.

2.3. Quantitative reverse transcription PCR (qRT-PCR)

For RNA preparation, 50–100 mg of hepatopanceas tissue from nine individuals of each treatment was homogenized using 1.0 ml RNAx Plus reagent (SinaClon BioScience, Iran), and treated with DNaseI (SinaClon BioScience, Iran) in accordance with the manufacturers' instructions. RNA quantification was carried out with a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) reading at 260/280 nm and the quality of the RNA was measured by electrophoresis on a 1.5% agarose gel. One microgram of total RNA was used to synthesize first-strand cDNAs using PrimeScriptTM RT reagent Kit (Takara, Tokyo, Japan) following the manufacturer's instructions. The transcripts of four immune-related genes including α2-M, integrin β, PX and LGBP, and the internal reference gene, beta-actin were measured by quantitative real-time PCR on an *QuantStudio*™12K Flex *Real-Time PCR* System (Applied Biosystems) using 1 μl of 1:10 diluted cDNA as a template, 100 nM of primers, 0.2 μl of ROX reference dye and 5 μl of a SYBR Green mix (Prime Q-Master mix, Genet Bio, Daejon, Korea). Each qPCR experiment was performed on five shrimp per treatment with the best RNA quality in triplicates using a programme of 95 °C for 10 min; 40 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s. The sequences of the primers that were used for the qRT-PCR were as follows: a2-M (F- GCACGTAATCAAGAT CCG, R- TGAAGGTGTTGGTCGCAGGTC) [24], integrin β (F- TTGGGC ATCGTGTTCGGACTC, R- TGAAGGTGTTGGTCGCAGGTC) [24], PX (F-ATCCAGCAGCCAGGTATG, R- CAGACTCATCAGATCCATTCC) [25], LGBP (F- CGGCAACCAGTACGGAGGAAC, R-GTGGAAATCATCGGCGA AGGAG) [26], and beta-actin (F- CCACGAGACCACCTACAAC, R- AGC GAGGGCAGTGATTTC [27]. QRT-PCR data were analyzed and amplification efficiencies were determined as previously described [28].

2.4. Statistical analysis

Data were analyzed using one way ANOVA and multiple comparisons performed by Turkey's post hoc based on significant effect at $P < 0.05$. Statistical analyses were conducted with SPSS (version 16) and the data were plotted using Sigma Plot (version 11).

3. Results

Transcripts of α2-M, integrin β, PX and LGBP were detected in *P. vannamei* fed diet with different levels of DSM. The results showed that the expression of α2-M was significantly lower in *P. vannamei* fed diets with 150 and 230 g/kg DSM compared to control group ($p < 0.05$). However, no significant differences were observed between control group and the 50 and 100 g/kg treatments (Fig. 1A). The transcript levels of integrin β were also lower in shrimp fed with 150 g/kg DSM compared to control group ($p < 0.05$), but here were no significant

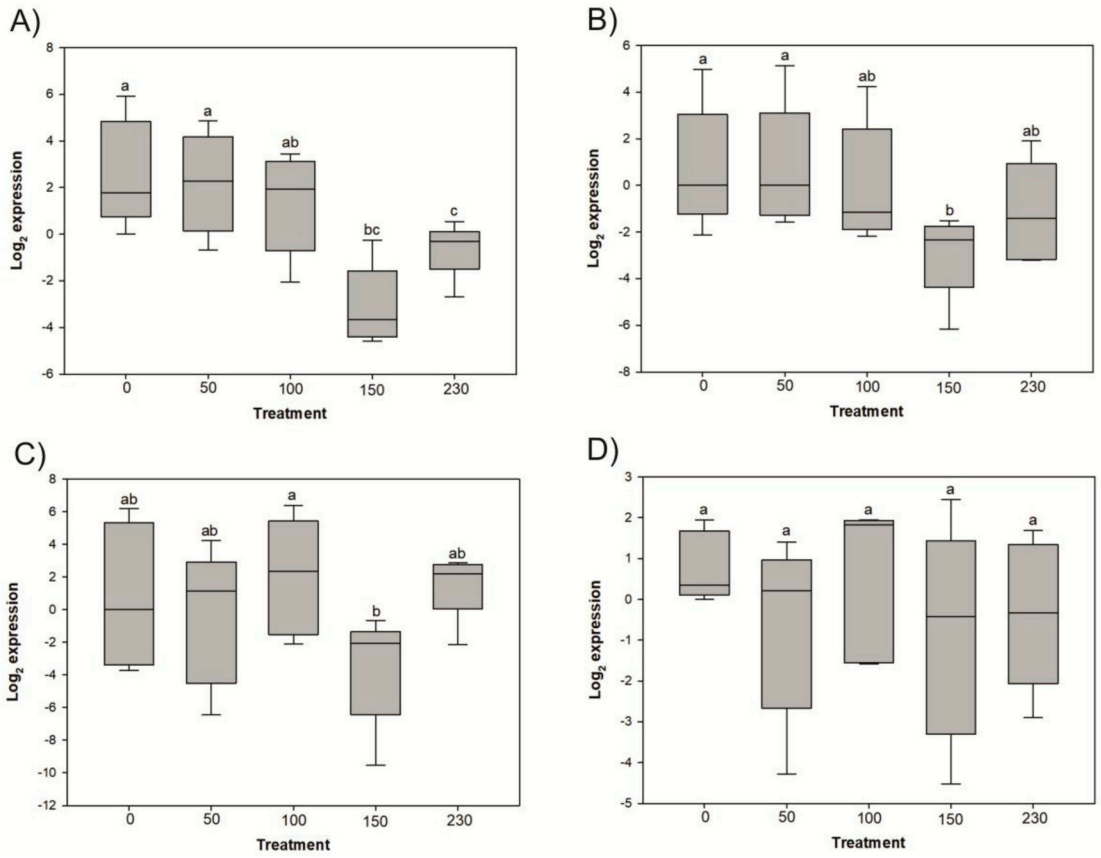

Fig. 1. Log_2 expression of (A) α2-macroglobulin (α2-M), (B) integrin β, (C) Peroxinectin, (D) lipopolysaccharide and beta-1,3-glucan-binding protein (LGBP), in hepatopancras of *Penaeus vannamei* fed diet with 0 (Control), 50, 100, 150 and 230 g/kg of date seed meal (DSM). Bars (mean ± SD, n = 5) with different letters are significantly different among treatments according to ANOVA test ($p < 0.05$).

differences among other treatments and control group (Fig. 1B). The expression of Peroxinectin and LGBP genes are shown in Fig. 1C and D. Results showed no significant differences in the expression of Peroxinectin and LGBP among shrimp fed different amounts of DSM and control group ($p > 0.05$), however, the expression of Peroxinectin was significantly lower in shrimp fed with 150 g/kg compared to that of 100 g/kg ($p < 0.05$).

4. Discussion

This study was performed to extend our understanding of the effects of DSM on the immune status of farmed aquatic species, particularly crustacean with lack of adaptive immune systems. Therefore, the expression of a panel of immune-related genes was investigated using the qRT-PCR in order to evaluate the immune status of shrimp after feeding diets with different levels of DSM. The results of the present study showed that up to 100 g/kg DSM in the diet caused no changes in the expression of immune-related genes in *P. vannamei*. However, 150 or 230 g/kg DSM in the diet showed suppressive effects on the expression of some immune-related genes in farmed shrimp. The effect of DSM on immune status was only studied in broilers where up to 6% supplementation of DSM resulted in enhancement of immune parameters [29].

Our results showed that the expression of α2-M was downregulated in shrimp fed diets containing more than 100 g/kg DSM. α2-macroglobulin is a non-specific protease inhibitor involved in innate immune system by inactivating proteases released by pathogens and dead or dying cells in both vertebrates and invertebrates [30–33]. Previous studies showed an increase in crustacean' a2M mRNA transcription upon bacterial, fungal and viral infection, as well as in response to some diets with immunostimulant properties [34,35]. On the contrary, our results showed a decreasing expression of shrimp a2M in response to higher levels of DSM. Therefore, higher amounts of DSM might decrease the protease inhibitor activity in shrimp. Similar to our results, excessive amounts of *Morinda citrifolia* leave extract also caused suppression of a2M gene expression in *Macrobrachium rosenbergii* [36]. In line with a2M expression, the transcripts of integrin β gene were also downregulated in response to higher dietary levels of DSM. Integrin β has a fundamental importance in innate immune responses as a cell adhesion receptor during the encounter of infections [37]. Integrin β mediates cell to cell, cell to extracellular matrix (ECM), and cell to pathogen interactions [38]. Recent studies demonstrated that integrin β might function as a cellular receptor for white spot syndrome virus (WSSV) infection in shrimp [39].

Crustaceans have mechanisms to recognize cell wall components of pathogens such as β-1,3-glucans (BG), lipopolysaccharides (LPS) and peptidoglycan (PG). Several pattern recognition proteins (PRPs) like LGBP recognize and respond to microbial intruders and are involved in activation of proPO (prophenoloxidase) system. Administering of polysaccharides like BG, PG, or LPS could increase the immune responses in shrimp and their resistance against pathogens [26]. For example, LGBP transcripts of *P. vannamei* fed diet containing β-1,3-glucan [40], carrageenan [41], and alginate [42] were upregulated within 72 h, and 3 weeks post-feeding, respectively. Our results showed that no remarkable changes in the expression of LGBP gene in shrimp fed different levels of DSM. It is likely that DSM contains no polysaccharide immunostimulants compounds to modulate the synthesis of PRPs in shrimp. Similarly, the expression of LGBP was not affected by administration of β-glucan and rutin in leshy shrimp (*Fenneropenaeus chinensis*) [43].

Consistent with LGBP expression, PX expression remained unchanged in *P. vannamei* fed diet containing different levels of DSM. Peroxinectin is also an important protein involved in proPO cascade in shrimp [44]. It is a multifunctional protein, promotes cell adhesion and encapsulation of bacteria in crustaceans [43,45]. Similar to our results, the levels of PX transcripts remained unchanged in freshwater crayfish species *Astacus astacus* and *Pacifastacus leniusculus* injected with laminarin [46] and in leshy shrimp, *F. chinensis* fed diets containing β-glucan and rutin [43]. On the other hand, feeding with diet containing sodium alginate for 5 months significantly increased PX expression in *P. monodon* [47].

In conclusion, the present study examined the effects of DSM on immune response in *P. vannamei* in order to obtain new basic information in their natural state prior to infection by pathogens. Our results showed that up to 100 g/kg incorporation of DSM as a low cost source of carbohydrate had no suppressive effects on the expression of immune-related genes in *P. vannamei*.

Conflicts of interest

The authors declare that they have no conflict of interest.

Ethical approval

The experiments were conducted in accordance with the Iranian Society for The Prevention of Cruelty to Animals and the Canadian Council on Animal Care.

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