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Effects of diets with fermented duckweed (*Lemna* sp.) on growth performance and gene expression in the Pacific white shrimp, *Litopenaeus vannamei*

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Abstract This study evaluated the effects of diets with fermented duckweed flour (*Lemna* sp.) (FDF) on growth performance and gene expression in Pacific white shrimp, *Litopenaeus vannamei*. Shrimp were cultured in an outdoor system during 50 days and fed diets containing 0, 5, 15, 25, and 35 % FDF replacing fishmeal (FM) (diets D0, D5, D15, D25, and D35, respectively). At the end of the bioassay, shrimp survival was 100 % in all treatments and growth performance was significantly better than D0 (100 % FM), especially in diet D35 with 35 % FDF. The mRNA expression of trypsin, chymotrypsin, cathepsin B, heat shock protein 70 (Lvhsp70), and heat shock protein 90 (Lvhsp90) was significantly increased at the highest FDF concentrations in diets (D15, D25, and D35) as compared to D0. Dietary FDF affected the immune system of shrimp only in diets D5 (superoxide dismutase and lysozyme) and D15 (lysozyme) where mRNA expression was significantly higher than D0. FM can be replaced with up to 35 % FDF without adversely affecting the survival and growth performance of cultured shrimp. The inclusion of FDF in diets affected the expression of stress and digestive genes, but, in immune-related genes, the effect did not show a clear trend.

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Introduction

The continuous growth and intensification of aquaculture worldwide demand high amounts of fishmeal (FM), an ingredient that has been used historically as a major source of dietary protein due to its suitable amino acid profile and palatability. As in other cultured animals, shrimp farming depends on FM supplementation to meet protein requirements. However, the reduction in the availability of FM due to its high cost and demand has led to the need of finding alternative protein sources to supplement the requirements of the aquaculture industry (Tacon and Metian 2008; Cheng et al. 2013).

Plant proteins represent a good option for replacing FM in aquaculture diets. Previous work has shown that the substitution of FM with plant-based protein is feasible without compromising growth and performance of cultured shrimp (Amaya et al. 2007; Suárez et al. 2009; Gamboa-Delgado et al. 2013).

Duckweed (*Lemna* spp.) is an aquatic macrophyte distributed worldwide (Landolt 1986). It has been used as fresh food for ducks and other animals (Leng et al. 1995) and in the preparation of diets for dogs (Brown et al. 2013), poultry (Anderson et al. 2011), and fish (Bairagi et al. 2002; Yilmaz et al. 2004; He et al. 2013). Duckweed can contain from 28 to 43 % crude protein, 5 % fiber in dry weight, high concentration of trace minerals, such as phosphorus and potassium, as well as xanthophylls and carotenes (Chaturvedi et al. 2003). However, it is important to consider that the presence of fiber and antinutritional factors (tannins and phytic acid, among others) in plants affects negatively their nutritional value and, consequently, growth of cultured animals (Edwards et al. 1985; Mukho-Padhayay and Ray 1999; Bairagi et al. 2002; Kumaraguru Vasagam et al. 2007). To this regard, the fermentation process of plants contributes to enhance flavor and texture as well as to decrease the antinutritional factors and crude fiber content, increasing plant digestibility (Bairagi et al. 2002; Nout 2009).

As a result of the inclusion of plant-based ingredients in shrimp diets, current research is now focused on shrimp responses (e.g., metabolic, physiological) to these dietary changes, as well as on the expression of genes responsible for such responses (Chávez-Calvillo et al. 2010). Regarding the gene expression, although there is no direct relationship between mRNA and the amount of protein eventually produced, mRNA provides some information about the "potential" for synthesis of a particular gene product (Sanchez-Paz et al. 2003). The expression levels of genes such as trypsin, chymotrypsin, and cathepsin B have been evaluated in response to source and level of protein of the diets for shrimp (Muhlia-Almazán et al. 2003; Chookird et al. 2010; Stephens et al. 2012). Furthermore, it is important to assess the immunostimulatory capacity of peptidoglycans provided to diet by bacteria during fermentation process.

The aim of this study was to evaluate the effects of FM replacement with different dietary levels of fermented duckweed on growth performance and gene expression in the Pacific white shrimp, *Litopenaeus vannamei*.

Materials and methods

Experimental diets

Duckweed (*Lemna* sp.) flour was fermented with two bacteria (*Bacillus pumilus* and *Pediococcus pentosaceus*), 10 % molasses, and 100 % moisture. Both bacteria were inoculated simultaneously at 1×10^6 CFU of each bacterium per gram of flour. The fermentation process was performed during 144 h at 35 °C (Flores-Miranda et al. 2014). Five experimental diets with fermented duckweed flour (FDF) were formulated to replace 0, 5, 15, 25, and 35 % of FM (Table 1).

Shrimp acclimation to laboratory conditions

Selection of healthy shrimp from a local farm was based on visible features. Shrimp were acclimated to ambient laboratory conditions for 3 days in 120-L indoor plastic tanks containing 80 L of filtered (20-μm mesh) seawater (30 ‰) and constant aeration in groups of 10 organisms per tank. Shrimp were fed twice daily at 09:00 and 17:00 h with commercial feed (Purina®, 35 % protein). Ration was according to commercial dietary tables (Purina®). At day 3, uneaten food was eliminated and half of the water was changed.

Feeding trial

Shrimp weighing 0.40 ± 0.2 g were fed with experimental diets (D0, D5, D15, D25, and D35) (Table 1). The feeding trial was carried out at the facilities of the Department of Aquaculture (CIIDIR-IPN, Sinaloa, Mexico) in an outdoor culture system in 120-L plastic tanks with 80 L of seawater and continuous aeration. Each treatment with three replicates had 12 shrimp per tank. Shrimp were fed twice daily at 09:00 and 17:00 h, and ration was adjusted every 10 days according to Purina tables. Uneaten food and waste matter were removed every 3 days before feeding, and 50 % of the water was exchanged. Values of pH (HI 98127 pHep, Hanna Instruments, Woonsocket, RI, USA), salinity (Refractometer W/ATC 300011, Sper Scientific, Scottsdale, AZ, USA), dissolved oxygen (YSI model 55 oxygen meter, Yellow Spring Instruments, Yellow Springs, OH, USA), and temperature were monitored every 3 days, before water exchange.

The bioassay was conducted for 50 days with five treatments, as follows: D0) diet without FDF (control); D5) diet with 5 % FDF; D15) diet with 15 % FDF; D25) diet with 25 % FDF; and D35) diet with 35 % FDF. During the bioassay, water temperature ranged from 23.9 ± 2.7 to 24.1 ± 2.6 °C, dissolved oxygen from 4.8 ± 0.5 to 4.9 ± 0.6 mg L^{-1}, salinity from 30.9 ± 1.1 to 31.1 ± 1.2 ‰, pH 8.3 ± 0.1, ammonium from 0.13 ± 0.02 to 0.23 ± 0.07 mg L^{-1}, nitrites from 0.3 ± 0.2 to 0.4 ± 0.1 mg L^{-1}, and nitrates from 0.62 ± 0.13 to 0.78 ± 0.12 mg L^{-1} in all treatments. Water parameters measured during the trial period remained within acceptable ranges for optimal growth of the whiteleg shrimp (Brock and Main 1994), except for the water temperature (23–30 °C) that fluctuated between 21.2 and 26.7 °C. At the end of the bioassay, weight and survival were determined. In addition, hemolymph and hepatopancreas samples were collected to determine the relative expression of stress, digestive, and immune-related genes.

Table 1 Ingredients (g kg^{-1}) and proximate composition (% dry weight) of experimental diets

Ingredients (g kg^{-1})	Experimental diets				
	D0	D5	D15	D25	D35
Fishmeal	250	237.5	212.5	187.5	162.5
Duckweed flour	0	50	150	250	350
Soybean meal	175	175	175	175	175
Wheat meal	500	462.5	387.5	312.5	237.5
Grenetin	40	40	40	40	40
Fish oil	17	17	17	17	17
Soybean lecithin	17	17	17	17	17
Vitamin premix[a]	0.1	0.1	0.1	0.1	0.1
Mineral premix[b]	1.1	1.1	1.1	1.1	1.1
Proximate composition (% dry matter)					
Protein	37.2 ± 0.06	37.1 ± 0.12	36.4 ± 0.0	34.9 ± 0.06	34.4 ± 0.00
Lipid	6.9 ± 0.06	6.4 ± 0.06	6.8 ± 0.06	6.3 ± 0.06	5.8 ± 0.00
Ash	6.4 ± 0.00	7.6 ± 0.00	9.1 ± 0.06	10.9 ± 0.00	12.5 ± 0.00
Crude fiber	0.6 ± 0.12	0.9 ± 0.12	1.4 ± 0.17	1.7 ± 0.06	1.0 ± 0.06
Moisture	9.6 ± 0.12	11.0 ± 0.06	12.1 ± 0.12	10.8 ± 0.12	9.1 ± 0.06
Gross energy (cal g^{-1})	4,425.03 ± 13.46	4,357.11 ± 9.55	4,282.25 ± 12.53	4,194.96 ± 15.03	4,144.46 ± 9.64
NFE	48.74	47.93	46.16	45.99	46.21

Diet D0 (fishmeal), diets D5–D35 (fishmeal replaced by FDF). Proximate analysis = mean ± SE

NFE nitrogen-free extract

[a] Vitamin premix (units in mg kg^{-1}, except when indicated): retinol, 5,000 IU; cholecalciferol, 4,000 IU; α-tocopherol acetate, 100; menadione, 5; thiamin, 60; riboflavin, 25; pyridoxine HCl, 50; pantothenic acid, 75; niacin, 40; biotin, 1; inositol, 400; cyanocobalamin, 0.2; folic acid, 10

[b] Mineral premix (g kg^{-1} diet): KCl, 0.5; $MgSO_4 \cdot 7H_20$, 0.5; $ZnSO_4 \cdot 7H_2O$, 0.09; $MnCl_2 \cdot 4H_2O$, 0.0234; $CuSO_4 \cdot 5H_2O$, 0.005; KI, 0.0005; $CoCl_2 \cdot 2H_2O$, 0.00025; Na_2HPO_4, 2.37

In vivo measurements and performance indexes

Shrimp were weighed at the beginning and at the end of the bioassay. Weight at harvesting (WH), absolute growth (AG), specific growth rate (SGR), feed conversion ratio (FCR), and protein efficiency ratio (PER) were calculated according to the following formulae (Ziaei-Nejad et al. 2006; Amaya et al. 2007):

$$\text{AG (g)} = W - W_0;$$

$$\text{SGR } \left(\% \text{ day}^{-1}\right) = (\ln W_t - \ln W_0) \times 100/t;$$

$$\text{FCR} = \text{feed intake/weight gain};$$

$$\text{PER} = \text{weight gain/protein intake};$$

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

where t is the culture period in days, ln W_0 is the natural logarithm of the weight of the shrimp at the beginning of the bioassay, and ln W_t is the natural logarithm of the weight of the shrimp at day t (W_0 and W_t are in grams).

Hemolymph and hepatopancreas

Two pools of hemolymph (four shrimp each one) from each replicate (six pools per treatment) were collected. Hemolymph (300 μL) of each intermolt shrimp was withdrawn from the pleopod base of the first abdominal segment with sterile 1-mL syringes (27G × 13 mm needle) loaded with 600 μL of a precooled (4 °C) solution (27 mM tri-sodium citrate, 385 mM NaCl, 115 mM glucose, at pH 7.5) used as an anticoagulant (Huang et al. 2010). Hemolymph was centrifuged at 800×g for 10 min at 4 °C. The plasma was removed, and the hemocyte pellet was rinsed with 250 μL of cold anticoagulant (4 °C) by centrifuging as above. The supernatant was removed, and the hemocytes were suspended in 250 μL of precooled Trizol Reagent® (Invitrogen, Carlsbad, CA, USA) and stored at −80 °C until use. Hepatopancreas of two shrimp per replicate (six per treatment) were dissected and placed individually in Trizol as above.

Total RNA isolation and cDNA synthesis

Total RNA of hemocytes and hepatopancreas was extracted with Trizol Reagent according to manufacturer's protocol. A nanophotometer (Implen, Inc., Westlake Village, CA, USA) was employed to analyze the RNA concentration and its purity. The RNA was treated with DNAse 1(1 U μL^{-1}, Sigma®-Aldrich, St. Louis, MO, USA). Reverse transcription was used to synthesize the first strand of cDNA using reverse transcriptase (Improm II, Promega®, Madison, WI, USA) with the oligo (dT_{20}) primer using 500 ng of total RNA. The cDNA was suspended in 80 μL of ultrapure water and stored at −80 °C until analysis. Five microliters of this cDNA dilution was used as template in qRT-PCRs.

Expression analysis of digestive, stress, and immune-related genes by qPCR

The expression of five immune-related genes [prophenoloxidase (proPO), transglutaminase (TGase), superoxide dismutase (SOD), lysozyme (Lys), and toll receptor (LvToll)] in

hemocytes, three digestive genes [trypsin (Try), chymotrypsin (Chymo), and cathepsin B (Cath B)] in the hepatopancreas, and two stress genes [heat shock protein 70 (Lvhsp70) and heat shock protein 90 (Lvhsp90)] in the hepatopancreas of experimental shrimp was measured by quantitative real-time PCR using a CFX96 system and the CFX Manager version 3.0 (Bio-Rad Laboratories, Hercules, CA, USA).

The expression of four genes was analyzed as reference genes (40S–S24, β-actin, EF1α, and L21). The **e**xpression of target genes was normalized to geometric mean of most stable reference genes. In order to determine the most stable reference genes, the relative stability was analyzed separately in samples obtained from hemocytes or hepatopancreas, using two algorithms: GeNorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004), using the RefFinder web application (http://www.leonxie.com/referencegene.php) that conveniently implements these algorithms. In hemocytes, the selected genes were EF1α and 40S–S24, whereas β-actin and L21 genes were chosen in the hepatopancreas. The primer sequences used for qPCR are shown in Table 2.

Amplifications were performed in duplicate in a 96-well plate in a 15-μL reaction volume containing 7.5 μL of 2× PCR Master Mix (1.5 μL of 10× reaction buffer, 0.75 μL of 50 mM $MgCl_2$, 0.3 μL of 10 mM dNTPs, 0.75 μL of EvaGreen® 20× [Biotium, Hayward, CA, USA]; 0.1 μL of 5 U μL^{-1} Biolase DNA Polymerase [Bioline™, Taunton, MA,USA], and 4.1 μL of ultrapure water), 0.35 μL of each primer (10 μM, Sigma®-Aldrich, St. Louis, MO, USA), 1.8 μL of ultrapure water, and 5 μL of cDNA. Amplification conditions were as follows: 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 15 s, 72 °C for 30 s, and 79 °C for 5 s (to acquire fluorescence), using a CFX96 Real Time PCR thermal cycler (BIO-RAD). After each reaction, a dissociation curve from 65 to 90 °C was recorded at increments of 0.5 °C and examined for unique and specific products.

Efficiency of the PCR was determined by calculating a slope with five serial dilutions (dilution factor of 5 or 10) of a representative pool of cDNA [E = 10(−1/slope) − 1]. To calculate expression of target genes, Cq values were transformed to relative quantities (RQ) using the equation $RQij = E^{[(Cqmean)-Cq(ij)]}$, where E is the gene-specific efficiency and [(Cq mean − Cq(ij)] is the absolute difference for each Cq sample against the mean Cq in the dataset for each gene. Relative expression was calculated with the equation RQ^{target}/Geometric mean of $RQ^{reference\ genes}$ (Hellemans et al. 2007).

Statistical analysis

One-way variance (ANOVA) analysis was applied to examine the differences in SGR, survival (%), and expression of immune, digestive, and stress-related genes among treatments. Survival data were arcsine transformed according to Daniel (1997). Where significant ANOVA differences were found, a post hoc test (Duncan's multiple range test) was used to identify these differences at $p < 0.05$.

Results

Survival and growth performance

During the experimental period, shrimp survival was 100 %. Growth performance showed a tendency to increase in fermented duckweed treatments as compared to D0 (100 % FM),

Table 2 Specific primers used for PCR amplifications of shrimp genes

Genes	Primers	Sequence (5′–3′)	References
Immune-related			
Prophenoloxidase	proPO-F	GAGATCGCAAGGGAGAACTG	Wang et al. (2010)
	proPO-R	CGTCAGTGAAGTCGAGACCA	
Transglutaminase	TGase-F	CCTCAGGATCTCCTTCACCA	Wang et al. (2010)
	TGase-R	TTGGGAAAACCTTCATTTCG	
Superoxide dismutase	SOD-F	ATCCACCACACAAAGCATCA	Wang et al. (2010)
	SOD-R	AGCTCTCGTCAATGGCTTGT	
Lysozyme	Lyz-F	GAAGCGACTACGGCAAGAAC	Wang et al. (2010)
	Lyz-R	AACCGTGAGACCAGCACTCT	
Toll receptor	LvToll-F	ATGTGCGTGCGGATACATTA	Wang et al. (2010)
	LvToll-R	GGGTGTTGGATGTCGAGAGT	
Digestive			
Trypsin	qLvTRY F	TCCAAGATCATCCAACACGA	Álvarez-Ruiz et al. (unpublished data)
	qLvTRY R	GACCCTGAGCGGGAATATC	
Chymotrypsin	ChymoLvFw1	GGCTCTCTTCATCGACG	Stephens et al. (2012)
	ChymoLvRv1	CGTGAGTGAAGAAGTCGG	
Cathepsin B	CatBFw2Lv	GGATGTAACGGAGGCTTC	Stephens et al. (2012)
	CatBRv1Lv	CTGTATGCTTTGCCTCCA	
Stress			
Lvhsp70	hsp70 F	GGCAAGGAGCTGAACAAGTC	Álvarez-Ruiz et al. (unpublished data)
	hsp70 R	TCTCGATACCCAGGGACAAG	
Lvhsp90	hsp90 F	TGGGGCTTCTACTCCGCCTACC	Qian et al. (2012)
	hsp90 R	ACGGTGAAAGAGCCTCCAGCA	
Housekeeping			
40S–S24	qLv40S_S24-F	CAGGCCGATCAACTGTCC	Álvarez-Ruiz et al. (unpublished data)
	qLv40S_S24-R	CAATGAGAGCTTGCCTTTCC	
L21	L21LvFw2	GTTGACTTGAAGGGCAATG	Stephens et al. (2012)
	L21LvRv2	CTTCTTGGCTTCGATTCTG	
EF1α	qLvEf-F	CTGTGGTCTGGTTGGTGTTG	Álvarez-Ruiz et al. (unpublished data)
	qLvEf-R	TCAGATGGGTTCTTGGGTTC	
β-Actin	qActin-Fw	CCACGAGACCACCTACAAC	Wang et al. (2010)
	qActin-Rv	AGCGAGGGCAGTGATTTC	

especially in D35 with 35 % FM substitution. WH values were from 2.76 ± 0.03 g in D0 to 3.45 ± 0.17 g in D35. In WH, significant differences were found in diets D5, D15, and D35 regarding to D0 ($p = 0.043$). SGR ranged from 3.89 ± 0.12 g day^{-1} in D25 to 4.32 ± 0.11 g day^{-1} in D5, without significant differences among treatments. AG ranged from 2.38 ± 0.07 g in D0 to 3.04 ± 0.17 g in D35. FCR values were from 2.06 ± 0.07 in D35 to 2.42 ± 0.09 in D25, and significant differences ($p = 0.223$) were found in D15 and D35 respect to D0. PER of the experimental diets ranged from 1.12 ± 0.05 in D0 to

Table 3 Survival and growth performance of Pacific white shrimp fed the experimental diets

Diets	Survival (%)	WH (g)	AG (g)	SGR (% day^{-1})	FCR	PER
D0	100 ± 0.00	2.76 ± 0.03^{a}	2.38 ± 0.07	3.98 ± 0.21	2.40 ± 0.11^{b}	1.12 ± 0.05^{a}
D5	100 ± 0.00	3.30 ± 0.29^{b}	2.92 ± 0.27	4.32 ± 0.11	2.18 ± 0.04^{ab}	1.24 ± 0.02^{ab}
D15	100 ± 0.00	3.43 ± 0.01^{b}	3.00 ± 0.01	4.18 ± 0.05	2.15 ± 0.02^{a}	1.27 ± 0.01^{b}
D25	100 ± 0.00	3.06 ± 0.12^{ab}	2.62 ± 0.11	3.89 ± 0.12	2.42 ± 0.09^{b}	1.19 ± 0.04^{ab}
D35	100 ± 0.00	3.45 ± 0.17^{b}	3.04 ± 0.17	4.28 ± 0.13	2.06 ± 0.07^{a}	1.41 ± 0.05^{c}

Experimental diets: D0) diet without FDF (control), D5) diet with 5 % FDF, D15) diet with 15 % FDF, D25) diet with 25 % FDF, and D35) diet with 35 % FDF. Values are mean ± SE. Values in the same column with different superscripts are significantly different ($p < 0.05$)

WH weight at harvesting, *AG* absolute growth, *SGR* specific growth rate, *FCR* feed conversion ratio, *PER* protein efficiency ratio

1.41 ± 0.05 in D35 with significant differences ($p = 0.0006$) among these two diets, which means a better quality protein in diet D35. Among treatments with FDF, the worst performance was observed in D25 and the best was found in D35 (Table 3).

mRNA relative expression of trypsin, chymotrypsin, and cathepsin B in the hepatopancreas

Results (Fig. 1) showed that the expression of trypsin in diet D25 (2.29 ± 0.44) was significantly higher than in D0 (0.90 ± 0.16, $p = 0.0019$), D5 (0.80 ± 0.26, $p = 0.0011$), D15 (1.06 ± 0.19, $p = 0.0043$), and D35 (1.43 ± 0.17, $p = 0.032$). The expression of chymotrypsin in diet D25 (2.63 ± 0.48) was significantly higher than in D0 (0.71 ± 0.12, $p = 0.0002$), D5 (0.85 ± 0.30, $p = 0.0003$), and D15 (1.24 ± 0.19, $p = 0.003$). The expression of chymotrypsin in diet D35 (1.83 ± 0.21) was significantly higher than in D0 (0.71 ± 0.12, $p = 0.017$), D5 (0.85 ± 0.30, $p = 0.030$). The expression of cathepsin B in diet D35 (2.90 ± 0.71) was significantly higher than in D0 (0.87 ± 0.32, $p = 0.013$) and D5 (0.97 ± 0.33, $p = 0.016$). The mRNA expression of trypsin and chymotrypsin reached it maximum level in D25, but they were down-regulated at the highest FDF concentration in feed (35 %). The mRNA expression of cathepsin B showed an increasing trend from D0 to D35.

mRNA relative expression of stress genes in hepatopancreas

The mRNA expression profiles of Lvhsp70 and Lvhsp90 in shrimp hepatopancreas are shown in Fig. 2. The expression of Lvhsp70 in diet D15 (1.83 ± 0.22) was significantly higher than in D0 (0.67 ± 0.14, $p = 0.003$) and D5 (0.6087 ± 0.20, $p = 0.003$). In diet D25, the expression of Lvhsp70 (2.49 ± 0.35) was significantly higher than in D0 (0.67 ± 0.14, $p = 0.0000$) and D5 (0.6087 ± 0.20, $p = 0.0000$). The expression of Lvhsp70 in diet D35 (2.11 ± 0.27) was significantly higher than in D0 (0.67 ± 0.14, $p = 0.0006$) and D5 (0.6087 ± 0.20, $p = 0.0006$). The expression of Lvhsp90 in diet D15 (1.54 ± 0.35) was significantly higher than in D0 (0.67 ± 0.14, $p = 0.008$). Shrimp fed with FDF (D5, D15, D25, and D35) were more stressed than animals fed with 100 % FM (D0); however, there is a decreasing trend with diet D35.

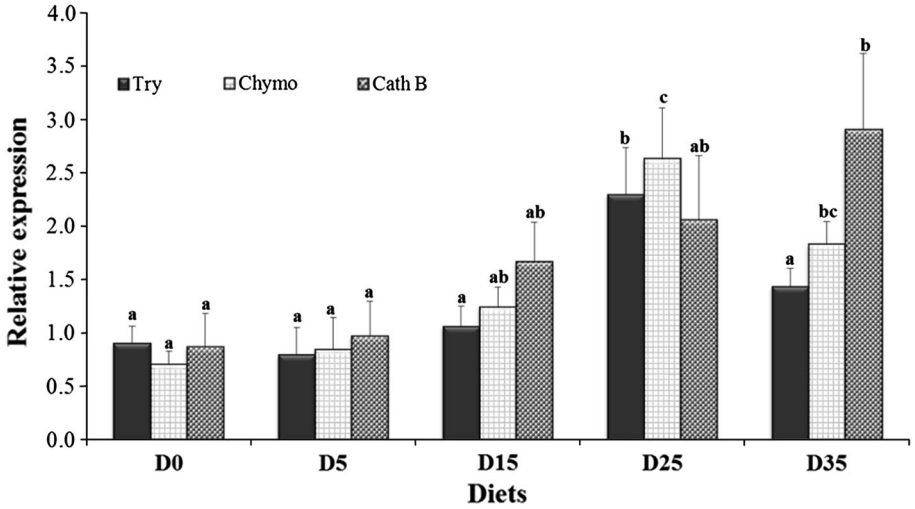

Fig. 1 Relative mRNA expression of trypsin, chymotrypsin, and cathepsin B (normalized to β-actin and L21) in hepatopancreas of Pacific white shrimp. Experimental diets: *D0* diet without FDF (control), *D5* diet with 5 % FDF, *D15* diet with 15 % FDF, *D25* diet with 25 % FDF and *D35* diet with 35 % FDF. The relative expression data represent mean ± SE. *Different letters* indicate significant differences ($p < 0.05$)

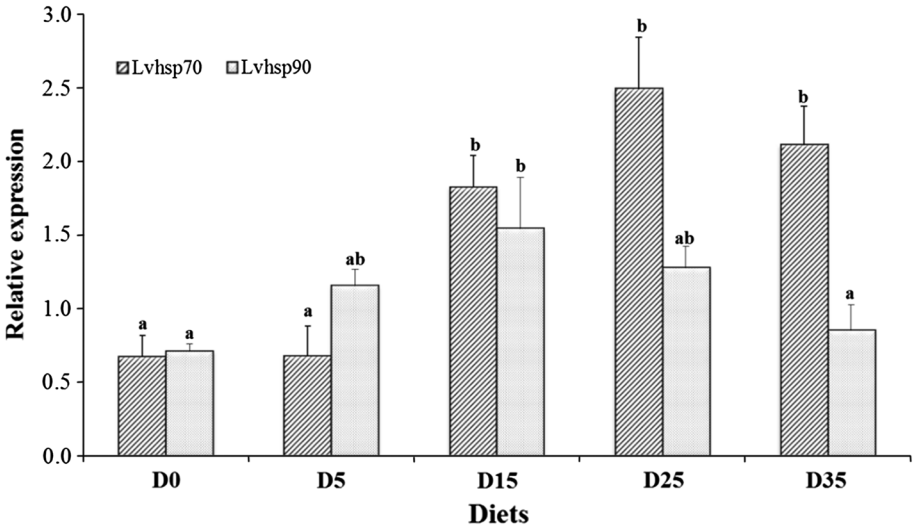

Fig. 2 Relative mRNA expression of Lvhsp70 and Lvhsp90 (normalized to β-actin and L21) in hepatopancreas of Pacific white shrimp. Experimental diets: *D0* diet without FDF (control), *D5* diet with 5 % FDF, *D15* diet with 15 % FDF, *D25* diet with 25 % FDF, and *D35* diet with 35 % FDF. The relative expression data represent mean ± SE. *Different letters* indicate significant differences ($p < 0.05$)

mRNA relative expression of immune-related genes in hemocytes

Figure 3 summarizes the results of mRNA relative expression of immune-related genes in hemocytes of Pacific white shrimp, along the feeding trial. The dietary FDF did not affect

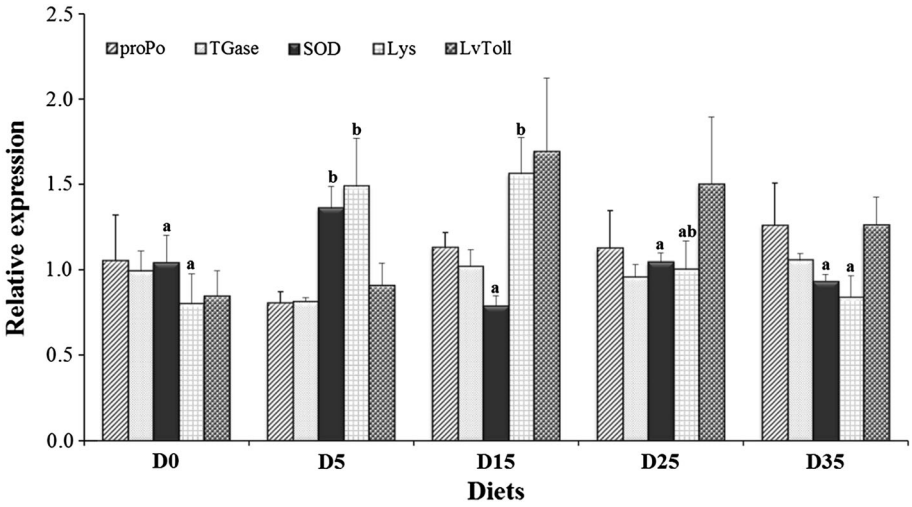

Fig. 3 Relative mRNA expression of proPO, TGase, SOD, Lys, and LvToll (normalized to EF1α and 40S–S24) in hemocytes of Pacific white shrimp. Experimental diets: *D0* diet without FDF (control), *D5* diet with 5 % FDF, *D15* diet with 15 % FDF, *D25* diet with 25 % FDF, and *D35* diet with 35 % FDF. The relative expression data represent mean ± SE. *Different letters* indicate significant differences ($p < 0.05$)

significantly the expression of proPO, TGase, and LvToll. For the SOD gene, the relative expression in diet D5 (1.36 ± 0.13) showed significant differences from the values in D0 (1.04 ± 0.16, $p = 0.04$), D15 (0.79 ± 0.06, $p = 0.0008$), D25 (1.04 ± 0.05, $p = 0.032$), and D35 (0.93 ± 0.04, $p = 0.008$). The expression of Lys in diet D5 (1.49 ± 0.28) showed significant differences in regard to D0 (0.80 ± 0.18, $p = 0.033$) and D35 (0.84 ± 0.13, $p = 0.038$). The expression of Lys in diet D15 (1.56 ± 0.21) showed significant differences as compared to D0 (0.80 ± 0.18, $p = 0.021$) and D35 (0.84 ± 0.13, $p = 0.025$). Dietary FDF affected positively the immune system of shrimp only in diets D5 (SOD and Lys) and D15 (Lys). SOD and Lys were down-regulated in diets D25 and D35 to expressions similar to those of the control diet D0.

Discussion

Fishmeal constitutes the most common protein source used in diets for shrimp culture (Cheng et al. 2013; FAO 2012; Oujifard et al. 2012), and it is well documented that it is possible to replace partially FM with plant protein sources without affecting growth performance of shrimp and fish (Bairagi et al. 2002; Richard et al. 2011; Sookying and Davis 2011). Additionally, fermentation processes are used to improve the nutritional quality of vegetable flour by lowering fiber and antinutritional factors (Bairagi et al. 2002; Saha and Ray 2011). According to the above, this study was conducted to assess the possibility of including FDF in diets for Pacific white shrimp by assessing growth performance and expression of digestive, stress, and immune-related genes.

In this work, FM was replaced up to 35 % (D35) with FDF in diets for juveniles of Pacific white shrimp without affecting survival. These results are consistent with those of Amaya et al. (2007), Suárez et al. (2009), and Bauer et al. (2012), who found no significant

differences in survival of *L. vannamei* fed diets where FM was partially or totally replaced with plant protein sources (non-fermented) such as soy and canola.

On the other hand, results indicate that substitution of 35 % FM with FDF has a positive effect on shrimp growth since animals grew better than those fed with 100 % FM. These results are better than expected considering the high quality of FM protein. Bauer et al. (2012) did not find significant differences in growth when replacing 25, 50, 75, and 100 % of FM with non-fermented soybean in diets for Pacific white shrimp. Kiron et al. (2012) found that growth performance of *L. vannamei* was not affected when animals were fed with up to 40 % non-fermented microalgae (*Nanofrustulum* and *Tetraselmis*). Likewise, Macias-Sancho et al. (2014) found no significant differences in growth of Pacific white shrimp, when they were fed with up to 75 % *Spirulina platensis* (non-fermented). Results showed that the fermentation process can improve protein quality of duckweed flour as observed in PER. However, it is important to mention that in addition to their role in fermentation, bacteria are an important source of high-quality protein with up to 70 % (Aas et al. 2006a, b).

Digestive proteinases from marine animals can be grouped on the basis of their similarity to well-characterized proteinases such as trypsin-like, chymosin-like, or cathepsin-like. Furthermore, based on the nature of the catalytic site, they may be classified into four groups, as acid or aspartate proteinases, serine proteinases, thiol or cysteine proteinases, and metalloproteinases (Simpson 2000; Klomklao 2008). In shrimp, protein digestion appears to be undertaken by serine proteinases, like trypsin and chymotrypsin, and by cysteine proteinases, like cathepsin B and cathepsin L (Muhlia-Almazán and García-Carreño 2002; Hu and Leung 2007; Omondi 2005; Stephens et al. 2012).

Although there is no direct relationship between mRNA and the amount of protein eventually produced, mRNA provides some information about the "potential" for synthesis of a particular gene product (Sanchez-Paz et al. 2003). In this study, the mRNA expression of trypsin and chymotrypsin reached its maximum level in D25, but it has a trend to decrease at the highest FDF concentration in feed (35 %), especially trypsin. On the other hand, the mRNA expression of cathepsin B showed an increasing trend from D0 to D35. Trypsin and chymotrypsin are the main digestive enzymes (endopeptidases) synthesized in the midgut gland of Pacific white shrimp (Muhlia-Almazán and García-Carreño 2002); however, it seems that cathepsin B is the main digestive enzyme when FM is replaced with 35 % FDF. In this sense, it has been reported that trypsin and chymotrypsin are substituted by cysteine proteinases such as cathepsin L in the shrimp *Crangon* spp. (Teschke and Saborowski 2005). Cathepsin B and cathepsin L play an important role during intracellular and extracellular protein hydrolysis (Hu and Leung 2007; Stephens et al. 2012). Finally, it is important to note that digestive gene expression correlates with the highest growth performance in diet D35.

Heat shock proteins (HSPs) are highly conserved proteins well known for their quick responses to environmental stresses (Qian et al. 2012). In the present study, we compared the expression patterns of Lvhsp70 and Lvhsp90 in the hepatopancreas of shrimp fed with different partial replacement of FM with FDF. Results revealed that shrimp fed with FDF were more stressed than animals fed with 100 % FM (D0), since they showed apparent upregulations at the mRNA level, although there is a decreasing trend with diet D35. In shrimp, there are no reports about the effect of plant proteins on the gene expression of HSPs.

If the expression of digestive and stress genes is compared, it is important to note that there is a positive correlation among them from D5 to D35; however, in D35, the expression of cathepsin B and stress genes correlates negatively. As results showed, FDF in diet for white shrimp regulates the expression of the mentioned genes.

The defense system in crustaceans includes (1) coagulation of the hemolymph; (2) melanization mediated by the prophenoloxidase system (proPO); (3) recognition and cell aggregation mediated by lectins; (4) antibacterial, antifungal, and antiviral systems mediated by the antimicrobial peptides, interference RNA, and pattern recognition proteins; (5) production of reactive forms of oxygen and nitrogen; and (6) phagocytosis and encapsulation mediated by hemocytes (Iwanaga and Lee 2005). The immune system of shrimp responds to both microbial attacks and to immunostimulants. Many of these active components are extracted from bacterial cell walls, as it is the case of the peptidoglycans present in Gram-positive bacteria (Le Moullac et al. 1998; McDonald et al. 2005).

Considering that FDF-contained bacteria used in the fermentation process, it is important to assess the potential impact of peptidoglycans on the immune system of Pacific white shrimp. In this work, mRNA expression of three immune-related genes in hemocytes (proPO, TGase, and LvToll) was not affected by the inclusion of fermented plant. However, regarding SOD and Lys, there was an increase in the relative expression in shrimp fed D5 (SOD and Lys) and D15 (Lys). Wang et al. (2008) used β-1,3-glycans from *Schizophyllum commune* in diets for Pacific white shrimp but did not observe changes in the expression of TGase and proPO. However, they found a rapid increase in the relative expression of Lys and SOD. In the same way, Subramanian et al. (2013) found an increase in the relative mRNA expression of SOD by the inclusion of phenolic extracts of *Rubus coreanus* in the diet. Results showed that there is no important effect of FDF as immunostimulant, which is very important since the continuous use of immunostimulants, even at an optimal dose, may suppress immunity in shrimp (Chang et al. 2000; Sajeevan et al. 2009; Flores-Miranda et al. 2011).

Conclusion

Fishmeal can be replaced with up to 35 % FDF without adversely affecting the survival and growth performance of cultured shrimp. Inclusion of FDF in diets affected the expression of stress and digestive genes, but in immune-related genes, the effect did not show a clear trend.

Further research is needed to better understand the effect of the inclusion of FDF, in the diet, on the expression of genes involved in the digestion of shrimp, as well as to determine other possible formulations including FDF, which provide the best nutrition and growth performance of the shrimp.

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References

Aas TS, Grisdale-Helland B, Terjesen BF, Helland SJ (2006a) Improved growth and nutrient utilisation in Atlantic salmon (*Salmo salar*) fed diets containing a bacterial protein meal. Aquaculture 259:365–376

Aas TS, Hatlen B, Grisdale-Helland B, Terjesen BF, Bakke-McKellep AM, Helland SJ (2006b) Effects of diets containing a bacterial protein meal on growth and feed utilisation in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 261:357–368

Amaya EA, Davis DA, Rouse DB (2007) Replacement of fishmeal in practical diets for the Pacific white shrimp (*Litopenaeus vannamei*) reared under pond conditions. Aquaculture 262:291–298
Andersen CL, Jensen JL, Orntoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64:5245–5250
Anderson KE, Lowman Z, Stomp AM, Chang J (2011) Duckweed as feed ingredient in laying hen diets and its effect on egg production and composition. Int J Poult Sci 10(1):4–7
Bairagi A, Sarkar Ghosh K, Sen SK, Ray AK (2002) Duckweed (*Lemna polyrhiza*) leaf meal as a source of feedstuff in formulated diets for rohu (*Labeo rohita* Ham.) fingerlings after fermentation with a fish intestinal bacterium. Bioresour Technol 85:17–24
Bauer W, Prentice-Hernandez C, Tesser MB, Wasielesky W Jr, Poersch LHS (2012) Substitution of fishmeal with microbial floc meal and soy protein concentrate in diets for the pacific white shrimp *Litopenaeus vannamei*. Aquaculture 342–343:112–116
Brock J, Main KL (1994) A guide to the common problems and diseases of cultured *Penaeus vannamei*. World Aquaculture Society, Baton Rouge
Brown WY, Choct M, Pluske JR (2013) Duckweed (*Landoltia punctata*) in dog diets decreases digestibility but improves stool consistency. Anim Prod Sci 53(11):1188–1194
Chang CF, Chen HY, Su MS, Liao IC (2000) Immunomodulation by dietary β-1,3 glucan in the brooders of the black tiger shrimp, *Penaeus monodon*. Fish Shellfish Immunol 10:505–514
Chaturvedi KMM, Langote DS, Asolekar RS (2003) Duckweed-fed fisheries for treatment of low strength community waste water. WWWTM Newsletter-Asian Institute of Technology, India
Chávez-Calvillo G, Perez-Rueda E, Lizamaa G, Zúñiga-Aguilar JJ, Gaxiola G, Cuzon G, Arena-Ortiza L (2010) Differential gene expression in *Litopenaeus vannamei* shrimp in response to diet changes. Aquaculture 300(1–4):137–141
Cheng W, Chiu CS, Guu YK, Tsai ST, Liu CH (2013) Expression of recombinant phytase of *Bacillus subtilis* E20 in *Escherichia coli* HMS174 and improving the growth performance of white shrimp, *Litopenaeus vannamei*, juveniles by using phytase-pretreated soy-bean meal-containing diet. Aquac Nutr 19:117–127
Chookird D, Tantikitti C, Pongdara A, Srichanun M (2010) Effect of hemoglobin powder substituted for fishmeal on growth performance, protein digestibility, and trypsin gene expression in *Litopenaeus vannamei*. Songklanakarin J Sci Technol 32(2):119–127
Daniel WW (1997) Bioestadística. Base para el análisis de las ciencias de la salud. D. F. Editorial Limusa, Mexico, pp 639–693
Edwards P, Kamal M, Wee KL (1985) Incorporation of composted and dried water hyacinth in pelleted feed for the tilapia *Oreochromis niloticus* (Peters). Aquac Fish Manag 1:233–248
FAO (2012) Food and agriculture organization of the United Nations. The State of World Fisheries and Aquaculture 2011 Food and Agriculture Organization of the United Nations, Rome
Flores-Miranda MC, Luna-González A, Campa-Córdova AI, González-Ocampo HA, Fierro-Coronado JA, Partida-Arangure BO (2011) Microbial immunostimulants reduce mortality in whiteleg shrimp (*Litopenaeus vannamei*) challenged with *Vibrio sinaloensis* strains. Aquaculture 320:51–55
Flores-Miranda MC, Luna-González A, Cortés-Espinosa DV, Cortés-Jacinto E, Fierro-Coronado JA, Álvarez-Ruiz P, González-Ocampo HA, Escamilla-Montes R (2014) Bacterial fermentation of *Lemna* sp. as a potential substitute of fish meal in shrimp diets. Afr J Microbiol Res 8(14):1516–1526
Gamboa-Delgado J, Rojas-Casas MG, Nieto-López MG, Cruz-Suárez LE (2013) Simultaneous estimation of the nutritional contribution of fishmeal, soy protein isolate and corn gluten to the growth of Pacific white shrimp (*Litopenaeus vannamei*) using dual stable isotope analysis. Aquaculture 380–383:33–40
He S, Liang XF, Li L, Sun J, Shen D (2013) Differential gut growth, gene expression and digestive enzyme activities in young grass carp (*Ctenopharyngodon idella*) fed with plant and animal diets. Aquaculture 410–411:18–24
Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol 8:R19
Hu KJ, Leung PC (2007) Food digestion by cathepsin L and digestion-related rapid cell differentiation in shrimp hepatopancreas. Comp Biochem Physiol B 146:69–80
Huang J, Yang Y, Wang A (2010) Reconsideration of phenoloxidase activity determination in white shrimp *Litopenaeus vannamei*. Fish Shellfish Immunol 28:240–244
Iwanaga S, Lee BL (2005) Recent advances in the innate immunity of invertebrate animals. Biochem Mol Biol 38:128–150

Kiron V, Phromkunthong W, Huntley M, Archibald JA, Scheemaker G (2012) Marine microalgae from refinery as a potential feed protein source for Atlantic salmon, common carp and whiteleg shrimp. Aquac Nutr 18:521–531

Klomklao S (2008) Digestive proteinases from marine organisms and their applications. Songklanakarin J Sci Technol 30(1):37–46

Kumaraguru Vasagam KP, Balasubramanian T, Venkatesan R (2007) Apparent digestibility of differently processed grain legumes, cow pea and mung bean in black tiger shrimp, *Penaeus monodon* Fabricius and associated histological anomalies in hepatopancreas and midgut. Anim Feed Sci Technol 132:250–266

Landolt E (1986) Biosystematic investigation on the family of duckweeds: the family of Lemnaceaea monograph study. Geobotanischen Institute ETH, StiftungRubel, Zurichbergstrasse, p 38

Le Moullac G, De Laborie LP, Saulnier D, Goarant C, Dehasque M (1998) Principles and problems involved in the evaluation of immunostimulants on juvenile shrimp. In: Cerecedo RC, Claudia BM, Pérez-Estrada J, Suarez LE, Ricque MD (eds) Avances de Nutrición Acuícola. Memorias del IV Simposium International de Nutrición Acuícola, La Paz, B.S.C, Mexico, pp 1–12

Leng RA, Stambolie JH, Bell R (1995) Duckweed a potential high-protein feed resource for domestic animals and fish. Livest Res Rural Dev 7:1–10

Macias-Sancho J, Poersch LH, Bauer W, Romano LA, Wasielesky W, Tesser MB (2014) Fishmeal substitution with Arthrospira (*Spirulina platensis*) in a practical diet for *Litopenaeus vannamei*: effects on growth and immunological parameters. Aquaculture 426–427:120–125

McDonald C, Inohara N, Nuñez G (2005) Peptidoglycan signaling in innate immunity and inflammatory disease. J Biol Chem 280(21):20177–20180

Muhlia-Almazán A, García-Carreño FL (2002) Influence of molting and starvation on the synthesis of proteolytic enzymes in the midgut gland of the white shrimp *Penaeus vannamei*. Comp Biochem Physiol B 133:383–394

Muhlia-Almazán A, García-Carreño FL, Sánchez-Paz JA, Yepiz-Plascencia G, Peregrino-Uriarte AB (2003) Effects of dietary protein on the activity and mRNA level of trypsin in the midgut gland of the white shrimp *Penaeus vannamei*. Comp Biochem Physiol B 135:373–383

Mukho-Padhayay N, Ray AK (1999) Utilization of copra meal in the formulation of compounded diets for rohu, *Labeo rohita* fingerlings. J Appl Ichthyol 15:127–131

Nout MJR (2009) Rich nutrition for the poorest–cereal fermentations in Africa and Asia. Food Microbiol 26:685–692

Omondi GJ (2005) Digestive endo-proteases from the midgut glands of the Indian white shrimp, *Penaeus indicus* (Decapoda: Penaeidae) from Kenya. West Indian Ocean J Mar Sci 4(1):109–121

Oujifard A, Seyfabadi J, Kenari AA, Rezaei M (2012) Fish meal replacement with rice protein concentrate in a practical diet for the Pacific white shrimp, *Litopenaeus vannamei* Boone, 1931. Aquac Int 20:117–129

Qian Z, Liu X, Wang L, Wang X, Li Y, Xiang J, Wang P (2012) Gene expression profiles of four heat shock proteins in response to different acute stresses in shrimp, *Litopenaeus vannamei*. Comp Biochem Physiol C 156:211–220

Richard L, Surget A, Rigolet V, Kaushik SJ, Geurden I (2011) Availability of essential amino acids, nutrient utilization and growth in juvenile black tiger shrimp, *Penaeus monodon*, following fishmeal replacement by plant protein. Aquaculture 322–323:109–116

Saha S, Ray AK (2011) Evaluation of nutritive value of water hyacinth (*Eichhornia crassipes*) leaf meal in compound diets for Rohu, *Labeo rohita* (Hamilton, 1822) fingerlings after fermentation with two bacterial strains isolated from fish gut. Turk J Fish Aquat Sci 11:199–207

Sajeevan TP, Rosamma P, Bright Singh IS (2009) Dose/frequency: a critical factor in the administration of glucan as immunostimulant to Indian white shrimp *Fenneropenaeus indicus*. Aquaculture 287:248–252

Sanchez-Paz A, Garcia-Carreño FL, Muhlia-Almazan A, Hernandez-Saavedra N, Yepiz-Plascencia G (2003) Differential expression of trypsin mRNA in the white shrimp (*Penaeus vannamei*) midgut gland under starvation conditions. J Exp Mar Biol Ecol 292:1–17

Simpson BK (2000) Digestive proteinases from marine animals. In: Haard NF, Simpson BK (eds) Seafood enzymes: utilization and influence on postharvest seafood quality, Editors. Marcel Dekker, New York, pp 531–540

Sookying D, Davis DA (2011) Pond production of Pacific white shrimp (*Litopenaeus vannamei*) fed high levels of soybean meal in various combinations. Aquaculture 319:141–149

Stephens A, Rojo L, Araujo-Bernal S, García-Carreño F, Muhlia-Almazan A (2012) Cathepsin B from the white shrimp *Litopenaeus vannamei*: cDNA sequence analysis, tissues-specific expression and biological activity. Comp Biochem Physiol B 161:32–40

Suárez JA, Gaxiola G, Mendoza R, Cadavid S, Garcia G, Alanis G, Suárez A, Faillace J, Cuzon G (2009) Substitution of fishmeal with plant protein sources and energy budget for white shrimp *Litopenaeus vannamei* (Boone, 1931). Aquaculture 289:118–123

Subramanian D, Jang YH, Kim DH, Kang BJ, Heo MS (2013) Dietary effect of *Rubus coreanus* ethanolic extract on immune expression in white leg shrimp, *Penaeus vannamei*. Fish Shellfish Immunol 35(3):808–814

Tacon AGJ, Metian M (2008) Global overview on the use of fishmeal and fish oil in industrially compounded aquafeeds: trends and future prospects. Aquaculture 285:146–158

Teschke M, Saborowski R (2005) Cysteine proteinases substitute for serine proteinases in the midgut glands of *Crangon crangon* and *Crangon allmani* (Decapoda: Caridea). J Exp Mar Biol Ecol 316:213–229

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3(7):0034

Wang YC, Chang PS, Chen HY (2008) Differential time series expression of immune-related genes of Pacific white shrimp *Litopenaeus vannamei* in response to dietary inclusion of β-1,3-glucan. Fish Shellfish Immunol 24:113–121

Wang KH, Tseng C, Lin H, Chen I, Chen Y, Chen Y, Chen T, Yang H (2010) RNAi knock-down of the *Litopenaeus vannamei* Toll gene (LvToll) significantly increases mortality and reduces bacterial clearance after challenge with *Vibrio harveyi*. Dev Comp Immunol 34:49–58

Yilmaz E, Akyurt I, Günal G (2004) Use of duckweed, *Lemna minor*, as a protein feedstuff in practical diets for common carp, *Cyprinus carpio*, fry. Turk J Fish Aquat Sci 4:105–109

Ziaei-Nejad S, Rezaei MH, Takami GA, Lovett DL, Mirvaghefi AR, Shakouri M (2006) The effect of *Bacillus* spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*. Aquaculture 252:516–524