

## 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  $1 \times 10^6$  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- $\mu$ m 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<sup>®</sup>, 35 % protein). Ration was according to commercial dietary tables (Purina<sup>®</sup>). At day 3, uneaten food was eliminated and half of the water was changed.

### Feeding trial

Shrimp weighing  $0.40 \pm 0.2$  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  $23.9 \pm 2.7$  to  $24.1 \pm 2.6$  °C, dissolved oxygen from  $4.8 \pm 0.5$  to  $4.9 \pm 0.6$  mg L<sup>-1</sup>, salinity from  $30.9 \pm 1.1$  to  $31.1 \pm 1.2$  ‰, pH  $8.3 \pm 0.1$ , ammonium from  $0.13 \pm 0.02$  to  $0.23 \pm 0.07$  mg L<sup>-1</sup>, nitrites from  $0.3 \pm 0.2$  to  $0.4 \pm 0.1$  mg L<sup>-1</sup>, and nitrates from  $0.62 \pm 0.13$  to  $0.78 \pm 0.12$  mg L<sup>-1</sup> 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<sup>-1</sup>) and proximate composition (% dry weight) of experimental diets

Ingredients (g kg <sup>-1</sup> )	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 <sup>a</sup>	0.1	0.1	0.1	0.1	0.1	
Mineral premix <sup>b</sup>	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 <sup>-1</sup> )	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						

<sup>a</sup> Vitamin premix (units in mg kg<sup>-1</sup>, except when indicated): retinol, 5,000 IU; cholecalciferol, 4,000 IU;  $\alpha$ -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

<sup>b</sup> Mineral premix (g kg<sup>-1</sup> diet): KCl, 0.5; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.09; MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.0234; CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.005; KI, 0.0005; CoCl<sub>2</sub>·2H<sub>2</sub>O, 0.00025; Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 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):

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

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

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

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

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

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  $\mu\text{L}$ ) of each intermolt shrimp was withdrawn from the pleopod base of the first abdominal segment with sterile 1-mL syringes (27G  $\times$  13 mm needle) loaded with 600  $\mu\text{L}$  of a precooled (4  $^\circ\text{C}$ ) 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  $800 \times g$  for 10 min at 4  $^\circ\text{C}$ . The plasma was removed, and the hemocyte pellet was rinsed with 250  $\mu\text{L}$  of cold anticoagulant (4  $^\circ\text{C}$ ) by centrifuging as above. The supernatant was removed, and the hemocytes were suspended in 250  $\mu\text{L}$  of precooled Trizol Reagent<sup>®</sup> (Invitrogen, Carlsbad, CA, USA) and stored at  $-80$   $^\circ\text{C}$  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  $\mu\text{L}^{-1}$ , Sigma<sup>®</sup>-Aldrich, St. Louis, MO, USA). Reverse transcription was used to synthesize the first strand of cDNA using reverse transcriptase (Improm II, Promega<sup>®</sup>, Madison, WI, USA) with the oligo (dT<sub>20</sub>) primer using 500 ng of total RNA. The cDNA was suspended in 80  $\mu\text{L}$  of ultrapure water and stored at  $-80$   $^\circ\text{C}$  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 (Lvhsps70) and heat shock protein 90 (Lvhsps90)] 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,  $\beta$ -actin, EF1 $\alpha$ , and L21). The expression 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 EF1 $\alpha$  and 40S–S24, whereas  $\beta$ -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- $\mu$ L reaction volume containing 7.5  $\mu$ L of 2 $\times$  PCR Master Mix (1.5  $\mu$ L of 10 $\times$  reaction buffer, 0.75  $\mu$ L of 50 mM MgCl<sub>2</sub>, 0.3  $\mu$ L of 10 mM dNTPs, 0.75  $\mu$ L of EvaGreen<sup>®</sup> 20 $\times$  [Biotium, Hayward, CA, USA]; 0.1  $\mu$ L of 5 U  $\mu$ L<sup>-1</sup> Biolase DNA Polymerase [Bioline<sup>™</sup>, Taunton, MA, USA], and 4.1  $\mu$ L of ultrapure water), 0.35  $\mu$ L of each primer (10  $\mu$ M, Sigma<sup>®</sup>-Aldrich, St. Louis, MO, USA), 1.8  $\mu$ L of ultrapure water, and 5  $\mu$ L 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 [ $E = 10(-1/\text{slope}) - 1$ ]. To calculate expression of target genes, Cq values were transformed to relative quantities (RQ) using the equation  $RQ_{ij} = E^{[(Cq_{\text{mean}}) - Cq_{(ij)}]}$ , where  $E$  is the gene-specific efficiency and  $[(Cq_{\text{mean}}) - Cq_{(ij)}]$  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  $RQ^{\text{target}}/\text{Geometric mean of } RQ^{\text{reference genes}}$  (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
<b>Immune-related</b>			
Prophenoloxidase	proPO-F	GAGATCGCAAGGGGAGAACTG	Wang et al. (2010)
	proPO-R	CGTCAGTGAAGTCGAGACCA	
Transglutaminase	TGase-F	CCTCAGGATCTCCTTCACCA	Wang et al. (2010)
	TGase-R	TTGGGAAAACCTTCATTTTCG	
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	
<b>Digestive</b>			
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	
<b>Stress</b>			
Lvhsps70	hsp70 F	GGCAAGGAGCTGAACAAGTC	Álvarez-Ruiz et al. (unpublished data)
	hsp70 R	TCTCGATACCCAGGGACAAG	
Lvhsps90	hsp90 F	TGGGGCTTCTACTCCGCCTACC	Qian et al. (2012)
	hsp90 R	ACGGTGAAAGAGCCTCCAGCA	
<b>Housekeeping</b>			
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	
EF1 $\alpha$	qLvEf-F	CTGTGGTCTGGTTGGTGTTC	Álvarez-Ruiz et al. (unpublished data)
	qLvEf-R	TCAGATGGGTTCTTGGGTTTC	
$\beta$ -Actin	qActin-Fw	CCACGAGACCACCTACAAC	Wang et al. (2010)
	qActin-Rv	AGCGAGGGCAGTGATTTC	

especially in D35 with 35 % FM substitution. WH values were from  $2.76 \pm 0.03$  g in D0 to  $3.45 \pm 0.17$  g in D35. In WH, significant differences were found in diets D5, D15, and D35 regarding to D0 ( $p = 0.043$ ). SGR ranged from  $3.89 \pm 0.12$  g day<sup>-1</sup> in D25 to  $4.32 \pm 0.11$  g day<sup>-1</sup> in D5, without significant differences among treatments. AG ranged from  $2.38 \pm 0.07$  g in D0 to  $3.04 \pm 0.17$  g in D35. FCR values were from  $2.06 \pm 0.07$  in D35 to  $2.42 \pm 0.09$  in D25, and significant differences ( $p = 0.223$ ) were found in D15 and D35 respect to D0. PER of the experimental diets ranged from  $1.12 \pm 0.05$  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 <sup>-1</sup> )	FCR	PER
D0	100 ± 0.00	2.76 ± 0.03 <sup>a</sup>	2.38 ± 0.07	3.98 ± 0.21	2.40 ± 0.11 <sup>b</sup>	1.12 ± 0.05 <sup>a</sup>
D5	100 ± 0.00	3.30 ± 0.29 <sup>b</sup>	2.92 ± 0.27	4.32 ± 0.11	2.18 ± 0.04 <sup>ab</sup>	1.24 ± 0.02 <sup>ab</sup>
D15	100 ± 0.00	3.43 ± 0.01 <sup>b</sup>	3.00 ± 0.01	4.18 ± 0.05	2.15 ± 0.02 <sup>a</sup>	1.27 ± 0.01 <sup>b</sup>
D25	100 ± 0.00	3.06 ± 0.12 <sup>ab</sup>	2.62 ± 0.11	3.89 ± 0.12	2.42 ± 0.09 <sup>b</sup>	1.19 ± 0.04 <sup>ab</sup>
D35	100 ± 0.00	3.45 ± 0.17 <sup>b</sup>	3.04 ± 0.17	4.28 ± 0.13	2.06 ± 0.07 <sup>a</sup>	1.41 ± 0.05 <sup>c</sup>

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).

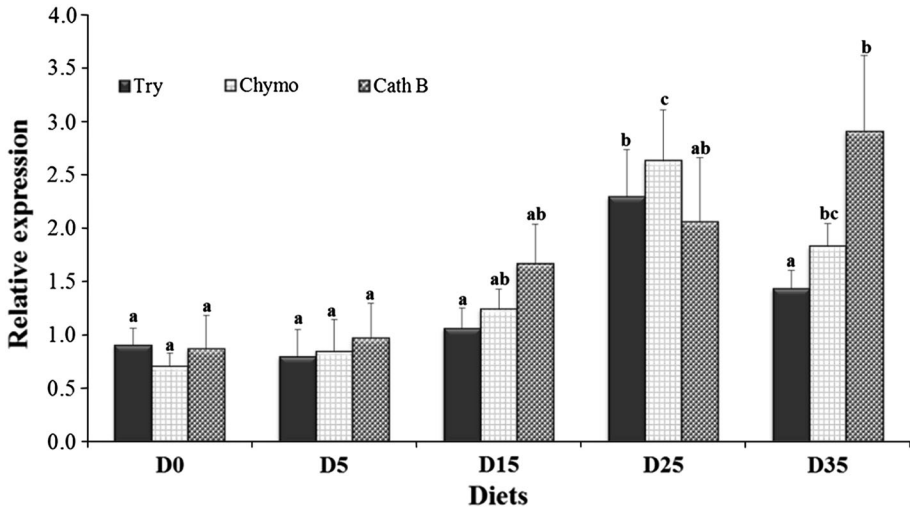
#### mRNA relative expression of trypsin, chymotrypsin, and cathepsin B in the hepatopancreas

Results (Fig. 1) showed that the expression of trypsin in diet D25 ( $2.29 \pm 0.44$ ) was significantly higher than in D0 ( $0.90 \pm 0.16$ ,  $p = 0.0019$ ), D5 ( $0.80 \pm 0.26$ ,  $p = 0.0011$ ), D15 ( $1.06 \pm 0.19$ ,  $p = 0.0043$ ), and D35 ( $1.43 \pm 0.17$ ,  $p = 0.032$ ). The expression of chymotrypsin in diet D25 ( $2.63 \pm 0.48$ ) was significantly higher than in D0 ( $0.71 \pm 0.12$ ,  $p = 0.0002$ ), D5 ( $0.85 \pm 0.30$ ,  $p = 0.0003$ ), and D15 ( $1.24 \pm 0.19$ ,  $p = 0.003$ ). The expression of chymotrypsin in diet D35 ( $1.83 \pm 0.21$ ) was significantly higher than in D0 ( $0.71 \pm 0.12$ ,  $p = 0.017$ ), D5 ( $0.85 \pm 0.30$ ,  $p = 0.030$ ). The expression of cathepsin B in diet D35 ( $2.90 \pm 0.71$ ) was significantly higher than in D0 ( $0.87 \pm 0.32$ ,  $p = 0.013$ ) and D5 ( $0.97 \pm 0.33$ ,  $p = 0.016$ ). The mRNA expression of trypsin and chymotrypsin reached its 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.

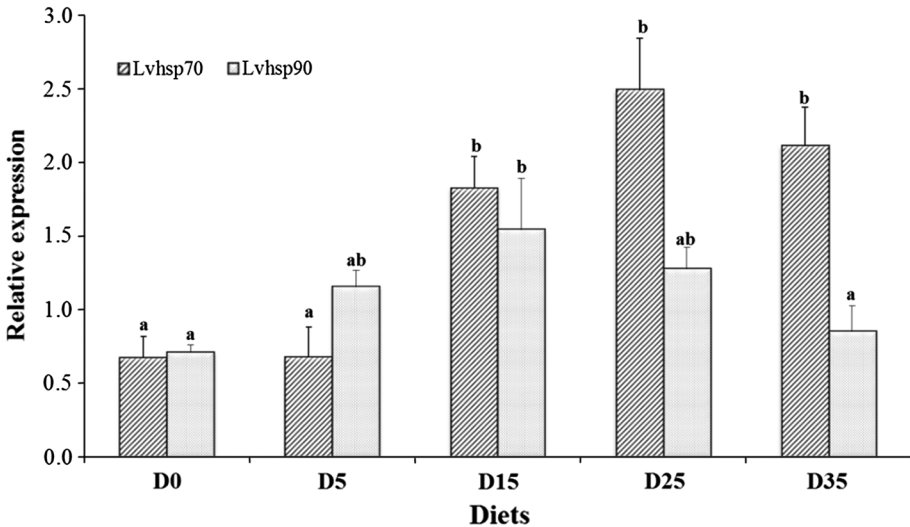
#### mRNA relative expression of stress genes in hepatopancreas

The mRNA expression profiles of Lvhs70 and Lvhs90 in shrimp hepatopancreas are shown in Fig. 2. The expression of Lvhs70 in diet D15 ( $1.83 \pm 0.22$ ) was significantly higher than in D0 ( $0.67 \pm 0.14$ ,  $p = 0.003$ ) and D5 ( $0.6087 \pm 0.20$ ,  $p = 0.003$ ). In diet D25, the expression of Lvhs70 ( $2.49 \pm 0.35$ ) was significantly higher than in D0 ( $0.67 \pm 0.14$ ,  $p = 0.0000$ ) and D5 ( $0.6087 \pm 0.20$ ,  $p = 0.0000$ ). The expression of Lvhs70 in diet D35 ( $2.11 \pm 0.27$ ) was significantly higher than in D0 ( $0.67 \pm 0.14$ ,  $p = 0.0006$ ) and D5 ( $0.6087 \pm 0.20$ ,  $p = 0.0006$ ). The expression of Lvhs90 in diet D15 ( $1.54 \pm 0.35$ ) was significantly higher than in D0 ( $0.67 \pm 0.14$ ,  $p = 0.008$ ). 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.





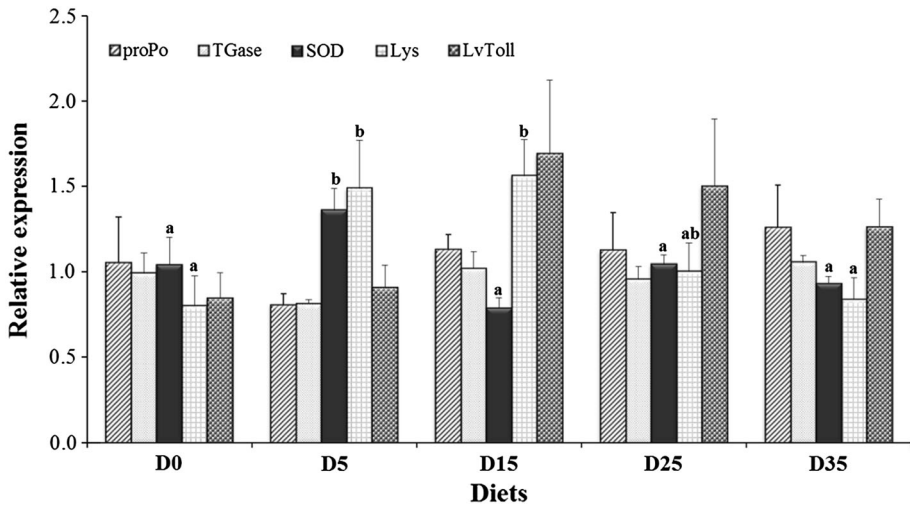
**Fig. 1** Relative mRNA expression of trypsin, chymotrypsin, and cathepsin B (normalized to  $\beta$ -actin and L21) in hepatopancreas of Pacific white shrimp. Experimental diets: *D0* diet without FDF (control), *D5* diet with 5 % FDF, *D15* diet with 15 % FDF, *D25* diet with 25 % FDF and *D35* diet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



**Fig. 2** Relative mRNA expression of Lvhs70 and Lvhs90 (normalized to  $\beta$ -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  $\pm$  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 EF1 $\alpha$  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  $\pm$  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 ( $1.36 \pm 0.13$ ) showed significant differences from the values in D0 ( $1.04 \pm 0.16$ ,  $p = 0.04$ ), D15 ( $0.79 \pm 0.06$ ,  $p = 0.0008$ ), D25 ( $1.04 \pm 0.05$ ,  $p = 0.032$ ), and D35 ( $0.93 \pm 0.04$ ,  $p = 0.008$ ). The expression of Lys in diet D5 ( $1.49 \pm 0.28$ ) showed significant differences in regard to D0 ( $0.80 \pm 0.18$ ,  $p = 0.033$ ) and D35 ( $0.84 \pm 0.13$ ,  $p = 0.038$ ). The expression of Lys in diet D15 ( $1.56 \pm 0.21$ ) showed significant differences as compared to D0 ( $0.80 \pm 0.18$ ,  $p = 0.021$ ) and D35 ( $0.84 \pm 0.13$ ,  $p = 0.025$ ). 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 (Muhlía-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 (Muhlía-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 Lvhs70 and Lvhs90 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  $\beta$ -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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