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Original Article

Effects of fishmeal quality on growth performance, protein digestibility and trypsin gene expression in pacific white shrimp (*Litopenaeus vannamei*)

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Abstract

Fishmeal quality is of great importance for production of aquatic animals particularly shrimp; however it varies widely depending on raw materials and processing methods. A seven week feeding trial was conducted using five diets to examine effects of fishmeal quality on growth performance, protein digestibility and expression of gene regulating trypsin in *Litopenaeus vannamei*. Each test diet was fed four times daily to four groups of shrimp with an average initial weight of 2.2 g shrimp^{-1}. The shrimp fed S1-premium grade fish meal (S1-PGFM) diet attained the highest weight gain, specific growth rate with the best feed conversion ratio (FCR) and protein utilization efficiency. Growth of shrimp fed S2-PGFM, grade 1 FM and grade 2 FM containing diets was inferior to those of S1-PGFM fed group. The imported FM diet fed group showed the slowest growth and feed utilization efficiency. FCR of shrimp fed grade 2 FM and imported FM was significantly the poorest ($p<0.05$).

In-vitro protein digestibility using crude enzyme extract from shrimp fed reciprocal diet was not significantly different among treatments but *in-vivo* protein digestibility of the S1-PGFM fed group was the highest (92.06+0.42%) in concordance with the highest trypsin gene expression.

Keywords: fishmeal quality, protein digestibility, growth, *Litopenaeus vannamei*, trypsin gene

1. Introduction

Fishmeal of high quality is a prime protein source for aquatic animal feed production that provides essential amino acid balance, essential fatty acids, phospholipids, excellent palatability property and high digestibility. In Thailand, fishmeal is categorized into premium grade, grade 1, grade 2 and grade 3 based on protein, fat and ash contents (Thai Fishmeal Producer Association, 2001) as shown in Table 1. The protein content ranges from 50 to >65 % and fat content from 4 to 20%. Ash content is highly variable from about 11-12% in anchovy meal to >23% in whitefish meals made from filleting waste. High quality fishmeal having high content and

Table 1. Classification of fishmeal quality in Thailand (Thai Fishmeal Producer Association, 2001)

Grade	Protein (%)	Ash (%)
Premium grade	> 65 %	
Grade 1	> 60 %	< 26 %
Grade 2	> 55 %	< 28 %

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suitable balance of essential amino acids is in high demand and vital for shrimp and carnivorous fish feed production, especially when used in combination with inferior alternative protein sources. However, incorporating premium grade fishmeal of similar protein level from different sources into shrimp diet does not guarantee good shrimp growth and production. This may be due to fish species, fishing methods, types of raw materials (whole fish, processing by-products or by-catch), raw material freshness and processing methods of fishmeal production that resulted in different essential amino acid composition, essential amino acid/non essential amino acid ratio and modification of protein structure affecting protein digestibility.

Effects of fishmeal quality on growth and feed utilization were observed in different species. In salmonids, an average of 15% increased growth and 10% reduced feed conversion ratios were obtained with the use of high quality fish meal in comparison with that of an average quality (Pike *et al*., 1990). A similar effect was also observed in wolf-fish (Moksness *et al*., 1995). In halibut, Aksnes and Mundheim (1997) experimentally produced different fishmeal qualities and found that both processing temperature and raw material freshness had an effect on growth and feed utilization efficiency which correlated with protein digestibility.

Protein digestibility is an important protein utilization assessment indicator of different protein sources (NRC, 2011; Oujifard *et al*., 2010). Fishmeal derived from different sources and produced differently has shown to have fluctuating protein digestibility to some extent such as the study in Atlantic salmon which varied from 86% to 98% (Anderson *et al*., 1997). In *Penaeus orientalis* shrimp, Kangsen (1986) showed that protein digestibility of Peruvian fishmeal was 88% while that of Chinese fishmeal was only 71%. Terrazas-Fierro *et al*. (2010) studied protein and amino acid digestibility of commercial fishmeal from different sources, batches and types of raw materials in whitelegged shrimp, *Litopenaeus vannamei,* and found that fish soluble protein concentrate exhibited the best apparent protein digestibility coefficient of 99.3% whereas those made from sardine varied among batches. The apparent amino acid digestibility coefficients differed among the fishmeal tested but was in line with protein digestibility. More importantly, Smith *et al*. (1985) found that there was a positive correlation between protein digestibility and growth rate in *L. vannamei*.

Protein digestion in shrimp is the function of different proteinases, but trypsin is the most abundant digestive proteinase that plays the major role (Sanchez-Paz *et al*., 2003). Trypsin and chymotrypsin encoding gene expression is controlled by hormonal and central nervous system. There have been reports in crustacean that the trypsin and chymotrypsin encoding genes can be induced by dietary protein levels in black tiger shrimp, *P. monodon,* (Muhlia-Almazan *et al*., 2003) and protein sources such as blood meal in *Anopheles gambiae* (Muller *et al*., 1995).

This study was carried out to examine different fishmeal qualities on growth performance, *in-vitro* and *in-vivo* protein digestibility, and trypsin encoding gene expression in *L. vannamei*.

2. Materials and Methods

2.1 Experimental diets and leaching tests

Five diets were formulated using different grades and sources of fish meal, imported FM (65.86% protein), S1-PGFM (65.25% protein) produced from sardine, S2-PGFM (71.11% protein) produced from round scad and sardine, grade 1 FM (64.91% protein) produced by mixing premium grade FM and grade 2 FM (55.22% protein) produced from surimi processing by-products. The diets contained crude protein and lipid at 42% and 8% of diet, respectively. The composition of experimental diets is shown in Table 2.

The coarse ingredients were finely ground then sieved through 30 mesh screen. Dry ingredients were mixed using Hobart mixer for 10 min then lecithin and oil were gently added and mixing was extended for 5 min. Distilled water was gradually added at 35% of diet and mixing was continued for another 10 min. The resulting mash was pelleted using a pellet mill with a 2 mm diameter pore size die and cut into 2 mm length. Pelleted diets were dried at 60°C for 24 h. The dried diets were sieved through a 2 mm diameter mesh screen and stored in polyethylene bags at -20°C until used. The proximate composition of ingredients and experimental diets was determined according to AOAC (1995) and amino acid profile was analyzed using HPLC (AOAC, 1995).

Diet leaching test was performed using three replicates according to the method modified from Aquacop (1978) and Cruz-Suarez *et al*. (2001). Five grams of the pellets were put in fine mesh baskets and submersed in seawater for 1 h with aeration simulating cultured condition in glass aquaria under the temperature of 27-28°C. The percent dry matter loss (% DML) shown in Table 2 was calculated as follows.

$$\% \text{ DML} = 100 \times (\text{DWd-DWwid})/\text{DWd}$$

Where DWd and DWwid = dry matter weights of the diet before and after submersion, respectively.

2.2 Growth trial

2.2.1 Shrimp and feeding trial

Juvenile *L. vannamei* were obtained from Somchai Farm, Satun province, Thailand. The shrimp were stocked and acclimatized in a cement tank for 15 days and fed a commercial feed which contained 40% protein and 4% lipid. Twenty-five shrimp with an individual initial weight of 2.29± 0.01 g were selected and randomly distributed into each of 20 glass aquaria (45x45x115 cm) containing 200 L of natural seawater (29-33 ppt) with the flow rate of 33.26 L/h and water temperature of 26-30°C. Five treatments were randomly assigned to four replicated aquaria and fed the respective

Table 2. Composition (g 100 g^{-1}), proximate composition (% as fed basis) and leaching loss (dry matter basis) of experimental diets

Ingredients	Experimental diets				
	Imported FM	S1-PGFM*	S2-PGFM*	Grade 1 FM	Grade 2 FM
Fishmeal	43.00	43.50	39.50	44.00	52.5
Squid meal [1]	8.00	8.00	8.00	8.00	8.00
Wheat flour [2]	20.00	20.00	20.00	20.00	20.00
Rice flour [3]	12.29	11.19	14.69	10.99	3.39
Wheat gluten meal	6.00	6.00	6.00	6.00	6.00
Lecithin [2]	2.00	2.00	2.00	2.00	2.00
Fish oil [2]	0.60	1.20	1.70	0.90	0.00
Vitamin mix[4]	0.33	0.33	0.33	0.33	0.33
Mineral mix[5]	4.00	4.00	4.00	4.00	4.00
Vitamin C [6]	0.10	0.10	0.10	0.10	0.10
Zeolite	1.50	1.50	1.50	1.50	1.50
BHT	0.02	0.02	0.02	0.02	0.02
Cholesterol [7]	0.50	0.50	0.50	0.50	0.50
Vitamin E [6]	0.15	0.15	0.15	0.15	0.15
CMC	1.00	1.00	1.00	1.00	1.00
Cr_2O_3	0.51	0.51	0.51	0.51	0.51
Proximate composition (% as fed basis) and leaching loss (dry matter basis)					
Crude protein	43.41	42.06	41.96	42.99	43.49
Crude fat	11.26	10.41	10.23	11.30	11.14
Ash	7.03	9.55	7.25	12.36	16.99
Leaching loss (%)	11.44	11.61	10.28	11.12	8.51

[1] dehydrated squid from the market
[2] donated from Charoen Pokphand Food Public Company Limited
[3] commercial rice flour from Tesco Lotus
[4] vitamin mix (in 1 kg of vitamin mix): retinol, 3500,000 IU; cholecalciferol, 800,000 IU; tocopherol, 40g; menaquinone, 15g; thiamine, 20g; riboflavin, 15g, pyridoxin, 20g; cyanocobalamine, 10mg; niacin 40g; panthothenic acid, 40g; folic acid, 4g; biotin, 400 mg; inositol, 150g.
[5] mineral mix (g kg^{-1} mineral): K_2HPO_4, 40; $Ca_3(PO_4)2$, 5.5; $MgSO_4$7H2O, 6.1; $NaH_2PO_4$2H_2O 16; cellulose 828.
[6] Rovithai Ltd.
[7] C75209 Sigma-Aldrich, MO, USA)
*S1-PGFM = premium grade fishmeal (sample 1), S2-PGFM = premium grade fishmeal (sample 2)

diets. Feeding was done by hand to satiation, determined by slow approach or no response of shrimp to the diet, four times daily at 8.00 am, 12.00 am, 5.00 pm and 10.00 pm for seven weeks. Amount of given feed was recorded daily and uneaten feed was collected for feed intake correction.

During the feeding period, shrimp were monitored for mortality and abnormality. After seven weeks feeding, shrimp were individually weighed to obtain the final weight.

2.2.2 Sampling

At the end of the seven week feeding period, six shrimp from each aquarium were sampled for proximate analysis. Two shrimp were decapitated and the hepatopancreas was dissected, fixed in TRIzol reagent and kept at -80°C for gene expression study. Another two shrimp were weighed, decapitated and hepatopancreas was taken then pooled from each replicated aquarium for *in-vitro* protein digestibility determination.

2.2.3 Growth performance and feed utilization evaluation

Initial weight, final weight, mortality, amount of eaten feed and proximate protein composition of shrimp were used for calculation of the following parameters:

Survival rate (%) =
Final number of shrimp x 100/Initial number of shrimp

Weight gain (g shrimp^{-1})
= Final weight (g shrimp^{-1}) – Initial weight (g shrimp^{-1})

Specific growth rate (SGR, % day^{-1})
= (ln W2-ln W1/T2-T1) x 100
W_1 = initial weight, W_2 = final weight,
T_2-T_1 = cultured period (days)

Feed conversion ratio (FCR)
= Feed intake (g shrimp^{-1}) /Weight gain (g)

Protein efficiency ratio (PER)
= Weight gain (g)/Protein intake (g)

Productive protein value (PPV, %)
= (Protein gain (g)/Protein intake (g) x 100

2.3 *In-vitro* protein digestibility

2.3.1 Enzyme extraction and activity determination

In-vitro protein digestibility of the experimental diets was determined using crude enzyme extract from the pooled hepatopancreas as described above. The preparation of crude enzyme extract was prepared and the *in-vitro* protein digestibility study was performed using the method modified from Bassompierre (1997). Crude enzymes were extracted from the hepatopancreas and homogenized (1:10 w/v) in 0.05 M Tris buffer pH 7.5 at 4°C. The homogenate was centrifuged twice at 12,000x*g* for 30 min at 4°C. The crude enzyme was obtained and kept at -80°C for further analysis. Protein in extracted enzyme was measured by a modified Lowry's method using bovine serum albumin (BSA) as a standard. Crude enzyme was diluted to 1 mg protein/mL before *in-vitro* digestion study. Trypsin activity of crude enzyme was determined using BAPNA as a substrate by mixing 950 µL of 0.1 M BAPNA and 50 µL of crude enzyme, incubated at 37°C for 10 min, then reaction was terminated by adding 100 µL of 30% trichloroacetic acid and measured for an absorbance at 410 nm. The enzyme activity was calculated as follows (Rathore *et al*., 2005):

Unit of enzyme activity (µmole/mL/mg protein)

$$= \frac{\text{(Abs at 410 nm/min) x 1000 mL x mL of reaction volume}}{\text{Extinction of choragen x mg protein in reaction mixture}}$$

The molar extinction coefficient of *p*-nitroanilide is 8800.

2.3.2 Preparation of diet for enzyme digestibility assay

A sample of each diet was ground and weighed to the exact weight at 30 mg protein calculated using dietary protein content. Forty mL of 0.01 M phosphate buffer (pH 7.8) and 1 mL of 0.5% chloramphenical (in 96% ethanol) were added and mixed thoroughly. The mixture was incubated at 30°C for 18 h in a shaking water bath.

2.3.3 Pre-digestion concentration

Prior to sample incubation, 0.5 mL of mixture from each treatment was sampled as a control, immediately heated at 100°C for 5 min to terminate the enzyme activity, rapidly frozen at -80°C for later determination of total reactive amino group using the trinitrobenzene sulfonic acid (TNBS) assay as described below.

2.3.4 Post-digestion concentration

The digestion process was performed by adding 0.5 mL of the crude enzyme extract (1 mg/mL of protein) into each treatment and incubated in a shaking water bath for 18 h at 30°C. At the end of the incubation time, 1 mL of digested mixture was sampled, immediately heated at 100°C for 5 min and rapidly frozen at -80°C for the later determination of free reactive amino group of the peptides using the trinitrobenzene sulfonic acid (TNBS) assay as described below.

2.3.5 Determination of free reactive amino acid groups

Dilution of 0.2 mL of either the undigested control or the digested mixture with 2 mL of 0.05 M phosphate buffer pH 8.2 were mixed thoroughly with 1 mL of 0.1% TNBS in 0.01 M phosphate buffer and incubated at 60°C for 1 h in the dark. The reaction was stopped by adding 1 mL of 1 N HCl and cooling to room temperature. The absorbance was measured at 420 nm and the concentration of free amino group was calculated using DL-alanine as the standard. *In-vitro* digestibility was expressed as mole alanine equivalent liberated reactive amino group per 200 µL sample.

Alanine equivalent (mole)
= alanine conc. (g/l) x (1/89.10 g/mole) x (0.2 mL/1,000 mL)

2.4 *In-vivo* protein digestibility

Apparent digestibility coefficients (ADC) of crude protein in diets were measured. Diets were prepared as described above with reducing chromic oxide (Cr_2O_3) as a marker at 0.5% of diet.

After growth trial termination, feeding was continued with chromic oxide containing diets for 30 days. Feces collection commenced 2 days after changing to chromic oxide diets by siphoning method twice a day at 2.00 pm and 8.00 pm. Feces were separated from feed particles manually, kept at -20°C and oven dried at 105°C. Determination of chromic oxide was carried out according to the method of Lall (1991). The ADC was estimated using the following equation.

$$\text{ADC} = \%\ \text{of protein digestibility} = 100 - (100 \times \frac{I\text{a}}{I\text{h}} \times \frac{P\text{h}}{P\text{a}})$$

ADC = apparent digestibility coefficient
Where I_a = % Cr_2O_3 in feeds; I_h = % Cr_2O_3 in feces;
P_a = % crude protein in food; P_h = % crude protein in feces.

2.5 Trypsin gene expression

Trypsin gene expression was studied using 2 step RT-PCR. In the first step cDNA synthesis, total RNA was extracted from the hepatopancreas of shrimp fed different diets using TRIzol reagent. Intact total RNA was used for reverse transcription; cDNA was synthesized from each individual sample using the Superscript III™ first-strand synthesis system for RT-PCR. Reverse transcription was performed using 8 µL (100 ng/iL) total RNA, 1 µL of (10 mM) dNTP mix and 1 µL of (50 ng/µL) random hexamer. The reaction mixtures were incubated for 5 min at 65°C then further for 2 min at 4°C. Then, the reaction mixtures were added with 10 µL of cDNA synthesis mixture containing 2-µl (10X) RT buffer, 4 µL of (25 mM) $MgCl_2$, 2 µL of (0.1 M) DTT, 1 µL of RNase out and 1 µL of (50 units) Superscript III™ RT. The total volume of the mixture was 20 µL. The reaction mixtures were incubated for 10 min at 25°C, 50 min at 50°C and 5 min at 85°C after that 1 µL of RNase inhibitor was added and incubated for 20 min at 37°C, then held at 4°C until used for the second step.

In the second step RT-PCR, trypsin primers for PCR amplification were based on three trypsin genes reported for *L. vannamei* (Klein *et al*., 1996). Primer sequences were Forward trypsin- CTCAACAAGATCGTCGGAGGAACTGA- and Reward trypsin–GACACTCGTCGTCAGAACACGATG- that matched 81-106 and 545-567 positions, respectively.

PCR amplifications were performed in a 25 µL final reagent mixture containing 12.5 µL of H_2O, 2.5 µL of (10X) PCR buffer, 1.5 µL of (25 mM) $MgCl_2$, 1 µL of (10 mM) dNTP mix, 1 µL (6 µM) of each primer, 5 µL of the obtained cDNA (100 ng/µL) of each sample and 0.5 µL of Tag DNA polymerase. A thermocycler was used with the following program: 5 min at 95°C, 1 min at 94°C, 1 min at 54°C and 1 min at 72°C (35 cycles); and over-extension step for 10 min at 72°C. The resulting PCR products for trypsin was analyzed in a single 1.5% agarose gel and stained with ethidium bromide (Sambrook and Russell, 2001)

The intensity of the bands in the gel images obtained were evaluated relative to that of Ef-1 alpha (Wongpanya *et al*., 2007) using Scion Images Program for Windows Version 4 (Pongdara *et al*., 2006).

2.6 Statistical analysis

Growth performance, survival rate, feed utilization efficiency, protein digestibility and trypsin gene expression data were analyzed using analysis of variance to determine if significant difference ($p<0.05$) existed among treatments. Tukey's HSD test was employed to compare differences between treatment means.

3. Results

3.1 Amino acid and proximate composition of experimental diets

S1-PGFM and grade 1 FM diets had higher levels of essential amino acids, particularly lysine and methionine than the other three diets. The ratio of EAA/NEAA of S1-PGFM and grade 1 FM diets was also at the same level of 0.73 and higher than those of imported FM, S2-PGFM and grade 2 FM diets which were 0.68, 0.69, and 0.65, respectively (Table 3).

Proximate composition of experimental diets was similar except ash content and leaching loss (Table 2). Grade 1 FM diet and grade 2 FM diet contained higher ash content than others at 12.36 and 16.99%, respectively. Leaching loss of the grade 2 diet at 8.51% was rather low in comparison with other diets which were in the range of 10.28-11.61%.

3.2 Growth and feed utilization

Shrimp fed S1-PGFM diet had the highest survival rate of 88.00±9.24% while the other groups ranged from 78.00±2.31% to 86.00±6.93% (Table 4) without statistical difference ($p>0.05$).

The highest final weight (10.28±0.39 g shrimp^{-1}), weight gain (7.99±0.38 g shrimp^{-1}) and SGR (2.98±0.08) were also attained in shrimp fed S1-PGFM followed by grade 1 FM, S2-PGFM and grade 2 FM fed shrimp (Table 4). Significantly, the lowest growth was in the group fed imported FM diet having final weight of 8.15±0.66 g shrimp^{-1} ($p<0.05$).

Feed intake of imported FM diet fed shrimp was significantly ($p<0.05$) the lowest at 8.79±0.52 g shrimp^{-1}, whereas that of other treatments ranged from 10.10±0.53 to 10.33±0.08 g shrimp^{-1}. The significantly highest feed intake ($p<0.05$) at 11.06±0.46 g shrimp^{-1} was grade 2 FM diet fed shrimp (Table 5). FCR of shrimp fed grade 2 FM containing diet was the highest (1.55±0.10) followed by imported FM, S2-PGFM and grade 1 FM. S1-PGFM diet gave significantly ($p<0.05$) the best FCR of 1.28±0.09 (Table 5). PER and PPV of different diets (Table 5) showed a similar trend as the FCR that S1-PGFM diet was significantly the best while grade 1 FM and S2-PGFM were the second and those of imported FM and grade 2 FM diet were significantly the worst ($p<0.05$).

The proximate composition of shrimp at the end of the trial (Table 6) was not different among treatments ($p>0.05$).

3.3 Protein digestibility

In-vitro and *in-vivo* protein digestibility of the diets in this study showed a similar trend that S1-PGFM based diet gave the best digestibility results (Table 7). Although products of the *in-vitro* digestibility assay using experimental diet induced enzyme were not significantly different among

Table 3. Amino acid composition of experimental diets (% of protein)

Ingredients	Experimental diets				
	Imported FM	S1-PGFM*	S2-PGFM*	Grade 1 FM	Grade 2 FM
Arginine	4.20	4.43	2.72	5.60	4.55
Histidine	1.90	2.37	1.81	2.43	1.51
Isoleucine	1.94	2.29	1.45	3.14	1.98
Leucine	5.21	5.64	3.91	7.25	4.44
Lysine	4.73	5.11	3.54	5.45	4.07
Methionine	1.95	2.14	1.44	2.44	1.78
Phenylalanine	3.17	3.48	2.38	4.06	2.97
Threonine	3.02	3.31	2.28	3.70	2.91
Tryptophan	0.64	0.62	0.54	0.54	0.54
Valine	2.30	2.70	1.72	3.39	2.37
Alanine	4.72	4.83	3.57	5.56	4.52
Aspartic acid	7.06	7.36	5.28	7.58	6.19
Cystine	0.56	0.56	0.35	0.79	0.45
Glutamic acid	14.43	14.88	10.67	18.27	12.98
Glycine	5.27	5.15	3.69	6.51	6.51
Proline	4.63	4.86	3.45	5.58	5.33
Serine	3.57	3.78	2.64	4.02	3.31
Tyrosine	2.47	2.64	1.91	3.62	2.35
EAA	29.07	32.09	21.79	38.00	27.12
NEAA	42.70	44.07	31.57	51.94	41.64
EAA/NEAA	0.68	0.73	0.69	0.73	0.65

*S1-PGFM = premium grade fishmeal (sample 1), S2-PGFM = premium grade fishmeal (sample 2)

Table 4. Growth performance of *L. vannamei* fed diets containing different fishmeal qualities for 7 weeks

Experimental diets	Final weight (g shrimp^{-1})	Weight gain (g shrimp^{-1})	SGR[2] (% day^{-1})	Survival rate (%)
Imported FM	8.15±0.66^{b1}	5.86±0.66^{b}	2.53±0.16^{b}	86±7
S1-PGFM*	10.28±0.39^{a}	7.99±0.38^{a}	2.98±0.08^{a}	88±9
S2-PGFM*	9.35±0.58^{a}	7.06±0.59^{a}	2.81±0.13^{a}	78±2
Grade 1 FM	9.46±0.32^{a}	7.16±0.31^{a}	2.83±0.06^{a}	81±4
Grade 2 FM	9.16±0.18^{a}	6.88±0.17^{a}	2.82±0.06^{a}	82±2

[1] Means within a column with different superscripts are statistically different ($p<0.05$, n=4)
[2] Specific growth rate = (ln W2-ln W1/T2-T1)x100, W_1 = initial weight, W_2 = final weight, T_2-T_1 = cultured period (days)
*S1-PGFM = premium grade fishmeal (sample 1), S2-PGFM = premium grade fishmeal (sample 2)

treatments, *in-vivo* digestibility of S1-PGFM was the highest and statistically higher than those of grade 1 and grade 2 FM containing diets ($p<0.05$). The apparent protein digestibility of imported FM and S2-PGFM was second to that of S1-PGFM while that of grade 1 FM diet was lower than the former two groups and that of grade 2 FM was significantly the lowest. In addition, shrimp fed grade 2 FM had significantly the highest amount of feces ($p<0.05$). Interestingly, specific trypsin activity of the S1-PGFM fed shrimp was distinctively higher than the rest of the experimental groups.

3.4 Gene expression

Trypsin gene expression is shown in Figure 1. Shrimp fed S1-PGFM diet exhibited the highest gene expression whereas those fed the imported FM, S2-PGFM and grade 1

Table 5. Feed utilization efficiency of *L. vannamei* fed diets containing different fishmeal qualities for 7 weeks

Experimental diets	Feed intake[2] (g shrimp^{-1})	FCR[3]	PER[4]	PPV[5](%)
Imported FM	8.79±0.52^{c1}	1.51±0.09^{a}	1.53±0.09^{b}	27.41±1.59^{b}
S1-PGFM*	10.10±0.53^{b}	1.28±0.09^{b}	1.80±0.13^{a}	32.83±2.38^{a}
S2-PGFM*	10.24±0.20^{ab}	1.46±0.15^{ab}	1.59±0.16^{ab}	28.64±2.86^{ab}
Grade 1 FM	10.33±0.08^{ab}	1.44±0.07^{ab}	1.60±0.07^{ab}	28.75±1.33^{ab}
Grade 2 FM	11.06±0.46^{a}	1.55±0.10^{a}	1.49±0.09^{b}	26.94±1.63^{b}

[1] Means within a column with different superscripts are statistically different ($p<0.05$, n=4)
[2] The reported feed intake was corrected for leaching loss
[3] Feed conversion ratio = Feed intake (g)/Weight gain (g)
[4] Protein efficiency ratio = Weight gain (g)/Protein intake (g)
[5] Productive protein value = (Protein gain (g)/Protein intake (g) x 100)
*S1-PGFM = premium grade fishmeal (sample 1), S2-PGFM = premium grade fishmeal (sample 1)

Table 6. Proximate composition (%) of shrimp fed diets with different fishmeal qualities for 7 weeks (wet weight basis)

Experimental diets	Moisture	Crude protein	Crude fat	Ash
Imported FM	75.93±0.78[1]	17.74±0.32[1]	2.08±0.09[1]	2.65±0.02[1]
S1-PGFM*	75.54±0.51	18.02±0.47	1.99±0.03	2.78±0.09
S2-PGFM*	75.93±1.11	17.87±0.55	2.02±0.02	2.66±0.10
Grade 1 FM	75.90±1.03	17.83±0.48	1.94±0.07	2.63±0.05
Grade 2 FM	75.70±1.02	17.93±0.24	2.16±0.15	2.58±0.10

[1] Means within a column are not statistically different ($p>0.05$, n=4) among treatment groups.
*S1-PGFM = premium grade fishmeal (sample 1), S2-PGFM = premium grade fishmeal (sample 2)

Table 7. *In-vitro* and *in-vivo* protein digestibility of shrimp fed diets with different fishmeal qualities

Experimental diets	AG liberated by extracted enzyme (10^{-7} mole ala/ 200 µL sample)	*In-vivo* digestibility (%)	Specific trypsin activity (unit/min/ mgprotein)	Feces (g/2 weeks)
Imported FM	1.29±0.43[1]	90.83±0.30^{a2}	1.29±0.04	2.53±0.71^{b}
S1-PGFM*	1.64±0.06	92.06±0.42^{a}	1.37±0.02	3.62±1.07^{b}
S2-PGFM*	1.61±0.06	90.56±0.54^{a}	1.32±0.13	3.13±0.56^{b}
Grade 1 FM	1.47±0.02	88.75±0.66^{b}	1.29±0.04	4.33±1.01^{b}
Grade 2 FM	1.68±0.10	85.07±0.13^{c}	1.29±0.01	7.88±1.85^{a}

[1] Means within a column are not statistically different ($p>0.05$, n=3) among treatment groups.
[2] Means within a column with different superscripts are statistically different ($p<0.05$, n=3)
*S1-PGFM = premium grade fishmeal (sample 1), S2-PGFM = premium grade fishmeal (sample 2)

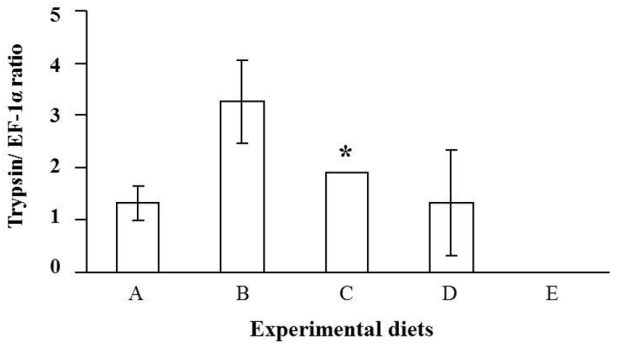

Figure 1. Trypsin gene expression of hepatopancreas extract of *L. vannamei* fed different diets (n=2, except * n=1): A = imported FM, B = S1-PGFM, C = S2-PGFM, D = Grade 1 FM, E = Grade 2 FM). Means are not statistically different ($p>0.05$, n=3) among treatment groups.

FM diets showed lower scores and those fed grade 2 FM was too low to detect. There was no statistical difference among the treatments ($p>0.05$).

4. Discussion

Growth responses and feed utilization efficiency in relation to protein quality and amino acid imbalance have been reported in many studies. Mengqing and Aksnes (2001) reported that shrimp (*P. chinensis*) and red seabream fed the diets formulated using good quality fishmeal showed significantly better feed conversion ratio, weight gain and protein digestibility than those fed low quality fishmeal and Peruvian fishmeal containing diets. Similarly, Atlantic salmon fed with the diet containing amino acid imbalance protein due to the processing high temperature gave lower growth than those of the control because of high disulfide groups formed during meal processing affected the utilization efficiency of the diet (Sunde *et al*., 2004).

The effects of different FM qualities on shrimp performance were observed in the present study. Overall, growth responses of shrimp fed local FM diets significantly outperformed those fed the imported FM. Although there were no statistical differences in weight gain and SGR within the local FM fed groups, shrimp fed the S1-PGFM diet showed the highest weight gain and SGR with the best FCR, PER and PPV. The best growth performance and feed utilization of S1-PGFM fed group was due to good quality protein with higher levels of essential amino acids, good amino acid balance and high EAA/NEAA ratio of the diet. Shrimp fed this diet also had good feed intake and protein digestibility, which would provide greater amount of amino acids for muscle growth. S2-PGFM diet had a similar level of feed intake and comparatively good protein digestibility but it had lower ratio of EAA/NEAA at 0.69, leading to compromised growth as compared to S1-PGFM diet. S1-PGFM was produced from fresh sardine while the S2-PGFM was produced from mixed species of round scad and sardine.

The shrimp fed grade 1 FM diet had a little higher feed intake than those of the aforementioned diets, but its lower protein digestibility was probably responsible for slower growth and higher FCR, despite having a high EAA/NEAA balance of 0.73. The good growth of grade 2 FM diet fed shrimp was most likely as a result of high feed intake, but having lower EAA/NEAA of 0.65 than the other diets. Therefore, the lowest EAA/NEAA ratio together with considerably less protein digestibility led to low ability to utilize the benefit of good feed intake to support maximum growth. The results demonstrated that the raw materials and process of fishmeal production have an effect on the shrimp growth and nutrient utilization. Grade 1 was a mixture of different sources of fish raw materials and grade 2 FM was produced from surimi processing by-products such as head, viscera and frame which had high bone/ash contents and did not contain sufficient levels of some essential amino acids. Growth depression due to amino acid imbalance of protein sources was also found in *L. vannamei* (Mente *et al*., 2002). The tested diets having a mixture of fishmeal, squid and shrimp powder as the protein source gave the best growth. Reduced growth occurred when half of the fish/squid/shrimp meal mixture was replaced by either soybean meal or casein. The reduction in shrimp growth that received the marine protein replacement diets was due to limiting amino acids, particularly low methionine and threonine level in soybean meal and casein, respectively (Mason and Castell, 1980).

Different growth responses in shrimp due to different fishmeal quality judged by raw material freshness were also reported by Ricque-Marie *et al*. (1998). In their study, fishmeal of different qualities were produced from anchovy, either fresh (12 h post capture), moderately fresh (25 h post capture) or stale (36 h post capture). Small *L. vannamei* of (0.9 g) average initial weight exhibited significantly higher feed consumption and 25% increased growth when fed the diet containing fresh raw material fishmeal as compared with the moderately fresh and stale raw material treatments whereas, larger *L. vannamei* (1.5 and 7.6 g) did not show any significant responses.

Significantly, the lowest growth of imported FM diet fed shrimp was due mainly to low feed intake, low protein digestibility and lower ratio of EAA/NEAA of 0.65 than other diets. In spite of high protein content FM that produced from pelagic fish, a long shipping and storage time necessitated inclusions of antioxidant to prevent lipid oxidation which may affect acceptance and intake of diet in shrimp. Laohabanchon, *et al*. (2009) reported that black tiger shrimp, *P. monodon*, fed the diet incorporated with long storage time and ethoxyquin treated FM had lower feed intake, slow growth and highest percentage of hepatopancreatic cell abnormality in comparison with the control group containing freshly produced and 1.5 month storage time FM without antioxidant treatment.

Considering protein digestibility, the results clearly demonstrated that fishmeal of different qualities even with similar protein level had different protein digestibility. Apparent protein digestibility coefficients, *in vitro* digesti-

bility products and specific trypsin activity of S1-PGFM and S2-PGFM were the highest which correspond with growth responses and feed utilization. Grade 2 FM diet had the lowest *in-vivo* protein digestibility coefficient while having a similar digestibility products in the *in-vitro* test as those of the S1-PGFM. The difference of digestibility values between the two techniques was probably due to the unique property of grade 2 FM. Grade 2 FM was produced from surimi processing by-product, consisting of head, frame and offal, which contained endogenous proteases that during storage could help digest protein leading to partial digestion. This might make the grade 2 highly digestible by *in-vitro* protein digestibility assay. The shrimp fed grade 2 FM also expelled highest amount of feces which indicated a high content of escape digesta. Therefore, the highly digestible grade 2 FM may not be an excellent source of protein as it may have too fast the transit time for efficient amino acid absorption. The varying protein digestibilities in response to different fishmeal qualities in this study was similar to the studies in the same shrimp species by Terrazas-Fierro *et al*. (2010). They evaluated the apparent protein digestibility of four commercial fishmeal (FMA, FMB, FMC and FMD) from different sources, batches or species. The apparent protein digestibility of FMA (sardine, 66% CP) was the highest at over 84%, while FMB (sardine, 70% CP), FMD (tuna 60% CP) and FMC (sardine 70% CP) were lower at 71%, 70% and 63%, respectively.

Trypsin gene expression in this study was related to amino acid balance of the diet. S1-PGFM diet fed shrimp with a high ratio of EAA/NEAA at 0.73 gave the highest expression of trypsin gene followed by those fed grade 1 FM, S2-PGFM, imported FM and grade 2 FM fed shrimp. Information in this area is very scarce. It could be deduced that free amino acids from the digestion process induced the expression of trypsin gene as described in *Aedes aegypti* by Noriega and Wells (1999). The free amino acids released from the digestion stimulated translation of early trypsin gene which then triggered protein digestion and after that products from the digestion stimulate the late trypsin gene. Protein digestibility of the balanced dietary amino acid profile then promoted good growth. Such mechanism might be the key factors affecting trypsin expression in *L. vannamei* in the present study. However, this suspected mechanism needs further investigation. The results in this study evidently indicate that fishmeal of good amino acid balance and good protein digestibility may induce trypsin gene expression which in association with good feed intake was responsible for good growth.

5. Conclusions

Growth performance and feed utilization of shrimp could be divided into three groups; the highest growth in S1-PGFM fed shrimp, medium growth in S2-PGFM, grade 1 FM and grade 2 FM diet fed shrimp, and significantly the lowest growth in imported FM fed shrimp. Domestic premium grade fishmeal produced from a single species gave the best growth performance, the highest protein digestibility and utilization, and the highest trypsin gene expression. The present study clearly demonstrated that fishmeal quality affected growth performance and feed utilization of juvenile *L. vannamei* mainly through protein digestibility, amino acid composition and feed intake.

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