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Evaluation of the potential of Andean lupin meal (*Lupinus mutabilis* Sweet) as an alternative to fish meal in juvenile *Litopenaeus vannamei* diets

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ABSTRACT

Two growth trials were conducted with juvenile *Litopenaeus vannamei* using experimental diets providing 35% protein and 11% lipid, where 0, 25, 50, 75 and 100% of fish meal protein (FM) was substituted by lupin kernel meal (LKM). Before grinding the lupin seeds, the alkaloids, hull and fat were removed by specific methods. In an indoor clear water aquarium trial, juvenile shrimp (initial weight 1.23 ± 0.22 g) were stocked at 8 per 50 L aquarium, with 6 replicate aquaria assigned to each treatment in a completely random design. At the end of the 57-day feeding trial, the average survival of the shrimp was >80% and there was no variation ($P > 0.05$) when FM was replaced partially nor totally with LKM. The results of this study showed that LKM can replace 50% of FM protein without significantly discouraging growth (6.7–7.0 g final weight) ($P > 0.05$), but the substitution of 75 and 100% resulted in lower growth (4.8–5.2 g final weight). The inclusion of LKM at any of the tested levels resulted in a statistical reduction ($P < 0.05$) of the apparent dry matter digestibility (ADMD) and apparent protein digestibility (APD) of the feed. The gradual increases of LKM in diets produced a significant decrease ($P < 0.05$) in ingestion rate. To demonstrate the inherent effects of water quality and natural food sources found in shrimp ponds, a growth trial was conducted in 1-m^2 bottomless cages in a single 1000-m^2 pond greenhouse. Juveniles weighing 5.84 ± 0.25 g (mean ± SD) were stocked in the cages at a density of 30 individuals per m^2. The feed was offered on a tray twice a day for 45 days. Five replicates were performed for each treatment. At the end of the 45-day field evaluation, no significant differences ($P > 0.05$) in final weight (11.1–12.2 g), specific growth rate (1.4–1.6 % day^{-1}), survival (69–79%) nor FCR (2.0–2.3) were found in any of the experimental shrimp diets. These findings show that *Lupinus mutabilis* Sweet has very good potential as an alternative protein source replacing at least 50% of protein from FM, equivalent to one third of the total protein in the diet for growth-out phase of *L. vannamei*. The study should be repeated under pond conditions to corroborate results obtained in cages and assess the cost benefit of including this ingredient in commercial feeds.

1. Introduction

The constant growth of aquaculture at a worldwide level to a rate of up to 9% year on year (Tacon and Metian, 2008), has produced an increase in the demand for feeds for commercial aquatic species. A fundamental part of the processing of these feeds has been fish meal, a protein source of high nutritional value and palatability that usually constitutes between 5 and 40% of the manufactured shrimp feeds (Tacon and Metian, 2008). Global exploitation of this resource, the average production of which in the last decade fluctuated between 5 and 7 million metric tons (Tacon and Metian, 2008) is becoming greater and greater, and if in addition we consider the fact that the processing of this raw material is affected by adverse climatologic conditions like El Niño, we could consider fish meal a marine protein source of high commercial value, as the lastest price data indicates, at around 2100 US$/ton (www.indexmundi.com). However, uncertain availability and fluctuations in its cost and quality have led to a worldwide search for new alternative protein sources for both shrimp and fish feeds.

With the intention of finding alternatives that allow the replacement of fish meal, numerous investigations have been carried out to evaluate protein substitutes in *L. vannamei* shrimp diets. Experimental aquafeeds have been formulated, where the marine protein is replaced by plant protein sources such as soy bean (Davis et al., 2004; Lim and Dominy, 1990, 1992), cottonseed (Lim, 1996), canola meal (Lim et al., 1997), pea meal (Davis et al., 2002; Martínez-Rocha et al., 2013), barley-based fermented grains (Molina-Poveda and Morales, 2004), a mixture of plant and poultry by-products (Amaya

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et al., 2007a,b), algae (Hanel et al., 2007; Ju et al., 2009), a mixture of plant protein soybean, canola and millo (Sookyin and Davis, 2011; Suárez et al., 2009), Jatropha kernel meal (Harter et al., 2011), soy protein concentrate (Bauer et al., 2012; Sá et al., 2013), and rice protein concentrate (Oujifard et al., 2012).

Likewise, some papers have been published studying several alternative ingredients in other shrimp species, soybean (Paripatananont et al., 2001; Piedad-Pascual et al., 1990; Rahman et al., 2010), fisheries by-products (Sudaryono et al., 1995), lupine meal (Smith et al., 2007a, b; Sudaryono, 2003, 2004; Sudaryono et al., 1999a,b,c), pea and canola (Bautista-Teruel et al., 2003; Cruz-Suárez et al., 2001), canola (Bulbul et al., 2012), and a mixture of soybean, corn gluten and rapeseed (Richard et al., 2011), sunflower (Syama-Dayal et al., 2011), or soybean and canola (Bulbul et al., 2013).

In this search, legumes/pulses have been used as the main source of vegetable protein, and soybean meal has been widely studied and used in commercial fish and shrimp feeds, obtaining inclusions up to 100% in *Penaeus monodon* with soybean meal supplemented by shrimp meal (Piedad-Pascual et al., 1990), and recently in *L. vannamei* by a plant mixture including soybean, millo and pea (Sookyin and Davis, 2011) and a mixture of soy protein concentrate and microbial floc meal (Bauer et al., 2012). However, soybean meal has the added disadvantage of high price due to its high demand for human food and animal feeds. There is therefore a need to explore another source of plant protein for aquatic feeds.

Among non-traditional sources used in feeds for aquaculture, several authors agree that the profile of lupine meal, with its high protein content and balanced levels of amino acids, makes it a potential candidate to reduce the dependence of fish and soybean meals. The pearl or Andean lupine (*L. mutabilis* Sweet) is a legume cultivated in Ecuador and other South American countries such as Argentina, Chile and Peru, potentially making it an excellent ingredient for aquafeeds. Previous studies of fish such as turbot, *Psetta maxima* (Burel et al., 2000), gilthead sea bream, *Sparus aurata* (Pereira and Oliva-Teles, 2004) and rainbow trout, *Oncorhynchus mykiss* (Borquez et al., 2011; De la Higuera et al., 1988) have shown that partial replacement of fishmeal by lupin and partial or total substitution of soybean is possible in tilapia, *Oreochromis niloticus* × *O. aureus* (Chien and Chiu, 2003). Likewise, in the shrimp *P. monodon*, a partial substitution of fish meal (Sudaryono et al., 1999a) or soybean meal (Sudaryono et al., 1999c) has been obtained with lupine meal.

These studies have allowed the development of low cost feed through partial or total replacement of fish meal without diminished growth, survival rates of animals nor feed efficiency. However, most of these studies have been carried out in aquarium or small plastic tanks, or production systems in which the natural productivity of the pond is not included, thus ignoring a resource that can generate a reduction in the production costs and a complementation of nutrients that could be covered up by the pond productivity. Nevertheless, some of experiments have been made in ponds (Amaya et al., 2007a; Sookyin and Davis, 2011) where shrimp can feed on macro invertebrates reducing feed conversion ratio and masking the effect of diet.

The present work was designed to assess the potential of the pearl or Andean lupin (*L. mutabilis* Sweet) as a source of plant protein in practical feeds for shrimp *L. vannamei* in laboratory aquaria and cages in earthen pond.

2. Material and methods

2.1. Diet formulation and preparation

L. mutabilis Sweet seeds (INIAP 450 Andino variety) were soaked in tap water for 48 h. Soaked grains were then cooked in boiling water for 40 min to remove the alkaloid. These were then drained, washed in tap water and soaked in running sea water for 4 days.

Lupin seeds were also subjected to a dehulling process in order to reduce fiber content. They were then dried in a fan-ventilated oven at 40 °C overnight. Oil was extracted by seven consecutive diethyl ether washings (2:1) under shaking at 135 rpm for 3 h at a time, followed by removal of solvent by discarding. Subsequent grinding produced lupin meal. The nutritional composition of the lupin recorded before and after processing (Table 1) was analyzed by AOAC (1990).

Experimental diets providing 35% protein and 11% lipid (Table 2) were prepared, where 0, 25, 50, 75 and 100% of fish meal protein (FM) was substituted by lupin kernel meal (LKM) on a protein basis. All diets contained 10% squid meal as an attractant. Only the corn starch (31.0 to 36.4%) and fish oil (5.3 to 7.1%) contents of the diets were varied to keep the protein and lipid content of the diets constant across treatments. Proximate analysis of the diets showed levels which closely mirrored the calculated values (Table 2), with the actual crude protein content ranging from 35.79% to 37.64% and lipid from 10.70% to 12.07%. The amino acid content of the experimental diets is presented in Table 3.

Once all of the dry ingredients were mixed by hand, soy lecithin and fish oil were added. Finally, water was gradually added (400–500 ml/kg) until the resulting dough could be easily extruded. The moist mixture was pelleted in a 2-mm diameter meat mincer. The 5–10-cm long "spaghetti-like" strands were dried in a fan-ventilated oven at 60 °C for 2 h. After drying, strands were broken up into pellets of about 1 cm in length, packed in sealed plastic bags and then stored at −10 °C until use.

2.2. Palatability of diets

During the sixth and seventh week, shrimps were fed *ad libitum* at 08:00 h and 16:00 h with the experimental diets. In order to estimate the acceptability based on the ingestion rate over 13 consecutive days, the uneaten feed was collected 2 h after each feeding by siphoning out through a previously weighted 300-μm mesh size net, dried at 60 °C for 24 h and then weighted to estimate the percentage of feed consumption according to biomass in each aquarium. A factor "F" was introduced to correct for feed losses due to water movement, aeration, siphoning and rinsing during the time that the feed was in the water. In order to determine this factor 10 aquaria with pellets but no shrimp were used. The amount of feed consumed by shrimp was expressed as a percentage of the biomass of shrimp in the aquaria and used as an indicator of diet palatability using the expressions:

$$\text{Palatability} = \frac{\text{Supplied feed} - (\text{Non consumed} \times \text{F})}{\text{Biomass in aquarium}} \times 100$$

$$\text{Correction Factor(F)} = \frac{\text{Supplied feed}}{\text{Retrieved feed}}$$

2.3. Pellet water stability

The pellet water stability (PWS) was determined by a horizontal shaking method using an EYELA heated circulating water bath with

Table 1
Nutrient composition (%dry weight) of *Lupinus mutabilis* Sweet.

Parameter (%)	Whole seed meal	Dehulled and deoiled lupin meal (kernel meal)
Moisture	5.73	10.30
Crude protein	50.51	61.45
Carbohydrate [a]	14.10	24.44
Crude lipid	28.22	1.24
Ash	1.44	2.57

[a] Calculated value: Carbohydrate = 100 − (ash + crude protein + moisture + crude lipid).

Table 2
Ingredients and proximate composition of the diets.

Ingredients	LKM0	LKM25	LKM50	LKM75	LKM100
Fish meal[a]	32.91	24.68	16.46	8.23	0.00
Lupin meal[b]	0.00	9.12	18.25	27.37	36.50
Corn starch[c]	36.44	35.12	33.75	32.40	31.05
Fish oil	5.28	5.71	6.17	6.63	7.08
Squid meal[d]	10.00	10.00	10.00	10.00	10.00
Wheat gluten[e]	5.00	5.00	5.00	5.00	5.00
Lecithin liquid	2.00	2.00	2.00	2.00	2.00
Cholesterol	0.50	0.50	0.50	0.50	0.50
Vitamins premix[f]	2.00	2.00	2.00	2.00	2.00
Minerals premix[g]	2.00	2.00	2.00	2.00	2.00
Antioxidant	0.02	0.02	0.02	0.02	0.02
Mold inhibitor	0.10	0.10	0.10	0.10	0.10
Carboxymethyl cellulose	3.00	3.00	3.00	3.00	3.00
Astaxantine	0.25	0.25	0.25	0.25	0.25
Chromic oxide	0.50	0.50	0.50	0.50	0.50
Proximate composition (%dry matter)					
Moisture (as was at mixing)	8.44	8.13	8.13	9.52	6.01
Crude protein (N × 6.25)	35.21	35.79	36.85	36.92	37.64
Crude lipid	11.23	12.07	11.08	11.51	10.70
Carbohydrate[h]	39.29	35.20	36.20	35.18	36.02
Ash	5.83	8.81	7.74	6.87	9.63
Energy (kJ g^{-1} dry matter)[i]	17.9	17.7	17.8	17.8	17.8

[a] Produced by steam dry method (69.7% crude protein c.p.; 6.9% fat). Polar, Salango, Ecuador.
[b] Dehulled lupin meal, solvent extracted (66.4% c.p.; 1.2% fat).
[c] Purchase from Sumesa S.A., Guayaquil, Ecuador.
[d] Processed in the laboratory by liophilization from commercial frozen baby squid *Loligo* sp (80.4% c.p.; 4.3% lipid).
[e] Purchase from Sigma Chemical Co., St. Louis, MO., USA.
[f] (mg 100 g^{-1} diet): p-aminobenzoic acid, 10; thiamin-HCl, 12; riboflavin, 20; pyridoxine-HCl, 12; choline chloride, 250; nicotinic acid, 75; calcium pantothenate, 50; inositol, 200; biotin, 0.5; folic acid, 1.5; ascorbic acid, 10; menadione, 4; α-tocopherol acetate, 40; cyanocolabamine, 0.03; cholecalciferol, 0.03; β-carotene, 1.15 10^{-3}.
[g] (mg 100 g^{-1} diet): calcium phosphate monobasic, 272; calcium lactate, 640.2; ferric citrate, 60; magnesium sulphate heptahydrate, 274; potasium phosphate, 480; sodium phosphate monobasic, 174; sodium chloride, 86; aluminium chloride, 0.4; potasium iodide, 0.3; cuprous chloride, 0.2; manganous sulfate monohydrate, 1.6; cobalt chloride hexahydrate, 2.1; zinc sulphate heptahydrate, 7.1; sodium selenite, 2.
[h] Calculated value: carbohydrate = total − (ash + crude protein + moisture + total lipid).
[i] Total calories was calculated using following factors: 23 kJ g^{-1} protein; 35 kJ g^{-1} lipid; 15 kJ g^{-1} carbohydrate.

shaker, Model NTS-120 (Japan). The shaker tray held up to six 200-ml flasks for each test run. Each flask was filled with 100 ml seawater (35 g L^{-1}) and 2 g of feed. The shaker speed was adjusted to 70 rpm and water temperature to 28 °C. After 2 h of continuous immersion, the pellets were recovered using a filtration apparatus with a 1-mm metallic mesh. The pellets were dried in a convection oven at 60 °C for 24 h and then cooled in a desiccator. The stability of the diets was expressed as the percentage of dry matter remaining of triplicates of pelleted the feed.

$$PWS = 100 - (\text{W dry}_{\text{before inmersion}} - \text{W dry}_{\text{after inmersion}}) \times 100$$

Table 3
Amino acid profile of the experimental diets (g AA/16 g Nitrogen).

	LKM 0	LKM 25	LKM 50	LKM 75	LKM 100
Arginine	5.4	5.3	5.6	6.3	6.6
Histidine	2.5	2.0	1.9	2.0	1.8
Isoleucine	4.0	3.6	3.5	3.7	3.4
Leucine	7.3	6.7	6.4	6.5	6.1
Lysine	5.4	4.4	4.0	3.9	3.6
Methionine	2.5	1.9	1.6	1.2	0.8
Phenylalanine	3.9	3.4	3.2	3.3	3.1
Threonine	3.9	3.5	3.1	3.2	2.8
Valine	4.6	3.4	3.7	3.7	3.3
Sum of analyzed EAA	39.5	34.2	33.0	33.8	31.5
Alanine	7.1	6.6	5.6	4.9	3.8
Aspartic acid	9.0	8.8	8.3	9.0	8.2
Cystine	1.8	1.2	0.9	0.7	0.2
Glutamic acid	19.2	21.0	21.7	24.7	23.4
Glycine	6.9	6.3	5.3	5.0	3.9
Proline	4.9	5.3	3.6	3.7	3.7
Serine	3.8	3.8	3.8	4.1	3.8
Tyrosine	3.0	2.7	2.5	2.6	2.4
Sum of analyzed AA	95.2	89.9	84.7	88.5	80.9

2.4. Digestibility assay

Seven days before the end of the growth trial, the shrimp were fed on their assigned diets that had been supplemented with 0.5% chromic oxide in order to acclimatize them to the new feed. The supplied feeds were left in the water for an hour and then uneaten feed and feces were removed. Collection of the fecal material was performed 2 h after feeding by siphoning. Feces from the same aquarium were collected every day at 10:00 and 16:00 h and pooled in 30 individual Eppendorf tubes. The feces were rinsed in distilled water to remove any salt and kept in an ice bath until the end of the day. They were then centrifuged for 5 min at 13,000 rpm in a refrigerated centrifuge at 4 °C to decant and discard excess water. Thirty pools of 10 days worth of feces were frozen in Eppendorf tubes at −80 °C. The feces were then dried for 48 h in a freeze drier. The dried feces were ground to a fine and homogeneous powder in Eppendorf tubes with a metallic piston. Afterwards the feces powder was oven dried for 24 h at 60 °C and kept in a dry atmosphere using silica gel to ensure complete dryness at the time of weighing. Protein and chromic oxide were analyzed in the feces and the six replicate diets following the procedure described by Foster and Gabbot (1971) and McGinnis and Kasting (1964), respectively.

The apparent digestibility (AD) of the test diets was calculated using the formula:

$$\text{AD} = 1 - \frac{(\%\ \text{nutrient}/\%\ \text{Cr}_2\text{O}_3)\ \text{feces}}{(\%\ \text{nutrient}/\%\ \text{Cr}_2\text{O}_3)\ \text{diets}} \times 100$$

2.5. Growth trial in the laboratory

Juveniles of less than 0.3 g/shrimp, free of white spot syndrome virus (WSSV), were obtained from the experimental facilities of the National Aquaculture and Marine Research Centre (CENAIM). These juveniles were reared in a system with a high sea water exchange rate of 1000% day^{-1}. The dissolved oxygen, salinity, and temperature averaged 5.1 mg L^{-1}, 34.7 g L^{-1}, and 28.1 °C, respectively. Prior to initiating the experiment, juvenile shrimp were only fed crumbled feed (40% crude protein; 10% lipid) until the average shrimp weighed 1 g.

Under laboratory conditions, juvenile *L. vannamei* with a mean body weight of 1.23 ± 0.18 g (mean ± SD), were randomly distributed with 8 shrimp (44 m^{-2}) in each 50-L 30 polyethylene aquaria (60 × 30 × 36 cm), each of which was supplied with aerated seawater.

Six replicate aquaria were assigned to each treatment in a fully randomized design and covered with 2 mm of mesh netting to prevent the shrimps from escaping. During the first two days, dead shrimp were replaced with shrimp of a similar weight. The experiment used a flow-through system. Seawater entering the aquaria was filtered through a sand filter and a 20-μm cartridge and exchanged at a rate of 1000% per day. A handheld oxygen meter WTW OXY3150i (Weilheim, Germany) was used to monitor the temperature and dissolved oxygen concentration twice a week and a refractometer was used to track salinity. Over the experimental period, the average temperature, dissolved oxygen and salinity were 27.3 °C (25.3–29.3 °C), 5.83 mg L^{-1} (3.19–7.00 mg L^{-1}) and 35 g L^{-1} (35–36 g L^{-1}), respectively. Light was provided by daylight fluorescent tubes (General Electric 40 W, USA). Light intensity was measured by a photometer Type 3281 (Yokogawa,

Japan) at the water surface. Water depth was 30 cm in the experimental aquaria. The daylight had an intensity of 1427 ± 103 lx. Photoperiod was 12 h light:12 h dark. Light was switched on at 09:00 h and off at 21:00 h.

The shrimps were fed *ad libitum* four times a day (08:00, 12:00, 16:00 and 20:00 h) during the first five weeks and then reduced to twice a day (08:00 and 16:00 h) for three weeks. Uneaten feed and feces were siphoned out every morning before the first feeding while moults were discarded throughout the day. Every 15 days the aquaria were thoroughly cleaned and the shrimps were weighed and counted in order to determine weight gain and survival rate, respectively.

2.6. Growth trial in the field

In order to confirm the results obtained in the laboratory bioassay and determine the contribution of natural productivity and experimental feeds to the overall nutritional budget of shrimp, an experiment under commercial conditions was carried out. The growth trial was conducted in bottomless cages (1 m^2 surface and 1.50 m height) sited in a single 1000-m^2 pond greenhouse. The cages were made with polyethylene mesh (15 × 20 mm aperture) and they were buried approximately 20 cm and spaced 40 cm apart. Following the density currently used in the farm, 30 juvenile shrimps *L. vannamei* were installed in each cage. The feed was offered on a tray at 6.5% (5.5 to 9 g), 6% (9 to 11.5 g) and 5.5% (11.6 g to harvest) of the biomass twice a day for 45 days. Five replicates were performed for each treatment. Additionally, five cages with shrimp, but without feed supply (NF), were placed in the pond. The five diets and the unfed control, as well as the animals, were randomly allocated to the 30 cages.

There was no water exchange, however, the loss of water through seepage and evaporation was recovered. Abiotic parameters such as dissolved oxygen concentration (8.7 ± 3 mg L^{-1}) and temperature (33 ± 1 °C) in the water were monitored daily at 06:00 h. Light intensity inside the greenhouse, measured by a light meter lux/fc (Model 840022, Sper Scientific Ltda., USA) every day during the experimental period, was 25,474.9 ± 19,287 lx. The cages were sampled on a weekly basis to estimate mean weight and adjust the amount of feed. At the end of the experimental period all the shrimps were counted and weighed to estimate the feed conversion ratio, survival, biomass gained and final mean weight.

2.7. Performance parameters

The growth parameter used to evaluate the quality of diets was calculated by the following equation:

$$\text{Specific growth rate } \left(\text{SGR};\% \text{ day}^{-1}\right): SGR = \left[\frac{\ln W_f - \ln W_i}{t(\text{days})}\right] \times 100$$

Daily feed intake (DFI, % d^{-1}) was calculated using the expression:

$$\text{DFI} = \left[\frac{\text{Supplied feed}}{\text{Average Biomass}} \times 100\right] / t(\text{days})$$

The feed conversion ratio (FCR) was estimated using the formula presented by Brand and Colvin (1977) for correcting dead shrimps:

$$\text{FCR} = \frac{\text{Total feed supplied}}{\text{Bf} + \left[\sum \frac{\text{Wi} + \text{Wf}}{2} \times \text{N}\right] - \text{Bi}}$$

Where B_f = final biomass, B_i = initial biomass, W_i = average initial weight in each period, W_f = average final weight in each period, and N = number of dead shrimp in each period.

An estimation of the percentage of Contribution of Natural Productivity (CNP) to nutritional requirements was made using the following equation:

$$\text{CNP} = 100 - [(W_f - W_i)\text{unfed shrimp} / (W_f - W_i)\text{fed shrimp}] * 100$$

2.8. Analytical methods

The feed ingredients and diets were milled to fine powder (300 μm) and their proximate compositions were analyzed using standard laboratory procedures (AOAC, 1990). Dry matter was calculated from weight loss after drying in an oven at 105 °C for 2 h. Crude protein (%N × 6.25) was measured using Kjeldahl method after acid digestion. Crude fat was calculated after extraction with diethyl ether extraction (Soxhlet technique). Ash was determined after ignition of the samples at 550 °C for 4 h in a muffle furnace. Amino acids were determined by high-performance liquid chromatography (Shimadzu, Japan) after hydrolysis of samples in 6 N HCl for 24 h at 110 °C. Then, samples were derivatized with o-phthaldialdehyde (OPA) according to Antoine et al. (1999).

2.9. Statistical analysis

The Anderson–Darling test was used to check for normality. Bartlett's test for homogeneity of variance was employed with P = 0.05 (Zar, 1999). All the data was subjected to the one-way analysis of variance (ANOVA) test ($P < 0.05$), considering initial weigh as covariate. Where ANOVA revealed significant differences, Student–Newman–Keul's multiple comparison test at $P < 0.05$ was applied to characterize and quantify the differences between treatments using Statgraphics (Statistical Graphics System, Version Centurion, Herndon, VA, USA).

3. Results

3.1. Stability and palatability of experimental diet

Pellet water stability was positively related ($P < 0.05$) to the level of dietary LKM (Table 4). The diet with the highest lupine inclusion level, LKM100, was the most stable ($P < 0.05$) after 2 h of immersion at 95%, and the diet without lupine, LKM0, presented the lowest value, at 82%. The rest of the diets had intermediate values of stability, between 85 and 90%.

The palatability results in the laboratory trial revealed a better acceptance of the LKM0 diet (8.4% d^{-1}) compared with the rest of the test diets. Feed intake for diets LKM75 and LKM100 were by far ($P < 0.05$) the lowest among all the other diets (3.4% d^{-1}).

3.2. Digestibility

The apparent dry matter digestibility (ADMD) decreased from 78.5 to 66.5% with the increase of LKM in the diet. The three highest replacement diets (50, 75 and 100%) had significantly ($P < 0.05$) lower ADMD than the two lowest replacement diets (0 and 25%) (Table 5). The apparent protein digestibility (APD) did not differ

Table 4
Pellet water stability (PWS) and palatability of diets containing different replacements levels of fishmeal by lupin.

Diet	LKM 0	LKM 25	LKM 50	LKM 75	LKM 100	SEM
Pellet water stability (%)	82.2^a	85.5^b	86.7^b	90.3^c	95.2^d	0.84
Palatability (% d^{-1})	8.40^a	7.15^b	4.47^c	3.37^d	3.47^d	0.27

Mean of three replicates for PWS and thirteen replicates for Palatability.
SEM = Standard Error of Mean.
Values in the same row with different superscripts are significantly different ($P < 0.05$).

($P > 0.05$) among the diets with 25, 50, 75, and 100% replacement, but was significantly ($P < 0.05$) lower with 0% replacement (control diet).

3.3. Survival, growth, and feed performance

The parameters for survival, growth, and feed performance for the aquaria experiment are shown in Table 6. No difference in initial individual weight using ANOVA indicated that the shrimp were homogeneously distributed between the treatments and replicates at stocking. There were no differences in survival, but there were statistical differences in SGR, DFI and FCR between the treatments. Shrimp fed diets LKM0, LKM25 and LKM50 (0, 25 and 50% replacement levels) exhibited similar results for SGR and FCR, with higher values than those fed on diets LKM75 and LKM100. In terms of daily feeding intake, this was highest for diet LKM0 and lowest for diet LKM100.

At the end of the cage experiment in the field, no significant differences ($P > 0.05$) in final weight, SGR, survival, DFI and FCR were found between any of the experimental shrimp diets (Table 7). In contrast, the control cages without feed supply presented a lower growth and survival rate than the average of the fed cages ($P < 0.05$).

The estimation of percentage contribution of natural productivity to the nutritional requirements of juvenile shrimp fed on different experimental diets varied between 26 and 31% and there were no statistical differences between them ($P > 0.05$) (Table 7).

4. Discussion

4.1. Diet composition and water stability

In the present study, *L. mutabilis* had a whole seed protein content of about 50% DM and a dehulled and deoiled lupin kermel protein content of 61% DM, both higher than that reported for others species. Glencross (2001) reported that, depending on the species, the protein content of dehulled lupin seeds (up to 57% DM) was higher than that of whole ones (44–45% DM). Like other lupin species and most legumes *L. mutabilis* protein contains low amounts of lysine and the sulfur amino acids, methionine and cystine, but has more arginine than soybean and a reasonably good balance of essential amino acids (Glencross, 2001). Nevertheless, as FM was replaced by increasing levels of LKM in the diets, there was a resulting increase in arginine and decrease in both lysine and methionine. When compared with published amino acid recommendations for juvenile *L. vannamei* (Akiyama et al., 1992), we found that threonine in three of diets (LKM50, LKM75, LKM100), methionine, lysine, phenylalanine and valine in all diets (except LKM0), and histidine in a 100% replacement diet were apparently deficient (Table 3). The other three amino acids exceeded the requirements in all the diets.

The lipid content of *L. albus* (7.6–11.8%), *L. angustifolius* (4.9–7.0%) and *L. luteus* (5.2–6.1%) reported by Sudaryono et al. (1999b) or Glencross (2001) were lower than *L. mutabilis* reported in this study (28%). Then, it was necessary to reduce this level in order to formulate diets with an adequate level of fish oil.

The water stability of experimental feeds was linked to the dietary level of LKM, with the percentage of dry matter remaining after 2 h immersion in seawater increasing significantly with increasing levels of LKM in the diets. Removing the seed coat to produce a kernel alters the composition of the resulting kernel meal, by decreasing the fiber content (Schoeneberger et al., 1982) contributing to a more binding diet. It is known that fiber levels affects water stability of diets, Akiyama et al. (1992) reported that feeds with high levels of fiber had reduced water stability which corroborates the work of Sudaryono et al. (1999b) who found that diets containing 40% whole lupin (*L. albus*) seed meal with 6.3% fiber, exhibited a lower percentage of retained dry matter than diets with 35% dehulled lupin containing 4.1% fiber. Nevertheless, Sudaryono et al. (1999a) reported a decrease in water stability in diets as a result of increased dietary levels of dehulled lupin seed meal and an increase in levels of fiber, but these results do not corroborate those obtained in the present trial. The differences in water stability between the Sudaryono et al. (1999a) and Sudaryono (2001) study and the present work are most likely to have been caused by the different carbohydrate content of the diets. A decrease in the amount of mainly starch sources (wheat flour: 18.5% to 9% and rice bran: 9% to 1%) in addition to an increase in the level of LKM of 10% (0–40%) may be responsible for the decreased water stability found in the former study. In contrast, the diets in the current study were formulated using corn starch as a filler (30–36%) and with an increasing level of lupin of 9% (0–36.5%). This could have improved the extrusion process, as Glencross et al. (2010) claimed that the inclusion of LKM increased the rate and degree of gelatinization, bulk density and pellet hardness of the mash starch content. The higher levels of water stability observed in the present study may be also attributed to the solvent used for fat extraction in the LKM affecting the properties of the lupin meal. The solvent, diethyl ether, used for fat extraction in the LKM, markedly affects functionality by removing non-polar lipids such as triglycerides and excluding polar lipids such as fatty acids and phospholipids. Sudaryono et al. (1999b) also reported that the highest percentage of remaining dry matter was for lupin protein concentrate as opposed to whole or dehulled lupin (*L. albus* and *L. augustifolius*) seed meal, which may explain why the pellet water stability was significantly affected by the inclusion of dehulled and defatted lupin seed meal in

Table 5
Apparent dry matter (ADMD) and protein (APD) digestibility of diets containing different replacements levels of fishmeal by lupin.

Diet	LKM 0	LKM 25	LKM 50	LKM 75	LKM 100	SEM
ADMD (%)	78.5^{a}	72.7^{b}	67.7^{c}	66.0^{c}	66.5^{c}	1.48
APD (%)	80.5^{a}	77.6^{b}	75.5^{b}	76.8^{b}	76.0^{b}	1.01

Mean of six replicates.
SME = Standard Error of Mean.
Values in the same row with different superscripts are significantly different ($P < 0.05$).

Table 6
Growth performance of white shrimp, *L. vannamei* reared for 57 days in 50-L indoor (clear water) aquaria and fed the different experimental diets.

Diet	LKM 0	LKM 25	LKM 50	LKM 75	LKM 100	SEM
Initial weight (g/shrimp)	1.19	1.18	1.24	1.20	1.27	0.06
Final weight (g/shrimp)	7.02^{a}	6.68^{a}	6.70^{a}	5.22^{b}	4.76^{b}	0.31
SGR (% day^{-1})	3.05^{a}	3.00^{a}	2.99^{a}	2.52^{b}	2.42^{b}	0.09
Survival (%)	82.3	81.8	96.8	90.4	86.1	5.68
DFI (%BW day^{-1})	6.48^{a}	5.31^{b}	5.99^{ab}	4.87^{b}	3.51^{c}	0.32
FCR	2.71^{a}	2.17a	2.48^{a}	2.24^{a}	1.66^{b}	0.15

Mean of six replicates using initial weight as covariate.
SME = Standard Error of Mean.
Values in the same row with different superscripts are significantly different ($P < 0.05$).

Table 7
Growth and nutritive performance of *Litopenaeus vannamei* reared for 45 days in 1-m^2 bottomless cages and fed diets containing different levels of lupin kernel meal (LKM) in replacement of fish meal.

Diet	LKM 0	LKM 25	LKM 50	LKM 75	LKM 100	SEM
Initial weight (g/shrimp)	5.87	5.98	5.63	5.85	5.79	0.11
Final weight (g/shrimp)	11.67	11.68	11.89	11.06	12.21	0.49
SGR (% day^{-1})	1.53	1.54	1.57	1.43	1.63	0.09
Survival (%)	77.1	80.4	70.8	78.6	69.2	5.1
DFI (%BW day^{-1})	2.87	2.73	3.12	2.90	2.77	0.17
FCR	2.17	1.99	2.35	2.32	2.04	0.20
Harvest biomass (g m^{-2})	268	281	250	261	249	12.6
CNP (%)	31.0	31.3	37.0	25.9	37.8	5.7

Mean of five replicates using initial weight as covariate.
SME = Standard Error of Mean.
Values in the same row with different superscripts are significantly different ($P < 0.05$).

the diets used in the present study. Protein losses were similar for all diets, Sudaryono (2001).

4.2. Digestibility

The ADMD results indicate that the lupin seed-based diets are not as well digested by juvenile *L. vannamei* as diets with no LKM. Sudaryono et al. (1996) reported that a low ADMD was found in diets of juvenile *P. monodon* containing 70% lupin meal made from the whole *Lupin* spp. seed, but Sudaryono et al. (1999a, c) observed that diets containing up to 40% dietary dehulled *L. albus* seed meal can be efficiently digested by *P. monodon*. These findings do not corroborate those of the present trial where dietary lupin levels ranged between 9 and 36%.

A significant decrease in apparent digestibility of dietary protein was observed when fish meal was replaced by any increment of LKM when compated to the control diet (80.5%). The APD values ranged from 75.5 to 77.6% in diets containing 9–36% LKM with no differences between the diets, and the values were lower than those of Sudaryono et al. (1996, 1999a). In juvenile *P. monodon*, however, APD gradually increased from 89.6% to 93.1% and was positively linked to lupin meal levels (Sudaryono et al., 1999a). When both dehulled and whole lupin seed meals were fed to *P. monodon*, it was found that the apparent digestibility of dry matter and crude protein of the dehulled lupin seed meal was higher than that of whole ones (Sudaryono et al., 1999b). These improvements are attributed to a reduction in indigestible insoluble non-starch polysaccharides (NSP, i.e. cellulose, hemicellulose, and lignins), which are composed of a soluble and an insoluble carbohydrate fraction (Sinha et al., 2011), and are primarily found in the seed coat. In the present study the lupin seed was dehulled before making meal and therefore the reduction of digestibility cannot be attributed to the NSP present in the seed coat. In lupins, the soluble NSP are predominantly oligosaccharides composed generally of α-galactosyl homologues of sucrose and ranging from 70 to 120 g kg^{-1} dry matter (Trugo and Almeida, 1988). At high concentrations, they could be considered anti-nutritional components for some species. In both *L. angustifolius* and *L. albus*, the ethanol extraction process notably removes soluble carbohydrate fractions comprised in a large proportion of oligosaccharides (70%) (Coon et al., 1990) and has also been reported to improve the nutritional value of lupins for trout. Glencross et al. (2003), when examining the influence of the oligosaccharide in *L. angustifolius* seed meal when fed to rainbow trout *O. mykiss*, demonstrated that oligosaccharide had a negative effect on the digestibility of the nutrients and energy of the diets evaluated. The removal of the ethanol-soluble component of the lupin meal had the greatest influence on the apparent digestibility of the energy, nitrogen, organic matter and nitrogen-free extractive components. NSP fractions can be detrimental to digestion because they alter gastric emptying, rate of passage, gut physiology and morphology, the native gut microflora and gut mucus layer, obstructing the digestive enzyme activity by changing digesta viscosity (Sinha et al., 2011). Although NSP was not determined in this study, the anti-nutritive potential of NSP could have an effect on *L. vannamei* because the dehulled LKM treatments had lower ADMD and APD than the control without LKM. This supports the hypothesis that oligosaccharides can interfere with digestion of other nutrients when fed to *L. vannamei*, and suggests that the oligosaccharide content of *L. mutabilis* may also be influencing the nutritional value of its own protein.

4.3. Growth and feed performance

The body weight gain in the shrimp reared in aquaria followed the trend of feed intake and decreased gradually with the increase in concentration of LKM in the feed. In the present study, *L. vannamei* displayed good growth with a 50% substitution of fish meal by de-fatted and dehulled *L. mutabilis* seed, but a significant decrease in weight gain and SGR when levels of 75% and above of the dietary fishmeal was replaced with lupin, meaning that 45% of the total dietary protein was from the LKM (27.4% inclusion level). This corroborates the findings of Sudaryono et al. (1999a) who found negative effects on the growth of juvenile *P. monodon* when dietary fishmeal was replaced totally by dehulled *L. albus* seed — an equivalent of 42% of the total dietary protein.

Better results with white shrimp growth were obtained when fish meal was substituted by several alternative sources; 80% fish meal by a co-extruded mixture of soybean and poultry meal (Davis and Arnold, 2000), 100% fish meal by mixtures of soybean and poultry meal (Samocha et al., 2004; Amaya et al., 2007b), 100% fish meal by a mixture of soybean and canola (Suárez et al., 2009), 50% fish meal by rice protein concentrate (Oujifard et al., 2012), 100% fish meal by soy protein concentrate (Sá et al., 2013), 100% fish meal by a mixture of soy bean meal and microbial floc meal (Bauer et al., 2012), 50% fish meal by a mixture of soy bean meal and animal meats (Ye et al., 2012). In general, it seems that mixtures of vegetable sources with animal meals give the best results.

The reductions in growth observed in experiments where fish meal was replaced by alternative protein sources have been attributed to anti-nutritive factors and an inadequate balance of amino acids and minerals in the tested sources (Lim and Dominy, 1991). Methionine and lysine are probably the most limiting and least-cost effective of the commercial feed formulae. The methionine level observed in 25%, 50%, 75% and 100% replacement diets is below the recommended level for shrimp diets (2.4% of protein) by Akiyama et al. (1992). The low level of methionine in lupin protein did not appear to limit the nutritional value of the feed when the dietary lupin inclusion level was 18.25% (LKM50), equivalent to about 32% of the total dietary protein. Despite the lower methionine level (1.6% of protein) in LKM50 diet, the average weight gain and SGR were not significantly different from those of the control diet. This can be attributed in part to the sparing effects of cysteine. Despite the fact that cysteine cannot be converted into methionine, the presence of cysteine lowered the use of methionine for protein synthesis and lessened the requirement for methionine (Goff and Gatlin, 2004; Moon and Gatlin, 1991). On the other hand, another reason may be that the amount of methionine recommended for shrimp feed by Akiyama et al. (1992) is overestimated, as Fox et al. (2006, 2011) suggested. The latter authors found that when feeding 35% crude dietary protein to *L. vannamei* shrimp the apparent methionine requirement was in fact 0.40% of the diet, equivalent to 1.14% protein; a lower amount that that reported (2.4%) by Akiyama et al. (1992). Likewise, Bauer et al. (2102) have reported optimum growth in white shrimp which have been fed diets containing lower dietary methionine levels than the requirements. From these results, it appears that the current dietary methionine recommendation is much higher than the actual requirement, when the diets contain 35% crude protein. This is clearly an issue that needs to be resolved.

According to Fox et al. (1995), the lysine level in 25%, 50%, 75% and 100% replacement experimental diets did not meet the requirements of *L. vannamei* (4.5% of dietary protein). Although diets LKM25 and LKM50 contained levels of lysine slightly below the requirement (4.4 and 4.0% of protein, respectively), the shrimps fed on these two diets did not show any significant difference ($P > 0.05$) in growth when compared to the control. Nevertheless, the growth of the animals fed on diets LKM75 and LKM100 was significantly lower than that of LKM0, LKM25 and LKM50. Recently, Xie et al. (2012) have re-evaluated the lysine requirements of *L. vannamei*, and the needs are higher (4.93% DP) than those suggested by Fox et al. (1995), meaning that the experimental LKM25 diet should clearly be deficient.

It is reasonable to assume that a replacement of 75 and 100% FM with LKM under isonitrogenous and isocaloric conditions would also have a negative effect on weight gain due to the deficiency of lysine, accentuated by the deficiency of methionine and the significantly lower levels of consumption of these two diets. The combination of low levels of methionine and lysine in lupin protein appears to limit

the nutritional value of the feed when the dietary lupin inclusion level is equivalent to 45% of the dietary protein. Hence, the optimal supplementation of lysine and eventually other essential amino acids such as methionine must be considered in future studies with shrimp.

The present study shows that a replacement of HP in shrimp feed adversely affects feed intake, especially with diets LKM75 and LKM100. This response seems to be linked to a decrease in palatability of the feed when HP is replaced with LKM. Although alkaloids levels of >100 mg kg^{-1} have been reported to cause palatability problems in rainbow trout diets (Serrano et al., 2008), there have been no reports of problems directly attributed to alkaloids in the diets of shrimp (Adler and Kittelson, 2004; Glencross, 2001; Smith et al., 2007b). The alkaloid levels of various cultivated lupin species range from 2.5 to 10% (Glencross, 2001; Petterson, 2000) and in the case of *L. mutabilis* the levels have usually been found to be >3% (Peralta et al., 2009). Although, the solution used in this study to reduce the alkaloid level in the lupin seeds was aqueous extraction, it seems that this was not enough to avoid the bitter taste that alkaloids impart. Smith et al. (2007b) examined the influence of the alkaloid gramine when it was included in diets fed to black tiger shrimp, *P. monodon*, and found that inclusion levels of up to 900 mg/kg of gramine did not significantly affect the daily feed intake over a 6 h period, probably due to slow feeding and rapid leaching of gramine from feed. It is possible that the difference in the response observed in the present study may have been influenced by the type alkaloid present in *L. mutabilis* or by the presence of some kind of repellent compound and by the fact the amount of feed eaten was determined over a period of 2 h. In every case, the intake of the experimental diets was clearly reduced by a high inclusion of LKM (27–36%), agreeing in part with Sudaryono et al. (1999a) who reported a smaller feed ingestion with a dehulled lupin meal inclusion of 30–40%. Saraç et al. (1998) obtained higher levels of inclusion of whole, dehulled and lupin meal concentrate without effects on intake, although the growth of tiger shrimp *P. monodon* was reduced.

Besides the poorer digestibility of diets LKM75 and LKM100 when compared to LKM25 and LKM50, and the lower essential amino acid balance in LKM75 and LKM100 than in the other diets (Table 3), the poorer growth seen in diets LKM75 and LKM100 than in LKM25 and LKM50 may also be attributed to lower levels of ingestion. Since these diets contained the same concentrations of protein, it is reasonable to accept that the growth rate under laboratory conditions was strongly related to feed intake, consequently resulting in a lower protein, methionine and lysine ingestion.

The variability of feed intake had an impact on the FCR values observed in this study with feed conversion values lower with LKM inclusions, but these results are not of any use, because growth was also much lower. Nevertheless, the FCR in the present trial were higher than those cited by some authors (Lim et al., 1997; Davis and Arnold, 2000; Samocha et al., 2004; Amaya et al., 2007b; Bauer et al., 2012; Ye et al., 2012), but similar to those reported by others (Davis et al., 2002; Molina-Poveda and Morales, 2004; Nunes et al. 2011).

No differences in mortality were found, and the main cause for the decrease in survival rate was the shrimp jumping out of the aquariums despite being covered with 2 mm of mesh.

The growth experiments conducted in cages in earthen ponds showed that, unlike the study conducted in aquaria, the gradual increase of LKM in diets did not produce a significant decrease in shrimp growth even when all the FM in the experiemental feed was replaced by LKM. This is because the bottomless cages allowed the shrimp free access to the substrate and the flora and fauna found there. This suggests that feeds with a lower biological value can be enhanced through the simultaneous shrimp consumption of the natural productivity present in an earthen pond (Leber and Pruder, 1988; Moorthy and Altaff, 2002). Others studies have also found that natural food contributed significantly (77–83%) to shrimp growth (Lawrence and Houston, 1993), even when using vegetable ingredients such as rice and wheat bran and soybean meal without marine meals.

Piedad-Pascual et al. (1990) conducted a growth trial in 1-m^2 cages in a 1-ha earthen pond using *P. monodon* juveniles shrimps, and after 3 months, the shrimps fed on diets containing 55% soybean meal (with a total replacement of fish meal) reached the same final weight as those fed on 15% soybean meal and 30% fish meal. According to Moss et al. (1992) pond water can enhance juvenile shrimp growth by as much as 89% over the growth rates attained in clear well water. The growth enhancement has been attributed to the shrimp's consumption of the microalgae and microbial–detrital aggregates present in pond water (Moss and Pruder, 1995), which in addition to the exogenous enzymes (Moss et al., 2001), supplies essential compounds lacking in the shrimp's diet (Tacon et al., 2002) and enhances by unknown levels the resulting growth factors (Leber and Pruder, 1988), out competing pathogenic bacteria.

Recent trials in *L. vannamei* reared in ponds (Amaya et al., 2007a; Sookyin and Davis, 2011) have shown the possibility of feeding white shrimp without fish meal with excellent conversion rates, even without dietary supplements of cholesterol. The requirements of this ingredient have been re-evaluated and reduced by Morris et al. (2011) and in fact some authors (Cruz-Suarez et al., 2007; Amaya et al., 2007b; Ye et al., 2011, 2012; Nunes et al., 2011; Sá et al., 2013) obtained goods results without any dietary cholesterol supplement, which would have an important effect on diet cost.

The natural productivity of ponds can be important in the feeding of shrimp. Although the unfed shrimp in bottomless cages grew, albeit less than fed shrimp (Table 8), survival was lower. Lawrence and Houston (1993) estimated a percentage contribution of natural productivity of 83% and 77% for *L. vannamei* stocked at 15 and 20 m^{-2} respectively. The estimation of percentage contribution of natural productivity from the data obtained in this study indicates that there was a nutritional demand of 26 to 31% for *L. vannamei* stocked at 30 m^{-2}. These differences are mainly due to lower stocking densities and shorter experimental periods (28 d) compared to the present work. Conceptually, as the stocking densities and the harvested biomass increase in an earthen pond, the percentage contribution to the nutritional requirements of shrimp would decrease.

Likewise, in recent laboratory experiments Moss et al. (2006) found that shrimp fed a 35%-protein diet minus vitamin premixes for 10 weeks in a flow-through shrimp pond water displayed a growth rate, 306% higher than those fed the same feed in flow-through well seawater. Shrimp in pond water with no feed additions survived and grew, indicating that shrimp derived some nutrition from web food.

Overall results have demonstrated that a low-cost feed made mostly from lupin meal as an alternative major protein source for imported fish meal and soybean meal can support production of *L. vannamei* stocked at 30 animals m^{-2} in cages under seawater pond conditions for a period of 45 days. A similar result was reported by Sudaryono (2004) in *P. monodon* stocked at 10 animals m^{-2} in cages under brackishwater pond conditions for a period of 60 days. The differences between the findings observed in aquarium and cages could be attributed to the trials being carried out in ponds where the natural productivity may have supplemented any amino acid imbalance in the diets tested. Although no cost–benefit analysis of shrimp production was conducted in this study, the inclusion of LKM produces a reduction in diet price of 56, 113, 169 and 225 US $ ton^{-1} respectively for the 25, 50, 75 and 100 diets, meaning an increase in profit would be achieved by using diets LKM75 or LKM100 in real conditions of shrimp production.

5. Conclusion

In summary, the experiments presented here reveal that feeds of varying levels of fish meal replacement perform significantly better in shrimp pond water than in clear seawater. Therefore and according to these findings, *L. mutabilis* Sweet could be a potential protein

Table 8
Comparison of growth performance of *Litopenaeus vannamei* reared for 45 days in 1-m^2 bottomless cages and fed experimental diets and fed natural productivity.

Diet	Diet	Natural
Initial weight (g/shrimp)	5.82 ± 0.05	5.92 ± 0.11
Final weight (g/shrimp)	11.68^a ± 0.23	9.84^b ± 0.52
SGR (% day^{-1})	1.53^a ± 0.05	1.13^b ± 0.10
Survival (%)	75.5^a ± 2.2	56.2^b ± 4.9
Harvest biomass (g/m^2)	262^a ± 5.3	161^b ± 11.9

Mean of twenty five replicates for diet and five for natural using initial weight as covariate.
Values in the same row with different superscripts are significantly different ($P < 0.05$).

sources to replace fish meal in commercial feeds for the growth phase of *L. vannamei*. The study should be repeated under pond conditions to see whether a total replacement of FM by LKM also results in no alteration to growth performance or feed utilization when compared to the control diet.

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