OPTIMISED FERMENTED LUPIN (*Lupinus angustifolius*) INCLUSION IN BARRAMUNDI *(Lates calcarifer)* JUVENILES DIETS

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ABSTRACT

The study aimed to investigate if fermentation of sweet lupin, *Lupinus angustifolius*, increases inclusion level of the lupin by replacing fishmeal content in the formulated diets of juvenile barramundi. Sweet lupin kernel was fermented by *Lactobacilli* (3.10^8 CFU/g) for 72 hours to be used to replace fishmeal at 0, 30, 45, 60 and 75% of inclusion levels. The results showed that the fermentation of lupin significantly reduced the amount of anti-nutrients and improved the amino acid profile of the lupin. The growth rates of the juvenile barramundi were not adversely affected by the different inclusion levels of fermented lupin in the diets. There was an even significant increase in the final weight and length of the barramundi fed 45% and 60% fishmeal replacement diets. The survival rates were more than 93% in all dietary treatments. Feed conversion rates (FCR) were unchanged among the diets, except significantly higher FCR shown in 75% fishmeal replacement diet. The differences of protein digestibility among diets were not significant. However, hydrolyzed fat and phosphorus in the diets significantly increased ($p<0.05$) when the fermented lupin inclusion level rose from 30% to75%. Fish carcass protein, fat and energy contents were not significantly affected by any diet, while essential amino acid profiles revealed a little change. In conclusion, the fermentation by *Lactobacilli* improved the lupin's nutritional quality, allowing higher inclusion level in barramundi diets.

Keywords: Lupin, fermented, barramundi, optimum level, diets

1. Introduction

The dependence of fishmeal based protein source for aqua-feed has long been realized as a significant limitation for sustainable development of aquaculture (Tacon, 1997; Tacon and Metian, 2008). Therefore, alternative high protein raw materials from animal by-product or plants are currently getting attention (Wanga *et al.*, 2006; Gatlin *et al.*, 2007). Lupins (*Lupinus* spp.), have been successfully tested as potential fishmeal replacements for salmonids and several other marine species (Carter and Hauler, 2000; Glencross *et al.*, 2004a; Glencross *et al.*, 2004b; Glencross and Hawkins, 2004; Glencross *et al.*, 2005; Glencross *et al.*, 2008; Katersky and Carter, 2009) and now are used in commercial diets (Glencross and Hawkins, 2004). Lupins at 40% inclusion level also produced unchanged growth and nitrogen retention in barramundi (*Lates calcarifer*) (Williams, 1998).

Although lupin and other legume seeds (*Phaseolus aureus, Cajanus cajan, Canavalia ensifomis*) contain a high amount of protein, their uses in food and aqua-feed are still limited due to their low protein digestibility and the presence of several anti-nutritional factors (ANFs) (Mubarak, 2005). Sweet lupin (*Lupinus angustifolius*), contains large amounts of soluble and insoluble non-starch polysaccharides, oligosaccharides, phytates, and tannins that have anti-nutritional effects including reduced digestion and absorption of amino acids (Barneveld, 1999; Glencross *et al.*, 2003). It has been suggested that lupins may also affect the structure of the gastrointestinal tract of salmonids (Farhangi and Carter, 2001; Refstie *et al.*, 2005) which might potentially affect amino acid flux and subsequent protein metabolism.

To enhance bioavailability of micronutrients in plant based diets by eliminating ANFs, several methods such as thermal and mechanical processes, fermentation, soaking and germination/malting can be applied (Hotz and Gibson, 2007). For improving utilization of plant protein in aqua-feed, fermentation seems to be cost effective method due to its simplicity and requirements for low operational energy and investment (Kang *et al.*, 2010). It is expected that lactobacilli fermentation of sweet lupin could improve its quality by reducing ANFs, improving amino acid balance and increasing digestibility thereby could increase its inclusion levels in the feed. However, pretreatment of lupin by fermentation to use as a source of protein for fish diets has never been investigated. Therefore, this study aims to evaluate the digestibility, growth performance and body composition of barramundi juveniles when fed different inclusion levels of lupin fermented by *Lactobacillus* sp.

2. Materials and methods

2.1. Experimental design

Barramundi (*Lates calcarifer*) juveniles were obtained from Northern National Marine Broodstock Centre, Haiphong, Vietnam and shipped to National Freshwater Breeding Centre (NBC), Haiduong, Vietnam where the juveniles were raised until they were adapted to salinity of 5 ppm. The fish were then acclimated for two weeks by feeding with Uni-President, Binhduong, Vietnam feed (45% protein, 12% fat). The juveniles were then graded, and those within the weight range of 7.0±1.6 g were selected and 600 fish were randomly delivered into fifteen tanks (40fish/tank) of 3.5 m^3, each attached to independent recirculating water system. The culture systems were set up out-doors in an open shed with a roof to protect from rain and direct sunlight. The natural temperature and photoperiod ranged between 28 – 31^oC and 12 hours of light respectively. After acclimation the experimental fish were fed for 61 days with 5 different pre-designed diets (Table 2). Every diet was fed in triplicate and three times daily (8 am, 12 am and 4 pm). Feeding was modified to 90% ASA-IM (American Soybean Association-International Marketing) satiation technique of which fish were fed to satiety for 20 minutes; the uneaten feed was collected immediately and measured in a calculation to determine amount of the feed intake; this amount was used for next 5 days and continued with another amount determined as outlined. After 61 days, the experiment was continued for another 7days to determine digestibility by feeding with the same diets after 1% Cr_2O_3 as an inert biomarker was added to them.

2.2. Fermentation of lupin

Sweet lupin, *Lupinus angustifolius* kernels, were provided by Co-operative Bulk Handling Grain, Perth, Western Australia. The kernels were grounded to less than 200 µm before fermenting by *Lactobacillus* spp. *Lactobacillus acidophilus*, *L. aporogenes* and *L. kefiri*, were obtained from a commercial product BIOLAC, BIOPHARCO, Nhatrang, Vietnam, and then mass incubated in MRS broth medium (Merck KgaA, Darmstadt, Germany) containing polysorbate, acetate, magnesium and manganese, which are known to act as special growth factors for *Lactobacillus spp*. To each 1000-ml of distilled water was added 55 g MRS broth and 250-ml soy extract. The combination was autoclaved at 121^oC for 15 minutes prior to the lactobacilli species being added. The incubation was carried out in a black glass jar with minimum oxygen for 24 hours at 37^oC in a refrigerated incubator (Scientifica, VELP, Usmate, Italy). After incubation samples were collected to check if bacterial density was > 10^8 CFU ml^{-1} (by diluted samples in 0.85% NaCl water to 10^{-5}, 10^{-6}, 10^{-7} and cultured in MRS algae at 30^oC for 24 hours), while the remaining part was mixed with autoclaved lupin in a plastic bag where commercial N_2 gas (obtained from Hai Duong Gas Company, HCM city, Vietnam) was filled to increase anaerobic conditions. The lupin fermentation was conducted at 37^oC for 72 hours. After fermentation, the samples of the lupin were collected to count bacterial density and nutritional profile.

2.3. Diets preparation

Diets were designed based on the nutritional composition of raw materials (Table 1) to meet 45% protein and 13% lipid levels. The five experimental diets having five inclusion levels, *viz*. 0%, 30%, 45%, 60% and 75%, of fermented lupin (FL) replacing fishmeal were prepared and labelled as 0FMR (control), 30FMR, 45FMR, 60FMR and 75FMR respectively (Table 2). Two sets of diets were prepared; one set was without chrome oxides (Table 2), and in the other set 1% of chrome oxide as an inert marker was added. The chrome oxide was added by replacing a part of cassava meal and wheat flour (for 75FMR) in the formulation thus protein content in diets was not affected (Table 2). The diet 0FMR contained 630 g kg^{-1} fishmeal. Diets were processed by addition of water to about 35% mash dry weight with well mixing to form a dough. This dough was then screw pelleted by a laboratory pelletizer to 1.2 – 2 mm pellets. These moist pellets were oven dried at 60^oC for 12 hours followed by cooling at room temperature before storing at – 20^oC till further use.

2.4. Nutritional analyses

Fermented and unfermented lupin samples were sent to LAREAL LAB in HCM city, Vietnam, for nutrients and anti-nutrients analyses. Nutrient parameters were analyzed in accordance with AOAC (1996). These consisted of crude protein (Kjeldalh), hydrolyzed fat (ISO 6492:1999), crude fiber (OACS Ba-6a-05), phosphorus (AOAC 965.17), and amino acid profile (HPLC), tannins (Spectrometry, Embaby, 2011) and phytic acid (Enzymatic, Haddad *et al*., 2007) and energy (Table 1; Table 3).

2.5. Fish handling and sampling

Before the commencement of the experiments, nine (9) fish were randomly selected and pooled into 3 groups for initial carcass analyses. The body parts, without tail, fins, intestine and head were collected for body composition analyses. The body parts were dried at 105^{o}C for 24 hours in a vacuum oven Shel Lab, Cornelius, USA (model 1445-2) at Environment and Disease Monitoring in Aquaculture, Bacninh, Vietnam, before sending to analyze crude protein and fat, energy and amino acid profile.

All fish handling activities were performed according to the Australian Code of Practice for the care of animals for science purposes, Approval No AEC_2014_14. Measurement of weight and length was carried out under an application of 2-phenoxyethanol anesthetic with a dose of 0.2 ml/l and 0.5ml/l to humanely kill the fish for body composition analyses (Tsantilas *et al.*, 2006). To evaluate growth, daily specific growth rate, feed conversion rates, and feed intake, all the fish at the beginning were measured for individual length and weight. At the end of experiments, 20 fish in each tank were randomly selected to measure length and weight. The digestibility analyses was performed by using fecal sedimentation method (Cho and Slinger, 1979). In every tank, a feeding tray was installed to collect all uneaten feed and if any feed escaped into the water column was siphoned immediately. After one hour of feeding, settled feces at the tank bottom were collected by siphoning, and frozen to -20^{o}C until further analyses. After 61 days of feeding test diets, one (1) fish from every tank was randomly selected to get 15 fish samples (3 samples/treatment) for final carcass analyses.

2.6. Calculations

Specific growth rate was calculated as: SGR = $[\ln W_2-\ln W_1)/(t_2-t_1)]*100$; where W_1 and W_2 are body weight at start and at the end of the experiments, respectively and t_2-t_1 is the culture period (days). Condition index (K) was determined based on length (L) and weight (W) using Fulton (1904) formulas as: $K = 100*L/W^3$ while skewness value was statistically calculated as: $S = [1/n\sum^{n}_{i=1}(x_i-\bar{})^3]/[\ [1/n\sum^{n}_{i=1}(x_i-\bar{x})^2]^{3/2}$; where x_i and n denote for the individual and observation, $\bar{x}$ was the sample mean. Apparent digestibility coefficients (ADC) of each nutritional component of the diets was calculated as: ADC=100-100*[Marker in feces(%)/Marker is diets (%)]*[Nutrient (i) in feces (%)/Nutrient (i) in diet (%)]; where (i) is a single nutrient like crude protein or hydrolyzed fat. Ingredient ADC was calculated as described by Forster (1999) and Glencross *et al.* (2007) as: $ADC_{ing}=[100-j)*(Nutr_{re}+Nutr_{ing}*j)*(ADC_{test}-((100-j)*Nutr_{re}*ADC_{re})]/[Nutr_{ing}*j]$; where j is percentages of FL replaced fishmeal proportion, $Nutr_{re}$ and $Nutr_{ing}$ are given nutrients in reference diet and FL, respectively and ADC_{ing}, ADC_{re} and ADC_{test} are digestibility coefficients of FL, reference diet and test diet respectively. Nutrient retention was calculated as % of nutrient intake.

2.7. Statistical analysis

The data were analyzed using SPSS for Windows version 18, IBM, Curtin University, Australia and Stata SE 12, Lakeway Drive, Texas, USA with the results expressed as the means and pooled standard errors of the mean (S.E.M). Paired-sample T Test was used to compare means of single nutritional parameter of the lupin before and after fermentation. One-way analysis variance (ANOVA) was used to compare effects of diets without and with different fermented lupin inclusions into the diets. Size distribution presented in the skewness values was performed together with normal distribution test. Levels of significance were determined for length and weight (Bonferroni), condition index, body composition (Tukey's HSD), digestibility, and growth performance (Least Significance Difference planned comparisons), with significant limits being set at $p<0.05$.

3. Results

3.1. Fermentation of lupin

Bacterial density found in fermented product was 3.10^8 CFU/g. There were differences in anti-nutrients and amino acid profile before and after the lupin was fermented. While two anti-nutrients, tannins and phytic acid were significantly reduced by 87.04% and 17.64% respectively, the amino acids, lysine, methionine, as well as phosphorus availability were increased (Table 1, Table 3).

3.2. Growth performance

There were some significant differences in final weight and length among fish fed the different diets (Table 4). Fish grew to a higher weight ($p<0.05$) when fed diets 45FMR and 60FMR than the control diet (0FMR), while those fed

30FMR and 75FMR did not show any growth increases. The juvenile barramundi length increased significantly when they were fed 60FMR and decreased when fish fed 75FMR than 0FMR, whereas 30FMR and 45FMR resulted in unchanged growth of the fish. There was no difference in specific growth rate (SGR) between control and test diets.

The survival of the fish of all diets was more than 93% (Table 4). Among them, diet 30FMR yielded the highest survival (98%) and was significantly higher than the control and other test diets. In contrast, feed conversion rates (FCR) were not significantly different among any diets except the 75FMR which produced the higher FCR ($p<0.05$).

The length and weight distribution (Figure 1, Figure 2) and skewness (Table 4) showed the various patterns in sizes of fish within each group fed different diets. The variations in length and weight were similar to the control and test diets, despite the fact that the shorter and lighter fish were found in 75FMR diet. In contrast, the K indices, indicating a fatness of the fish, were significantly different among fish fed various diets. The fatness calculated from condition indices of fish fed FL diets was not significantly different with the control diet. However, in general, fish were significantly less fat when fed diets 30FMR, 45FMR and 60FMR than fed 75FMR.

3.3. Digestibility

Apparent digestibility coefficient (ADC) of protein was not significantly different among diets. Whereas, there were significant differences in ADC of hydrolyzed fat, energy, fiber and phosphorus levels between control and test diets (Table 5). While, 30FMR diet resulted in significantly lower ADC of hydrolyzed fat and energy than control, 45FMR, 60FMR and 75FMR diets produced higher ADC of hydrolyzed fat and energy. When the FL inclusion levels increased in diets, the ADC of phosphorus correspondingly increased.

The ADC of ingredient-FL showed no significant differences of ADC of protein and phosphorus (Table 5). However, ADC of hydrolyzed fat and energy of ingredient-FL was higher in 60FMR and 75FMR diets than of 30FMR and 45 FMR diets. Meanwhile, ADC of fiber of ingredient-FL was significantly different between 75FMR and the lowered inclusion level diets.

3.4. Body composition

Proximate protein content (%) in initial fish and fish fed control diet were higher ($p<0.05$) than fish fed FL diets (Table 6). All FL diets resulted in the similar carcass proximate protein levels while the fish fed the control diet did not change in carcass protein compared to the fish before the experiment commenced. The carcass fat and energy levels of the initial fish were significantly higher than those of the fish fed any test diets. No significant difference in carcass fat and energy levels were found in any fish fed test diets. The percentages of essential amino acids (EAA) such as histidine (His) and tryptophan (Try) of carcass of initial fish and fish fed test diets were similar, whereas remaining EAA were significantly different. Met of fish fed 0FMR was higher than the initial fish and fish fed FL inclusion diets (Table 6).

There was significant differences in protein retention between fish fed control (0FMR) diet and FL inclusion diets (Table 7). However, when FL inclusion level increased, protein retention was unchanged. Neither fish fed fishmeal nor different inclusions of FL in diets significantly resulted in the change of fat and energy retentions.

3.5. Interactions

There was no significant interaction between inclusion levels of FL and blood meal; tannins and phytates; and FL and cassava. A closed significant ($p=0.07$) interaction was observed between FL and inclusion levels of wheat flour. The variations in FCR and ADC of phosphorus were significantly related to FL inclusion levels and concentration of ANFs (Table 8, Figure 3).

4. Discussion

A number of ANFs are present in protein-rich plants (Francis *et al.*, 2001) including lupins (Dupont *et al.*, 1994). Sweet Australia lupin is low in alkaloids (Dupont *et al.*, 1994) however phytates and tannins are major factors influencing the digestibility and thus reduces growth performance in aquatic species. Tannins contents are 1.17 and 2.64 $\mu g\ g^{-1}$ in sweet and bitter lupins respectively (Dupont *et al.*, 1994) that influence the protein utilization and digestion (Francis *et al.*, 2001). These ANFs are rather stable under heat treatment (Boland *et al.*, 1975) but can be efficiently removed by fermentation (Nnam and Obiakor, 2003). Lactic acid fermentation has been shown to give a significant reduction in phytic acid in cereals and sesame seed (Marklinder *et al.*, 1996; Mukhopadhyay

and Ray, 1999; Skrede *et al.*, 2002). Bartkiene *et al.* (2013) indicated that Lacto-fermentation of sweet lupin (*L. angustifolius*) could reduce acrylamide in enriched bread with high quality protein. Phytic acid and tannins in fermented lupin were reduced by 27.3% and 10.7%, respectively after 9 hours of fermentation by traditional method (Dhankher and Chauhan, 1987). Fermentation is the most effective way in decreasing the 56-96% of phytic acid than soaking and germination of brown rice (Liang *et al.*, 2008).

In this study, the fermentation by *Lactobacillus* sp. significantly decreased the levels of phytic acid and tannins by 87.04% and 17.64% respectively. These reductions are crucial to increase the inclusion levels of FL diets as high concentration of these ANFs can be detrimental to growth, for instance 0.5% purified phytic acid supplemented in feed can reduce 10% growth rate in rainbow trout (Spinelli *et al.*, 1983). Although other ANFs such as saponins, oxalate and cyanogenic glycosides, were not evaluated in the present study, they are also reduced when raw materials are fermented (Ketiku *et al.*, 1978; Eka, 1980; Fenwick and Oakenfull, 1983).

Sweet lupin had a little effect on the palatability of fish. The mixture of lupin kernel and lupin concentrate in barramundi juvenile diets did not influence to palatability (Katersky and Carter, 2009). Similarly, Glencross *et al.* (2011) demonstrated that a threshold where diets' palatability was maintained at 150 g kg^{-1} fishmeal with lupin, contributed 425 g kg^{-1} diet. In the present study feed intake was not reduced in any fish, with the highest inclusion level of FL was at 400 g kg^{-1} (60FMR) diet. This could be due to low alkaloids presence in the lupin, as the alkaloids result in a bitter taste and fermentation of lupin can improve aroma for the diets (Schindler *et al.*, 2011b).

After 61 days of culture, the barramundi juveniles fed all diets gained greater than 30 g from an initial average of 7g. Unfermented lupin as a fishmeal replacing single ingredient has been evaluated in other marine species. In rainbow trout 50% inclusion of lupin (*L. angustifolius*) in the diet resulted in significant reduction in the growth (Farhangi and Carter, 2001). In Atlantic salmon, the same replacement at a inclusion levels of 25 – 33% , resulted in lower utilization (Carter and Hauler, 2000). In this study, up to 60% of fishmeal was replaced by FL which resulted in higher growth than the control diet where only fishmeal was a main source of protein.

Dependence on only fishmeal source presents considerable risks associated with supply, price and quality fluctuations (Glencross *et al.*, 2007). Therefore, proportion of fishmeal should be reduced in a diet while maintaining a balanced nutritional formulation and thereby producing an acceptable good growth and low FCR. When fishmeal is replaced by a lower protein sources such as lupin, the blood meal has been used in accordance with the levels of FL included into diets. Concomitantly, the wheat flour and cassava meal were also used to balance the nutrients in the diets. Blood meal can be well utilized by barramundi and its added levels in these test diets were in the range that did not negatively influence to growth and FCR (Williams *et al.*, 2003). In contrast, the carbohydrate derived from wheat flour and cassava meal could influence the growth performance as carbohydrate was used limitedly by only marine fish (McMeniman, 2003). In the present study, interaction among ingredient inclusion levels was not observed, proved in better growth rate and high digestibility of test and control diets. Additionally, after fermentation, the lupin's EAA profile was modified which formed the diets more close to EAA profile found in barramundi (Glencross, 2006). This suggests that all formulated diets in the current study were nutritionally balanced.

Length-weight composition and K indices are important to determine the fitness and health of the fish population (Fulton, 1904), which is also referred as a return rate of operation cost in fish culture (Engle *et al.*, 2011). Size composition reflected by the fitted or skewed frequencies of the size (Ohlberger *et al.*, 2013) are strongly influenced by food quantity and quality (Fuiman, 2002), and feeding regime (Wang *et al.*, 1998). The fish in this study were more uniform when fed all FL inclusion diets than the control diet which is desirable from marketing viewpoint. The more uniform size could be explained by the feed intake, since fish eat more feed of 30FMR, 45FMR and 60FMR diets than that of control and 75FMR diets. As high inclusion level of FL in the diet formulation can reduce the feed production cost, a minimum size variation in the harvested fish size is critical for feed producers to reduce the feed costs.

Very few studies have attempted to evaluate the nutrient digestibility of FL. However, the bio-processed pre-treatment for plant ingredients have proven to increase digestibility. Lactic acid (*Lactobacillus* sp) fermentation of oil-extracted soybean meal partly eliminates and inactivate ANFs restricting the absorption of lipids by Atlantic salmon which then leads to a higher digestibility of total dietary energy, and subsequently improved feed efficiency (Refstie *et al.*, 2005). The addition of lupin protein concentrate and wheat gluten, exposed to certain extent of bio-processing, increases protein digestibility in diets for Atlantic salmon (Storebakken *et al.*, 2000; Refstie *et al.*, 2006). The digestibility in this study was higher than the study of Carter and Hauler (2000) partly due to the fecal collection method by sedimentation which can overestimate the digestibility of the nutrients

(Glencross *et al.*, 2007), however the main reason for the increase could be attributed to the fermentation process that reduced tannins and phytates, and others ANF's (Refstie *et al.*, 2005), improved amino acid profile (Yabaya *et al.*, 2009) and aroma (Schindler *et al.*, 2011a).

The results in this study were in agreement with Carter and Hauler (2000) where inclusion of sweet lupin resulted in a significant increase in digestibility of crude protein but no changes in energy levels. An combination of different plant ingredients also increased digestibility in juvenile barramundi (Glencross *et al.*, 2011). Apparent digestibility of phosphorus was affected by the inclusion levels of FL in diets with a strong regression (R^2 = 0.97). This was explained by the content of digestible phosphorus which was high in lupin ingredient and the fermentation process leads to increase in digestibility of phosphorus as shown in pigs (Almeida and Stein, 2012).

There was no change in proximate compositions among fish fed test diets. The results in this study were similar to the finding on cuneate drum (*Nibea miichthioides*) fed soybean substituting fishmeal (Wang *et al.*, 2006) where carcass protein also remained unchanged. The higher level of protein, fat and energy contents in initial carcass in the present study could be explained by the age and the diets. Initial fish were smaller and were fed on both trash fish and commercial feed before they were stocked to the test facility. The EAA in barramundi carcass in this study was similar as reported by Glencross (2006). In general, there was little relationship between EAA in FL which reflected in test diets and the EAA in carcass. Some EAA, Iso, Leu, Lys, Phe and Val were higher in FL than fishmeal, but these were not differences in carcass between fish fed 0FMR and FL inclusion diets. Met in FL was higher than fishmeal, however Met in carcass of 0FMR was higher with FL inclusion, suggesting that the Met was not well utilized by the fish.

Fermentation of sweet lupin, *L. angustifolius by Lactobacillus sp*, resulted in the elimination and/or inactivation of ANFs that restrict the absorption of nutrients by barramundi juvenile. This led to higher digestibility of crude protein, hydrolyzed fat and phosphorus which in turn resulted in an improved feed efficiency. The fermentation also improved lupin quality, reflecting in the acceptance of the fish with high inclusion level of the FL in test diets. Even though the protein retention in control diet was higher than in test diets, increased levels of FL inclusion did not change the retentions of protein, fat and energy. In addition, the body composition was the same among any fish fed any test diet, suggesting that high inclusion of FL, up to 60% could result in higher growth in barramundi juveniles.

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Table 1. Ingredients and diets' chemical analyzed. DM, GE, DE, CP, LOA, LNA, ARA, EPA, and DHA denote for dry matter, gross energy, digestible energy, crude protein, inolenic acid, linoleic acid, arachidonic acid, essential fatty acid, and docosahexaenoic acid.

Parameters	Fermented lupin	Lupin	0FMR	30FMR	45FMR	60FMR	75FMR
DM (g/100 g)	83.00	89.50					
Ash(g/100 g)	2.58	2.60					
GE MJ/kg	20.45	17.10					
DE MJ/kg	13.70	12.00					
CP(g/100 g)	40.00	38.55					
Dig CP(g/100 g)	38.20	35.70					
Lipid (g/100 g)	5.87	7.80					
Fibre (g/100 g)	2.58	4.20					
Arginine (g/100 g)	3.90	4.14	2.56	2.83	2.96	2.98	3.09
Histidine (g/100 g)	1.33	0.78	1.20	1.32	1.41	1.56	1.65
Isoleucine (g/100 g)	1.84	1.42	2.00	1.92	1.84	1.63	1.56
Leucine (g/100 g)	2.93	2.55	3.57	3.61	3.65	3.77	3.82
Lysine (g/100 g)	2.23	1.73	3.39	3.25	3.15	3.08	3.02
Methionine (g/100 g)	0.32	0.25	1.26	1.05	0.92	0.74	0.63
M+C (g/100 g)	0.90	1.00	1.69	1.52	1.41	1.25	1.15
Phenylalanine (g/100 g)	1.70	1.40	1.99	2.03	2.07	2.15	2.18
P+T (g/100 g)	3.40	3.20	3.47	3.57	3.62	3.67	3.72
Threonine (g/100 g)	1.63	1.34	1.91	1.90	1.88	1.87	1.86
Tryptophan (g/100 g)	0.33	0.33	0.52	0.50	0.48	0.47	0.46
Valine (g/100 g)	1.90	1.40	2.47	2.47	2.47	2.54	2.56
Available P (g/100 g)	0.23	0.09	2.96	2.28	1.84	1.25	0.87

Table 2. Ingredients composition of diets' formulation for growth, FCR and feed intake determination.

	Formula %				
Ingredient	**0FMR**	**30FMR**	**45FMR**	**60FMR**	**75FMR**
Fish meal	63.00	48.00	38.00	25.00	16.50
Lupin	0.00	20.00	31.00	40.00	49.50
Fish oil, Salmon	8.20	8.80	9.20	9.90	10.20
Wheat flour	12.00	10.00	10.00	6.50	5.00
Blood meal	4.50	6.50	8.60	14.00	16.00
Cassava meal	10.44	4.84	1.34	2.74	0.94
Soy lecithin	1.00	1.00	1.00	1.00	1.00
Vitamin PMX-F2	0.50	0.50	0.50	0.50	0.50
Mineral PMX-F1	0.25	0.25	0.25	0.25	0.25
Mold Inhibitor	0.05	0.05	0.05	0.05	0.05
Stay C - 35%	0.03	0.03	0.03	0.03	0.03
Antioxidant	0.02	0.02	0.02	0.02	0.02
Diets analyses					
DM (g/100 g)	88.76	87.46	87.70	86.51	98.84
Ash (g/100 g)	20.69	17.37	14.82	13.71	12.74
GE MJ/kg	21.26	21.65	21.85	22.17	22.37
DE MJ/kg	17.80	17.63	17.47	17.12	16.96
CP (g/100 g)	44.77	44.55	43.97	44.21	44.01
Dig CP (g/100 g)	38.98	39.20	39.02	37.97	37.86
Lipid%	13.04	14.72	13.62	13.83	13.04
Fibre (g/100 g)	1.32	1.50	1.59	1.68	1.77

Notes: Vitamin and mineral premix per kg: Vitamin A (UI) 1335000, vitamin D3 (UI) 500000, vitamin E (UI) 16670, vitamin K3 (mg) 3335, vitamin B1 (mg) 6670, vitamin B2 (mg) 5835, vitamin B6 (mg) 6670, vitamin B12 (mg) 3.35, folic acid (mg) 835, d-calpan (mg) 20000, vitamin C mono-phosphate (mg) 33335, inositol (mg) 45000, iron (mg) 8335, zinc (mg) 16670, manganese (mg) 3000, copper (mg) 8335, cobalt (mg) 670, iodine (mg) 167.5 and selenium (mg) 67.5.

Similar diets of which added chrome oxide (1%) (by replacing apart of cassava meal and wheat flour (for 75FMR) were formulated to determine digestibility

Table 3. ANFs presence (%) in lupin and its fermentation product

Ani-nutritional factors	Lupin	Fermented lupin	Pooled S.E.M
Phytic acid (phytate salt)	0.54[a]	0.07[b]	0.006
Tamins	0.17[a]	0.14[b]	0.003

Note: Within rows, values followed by the same letters are not significantly different ($p<0.05$, pair T test)

Table 4. Growth performance, SGR and feed intake of fish fed fishmeal diet and fishmeal partly replaced by fermented lupin diets. SGR and FCR denotes for specific growth rate and feed conversion rate.

Parameters	0FMR	30FMR	45FMR	60FMR	75FMR	Pooled S.E.M
Initial weight (g)	6.8[a]	7.2[a]	6.9[a]	6.9[a]	6.9[a]	0.68
Initial length (cm)	7.7[a]	7.9 [a]	7.8[a]	7.8[a]	7.8[a]	0.45
Final weight (g)	30.3[a]	33.3 [a]	34.6[b]	34.6[b]	31.4[ab]	0.38
Final length (cm)	13.0[a]	13.8 [ab]	13.8[ab]	14.0[b]	12.9[c]	0.58
SGR (%)	2.45	2.50	2.61	2.63	2.47	0.33
Feed intake (g)	991.7[a]	1080.7[b]	1085.0[b]	1085.4[b]	903.7[c]	0.36
FCR	1.11[a]	1.06[a]	1.05[a]	1.08 [a]	1.21[b]	0.28
Survival (%)	96.0[a]	98.0[a]	96.0 [a]	93.0[b]	93.0[b]	0.07
Size distribution statistics						
Skewness for weight	-0.179	-0.096	-0.091	-0.124	0.062	
Skewness for length	-0.126	0.169	-0.360	-0.262	-0.408	

Note: Within rows, values followed by the same letter are not significantly different ($p<0.05$, LSD test)

Table 5. Digestibility (%) of diets containing different FL inclusion levels and FL ingredient in test diets

	Diets					Pooled
AD (%)	**0FMR**	**30FMR**	**45FMR**	**60FMR**	**75FMR**	**S.E.M**
Diets						
Protein	91.37[a]	89.79[a]	94.79[a]	94.78[a]	96.59[a]	1.82
Hydrolyzed fat	92.14[a]	89.20[b]	94.96[ac]	96.22[ac]	97.81[c]	1.54
Energy	88.25[a]	87.57[a]	93.63[ab]	94.43[ab]	96.48[b]	1.09
Fiber	40.53[a]	47.14[a]	54.76[a]	48.07[a]	89.10[b]	4.90
Phosphorus	49.09[a]	69.70[b]	89.81[c]	92.23[c]	96.19[c]	4.75
Ingredient-FL						
Protein		86.14	68.10	98.32	97.72	6.47
Hydrolyzed Fat		59.24[a]	67.28[a]	98.94[b]	99.18[b]	5.62
Energy		86.00[a]	70.43[b]	98.54[c]	99.21[c]	3.64
Fiber		62.42[a]	58.71[a]	53.10[a]	108.64[b]	6.78
Phosphorus		117.85	123.73	120.96	111.82	1.83

Note: within rows, values followed by the same letter are not significantly different ($p<0.05$, Tukey's HSD test)

Table 6. Body composition (%) and essential amino acids (histidine (His), threonine (Thr), arginine (Arg), valine (Val), methionine (Met), lysine (Lys), Isoleucine (Iso), leucine (Leu), phenylalanine (Phe) and tryptophan (Try)) of initial fish and fish fed test diets after 61 days.

Diets	Body proximate				Essential amino acids									
	Moisture	Protein	Fat	Energy	His	Thr	Arg	Val	Met	Lys	Iso	Leu	Phe	Try
Initial	78.27	17.21^{a}	1.00^{a}	0.79^{a}	2.52	4.78^{a}	7.13^{a}	6.88^{abcde}	4.61^{a}	11.31^{abf}	6.22^{ade}	9.47^{ae}	10.65^{a}	1.31^{a}
0FMR	79.00	16.38^{a}	0.50^{b}	0.70^{a}	2.99	6.39^{b}	7.39^{ab}	4.61^{abcef}	8.24^{b}	14.34^{be}	7.02^{b}	10.56^{abe}	13.42^{b}	1.26^{a}
30FMR	78.07	15.40^{b}	0.43^{b}	0.67^{b}	2.55	5.76^{b}	7.93^{ab}	6.29^{abcef}	4.47^{a}	11.26^{acf}	5.95^{cde}	9.17^{ce}	12.36^{c}	1.30^{a}
45MFR	75.53	14.43^{b}	0.40^{b}	0.62^{b}	2.65	5.69^{b}	6.40^{c}	6.01^{adef}	4.68^{a}	20.73^{d}	5.54^{de}	7.57^{d}	7.25^{d}	1.09^{b}
60FMR	78.60	15.10^{b}	0.46^{b}	0.65^{b}	2.93	6.13^{b}	7.11^{abcd}	6.90^{abcde}	5.11^{a}	13.72^{be}	6.24^{abcde}	7.61^{C}	9.10^{e}	1.30^{a}
75FMR	79.23	15.73^{b}	0.52^{b}	0.67^{b}	2.85	2.57^{c}	8.26^{ce}	6.49^{bf}	4.58^{a}	11.18^{acf}	5.87^{e}	9.38^{e}	12.22^{c}	1.25^{a}
Pooled S.E.M	0.53	0.23	0.05	0.01	0.05	0.32	4.48	0.19	0.33	0.83	0.13	0.27	0.52	0.02
p<F	0.47	0.00	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.01

Note: Within columns, values followed by the same letter are not significantly different ($p<0.05$, Tukey's HSD test)

Table 7. Nutrient retention (%) in different FL inclusion levels in diets

Diets	Protein	Lipid	Energy
0FMR	37.07[a]	0.478	2.98
30FMR	31.71[b]	0.54	2.64
45MFR	29.27[b]	0.51	2.62
60FMR	30.47[b]	0.63	2.77
75FMR	31.70[b]	0.71	3.03
Pooled S.E.M	0.75	0.28	0.68
p<F	0.00	0.33	0.42

Note: within columns, values followed by the same letter are not significantly different ($p<0.05$, Tukey's HSD test)

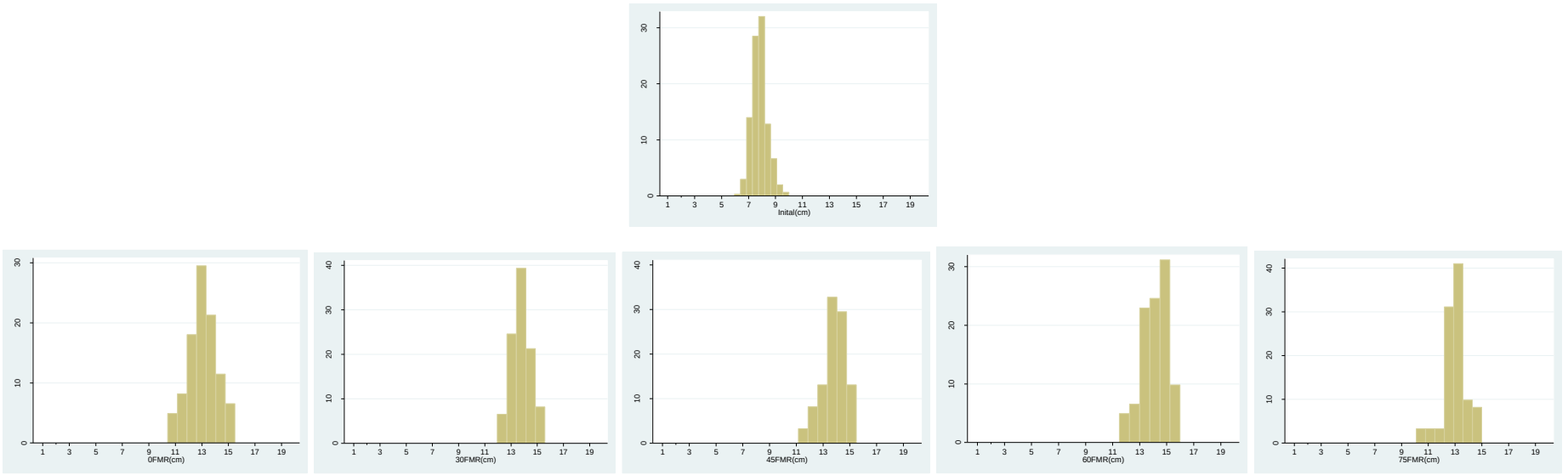

Figure 1. Histogram of length distribution of the fish of initial group and groups fed different FL inclusion levels in diets after 61 days.

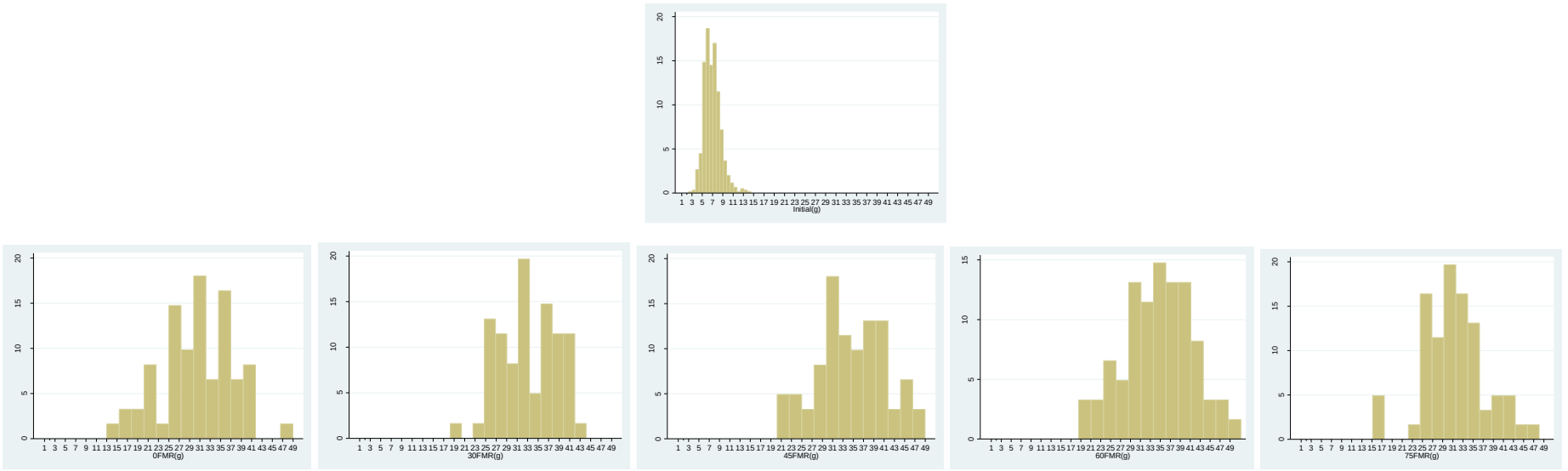

Figure 2. Weight distribution of the fish of initial group and groups fed different FL inclusion levels in diets after 61 days.

Table 8. Regression relationships betweentwo phytates level and FCR, ADC of protein, ADC of fat, ADC of energy, ADC of fiber and ADC of phophorus. In equations, y denotes for phytates and x denotes for the parameters in the same row.

Parameters	Equations	R^2	p
FCR	$y = 0.112x^2 - 0.189x + 1.1028$	0.96	0.03
Protein	$y = 0.0002x^2 - 0.0334x + 2.1754$	0.77	0.2
Hydrolyzed fat	$y = 2.6042x^2 - 2.1167x + 91.594$	0.76	0.2
Energy	$y = 1.5655x^2 + 1.1773x + 87.685$	0.85	0.1
Fiber	$y = 15.483x^2 - 13.733x + 42.574$	0.77	0.2
Phosphorus	$y = -6.7333x^2 + 37.566x + 48.054$	0.96	0.04

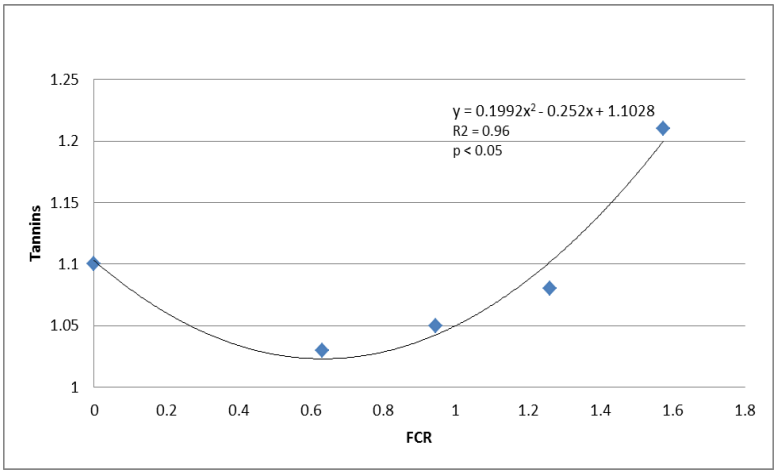

Figure 3. Regression of Tannins concentration in diets and FCR. The concentration was calculated based on the Tannins concentration in the lupin and inclusion levels of each test diets