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Original Article

Replacing moringa leaf (*Moringa oleifera*) partially by protein replacement in soybean meal of fancy carp (*Cyprinus carpio*)

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Abstract

Moringa oleifera Lam (Moringaceae) is a highly valued plant, distributed in many countries of the tropics and subtropics. The leaves are the protein source with an adequate profile of amino acids. The present study was undertaken in order to determine the effect of a dietary of moringa leaves on digestibility and growth performance of fancy carp. Fish were fed with diets containing isonitrogenouse and isoenergetic formulated by 20 and 50 g kg^{-1} of moringa leaves to replace protein in soybean. Fish were distributed in 500-liter tanks with flow-through water. Every fish was weighed and after the terminal experiment, all groups' livers and distal intestines were sampled. All fish grew normally ($p>0.05$) but fish fed with protein-replacing moringa leaves at 50 g kg^{-1} were noted to exhibit slightly poor growth performance and feed utilization. The study indicated that the tested moringa leaf diet contains ingredients that could be used for fancy carp diets with possibly not over up to 20 g kg^{-1} soybean protein replacement without negative effect on growth and digestibility.

Keywords: fancy carp, moringa, growth, digestibility

1. Introduction

Since farming aquatic animals in Thailand was broadly adopted and improved, it has caused a problem of high-priced feed as well as insufficient nutrition. A significant proportion of fish meal possesses a broad range of amino acids, and hence high-priced. There has been an attempt to replace fish meal with soybean meal which possesses good quality of essential amino acids (EAA). As a result, soybean meal, both imported and locally made, is utilized with the hope to help decreasing the costs, but as it turns out, it is also quite expensive. Herdsmen, no matter of undersized or oversized farms, are looking for a new cheaper raw material to decrease the cost though, however, it might not be as preferable as fish meal or soybean meal. This new material should be able to be produced locally, should be inexpensive and should provide high nutrition. Certain plant materials offer the most promising alternative aqua feed ingredients and in fact locally produced materials have already been used in Thailand.

Thailand is an agricultural country with a huge variety of plants in nature. The moringa (*Moringa oleifera*) is a fast-growing plant widely available in tropics and subtropics with several economic-important industrial and medicinal uses, and is a native food in Southeast Asia. The moringa leaves have been known to be effective for certain medicinal purposes. Based on a number of reports of the nutritional or medicinal values of a natural product, there are a staggering number of purveyors of "healthful" food who are now promoting moringa as a panacea. In addition, the products from the moringa hold considerable potential for becoming animal

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and fish feed ingredients because of their high nutritional quality. However, there is no information regarding the utilization of moringa in fish feed. An alternative is to use moringa leaves to replace soybean meal since they provide 260.0 g kg^{-1} protein (Makkar and Becker, 1996). EAA composition in moringa leaves is sulfur amino acid such as methionine, cystine, tryptophan (Makkar and Becker, 1996), as required by EAA for aquatic animals (WHO, 1985). These amino acids, however, have very low concentrations in soybean meal. It is found that methionine acid allows protein synthesis as well as being a reactant for homocysteine, cystine, carnitine, creatine, and choline. To some degree, most plant proteins contain some anti nutritional factors that vary with the processing, type and quality of the plant protein. Formulators should keep these concerns in mind so as to find the correct quality and type of plant protein for their purposes. Proteins of animal origin are generally more digestible than those of plant origin. Some technological treatments applied to plant proteins can bring an improvement in the apparent digestibility coefficient (ADC) by destroying antinutritional factors (Guillaume *et al.*, 2001). This study offers an alternative of utilizing moringa leaves as a protein source to replace soybean meal in carp diet and add value to the raw materials. The aims of this study were to investigate protease activity and the in vitro digestibility, and the effect of supplemented moringa leaves in fancy carp on growth performance in fancy carp.

2. Materials and Methods

2.1 Fish, diets and feeding protocol

Mixed sexes of fancy carp were maintained on a commercial diet of gold fish with 300.0 g kg^{-1} protein for four weeks prior to feeding the three experimental diets. Each treatment with an initial mean wet weight of 18.08±0.26 g per fish, were randomly distributed to each of 500-liter tanks with flow-through water. The diets were substituted by moringa leaves, and protein in soybean is replaced as follows: 1. Control diet; without moringa leaves. 2. Diet substituted by moringa leaves with 200 g kg^{-1} replacement of soybean protein. 3. Diet substituted by moringa leaves with 500 g kg^{-1} replacement of soybean protein.

Three isonitrogenouse and isoenergetic diets were formulated to contain approximately 350 g kg^{-1} protein and 13.12 kJ g^{-1} to meet the known nutrient requirements of carp. All diets were supplemented with L-methionine, to balance of this amino acid in the diets was similar in all case. The daily feed was done by hand-fed method to apparent satiation twice a day (09.00 and 16.00), six days a week for six weeks. Total feed was recorded weekly. Fish from each tank was weighed to measure growth at the end of the experiment at six weeks and growth performance was calculated.

2.2 Proximate analysis

Proximate analysis of diets were analyzed as follows: dry matter after drying in an oven at 105°C until constant weight; ash content by incineration in a muffle furnace at 600°C for 6 hrs; crude protein (N x 6.25) by Kjeldahl method after acid digestion; lipid by petroleum erher extraction in a Soxlet apparatus by AOAC (1990) method. The amino acids of fish carcass and diets were analyzed with an ultra fast liquid chromatography (UFLC), Shimadzu system (Shimadzu, Kyoto, Japan) (shown in Table 1).

2.3 Crude enzyme preparations

The upper, lower and whole intestine were homogenized (1:2 w/v) with 50 mM Tris–HCl buffer at pH 7.5 (Fisher Scientific, USA) in an ice water bath, using a tissue homogenizer. The preparation was centrifuged at 10,000 x g for 15 min at 4°C. The floating lipid fraction was removed and the aqueous supernatant was recovered and kept at -20°C until analysis was completed (Gimenez *et al.*, 1999).

2.4 Protease activity

Protease activity was monitored in triplicate by measuring the increase in cleavage of short chain polypeptide (Bezerra *et al.*, 2005). The total protease activity was determined by using 1 g L^{-1} (w/v) azocasein (Sigma-Aldrich, U.S.A.). The substrate (500 ml) was incubated with crude extract (20 ml) and buffer solution (200 ml) for 60 min at 30°C. Then, 500 ml of 200 g L^{-1} (w/v) trichloroacetic acid (Sigma-Aldrich, U.S.A.) was added to stop the reaction. After 15 min, centrifugation was carried out at 10,000 g for 10 min. The supernatant (1.0 ml) was added to 1 M NaOH (1.5 ml; Qrec, New Zealand) and the absorbance was measured at 440 nm against a blank similarly prepared but without the crude extract sample. The protease specific activity was expressed as unit of change in absorbance per min per mg protein of the enzyme extract (DAbs min^{-1} mg $protein^{-1}$).

2.5 Protein concentration

Protein concentration was determined by using bovine serum albumin (Sigma-Aldrich, U.S.A.) as a standard (Lowry *et al.*, 1951).

2.6 *In vitro* protein digestibility

Three experimental diets were measured in triplicate by the cleavage peptides (Gimenez *et al.*, 1999). Twenty milligrams of each diet was added with 12 ml of 50 mM phosphate buffer (pH 7.5) and incubated overnight at 30°C. *In vitro* digestion was started by adding 500 µl of the crude

Table 1. Ingredients and chemical composition of experimental diets.

Ingredient (g kg^{-1})	Protein replacement in soybean meal by moringa leaves (g kg^{-1})		
	0	200	500
Fish meal	320	320	320
Soybean meal	230	184	115
Moringa leaves	0	88	220
Wheat Flour	120	120	120
Cellulose	160	117.6	54.1
Fish oil	35	35	35
Soybean oil	35	35	35
Guar gum	10	10	10
Dicalcuim phosphate	20	20	20
Premix	70	70	70
L-Methionine	0	4	9
Total	1000	1000	1000
Nutrient composition by analysis (g kg^{-1} dry weight on basis)			
Protein	35.63 ± 1.95	34.67 ± 0.03	35.12 ± 1.07
Fat	9.39 ± 0.01	9.42 ± 0.04	9.37 ± 0.14
Fiber	2.11 ± 0.78	2.10 ± 0.17	2.18 ± 0.25
Dry matter	67. 60 ± 0.23	68.04 ± 0.28	67.68 ± 0.25
Ash	11.79 ± 0.09	11.52 ± 0.05	11.93 ± 0.45
Amino acid composition (g kg^{-1}dry weight on basis)			
Histidine	2.50 ± 0.01	2.58 ± 0.02	2.74 ± 0.01
Arginine	20.38 ± 0.01	18.50 ± 0.01	15.86 ± 0.10
Asparagine	3.28 ± 0.03	3.83 ± 0.02	5.88 ± 0.03
Glutamic acid	3.19 ± 0.01	3.44 ± 0.04	4.45 ± 0.04
Alanine	4.36 ± 0.09	4.08 ± 0.01	4.03 ± 0.01
Proline	2.52± 0.02	3.30 ± 0.04	3.87 ± 0.05
Methionine	1.27 ± 0.03	1.08 ± 0.01	0.79 ±0.01
Valine	3.33 ± 0.03	3.30 ± 0.03	3.52 ± 0.04
Trytophane	n.d.	n.d.	n.d.
Leucine	9.33 ± 0.06	9.30 ± 0.04	9.53 ± 0.04
Lysine	6.11 ± 0.02	9.08 ± 0.01	10.53 ± 0.02
Cysteine	10.06 ± 0.10	8.62 ± 0.03	8.86 ± 0.07

enzyme extract and incubated for 24 hrs at 30°C. After digestion, 1 ml of each digested mixture was determined of cleavage peptides by measuring the absorption at 750 nm and converted in to mg protein using a standard curve (Lowry *et al*., 1951).

2.7 Pepsin digestibility test

To assess the quality of experimental diets, digestible crude protein was determined by pepsin digestibility test (AOAC, 1990), using pepsin (Sigma-Aldrich, U.S.A.).

2.8 Statistical analysis

Mean value and standard deviation (S.D.) were calculated from the results. One way analysis of variance (ANOVA) was applied for comparison of the mean values, $P<0.05$ was established as significant.

3. Results

3.1 Total protease activity

Among the three parts of intestinal tract in initial fish, total protease activity was significantly ($p<0.05$) higher in upper tract (1.77±0.06 U mg protein^{-1} min^{-1}), followed by whole and lower tract (1.48±0.15 and 1.15±0.15 U mg protein^{-1} min^{-1}) (Table 2).

Digestive protease activity of fish after the end of the experiment is displayed in Figure 1, which showed higher activity of upper tract followed by whole and lower tract. Total protease activity of upper tract was significantly ($p<$

Table 2. Protein content and total protease activities of digestive tract of initial fish.

Digestive tract	Protein content (mg ml^{-1})	Total protease activities (U mg protein^{-1} min^{-1})
Whole tract	1.17 ± 0.04	1.48 ± 0.15^{a}
Upper tract	1.15 ± 0.03	1.77 ± 0.06^{b}
Lower tract	1.13 ± 0.03	1.17 ± 0.15^{c}
P–value	0.0749	0.0059

Values were means of triplicate analyses. Mean value within the columns with different letters were significantly different at $p<0.05$.

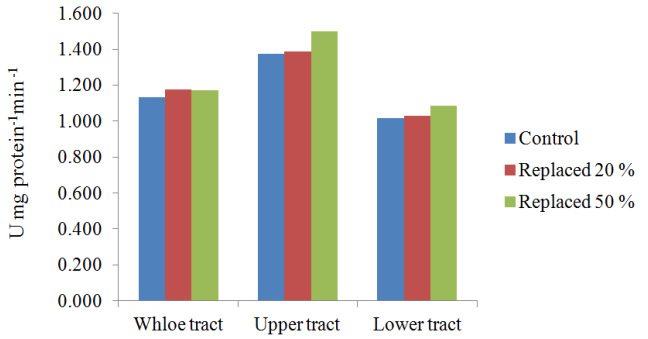

Figure 1. Total protease activities of digestive tract at terminal of experiment.

0.05) higher in fish fed with diet substituted by moringa leaves to replace protein in soybean at 50 g kg^{-1} (1.50±0.11 U mg protein^{-1}min^{-1}).

3.2 *In vitro* protein digestibility and Pepsin digestibility study

The study of *in vitro* protein digestibility by crude enzyme extract from intestine of carp and pepsin digestibility on three experimental diets were shown in Table 3. The results showed no difference ($p>0.05$) in *in vitro* protein digestibility assay and pepsin digestibility study of all experimental diets.

3.3 Growth performance and feed utilization

Growth parameters of fancy carp were shown in Table 4. The results indicated that there were no significant differences in weight gain (WG) and average daily gain (ADG) ($p>0.05$). Nevertheless, the higher WG and ADG were found in fish fed with the diets supplemented with moringa leaves to replace protein in soybean at 200 g kg^{-1} and control diet. WG ranged from 22.93 to 16.70 g, while ADG ranged from 0.81 to 0.56 g/fish/day. In terms of feed utilization, the data showed that there were also no significant differences in feed conversion ratio (FCR) and protein efficiency ratio (PER) ($p>0.05$) among all groups. FCR ranged from 0.72 to 0.43 and PER ranged from 0.59 to 0.41. All fish grew normally, and no specific signs of disease were observed. No mortality occurred throughout the experiment.

3.4 Amino acid composition in fish flesh

The amino acid composition of fish at the end of experiment is given in Table 5. With higher inclusion of moringa leaf meal in the diets, the amino acid content remained constant in all experimental groups. All groups showed no statistical difference ($p>0.05$) in muscle tissue amino acid content when compared to fish fed with the reference diet.

4. Discussion

Nowadays, plant sources have been used to replace the protein in fish meal and soybean meal, either partially or totally. Practical fish feed has been an area of focus in aquaculture nutrition research recently (Gomes *et al.*, 1995; Hossain *et al.*, 2001; Ogunji and Wirth, 2001; Siddhuraju and Becker, 2003). Moringa leaf has been widely studied as an alternative protein source in fish diet and seems to be a

Table 3. *In vitro* protein and pepsin digestibility study in experimental diets.

Digestibility (%)	Protein replacement in soybean meal by moringa leaves (g kg^{-1})			P-value
	0	200	500	
In vitro protein digestibility	65.65 ± 2.92	66.93 ± 2.54	64.63 ± 5.41	0.6571
Pepsin digestibility	76.18 ± 1.35	75.02 ± 4.34	74.74 ± 0.97	0.8958

Values were means of triplicate analyses. Mean value within the row with different letters were significantly different at $p<0.05$.

Table 4. Growth performance and feed utilization of fancy carp fed with experimental diets supplemented with moringa leaves at terminal period.

Parameters	Protein replacement in soybean meal by moringa leaves (g kg^{-1})			P-value
	0	200	500	
WG	22.93 ± 3.71	24.16 ± 3.16	16.70 ± 0.73	0.144
ADG	0.77 ± 0.12	0.81 ± 0.11	0.56 ± 0.73	0.137
SR	100	100	100	-
FCR	0.72 ± 0.40	0.62 ± 0.23	0.43 ± 0.01	0.583
PER	0.54 ± 0.12	0.59 ± 0.17	0.41 ± 0.01	0.403

Mean with the different letters in same row are significantly different at $p < 0.05$.
Note: WG: Weight gain (g), ADG: Average daily gain (g/fish/day), FCR: Feed conversion ratio (FCR), SR: Survival (%).

Table 5. Amino acid composition (g kg^{-1} dry weight on basis) in muscle of fancy carp at terminal of the experiment.

Amino acid	Protein replacement in soybean meal by moringa leaves (g kg^{-1})			P-value
	0	200	500	
Histidine	8.82 ± 0.08	8.58 ± 0.08	9.27 ± 1.55	0.200
Arginine	27.94 ± 4.35	25.28 ± 5.71	26.11 ± 5.15	0.924
Asparagine	3.08 ± 0.59	2.72 ± 0.40	3.04 ± 0.25	0.209
Glutamic acid	2.81 ± 0.34	2.82 ± 0.58	3.17 ± 1.85	0.908
Alanine	5.28 ± 0.89	4.58 ± 0.06	4.43 ± 0.86	0.069
Proline	3.00 ± 0.53	2.71 ± 0.30	2.80 ± 0.51	0.465
Methionine	3.21 ± 1.81	2.84 ± 0.59	2.94 ± 0.22	0.136
Valine	2.97 ± 0.86	2.81 ± 0.35	2.12 ± 0.57	0.180
Trytophane	n.d.	n.d.	n.d.	-
Leucine	4.43 ± 1.94	3.85 ± 1.28	3.14 ± 0.61	0.377
Lysine	1.31 ± 0.31	0.97 ± 0.44	0.93 ± 0.35	0.393
Cysteine	2.69 ± 0.44	0.45 ± 0.32	0.99 ± 0.38	0.003

promising protein source. Moringa leaf can partially replace conventional diets without any depression in growth performance of Nile tilapia (*Oreochromis niloticus* L.) (Afuang *et al.*, 2003; Richter *et al.*, 2003). In the present study, total protease activity from intestine of different parts of carp was investigated, as presented in Table 1. The activity of protease in upper tract was higher than those in the other parts ($p < 0.05$) and higher in fish fed with diet substituted by moringa leaves that replace protein in soybean at 500 g kg^{-1} (1.50± 0.11 U mg protein^{-1} min^{-1}). The results agreed with a report, which also found that the protease activity of three carps were higher in rohu (1.219±0.059 U mg protein^{-1} min^{-1}) followed by silver carp (1.084±0.061 U mg protein^{-1} min^{-1}), and catla (0.193±0.006 U mg protein^{-1} min^{-1}) (Kumar *et al.*, 2007). The digestive protease activity was different in other species (Chakrabarti and Sharma, 2005), which may greatly depend upon their digestive capability, feeding habits and environment. Regarding food quality, a complete diet with essential amino acids, fatty acids and vitamins is required for high growth rate in fish including available proteins in their diet by increasing consumption rate and enzyme production (Hofer, 1982). From the results, the activities of protease, which are essential for the utilization of protein from feed, contribute to high growth rate in fish.

In vitro methods of evaluating protein digestibility are important as they are rapid, less expensive, and allow close observation of the dynamics of the breakdown of protein by using only small amounts of raw materials (Grabner, 1985). Thus, characterization of digestive proteases is essential along with the quantitative estimations for the better understanding of digestive capability of the cultured species and for assessing protein ingredients in feed formulations (Moyano *et al.*, 1996). None of the diets adversely affected the *in vitro* protein digestibility and pepsin digestibility compared to the control diet without moringa leaves, but the diets supplemented with moringa leaves seemed to offer lower digestibility compared to the control diet. Plant ingredients (bean meal, groundnut oilcake and sunflower oilcake) can efficiently substitute fishmeal at 250 g kg^{-1} in African catfish diets, and there were no significant differences in

protein ADCs (88–90) with increased levels of dietary plant-based protein in diets (Nyina *et al.*, 2010). The ADC in protein of plant leaf ingredients was determined and barnyard grass and dried maize leaves were found not only to offer poor digest but also yield negative impact on the digestibility of the reference diet. On the contrary, fresh maize leaves were well digested for grass carp; with percentage 60.9, 70.5 and 84.7, ADC respectively in protein compared to 94.1 in control diet. This indicated that dry plant materials seem to be low digestible and could even inhibit fish utilization of other nutrients contained in diet (Dongmeza *et al.*, 2010).

In this study, neither growth nor feed conversion efficiency were affected significantly by dietary treatment for all treatments diets. Growth parameters, namely weight gain, feed conversion ratio and survival were similar ($p>0.05$). This agrees with the study that shows no effects of dietary supplement of methanol-extracted leaf meal containing 11, 22, and 33 g kg^{-1} found on the growth of Nile tilapia (*Oreochromis niloticus* L.) (Afuang *et al*., 2003). Tilapia fed with raw moringa leaf meal revealed that 10% of replacement of fish-meal-based dietary protein did not cause any adverse effect on growth performance (Richter *et al*., 2003). Most published research on the use of plant protein as a substitute of SBM in fish feeds has focused on the inclusion of palm kernel meal (Ng and Chen, 2002), cotton seed meal (Yue and Zhou, 2008) and Faba beans (Azaza *et al*., 2009) with the goal to increase inclusion of sustainable plant-based diet for fish and all results show that dietary protein source from plant origins did not affect growth and survival of fish. The amino acid compositions in all experimental diets of this present study were generally similar, but substantial differences existed in methionine content. This was because lower level of methionine in diets supplemented with moringa leaf (Diets 2 and 3) were more limited in this amino acid than in the control diet. In the present study, low dietary levels of methionine have been shown to suppress growth and feed utilization. Methionine content of the experimental diets supplemented with moringa leaf gradually decreased at 14.96 and 37.79 g kg^{-1} compared to the control diet. Methionine is generally the limiting amino acid and methionine deficiency frequently causing reduced growth (Jackson *et al*., 1982; Gaber, 2006).

This indicates that methionine deficiency may be one of the reasons responsible for the lower growth performance and poorer diet utilization of the groups fed the diets supplemented with moringa leaves. Similar to a study report of low dietary levels of methionine, growth of juvenile hybrid striped bass and increased mortality has been shown (Keembiyehetty and Gatlin, 1993). However, essential amino acid (EAA) composition in moringa leaves is sulfur amino acid such as methionine, cystine and tryptophan which should be used as supplementation only (Goff and Gatlin, 2004).

5. Conclusions

In conclusion, this study indicated that moringa leaves used as a plant protein sources for replacing soybean meal could support the growth, adversely affected and digestibility of fancy carp. Thus, moringa leaf could possibly replace not over up to 200 g kg^{-1} of protein in soybean and become an alternative plant protein source in fish diet to lower the production cost of fish diets and add value to a plant origin.

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