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Replacement of fish meal with *moringa* diets on digestive enzymes activities in carp, *labeo rohita* (Hamilton, 1822)

T Dyson[1], R James[2]
[1-2] P.G. and Research Department of Zoology, V.O.C. College, Tuticorin, Tamil Nadu, India

Abstract
Replacement of fish meal with different levels of *Moringa oleifera* diets (10, 15, 20, 25 and 30%) on digestive enzymes viz., amylase, protease and lipase were studied in carp, *Labeo rohita* for 80 days. The digestive enzyme, amylase activity was increased with extension of rearing period (0 – 80 days) and inclusion of *Moringa* diet in the place of fish meal. Amylase activity was gradually enhanced with inclusion of *Moringa* diet upto 25% and thereafter it declined in the foregut, midgut and hindgut of *L. rohita.* Duncan multiple range test revealed that, 25% MS diet elicited the maximum amylase activity in the tested digestive regions as compared to other MS diets. A significant ($P < 0.01$) and positive correlations were obtained between the inclusion of MS diets and amylase activity in *L. rohita.* The 'b' values obtained for amylase activity in foregut of *L. rohita* related to the chosen MS diets was 1.19, 1.29, 1.46, 1.76 and 1.37. It revealed that, high 'b' value in fish fed with 25 MS diet (1.76) as compared to other diets. The similar relationships were also obtained in midgut and hindgut of *L. rohita*. The results obtained for protease and lipase activities were similar to those of amylase activity. *L. rohita* fed with 25% MS diet registered the more protease and lipase activities in midgut and hindgut as compared to other diets. The present study also elicited that, the low (10 – 20%) and high level (30%) of *Moringa* inclusion in the place of fish meal, reduced the digestive enzymes, amylase, protease and lipase in *L. rohita.* The present investigation concludes that, inclusion of 25% *M. oleifera* leaf meal in the place of fish meal enhances the digestive enzymes (amylase, protease and lipase) in the chosen digestive tracts (fore, mid and hind guts) of carp *L. rohita.*

Keywords: replacement of fish meal, *Moringa diet*, amylase, protease, lipase, *Labeo rohita*

Introduction
Fish meal is the major source of protein in the commercial fish feed elsewhere in the world. However, the use of fish meal as a sole source of protein is not feasible and expense of conventional pelleted feed put severe constraint in the development of low cost aquaculture systems. It is possible to replace costly fish meal by inexpensive indigenous ingredients such as *Moringa* meal which is rich in protein, carotenoids, ascorbic acid and iron (Sanchez *et al.,* 2006) [16]. The leaves of *Moringa* are also used as nutritional supplement and growth promotes due to the significant presence of protein, Se, P, Ca, β-carotene and α-tocopherol (Foidl *et al.,* 2001) [4]. Besides, many researchers have reported the beneficial effects of soybean meal, poultry meal (Hasan *et al.,* 1990; Hashim *et al.,* 1994; Hasan and Roy, 1994; Mazid *et al.,* 1994) [7, 8, 6, 13] in fishes and less research on the effect of *Moringa* diet in the fish (Sirimongkolvorakul *et al.,* 2011) [22]. Hence the present study has been undertaken to study the digestive and respiratory enzymes activities with the incorporation of *Moringa* diet in a simple replacement of fish meal in carp, *Labeo rohita.*

Materials and methods
Healthy and active juveniles of *L. rohita* (375 Nos.) were collected from the acclimation tanks and fasted for 24 hr prior to the commencement of the experiment. They were divided into five groups corresponding to the supplementation of MS diets viz. 10, 15, 20, 25 and 30% in the place of fish meal. Each group consisting of 25 individuals was reared in circular cement tank containing 100 l of water. Triplicates were maintained for each MS diets. The tanks were filled with dechlorinated well water and drained twice in a week and replenished with freshwater to remove accumulated feces from the bottom. The averaged hydrological parameters of the medium were : temperature : 28.1 ± 0.8 ^{0}C; hardness : 340.00 ± 13 mg $CaCO_3$ l^{-1}; pH : 7.6 ± 0.08; dissolved oxygen : 4.18 ± 0.20 ml l^{-1} and salinity : 0.47 ± 0.03 ppt. Fish were fed *ad libitum* twice a day for a period of 1 hr each and after which unconsumed feed was removed from the experimental tanks. The experiment was lasted for 80 days.

Feed formulation
Feed formulation was done by Square method (Hardy, 1980) [9] and 30% basal protein diet was prepared by using ingredients like fish meal, ground nut oil cake, tapioca flour, maida, cod liver oil (lipid source) and vitamin and mineral mixtures (Table 1). At first, dried and powdered ingredients were blended to make a homogenous mixture. Subsequently, the feed ingredients were mixed with suitable levels of dried powdered *Moringa* diets (10, 15, 20, 25 and 30%) an aliquot of boiled water and then cooked in steam for 20 minutes. The pellets (2 mm size) were prepared with a hand operated pelletizer and dried in sunlight. The dried diets were stored in a refrigerator until use. The composition of the experimental diet is given in Table 1.
Three fishes were separately selected from the each experimental tanks at an interval of 20 days. Test fishes were sacrificed to isolate the fore, mid and hind guts

separately and thereafter, they were subjected to the estimation of chosen digestive enzymes. Amylase, protease and lipase enzymes were estimated following the method of Bernfield (1955), Jony (1976) [11] and Teitz and friedrick (1966) [23] respectively.

Table 1: Formulation and percentage composition of experimental diets. Each value is the mean ($\overline{X}$ ±SD) of three estimations.

Ingredients (g / 100 g diets)	Experimental diets (%)				
	10	**15**	**20**	**25**	**30**
Fish meal	28.0	25.0	22.0	19.0	16.0
Ground nut oil cake	24.5	22.5	20.5	18.5	16.5
Tapioca flour	18.5	18.5	18.5	18.5	18.5
Rice bran	17.0	17.0	17.0	17.0	17.0
Moringa leaf meal	10	15	20	25	30
Vitamin and mineral mixture	1	1	1	1	1
Cod liver oil (ml)	1	1	1	1	1
	Proximate composition (%)				
Protein	30.01± 1.25	31.50 ± 1.28	32.55 ± 2.33	35.51 ± 3.46	35.05 ± 2.51
Lipid	6.38 ± 0.98	3.16 ± 0.71	2.52 ± 0.53	2.11 ± 0.35	1.21 ± 0.19
Ash	17.67 ± 1.15	13.99 ± 1.23	12.87 ± 1.13	14.37 ± 1.30	16.25 ± 1.18
Nitrogen free extract	45.94 ± 6.20	51.35 ± 5.33	51.06 ± 5.65	48.01 ± 3.11	47.49 ± 4.89

Preparation of enzymes source

Prior to the estimation of digestive enzymes (amylase the protease and lipase) on sampling days the tested fishes were removed from each experimental groups and starved for 24 hrs and sacrificed. The whole alimentary tract was dissected out in ice cold fish ringer solution and thoroughly washed externally. The tissue was rinsed with cold distilled water and a portion of alimentary canal, foregut midgut and hindgut was ligatured, split open and washed thoroughly. The tissues were homogenised separately with distilled water using mechanical dispencer. The homogenate was centrifuged at 40,000 rpm for 15 min at 0^0C using high speed refrigerated centrifuge (Remi Model K-II) to prepare 1 to 10% of aqueous extracts. The clear supernatant was used as the crude enzyme extract for subsequent assay.

Statistics

Duncan multiple range test was used to determine multiple variations of mean values between control and experimental groups to assess the impact of replacement of fish meal with *Moringa* diet in *L. rohita*. Correlation and regression were applied following the least square method (Zar, 1984) [26]. Two way ANOVA test was applied to find the significant effects of replacement of fish meal with *Moringa* diet and rearing period on chosen digestive enzymes.

Results

The digestive enzyme, amylase activity was increased with extension of rearing period (0 – 80 days) and inclusion of *Moringa* diet in the place of fish meal. Amylase activity was gradually enhanced with inclusion of *Moringa* diet upto 25% and thereafter it declined in the foregut, midgut and hindgut of *L. rohita*. For instance, amylase activity was 305, 315, 330, 362 and 325 µg maltose / mg of protein / hr in foregut of *L. rohita* fed with 10, 15, 20, 25 and 30% MS diet respectively. Duncan multiple range test revealed that, 25% MS diet elicited the maximum amylase activity in the tested digestive regions as compared to other MS diets (Table 2). The amylase activity occurred in the following order: foregut > midgut > hindgut. Two-way ANOVA showed that, amylase activity was significantly ($P < 0.05$) related to the inclusion of MS diets and rearing period (Table 3) in *L. rohita.* A significant ($P < 0.01$) and positive correlations were obtained between the inclusion of MS diets and amylase activity in *L. rohita.* The 'b' values obtained for amylase activity in foregut of *L. rohita* related to the chosen MS diets was 1.19, 1.29, 1.46, 1.76 and 1.37. It revealed that, high 'b' value in fish fed with 25 MS diet (1.76) as compared to other diets. The similar relationships were also obtained in midgut and hindgut of *L. rohita* (Fig. 1).

The results obtained for protease and lipase activities were similar to those of amylase activity. However, protease and lipase activities were maximum in midgut and hindgut of *L. rohita* respectively (Table 4 and 5). *L. rohita* fed with 25% MS diet registered the more protease and lipase activities in midgut and hindgut as compared to other diets. Duncan multiple range test and 'b' values obtained from regression line also revealed that, inclusion of 25% MS diet triggered the digestive enzymes activities as compared to other tested diets. However, high level inclusion of MS diet (30%) decreased the digestive enzymes in *L. rohita.* Besides, two-way ANOVA also elicited that, MS diets and rearing period hold significant ($P<0.05$) impact on the tested digestive enzymes activities in *L. rohita*. The enzymes activities occurred in the following order in different regions of *L. rohita.* Protease: midgut >hindgut > foregut; Lipase: hindgut > midgut > foregut.

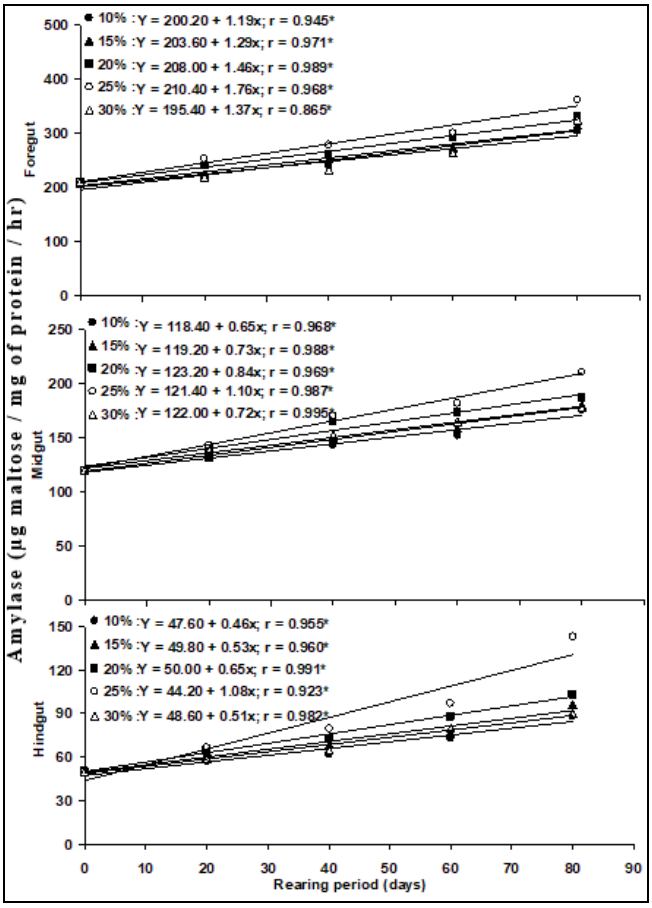

Fig 1: Regression lines on replacement of fish meal with various levels of *Moringa* diets on amylase (µg maltose / mg of protein / hr) enzyme activities in different tissues of *Labeo rohita* as a function of time. *P < 0.01

Table 2: Replacement of fish meal with different levels of *Moringa* diets on amylase (µg maltose / mg of protein / hr) enzyme in different tissues of *Labeo rohita* as a function of time. Each value is the mean $\bar{X} \pm SD$ of three estimations.

Rearing period (days)	*Moringa* diets (%)				
	10	15	20	25	30
	Foregut				
0	210 ± 3.5	210 ± 3.5	210 ± 3.5	210 ± 3.5	210 ± 3.5
20	^{a}218 ± 1.2	^{b}225 ± 1.0	^{c}240 ± 1.2	^{d}253 ± 1.4	^{a}220 ± 1.8
40	^{b}240 ± 1.8	^{c}253 ± 1.0	^{d}260 ± 1.2	^{e}278 ± 2.1	^{a}232 ± 2.0
60	^{a}265 ± 2.3	^{b}273 ± 1.2	^{c}292 ± 1.5	^{d}301 ± 1.0	^{a}263 ± 1.9
80	^{a}305 ± 0.8	^{b}315 ± 1.4	^{d}330 ± 1.0	^{e}362 ± 1.7	^{c}325 ± 1.5
	Midgut				
0	120 ± 1.7	120 ± 1.7	120 ± 1.7	120 ± 1.7	120 ± 1.7
20	^{a}132 ± 1.0	^{a}133 ± 1.5	^{b}139 ± 1.1	^{c}143 ± 1.5	^{b}138 ± 1.2
40	^{a}143 ± 1.6	^{b}150 ± 2.1	^{c}165 ± 1.8	^{d}171 ± 1.1	^{b}152 ± 0.9
60	^{a}152 ± 1.3	^{b}159 ± 1.5	^{d}173 ± 4.2	^{e}182 ± 1.1	^{c}165 ± 2.1
80	^{a}175 ± 1.9	^{b}180 ± 2.0	^{c}187 ± 0.5	^{d}210 ± 1.0	^{b}178 ± 1.2
	Hindgut				
0	50 ± 1.2	50 ± 1.2	50 ± 1.2	50 ± 1.2	50 ± 1.2
20	^{a}57 ± 1.9	^{b}63 ± 1.2	^{bc}65 ± 1.5	^{c}67 ± 1.5	^{a}59 ± 1.8
40	^{a}62 ± 1.5	^{c}69 ± 1.8	^{d}73 ± 1.0	^{e}80 ± 1.1	^{b}65 ± 2.1
60	^{a}73 ± 1.8	^{b}77 ± 1.4	^{d}88 ± 1.3	^{e}97 ± 1.5	^{c}80 ± 1.2
80	^{a}88 ± 7.1	^{b}96 ± 1.0	^{c}103 ± 1.5	^{d}143 ± 0.8	^{ab}90.1 ± 1.4

Duncan multiple range test: Values (mean ± SD) with different superscript in the same row are significant at P < 0.05

Table 3: Two-way ANOVA for amylase enzyme activity in different tissues of *Labeo rohita* fed with inclusion of *Moringa diets* and its replacement with fish meal.

Source of Variation	SS	df	MS	F-value	P-value
Foregut					
Between *Moringa* levels	4690.000	4	1172.500	28.71	P < 0.01
Between rearing period	25777.000	3	8592.333	210.42	P < 0.01
Error	490.000	12	40.833		

Total	30957.000	19			
Midgut					
Between *Moringa* levels	1650.800	4	412.700	16.14	P < 0.01
Between rearing period	6252.950	3	2084.317	81.52	P < 0.01
Error	306.800	12	25.567		
Total	8210.550	19			
Hindgut					
Between *Moringa* levels	1765.252	4	441.313	5.86	P < 0.01
Between rearing period	5033.002	3	1677.667	22.28	P < 0.01
Error	903.556	12	75.296		
Total	7701.810	19			

Table 4: Replacement of fish meal with different levels of *Moringa* diets on protease (µg tyrosine / mg of protein / hr) enzyme in different tissues of *Labeo rohita* as a function of time. Each value is the mean $(\bar{X} \pm SD)$ of three estimations.

Rearing period (days)	*Moringa* diets (%)				
	10	15	20	25	30
	Foregut				
0	133 ± 0.5	133 ± 0.5	133 ± 0.5	133 ± 0.5	133 ± 0.5
20	^{a}138 ± 1.1	^{a}140 ± 1.5	^{b}145 ± 1.2	^{c}153 ± 1.0	^{b}145 ± 1.5
40	^{a}147 ± 1.1	^{a}150 ± 2.8	^{b}156 ± 4.1	^{d}173 ± 2.9	^{c}165 ± 2.1
60	^{a}168 ± 1.0	^{b}173 ± 1.2	^{c}180 ± 1.1	^{d}195 ± 1.4	^{c}180 ± 1.2
80	^{a}183 ± 1.4	^{b}190 ± 1.8	^{c}195 ± 1.3	^{d}212 ± 1.4	^{c}195 ± 1.5
	Midgut				
0	182 ± 1.5	182 ± 1.5	182 ± 1.5	182 ± 1.5	182 ± 1.5
20	^{a}190 ± 1.0	^{ab}193 ± 1.2	^{b}195 ± 1.4	^{c}199 ± 2.3	^{a}190 ± 2.5
40	^{a}203 ± 1.2	^{b}210 ± 3.0	^{c}218 ± 1.3	^{d}225 ± 1.4	^{b}210 ± 1.4
60	^{a}210 ± 1.0	^{b}225 ± 5.0	^{c}230 ± 0.8	^{d}243 ± 1.2	^{c}230 ± 1.6
80	^{a}235 ± 1.3	^{b}243 ± 1.2	^{d}255 ± 3.0	^{e}268 ± 0.5	^{c}250 ± 0.9
	Hindgut				
0	160 ± 1.0	160 ± 1.0	160 ± 1.0	160 ± 1.0	160 ± 1.0
20	^{a}163 ± 1.8	^{a}165 ± 1.3	^{b}172 ± 1.3	^{c}180 ± 1.4	^{b}170 ± 1.2
40	^{a}170 ± 1.0	^{b}178 ± 1.5	^{c}183 ± 1.5	^{d}195 ± 1.0	^{c}183 ± 1.5
60	^{a}183 ± 1.0	^{b}189 ± 1.0	^{d}199 ± 1.0	^{e}205 ± 1.0	^{c}195 ± 1.4
80	^{a}190 ± 0.9	^{b}201 ± 0.9	^{c}220 ± 0.9	^{d}258 ± 3.0	^{c}219 ± 1.4

Duncan multiple range test: Values (mean ± SD) with different superscript in the same row are significant at P < 0.05

Table 5: Replacement of fish meal with different levels of *Moringa* diets on lipase (µg lipase / mg of protein / hr) enzyme in different tissues of *Labeo rohita* as a function of time. Each value is the mean $(\bar{X} \pm SD)$ of three estimations.

Rearing period (days)	*Moringa* diets (%)				
	10	15	20	25	30
	Foregut				
0	80 ± 0.7	80 ± 0.7	80 ± 0.7	80 ± 0.7	80 ± 0.7
20	^{a}83 ± 1.0	^{b}86 ± 1.5	^{bc}88 ± 1.3	^{d}93 ± 1.0	^{c}90 ± 1.7
40	^{a}90 ± 1.6	^{b}95 ± 1.0	^{c}99 ± 1.2	^{d}105 ± 0.5	^{c}99 ± 1.4
60	^{a}95 ± 1.5	^{b}102 ± 1.7	^{d}118 ± 1.3	^{e}123 ± 1.5	^{c}110 ± 1.0
80	^{a}110 ± 1.1	^{b}123 ± 1.0	^{c}130 ± 1.5	^{d}158 ± 0.9	^{b}125 ± 1.6
	Midgut				
0	210 ± 1.0	210 ± 1.0	210 ± 1.0	210 ± 1.0	210 ± 1.0
20	^{a}215 ± 1.7	^{b}219 ± 1.5	^{c}223 ± 1.0	^{d}228 ± 0.9	^{b}220 ± 1.6
40	^{a}222 ± 1.2	^{b}225 ± 1.8	^{d}233 ± 1.2	^{e}245 ± 0.5	^{c}230 ± 0.3
60	^{a}230 ± 1.5	^{b}235 ± 1.2	^{c}241 ± 1.0	^{e}252 ± 1.9	^{d}245 ± 1.5
80	^{a}247 ± 1.8	^{b}250 ± 1.7	^{c}258 ± 0.3	^{d}280 ± 1.5	^{c}260 ± 1.2
	Hindgut				
0	230 ± 1.2	230 ± 1.2	230 ± 1.2	230 ± 1.2	230 ± 1.2
20	^{a}236 ± 1.0	^{b}241 ± 1.8	^{c}245 ± 2.0	^{d}253 ± 1.2	^{c}245 ± 1.8
40	^{a}241 ± 1.5	^{b}246 ± 2.3	^{c}253 ± 1.2	^{d}261 ± 1.7	^{c}252 ± 1.2
60	^{a}255 ± 2.3	^{b}262 ± 1.8	^{c}270 ± 2.5	^{d}278 ± 1.0	^{b}260 ± 1.3
80	^{a}268 ± 1.8	^{b}273 ± 1.1	^{c}280 ± 1.1	^{d}313 ± 0.9	^{c}282 ± 1.2

Duncan multiple range test: Values (mean ± SD) with different superscript in the same row are significant at P < 0.05

Discussion

The present study revealed that, 25% MS diet increased the chosen gut enzymes while 10 – 20% and 30% MS diets registered the low enzyme activities in *L. rohita*. *Moringa* diet stimulates the production of enzymes that transport fats within the fish body and it is utilized for growth instead of just storing and becoming flabby. The increased activities of digestive enzymes due to MS diet improve the intestinal

flora in fish by breaking indigestible feed components, thereby extracting more nutrients from the feed. The benefits of intestinal flora or bacteria are that they produce vitamins and displace harmful substances (Bclay, 2002) [2]. Khetral *et al.* (2018) found that, weight gain and amylase activities were improved in *L. rohita* fed with *M. oleifera* diet by replacing with fish meal. They further reported that, *L. rohita* fed with 30% *M. oleifera* diet enhanced the amylase and cellulase activities as compared to fish fed with 15% or 45% replaced MS diets, which supports the present investigations. Vasudhevan *et al.* (2007) reported that, 30 mg *Spirulina* / kg, 200 mg vitamin C / kg and 300 mg vitamin K / kg diets significantly ($P < 0.05$) increased the level of digestive enzymes (amylase, protease and lipase) than those fed with other low or higher levels of respective nutrients.

The present study also revealed that, digestive enzymes amylase, protease and lipase were significantly ($P < 0.05$) higher in foregut, midgut and hindgut respectively than other regions in *L. rohita.* Working on gold fish, *Carassius auratus*, Vasudhevan *et al.* (2007) observed that, the digestive enzymes amylase, protease and lipase were highly active in fore, mid and hind guts respectively than other regions, which supports the present investigations. They also observed that, the nutrients like *Spirulina*, vitamins C and E were influenced amylase, protease and lipase in fore, mid and hind guts in *C. auratus.* Field beans and groundnut leaf meals increased the amylase activity in the foregut and midgut; prawn head meal and chicken intestine diets showed an elevated amylase secretion in the foregut but decreased gradually in the mid and hind guts of *Labeo rohita* (Sethuramalingam and Haniffa, 2002) [18]. They also reported that prawn head meal and chicken intestine meals produced the higher secretion of protease in the mid gut of *L. rohita.* The maximum lipase activity was observed in hind gut of gold fish, *C. auratus* fed with three different nutrients confirmed the findings of the present investigation. On the contrary, lipase activity was high in mid gut followed by hind gut in cultivable fishes (Sastry, 1974; Sethuramalingam and Haniffa, 2002) [18].

The present study also elicited that, the low (10 – 20%) and high level (30%) of *Moringa* inclusion in the place of fish meal, reduced the digestive enzymes, amylase, protease and lipase in *L. rohita.* Nandeesha *et al.* (1998) [14] found that higher levels of *Spirulina* (60 – 100 mg / kg diet) supplement reduced the intestinal protease and lipase in fish common carp, *Cyprinus carpio* and it supports the observations made in the present study. The present study also indicated that, indiscriminate inclusion of MS diet (either lower or higher levels) may reduce the physiological and biochemical functions in *L. rohita.* Hence, the present investigation concludes that, inclusion of 25% *M. oleifera* leaf meal in the place of fish meal enhances the digestive enzymes (amylase, protease and lipase) in the chosen digestive tracts (fore, mid and hind guts) of carp *L. rohita.*

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