Effect of dietary supplements on digestive enzymes and growth performance of rainbow trout (*Oncorhynchus mykiss,* Walbaum)

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Abstract: Rainbow trout, *Oncorhynchus mykiss* (Walbaum), (average weight = 18 ± 0.2 g) were fed for two months with diet supplemented with 1 g (= 1%) and 2 g (= 2%) 100 g^{-1} of lupin (*Lupinus perennis)*, mango (*Mangifera indica*) and stinging nettle (*Urtica dioica*), and with normal diet as controls. Digestive enzymes (in the stomach and intestine), growth performance and body composition were examined following each treatment with results revealing that there was as statistically significant enhancement only in pepsin activity compared with the controls. There was a significant enhancement in weight gain, fish length and specific growth rate (SGR) of the treatment groups compared to controls.
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1. Introduction

The growth rate of fish is dependent on the digestive capacity, oxygen availability and the metabolic capacity required to support protein synthesis (1), diet composition and the duration of feeding (2). Breakdown of large nutrients into small absorbable subunits in the digestive tract of animals depends largely on the available enzymes (3). The distribution and intensity of intestinal enzymes varies with feeding habits and intestinal morphology (4 &5). Utilization of dietary nutrients is reflected in the metabolic profile in fish tissues, as metabolic efficiency determines the growth characteristics (6). Studies have been aimed at replacing fish meal in salmonid diets with other cheaper source of protein, such as from plants, e.g. peas and faba beans (7 & 8), lupin (7, 9, 10, 11 &12) and rapeseed and canola (9 & 13). However, the complete replacement of fish meal with plant material is not recommended as some authors reported a decrease in growth with an increasing proportion of plant products in the diet (14, 15 & 10). Workers considered that the higher levels of crude fiber, protease inhibitors and anti-nutritional compounds in plant material affects protein digestibility, causing adverse physiological effects and reduced growth (16, 17 & 18). However, replacing half fish meal with an equivalent amount of plant material may be appropriate. In this connection, 19 found that replacement of fish meal by lupin, corn gluten and wheat gluten meal, in the diets of juvenile turbot (*Psetta maxima,* L.) by up to 50%, did not significantly affect growth rate, whereas a 75 or even 100% replacement significantly reduced growth.

Lupin has been used previously as an alternative source of protein in fish diets (11, 15, 20 & 21). Mango and stinging nettle have been studied before as immunostimulants in fish (22 & 23). Following on from previous work in which the benefit of lupin (*Lupinus perennis)*, mango (*Mangifera indica*) and stinging nettle (*Urtica dioica*) was identified for the control of *Aeromonas hydrophila* infection in rainbow trout (*Oncorhynchus mykiss,* Walbaum*)* (24), this study has sought to determine the effect of these plant materials on digestive enzymes and growth performance .

2. Materials and methods:

Fish

Rainbow trout fingerlings were obtained from commercial fish farms in Scotland, and acclimatized in aerated biologically filtered recirculating freshwater at a flow rate of 1-2 L min^{-1} for 14 days before use. During the acclimatization period, fish were fed twice daily with a Biomar (Grangemouth, Scotland, UK) commercial diet (Table 1). At the start of experiment, the average weight of the rainbow trout population was 18 ± 0.2 g. The fish were distributed randomly into 7 tanks, with 60 fish per tank (1 m^2 tank containing 250 L of water). Fish were fed for 2 months with diet supplemented with 1 g and 2 g 100 g^{-1} of lupin (seeds were obtained from a supermarket in Edinburgh, Scotland), mango (fresh fruit), stinging nettle (dried leaves from a health food shop in Edinburgh), and with commercial diet as control. During the experiment, average water temperature ranged from approximately 8-14˚C,

oxygen saturation was from 7-9 mg l^{-1}, and the pH was 6.0- 6.5. The photoperiod was adjusted at 14 h light and 10 h dark cycles.

Determination of digestive enzyme levels

Sub-groups of 7 fish were starved for 2 days prior to sampling to determine enzyme levels. Then, the fish were killed by overdose of anaesthetic (tricaine methane sulphonate; MS222; Sigma-Aldrich, Basingstoke, England) followed by immediate dissection. The stomach and whole intestines were removed and rinsed separately with cold distilled water, and homogenized using an electrical homogenizer. The homogenate was centrifuged at 15000 x *g* for 20 min at 4˚C before the supernatant was removed and stored at -80˚C until needed. The total protein content of the supernatant was determined using the Bradford assay (25).

Pepsin activity

Pepsin activity was estimated by using 2% haemoglobin (Sigma-Aldrich) in 0.06 N HCl as substrate, according to 26. Briefly, 100 µL of enzyme extract (= homogenate) in 0.01 N HCl and 500 µL of substrate were incubated for 10 min at 37◦C. The reaction was stopped by the addition of 1 mL of 5% trichloroacetic acid (TCA; Sigma-Aldrich) and left for 5 min before centrifuging for 5 min at 12000 x *g*. Absorbance (A) was recorded at 280 nm. In the blank, TCA was added to the substrate prior to the addition of enzyme extract. Specific activity (U) was expressed as:

$$\frac{\text{A (supernatant) - A (blank) x 1000}}{\text{10 min x mg protein}}$$

Amylase activity

Amylase activity was evaluated according to 27 by using 1% starch in 20 mM sodium phosphate buffer (pH 6.9, containing 6.0 mM NaCl) as substrate. For this, 0.5 mL of substrate was added to 0.5 mL of enzyme extract, and incubated for 3 min at 55°C. This was followed by the addition of 0.5 ml dinitrosalicylic acid (Sigma-Aldrich) and incubation in a boiling water bath for 15 min. Absorbance at 540nm was recorded. The amount of maltose released was determined from a standard curve prepared from maltose solution. One unit was defined as the quantity of enzyme that released one mmol of maltose in 1 min.

Lipase activity

Lipase activity was determined with a rapid colorimetric kit (BioAssay Systems, Hayward, CA, USA). Thus, 150 µL H_2O, and 150 µL calibrator were pipetted into wells of a clear bottom 96-well microtitre plate (Nalge-Nunc; Thermo Fisher Scientific, Roskilde, Denmark). Then, 10 µL samples and 140 µL of the Working Reagent were added to each well with brief mixing. A_{412} was recorded after 10 min (OD_{10}min) and 20 min (OD_{20}min). The lipase activity was calculated as follows:

$$\textbf{Lipase Activity} = \frac{OD_{20min} - OD_{10min}}{OD_{Calibrator} - OD_{H2O}} \times 735\ (U\ L^{-1})$$

Growth performance

Growth performance was measured in fish, which were deprived of food for 24 h before weighing and sampling. Thus, subgroups of 10 fish were taken in triplicate for each group and the following parameters were measured:

Weight gain (g) = final body mass – initial body mass

Specific growth rate (SGR) (%) = (Final mean body mass – initial mean body mass (g))/Time interval (days)

Condition factor (CF) = Mass (g)/ Length $(cm)^3$

Feed intake (g $fish^{-1}$) =Dry feed intake (g)/ Number of fish

Feed conversion ratio (FCR) (%) = Feed intake (g)/ Gain in body mass (g)

Daily intake rate (DIR) = ([feed intake /mean body mass] / no. days) ×100

Determination of body composition

To determine body composition at the end of feeding period, all fish were starved for 24 h before subgroups of 7 animals were sacrificed and dried intact in an oven at 105ºC for 24 h before the whole body was crushed for body composition analysis according to AOAC guidelines (28), Samples were weighed before returning to the oven for another 24 h at 105°C, and reweighting to estimate the moisture content. Crude protein was determined by using a micro Kjeldahl method (28) by measuring the total nitrogen content of the sample multiplied by the empirical factor, i.e. 6.25. This method includes three stages, namely digestion by sulphuric acid, distillation and titration. Crude lipids were extracted by a Soxhlet method (28), using diethyl ether as solvent at 60-80°C for 12 h. The sample together with the filter paper was air dried and reweighed; the difference between sample weights indicating the total lipid content in the sample. Ash was combusted at 550°C.

Statistical examination of the data

Data were analyzed by one-way analysis of variance (ANOVA). When differences were found among treatments, Tukey's test was used to compare means by SPSS software (SPSS; IBM, Somers, NY, USA). Differences were considered significant at $P < 0.05$.

3. Results

Amylase activity

In the stomach, feeding with 2% lupin and 2% stinging nettle for 2-months resulted in 0.122 and

0.267 U/ mg protein compared to 0.017 and 0.029 U/ mg protein for 1% of the plant material. In comparison 1% mango led to higher amylase activity than the 2% dose. Moreover, 2% of all three dietary supplements led to high amylase activity in the intestine compared to the 1% dose (Fig.1A). Amylase activity in the controls was 0.012 and 0.031 U/ mg protein in the stomach and intestine, respectively. However, the data were not statistically significant.

Lipase activity

There was not any significant difference between controls and treated groups in the stomach and intestine in terms of lipase activity. Nevertheless, in the stomach, 2% lupin and stinging nettle resulted in 2.42 and 17.78 U/mg protein (Fig. 1B**),** which was higher than the 1% dose (1.27 and 6.44 U/mg protein, respectively). Conversely, the higher lipase activity resulted with feeding 1% rather than 2% mango (3.16 U/mg protein for the 1% dose in the stomach). The controls revealed 0.83 U/mg protein. In the intestine, the highest lipase activity was recorded with the 2% dose of all three plants (Fig. 1B).

Pepsin activity

Generally, pepsin activity was highest in the stomach rather than the intestine. In stomach, the 2% dose of lupin and stinging nettle led to higher pepsin activity than the 1% amount. Conversely with mango, the 1% dose resulted in higher pepsin activity than the 2% amount (Fig.1C, D**)**. Opposite observation was recorded in intestine. Moreover, the controls recorded the lowest value in both stomach and intestine (19.45 and 0.18 U/mg protein, respectively). The data were significantly different ($p < 0.05$) for the 2% dose of lupin and stinging nettle and with 1% mango (169.82, 170.25 and 127.91 U/mg protein, respectively).

Body mass and growth rate

The final weight, weight gain and specific growth rate (SGR) of treatment groups was significantly higher than the controls ($p < 0.05$). In contrast, feeding with 1% stinging nettle and mango led to the highest level than 2%. Conversely with lupin, the 2% dose resulted in higher values than the 1% dose (Table 2). However, feeding with 1% lupin, mango and stinging nettle for 2-months led to the highest length and condition factor compared to the controls ($p < 0.05$). For the feed conversion ratio (FCR), 2% of stinging nettle and mango was higher than 1% and the opposite was true in lupin. Moreover, the control did not show the lowest value instead this was recorded with 2% of mango. The controls showed the lowest value in terms of feed intake, without a significant difference compared to treatments. The 2% doses of stinging nettle and mango were higher than the 1% doses in terms of feed intake. Conversely 1% lupin led to higher values than the 2% dose. Yet, there was not any significant difference between any of the data. In addition, 2% of mango, nettle and control were the highest in terms of the day intake ratio (DIR).

Crude protein levels

The crude protein levels for whole dried fish were highest after feeding with 1% and 2% of lupin and 1% of mango compared to the controls (Table 3). In whole dried fish, the lipid was higher after administering 1% than 2% lupin, mango and stinging nettle. Although, the controls revealed the lowest levels, there was not any significant difference between each treatment group and the controls. With moisture and ash, 2% stinging nettle and mango led to higher levels than the corresponding 1% doses. The reverse occurred with lupin when 1% led to higher levels of moisture and ash than the 2% dose. Again, there was not any significant difference between the treatment and control groups.

Table 1. Diet composition of commercial diet (Biomar)

Diet composition	**%**
Protein	53
Oil	19
Ash	10
Moisture	7.5
Phosphorus	1.2
Nitrogen	8.4
N.F.E.	10.5

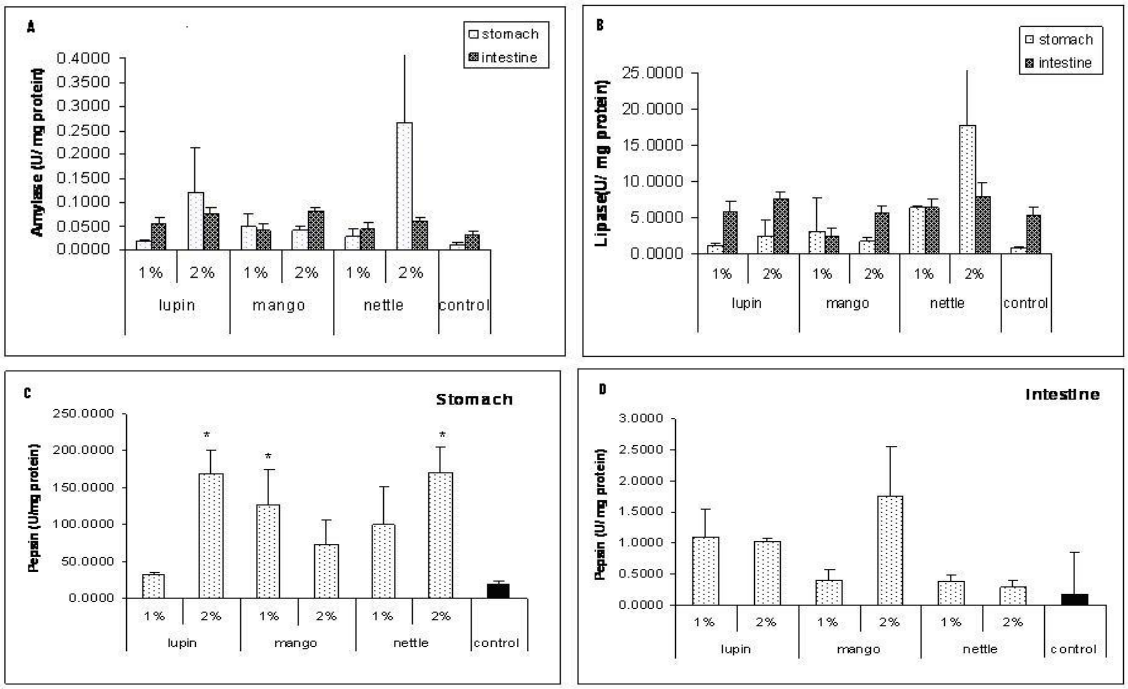
Table 2. Growth performance of *O. mykiss* fed for 2 months with 1% and 2% lupin, mango and stinging nettle (mean ± SE). The average weight of the fish at the start of the experiment was 18 ± 0.2 g

Plant	Dose	final weight	Weight gain	SGR %	length	CF	FCR	feed intake	DIR
		g	g		cm	g cm^{-3}	%	g $fish^{-1}$	
	1%	48.81 ± 0.63*	30.81± 0.63*	0.51 ± 0.01*	15.9 ± 0.14*	3.07 ± 0.03	0.75 ± 0.04	23.16 ± 0.01	0.79 ± 0.01
Lupin	2%	49.39 ± 1.02*	31.39 ± 1.02*	0.52 ± 0.02*	15.67 ± 0.04*	3.15 ± 0.06	0.71 ± 0.05	22.17 ± 0.1	0.75 ± 0.02
	1%	49.17 ± 0.93*	31.17 ± 0.93*	0.52 ± 0.02*	15.77± 0.04*	3.12 ± 0.06	0.71± 0.06	22.16 ± 0.05	0.75 ± 0.01
Mango	2%	45.52 ± 1.09*	27.53 ± 1.09*	0.46 ± 0.004*	15.37 ± 0.11*	2.96 ± 0.05	0.92 ± 0.02	25.44 ± 0.06	0.93 ± 0.02
	1%	50.34 ± 0.27*	32.34 ± 0.27*	0.54 ± 0.01*	16.11 ± 0.05*	3.12 ± 0.01	0.66 ± 0.05	21.29 ± 0.02	0.71 ± 0.01
Stinging nettle	2%	49.55 ± 0.53*	31.55 ± 0.53*	0.53 ± 0.008*	15.9 ± 0.04*	3.12 ± 0.03	0.78 ± 0.03	24.63 ± 0.03	0.83 ± 0.01
Control		36.9 ± 2.46	20.9 ± 2.46	0.35 ± 0.05	14.63 ± 0.31	2.52 ± 0.12	1.00 ± 0.05	20.91 ± 0.03	0.87 ± 0.06

(*) =significant difference $p < 0.05$ compared with control

Table 3. Composition of the whole body of *O. mykiss* after feeding for 2 months with 1% and 2% of lupin, mango and stinging nettle (mean ± SE)

Plant	Dose	Crude protein	Crude Lipid	Moisture	Ash
Lupin	1%	62.45 ± 6.74	34.42 ± 2.58	72.34 ± 0.56	7.94 ± 0.89
	2%	58.67 ± 3.27	32.25 ± 0.37	70.02 ± 1.18	7.25 ± 0.47
Mango	1%	58.98 ± 2.03	35.28 ± 0.43	68.12 ± 4.43	7.34 ± 0.13
	2%	56.5 ± 3.5	28.75 ± 0.92	72.77 ± 0.68	7.47 ± 0.53
Stinging Nettle	1%	56.8 ± 1.58	32.55 ± 1.7	72.33 ± 0.89	7.87 ± 0.47
	2%	56.28 ± 1.68	32.21 ± 1.39	72.73 ± 0.55	8.35 ± 0.55
Control		57.54 ± 1.04	27.77 ± 2.11	72.97 ± 0.34	8.62 ± 0.4



Fig, 1. Digestive enzymes in the stomach and intestine after feeding for 2 months. A = Amylase activity; B = lipase activity; C = pepsin activity in stomach; D = pepsin activity in intestine. Data represented as mean ± S

4. Discussion

The digestion of nutrients begins with actions of the digestive enzymes in the stomach and continues in the intestine with enzymes secreted by the pancreas, including trypsin, chymotrypsin, amylase and lipase (29, 30 & 31). Amylase is stimulated by glycolytic chains, glycogen and starch in larval and juvenile fish (32). Several studies have reported amylase activity in carnivorous fish (33, 34; 27; 35). 5 and 33 documented low amylase activity in carnivorous fish (with stomach) and high activity in omnivorous fish (without a stomach). It has been considered that amylase activity depends on the natural diet of each species, with herbivorous and omnivorous fish having more activity than carnivores (33). This study recorded an enhancement in amylase activity in all treatment groups as compared with the controls. However, the differences were not statistically significant. Similar results were reported by 36, who recorded higher increase in amylase activity in animals fed diets containing plant-based ingredients. For example, amylase activity in animals fed with lupin meal was 4.87 U/mg protein whereas use of fish meal led to 3.63 U/mg protein. Interestingly, 37 did not find any correlation between amylase activity and carbohydrate content in the diet of redclaw (*Cherax quadricarinatus,* Von Martens). Certainly, amylase activity was found to increase in rainbow trout fed diets containing increased amounts of dietary plant protein (38).

Lipase is secreted mainly by the pancreas, and exerts a major role in breaking down fats, especially the triacylglycerols, leading to digestion. Generally, the treatment groups recorded higher lipase activity compared to the controls, although there was not any significant difference in the results. However, it was not surprising that intestine recorded higher lipase activity compared with the stomach because the site

of action for lipase is in the intestine. Certainly, the data for lipase activity matched those for *C. quadricarinatus* fed with soybean meal and lupin meal (36). Moreover, 37 observed significant differences in lipase activity in animals fed a sorghum diet compared to those receiving red crab (*Gecarcoidea* sp.) meal and sardine (*Sardina pilchardus,* Walbaum) meal. In contrast, tilapia (*Oreochromis* sp.) revealed limited distribution and level of lipase activity in the intestinal tract, possibly due the low intake of lipid from plant-related diets (39).

When chyme arrives at the intestine, several proteases secreted by the pancreas continue hydrolysis (34). This study recorded an increase in pepsin activity in stomach more so than the intestine. Certainly, several forms of pepsin reacting at different pH values have been reported (40 & 41). For example, 42 recorded a strong pepsin-like activity at pH 3 in adult halibut (*Hippoglossus hippoglossus,* L.) and turbot (*Scophthalmus maximus,* L.). Also, higher pepsin activities have been reported in the carnivorous sea bass (*Lates calcarifer,* Bloch) as compared to the herbivorous rabbitfish (*Siganus canaliculatus,* Park), (5).

There was a remarkably increase in weight gain, length and SGF in treated groups compared with the controls. Certainly, 43 reported an increase in body weight of Jain carp (*Cyprinus carpio* var. Jain, L.) after feeding with traditional Chinese medicine (TCM) formulation from *Astragalus* root (*Radix astragalin seu Hedysari*) and Chinese Angelica root (*R. Angelicae Sinensis*) at a ratio of 5:1. Also, the weight gain was significantly improved in Japanese flounder (*Paralichthys olivaceus,* Temminck & Schlegel) fed with 0.5 % of herbal mixture (44). In contrast, growth in rainbow trout fed with plant meal diet was significantly less than fish fed the fishmeal diet (45). Yet, 46 reported elevated specific growth rates of rohu (*Labeo rohita,* Hamilton) after feeding with the medicinal plant *Achyranthes* as compared with control. *Labeo rohita* fed with mango kernel showed an increase in SGR and FCF, although there was not any significant difference between each treatment and the controls (23). Similarly, 47 reported that the weight gain, SGR, feed intake and nitrogen deposition in Mozambique tilapia (*Oreochromis mossambicus,* Peters) were best with low levels, i.e. 15-20%, of alfalfa protein inclusion.

Feeding with the medicinal plant *Achyranthes aspera* incorporated diets improved the food conversion ratio of *L. rohita* (46). In contrast, FCR was significantly lower in common carp (*C. carpio* L.) fed with diet supplemented with 150 mg kg^{-1} the tree *Quillaja* saponins as compared with the controls (48). Indeed, 49 recorded an improvement in FCR, weight gain and SGF in greasy grouper (*Epinephelus tauvina,* Forsskål) juveniles fed with diets incorporating 100 and 200 mg kg^{-1} diet of holy basil (*Ocimum sanctum)* and Indian ginseng (*Withania somnifera)*, whereas there was not any improvement in the groups fed with nutmeg (*Myristica fragrans)*. Of interest, 10 declared that incorporation of yellow lupin at 12.5% into fish diets of *O. mykiss* enhanced FCR.

Interestingly, feeding with lupin, mango and stinging nettle led to higher feed intake compared to the controls, and this may be attributed to the actual ingredients. However, further work could be carried out on these plants to determine the precise nature of the active ingredient(s). Indeed, other studies have recorded a significant decrease in feed intake values of *O. niloticus* fed diets containing 33% and 66% plant protein compared to fish fed with 100% plant protein (14). In previous work with *O. mykiss*, there was not any effect following an increase in the level of lupin in the diets (10, 11& 21).

This study demonstrated a slight increase in body composition in treatment groups compared with the controls, albeit without significant differences between each group and the control. Moreover, the higher crude protein level which was recorded in lupin could be attributed to the high protein content, which reaches 32-36% of the whole seed (50). Similar results were observed in rainbow trout fed with 12.5% yellow lupin meal (10). Also, 18 noticed an increase in body composition of *O. niloticus* fed with diets containing 5% and 10% alfafa meal, whereas 15% and 20% led to a non significant decrease as compared with controls.

In conclusion, this investigation showed a marked increase in growth performance, especially weight gain, SGF and digestive enzymes of *O. mykiss* and slight enhancement in body composition in diets containing lupin, mango and stinging nettle, which extends the earlier work on the health benefits of these plants (24).

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References

1- Blier, P., Pelletier, D. & Dutil, J.D. (1997) Does aerobic capacity set a limit on fish growth rate? *Rev. Fish. Sci*., 5: 323–340.
2- Subhadra, B., Lochmann, R., Rawles, S. & Chen, R. (2006) Effect of dietary lipid source on the growth,

tissue composition and hematological parameters of largemouth bass (*Micropterus salmoides*). *Aquaculture,* 255: 210–222.
3- Cho, C.Y. (1987) La energía en la nutrición de los peces. In: Nutrición en Acuicultura (Espinosa de los Monteros J. & Labarta, U. eds.), Vol II., pp. 197-244.CAICYT, Madrid, Spain.
4- Kuz'mina, V.V. & Smirnova, Y.G. (1992) Distribution of alkaline phosphatase activity along the length of the intestine of freshwater teleosts. *J. Ichthyol.,* 32: 1–9.
5- Sabapathy, U. & Teo, L.H. (1993) A quantitative study of some digestive enzymes in rabbitfish, *Siganus canaliculatus* and the sea bass, *Lates calcarifer*. *J. Fish Biol.,* 42: 595–602.
6- Debnath, D., Pal, A.K., Sahu, N.P., Yengkokpam, S., Baruah, K., Choudhury, D. & Venkateshwarlu, G. (2007) Digestive enzymes and metabolic profile of *Labeo rohita* fingerlings fed diets with different crude protein levels. *Comp. Biochem. Physiol.,* 146B: 107–114.
7- Gouveia, A., Oliva Teles, A., Gomes, E. & Rema, P. (1993) Effect of cooking/expansion of three legume seeds on growth and food utilisation by rainbow trout. In: Fish Nutrition in Practice (Kaushik, S.J. & Luquet, P. eds.), pp. 933-938. INRA, Paris, France.
8- Pfeffer, E., Kinzinger, S. & Rodehutscord, M. (1995) Influence of the proportion of poultry slaughter by-products and of untreated or hydrothermically treated legume seeds in diets for rainbow trout, *Oncorhynchus mykiss* (Walbaum), on apparent digestibilities of their energy and organic compounds. *Aquacult. Nutr.,* 1: 111-117.
9- Bangoula, D., Parent, J.P. & Vellas, F. (1993) Nutritive value of white lupin (*Lupinus albus var Lutop*) in rainbow trout (*Oncorhynchus mykiss*). Effects of extrusion cooking. *Reprod. Nutr. Devel.,* 33: 325-334.
10- Glencross, B., Evans, D., Hawkins, W. & Jones, B. (2004) Evaluation of dietary inclusion of yellow lupin (*Lupinus luteus*) kernel meal on the growth, feed utilisation and tissue histology of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture,* 235: 411 –422.
11- Burel, C., Boujard, T., Corraze, G., Kaushik, S.J., Boeuf, G., Mol, K.A., Van der Geyten, S. & Kuhn, E.R. (1998) Incorporation of high levels of extruded lupin in diets for rainbow trout (*Oncorhynchus mykiss*): nutritional value and effect on thyroid status. *Aquaculture,* 163: 325–345.
12- Burel, C., Boujard, T., Tulli, F. & Kaushik, S. (2000) Digestibility of extruded peas, extruded lupin, and rapeseed meal in rainbow trout (*Oncorhynchus mykiss*) and turbot (*Psetta maxima*). *Aquaculture,* 188: 285–298.
13- Stickney, R.R., Hardy, R.W., Koch, K., Harrold, R., Seawright, D. & Massee, K.C. (1996) The effects of substituting selected oilseed protein concentrates for fish meal in rainbow trout (*Oncorhynchus mykiss*) diets. *J. Wld. Aquacult. Soc.,* 27: 57- 63.
14- Fontainhas-Fernandes, A., Gomes, E., Reis-Henriques, M. & Coimbra, J. (1999) Replacement of fish meal by plant proteins in the diet of Nile tilapia: digestibility and growth performance. *Aquacult. Int.,* 7: 57–67.
15- Gomes, E.F., Rema, P. and Kaushik, S.J. (1995) Replacement of fishmeal by plant proteins in the diet of rainbow trout (*Oncorhynchus mykiss*): digestibility and growth performance. *Aquaculture,* 130: 177–186.
16- Olli, J., Hjelmeland, K. & Krogdahl, A. (1994) Soybean trypsin inhibitor in diets for Atlantic salmon (*Salmo salar*, L): Effects on nutrient digestibilities and in pyloric caeca homogenate and intestinal content. *Comp. Biochem. Physiol.,* A 109: 923–928.
17- Vielma, J., Makinen, T., Ekholm, P. & Koskela, J. (2000) Influence of dietary soy and phytase levels on performance and body composition of large rainbow trout (*Oncorhynchus mykiss*) and algal availability of phosphorus load. *Aquaculture,* 183: 349–362.
18- Ali, A., Al-Asgahn, N.A., Al-Ogaily, M.S. & Ali, S. (2003) Effect of feeding different levels of alfalfa meal on the growth performance and body composition of Nile tilapia (*Oreochromis niloticus*) fingerlings. *Asian Fish. Sci.,* 16: 59-67.
19- Fournier, V., Huelvan, C. & Desbruyeres, E. (2004) Incorporation of a mixture of plant feedstuffs as substitute for fish meal in diets of juvenile turbot (*Psetta maxima*). *Aquaculture, 2*36: 451-465.
20- Farhangi, M. & Carter, C.G. (2001) Growth, physiological and immunological responses of rainbow trout (*Oncorhynchus mykiss*) to different dietary inclusion levels of dehulled lupin (*Lupinus angustifolius*). *Aquacult. Res.,* 32: 329– 340.
21- Glencross, B., Curnow, J.G., Hawkins, W.E. & Felsing, M. (2002) Preliminary evaluation of yellow lupin (*Lupinus luteus*) kernel meal as an alternative protein resource in the diet of sea-cage reared rainbow trout, *Oncorhynchus mykiss*. *J. Wld. Aquacult. Soc.* 33: 287– 296.
22- Dügenci, S.K., Arda, N. & Candan, A. (2003) Some medicinal plants as immunostimulant for fish. *J. Ethnopharmacol.,* 88: 99– 106.
23- Sahu, S., Das, B. K., Pradhan, J., Mohapatra, B.C., Mishra, B.K. & Sarangi, N. (2007) Effect of *Magnifera indica* kernel as a feed additive on immunity and resistance to *Aeromonas hydrophila* in *Labeo rohita* fingerlings. *Fish Shellfish Immunol.,* 23: 109 – 118.
24- Awad, A. & Austin, B. (2010) Use of lupin *(Lupinus perennis),* mango *(Mangifera indica)* and stinging nettle *(Urtica dioica)* as feed additives to prevent *Aeromonas hydrophila* infection in rainbow trout *(Oncorhynchus mykiss,* Walbaum). *J. Fish Dis.,* 33: 413-420.
25- Bradford, M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye-binding. *Anal. Biochem.,* 72: 248.
26- Worthington, V. (1993). Worthington Enzyme Manual. Enzymes and Related Biochemicals Worthington Chemical, New Jersey, USA. 399 pp.
27- Natalia, Y., Hashim, R., Ali , A. & Chong, A. (2004) Characterization of digestive enzymes in a carnivorous ornamental fish, the Asian bony tongue

Scleropages formosus (Osteoglossidae). *Aquaculture,* 233: 305–320.

28- AOAC (Association of Official Analytical Chemists), (1990) Official Methods of Analysis of the Association of Official Analytical Chemists, 15th ed. Association of Official Analytical Chemists, Washington, DC, USA.

29- Nagase, G. (1964) Contribution to physiology of digestion in *Tilapia mossambica* Peters: digestive enzymes, and the effects of diets on their activity. *Zeit. Vergleichende Physiol.,* 49: 270–284.

30- Cockson, A., & Bourne, D. (1972) Enzymes in the digestive tract of two species of euryhaline fish. *Comp. Biochem. Physiol.,* A 41: 715–718,

31- Moriarty, D.J. (1973) The physiology of digestion of blue green algae in the cichlid fish, *Tilapia nilotica*. *J. Zool. Lond.,* 171: 25–39.

32- Krogdahl, Å., Hemre, G-I. & Mommsen, T.P. (2005) Carbohydrates in fish nutrition: digestion and absorption in post larval stages. *Aquacult. Nutr.,* 11: 103-122.

33- Hidalgo, M.C., Urea, E. & Sanz, A. (1999) Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. *Aquaculture,* 170: 267–283.

34- Alara'con, F.J., García-Carreño, F.L. & Navarrete del Toro, M.A. (2001) Effect of plant protease inhibitors on digestive proteases in two fish species, *Lutjanus argentiventris* and *L. novemfasciatus*. *Fish Physiol. Biochem.,* 24: 179–189.

35- Suzer, C., Çoban, D., Okan, K.H., Saka, Ş., Firat, K., Otgucuoğlu, Ö. & Küçüksari, H. (2008) *Lactobacillus* spp. bacteria as probiotics in gilthead sea bream (*Sparus aurata*, L.) larvae: Effects on growth performance and digestive enzyme activities. *Aquaculture,* 280: 140–145.

36- Pavasovic, A., Anderson, A.J., Mather, P.B. & Richardson, N.A. (2007) Effect of a variety of animal, plant and single cell-based feed ingredients on diet digestibility and digestive enzyme activity in redclaw crayfish, *Cherax quadricarinatus* (Von Martens 1868). *Aquaculture,* 272: 564–572.

37- Lopez-Lopez, S., Nolasco, H., Villarreal-Colmenares, H. & Civera- Cerecedo, R. (2005) Digestive enzyme response to supplemental ingredients in practical diets for juvenile freshwater crayfish *Cherax quadricarinatus*. *Aquacult. Nutr.,* 11: 79–85.

38- Kawai, S. & Ikeda, S. (1973) Studies in digestive enzymes of fishes. III. Development of the digestive enzymes of rainbow trout after hatching and the effect of dietary change on the activities of digestive enzymes in the juvenile stage. *Bull. Jap. Soc. Sci. Fish.,* 38: 265–270.

39- Tengjaroenkul, B., Smith, B.J., Caceci, T. & Smith, S.A. (2000) Distribution of intestinal enzyme activities along the intestinal tract of cultured Nile tilapia, *Oreochromis niloticus* L. *Aquaculture,* 182: 317-327.

40- Tanji, M., Kageyama, T. & Takahashi, K. (1988) Tuna pepsinogens and pepsins. *Eur. J. Biochem.,* 177: 251– 255.

41- Chiu, S.T. & Pan, B.S. (2002) Digestive protease activities of juvenile and adult eel (*Anguilla japonica*). *Aquaculture,* 205: 141–156.

42- Glass, H.D., MacDonald, N.L., Moran, R.M. & Stark, J.R. (1989) Digestion of protein in different marine species. *Comp. Biochem. Physiol.,* 94B: 607– 611.

43- Jian, J. & Wu, Z. (2004) Influences of traditional Chinese medicine on non-specific immunity of Jian carp (*Cyprinus carpio* var. Jian). *Fish Shellfish Immunol.,* 16: 185–191.

44- Seung-Cheol, J.I., Jeong, G., Gwang-Soon, I.M., Lee, S., Yoo, J. & Takii, Kenji. (2007) Dietary medicinal herbs improve growth performance, fatty acid utilization, and stress recovery of Japanese flounder. *Fish. Sci.,* 73: 70–76.

45- Pierce, L.R., Palti, Y., Silverstein, J.T. Barrows, F.T., Hallerman, E.M. & Parsons, J.E. (2008) Family growth response to fishmeal and plant-based diets shows genotype × diet interaction in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture,* 278: 37–42.

46- Rao, V.Y., Das, B.K., Jyotyrmayee, P. & Chakrabarti, R. (2006) Effect of *Achyranthes aspera* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila. Fish Shellfish Immunol.,* 20: 263 – 273.

47- Olvera-Novoa, M.A., Campos, S.G. Sabido M.G. & Martinez-Palacios, C.A. (1990) The use of alfalfa leaf protein concentrates as a protein source in diets for tilapia (*Oreochromis mosssambicus*). *Aquaculture,* 90: 291-302.

48- Francis, G., Makkar H.P. & Becker, K. (2002) Dietary supplementation with a *Quillaja saponin* mixture improves growth performance and metabolic efficiency in common carp (*Cyprinus carpio* L.). *Aquaculture,* 203: 311–320.

49- Sivarama, V., Babua, M.M., Immanuela, G., Murugadassa, S., Citarasub, T. & Marian. M.P. (2004) Growth and immune response of juvenile greasy groupers (*Epinephelus tauvina*) fed with herbal antibacterial active principle supplemented diets against *Vibrio harveyi* infections. *Aquaculture,* 237: 9 –20.

50- Petterson, D.S. & Mackintosh, J.B. (1994) The chemical composition of lupin seed grown in Australia. In: Proceedings of the First Australian Lupin Technical Symposium, Perth, Western Australia, 17–21 October 1994 (Dracup, J. ed.), pp. 39.