Histological and physiological changes induced by Red Kidney Bean lectins in the digestive system of rainbow trout, *Oncorhynchus mykiss* (Walbaum)

Modificazioni istologiche e fisiologiche indotte da lectine del fagiolo nell'apparato digerente della trota iridea, Oncorhynchus mykiss *(Walbaum)*

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SUMMARY - The use of a plant protein sources as a substitute for fishmeal is limited by the content of anti-nutritional factors. The aim of this work is to evaluate the enzymological and histological effects of lectins on the rainbow trout intestine associated with a plant protein source. In the present experiment a high level of inclusion of lectins has been used in order to induce a clear effect and a lupin meal has also been introduced to study the interactions between the lectins and other proteins. Twenty-four juvenile rainbow trout were hand-fed twice a day for 28 days with experimental diets. The daily ration was 1.7% of bulk weight. Enzymological analyses were performed on different digestive tracts. Histological assays were conducted on the digestive tracts and liver. Lectins interfere with protease enzymes activity in the stomach and intestine. As far as the histology is concerned, PHA lectins induce local inflammatory reactions on the intestinal mucosae and normal liver histological pattern. In order to preserve fish health, it is important to evaluate the utilisation of different vegetable protein sources. The interaction among different ANFs suggest the use of a mixture of several plant protein sources in fish feedstuff formulations.

RIASSUNTO - L'utilizzo delle proteine vegetali nell'alimentazione in acquacoltura è limitato dal contenuto di fattori antinutrizionali presenti nelle piante utilizzate. Lo scopo di questo lavoro è valutare gli effetti enzimologici ed istologici delle proteine di origine vegetale sull'intestino della trota iridea. In questa ricerca è stato utilizzato un alto livello di inclusione di lectine e la farina di lupino nell'alimentazione della trota iridea per ottenere un effetto chiaramente studiabile. 24 trotelle sono state alimentate due volte al giorno per 28 giorni con le diete sperimentali. Il razionamento alimentare adottato è stato dell'1,7% del peso vivo. Le analisi enzimologiche sono state fatte in diversi punti dell'apparato digerente, mentre le analisi di istologia sono state effettuate sull'intestino e sul fegato. Le lectine interferiscono con l'attività delle proteasi nello stomaco e nell'intestino. Per quanto riguarda l'istologia le lectine utilizzate in questa sperimentazione inducono reazioni infiammatorie locali nella mucosa intestinale e a livello epatico. Considerando i risultati ottenuti e le interazioni che sono emerse tra i diversi fattori antinutrizionali presenti nelle materie prime valutate in questa sperimentazione, per il futuro potrebbe essere utile valutare una miscela di materie prime di origine vegetale nell'alimentazione dei pesci di allevamento.

Key words: Rainbow trout, *Oncorhynchus mykiss*, Feeding, PHA lectins, Digestive enzymes

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INTRODUCTION

An increasing world demand for ingredients used in aquaculture feeds has resulted in increased research oriented towards the nutritive evaluation of plant protein sources.

The use of plant protein sources in aquaculture as a substitute for fishmeal is a very extensively studied subject (Bakke-McKellep, 1999; Bakke-McKellep *et al.*, 2000; Francis *et al.*, 2001; Naylor *et al.*, 2001; Krogdhal *et al.*, 2003; Palmegiano *et al.*, 2005), as because food alimentation fishmeal will be a limit resource in the future.

The main limitation to the use of plant protein sources is the existence of anti-nutritional factors (ANFs) that can reduce the activity of fish digestive enzymes (Huisman & Tolman, 1992). Some of the best known of these compounds are protease inhibitors which are present above all in legume seeds (Hendricks & Bailey, 1989; Liener, 1989; Refstie *et al.*, 1998). Regardless of the utilised plant source, there are some clear anti-nutritional effects on fish caused by a group of heterogeneous substances normally present in vegetables. All ANFs could cause pathomorphological changes in the intestinal epithelium of fish. These alterations negatively affect food absorption and cause stunted growth in fish, there for the study of pathomorphological and physiological changes are important as some modifications could be utilised in future as a signal of less efficiency of diet.

Among these ANFs, plant lectins are molecules that have been widely studied. Lectins are carbohydrate-binding glycoproteins, which are ubiquitous in nature; one of the most nutritionally important features of plant lectins is their ability to survive digestion in the gastrointestinal tract of consumers. This characteristic allows lectins to bind the membrane glycosyl groups of the cells that line the digestive tract. As a result of this interaction a series of harmful local and systemic reactions are triggered, making this class of molecules anti-nutritional and/or toxic substances (Vasconcelos & Oliveira, 2004). Locally, they can affect the turnover and loss of gut epithelial cells, damage the luminal membranes of the epithelium and interfere with nutrient digestion and absorption (Vasconcelos & Oliveira, 2004). Systemically, they can disrupt lipid, carbohydrate and protein metabolism, promote enlargement and/or atrophy of key internal organs and tissues and alter the hormonal and immunological status. At high intakes, lectins can seriously threaten the growth and health of consuming animals (Vasconcelos & Oliveira, 2004).

In a previous research (unpublished data) a lupin meal was tested; the negative results of that trial induced the authors to study in depth and to explain the effects of plant protein ANFs on digestive tract.

In the present experiment kidney bean lectins (*Phaseolus vulgaris* L.) (PHA) were introduced into fish diets in order to clearly recognize what the effect of lectins are, on the rainbow trout digestive system and to obtain a clear histological and enzymological pattern. PHA toxic effects on monogastric animals have been well documented, as reported by Pusztai (1991), Pusztai *et al.* (1995a).

A reference point could be very interesting in the future of fish feeding as these alterations could precociously indicate the effectiveness of new fish diets.

The aim of this work is to evaluate the enzymological and histological effects of lectins associated to a plant and animal protein source on the digestive tract of rainbow trouts.

In the present challenge a high level of inclusion of lectins has been utilised to provoke a clear effect and a lupin (*Lupinus luteus* L.) meal has also been introduced to study the possible interactions between lectins and other plant feedstuff.

MATERIALS AND METHODS

Fishes and diets –

The experiment was performed at the experimental fish farm (Carmagnola (TO), NW Italy) of the Department of Zootechnical Sciences, University of Turin. A stock of 10 kg of common commercial juvenile rainbow trout was bought from a fish breeder near Carmagnola. Some trout were randomly sampled to perform the virological, parasitological and bacteriological analysis, to exclude the presence of possible pathologies. These pathological examinations was performed at the Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Fish Diseases Laboratory, and the results were negative. Subsequently, twenty-four juvenile rainbow trout (*Oncorhynchus mykiss* Walbaum) of the checked stock (mean body weight 110 g.) were equally divided into four 400 liters tanks and hand-fed twice a day with four experimental diets.

The water temperature and dissolved oxygen were monitored systematically and resulted optimal for the rainbow trout rearing (temperature 13.3-13.8° C; O_2 7.5-8.7 ppm).

The daily ration was equal to the 1.7% of bulk weight and the fish were fed for 28 days. Four experimental diets were prepared with the formulations shown in Table 1 and the proximate compositions of these diets were determined according to AOAC (1990). In the first diet (A) fish meal was the main protein source, in the third diet (C) 55% of lupin seed meal was the main plant protein source. This amount of lupin meal provides around 50% of the whole protein diet. The second diet (B) and the last diet (D) had the same composition of fishmeal as diet A and of lupin meal as diet C respectively, but both diets had added lectins.

The lectins utilised for the experimental diets were bean lectins (red kidney bean), the inclusion level was 0.1% (w/w) on a dry matter basis for both diets (B and D).

	Diets			
Ingredients (%)	**A**	**B***	**C**	**D***
Herring meal	57	57	27	27
Lupin meal	0	0	55	55
Fish oil	6	6	8	8
Corn starch	9	9	7	7
Toasted barely	23.5	23.5	0	0
Brewer's Yeast	2	2	0	0
Binder	0.5	0.5	0.5	0.5
Vitamin mixture	1	1	2	2
Mineral mixture	1	1	0.5	0.5
Crude protein	48.06	48.06	44.02	44.02
Ether extract	14.34	14.34	16.61	16.61
NFEs	25.5	25.5	33.04	33.04
Ash	10.12	10.12	6.33	6.33
Gross energy	20.61	20.61	21.74	21.74

Legenda:
A = fishmeal diet; B = fishmeal with added lectins diet; C = lupin meal diet; D = lupin meal with added lectins diet; * Lectins (0.1% w/w), purchased from Sigma-Aldrich (L 8754), were added to diets B and D.

Table 1 - Feed formulation and chemical composition (on a dry matter basis) of the four experimental diets.
Tavola 1 – Formulazione e composizione centesimale dei mangimi (su base secca) relative alle quattro diete sperimentali.

Enzyme analyses –

Sampling:

At the end of the experiment, the fish were killed six hours after the last meal. This time was chosen after previous investigations in order to obtain an adequate gastric empting and intestinal fulfil status. The stomach, pyloric caeca, middle and distal intestine from three fish for each treatment were separated and the tissue contents removed and rinsed with a cold physiological solution. The tissues, from 3 fish, were quickly collected and immediately frozen in liquid nitrogen for the successive enzymatic analyses. Finally 48 tissue samples were collected.

The tissues from each fish were homogenized in a cold Tris-HCl 50mM (pH 7.0) buffer with a 1:10 (w/v) ratio for the middle and distal intestine and 1:5 (w/v) for the stomach and pyloric caeca, using a polytron homogeniser. The homogenate was then centrifuged at 4° C at 7500 (xg) for 10 min. The supernatant containing the crude extracts was picked-up and stored at –20° C before analysis. The soluble protein content of the enzyme extract was measured according to Bradford (1976) using a protein assay kit purchased by Bio-Rad TM.

Total proteases:

The effect of different pH incubations on the proteolytic activities of the crude enzyme extract was determined on the bases of the casein hydrolysis assay by Kunitz (1947). The pH values of different buffers were previously optimised for each gastrointestinal section: 0.1 M KCl–HCl (pH 1.0–2.0) and 0.1 M citrate–HCl (pH 3.0) for the stomach, 0.2 M phosphate buffer (pH 8.0) and 0.1 M glycine–NaOH (pH 9.0–10.0) for the pyloric caeca, middle intestine and distal intestine. The enzyme-substrate mixture consisted of 0.75 ml 2% (w/v) casein in water, 0.75 ml selected buffer and 0.1 ml crude enzyme extract incubated in a water bath for 1 h at 37° C. A total of 2.25 ml trichloroacetic acid (TCA), 5% (w/v) was then added to the reaction mixture to stop the reaction. This mixture was then allowed to stand for 0.30 h at 4° C before centrifuging at 3500 rpm for 15 min. Absorbance of the supernatant was recorded at 280 nm to measure the amount of tyrosine produced. The blank used for this assay was prepared by incubating a mixture of the casein, water and buffer for 1 h at 37° C, followed by the addition of a crude extract and TCA. One unit of specific activity was defined as the amount of enzyme needed to produce 1 µg tyrosine per min per mg of soluble protein of enzyme extract (U mg protein^{-1}) .

Trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) activities:

The trypsin activity of the enzyme extract was assayed using p-tosyl-L-arginine methyl ester (TAME) as a substrate according to Hummel (1959). A freshly prepared substrate comprising of 10 mM TAME (Sigma Aldrich) in 46 mM Tris–HCl buffer containing 11.5 mM of $CaCl_2$, pH 8.0 was used for the assay. Fifty microliters of enzyme extract was mixed with 150 µl of freshly prepared TAME substrate solution and 1.30 ml of buffer solution with a reaction temperature of 25° C. The increase in the absorbance value at 247 nm was recorded every min for 3 min. The trypsin activity was then expressed as TAME units per mg of soluble protein of enzyme extract (U mg protein^{-1}).

The chymotrypsin activity was assayed using N-benzoyl-L-tyrosine ethyl ester (BTEE) dissolved in a water/methanol solution at a ratio of 1:1 (v/v) as a substrate according to Hummel (1959). A freshly prepared substrate comprising of 1.07 mM BTEE (Sigma Aldrich) in 80 mM Tris–HCl containing 1mM $CaCl_2$ at pH 7.8 was used for the assay. Fifty microliters of enzyme extract were mixed with 700 µl of freshly prepared BTEE substrate solution and 750 µl buffer solution with reaction temperature of 25° C. The increase in absorbance value at 256 nm was recorded every min for 3 min., the chymotrypsin activity

was then expressed as BTEE units per mg of soluble protein of enzyme extract (U mg protein^{-1}).

Histological analyses –
Sampling and stains:
At the end of the trial, the fish were killed six hours after the last meal. Three trouts from each treatment were sampled and the gut and liver were isolated from the rest of the body. The visceral pack was stretched to separate the gut from the inter-visceral fat. Samples of the liver, stomach, pyloric caeca, middle and the distal intestine were sampled and fixed in 4% buffered (pH 7.2) and isotonic formalin cooled at 4° C. Finally 60 tissue samples were collected.
Sample vials were stored at a temperature of 4° C before the analyses.
After one week, the fixed pieces were embedded in paraffin wax, following normal histological procedures. Three µm thick paraffin sections were cut on a microtome and collected on microscope slides for successive stains.
All the tissue samples were stained with the normal Mayer hematoxylin-eosin method and more stains were on the liver sections performed, in particular PAS, PAS diastase and Sudan Black stains. The PAS diastase stain was necessary to discriminate the PAS positive reaction due to glycogen from other PAS positivity, such as mucopolysaccaridic substances and glycoproteins. The Sudan Black stain was performed to observe and confirm the possible presence of ceroid substances.

Statistical analysis –
In order to verify the assumptions of ANOVA utilisation, a normality of data distribution was performed by Shapiro-Wilk normality test (Venables & Ripley, 2002), (R statistical package, release 1.7.1). The data expressed as ratios were transformed in arcsin of roots; ANOVA variance analyses was performed by means of SPSS package (proc GLM), release 6.1. SPSS Inc. (1995).; the significant differences among the treatments were ranged according to the Scheffé test.

RESULTS

Enzymological results –
Total protease results:
The results obtained from total proteases assay are shown in Table 2. These values show that the PHA lectins clearly affected the total protease activities in all the analysed tissues. In the stomach, where the pHs are acids, both diets A and C show higher proteolytic activities than diets B and D. In the pyloric caeca, alkaline pH, the results show higher values for diet B and D than diets A and C; these values are in contrast with the stomach results. In the different intestine parts (middle and distal intestine), two completely opposite trends were observed, in fact diet A (fishmeal based) shows higher values than diet B (fishmeal based with added lectins) (2.90 vs 1.04) in the middle intestine, whereas in the distal, a slight difference appeared (0.99 vs 0.93). On the other hand, diet C, consisting of fish meal and a lupine meal mixture, shows lower values than diet D with added lectins in both intestine tracts (1.42 vs 2.06 and 0.72 vs 1.05).

Section	Experimental diets			
	A	B	C	D
S.	0.52±0.16a	0.17±0.05b	0.63±0.12a	0.25±0.07b
P.C.	2.69±0.05b	3.15±0.08a	2.79±0.22b	3.21±0.13a
M.I.	2.90±0.22a	1.04±0.08d	1.42±0.11c	2.06±0.16b
D.I.	0.99±0.04ab	0.93±0.04b	0.72±0.02c	1.05±0.07a

Legenda:
S. = stomach; P.C. = pyloric caeca; M.I. = middle intestine; D.I. = distal intestine.
A = fishmeal diet; B = fishmeal with added lectins diet; C = lupin meal diet; D = lupin meal with added lectins diet.
The means (n = 6) are indicated with the standard deviation.
Different letters in the same row indicate significant differences p< 0,05

Table 2 - Total protein activities (U/mg protein) in the gastrointestinal sections of rainbow trout fed different diets.
Tabella 2 – Attività proteica totale (U/mg di proteina) nelle porzioni gastrointestinali di trote iridea alimentate con le differenti diete.

Trypsin and chymotrypsin results:

The results obtained in the trypsin and chymotrypsin analyses are shown in Tables 3 and 4. The analysed trypsin and chymotrypsin values show the same trend that was reported for the total proteases with lower values for diet B (fish meal plus lectins) and diet C (lupine meal without lectins), respectively. The only differences that were noticed concern the trypsin values of diets A and C in the stomach (equal in both diets) and the distal intestine (higher in diet A than C); in these tissues, the trypsin activities are in contrast with the proteolityc activity values.

In spite of the trypsin and chymotrypsin activities in the diets containing lupin meal, diet B shows higher values than diet D which contains lectins in the pyloric caeca tissue. This is in contrast with results obtained for the total proteases activities.

Section	Experimental diets			
	A	B	C	D
S.	0.10±0.04	0.09±0.02	0.19±0.07	0.03±0.02
P.C.	0.07±0.03	0.11±0.01	0.38±0.30	0.01±0.03
M.I.	0.17±0.08	0.05±0.03	0.10±0.06	0.11±0.05
D.I.	0.07±0.02	0.04±0.02	0.02±0.01	0.06±0.04

Legenda:
S. = stomach; P.C. = pyloric-caeca; M.I. = middle intestine; D.I. = distal intestine.
A = fishmeal diet; B = fishmeal with added lectins diet; C = lupin meal diet; D = lupin meal with added lectins diet.
The means (n = 3) are indicated with the standard deviation.

Table 3 - Trypsin activities (U/mg protein) (mean ± s.d. n = 3) in the gastrointestinal sections of rainbow trout fed with different diets.
Tabella 3 – Attività della tripsina (U/mg di proteina) (media ± deviazione standard, con n = 3) nelle porzioni gastrointestinali di trote iridea alimentatate con le differenti diete.

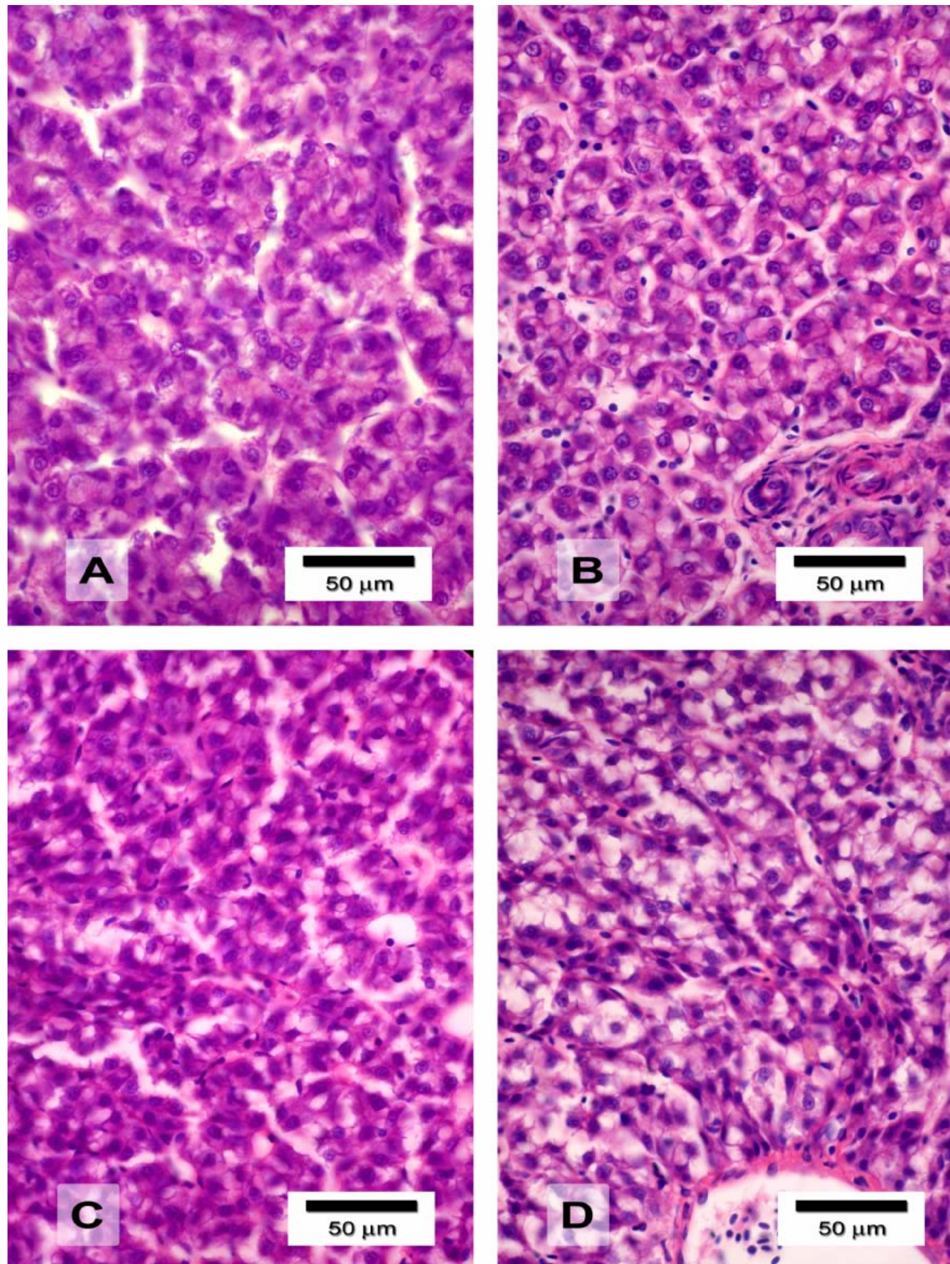

Figure 1 - Liver histological pictures, hematoxylin-eosin stained, of all diets. A - fish meal diet; B - fish meal with added lectins diet; C - lupin meal diet; D - lupin meal with added lectins diet.

Figura 1 – Sezione istologica dei fegati dei pesci alimentati con tutte le diete sperimentali, colorazione ematossilina-eosina. A - dieta a base di farina di pesce; B - dieta a base di farina di pesce e lectine; C - dieta a base di farina di lupino; D - dieta a base di farina di lupino con lectine.

Section	Experimental diets			
	A	**B**	**C**	**D**
S.	0.18±0.11	0.10±0.01	0.22±0.03	0.13±0.01
P.C.	2.31±0.05	3.02±0.01	2.82±0.01	2.62±0.25
M.I.	3.33±0.13	0.89±0.07	1.28±0.01	1.49±0.13
D.I.	0.47±0.17	0.49±0.04	0.32±0.10	0.99±0.09

Legenda:
S. = stomach; P.C. = pyloric-caeca; M.I. = middle intestine; D.I. = distal intestine.
A = fishmeal diet; B = fishmeal with added lectins diet; C = lupin meal diet; D = lupin meal with added lectins diet.
The means (n = 3) are indicated with the standard deviation.

Table 4 - Chymotrypsin activities (U/mg protein) (mean ± s.d. n = 3) in the gastrointestinal sections of rainbow trout fed different diets.
Tabella 4 – Attività della chimotripsina (U/mg di proteina) (media ± deviazione standard, con n = 3) nelle porzioni gastrointestinali di trote iridea alimentatate con le differenti diete.

Histological results –
Liver histology:

Samples from the fish meal diet (diet A) show histological pictures with a normal content of glycogen and small intra-cytoplasmatic vacuoles of ceroid substances. Only one sample of liver presented some hepatocytes with a large positive vacuole on the Sudan Black stain.

The fish fed with a lupin seed meal (diet C) showed slight changes in the liver pattern. The patterns of the alterations showed blood cell congestion in the sinusoidal blood vessels. Two samples showed a reduction in the glycogen content but the lipids content decreased slightly. In the last sample, there was less glycogen and fats.

All the livers from the fish fed with fishmeal added lectins (diet B) present blood cell congestion in the blood vessels and a reduction in the glycogen content. In two samples, the ceroid substance was equal to the liver from diet A; the last one showed an increase in content of this substance. Large transparent vacuoles in cytoplasm were present in all the samples due to initial phase Lipid Liver Disease.

All the livers of the fish fed with lupin seed meal with added lectins (diet D) present a less intense blood vessel congestion than the livers from fish fed with fish meal diet with added lectins. All the samples show a reduction or absence of glycogen and, a diminished presence of the ceroid substance. (Figures 1-3).

Intestine histology:

The fishmeal diet: only one sample of pyloric caeca presented a slight change, principally lymphocyte infiltration in the villi lamina propria and mucous membrane. The remaining samples were normal. In the mid intestine tract, one fish presented a slight change, lymphocyte infiltration and a reduction of the absorptive vacuoles. In the distal intestine, that histological pattern was normal and only one sample showed slight infiltration.

The lupin seed meal diet: there was only one sample from the mid intestine tract with a pattern of slight infiltration. The remaining samples were normal.

The fishmeal with added lectin: the pyloric caeca presents two samples with a slight change in pattern but the last one was normal. In the mid intestine tract one fish showed slight

infiltration and the other two samples present a pattern of severe change. The absorptive vacuoles and brush border were lost, the villi lamina propria were under thickening, dense cytoplasm and the nuclei shifted in the mid of the enterocytes, with strong lymphocyte infiltration of the villi. The distal portion of the intestine had one slightly changed sample, one severly changed and one normal sample.

The lupin seed meal with added lectins: the number of altered samples were higher than those in a diets A and C; one sample from the pyloric caeca, two from the mid intestine and one from the distal intestine showed only a slight change. The other samples were normal (Figure 4).

DISCUSSION AND CONCLUSION

The lowest activities were observed in the stomach and distal intestine in the anatomical sites along the digestive tract, on the other hand, high protease activity was observed in the pyloric caeca and mid intestine. This trend is common in all the considered experimental groups, regardless of the diet, therefore the successive considerations focus on the differences between the diets.

The proteolytic enzyme activities in the stomach were lower in fish fed diets with added lectins than in diets A and C, without lectins. Although the reaction between most lectins and their specific ligands is eliminated at pH 3 or less, lectins are able to bind the stomach epithelium in vivo as reported by Bardocz *et al.* (1995). The presence of lectins in a diet slows down stomach emptying in rats (Van Damme *et al.*, 1998), but no data are available for fish species.

The activities of the proteolytic enzymes were similar in diets A and C in the pyloric caeca of fish fed a diet without lectins. The highest values were detected in diets B and D, which confirms that lectins have a stimulatory effect on proteases activities in this gut region.

In the mid intestine, enzyme activities appear similar in diets B and C and are lower than those in diets A and D. In this portion lectins showed an opposite effect rather in the pyloric caeca. Lectins had strong inhibitory effect on protease activities when fish meal is the major protein source. Protein mixture (fish and lupin meal protein) had an inhibitory effect caused by lectins. In the case of the protein mixture added with lectins (diet D) the enzymatic activities resulted in the midway compared to the control diet (diet A) vs protein mixture (diet C); this fact could be explainable with an neutralization effect between lectins and lupin compounds. Lectins probably interfere with some lupin compounds, since lectins are able to make glyco-protein complexes, as demonstrated by Van Damme *et al.* (1998).

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Figure 2 - Liver histological pictures, PAS and PAS diastase stained, of all diets. A - fish meal diet; B - fish meal with added lectins diet; C - lupin meal diet; D - lupin meal with added lectins diet. The number near the letter indicate the stain: 1 - PAS, 2 - PAS diastase. The difference in chromatic density between 1 and 2 indicate the glycogen content. In PAS diastase images there are fine PAS+ reaction also after diastase process, these are not glycogen substance.

Figura 2 - Quadri istologici di fegato, colorati con la PAS e PAS-diastasi, di tutte le diete. A - dieta a base di farina di pesce; B - dieta di farina di pesce addizionata di lectine; C - dieta a base di farina di lupino; D - dieta con farina di lupino addizionata di lectine. I numeri affianco alle lettere indicano la colorazione: 1 - PAS, 2 - PAS-diastasi. La differenza di intensità cromatica tra le foto con i numeri 1 e 2 indica il contenuto in glicogeno. Nelle immagini della PAS-diastasi ci sono reazioni PAS+ anche dopo il trattamento di diastasi dove non ci dovrebbe essere glicogeno.

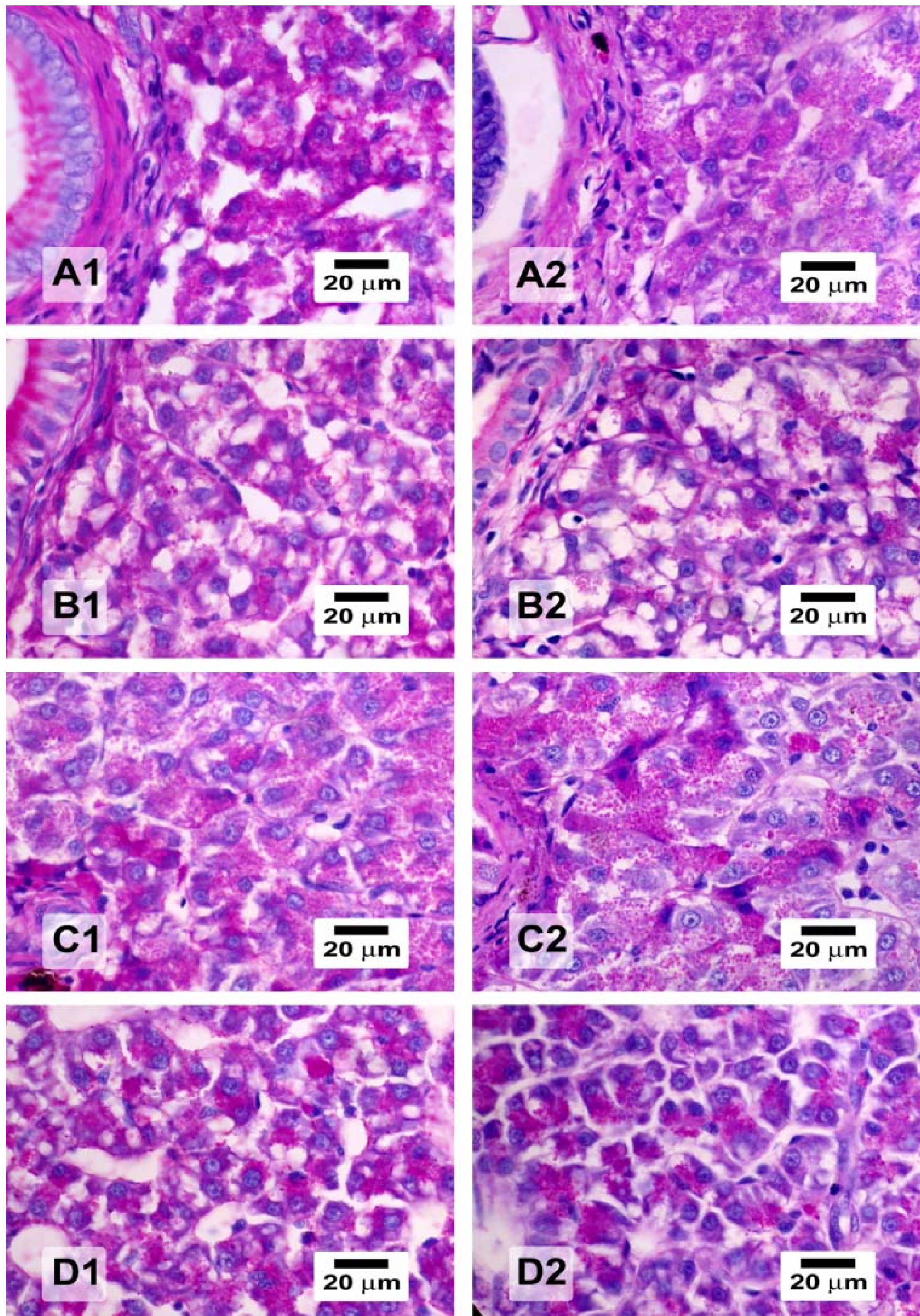

Figure 2

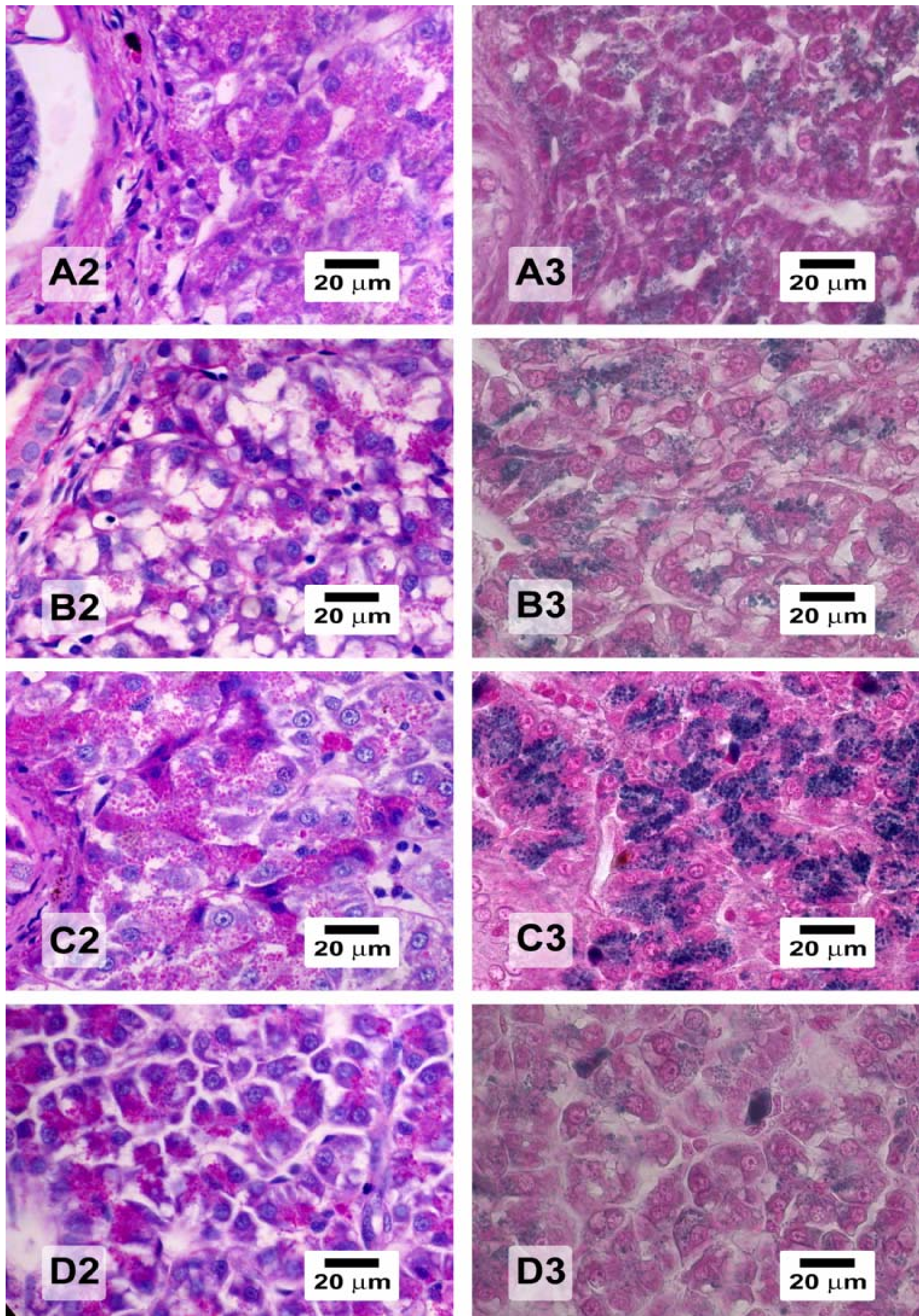

Figure 3

Figure 3 - Liver histological pictures, PAS diastase and Sudan Black stained, of all diets. A - fish meal diet; B - fish meal with added lectins diet; C - lupin meal diet; D - lupin meal with added lectins diet. The number near the letter indicate the stain: 2 - PAS diastase and 3 - Sudan Black stain. The fine PAS+ granulation (gray colour) in photos with number 2 have the same dimension of black granulation in photos with number 3. Besides, more dense and big PAS+ granulation corresponding to more big black granulation, to confirm that PAS+ reaction in PAS-diastase is due at the ceroid substance.

Figura 3 - Quadri istologici di fegati, colorati con PAS-diastasi e Sudan Nero, di tutte le diete. A - dieta a base di farina di pesce; B - dieta di farina di pesce adizionata di lectine; C - dieta a base di farina di lupino; D - dieta con farina di lupino addizionata di lectine. I numeri affianco alle lettere indicano la colorazione: 2 - PAS-diastasi e 3 - Sudan Nero. Le fini granulazioni PAS+ (in color grigio) nelle foto con il numero 2 hanno stesse dimensioni delle granulazioni nere nelle foto con il numero 3. Oltretutto, le granulazioni più dense e grosse PAS+ corrispondono a granulazioni nere più grandi, a conferma che le reazioni PAS+, positive nelle colorazioni PAS-diastasi sono dovute a sostanza ceroide.

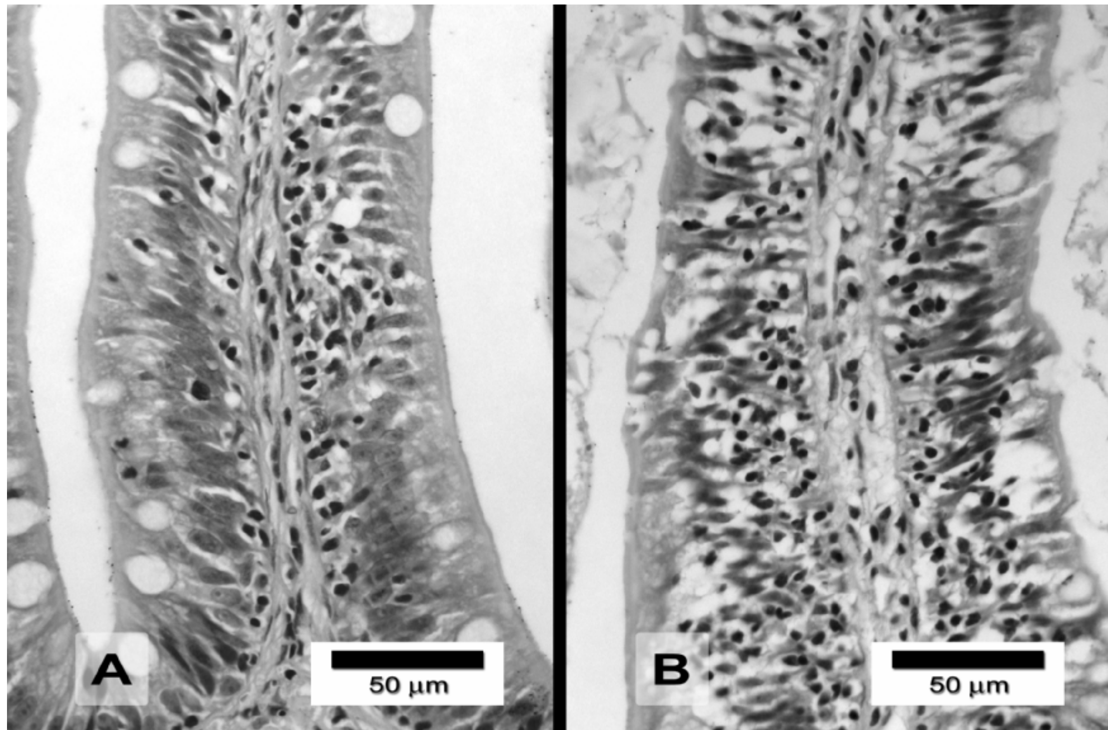

Figure 4 - Picture of middle intestine hematoxylin-eosin stained tracts. Letter A - fish meal diet and B - fish meal with added lectins diet. On the left a normal villus from fish fed with diet A, on the right the pattern of severe change in villus mucosa from fish fed with diet B. The lamina propria of photo B is thick, with cytoplasmatic vacuolisation and congestion of the blood vessel. In the mucosa layer there is a strong presence of lymphocyte, cytoplasm of mucosal cells are more dense than in the photo A and nuclei of the same cells are dense and shifted in the mid of the cell body.

Figura 4 - Sezione di intestino medio colorata con ematossilina-eosina. A - dieta a base di farina di pesce; B - dieta a base di farina di pesce e lectine. Sulla sinistra un villo normale di un pesce alimentato con la dieta A, sulla destra si nota un quadro istologico con gravi cambiamenti della mucosa del villo di un pesce alimentato con la dieta B. La lamina propria della foto B è molto ispessita, con vacuolizzazioni citoplasmatiche e congestione dei vasi sanguigni. Nello strato della mucosa vi è un forte infiltrato linfocitario, il citoplsma delle cellule della mucosa è più denso ed i nuclei delle cellule sono spostati nel mezzo del cariosoma rispetto alla foto A.

This possible lectin to lupin-carbohydrate interaction can explain the above statement. The pattern of protease activities, for the different intestinal tracts described above, it is unusual inasmuch as opposite (stimulatory/inhibitory) compared to the control and related to the lectins presence. Lacking of scientific literatures about the previous supposition does not permit to us a more deeply discussion of this findings.

Moreover, if diets A and B (fish meal based) are compared, it can be seen that diet B shows a marked decreased protease activity, demonstrating that the harmful lectin effect is due to the above described effect.

Glencross *et al.* (2003) demonstrated that processing lupin meal by ethanol extraction, reduce the Not-Starch-Polisaccharides (NSP) content and improves the nutritional value of the same protein. The same authors reported that NSP has a negative influence on the lupin nutritional value. This is confirmed by our results on intestine proteases activities in which the values in diet C were similar to the values obtained from diet B.

As far as the distal intestinal enzyme activity are concerned, the trend was similar to the mid intestine activities. In this portion, there are no differences between the diet A and D values, but instead, the differences between the values are closer.

The total proteases reduction in the diet containing lectins compared to the fishmeal diet can be justified by the fact that PHA lectins strongly binds to the brush border membrane of intestinal cells, forming glyco-protein complexes with intestinal cell receptors, as reported in other species by Pusztai *et al.* (1990) and Pusztai *et al.* (1995b). It is possible that lectins bind to the brush border and cause an inhibition of cholecystokinin (CCK) secretion, as reported by Pusztai *et al.* (1992) and Herzig *et al.* (1997). In mammals, lectins reduce the insulin concentration and exocrine pancreatic secretion activity (Van Damme *et al.*, 1998).

Our results did not show a clear or obvious alteration of the trypsin and chymotrypsin activity among the treatments, however a wide variation was found between the enzyme activities of the experimental groups, in agreement with a previous study on gilthead seabream (Robaina *et al.*, 1995). This inhibition effect in rainbow trout is probably not so evident as in mammals or it is due to a wide subjective variability.

Lectins cause histological changes in the mid intestine of rainbow trout, Bakke McKellep *et al.* (2000) stated that in salmon fed with soybean meal there is a reduction of functionality of the distal intestine and an increased presence of lymphocytes in the lamina propria.

The increased number of lesions in the middle intestinal tract with diets B and D indicates morphological alterations induced by lectins. As far as the experimental treatments are concerned, the seriousness of lesions is higher in the middle intestine tract with diet B, followed by diets D and C. The distal intestine is less involved than the mid intestine, in contrast with what has been reported by other authors (Bureau *et al.*, 1998) where soybean meal causes an acme of alteration in the distal intestine in chinook salmon. Lectins are very resistant vegetable proteins, with high stability in a large pH range (pH 1 to pH 12); they are more resistant than other proteins to enzyme activity (Peumans *et al.*, 1996). A hypersensitize immune reaction to lectins, probably occurs in fish, as has been demonstrated in rats (Van Damme *et al.*, 1998).

There is a correlation between the lower enzyme activities in the mid intestine and the histological lesions on the same portion.

As far as the liver histology is concerned, the different quality of proteins seems to interfere with the biochemical hepatocytes activity. Lupin seed meal in general reduces the lipid droplets in the hepatocytes, which is in agreement with other authors (Glencross *et al.*, 2004). Similar results were found in gilthead seabream fed with corn gluten meal as reported by Robaina *et al.* (1997). As far as the ceroid substance is concerned, a reduction in the content was shown in the hepatocytes of fish fed with diets B and D. This effect is more

evident in livers obtained from fish fed with diet D. Instead the alcohol soluble lipid content was higher in fish fed with diet B.

Lipoid Liver Disease (LLD) may be related to a non-infectious disorder chiefly linked to fat rancidity (Halver, 1972; Ghittino, 1983; Leatherland & Woo, 1998). Our experimental diets were produced using high quality raw materials at the start of the trial and then these were stored in a cold room at 4° C during the feeding period, therefore this eventuality can be excluded. Ghittino (1983) reported that fish feeding LLD pathogenesis for rainbow trout is comparable with mouse LLD. In human pathology; rats and mice are utilised for the study of pathogenesis processes and it can be supposed that the possible causes of LLD are the same as those studied in human pathology, where LLD is known as steatosis. An unbalanced dietary protein/fat ratio, carential status of choline, hypovitaminosis E, essential amino acid carential status, and hypossihemic status, could therefore be the causes of LLD in trout, as reported in human pathology by Dianzani (1995). Pereira *et al.* (2002) found that PAS positive-like vacuole structures correspond to a glycogen accumulation. In our histological investigations, the same pattern was found, but in order to discriminate the real nature of PAS+ reactivity, the same samples were treated with an enzymatical diastase process, in order to remove the glycogen and/or other polysaccharide matter. Sudan Black stainings performed on the same sample slices confirmed our suspicions that PAS positive-like vacuole structures are not due to glycogen accumulation but are lipid-ceroid substances.

Our observations are in partial agreement with Robaina *et al.* (1995, 1997), who reported a decrease in the liver glycogen level in gilthead seabream fed on alternative protein sources.

Some authors have reported that the distal intestine of fish is able to absorb complex compounds, for instance polypeptides and oligosaccharides, as reported for salmon by Bakke-McKellep (1999). The distal intestine could probably absorb the lectins, which arrive in the liver and cause an alteration of the hepatocytes metabolism. The PHA lectins inhibit proteic synthesis (Bureau *et al.*, 1998), therefore liver lipoproteins synthesis could be reduced in the hepathocytes, so fat exchange is reduced and subsequently a lipid deposition is induced. Furthermore, it is possible that the alteration of protein digestion could be due to a reduction in the digestive enzyme activity. Another cause of the alteration of the protein digestion could be due to a reduced absorption activity of enterocytes correlated to the agglutination effect of lectins on the brush border mucosa. Both physiological mechanisms result in a lower absorption of amino acids. In both cases, these effects lead to a nutritional sub-deficiency status of amino acids. The lack of amino acids can mime the nutritional unbalance of dietary protein and the lipid ratio, inducing a liver steatosis process (Ghittino, 1983; Dianzani, 1995; Van Damme *et al.*, 1998). A correct digestible protein to digestible energy ratio is important for optimal fish growth, as already described for different fish species by some authors (Pusztai *et al.*, 1990; NRC, 1993; Robert *at al.*, 1993; Médale *et al.*, 1995; Grisdale-Helland & Helland, 1997; Dias *et al.*, 1998; Refstie *et al.*, 1998).

In conclusion, our trial results lead us to conclude that PHA lectins interfere in the normal physiological digestive process of rainbow trout. PHA lectins are able to inhibit the trout stomach protease enzymes. On the other hand, these antinutritional factors (ANFs) showed a weak activity in the intestinal tract, in diets containing a high inclusion level of vegetable protein sources with respects to fishmeal based diets. As far as the intestinal histology is concerned PHA lectins induce local inflammatory reactions; particularly in the mucosa of the middle intestinal tract. Another clear histological effect caused by these ANFs, is the interaction with the liver, which leads to an alteration of its metabolism and provokes a lipoid liver disease. An analysis of the data collected in this trial, highlights that there is a correlation between the hystological inflammatory pattern and the enzymatical proteases activity, in the intestine of trout.

The evidence of interaction among different ANFs would suggest the use of a mixture of several protein plant sources in fish feedstuff, to achieve a partial neutralization of the detrimental effect of ANFs, as reported by Francis *et al.* (2001).

Refstie *et al.* (1998) demonstrated the need to monitor several factors that may affect nutrient absorption and thereby fish growth when evaluating feed ingredients in fish feeds. Further studies are necessary to better understand fish pathophysiological mechanisms due to vegetable ANFs, and to prevent diseases based on feedstuffs.

During the planning of nutritional trial, when a new ingredient is introduced in the diet formulation, beyond the common zootechnical analyses an enzymological and histological evaluation is necessary.

ACKNOWLEDGEMENTS

Prof. Marco Galloni, Dr. Cristina Vignolini, Dr. Karine Gabiale and Mr. Mimmo Falzone (Dep. of Veterinary Morphophysiology, University of Torino), to have allowed the utilization of lab. B01 and histological section and stain support.

Dr. Laura Gasco (Dep. of Animal Science, University of Torino) and Dr. Ivan Ferro (Dep. of Animal Production, Epidemiology and Ecology, University of Torino) for support in fish managements.

REFERENCES

Association of the Official Analytical Chemists (1990). Official Methods of Analysis, 15th edn. *Association of Official Analytical Chemists,Washington, DC, USA.*

Bakke-Mckellep A.M. (1999). Intestinal nutrient absorption in Atlantic salmon (*Salmo salar* L.) and pathophysiological response of the intestinal mucosa to dietary soybean meal. *PhD Thesis. Norwegian School of Veterinary Science, Oslo, Norway. ISBN 82-7725-059-2.*

Bakke-McKellep A.M., Press C.M.L., Baeverfjord G., Krogdahl Å. & Landsverk T. (2000). Changes in immune and enzyme histochemical phenotypes of cells in the intestinal mucosa of Atlantic salmon, *Salmo salar* L., with soybean meal-induced enteritis. *J. Fish Dis*., 23: 115-127.

Bardocz S., Grant G., Ewen S.W.B., Duguitd T.J., Brown D.S., Englyst K. & Pusztai A. (1995). Revesible effect of phytohaemagglutinin on the growth and metabolism of rect gastrointestinal tract. *Gut*, 37: 353-360.

Bradford M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.*, 72: 248-254.

Bureau D.P., Harris A.M. & Young C.C. (1998). The effects of purified alcohol extracts from soy products on feed intake and growth of chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, 161: 27-43.

Dianzani M.U. (1995). Istituzioni di Patologia generale, 3rd Edition. *UTET, Torino*: 357-381.

Dias J., Alvarez M.J., Diez A., Arzel J., Corraze G., Bautista J.M. & Kaushik S.J. (1998). Regulation of hepatic lipogenesis by dietary protein energy in juvenile European seabass (*Dicentrarchus labrax*). *Aquaculture*, 161: 169-186.

Francis G., Makkar H.P.S. & Becker K. (2001). Antinutritional factor present in plant-derived alternate fish feed ingredients and their effect in fish. *Aquaculture*, 199: 197-227.

Ghittino P. (1983). Tecnologia e patologia in acquacoltura. 2nd volume. *Ed. Bono, Torino*: 337-341.

Glencross B.D., Boujard T. & Kaushik S.J. (2003). Influence of oligosaccharides on the digestibility of lupin meals when fed to rainbow trout, *Oncorhynchus mykiss*. *Aquaculture*, 219: 703-713.

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.

Grisdale-Helland B. & Helland S.J. (1997). Replacement of protein by fat and carbohydrate in diets for Atlantic salmon (*Salmo salar*) at the end of the freshwater stage. *Aquaculture*, 152: 167-180.

Halver J.E. (1972). Fish nutrition. *Edited by Academic Press*: 422-424.

Hendricks J.D. & Bailey G.S. (1989). Aventitious toxins. *In: Halver J.E., editor. Fish Nutrition, 2nd ed. New York: Academic Press*: 605-651.

Herzig K.-H., Bardocz S., Grant G., Nustede R., Fölsch U.R. & Pusztai A. (1997). Red kidney bean lectin is a potent cholecystokinin releasing stimulus in the rat inducing pancreatic growth. *Gut*, 41: 333-338.

Huisman J. & Tolman G.H. (1992). Antinutritional factors in the plant proteins of diets for non-ruminants. *In: Garnsworthy P.C., Haresign W.& Cole D.J.A., (ed. by Recent Advances in Animal Nutrition). Oxford: Butterworth-Heinemann*: 3-31.

Hummel B.C.W. (1959). A modified spectrophotometric determination of chymotrypsin, trypsin, and thrombin. *Can. J. Biochem. Physiol.,* 37: 1393-1399.

Krogdahl Å., Bakke-McKellep A.M. & Baeverfjord G. (2003). Effects of graded levels of standard soybean meal on intestinal structure, mucosal enzyme activities, and pancreatic response in Atlantic salmon (*Salmo salar* L.). *Aquacult. Nutr.,* 9, 6: 361-371.

Kunitz M. (1947). Crystalline soybean trypsin inhibitor II. General properties. *J. Gen. Physiol.*, 30: 291–310.

Leatherland J.F. & Woo P.T.K. (1998). Fish disease and disorders; 2nd volume. *Edited by Cabi Pubblishing*: 317.

Liener I.E. (1989). Antinutritional factors in legume seeds: state of the art. *In: Huisman J, Van der Poel T.F.B. & Liener I.E., editors. Recent Advances of Research in Antinutritional Factors in Legume seeds. Wageningen, Pudoc*: *6-13.*

Médale F., Brauge C., Vallée F. & Kaushik S.J. (1995). Effect of dietary protein/energy ratio, ratio size, dietary energy source and water temperature on nitrogen excretion in rainbow trout. *Water Sc. Tech*., 31, 10: 185-194.

National Research Council (1993). Nutrient requirements of fish. *National Academic Press, Washington DC*: 114.

Naylor R.L., Goldburg R.J., Primavera J.H., Kautsky N., Beveridge M.C.M., Clay J., Folke C., Lubchenco J., Mooney H. & Troell M. (2001). Effect of aquaculture on world fish supplies. *Nature*, 405: 1017-1024.

Palmegiano G.B., Agradi E., Forneris G., Gai F., Gasco L., Rigamonti E., Sicuro B. & Zoccarato I. (2005). Spirulina as a nutrient source in diets for growing sturgeon (*Acipenser baeri*). *Aquacult. Res.*, 36: 188-195.

Pereira O., Rosa E., Pires M.A. & Fontaìnhas-Fernandes A. (2002). Brassica by-products in diets of rainbow trout (*Oncorhynchus mykiss*) and their effects on performance, body and liver histology. *Anim. Feed Sci. Tech*., 101: 171-182.

Peumans W.J., Verhaert P., Pfuller U. & Van Damme E.J.M. (1996). The chitin-binding mistletoe (*Viscum album*) agglutinin. *COST 98 - Effect of anti-nutrients value of legume diets, Vol. 5*: 63-68.

Pusztai A. (1991). General effects on animal cells. *In: Plant Lectins (ed. Pusztai A.), Cambridge University Press, Cambridge*: 167-180.

Pusztai A., Ewen S.W.B., Carvalho A.F.U., Grant G., Brantner K. & Bardocz S. (1995a). Dietary lectins affect hormone balance of body and modulate its general metabolism. *In: Gastrointestinal Tract and Endocrine System (ed. M.V. Singer, R. Ziegller & G. Rohr) Kluwer Academic Publishers, Lancaster*: 457-463.

Pusztai A., Ewen S.W.B., Grant G., Brown D.S., Peumans W.J., Van Damme E.J.M. & Bardocz S. (1992). Stimulation of growth and polyamine accretion in the small intestine and pancreas by lectins and trypsin inhibitors. *In: Polyamines in the gastrointestinal tract (ed. by R.H. Dowling, U.R. Folsch & C. Loser) Kluwer academic, Dordrecht:* 473-483.

Pusztai A., Ewen S.W.B., Grant G., Peumans W.J., Van Damme E.J.M., Coates M.E. & Bardocz S. (1995b). Lectins and also bacteria modify the glycosylation of gut receptors in the rat. *Glyconjugate J.*, 12: 22-35.

Pusztai A., Ewen S.W.B., Grant G., Peumans W.J., Van Damme E.J.M., Rubio L. & Bardocz S. (1990). Relationship between survival and binding of plant lectins during small intestinal passage and their effectiveness as growth factors. *Digestion*, 46: 308-316.

Refstie S., Storebakken T. & Roem A.J. (1998). Feed consumption and conversion in Atlantic salmon (*Salmo salar*) fed diets with fishmeal, extracted soybean meal or soybean meal with reduced content of oligosaccharides, trypsin inhibitors, lectins and soy antigens. *Aquaculture*, 162: 301-312.

Robaina L., Izquierdo M.S., Moyano F.J., Soccoro J., Vergara J.M., Montero D. & Fernandez-Palacios H. (1995). Soybean and lupin seed meals as protein sources in diets for gilthead seabream (*Sparus aurata*): nutritional and histological implications. *Aquaculture*, 130: 219-233.

Robaina L., Moyano F.J., Izquierdo M.S., Socorro J., Vergara J.M. & Montero D. (1997). Corn gluten and meat and bone meals as protein sources in diets for gilthead seabream (*Sparus aurata*): nutritional and histological implications. *Aquaculture*, 157: 347-359.

Robert N., Le Gouvello R., Mauviot J.C., Arroyo F., Aguirre P. & Kaushik S.J. (1993). Use of extruded diets in intensive trout culture: effect of protein to energy ratios on growth, nutrient utilization and on flesh and water quality. *Fish nutrition in practice, S.J. Kaushik and P. Luquet, edited by INRA Paris*: 497-500.

SPSS Inc. (1995). SPSS Professional Statistic, release 6.1. *SPSS Inc. Chicago IL, USA*.

Van Damme E.J.M., Peumans W.J., Pusztai A. & Bardocz S. (1998). Handbook of plant lectins: properties and biomedical application. *Ed. by Wiley & Sons*: 3-50.

Vasconcelos I.M. & Oliveira J.T.A. (2004). Antinutritional properties of plant lectins. Review. *Toxicon*, 44: 385-403.

Venables W.N. & Ripley B.D. (2002). Modern applied statistics with S. *Ed. Springer-Verlag*: 1-497.