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The Histological Evaluation of Sea Cucumber Meal as a Potential Ingredient in Rainbow Trout Diet

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Abstract

Sea cucumber meal is proposed as ingredient for rainbow trout diet using an histological approach. Three isoproteic (crude protein 39.5%) and isoenergetic (18 MJ/kg DM) diets were formulated with increasing level of sea cucumber meal (FO10, FO15 and FO20; with an inclusion of 10, 15 and 20% of sea cucumber meal), these diets were tested against a fish meal based diet (FP). A monofactiorial balanced experimental design (3 x 4) was adopted. 48 trout, (110±7.5 g) of initial fish body weight, were utilized. The fish feed trial lasted in 49 days. At the end of experiment, histological sections of intestine and liver were sampled. All the tissue samples were stained with the Mayer hematoxylin-eosin (HE) stain while on the liver samples PAS, PAS diastase and Alcian Blue stains were carried out. The enteritis score showed a slight enteritis status in fish fed with sea cucumber meal, independently from inclusion level (FP: 6.13±0.35; FO10: 7.0±1.6; FO15: 7.38±1.77; FO20: 7.88±1.89). The inclusion of sea cucumber meal did not cause relevant histological changes in the intestine of rainbow trout, thus showing positive perspectives for its future utilization in fish feeds.

Keywords: Fish feeds, histology, fish enteritis, fish nutrition.

Introduction

The substitution of fish meal in fish feeds is a paramount issue for modern aquaculture and the list of possible alternative feedstuffs to fish meal is constantly updated in aquafeed scientific literature. Being naturally consumed by benthophagus fish, sea cucumber is a candidate for partial fish meal substitution, moreover sea cucumbers are worldwide harvested and reared for human consumption. Sea cucumbers are very common along Mediterranean coasts and they are never been considered as potential ingredient in fish feeds. Considering that sea cucumbers have natural toxic compounds, as holoturins, that in the wild can exert repulsive activity against natural predators, in this research the potential anti - nutritional effect of sea cucumber meal has been studied in rainbow trout feeds utilizing the histological approach previously adopted for soybean meal. In the Mediterranean Sea an increasing commercial interest for fishery and potential aquaculture of sea cucumbers has been registered (Aydin, 2008). Sea cucumbers are detritivorous benthonic invertebrates, their presence greatly increases in proximity of sea farms and they are used for integrated aquaculture productions (Sicuro and Levine, 2011). The principal physiological consequence of anti-nutritional factors in the novel ingredients used as fish meal substitute in fish feeds is the inflammation of distal intestine (Refstie *et al*., 2000; Nordrumet *et al*., 2000). A typical pathological alteration of fish digestive tract is the soybean - induced enteritis that has been studied and depicted primarily by Van den Ingh *et al*., 1991. This enteritis has been extensively investigated in salmonids for its effect on fish productive parameters (Urán *et al*., 2008; Uran, 2008) and it can be also considered a reference model in the studies on fish digestive system anatomical alterations. The aim of this work is to study primarily the intestine modification caused by sea cucumber meal inclusion in rainbow trout feeds and secondarily verify the histological approach already used with soybean meal inclusion in fish feed.

Materials and Methods

Fish Feed Trial

Fish feed trial was conducted at the Experimental Station of Department of Animal Husbandry of the University of Turin. The experimentation design was mono-factorial, balanced

with four levels of treatment, a control group and 3 replicates each (4x3), the experimental factor tested was fish diet. Forty eight trout, (110±7.5 g) of initial fish body weight, were reared in 12 tanks, 50 L volume each. Water quality and temperature were weekly monitored. After 10 days of acclimatization with a commercial diet, fishes were fed by hand twice a day, 6 days per week. The fish feed trial lasted in 49 days. Fish were fed at visual satiety and feed intake was checked each time even all the supplied feed was consumed. Water temperature ranged between 11 and 13°C and dissolved oxygen was at saturation level during the experimentation. At the end, hepatosomatic (HSI) and viscerosomatic (VSI) indexes were measured on 8 fishes per treatment as follows:
HSI = (liver/body in weight) x 100; VSI = (viscera/body in weight) x 100.

Fish Diets

Three different diets were formulated with increasing level of sea cucumber meal (FO10, FO15 and FO20; with an inclusion of 10, 15 and 20% of sea cucumber meal respectively), these diets were tested against a control diet fish meal based (FP). Sea cucumber meal utilized was composed of a mixture of two species of sea cucumber (*Holoturia tubulosa*, and *Holoturia forkalii*), 50% each, that were dehydrated in the oven at temperature of 75°C for 12 h and successively grounded. Diets analyzed by proximate composition according to standard methods (AOAC, 1995) showed that all diets were isoproteic (crude protein 39.5%) and isoenergetic (18 MJ/kg DM) (Table 1). The feeds were manufactured in the laboratory, all the dry ingredients and the oil were thoroughly mixed; water was then blended into the mixture to attain a consistency appropriate for pelleting using a 3.5 mm die meat grinder. After pelleting, the diets were dried in a stove overnight at 50°C and refrigerated at 6°C until utilization. Diets formulations and proximate composition are reported in Table 1.

Histological Methods

At the end of experiment, immediately after gutting, histological sections were excised on 8 fish per experimental group and fixed in buffered formalin prior to assess the eventual enteritis induced by experimental diets and its degree of intensity. After dehydration and embedding in paraffin wax following standard histological techniques, 3 sections (5 Am thickness) of digestive tract were mounted per each fish: proximal, distal intestine and liver (with the exception of one fish of FP group where liver and intestine was not sampled) were stained with haematoxylin and eosin. 94 histological samples were analyzed at all: 3 samples per 8 fish in 4 experimental groups. Evaluated parameters consisted of the widening and shortening of the intestinal folds, the enterocyte supra nuclear vacuolization extent, the lamina propria widening status villi and lymphocyte infiltration in the lamina propria and submucosa. The perivisceral fat was gently separated from the visceral pack. Samples of the liver, pyloric caeca, proximal and the distal intestine were taken and fixed in 4% buffered (pH 7.2) and isotonic formalin cooled at 4°C. Sample vials were stored at 4°C before analyses. After one week, the fixed tissues were embedded in paraffin wax, following standard histological procedures. Five µm thick paraffin sections were cut and collected on microscope slides for the histological

Table 1. Ingredients and proximate composition of experimental diets (g/kg)

Ingredients	FO20	FO15	FO10	FP
Herring fish meal	450	491	520	651
Haemoglobin meal	60	50	50	0
Sea cucumber meal	200	150	100	00
Corn starch	140	164	185	214
Cod liver oil	110	105	105	95
Celite	5	5	5	5
Lygnumsulphyte	10	10	10	10
Mineral mixture [1]	5	5	5	5
Vitamine mixture[2]	10	10	10	10
Liver-protector integrator[3]	10	10	10	10
Proximate composition (% DM; mean±sd)				
Moisture	5.01±0.1	4.81±0.0	4.59±0.1	4.41±0.2
Crude protein	46.51±0.0	47.43±0.11	47.43±0.18	48.15±0.03
Ether extract	15.32±0.1	15.22±0.26	15.21±0.06	14.73±0.25
Ash	15.57 ±0.18	14.35±0.15	12.61±0.15	10.39±0.02
Gross energy (MJ/kg DM)	19.3±0.33	19.8±0.47	20.0±0.26	20.7±0.33

[1] Mineral mixture (mg/kg diet): bicalcium phosphate, 500 g; calcium carbonate, 215 g; sodium salt, 40 g; potassium chloride, 90 g; magnesium chloride, 124 g; magnesium carbonate, 124 g; iron sulphate, 20 g; zinc sulphate, 4 g; copper sulphate, 3 g; potassium iodide, 4 mg; cobalt sulphate, 20 mg; manganese sulphate, 3 g; sodium fluoride, 1 g (Granda Zootecnica, Cuneo, Italy).
[2] Vitamin mixture (IU or mg/kg diet): dl-a-tocopherol acetate, 60 IU; sodium menadione bisulphate, 5 mg; retinyl acetate, 15 000 IU; dlcholecalciferol, 3000 IU; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; B12, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium pantothenate, 50 mg; choline chloride, 2000 mg (Granda Zootecnica, Cuneo, Italy).
[3] Liver-protector integrator: Inve Technologies, Dendermonde, Belgium

stains. All the tissue samples were stained with the Mayer hematoxylin-eosin (HE) stain while on the liver samples PAS, PAS diastase and Alcian Blue stains were also carried out. PAS stain was used in order to observe complex carbohydrates such as glycogen, mucopolysaccarides and glycoproteins. PAS diastase stain was necessary to discriminate the PAS positive reaction due to glycogen from other PAS positivity. The Ceroid substances Presence Index (CPI) was calculated as a new parameter as follows: CPI = (number of livers with ceroid substances / total sampled livers). Alcian blue stained acid mucopolysaccharides (as cartilages) acetic mucins, moreover this stain improve the contrast between supranuclear vacuoles and goblet cells.

Scoring Method

In order to adopt an objective measurement of histological modification of intestinal epithelium, two indexes have been used: a semi-quantitative and a quantitative indexes.

Semi-Quantitative Method

The semi-quantitative index has been based on Morris *et al.* (2005) index and six parameters of enteritis were quantified independently, according to: 1) the appearance and length of the mucosa folds (PM); 2) the presence and size of supranuclear vacuoles (VSN); 3) number and position of goblet cells (CCM); 4) the degree of infiltration abundance and of eosinophilic granulocytes into the lamina propria and into the sub-epithelial mucosa (EG); 5) the degree of widening of the lamina propria (LP); 6) the degree of thickening of the sub-epithelial mucosa (SM). Each of these parameters was scored on a scale from 1 to 5. Scores of 1–2 were considered to fall within normal bounds while scores ranging between 3 and 5 were considered to represent significant, well established and substantial enteritis, respectively.

Quantitative Method

The quantitative index has been based on morphometrical evaluations of digital pictures. Sample sections were examined through stereo and light microscopy (Nikon Eclipse 80i), then digital images were taken to document morphology (Leica Q Imaging QICAM Fast 1394 digital camera; Image Proplus ver. 6.0 imaging software). Morphometrical evaluations were carried out on the digital pictures with Image-Pro Plus ver. 6.0, Media Cybernetics. Quantitative index took in consideration following parameters: 1) lamina propria / mucosa folds width ratio; 2) the number of goblet cells in 100 µ of lamina propria; 3) enterocytes / supranuclear vacuoles height ratio. Each of these parameters was analyzed as a ratio.

Statistical Methods

In the first part of data elaboration normal distribution of data was tested using Shapiro Wilks test and homogeneity of variance with Bartlett test. Where utilizable, one-way ANOVA was used for inferential statistics, otherwise non parametric test, Kruskal Wallis test. After the ANOVA, differences among means were determined by the Duncan test comparisons of means, using the significant level of $P<0.05$. For statistical elaboration R software was used (R version 2.5.0, 2007-04-23).

Results

Field Experimentation and Morphological Indexes

No feed reject events were recorded during the trial. During the feed fish trial no mortality was recorded related with the experimental conditions. Sea cucumber meal is characterized by an high percentage of protein and low fat content (Table 2), moreover its high ash content make difficult high level inclusion in fish feed. Considering the morphology of internal organs, no differences have been observed on hepatosomatic index and small difference was found in perivisceral fat deposition (Table 3).

Histology

During the first part of application of semi–quantitative method the six considered parameters has been compared for the 4 experimental groups, following the scores have been summed in order to indicate a total indication of enteritis. Mucosa folds were not different in the experimental groups, consequently data were not shown in the table. This parameter was not affected by experimental treatments. Considering sopranuclear vacuoles, quantitative scores (Table 3) indicate a progressive enterocyte alteration proportional with sea cucumber meal inclusion in the diet and sea cucumber meal fed fish were similar between them and differ from fish meal fed fish. Semi - quantitative scores (Table 3) showed similar difference in sopranuclear valcuoles score, even if not statistically significant. In Figure 1 the enterocyte vacuolization is shown in a distal intestine section, Figure 2 shows sopranuclear vacuoles in the mucosa fold of F20 fed fish intestine.

Table 2. Sea cucumber meal composition

Proximate composition (% DM)	*H. polii*	*H. tubulosa*
Dry matter	22.03±3.07	16.09±1.51
Crude protein	36.99±0.62	44.58±1.01
Ether extract	0.55±0.12	0.71±0.12
Ash	48.22±1.09	46.43±0.51
Gross energy (MJ/kg DM)	8.1±0.2	9.7±0.21

Mean±sd; n =4

Table 3. Semi – quantitative and quantitative scores in histological measures and morphometric indexes

	FP	FO10	FO15	FO20
Sopranuclear vacuoles (VSN)	1.00±0.0 $^{n.s.}$	1.33±0.5 $^{n.s.}$	1.38±0.5 $^{n.s.}$	1.25±0.5 $^{n.s.}$
Goblet cells (CCM)	1.13±0.4 $^{n.s.}$	2.00±0.9 $^{n.s.}$	1.63±0.7 $^{n.s.}$	2.13 ±1.0 $^{n.s.}$
Eosinophil granulocytes (EG)	1.00±0.0 $^{n.s.}$	1.00±0.0 $^{n.s.}$	1.00±0.0 $^{n.s.}$	1.13±0.4 $^{n.s.}$
Lamina propria (LP)	1.13± 0.4 $^{n.s.}$	1.33±0.5 $^{n.s.}$	1.5±0.8 $^{n.s.}$	1.38±0.7 $^{n.s.}$
Sub-epithelial mucosa (SM)	1.00±0.0 $^{n.s.}$	1.33±0.5 $^{n.s.}$	1.25±0.7 $^{n.s.}$	1.00±0.0 $^{n.s.}$
Enterocytes / supranuclear vacuoles	46.4±11.8^{a}	35.9±6.4^{b}	36.1±7.7^{b}	36.1±8.1^{b}
Number of goblet cells /100 µ of lamina propria	0.18 ±0.1$^{n.s.}$	0.16±0.0 $^{n.s.}$	0.26±0.2 $^{n.s.}$	0.24±0.1 $^{n.s.}$
Lamina propria / mucosa folds	33.1±16.4 $^{n.s.}$	34.8±3.9 $^{n.s.}$	33.9±11.5 $^{n.s.}$	32.02±10.8 $^{n.s.}$
Enteritis score	6.13±0.35 ^{a}	7.0±1.6 ^{b}	7.38±1.77 ^{b}	7.88±1.89 ^{b}
HSI (hepatosomatic index)	0.01±0.003^{ns}	0.01±0.006^{ns}	0.01±0.003 ^{ns}	0.01±0.003 ^{ns}
VSI (viscerosomatic index)	0.07±0.02^{a}	0.06±0.01^{b}	0.06±0^{b}	0.07±0.01^{ab}

In the rows, different letters mean statistical difference at P<0.05
Mean±sd; n=8

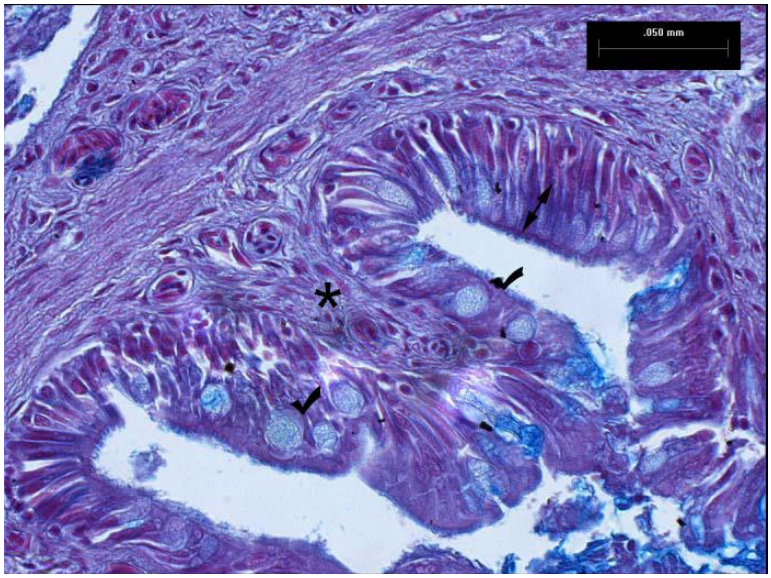

Figure 1. Distal intestine section of fish fed FO20 diet (Alcian Blue stain); asterisk indicates lamina propria; "✓ " indicates mucipar caliciphorm cells that are regularly disposed between enterocytes; double arrow indicates enterocyte vacuolization.

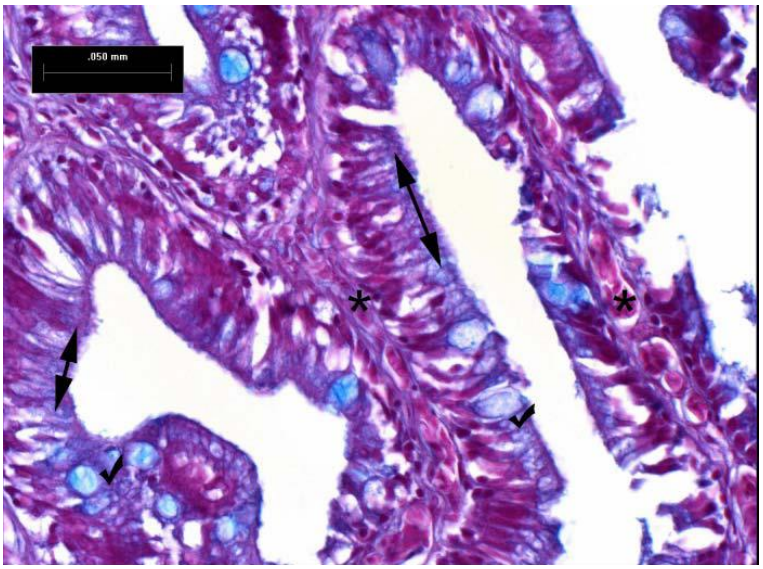

Figure 2. Intestine section of FO20 diet fed fish (Alcian Blue stain). Asterisk indicates lamina propria layer; flag symbol indicates caliciphormes mucipar cells and the surrounding enterocytes; double arrows indicate sopranuclear vacuoles longitudinally disposed along the mucosa fold.

The number of goblet cells increased in the fish fed with sea cucumber meal and this is visible both in the semi–quantitative and in the quantitative scores, even if values were not proportional with sea cucumber meal inclusion in fish feed. Eosinophil granulocytes did not show particular differences in the experimental groups with the exception of FO20 group where some fish showed a small increase (Table 3; Figure 3).

All the considered samples did not show any remarkable alteration of lamina propria as showed by semi – quantitative and quantitative scores (Table 3), a small difference is only visible in the relation between lamina propria and mucosa folds in the FO10

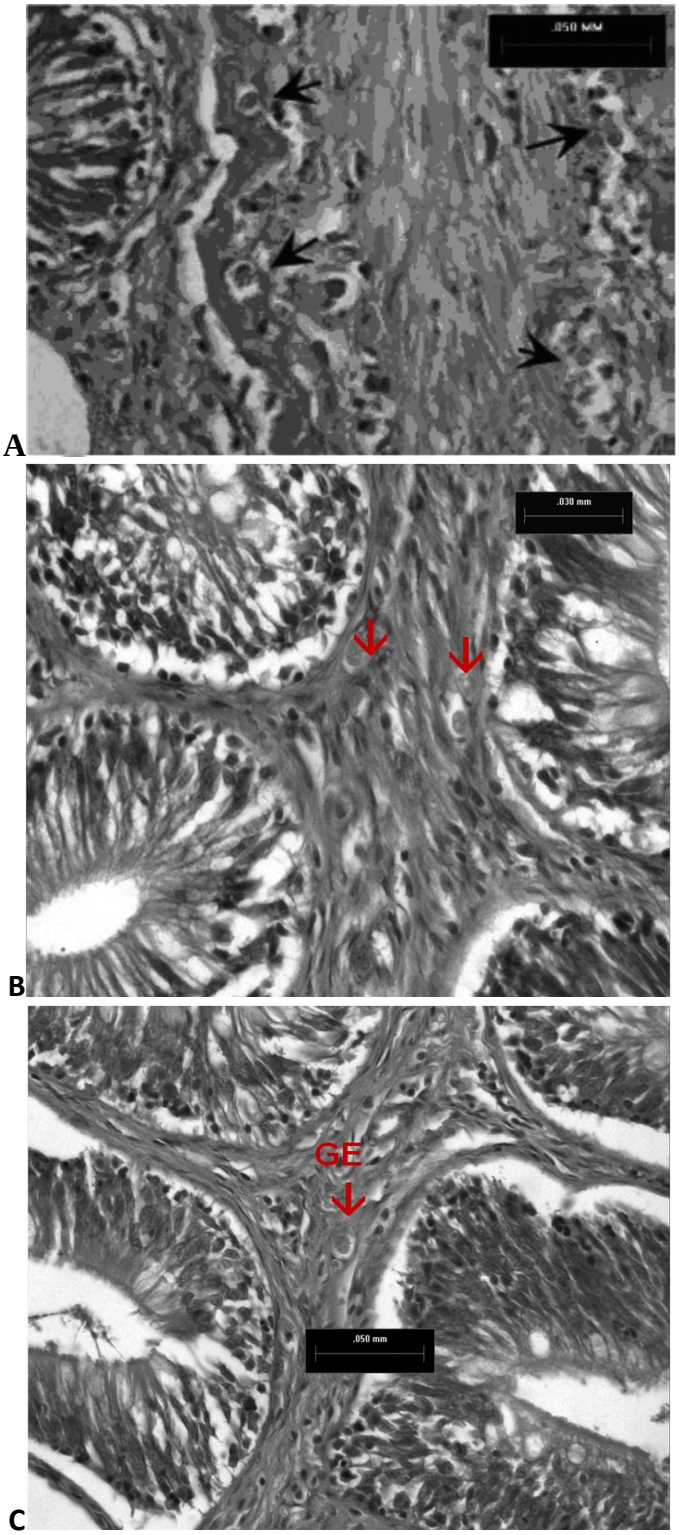

Figure 3. Distal intestine sections from fish fed FO20 diet. Black arrows indicate eosinophil granulocytes with evident granules in the submucosa layer (HE stain).

and FO15 groups (Table 3; Figure 4) that could indicate a slight inflammation.

Figure 4b shows a histological section of distal intestine of FO10 fed fish, where mucipar caliciphorm cells are grouped. Sub–epithelial mucosa status showed a little widening in the FO15 and F10 group of fish (Table 3) even not statistically significant. Liver histological sections did not show any difference between experimental treatments. Finally the enteritis score, based on the histological scores previously measured, has been calculated and this parameters showed a slight enteritis status in fish fed with sea cucumber meal, independently from inclusion level (Table 3).

Discussion

The major finding of this experimentation was that dietary supplementation of sea cucumber meal obtained by most common Mediterranean species of sea cucumber did not induce severe histopatological change in the rainbow trout intestinal tract. Currently, soybean meal is the main substitute of fish meal in aquafeeds (Ustaoglu and Rennert, 2006) and considering that soybean induced enteritis represents a physiological reference model for the studies on inflammation of digestive system of farmed fish, in this study the same histological approach and quantitative methods (Uran, 2008; Uran *et al*., 2008; Refstie *et al*., 2010) has been used. In particular, considering its effectiveness, the scoring method proposed by Urán *et al*. (2004) has been used in this study with a little modification that considered the relation between sopranuclear vacuoles and enterocytes. This modification has been introduced here in order to decrease the variability of sopranuclear vacuoles that is highly influenced by fish age and diet. Sea cucumbers, as other echinoderms, are characterized by the presence of internal endosckeleton made by microscopic ossicles. These ossicles are embebbed in the sea cucumber body and are responsible of high quantity of ash content in sea cucumber meal. Even if its high protein, low fat and antioxidant content (Zhong *et al*., 2007) make sea cucumber meal a potential protein source for fish feeds, the calcareous ossicles of sea cucumber could

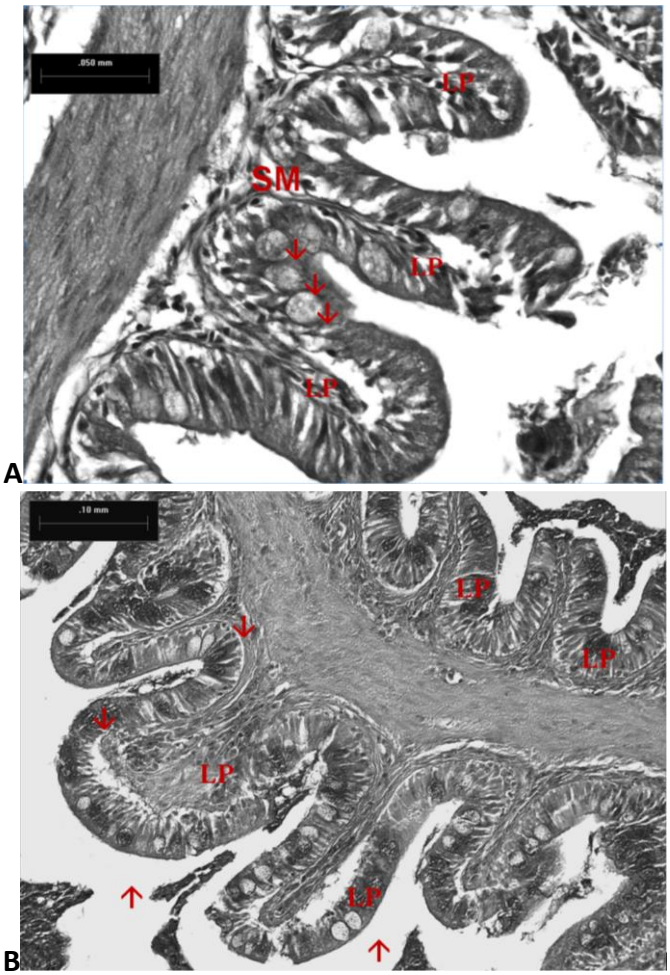

Figure 4a. Distal intestine section sampled from fish fed FO10 diet (Alcian Blue stain). Black arrows indicate caliciphormes mucipar cells where mucins absorbed Blue stain. LP indicate lamina propria. SM indicates submucosa layer comprised between basal portion of mucosa folds and muscularis mucosae. **4b.** Distal intestine of FO10 diet fed fish (Alcian blue stain). LP indicate lamina propria of normal intestinal villi, black arrows indicate mucipar caliciphorm cells that are grouped and more numerous.

exert an anti-nutritional effect on fish. Sea cucumber meal in this moment is not commercially available, the use of this feedstuff for fish feeds proposed in this research could appear a curious hypothesis, but sea cucumbers utilized in this research are very common around Mediterranean coasts and in this moment this is almost unexploited and low cost resource. Considering that they are animal marine organisms it is likely that their inclusion in fish feeds will be more feasible respect to vegetal protein that are commonly used in fish feeds in this moment. The only perplexity related with sea cucumber meal utilization is the high ash content and consequent negative effect of high concentration of mineral in fish feeds, even if the effect of sea cucumber inclusion could potentially decrease the mineral inclusion in future fish feeds. In this experimentation the size of liver and intestine did not show relevant differences between fish groups and this was first indication of absence of negative effect of sea cucumber meal inclusion in trout feeds. Previous researches stated that soybean enteritis in Atlantic salmon induces a decrease of the supranuclear vacuoles present in normal enterocyte (Kroghdal *et al*., 2003; Knudsen *et al*., 2007; Uran *et al*., 2008), in this case sopranuclear vacuolization was a little bit higher in the sea cucumber fed fish, however the average score measured for sopranuclear vacuoles in this study (1, 2) is in the physiological range and was lower than 3, 8, which is considered the minimum sign of enteritis in fish (Uran *et al*., 2008). In general, the scores obtained in this study are comparable with those found in control treatments in similar studies (Sanden *et al*., 2005; Knudsen *et al*., 2007; Uran *et al*., 2009). Considering goblet cells, it is clear that there is a score increase proportional with sea cucumber inclusion in the fish feed, but the average score measured (1, 7) is still the half of that considered enteritis sign (3, 5) (Uran *et al*., 2008). The increase of goblet cells, mainly in the distal intestine, can be related with a mechanical irritation caused by the presence of calcareous ossicles in sea cucumber meal. Similarly to previous researches where absence of inflammation was recorded (Morris *et al*., 2005), in this study, lamina propria, sub – epithelial mucosa and mucosa folds did not change in the experimental treatments, thus confirming the absence of inflammation. The main results obtained in this research indicate that this sea cucumber meal could be used in future fish feeds. Secondarily, this research offers some interesting indications for future application of quantitative methods used in the histology of fish intestinal tract, particularly in the investigation of diet-induced inflammations. From the point of view of fish feed formulation, the high ash content hampers the future sea cucumber meal inclusion in fish feed and 20% inclusion utilized in this research is the maximum level possible for a balanced feed for fish feed, further researches are necessary in order to by pass this obstacle.

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