FINAL REPORT

Determine optimal levels of fishmeal/fish oil replacement with soy products (soybean meal, soybean oil and soy protein concentrate) in practical feeds for Gilthead sea bream (*Sparus aurata*)

Scientific team

Caditec Testing – Spain:
- Rocio Robles, Lic – Technical director
- Jurgen Adriaen, Ir. - Biologist

INVE Aquaculture Nutrition – Belgium
- Sam Ceulemans, Ir. – Coordinator R&D Feedmill Nutrition
- Alexander Van Halteren, Ir. – Project Coordinator Feedmill Projects
- Peter Coutteau, PhD – Product Manager Feedmill Nutrition

Executive summary

A feeding trial with Gilthead seabream, the main species of marine fish produced in the Mediterranean region (>120 000 MT per year) was performed to evaluate the effect of different inclusion levels of soybean products (defatted soybean meal, soybean protein concentrate and soybean oil) as fish meal/oil replacement. The inclusion levels of soybean products were 18% for the control diet (high quality feed with 65% of the protein originating from fishmeal), 31-34% for the treatments with 50% of the protein coming from fishmeal and up to 46% for the treatments where only 35% of the protein was provided by fishmeal. Dietary formulation was adapted to compensate for effects of soybean inclusion on nutritional and palatability characteristics of the feeds.

Seabream were fed the experimental feeds during 8 weeks and parameters followed included growth, survival, food conversion, protein efficiency ration, carcass composition, filet composition, hepatosomatic and viscerosomatic indices, filet index, liver composition and gut histology.

Results indicated that none of the evaluated parameters was significantly affected by the different replacement levels compared to the control. Neither growth nor food conversion were affected by the inclusion of high levels of soybean products. Histological study of the intestinal epithelium did not show any pathological sign due to the replacement.
It can be concluded that for Gilthead seabream, the replacement of fish meal with soybean meal under the conditions of the trial, is perfectly feasible without affecting the performance of the fish during the culture.

1 Objective

Gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) are the main species of marine fish produced in the Mediterranean region, totalling close to 200,000 MT in 2006 with about 60/40 % distribution among seabream and bass.

The main objective of the present study is to determine the optimal inclusion levels and value of soy bean products (defatted soybean meal SBM, soybean oil SBO and soy protein concentrate SPC of U.S. origin) as fish meal/fish oil replacers in practical diets for *S. aurata*. The effect of fish meal replacement with soy products on growth, survival, HSI, VSI, liver histology, FCR and PER will be evaluated under controlled lab scale conditions.

2 Materials and methods

2.1 Feed Preparation

Feed preparation was subcontracted to INVE Aquaculture N.V. Belgium which is producing the feeds in their pilot extrusion plant for specialty fish feed, including:

- Fresh ingredients from the factory stock passed through INVE quality control (physical, chemical, legal aspects..)
- Pulverization of coarse ingredients
- Mixing ingredients with double shaft paddle mixer
- Cooking-extrusion/drying on twin-screw Buhler extruder line on required pellet size
- Vacuum coating of oil phases
- Quality control (including analysis of feed specifications : C Protein/C Fat after hydrolysis/ash/moisture)
- Packaging and transport by courier

Formulations tested were based on:

- 45/20 protein/fat specification; although there is wide variation among regions/farmers, this levels are generally accepted for high performance feeds for marine fish (bass and bream) in European cage farming
- actual average ingredient prices offered by suppliers to European feed mills
- experience with different qualities/performances of practical feed formulations in the marine fish feed market in Europe
- the general state-of-the-art in the field and academic studies on the limits of using SBM as a fishmeal replacement and the expectations that a mixture of SPC/SBM is a promising FM replacer
- practical formulation experience to compensate low fishmeal formulations with amino acids, available phosphorous and attractants

It should be noted that the cost comparisons between formulations may vary in function of market fluctuations of ingredient prices. Detailed formulations and cost structure information is presented in Table 1.

Table 1: Feed formulations and cost structure:

Ingredient CP/CF	Ingredient cost €/MT	Inclusion %					
		FM65	FM50 1	FM50 2	FM35 1	FM35 2	FM35 3
S Am Fish Meal 64.5/8.6	1,270	45.3%	34.8%	34.8%	24.4%	24.4%	24.4%
Defatted Soybean Meal 49.2/1.7 - Provasoy	230	18.3%	22.5%	10.0%	35.0%	22.5%	
Soya Protein Concentrate 65/1.5 - DANPRO A IP (Solae)	700		7.2%	16.7%	4.6%	14.1%	31.2%
Corn Gluten 60/7	510	4.0%	4.0%	2.0%	4.3%	5.0%	4.2%
Wheat Gluten 78.6.6	860	3.8%	1.1%	1.0%	1.0%	1.0%	1.0%
Pea Protein Concentrate 78/8.5	850				4.7%	2.0%	
Rapeseed Meal 36/2.4	200		5.9%	9.0%		4.9%	9.7%
Wheat Flour	180	13.1%	7.0%	8.9%	7.0%	7.0%	10.3%
Vitamin/Mineral Premix	3,500	0.50%	0.50%	0.50%	0.50%	0.50%	0.50%
Lysine 78%	1,350		0.10%	0.10%	0.15%	0.15%	0.15%
Methionine	2,300		0.10%	0.10%	0.10%	0.10%	0.10%
Mono Calcium Phosphate	380		0.20%	0.20%	0.40%	0.40%	0.40%
Attractant (Aquaflavour MF)	3,200		1.00%	1.00%	2.00%	2.00%	2.00%
Fish Oil	900	15.0%	11.5%	11.7%	9.8%	9.9%	10.1%
Soybean Oil	650		4.0%	4.0%	6.0%	6.0%	6.0%
SUM		100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
€/MT		847	783	820	721	750	813
formula cost difference with FM65 - €/MT			**-64**	**-26**	**-126**	**-97**	**-34**
formula cost difference with FM65 - % of formula cost FM65			**-7.5%**	**-3.4%**	**-15.3%**	**-13.5%**	**-4.5%**
inclusion soybean products - %		**18%**	**34%**	**31%**	**46%**	**43%**	**37%**
value soybean products - €/MT		42	128	166	152	190	257
value soybean products - % of value in FM65			**305%**	**394%**	**362%**	**451%**	**612%**
value soybean products - % total formula cost		**5%**	**16%**	**20%**	**21%**	**25%**	**32%**
Analytical values (formulation matrix per kg)							
DM g		924	925	923	929	926	920
CP g		450	450	450	450	450	450
CFIBRE g g		10	21	25	16	23	30
CFAT hydr. g		200	200	200	200	200	200
CASH g		95	91	92	81	82	82
STARCH g		91	64	74	66	70	89
LYS g		30	30	31	30	30	30
MET g		11	11	11	11	11	11
n-3 HUFA g		50	39	39	32	32	32
PHOSPHORUS g		13	13	13	12	12	12
Ca g		17	15	15	11	12	12
Vit E per kg IE		250	250	250	250	250	250
VIT C mg		250	250	250	250	250	250
Protein distribution by origin (% of total protein)							
Fishmeal CP %		65.0%	50.0%	50.0%	35.0%	35.0%	35.0%
Soya CP %		20.0%	35.0%	35.0%	45.0%	45.0%	45.0%
Viten CP %		6.6%	2.0%	1.7%	1.7%	1.7%	1.7%
Corn %		5.3%	5.3%	2.6%	6.0%	6.6%	5.5%
Pea Prot %					8.2%	3.5%	
Canola Prot %			4.9%	7.3%		4.1%	8.0%

2.2 Experimental design

The set up used for the trial consisted in a R.A.S (recirculating aquaculture system) with 18 cylindrical fibre glass tanks with a working volume of 600 l, with well seawater of 22 ± 1°C and salinity of 37 ± 1 g/l. The system had a renewal of 10-15 % per day depending on the water quality values and a flow rate of 6-7 l/tank/min. Initial density was 1.91 Kg/m^3, with 40 individual per tank. The tanks were connected to a biofiltration unit of 3 tanks with different biofilter substrates (rigid plastic mesh, blue sponge and moving plastic beds) and to a protein skimmer; a swirl separator is also part of the RAS. Photoperiod was set to have 12 h of light (12L/12D); feeding (with automatic belt feeders) lasted also 12 h (8.00 h to 20.00 h). Experimental fish, *S. aurata* fingerlings, were obtain from a local hatchery and grown up till test size in Caditec Testing S.L. facilities.

Trial duration was 10 weeks. Water quality (T.A.N, nitrite) was checked three times per week. Temperature and dissolved oxygen was checked daily.

- Feeding

The fish were fed with automatic belt feeders, depositing the daily feed ration on the belt feeder at first hour in the morning (working days and Saturdays). Automatic belt feeder was set to work during 12h /day (8.00- 20.00) together with illumination.. The first two weeks the fish have received an acclimation diet, the same for all the tanks.

The daily feed ration was calculated following feeding tables for this size of fish. Each tank had received a fixed % feeding ration in function of its biomass; the same % of tank biomass was applied to all the tanks.
Not eaten pellets were collected per tank twice per day and the feed intake was corrected accordingly.

- Stocking and sampling

For all the handling of the animals, phenoxyethanol has been used as anaesthetic (recommended dose 0.25ml/l) .Before stocking fish were sampled (weight in water) to determine size distribution. Stocking has been done sequentially. Diets were assigned to the tanks at random.
Sampling happened every two weeks; for the sampling, all the fish/tank have been weighed in groups and carefully observed to check the health status of the animal. From this sampling, average weight and survival have been determined and feed ration adjusted accordingly every two weeks.
At the final sampling fish have been weighed individually and 3 individuals per tank (9 per treatment) have been picked randomly for dissection to determine HSI/VSI index and collect samples for liver and carcass for further analysis (for carcass analyses 5 individuals per tanks were selected).

- Evaluation Parameters

- Specific Growth Rate (SGR): Ln(final weight/initial weight)*100/days of feeding), (%/day)
- Survival , %
- Food conversion ration FCR (feed intake/wet weight gain)
- Protein efficiency ration, PER (wet weight gain/protein intake)
- Carcass composition at the end of the trial (Protein, Fat after Hydrolysis, Moisture, Ash content)
- Filet composition (Protein, Fat after Hydrolysis, Moisture)
- Hepatosomatic index HSI (liver weight*100/total weight) and Viscerosomatic index, VSI (viscera weight*100/total weight)
- Filet index, FI (filet weight*100/total weight)
- Liver analysis
- Gut histology
- Water quality parameters

3 Results

Growth results

The fish were 10 weeks in the experimental system; the first two weeks have been considered as acclimation period and experimental feeds have been provided during the next 8 weeks.

The results presented here correspond to the 8 weeks of feeding experimental diets. The data presented are the average of three replicates per treatment.

Concerning growth all the treatments have shown very similar results in terms of weight gain (g/fish), SGR (specific growth rate) and FCR (see Fig.1 and Table 1). The growth results presented in the graph are a confirmation that at any moment of the trial, a difference among diets could be found.

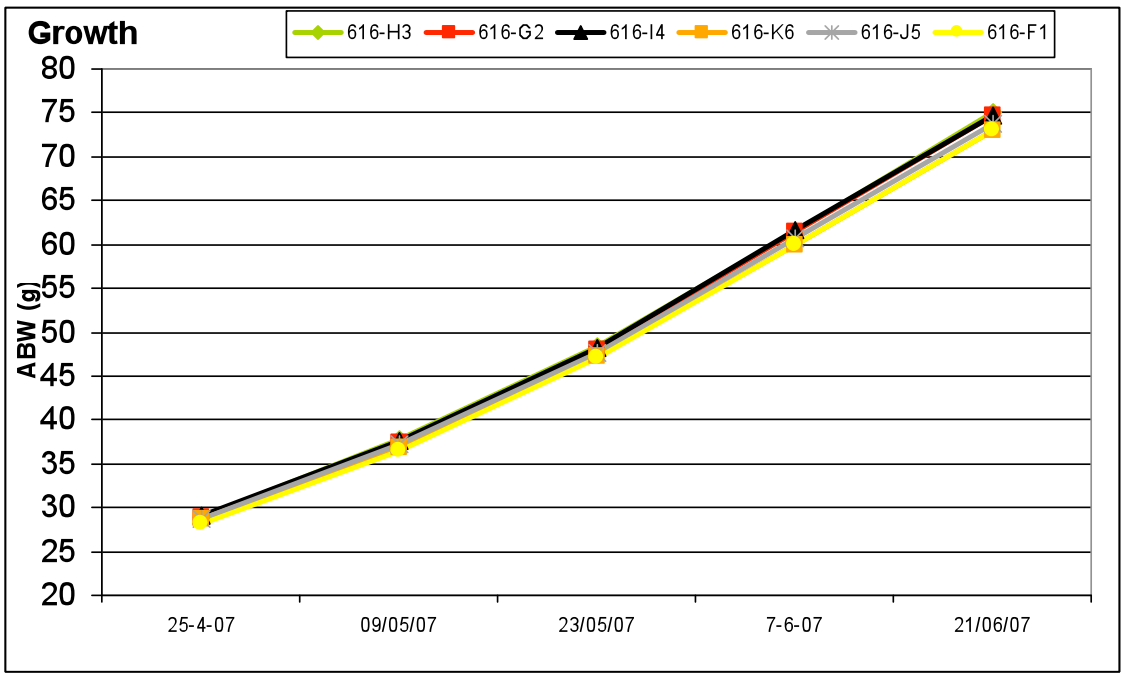

Fig. 1.-Growth curve of the seabream during 8 weeks of feeding experimental diets

No mortality was observed during the trial and the fish did not show any signs of pathologies or damage at any moment. The fish presented a very good external aspect and colouration (Table 1). Feeding behaviour was good and the amount of not eaten feed was nil. Feeding tables were adapted after each sampling. FCR was quite similar among the treatments (Table 1). After 10 weeks of trial (8 weeks feeding experimental diets) and for this size of fish, the general performance can be considered as very good in terms of SGR and FCR. Concerning HSI and VSI, the values are within the range of the normal values for the species and no statistical difference among treatments was found (Table 2). For the filet index, FI, the values found were similar for all the treatments and no significant difference.

Table 2

Diet	616F1 FM65	616G2 FM50-1	616H3 FM50-2	616I4 FM35-1	616J5 FM35-2	616K6 FM35-3
Survival (%)	99.33±1.15	99.33±1.15	100.00±0.0	100.00±0.0	100.00±0.0	99.33±1.15
Initial weight (g)	28.09±0.86	28.88±1.13	28.96+0.33	29.07±0.99	28.74±0.58	28.65±0.38
Final weight (g)	72.91±1.26	74.77±2.61	75.02±0.89	74.62±2.61	73.71±2.76	72.98±0.54

Weight gain (g)	44.83±0.60	45.89±2.65	46.06±0.85	45.55±1.66	44.97±2.30	44.33±0.26
SGR (%/d)/ind	1.67±0.03	1.67±0.03	1.67±0.02	1.65±0.01	1.65±0.04	1.64±0.01
Total feed/ind (g)	49.11±0.93	50.17±2.55	50.23±0.65	50.22±1.44	50.18±1.03	49.37±0.67
Feed intake (%ABW/d)*	1.71±0.02	1.70±.0.01	1.69±0.01	1.70±0.01	1.72±0.03	1.70±0.01
FCR	1.10±0.02	1.09±0.02	1.09±0.02	1.10±0.01	1.12±0.04	1.11±0.01
PER	1.89±0.04	1.88±0.02	1.86±0.02	1.86±0.00	1.88±0.02	1.88±0.01
HSI	1.27±0.07	1.23±0.12	1.09±0.07	1.16±0.19	1.16±0.17	1.28±0.03
VSI	5.30±0.21	5.39±0.32	5.55±0.46	5.62±0.16	5.68±0.45	5.33±0.41
Filet index	19.95±0.43	20.08±0.52	20.07±0.76	19.34±0.37	19.63±0.82	19.76±0.34

* Feed intake expressed as percentage of average body weight per day

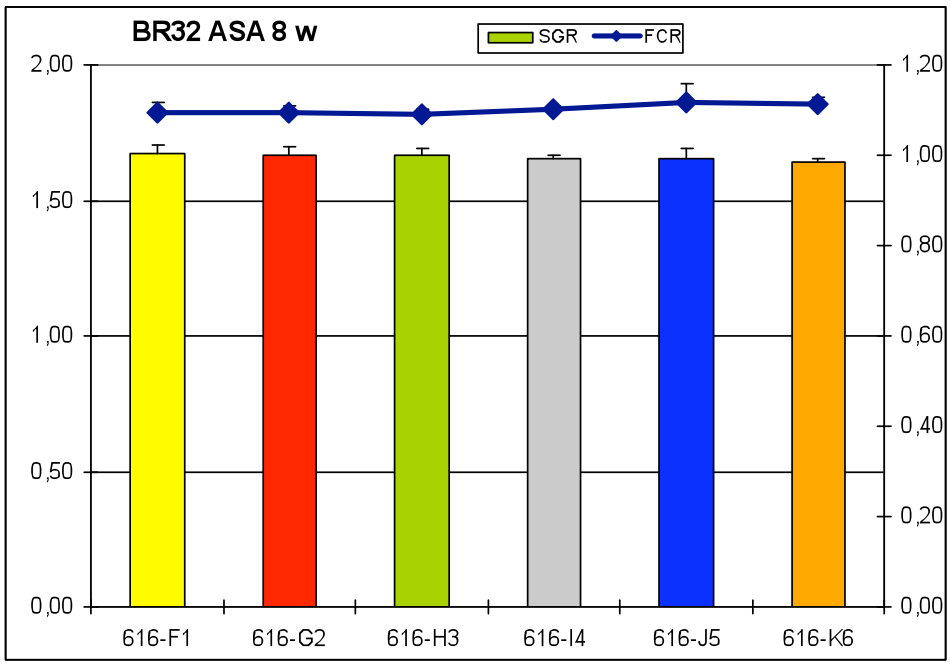

Fig.2- SGR and FCR of seabream fed the different diets

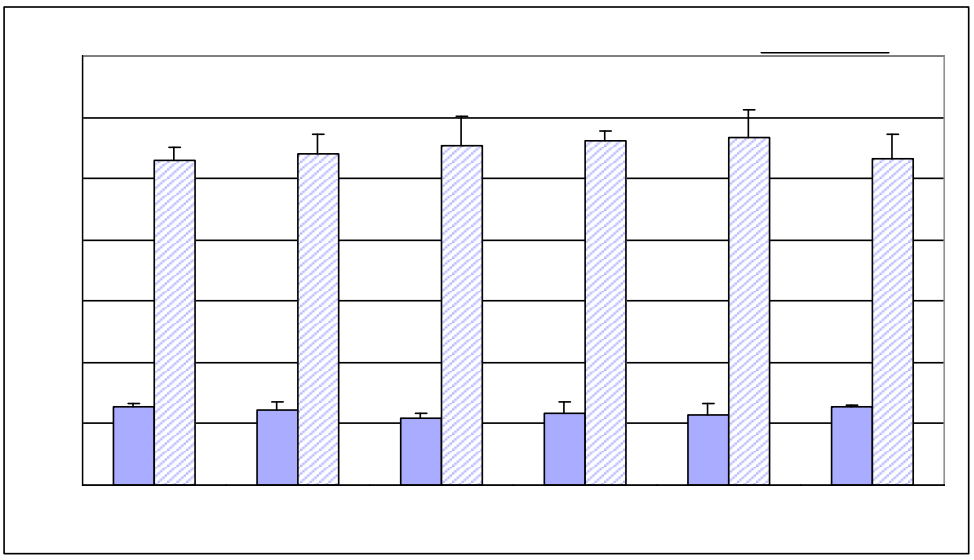

Fig. 3.- HSI and VSI of the seabream fed the different diets; values are the mean of 9 fish per treatment for both indices.

Filet and liver analyses

Concerning proximal composition of the filet and liver the results are presented in Table 3. No significant difference was found among the treatments in filet composition or in liver composition (*p<0.05)*. DM value for the filet of 616F1 diet is lower (29.89 % ww) than for the rest of the diet.

Table 3

Diet		616F1 FM65	616G2 FM50-1	616H3 FM50-2	616I4 FM35-1	616J5 FM35-2	616K6 FM35-3
FILET %ww							
	Dry matter	29.89±0.37	31.35±1.33	31.29±0.73	30.88±0.69	30.81±0.90	31.11±1.62
	Crude Protein	19.43±0.37	19.43±0.39	19.64±0.36	19.40±0.53	19.92±0.30	19.72±0.48
	Crude fat	10.06±1.18	10.10±1.56	9.85±0.48	10.05±1.48	10.06±2.07	10.67±2.07
	Crude ash	1.61±0.05	1.64±0.20	1.72±0.17	1.67±0.16	1.66±0.23	1.59±0.07
LIVER %ww							
	Dry matter	36,04±3.22	38,22±3.01	39,64±2.65	38,77±0.10	38,03±1.01	39,66±1.40
	Crude fat	14,51±3.16	16,74±3.77	19,11±3.77	18,70±0.26	17,31±0.91	19,70±2.18

Regarding DM in the liver, the values ranged from 36.04 % ww for 616-F1 (FM65 diet) to 39.66 % ww for the diet 616-K6 (FM35-3 diet). CF in the liver, ranged from 14.51 % (616-F1 diet) to 19.70 % (616-K6). Although there is an increase of fat content in the liver with the increased FM replacement, no statistical difference was found among treatments.

Histological analyses

In general the histological analysis does not show major morphological changes due to the inclusion of SBM in the diet for seabream (full report is included as Annex I). Nine samples per diet were evaluated and the general overview of the intestine samples is good and can be considered normal for the species. Only in same specific cases, some alterations were found in the thickness of the submucosa but none of the findings can be regarded as criterion of intestinal damage.

ANNEX I

SDPP
SERVEI DE DIAGNÒSTIC PATOLÒGIC EN PEIXOS
FACULTAT DE VETERINÀRIA DE BARCELONA

Servicio de Diagnóstico Patológico en Peces
Facultad de Veterinaria.
Universitat Autònoma de Barcelona
08193 Bellaterra (Barcelona)
Tel. (+34) 93 5812817
Fax (+34) 935812006
e-mail: francesc.padros@uab.es

Samples arrival date: 26 /07/2007
Report submission date: 15/10/2007
Sample code: CADITEC TESTING VEGETAL FLOUR SUBSTITUTION TRIAL
Customer code CADT0707(1)
Sample type: intestine
Species: dorada / sea bream *Sparus aurata*

PREVIOUS REPORT

A total number of 54 samples, distributed in 18 trial groups (Tanks 1 to 18) were submitted to our laboratory. Each test group was composed by 3 different subsamples, corresponding to sea bream intestines fixed in 10% buffered formalin.

MATERIAL AND METHODS

Each intestine sample was cut in 6 portions of about 5 mm each, taken at the same distance between the two edges (anterior and posterior intestine). All the six intestine pieces were allocated in a histology cassette with the corresponding sample code and processed in paraffin according to the routine laboratory protocols. Blocks were sectioned at 4 microns and stained with haematoxylin-eosin.

The samples were evaluated using a random and "blind" system, this means, the code of the sample was not known at the moment of the evaluation of the sample.

Once the results were obtained, the codes were reassigned to the submission codes and grouped again for the final evaluation. Finally, when the final evaluation was done, Caditec

Testing sent to this laboratory the original codes in order to group the results in the different feeding tests.

STUDY RESULTS

TANKS 6, 12 and **18 DIET 616-F1 (FM65)**

- TANK 6: in two of the samples, an increased thickness in the apical zone of the intestinal folds was noticed. These modifications were similar to those observed in other samples, like in T1. The other sample shows a normal aspect.
- TANK 12: no significant alterations. Normal
- TANK 18: no significant alterations. Normal

TANKS 2,8 and 14: **DIET 616-G2 (FM50-1)**

- TANK 2: similar to the description given in Tank 1, in 2/3 samples, an increase of the thickening of the submucosa (oedema?) was noticed in the apical tips of the intestinal folds. In one of these samples, a certain epithelial desquamation was also observed.
- TANK 8: no significant alterations. Normal.
- TANK 14: Two of the subsamples show a normal structure, but one of the samples present clear alterations (mainly necrotic and degenerative changes, with epithelial desquamation) in the apical zone of the folds. The basal area of the epithelium does not present relevant changes.

TANKS 1,7 and 13: **DIET 616-H3 (FM 50-2)**

- TANK 1: In the 3 samples, a certain thickening (oedema?) in the apical zone of the folds was noticed, together with some epithelial desquamation. The main characteristics of the lesions do not indicate that these alterations are an artefact-related change and should be considered as a potential criterion for intestinal damage.
- TANK 7: In this case, thickening of the folds is only observed in one sample. The other two subsamples do not show significant alterations.
- T13: No significant alterations. Normal

TANKS 3,9 and 15: **DIET 616-I4 (FM35-1)**

- TANK 3: Only in one of the subsamples some epithelial alterations have been found. The epithelium shows some discontinuities in the arrangement of the enterocytes and some cellular desquamation, showing a slightly different aspect when compared to the other two subsamples. (Figure A)
- TANK 9: No significant alterations. Normal
- TANK 15: No significant alterations. Normal

TANKS 5, 11 and 17: **DIET 616-J5 (FM35-2)**

- TANK 5: No significant alterations. Normal
- TANK 11: No significant alterations. Normal
- TANK 17: In one of these samples, a hyaline deposit is observed in the intestinal submucosa (figures E and F). This material is found in only one of the six portions of intestine. This material is surrounded by a mild lymphocytic inflammatory reaction and also by a slight granulomatous reaction. As it is only a very circumscribed lesion, it seems that could be related to some kind of foreign body or bodies in a reabsortive process that maybe penetrated into the submucosa from the intestinal lumen. These findings, like torsions, volvulus and invaginations are not unusual in sea bream.

TANKs 4,10 and 16: **DIET 616-K6 (FM35-3)**

- TANK 4: No significant alterations. Normal.
- TANK 10: No significant alterations. Normal.
- TANK 16: No significant alterations. Normal.

COMMENTS ABOUT THIS STUDY

As a general comment, it should be stressed that no clear and significant alterations has been found in the intestine samples of the fish fed with the different diets and the changes that occasionally has been observed does not seem to have important presentation frequencies in the diet groups.

In the A group (control diet) in two of the samples (2/9) a certain increase of the thickness of the submucosa was found. These observations are usually observed in the diagnostic on fish intestine samples and in our opinion, these alterations are probably due to an artefact during the fixation process or sample processing. In this experiment, the same type of alteration has been found in the intestine of fish fed with other diets (Figure A), so the diagnostic value of this modification seems to be low.

In the diet group B, even though most of the samples does not show alterations (including submucosal thickness), in two of these samples we have found some kind of modification in the intestinal mucosa that can be associated to necrotic (or even apoptotic) phenomena, associated to intestinal desquamation (Figures B, C and D). Although these lesions are only observed in two of the subsamples, similar lesions have been found in problems associated to enteropathic lesions induced by the inclusion of several levels of soya meals in the diet f the Atlantic salmon (Baeverfjord & Krogdhal, 1996). In similar experiences, increasing levels of PCNA (*proliferating cell nuclear antigen*) (Sanden et al, 2005) has been found associated with toxic-type changes in the intestinal mucosa due to these diets. In any case, these observations should not be considered as clear indications of diet-associated alterations if other dysfunction indicators such as changes in growth ratio, conversion index, etc.) are found in the same feeding regime groups.

In diets C and D, only in one case, lesions suggesting damage or alteration of the intestinal mucosa have been found. The comments in this case are the same as in diet B, but in this case with a lower occurrence. In any case, in all the cases, these alterations were not extensive to all the different sections of intestine and usually only one or two portions showed these features, so artefacts can not be discarded. However it should be stressed that in an overall evaluation, the quality of the samples for histology was excellent.

In E and F diets, no significant alterations were observed.

To sum up, we can indicate that, except for some few specific cases in diets B, C and D, the general overview of the samples of intestine is good and can be considered as normal. No important morphological changes similar to those described concerning the potential toxic or allergic changes induced by some row material in the diets for Atlantic salmon, such as presence of vacuoles or lipid deposits into the cytoplasm of enterocytes, increase of the

number of mucous cells or alterations of the microvilli (van den Ingh et al (1991) and Bakke-McKellep et al. (2000)) have been observed in these samples. No significant changes in the submucosa such as increase of the presence of inflammatory cells (macrophages, granulocytes or lymphocytes) have been found.

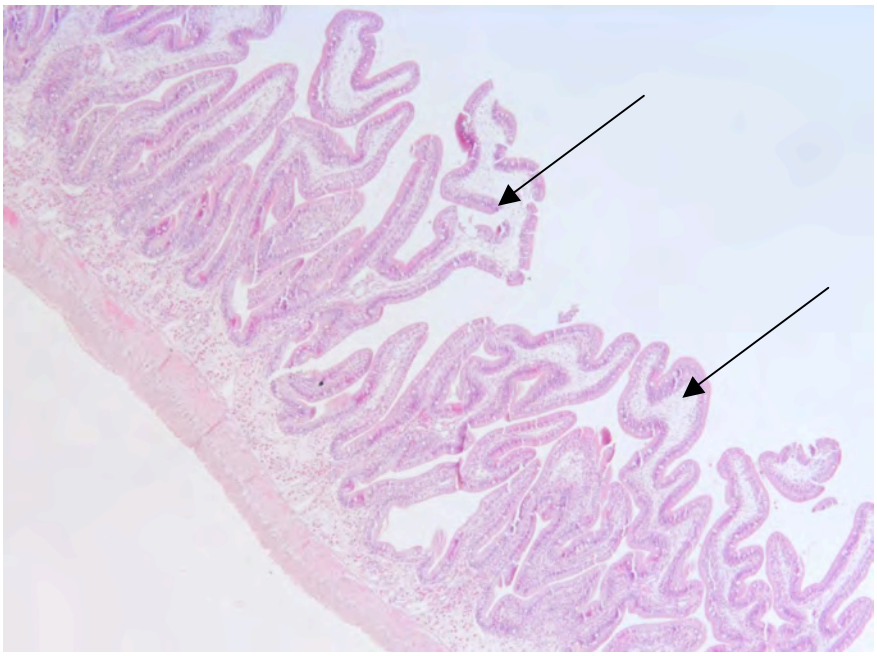

Figure A: AT9F3. Increased thickness (arrows) in the apical zone of the intestinal mucosa (X40)

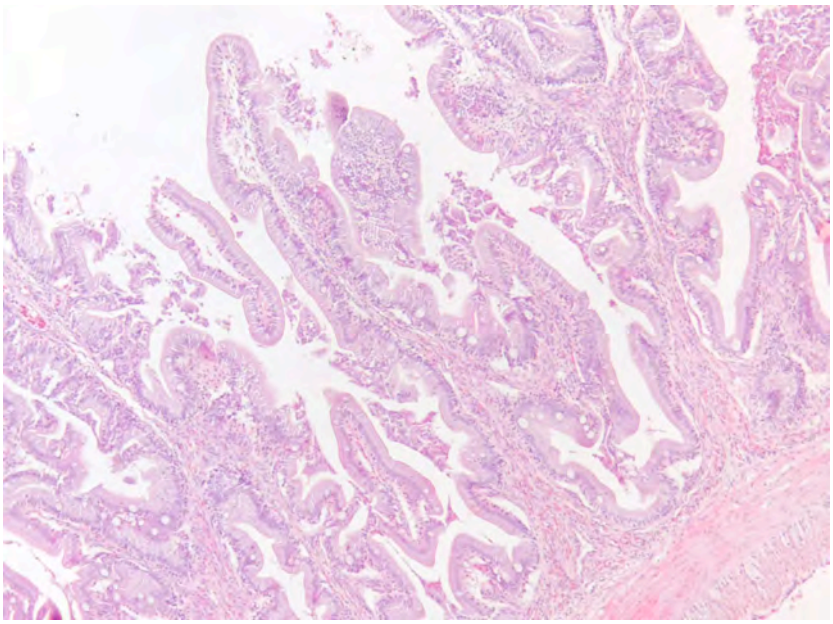

Figure B: AT3F3: Extensive epithelium alteration. Notice the groups of desquamated intestinal cells. The general structure of the epitheliums seems to be altered. (x40)

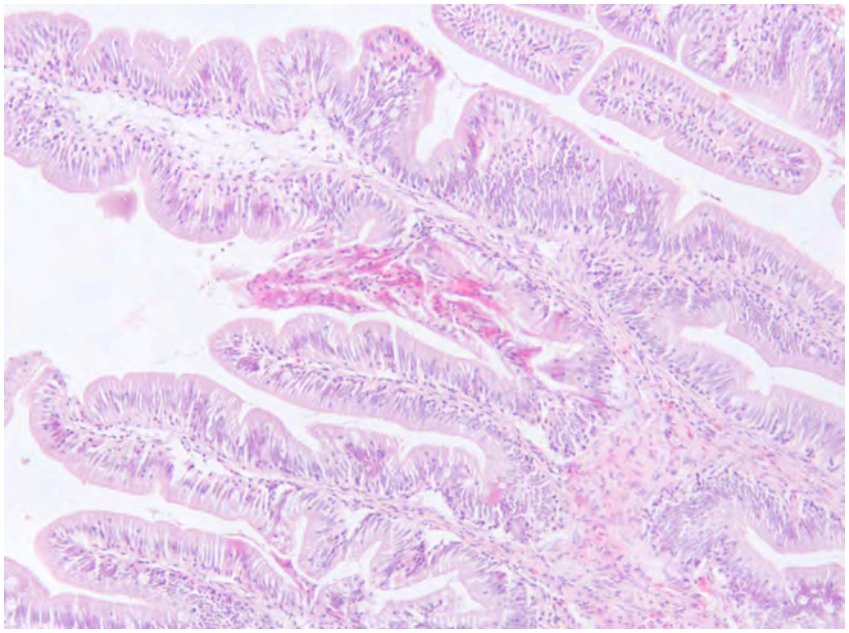

Figure C: AT7F3: presence of small necrotic areas within the epithelium. Notice that in this case, the surrounding epithelium does not present signs of alteration (x100)

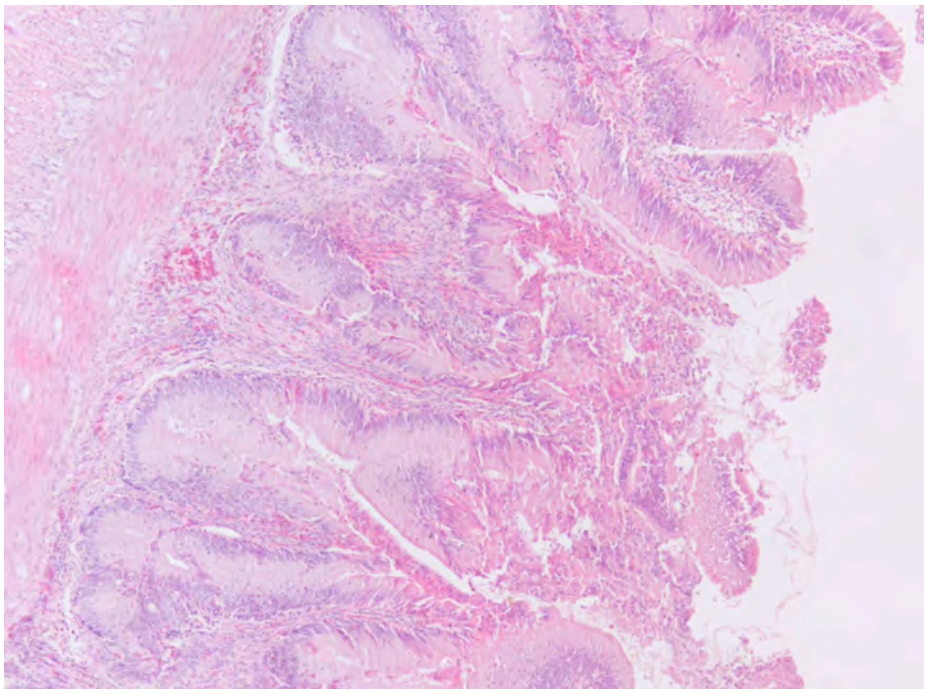

Figure D: AT14F2: large areas of epithelial necrosis / degeneration. (x100).

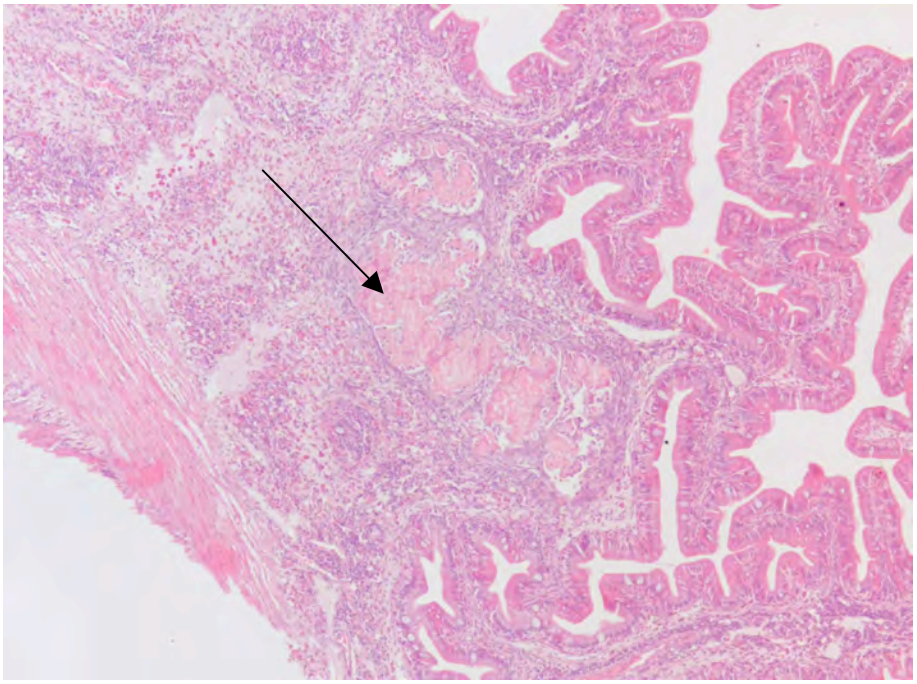

Figure E (AT17F1): Presence of hyaline material (arrow) in the intestinal submucosa. Moderate general inflammatory reaction and mild granulomatous reaction. (x100)

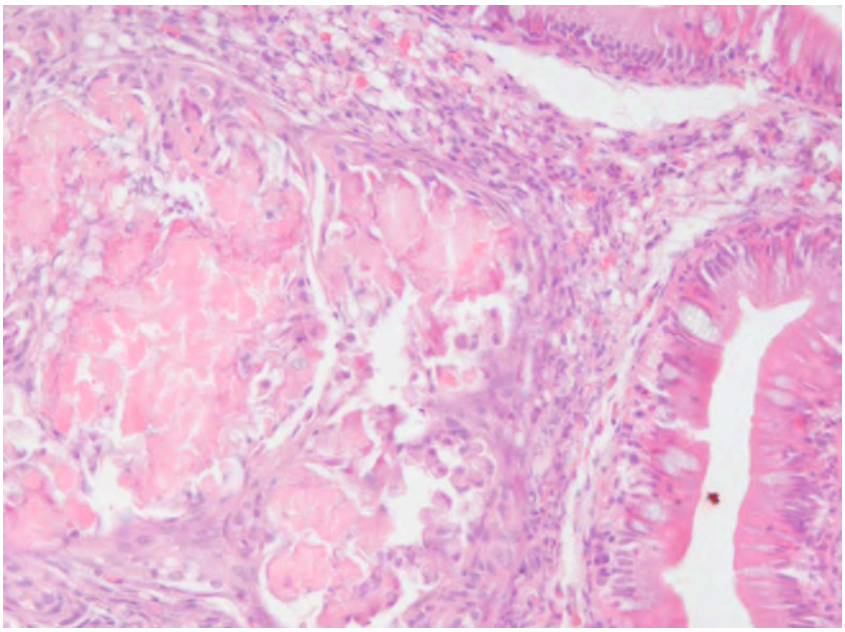

Figure F: the same image in D, but at higher magnification (X400)

References:

- Baeverfjord G. & Krogdahl A. (1996) Development and regression of soybean meal induced enteritis in Atlantic salmon, *Salmo salar* L., distal intestine: a comparison with the intestines of fasted fish. ***Journal of Fish Diseases*** 19, 375–387.
- Bakke-McKellep A.M., Press C.M., Baeverfjord G., Krogdahl A. & 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. ***Journal of Fish Diseases*** 23, 115–127.
- Sanden M., Berntssen M.H.G., Krogdahl A., Hemre G.I. & Bakke-Mckellep A.M. (2005) An examination of the intestinal tract of Atlantic salmon, *Salmo salar* L., parr fed different varieties of soy and maize. ***Journal of Fish Diseases*** 28, 317–330.
- van den Ingh T., Krogdahl A., Olli J.J., Hendriks H. & Koninkx J. (1991) Effects of soybean-containing diets on the proximal and distal intestine in Atlantic salmon (*Salmo salar*): a morphological study. ***Aquaculture*** 94, 297–305.