Aquaculture Nutrition
April 2015, Volume 21, Issue 2, Pages 223-233
http://dx.doi.org/10.1111/anu.12162

Influence of lupin and rapeseed meals on the integrity of digestive tract and organs in gilthead seabream (*Sparus aurata* L.) and goldfish (*Carassius auratus* L.) juveniles

M.H. Omnes[1,*], F.C.P. Silva[2], J. Moriceau[1], P. Aguirre[3], S. Kaushik[3] and F. J. Gatesoupe[3]

[1] Ifremer, UMR 6539, Unité PFOM, Laboratoire Adaptation Reproduction Nutrition des poissons marins, Plouzané, France
[2] Instituto de Ciências Biológicas, Departamento de Microbiologia, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil
[3] INRA, UR 1067, Nutrition Métabolisme, Aquaculture, Pôle Hydrobiologie, Saint-Pée-sur-Nivelle, France

*: Corresponding author : M. H. Omnes, email address : mhomnes@ifremer.fr

Abstract:

Two rearing trials were conducted to evaluate the effects of dietary lupin (LM) and rapeseed (RM) meals in gilthead seabream (*Sparus aurata* L.) and goldfish (*Carassius auratus* L.) juveniles. Each plant meal was incorporated at the rate of 200 g kg^{-1} in two distinct diets, which were compared with a fishmeal-based diet as control. After 1 month, the two plant diets did not influence the whole body growth, but the digestive systems were affected. The splenosomatic index was reduced with two plant meals in goldfish and with RM in seabream. The hepatosomatic index was only reduced in LM-fed seabream. Cellular characteristics were also affected. The largest liver cells were observed in RM-fed goldfish suggesting changes in metabolic function. The LM and RM diets stimulated in seabream, especially the reaction in haematopoietic tissues with the proliferation of melano-macrophages centres, and a tendency for elongated villus height in the anterior intestine thus that possibly compensated for a reduction in digestive function. Such adaptive structural modifications and the absence of degenerative signs allowed concluding that the integrity of the digestive system was maintained in fish fed plant meals.

Keywords: digestive tract ; gilthead seabream ; goldfish ; histology ; lupin ; rapeseed

1. Introduction

To support fast-growing and sustainable aquaculture production, the reduction in fishmeal and fish oil availability has proven to be a challenge for the development of aquafeeds (FAO 2012). A greater use must therefore be made of a broader range of ingredients. A variety of plant meals have been tested as possible substitutes for fishmeal in a range of finfish and crustacean species (Gatlin et al. 2007; Tacon & Metian 2008). Plant protein meals and fishmeal, however, differ in their nutrient composition and because the former contains varying levels of antinutritional factors, it is pertinent to study the gut health of the animals being fed with these ingredients (Krogdahl et al. 2010). Such discrepancies with marine-based resources limit their routine use for fish nutrition despite the implementation of processing technology to improve the nutritional value of plant feedstuffs (Drew et al. 2007). Therefore, much research has been undertaken in an attempt to solve these limitations and to provide a viable alternative to substitute fishmeal.

Fish species adapt differently to the replacement of fishmeal by plant protein feed ingredients, in terms of growth performance, nutrient digestion and digestive system adaptations. Some plant protein products in aquafeeds have been reported to cause digestive alterations in fish (Krogdahl et al. 2010). In particular, there is some evidence of changes in the morphology of the digestive tract with potential consequences for the digestive physiology and the nutrient absorption. The main changes in fish gut summed up by Hansen and Hemre (2013) concerned the impairment of the distal intestine in Atlantic salmon (*Salmo salar* L.) when feed included soybean meal. By contrast, this ingredient caused no apparent changes upon the histological features of the channel catfish (*Ictalurus punctatus* Rafinesque) and European seabass (*Dicentrarchus labrax* L.) intestines (Evans et al. 2005; Bonaldo et al. 2008). In gilthead seabream (*Sparus aurata* L.), only moderate changes were triggered in the distal intestine of fish (Bonaldo et al. 2008).

The aim of this study is to assess the effects of two other plant protein sources on the digestive system integrity of two fish species from contrasted environments and having different feeding habits. Sweet white lupin (*Lupinus albus* L.) and canola/rapeseed (*Brassica napus* L.), the major oilseed, are worldwide grown in temperate regions, and their protein-rich meals are much appreciated in animal feeds. One or the other of the ingredients replaces up to 40% of the traditional fishmeal but they differ in their nature, composition and specific compounds (Sauvant et al. 2004). For instance, lupin dietary fibre contains soluble non-starch polysaccharides (NSP) like pectin-like substance highly fermentable, and low level of cellulose and hemicellulose, while the fibre components of canola include large amount of lignin, cellulose and other polysaccharides (Bach Knudsen 1997; Glencross 2009). Among plant secondary metabolites, the lupin alkaloids cause a bitter taste and α-galactosides, antinutritive effect (Serrano et al. 2011), and canola/rapeseed glucosinolates and their hydrolytic products depressed palatability and reduced the ingredient nutritive value (Mwachireya et al. 1999). However, the application of biotechnology and processing had shown to inactivate or reduce antinutritive compounds (Pereira & Oliva-Teles 2004; Drew et al. 2007; Farhangi & Carter 2007). The inclusion of lupin or rapeseed meals in fish diets has generated mixed results in terms of morphological changes of the digestive system. Indeed, differences between the biological value of different species and cultivars of lupin make it difficult to understand the various study results (Pereira & Oliva-Teles 2004). In rainbow trout (*Oncorhynchus mykiss* Walbaum) fed dehulled lupin (*Lupinus angustifolius*), Farhangi and Carter (2001) observed a non-significant trend towards shorter villus height in the proximal intestine, whereas the use of this lupin species together with exogenous enzyme supplements (Farhangi & Carter 2007) led to no negative changes in the gut. On the other hand, Glencross et al. (2004) described a tendency of longest gastrointestinal tract in *O. mykiss* fed yellow lupin (*Lupinus luteus*) with increasing levels in diets. In addition, Borquez et al.'s (2011) histological examinations of *O. mykiss* fed whole grains of *Lupinus albus* did

not show gut detrimental effects, but distal displacement of enterocyte nucleus and lipid infiltration detected in both hepatocytes and enterocytes, were only noticed at 40% inclusion level. In *S. salar* fed either *L. luteus* or *L. angustifolius* in diets, Refstie et al. (2006) observed fish stomacal ulcer-like lesions whatever the feeding group. Conversely, Aslaksen et al. (2007) observed no stomacal changes in salmon fed *L. angustifolius*. Studies on the use of rapeseed in fish feeds have been focused mostly on fish growth performance, nutrient digestibility (Thiessen et al. 2004; Drew et al. 2007; Burel & Kaushik 2008; Enami 2011) and fish thyroid disorders resulting from the presence of glucosinolate products (Burel & Kaushik 2008), but little is known about some effects of rapeseed meal on the digestive tract integrity. The potential use of lupin and rapeseed meals in aquafeeds has been already assessed as protein sources in feeding trials with several species, among them *S. aurata* (Robaina et al. 1995; Kissil et al. 2000; Pereira & Oliva- Teles 2004; Sitja-Bobadilla et al. 2005; Santigosa et al. 2008) and a subspecies of goldfish, gibel carp (*Carassius auratus* gibelo) reported by Xie et al. (2001). The temperate fish *S. aurata* is one of the relevant finfish species cultured in marine waters and the most produced in the Mediterranean area. The warm freshwater fish, goldfish (*Carassius auratus* L.), introduced into temperate environments as a food or ornamental fish, belongs to the cyprinid family that has considerably increased the worldwide aquaculture production particularly in Asia, such as the production of freshwater fishes, has been dominated by carps (FAO 2012). These fish exhibit a species-specific morphology of the digestive tract, short in the carnivorous stomach-containing teleost *versus* long in the herbivorous stomachless teleost, previously described in several studies (*S. aurata*, Cataldi et al. (1987); *C. auratus*, McVay & Kaan (1940); Yamamoto (1966), respectively).

As part of the studies on the effects of lupin and rapeseed meals on the digestive enzyme activities of the intestinal brush-border membrane (Silva et al. 2010) and on the microbial diversity in fish guts (Silva et al. 2011), the purpose of the present work was to determine the effects of the same ingredients on the *S. aurata* and *C. auratus* gut integrity by structural analysis. The influence of fish exposure to diets, including 200 g kg^{-1} of plant meals that partially substitute fishmeal or only fishmeal, has been investigated on the morphology of gastrointestinal tracts and associated glands highlighting by histological changes of tissues.

2. Materials and methods

2.1. Fish husbandry and feeding

In the first trial, *S. aurata* juveniles were obtained from Ferme Marine de Douhet (FMD, La Brée les Bains, France). The experiment was carried out at the Ifremer facilities (Centre Bretagne, France). The fish were randomly allocated to 12 cylindrical-conical fibreglass tanks (60 L useful capacity), assigned to each of the three experimental diets (four replicate groups, n = 40 fish per tank). Fish were reared in an open seawater system of 35 g l^{-1} salinity and at 20 ± 2 °C. In the second trial, *C. auratus* juveniles were reared at INRA facilities (St Pée sur Nivelle, France). After the acclimation period, fish were randomly allocated to nine cylindrical-conical fibreglass tanks assigned to each of the three experimental diets (triplicate groups, n = 30 fish per tank). Fish were reared in an open freshwater system at 23.0 ± 2 °C. A 12– 12 h light/dark photoperiod was applied in the two experiments. The diets were prepared at the corresponding experimental laboratories. Briefly, three isoproteic diets (420 g kg^{-1} on a dry matter basis) contained fishmeal (FM) alone as control diet or 800 g kg^{-1} fishmeal and 200 g kg^{-1} on a dry matter basis, dehulled sweet white lupin (*Lupinus albus* cultivar Ares) or rapeseed (*Brassica napus*) meals, named LM and RM diets (Table 1). Fish were initially fed at a feeding rate of 2% fish biomass per tank, then feeding was adjusted during the experiments, and fish were fed the experimental diets *ad libitum* twice daily for 1 month.

2.2. Biometry, tissue sampling and calculations

Fish were weighed under anaesthetic (2-phenoxyethanol) procedure. At the beginning of the experiment, several batch (n = 10) of gilthead seabream (mean initial body weight 18.80 ± 2.1 g) and a pool of goldfish (initial body weight 21.5 ± 0.3 g) were weighed and randomly distributed in tanks allocated to the respective treatments. At the end of the experiments, fish were randomly collected for biometric measures *S. aurata*, three fish per tank (12 per treatment); *C. auratus*, four or three fish per tank [21 (FM) or 18 (LM and RM) per treatment)]. Biometry was performed for the evaluation of fish condition by calculating Fulton's condition factor (K) determined from the length and weight data for each individual:

K = 100 x (body weight/body length^3)

and for somatic index calculation to overcome the problem of variability in organ mass. Fishes were cleaned aseptically with ethanol (70%), the ventral surface was opened and emptied digestive tracts and organs were rapidly removed (Fig. 1) and weighed separately to the nearest 0.001 g, using preweighed aluminium cupules. Somatic indexes were calculated:

HSI (%) = 100 x (liver weight/body weight)
HSI, hepatosomatic index

SSI (%) = 100 x (spleen weight/body weight)
SSI, splenosomatic index

NSI (%) = 100 x (kidneyweight/bodyweight)
NSI, nephrosomatic index

2.3. Histological procedure

For histological investigations (three fish per tank), biopsies including *S. aurata* stomach sections and in both species spleen, kidneys and transverse sections from the anterior and posterior intestinal segments of guts were immediately placed in Bouin's fixative solution. Tissue samples were embedded in paraffin wax (Miles Tissue tek, Delta microscopies, Ayguesvives, France) after general dehydration procedures before being sectioned at 5 µm (Minot 1130) and mounted on glass slides coated with poly-L-lysine solution (Sigma-Aldrich, Saint-Quentin Fallavier, France). Paraffin was extracted with MicroClear solvent (Microm Microtech, Francheville, France), and rehydrated tissue sections were stained either with haematoxylin–eosin (H&E) or periodic acid-Schiff's reagent (PAS), for general structure morphology, and mucus secretion and goblet cell observations, respectively. Spleen and kidney sections were placed in Perls' reagent according to the method for assessing the distribution of macrophages and macrophage aggregates the so-called melano-macrophage centres (MMCs; Meseguer et al. 1994). Investigations of morphometric and epithelium changes related to diets were performed on sections (n = 2 per fish) by light microscope and photographed [Olympus (Rungis, France) BX41 and Olympus C-3030 Zoom; magnification 9100 and 9400]. Hepatocyte area (global average, n = 22 measures per section) was assessed to determine liver changes, and data calculated for several fish were expressed as mean hepatocyte size for a given treated group. Macrophages and MMCs were counted in the kidney and spleen tissues; count was attributed an abundance score, which extended from 1 = few to 4 = abundant, adapted after Glencross et al.'s (2004) examination method for the presence or absence of granular substance in haematopoetic tissue. The measure of villus height from the apex to the base (Farhangi & Carter 2001; Samanya & Yamauchi 2002)

was taken on traverse sections in two gut segments. Data expressed in arbitrary units (AU), obtained using ImageJ 1.36 (NIH, National Institute of Health, USA, http://rsb.info.nih.gov/ij/), were exported to a Microsoft Excel file for further statistical analysis.

2.4. Statistical analysis

Globally, variable data are expressed as means ± SD per dietary group in tables and means ± SEM on graphs. The morphometric and somatic index (arcsine square root transformed) data were analysed by one-way analysis of variance (ANOVA), with Newman–Keuls' *post hoc* multiple test for comparing differences found in response to dietary meals, at a $P < 0.05$ significant level. The statistical analyses were performed with Statistica 6 (StatSoft, Maisons-Alfort, France) for Windows.

3. Results

No mortality was observed within the two trials during the experimental feeding periods, neither significant difference was observed in growth parameters between dietary treatments. All diets were palatable for juveniles of both species. In *S. aurata*, the body weight almost doubled over the course of the experiment, whereas in *C. auratus*, body gain was much lower, which is a trait of this species. *K* ranged between an average of 1.83 for the former and 2.74 for the latter (Table 2).

Carassius auratus splenosomatic index (SSI) at 0.35%, 0.21% and 0.25% in FM, LM and RM group, respectively, was 40% significantly ($P < 0.05$) reduced for LM and 28% for RM in comparison with FM index. *S. aurata* SSI at 0.08%, 0.07% and 0.06% in FM, LM and RM group, respectively, was 25% significantly ($P < 0.05$) lowest in RM-fed fish, whereas the LM group showed only a 12.5% noticeable reduction trend compared with the FM index. *C. auratus* hepatosomatic index (HSI) at 4.40%, 4.10% and 4.17% in FM, LM and RM group, respectively, did not differ significantly among treatments. Moreover, *S. aurata*- HSI at 3.02% in LM-fed fish was significantly ($P < 0.05$) lower than FM-HSI at 3.79% and RM-HSI at 3.49%, respectively. No significant differences in nephrosomatic index (NSI) were detected between dietary treatments, but there was still a non-significant NSI reduction in fish fed plant meal diets (*S. aurata*-NSI at 2.20%, 2.17% in LM- and RM-treated groups *versus* 2.24% in FM-NSI; *C. auratus*-NSI at 0.50%, 0.46% in LM- and RM-treated groups *versus* 0.58% in FM-NSI).

The experimental diets induced only few changes in the histological features of the guts and digestive organs, without lesions or pathological signs in tissues. Examinations in livers revealed no structural disorders. The hepatocytes were almost polygonal and mostly had central located nuclei. Glycogen and lipids were stored in the cytoplasm. In liver sections from *S. aurata*, most of the hepatocytes had a typical shape, with visible nuclei and hepatic cells separated by sinuses. The hepatic cell area tended to a non-significant decrease in LM-fed *S. aurata* concomitant to a significant ($P < 0.05$) HSI reduction in this group (Fig. 2A). *C. auratus* livers contained regular round hepatocytes with central located nuclei and showed in RM-fed fish significant ($P < 0.05$), 15.0% and 27.7% hepatic cell enlargement than cell area in FM- and LM-fed fish (Fig. 2B). No steatosis (vacuole formation containing excess lipids in the cell cytoplasm) was observed in the hepatocytes of both species.

Histopathological assessment of spleen and kidneys revealed different patterns of pigment deposition between the two species studied. The tissues of *S. aurata* were pale and slightly brownish (Fig. 3). Aggregates of pigmented cells forming melano-macrophage centres were

observed in most cases of all groups, but were more abundant in *S. aurata* fed diets containing plant products. By contrast, histological examinations of *C. auratus* revealed the presence of only few small macrophages, with no obvious aggregation into MMCs (Fig. 4). Pigmentation was not observed on hepatic sections for both species.

The stomach mucosa in gilthead *S. aurata* was seen indented with folds and cavities, and distinct tubular gastric glands, visible in the lower part of the mucosal folds (Fig. 5). An active mucus secretion from the epithelial cells was PAS pink stained. Nevertheless, individual variability in staining intensity could not be attributed entirely to the dietary effects.

Villus height followed a bimodal distribution of classes, short, less frequent and long villi. In both species, the villi height was significantly ($P < 0.05$) longer in the anterior intestinal segment than in the posterior segment. In *S. aurata* (long villi $n = 63$, short villi $n = 32$ measures, Fig. 6A), the anterior intestine from RM-fed fish had significantly ($P < 0.05$) the longest long villi, whereas the LM diet tended to increase long villus length. Both plant protein diets led to significantly ($P < 0.05$) longer short villi than in fish fed the FM diet. Diet had no effect on villus height in the posterior segment of the intestine. In *C. auratus* (average of long villi $n = 70$, short villi $n = 13$ measures, Fig. 6B), diets had thus no effect on villus height, albeit only long villi were observed in fish fed RM diet. The goblet cells were very numerous within intestinal villi for both species. Furthermore, they seemed more abundant in the posterior than in the anterior part of the intestine. Although *S. aurata* posterior intestine shown a slight increasing trend related to RM diet, no conclusive differences were apparent.

4. Discussion

The relevant subject of this study was focused upon the effects of two plant meals of highly contrasted types of components, on the morphology and integrity of the digestive tracts and internal organs of two fish species *S. aurata* and *C. auratus* having different natural environments and feeding habits. So, we anticipated possible morphological changes as an adaptive response to plant meals present in the experimental diets. No serious adverse effects were observed in the guts. However, some fluctuations of somatic indexes and cellular volumes, in particular, were noticed in the course of the gastrointestinal tract examinations.

The rapeseed meal triggered an increase in liver cell size in *C. auratus* not related to HSI value, suggesting that the liver mass was underestimated in some cases of measures. We have hypotheses explaining liver cell hypertrophy by changes in metabolic function acting on either glycogen fraction or enzyme activities. Feeding habits that differed between species (Tan et al. 2006) and/or rapeseed compounds (Enami 2011) may influence the glycogen fraction. Tan et al. (2006) described the hepatic glycogen storage in carp species with contrasted feeding habits, such as the omnivore *C. auratus gibelo* shows the higher glycogen synthesis capacity and HSI value than the carnivore Chinese catfish (*Leiocassis longirostris* Günther). This appears to be consistent with our findings for *C. auratus* and *S. aurata*. On the other hand, changes in hepatocyte size may reflect the ingestion of rapeseed by our omnivore fish, although by contrast, no such cell enlargement was observed in the RM-treated carnivore *S. aurata*. Indeed, the consumption of rapeseed meal in terrestrial animals has been shown to induce changes of the liver, characterized by a 35% increasing hepatic mass compared with the control, in rats fed 120 g rapeseed kg^{-1} diet (Roland et al. 1996) even more pronounced if the diet was supplemented with 70 g fibre kg^{-1} diet in the form of inulin or oat, and by around 33% in broiler chicks (Taraz et al. 2006) with the increasing of RM proportions in the diet (58 to 231 g rapeseed kg^{-1} diet). However, these last authors suggested that the hepatic hypertrophy was probably due to the presence of hydrolytic products of glucosinolate (Gls), at levels toxic to the birds that induced changed activities of

the liver enzymes, rather than an effect of the ingestion of rapeseed dietary fibre itself. Tripathi & Mishra (2007) reported that diets containing various amounts of GIs induced various physiological disorders, in both livestock and some fishes. In our study, GI products are highly unlikely to account for the observed liver cell enlargement in *C. auratus*. The RM used had a low seed GI content (<30 μmol kg^{-1}). Furthermore, only 200 g kg^{-1} diet of rapeseed had substitute the fishmeal, so the fish were exposed to these compounds or their metabolites, to less than 0.01 μmol kg^{-1} diet per day. A lack of toxicity might therefore indicate no negative effects, and our results suggested that RM had only a minor metabolic effect on *C. auratus* liver. A total GIs level of 1.4 μmol kg^{-1} diet may be considered safe, and limited amounts of canola/rapeseed meals are already included in feedstuffs (Tripathi & Mishra 2007; Burel & Kaushik 2008; Enami 2011). At the opposite, lupin meal in *S. aurata* induced a slight decreasing of hepatocyte size and a significant HSI reduction. Nevertheless, these observations could not be consolidated being connected to a lower gluconeogenesis in fish. According to Robaina et al. (1995), no histological alterations of liver were observed in *S. aurata*, but fish fed *L. angustifolus* at 200 or 300 g lupin meal kg^{-1} diet shown small lipid droplets together with reduction in glycogen deposition in liver. In rainbow trout fed *L. albus* (Borquez et al. 2011), a similar HSI reduction by lupin treatments than in our study was observed, with no changes in hepatocyte contents.

Splenosomatic index values of both species fed plant products were lower than those of fish from the control diet. Similarly, a decreasing splenic mass and index had been described in a rat study when animals fed algae (Bocanegra et al. 2003). The authors involved an effect of the ratio of dietary electrolytes on the splenic blood pressure. We did not determine the mechanism by which certain electrolytes of ingredients might trigger these consequences on the spleen. In *S. aurata*, the dietary LM and RM exposure revealed a weak decrease in splenic parameters in contrast to *C. auratus*; then, other factors such as rearing conditions, that is, marine waters *versus* freshwater, could explain these fish physiological changes. It therefore seemed likely that the consumption of plant products had only indirect physiological effects within the spleen and probably the kidneys. However, morphological changes were observed in the *S. aurata* spleen, with the occurrence of macrophages detected by histology in all groups, more frequent in haematopoietic tissues of fish fed plant diets. Several studies have reported early the relative abundance of melano-macrophage pigments in *S. aurata* (Meseguer et al. 1994; Montero et al. 1999; Manera et al. 2000), assuming that this species may be particularly sensitive to variations in environment, to disease or food status. Conversely, the spleen tissue of the *C. auratus* contained only few macrophages, detected as small pigments apparently unrelated to diet. This observation agrees with the findings in other omnivore, *I. punctatus* by Evans et al. (2005), when fish fed soy products.

The *S. aurata* stomach epithelium did not seem to be affected structurally by diets. Some variations in staining intensity revealed the epithelial mucus production, but no firm conclusions could be drawn about their importance. No overt signs of pathological effects were observed. Nevertheless, in *S. salar*, lupin (*L. luteus*) a 300 g kg^{-1} diet inclusion level induced gastric lesions resembling ulcers (Refstie et al. 2006). The proportion of *L. albus* used in our experiment was lower than this of *L. luteus* in the Refstie's study, and this may account for the absence of such gastric reactions. An interesting morphological adaptation to diets was observed in *S. aurata* with the stimulation of the villus development of the proximal intestine epithelium from plant-products-fed fish, accented by the rapeseed consumption. Thus, as response adaptation, it suggests a probable enhancement of the absorptive function. This surface increase could compensate for the inferior specific activity of intestinal brush-border membrane enzymes, noted in seabream in the present experiment (Silva et al. 2010). The digestive capacity seemed thus to be maintained, without detrimental consequences on growth performance. Such adaptive mechanism was not observed in the *C. auratus* anterior segment nor was observed enzymatic activity decrease. These findings differed with other observations of *S. aurata* fed diets including the same plant meals (Santigosa et al. 2008). Indeed, higher proportions of rapeseed and lupin mixture in diets

substituted partially or totally fishmeal compared with that used here for each ingredient. Furthermore, fish feeding trial ran over a 12-week period, and the relative intestinal length and damages, characterized by villus atrophy, have gradually increased with the elevating proportion of plant products used (Santigosa et al. 2008). The morphology of fish digestive system might be expected to reflect their adaptation to various diets. The phenotypic response to feed on a natural diet described in juvenile grass carp (*Ctenopharyngodon idella* Valenciennes), redbelly tilapia (*Tilapia zilli* Gervais) and blue tilapia (*Tilapia aurea* Steindachner) concerned mostly the anterior intestine (Stroband 1977; Frierson & Foltz 1992). An increase in the epithelium available for the digestion was identified as a key adaptive feature to diet. Such intestinal expansion showed a higher degree of fold development (convolutions) and taller microvilli in blue tilapia (observed there, using scanning electron microscope, Frierson & Foltz 1992). Although a recent study described *S. aurata* carnivorous and opportunistic (Hadj Taieb et al. 2013), in the natural environment, juvenile stages have also shown, a preference to eat macrobenthos and macrophyte detritus (Ferrari & Chieregato 1981) as well as an ability to utilize natural food resources, like non-negligible quantities of macrophytes, seagrass and algae, then, in connection with the occupation of the feeding grounds and their natural habitats (Arechavala-Lopez et al. 2012). Trophic flexibility of *S. aurata* could corroborate a plausible adaptation of the proximal intestine to diets incorporating plant meals.

In animal models, the dietary supplementation with pectin induced changes in small intestinal morphology, with elongated villus height at level of 25 g kg^{-1} diet in male rats concomitant to crypt depth increase (Andoh et al. 1999) and at 80 g kg^{-1} in pigs, both in the duodenum and ileum (Buraczewska et al. 2007). Here, lupin meal brought amounts of pectin no more than 10 g kg^{-1} in LM diet, which appeared to be a too low level of soluble NSP to induce changes on the epithelial morphology, unless our marine fish is much more sensitive to this compound than the terrestrial animals. Chyme and digesta in LM-fed *S. aurata* seemed fluid probably related to high water contents in faeces, resulting from the additive effects of osmotic active pectic polysaccharides with a high water absorption capacity, and the release of pancreatic juices into the small intestine that could influence morphology as intestinal adaptation (Johnson 1990). Even if dietary fibre stimulates the extension of the absorptive epithelium, this process would be not continuous and occurs in a few days (Johnson 1990). The villus height was probably stimulated likely through a dynamic process of cell turnover coupling crypt cell proliferation and cell apoptosis from the villi apex. The mechanisms and location of such adaptive processes should therefore be more investigated in details. Moreover, dietary fibre remained mostly unchanged after passage through the gastrointestinal tract, with an apparent moderately or lack of fermentation. Indeed, it has been reported in several studies that most of the hemicellulose fraction is not digested by fish (Davies 1985). In the present work, small amounts of rapeseed fibre were identified by a direct visual observation within faeces from RM-fed *C. auratus* and *S. aurata*. One of the properties of insoluble dietary fibre, such as the lignin present in the rapeseed, is to decrease the transit time in the digestive tract with increasing the faecal bulk. Aslaksen et al. (2007) has even observed in *S. salar* that the viscosity of the digested matter was significantly higher in fish fed rapeseed and lupin meals in comparison with fishmeal. In broiler chickens, Baurhoo et al. (2007) reported that purified lignin included at 12.5 g kg^{-1} in the diet improved the morphological structure of the small intestine with an increase of villus height and goblet cell number. In addition, these changes together with the richness of beneficial bacteria reinforced that lignin had benefits in animal fed this component as a natural food additive (Baurhoo et al. 2007, 2008). Here, the lignin-rich plant product induced the longest intestinal fold in *S. aurata*. It was not possible however to determine whether this compound achieved a direct and indirect effect on the RM-gut health, and this aspect requires further investigations.

5. Conclusion

This study provides complementary advances for the selection of ingredients for cultured fish species. A histological descriptive approach was useful to characterize the consequences of the consumption of plant products within key organs. These observations suggested a species-specific phenotypic response to the inclusion of the ingredients in diets: metabolic and physiological changes of liver and spleen in *C. auratus* and noticeable morphological changes of spleen and proximal intestine in *S. aurata*. This last intestinal feature seemed to contribute to the digestive capacity function. As our study did not reveal any detrimental effect such as pathological lesions in the tissues, we conclude that the integrity of the digestive system was maintained in fish fed diets with these plant ingredients.

Acknowledgements

We thank ANSES (formerly known as AFSSA, Plouzané, France) for providing access to the laboratory in which the histological procedures were carried out, and A. Le Ven and F. Lamour, for technical assistance. For tissue sampling and diet composition analysis, we thank our colleagues E. Desbruyères, M.M. Le Gall, A. Sévère and H. Le Delliou from PFOM-ARN Unit, at Ifremer (Plouzané, France), and for fish handling, D. Bazin from INRA (St Pée-sur-Nivelle, France). The work of F.C.P. Silva was supported by Fundacao de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG CBB 804/06) and coordinated by CAPES, CNPq (Brazil), Ifremer and INRA (France).

References

Andoh, A., Bamba, T. & Sasaki, M. (1999) Physiological and anti-inflammatory roles of dietary fiber and butyrate in intestinal functions. *J. Parenter. Enteral Nutr*., **23**, S70–S73.

Arechavala-Lopez, P., Uglem, I., Fernandez-Jover, D., Bayle-Sempere, J.T. & Sanchez-Jerez, P. (2012) Post-escape dispersion of farmed seabream (*Sparus aurata* L.) and recaptures by local fisheries in the Western Mediterranean Sea. *Fish. Res*., **121–122**, 126–135.

Aslaksen, M.A., Kraugerud, O.F., Penn, M., Svihus, B., Denstadli, V., Jorgensen, H.Y., Hillestad, M., Krogdahl, A. & Storebakken, T. (2007) Screening of nutrient digestibilities and intestinal pathologies in Atlantic salmon, *Salmo salar*, fed diets with legumes, oilseeds, or cereals. *Aquaculture*, **272**, 541–555.

Bach Knudsen, K.E. (1997) Carbohydrate and lignin contents of plant materials used in animal feeding. *Anim. Feed Sci. Technol*., **67**, 319–338.

Baurhoo, B., Phillip, L. & Ruiz-Feria, C.A. (2007) Effects of purified lignin and mannan oligosaccharides on intestinal integrity and microbial populations in the ceca and litter of broiler chickens. *Poult. Sci*., **86**, 1070–1078.

Baurhoo, B., Ruiz-Feria, C.A. & Zhao, X. (2008) Purified lignin: nutritional and health impacts on farm animals – A review. *Anim. Feed Sci. Technol*., **144**, 175–184.

Bocanegra, A., Nieto, A., Blas, B. & Sanchez-Muniz, F.J. (2003) Diets containing a high percentage of Nori or Konbu algae are well-accepted and efficiently utilised by growing rats

but induce different degrees of histological changes in the liver and bowel. *Food Chem. Toxicol*., **41**, 1473–1480.

Bonaldo, A., Roem, A.J., Fagioli, P., Pecchini, A., Cipollini, I. & Gatta, P.P. (2008) Influence of dietary levels of soybean meal on the performance and gut histology of gilthead seabream (*Sparus aurata* L.) and European sea bass (*Dicentrarchus labrax* L.). *Aquac. Res*., **39**, 970–978.

Borquez, A., Serrano, E., Dantagnan, P., Carrasco, J. & Hernandez, A. (2011) Feeding high inclusion of whole grain white lupin (*Lupinus albus*) to rainbow trout (*Oncorhynchus mykiss*): effects on growth, nutrient digestibility, liver and intestine histology and muscle fatty acid composition. *Aquac. Res*., **42**, 1067–1078.

Buraczewska, L., Swiech, E., Tusnio, A., Taciak, M., Ceregrzyn, M. & Korczynski, W. (2007) The effect of pectin on amino acid digestibility and digesta viscosity, motility and morphology of the small intestine, and on N-balance and performance of young pigs. *Livest. Sci*., **109**, 53–56.

Burel, C. & Kaushik, S. (2008) Use of rapeseed/canola in diets of aquaculture species. In: Alternative Protein Sources in Aquaculture Diets (Lim, C., Webster, C.D. & Lee, C.S. eds), pp. 343– 408. The Haworth Press Inc, New York, USA.

Cataldi, E., Cataudella, S., Monaco, G., Rossi, A. & Tancioni, L. (1987) A study of the histology and morphology of the digestive tract of the sea bream, *Sparus aurata*. *J. Fish Biol*., **30**, 135–145.

Davies, S.J. (1985) The role of dietary fibre in fish nutrition. In: Recent Advances in Aquaculture. Vol. 2 (Muir, J.F. & Roberts, R.J. eds), pp. 219–249. Westview Press, Boulder, CO; London, UK.

Drew, M.D., Borgeson, T.L. & Thiessen, D.L. (2007) A review of processing of feed ingredients to enhance diet digestibility in finfish. *Anim. Feed Sci. Technol*., **138**, 118–136.

Enami, H.R. (2011) A review of using canola/rapeseed meal in aquaculture feeding. *J. Fish. Aquat. Sci.*, **6**, 22–36.

Evans, J.J., Pasnik, D.J., Peres, H., Lim, C. & Klesius, P.H. (2005) No apparent differences in intestinal histology of channel catfish (*Ictalurus punctatus*) fed heat-treated and non-heat-treated raw soybean meal. *Aquacult. Nutr*., **11**, 123–129.

FAO (2012) The State of World Fisheries and Aquaculture. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy, 230.

Farhangi, M. & Carter, C.G. (2001) Growth, physiological and immunological responses of rainbow trout (*Oncorhynchus mykiss*) to different dietary inclusion levels of dehulled lupin (*Lupinus angustifolius*). *Aquac. Res*., **32**, 329–340.

Farhangi, M. & Carter, C.G. (2007) Effect of enzyme supplementation to dehulled lupin-based diets on growth, feed efficiency, nutrient digestibility and carcass composition of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquac. Res*., **38**, 1274–1282.

Ferrari, I. & Chieregato, A.R. (1981) Feeding habits of juvenile stages of *Sparus aurata* L., *Dicentrarchus labrax* L. and Mugilidae in a brackish embayment of the Po River Delta. *Aquaculture*, **25**, 243–257.

Frierson, E.W. & Foltz, J.W. (1992) Comparison and estimation of absorptive intestinal surface areas in two species of Cichlid fish. *Trans. Am. Fish. Soc*., **121**, 517–523.

Gatlin III, D.M., Barrows, F.T., Brown, P., Dabrowski, K., Gaylord, T.G., Hardy, R.W., Herman, E., Hu, G., Krogdahl, A., Nelson, R., Overturf, K., Rust, M., Sealey, W., Skonberg, D., Souza, E.J., Stone, D., Wilson, R. & Wurtele, E. (2007) Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquac. Res*., **38**, 551-579.

Glencross, B. (2009) The influence of soluble and insoluble lupin non-starch polysaccharides on the digestibility of diets fed to rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, **294**, 256– 261.

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 (*Onchorhynchus mykiss*). *Aquaculture*, **235**, 411–422.

Hadj Taieb, A., Sley, A., Ghorbel, M. & Jarboui, O. (2013) Feeding habits of *Sparus aurata* (Sparidae) from the Gulf of Gabes (central Mediterranean). *Cah. Biol. Mar.*, **54**, 263–270.

Hansen, A.C. & Hemre, G.I. (2013) Effects of replacing fish meal and oil with plant resources in on-growing diets for Atlantic cod *Gadus morhua* L. *Aquacult. Nutr*., **19**, 641–650.

Johnson, I.T. (1990) The biological effects of dietary fibre in the small intestine. In: Dietary Fibre: Chemical and Biological Aspects. Proceedings of Fibre 90: Chemical and Biological Aspects of Dietary Fibre, Norwich, England (Soutghate, D.A.T., Waldron, K., Johnson, I.T. & Fenwick, G.R. eds), pp. 151–163. The Royal Society of Chemistry, Cambridge, UK.

Kissil, G.W., Lupatsch, I., Higgs, D.A. & Hardy, R.W. (2000) Dietary substitution of soy and rapeseed protein concentrates for fish meal, and their effects on growth and nutrient utilization in gilthead seabream *Sparus aurata* L. *Aquac. Res*., **31**, 595–601.

Krogdahl, A., Penn, M., Thorsen, J., Refstie, S. & Bakke, A.M. (2010) Important antinutrients in plant feedstuffs for aquaculture: an update on recent feedings regarding responses in salmonids. *Aquac. Res*., **41**, 333–334.

Manera, M., Serra, R., Isani, G. & Carpené, E. (2000) Macrophage aggregates in gilthead sea bream fed copper, iron and zinc enriched diets. *J. Fish Biol*., **57**, 457–465.

McVay, J.A. & Kaan, H.W. (1940) The digestive tract of *Carassius auratus*. *Biol. Bull*., **78**, 53–67.

Meseguer, J., Lopez-Ruiz, A. & Esteban, M.A. (1994) Melanomacrophages of the seawater teleosts, sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*): morphology, formation and possible function. *Cell Tissue Res*., **277**, 1–10.

Montero, D., Blazer, V.S., Socorro, J., Izquierdo, M.S. & Tort, L. (1999) Dietary and culture influences on macrophage aggregate parameters in gilthead seabream (*Sparus aurata*) juveniles. *Aquaculture*, **179**, 523–534.

Mwachireya, S.A., Beames, R.M., Higgs, D.A. & Dosanjh, B.S. (1999) Digestibility of canola protein products derived from the physical, enzymatic and chemical processing of commercial canola meal in rainbow trout *Oncorhynchus mykiss* (Walbaum) held in fresh water. *Aquacult. Nutr*., **5**, 73–82.

Pereira, T.G. & Oliva-Teles, A. (2004) Evaluation of micronized lupin seed meal as an alternative protein source in diets for gilthead sea bream *Sparus aurata* L. juveniles. *Aquac. Res*., **35**, 828–835.

Refstie, S., Glencross, B., Landsverk, T., Sorensen, M., Lilleeng, E., Hawkins, W. & Krogdahl, A. (2006) Digestive function and intestinal integrity in Atlantic salmon (*Salmo salar*) fed kernel meals and protein concentrates made from yellow or narrow leafed lupins. *Aquaculture*, **261**, 1382–1395.

Robaina, L., Izquierdo, M.S., Moyano, F.J., Socorro, 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.

Roland, N., Rabot, S. & Nugon-Baudon, L. (1996) Modulation of the biological effects of glucosinolates by inulin and oat fibre in gnotobiotic rats inoculated with a human whole faecal flora. *Food Chem. Toxicol*., **34**, 671–677.

Samanya, M. & Yamauchi, K. (2002) Histological alterations of intestinal villi in chickens fed dried *Bacillus subtilis* var. natto. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.*, **133**, 95–104.

Santigosa, E., Sanchez, J., Médale, F., Kaushik, S., Perez-Sanchez, J. & Gallardo, M.A. (2008) Modifications of digestive enzymes in trout (*Oncorhynchus mykiss*) and sea bream (*Sparus aurata*) in response to dietary fish meal replacement by plant protein sources. *Aquaculture*, **282**, 68–74.

Sauvant, D., Perez, J.M. & Tran, G. (2004) Tables de composition et de valeur nutritive des matières premières destinées aux animaux d'élevage. 2nd edn. INRA, Paris, France.

Seiliez, I., Bruant, J.S., Zambonino Infante, J.L., Kaushik, S. & Bergot, P. (2006) Effect of dietary phospholipid level on the development of gilthead sea bream (*Sparus aurata*) larvae fed a compound diet. *Aquacult. Nutr*., **12**, 372–378.

Serrano, E., Storebakken, T., Penn, M., Øverland, M., Hansen, J.O. & Mydland, L.T. (2011) Responses in rainbow trout (*Oncorhynchus mykiss*) to increasing dietary doses of lupinine, the main quinolizidine alkaloid found in yellow lupins (*Lupinus luteus*). *Aquaculture*, **318**, 122–127.

Silva, F.C.P., Nicoli, J.R., Zambonino-Infante, J.L., Le Gall, M.M., Kaushik, S.J. & Gatesoupe, F.J. (2010) Influence of partial substitution of dietary fish meal on the activity of the digestive enzymes in the intestinal brush border membrane of gilthead sea bream, *Sparus aurata* and goldfish, *Carassius auratus*. *Aquaculture*, **306**, 233–237.

Silva, F.C.P., Nicoli, J.R., Zambonino-Infante, J.L., Aguirre, P., Kaushik, S.J. & Gatesoupe, F.J. (2011) Influence of the diet on the microbial diversity of faecal and gastrointestinal contents in gilthead sea bream (*Sparus aurata*) and intestinal contents in goldfish (*Carassius auratus*). *FEMS Microbiol. Ecol*., **78**, 285–296.

Sitja-Bobadilla, A., Pena-Llopis, S., Gomez-Requeni, P., Médale, F., Kaushik, S. & Perez-Sanchez, J. (2005) Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*). *Aquaculture*, **249**, 387–400.

Stroband, H.W.J. (1977) Growth and diet dependant structural adaptations of the digestive tract in juvenile grass carp (*Ctenopharyngodon idella*, Val.). *J. Fish Biol*., **11**, 167–174.

Tacon, A.G.J. & Metian, M. (2008) Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: trends and future prospects. *Aquaculture*, **285**, 146–158.

Tan, Q., Xie, S., Zhu, X., Lei, W. & Yang, Y. (2006) Effect of dietary carbohydrate sources on growth performance and utilization for gibel carp (*Carassius auratus gibelio*) and Chinese longsnout catfish (*Leiocassis longirostris* Gunther). *Aquacult. Nutr*., **12**, 61–70.

Taraz, Z., Jalali, S.M.A. & Rafeie, F. (2006) Effects of replacement of soybean meal with rapeseed meal on organs weight, some blood biochemical parameters and performance of broiler chicks. *Int. J. Poult. Sci*., **5**, 1110–1115.

Thiessen, D.L., Maenz, D.D., Newkirk, R.W., Classen, H.L. & Drew, M.D. (2004) Replacement of fishmeal by canola protein concentrate in diets fed to rainbow trout (*Oncorhynchus mykiss*). *Aquacult. Nutr*., **10**, 379–388.

Tripathi, M.K. & Mishra, A.S. (2007) Glucosinolates in animal nutrition: a review. *Anim. Feed Sci. Technol.*, **132**, 1–27.

Xie, S., Zhu, X., Cui, Y. & Yang, Y. (2001) Utilization of several plant proteins by gibel carp (*Carassius auratus* gibelio). *J. Appl. Ichthyol.*, **17**, 70–76.

Yamamoto, T. (1966) An electron microscope study of the columnar epithelial cell in the intestine of fresh water teleosts: goldfish (*Carassius auratus*) and rainbow trout (*Salmo irideus*). *Z. Zellforsch. Mikrosk. Anat.*, **72**, 66–87.

Tables

Table 1. Formulation (g kg $^{-1}$ diet) and proximate composition (g kg $^{-1}$ DM) of the fishmeal (FM), lupin (LM) and rapeseed (RM) experimental diets.

Ingredients	FM	LM	RM
Fishmeal[1]	570	451	478
Lupin[2]	0	200	0
Rapeseed[3]	0	0	200
Cod liver oil[1]	124	124	124
Starch	276	193	168
Carob gum E410	5	5	5
Xanthan gum E415	5	5	5
Vitamin premix[4]	10	10	10
Mineral premix[4]	10	10	10
Amino acid mixture[5]	0	2	0
Proximate composition			
Dry matter (g kg^{-1})	978	947	976
Crude protein	457	454	455
Crude fat	190	205	186

[1]Norse-LT 94 supplied by La Lorientaise, Sopropêche, Lorient, France.

[2]Terrena Lup'Ingrédients, Martigné-Ferchaud, France.

[3]Saipol, Grand Couronne, France.

[4]Composition and concentration of the vitamin and mineral premix, Seiliez *et al.* (2006).

[5]Leucine 368 g kg^{-1} amino acid mixture, lysine 316 g kg^{-1}, methionine 366 g kg^{-1} [diet amino acid composition, Silva *et al.*, (2010)].

Table 2. Initial and final mean weights (mean values ± SD) and condition factor of juvenile gilthead seabream *Sparus aurata* and goldfish *Carassius auratus* fed fishmeal (FM), lupin (LM) and rapeseed (RM) experimental diets.

Diets	FM	LM	RM
Gilthead seabream			
Initial body weight	18.50±5.10	19.00±7.20	19.00±8.30
Final body weight	31.90±4.11	34.20±5.65	35.20±6.63
Condition factor	1.85±0.06	1.83±0.17	1.83±0.43
Goldfish			
Initial body weight	21.50±0.30	21.50±0.30	21.50±0.30
Final body weight	27.72±6.44	26.53±5.51	33.44±10.1
Condition factor	2.81±0.41	2.68±0.54	2.75±0.44

Figures

Figure 1. Digestive tracts: (A & B) in *Sparus aurata*: (A) the gut still in place: (a) liver, (b) stomach, (c) kidney, (d) pyloric caeca, (e) spleen, (f) intestine; (B) digestive tract: (a) stomach, (b) pyloric caeca, (c) anterior intestine, (d) posterior intestine, (e) rectum; (C & D) in stomachless *Carassius auratus*: (C) gut in place: (a) kidney, (b) spleen, (c) anterior intestine, (d) posterior intestine; (D) digestive tract: (a) digestive bulb, (b) anterior intestine, (c) posterior intestine, (d) rectum. (B & D) Symbol (–) locates biopsy; (D); dashed line (- -) symbolizes goldfish total length.

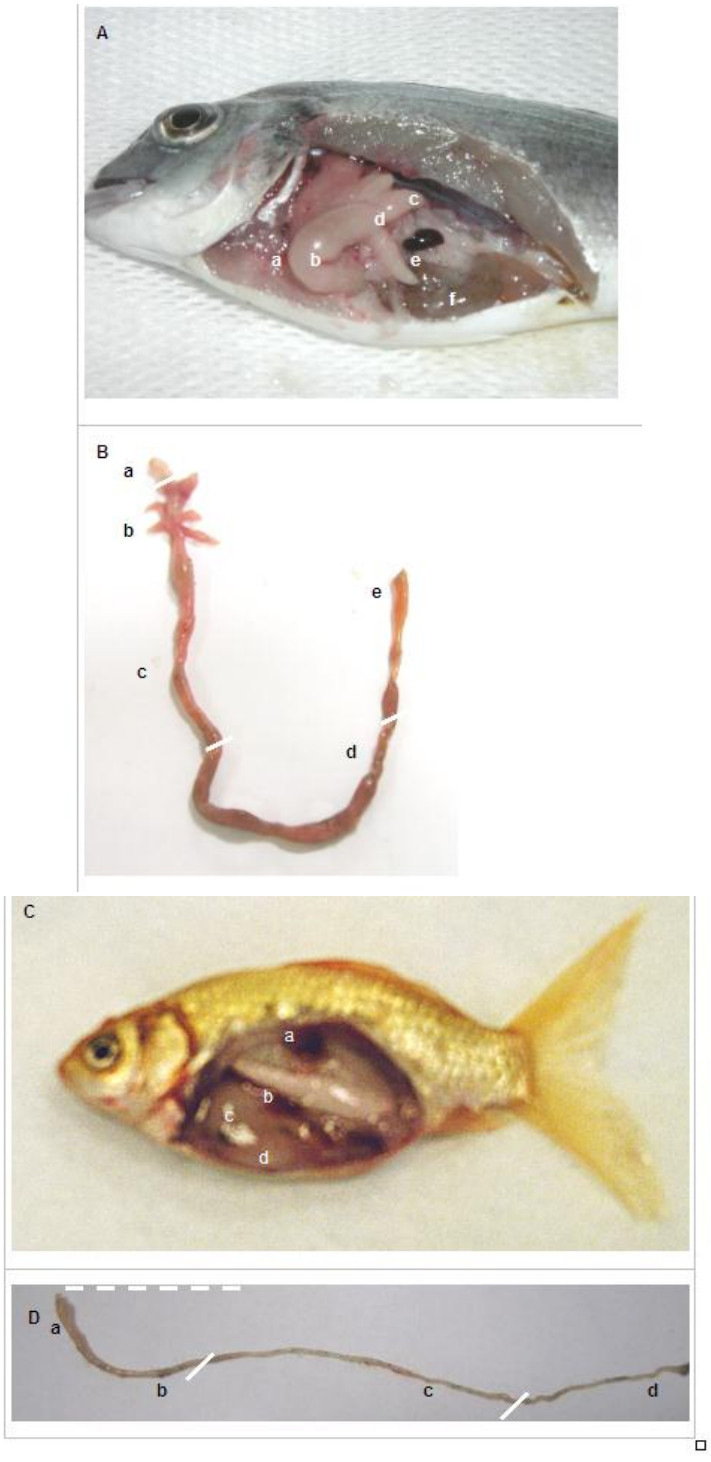

Figure 2. Hepatocyte size in livers of (A) *Sparus aurata* and (B) *Carassius auratus* fed fishmeal (FM), lupin (LM) and rapeseed (RM) diets, means ± standard error of the mean (SEM). Different superscript letters indicate non-significant (ns) or significant difference between treatments ($P < 0.05$).

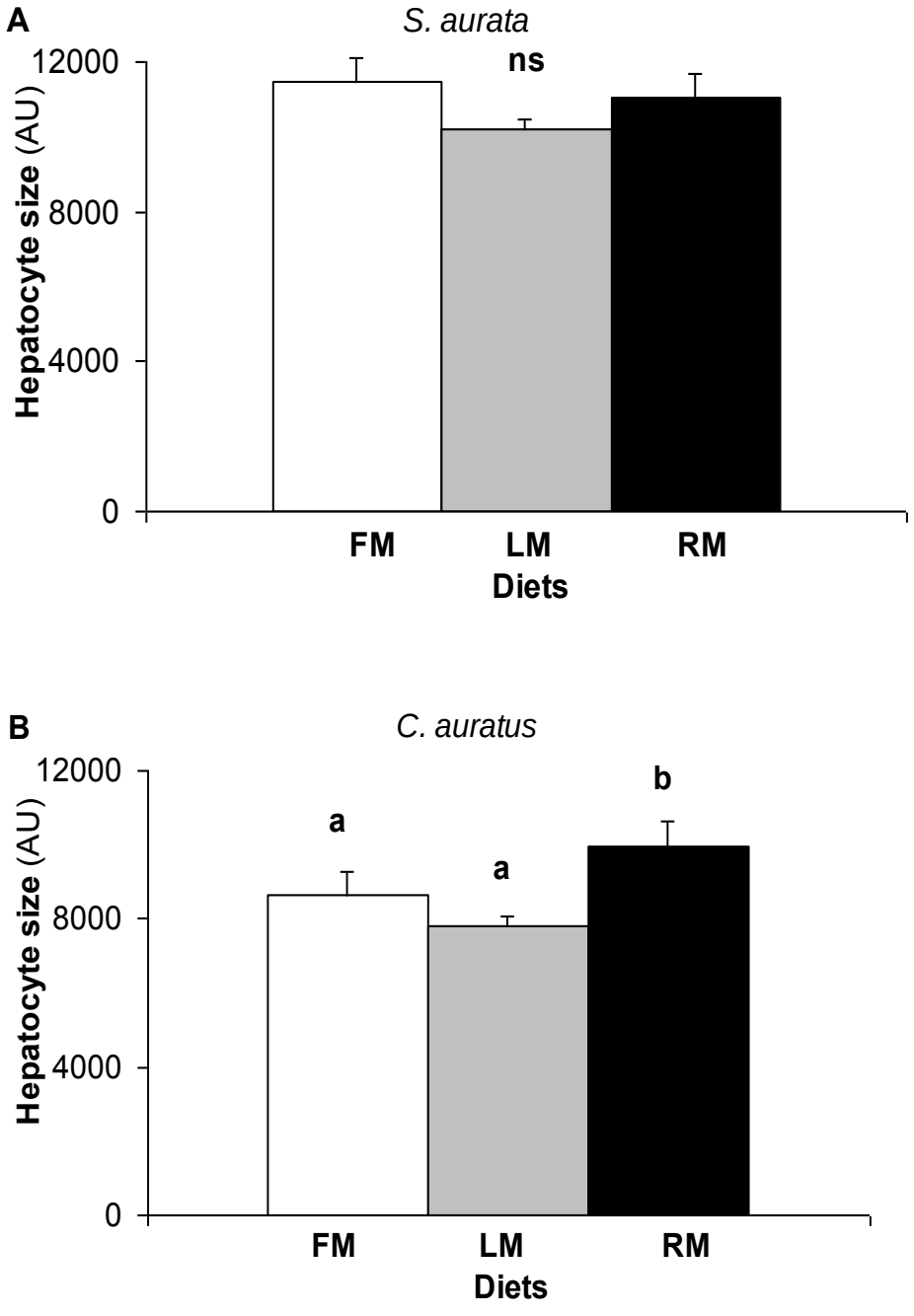

Figure 3. Spleen and kidneys showing macrophages (arrows) and melanomacrophage centres (MMC) in *Sparus aurata* fed plant protein diets: (A) lupin–spleen, (B) rapeseed–spleen sections and (C) lupin–kidney, (D) rapeseed–kidney sections (Perls stained x100).

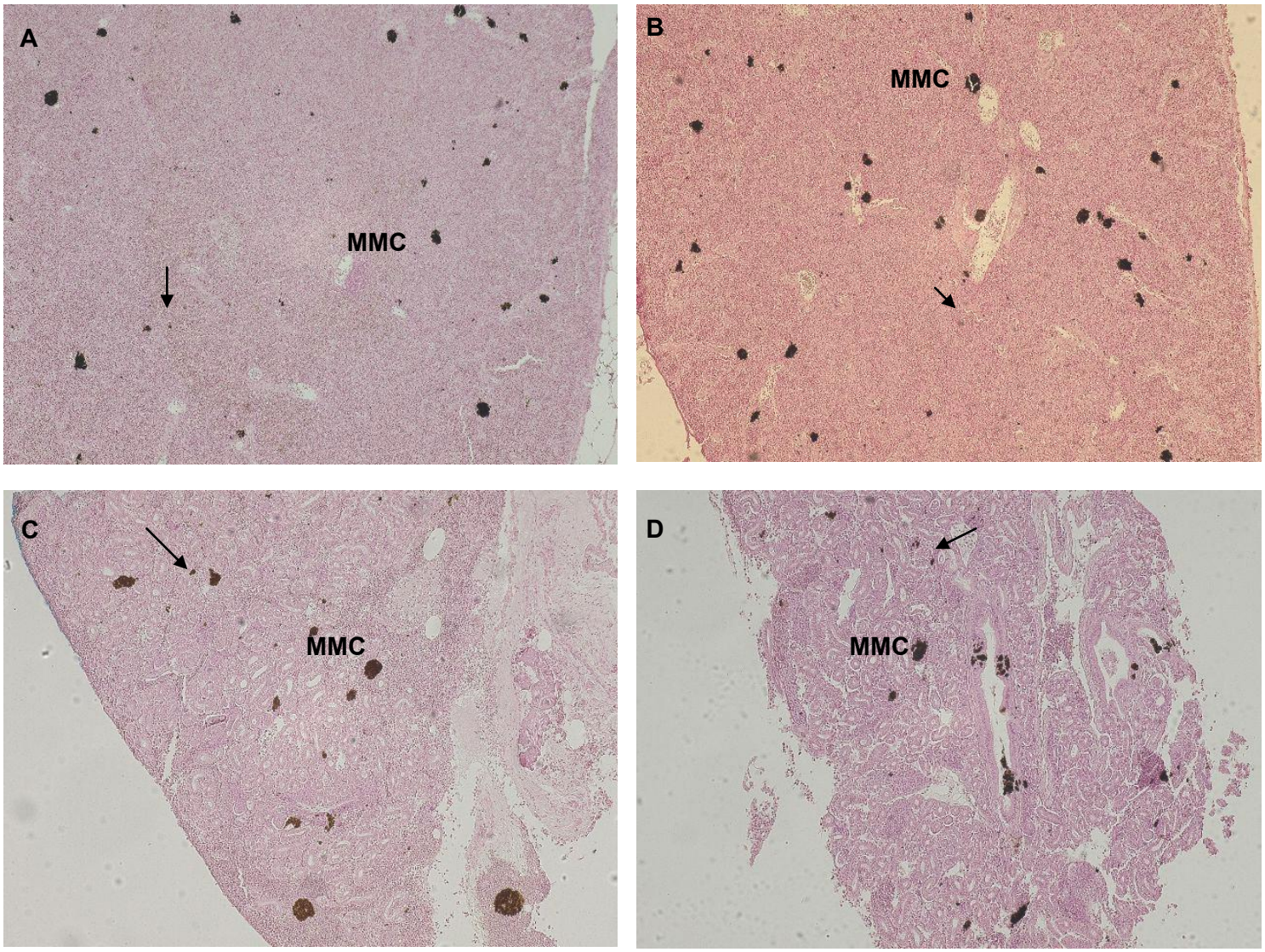

Figure 4. Spleen and kidneys showing small macrophages (arrows) in *Carassius auratus* fed plant protein diets: (A) lupin–spleen, (B) rapeseed–spleen sections and (C) lupin–kidney, (D) rapeseed–kidney sections (Perls stain x100).

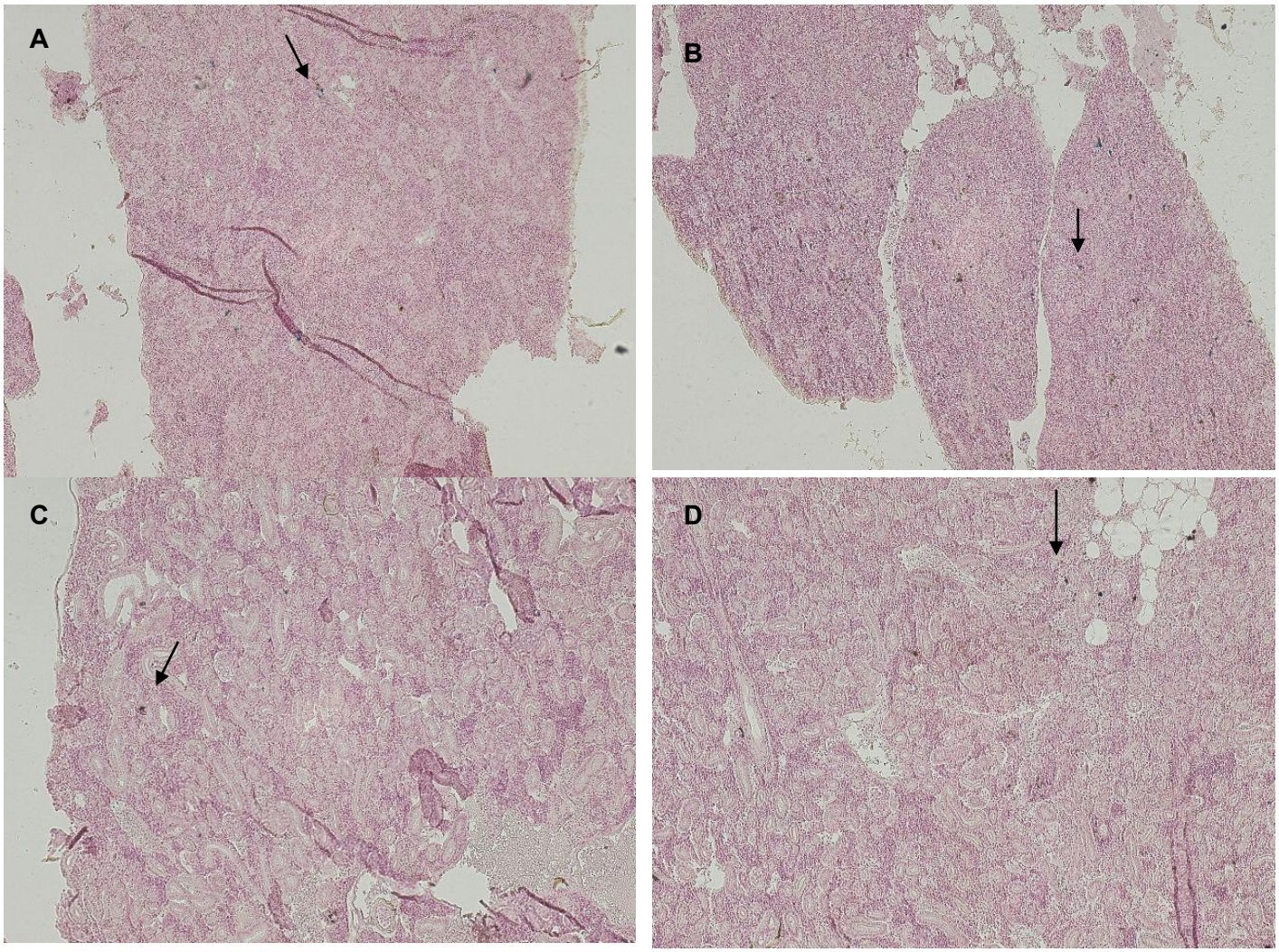

Figure 5. Representative histological sections in *Sparus aurata* stomach fed: (A) fishmeal (FM, control), (B) lupin and (C) rapeseed diets. (CE) columnar epithelium, (GG) gastric glands, (SM) submucosa, (L) lumen. (PAS stain x400) PAS, periodic acid-Schiff.

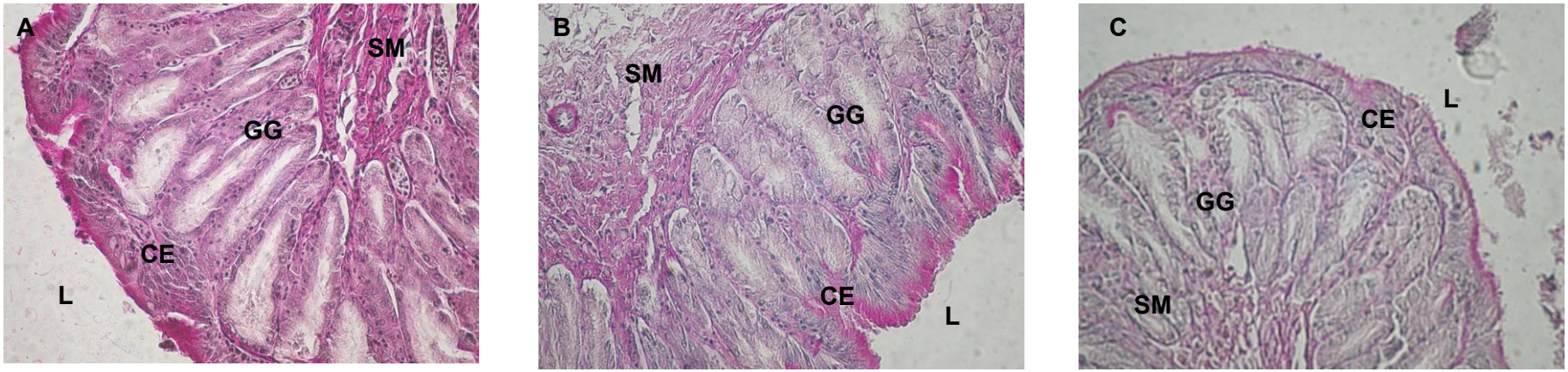

Figure 6. Villus height assessment in anterior and posterior intestinal segments of fish fed fishmeal (FM), lupin (LM) and rapeseed (RM) diets from replicate-treated groups, in (A) *Sparus aurata* (*n* = 4) and (B) *Carassius auratus* (*n* = 3), means ± standard error of the mean (SEM). Different superscript letters indicate non-significant (ns) or significant difference between treatments and between intestinal segments ($P < 0.05$).