Plant oils inclusion in high fish meal substituted diets. Effect on intestinal
digestion and nutrient absorption in sea bream (*Sparus aurata*)

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Running title: Digestive response of sea bream to FM- and FO-replaced diets

1. ABSTRACT

Here we performed an 11-week growth trial in juvenile sea bream to study the
effects of four experimental diets on digestion, nutrient absorption and
histological intestinal integrity. For this purpose, all diets formulated had a low
FM content (25%) and were rich (75%) in plant protein sources (corn gluten
meal, soybean meal and extruded wheat). FO was replaced at 0, 33, 66 and
100% (referred to as FO, 33VO, 66VO and 100VO) by graded levels of a blend
of vegetable oils (VOs; rapeseed, linseed and palm oils).

Protease activity was increased in pyloric caeca and decreased in the proximal
intestine of the 66VO and 100VO groups in response to FO replacement by VO,
while lipase and α-amylase activites were not modified. The capacity of brush
border membrane vesicles obtained from pyloric caeca, proximal intestine and
distal intestine to absorb amino acids was not modified; however, D-glucose
and linoleic acid absorption diminished when VO was included in the diets.

These results indicate a progressive delay in transit rate when up to 66% of FO
is replaced by VO. Only the 100VO group showed impaired digestion, with
reduced total protease activity and the accumulation of lipid droplets in the
enterocytes of the proximal intestine.
Keywords: protease; amylase; lipase; brush border membrane vesicles; vegetable oil.
2. INTRODUCTION

The limited availability of fish oil (FO) and fish meal (FM), together with the increase in aquaculture (SOFIA, 2008), calls for the identification of alternative lipid and protein sources for the development of sustainable fish farming activities. Plant ingredients are among the alternatives (Bell & Waagbø, 2008; Turchini et al., 2009).

The inclusion of vegetable oils (VOs) in the diets of carnivorous fish yields growth rates similar to those obtained under FO diets (Glencross et al., 2003; Mourente et al., 2005). However, digestive and absorptive processes can be affected. Modifications in the composition of enterocyte membranes have been described in fish fed VOs (Caballero et al., 2003; Sitjà-Bobadilla et al., 2005). These alterations can compromise intestinal function by reducing lipid (Francis et al., 2007; Geruden et al., 2009) and protein (Francis et al., 2007) digestibility. Similarly, changes in brush-border enzyme activities have been reported in response to diet fatty acid (FA) composition (Cahu et al., 2000), and it has also been hypothesized that the digestibility of a given FA can be altered by the presence of another FA (Torstensen et al., 2000). Moreover, in rat jejunum, dietary fat modulates disaccharidase activities (Goda and Takase, 1994). Furthermore, the modification of membrane fluidity may alter nutrient transport (Drozdowski & Thomson, 2006). In this regard, higher uptake rates for amino acids and free FAs have been found in fish fed VO than in those fed FO (Jutfelt et al., 2007). However, in spite of these drawbacks and given the decrease in FO availability, alternative sustainable fat sources that allow satisfactory growth rates and an acceptable quality of the final product are required (Rosenlund et al., 2001).

Concerning FM replacement, it has been demonstrated that in sea bream (Sparus aurata) up to 75% of FM can be replaced by plant protein sources when diets are balanced to match fish amino acid requirements. FM replacement initially leads to a slight decrease in growth rate (Gomez-Requeni et al., 2004), which is overcome at the end of a complete culture cycle (De Francesco et al., 2007). However, previous studies on this species have reported that digestion processes (Santigosa et al., 2008) and protein retention...
(Kissil et al., 2000) are affected by plant protein diets because of the presence of anti-nutritional factors (ANFs) in meal (Francis et al., 2001; Gatlin et al., 2007).

Less information is available on the effects of simultaneous FO and FM replacement on sea bream (Bouraoui et al., 2006; Benedito-Palos et al., 2007; Benedito-Palos et al., 2008) or other species (Subhadra et al., 2006; Drew et al., 2007; Torstensen et al., 2008; Panserat et al., 2009; Salze et al., 2010). To our knowledge, no study has addressed the modifications of digestive enzymes and intestinal nutrient absorption caused by diets in which FM and FO have been replaced by proteins and lipids derived from plants, respectively.

In vertebrates, the gastrointestinal tract is the main site of food digestion and nutrient uptake. Digestion depends on the signalling pathway of the peptide hormone cholecystokinin (CCK). This hormone is synthesized by I-cells in the mucosal epithelium of the small intestine and secreted in the proximal intestine, where it stimulates the digestion of fat and protein by triggering the release of pancreatic enzymes that are active in the lumen. Serine proteases, glucosidases and lipases then break down the ingested proteins, carbohydrates and fats, and produce metabolites, which are then absorbed by enterocytes. Absorption occurs by diffusion, facilitated transport or active transport. In mammals, the apical translocation of lipolytic products into the enterocyte seems to occur by passive or active diffusion at high and low FA concentrations, respectively, by means of specialized carrier proteins, such as FATP (Stremmel et al., 1985). The uptake of amino acids, oligopeptides and glucose, which is mediated by both facilitated and active transport, follows asymptotic kinetics and depends on the presence of protein transporters immersed in the lipid bilayer (Casirola et al., 1995). Fish absorption mechanisms have been widely studied (Ferraris & Ahearn, 1984; Collie & Ferraris, 1995) and are similar to those of mammals, although fish show lower rates (Reshkin & Ahearn, 1987). These rates are programmed to match diet composition, and transport proteins are modulated to fit lumen nutrient concentration (Ferraris & Ahearn, 1984; Buddington et al., 1987).
Here we performed an 11-week growth trial in sea bream to study the effects of four experimental diets, in which FM and FO were simultaneously replaced by plant proteins and VO respectively, on the following parameters: 1) luminal protease, α-amylase and lipase activities; 2) intestinal absorption capacities and 3) intestinal absorption regionalization pattern. The experimental diets contained 75% plant protein and graded (0, 33, 66 and 100%) levels of a blend of VOs.

3. MATERIAL AND METHODS

3.1. Diets

Four experimental diets with a low level (25%) of FM and rich (75%) in plant protein sources (corn gluten meal, soybean meal and extruded wheat) were formulated. FO was replaced at 0, 33, 66 and 100% (FO, 33VO, 66VO and 100VO) by graded levels of a blend of vegetable oils (VOs; rapeseed, linseed and palm oils). Diets were supplemented with L-lysine and the DHA/EPA ratio (1.1-1.2) was kept constant (Table 1). For a more detailed description (including the FA profile) of the diets see Benedito-Palos et al., 2007.

3.2. Fish and sampling

Nine hundred and sixty gilthead sea bream (Sparus aurata) obtained from Ferme Marine de Douhet (Ile d'Olérn, France) were acclimatized at the Instituto de Acuicultura de Torre de la Sal –CSIC (Castellón, Spain) for 20 days. At the start of the experiment, fish weighing 16.2 ± 0.05 g were randomly distributed in 12 fibreglass tanks (500-L). The water (37.5% salinity) flow was 20 L·min⁻¹ and the oxygen content of outlet water was higher than 85% saturation. During the 11-week trial, triplicate groups of animals were manually fed the corresponding diet twice a day (9:00 and 14:00 h) until visual satiety. Day length and water temperature followed natural changes over the course of the trial (May-August). Growth coefficients and feed efficiency at the end of the growth trial were similar in the FO, 33VO and 66VO groups. The 100VO group showed reduced feed intake and growth (Benedito-Palos et al., 2007). At the end of the experiment, four animals per tank (12 fish per condition) were anesthetized (MS-222 0.1g L⁻¹.
weighed and sacrificed by severing the spinal cord 5 h after the morning manual feeding. Digestive tracts were isolated on ice and adherent tissue was removed. A portion of pyloric caeca (PC) and the first proximal segment (0.5 cm), including the intestinal content, were rapidly frozen in liquid nitrogen and maintained at –80ºC until enzymatic studies. A second segment of the proximal intestine (PI) (0.5 cm) was pre-conserved in 4% buffered formol for histological observation. The remaining PC and PI, and the whole distal intestine (DI) were cut lengthwise, washed in isosmotic saline solution containing 0.1 M protease inhibitor (phenyl-methyl-sulphonyl-fluoride, PMSF), and frozen in liquid nitrogen until nutrient absorption experiments.

3.3. Digestive enzyme activity

Preparation of enzyme extracts: Samples were individually homogenized in 50 mM TrisHCl; pH 7.5 (Politron 2000, Sorvall TC; 4ºC) to a final concentration of 250 mg tissue·mL⁻¹ for lipase activity analyses and to a final concentration of 50 mg tissue·mL⁻¹ for protease and α-amylase activity measurement. Homogenates were then centrifuged (1100xg, 15 min, 4ºC. Jouan CR 411) and supernatants were recovered and stored at –20ºC.

Total protease activity measurement: Acid protease activity in stomach samples was measured following Alarcón et al. (1998) using 0.5% haemoglobin in 0.1 M glycine-HCl pH 2.0 (30 min, 20ºC). Alkaline protease activity in PC and PI extracts was measured by the casein method described by Kunitz (1947), as modified by Walter (1984), using 1% casein in TrisHCl 50 mM pH 9.0 (30 min, 20ºC). Both reactions were stopped by adding 12% trichloracetic acid (TCA). Samples were then centrifuged (7500g, 5 min, 4ºC. Sigma 2-15) and supernatant absorbance at 280 nm was recorded (UV-1603, Shimadzu). All measurements were analyzed in triplicate and the blank for each sample was measured separately. Activity was referred to the soluble protein concentration of the extract, which was determined following Bradford (1976) using bovine serum albumin as standard. Alkaline protease activity was measured as BAEE units.

Modifications of individual protease activities: By means of zymography (García-Carreño et al. 1993), we identified changes in individual protease
activity in PC and PI. This method allowed the viewing of bands with protease activity in polyacrylamide (12%) gels (10 x 10.5 x 0.1 cm). Electrophoresis was performed at a constant current of 15 mA per gel (100 min, 4°C. EPS 301 Power Supply) and gels were incubated in a TrisHCl buffer pH 8.2 containing 2% casein (30 min, 4°C and 90 min 25°C). They were then washed and stained in a methanol:acetic:water solution (40:10:40) with 0.1% of Coomassie brilliant blue R-250. Distaining was done using the same solution without colorant. Molecular weights were established using a molecular weight marker (RPN800, Amersham Pharmacia Biotech, Spain). The combination of the extracts in polyacrylamide gels with specific inhibitors allowed identification of the specific activities of the bands (Santigosa et al., 2008). Individual PC and PI fish extracts were analyzed by substrate-SDS-PAGE.

**α-Amylase activity**: We analyzed α-amylase activity in PC and PI extracts following the method described by Dold et al. (1995), as modified by Santigosa et al. (2008). This method involves the use of the tracer material amylose azure, a potato starch covalently labelled with Ramazol Brilliant Blue R dye. Briefly, 100 µL of homogenate was combined with 400 µL of 0.5% tracer solution. Soybean trypsin inhibitor (SBTI, 0.04 mg mL$^{-1}$) was added to prevent proteolysis. The reaction was stopped after 30 min by adding 6% acetic acid. After 30 min at 4°C, the tubes were centrifuged (13,000 g, 10 min, 4 °C). Supernatant absorbance was recorded at 595 nm. Each sample was analyzed in triplicate and individual blanks were established. α-Amylase was used as standard. One unit of enzyme activity was defined as 1.0 mg of maltose released from starch in 3 min at pH 6.9 at 20 °C.

**Lipase activity**: Lipase activity was analysed in PI extracts using a modification of the method described by Panteghini (2001). Briefly, 50 µL of homogenate was combined with 950 µL of a buffer containing (in mM) 20.5 Tris, 3.6 taurodeoxicolate, 0.9 deoxicolate, 0.8 tartrate, 0.12 DGGR (1,2-o-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin) ester), 0.05 CaCl2, 30 mannitol, and 1 mg L$^{-1}$ colipase (pH 8.3), and the increase in absorbance was recorded at 580 nm between 10 and 20 min. Each sample was analyzed in triplicate and individual blanks were established. Lipase was used as standard. One unit
hydrolyzes 1.0 microequivalent of fatty acid from triacetin in 1 h at pH 7.4 at 37°C.

3.4. Nutrient uptake analysis

Brush border membrane vesicles (BBMVs) from PC, PI and DI were obtained as described by Sala-Rabanal et al. (2004). Briefly, samples were thawed in a hypoosmotic buffer (in mM: 100 mannitol, 2 HEPES, pH 7.4) and placed in a Waring blender at low speed to separate mucose from the muscular layers. The resulting eluate was filtered through a Büchner funnel and homogenized at high speed. The homogenate was subsequently filtered through nylon stocking material, and MgCl$_2$ was added to the eluent to a final concentration of 10 mM, for selective precipitation of most cellular membranes except for the brush border. Subsequent selective centrifugations allowed the purification and concentration of apical enterocyte membranes, which were vesiculated using an insulin syringe.

Validation of the BBMV suspensions: To ensure the enrichment in brush border membrane, as well as the depletion in other cellular fractions, the activity of membrane enzymes considered suitable cellular markers (Scalera et al., 1980) was measured (20°C) in the initial homogenate and in the final BBMV preparations. Thus, alkaline phosphatase activity was measured following Weiser (1973) to ensure an increase in brush border membrane during vesiculation. The decrease in Na$^+$/K$^+$-ATPase activity (Sala-Rabanal et al. 2004) and in citrate synthase activity (Srere, 1969) ruled out possible contaminations with baso-lateral and mitochondrial membranes, respectively.

The orientation of BBMVs was studied using a modification of the approach described by Del Castillo & Robinson (1982). Thus, vesicle preparations were incubated in the presence and absence of a solution of 2 mM sodium deoxycholate containing EDTA 15 mM, which disrupts cellular membranes. The total saccharase activity of the solution was then measured by adding 100 μM sacarose to the preparations. An increase in saccharase activity in disrupted versus non-disrupted BBMVs indicates the presence of vesicles in a non-physiological orientation.
**BBMV uptake of amino acids, glucose and linoleic acid:** The capacity of BBMVs to absorb 3 essential amino acids (L-leucine, L-lysine and L-phenylalanine) and 3 non-essential amino acids (L-alanine, L-glutamic and L-proline) was studied. Glucose and linoleic influxes were also determined. In all cases, we used the technique described by Sala-Rabanal et al. (2004).

For this measurement, 10μL of BBMVs was mixed with 40μL of incubation buffer (in mM: 250 NaSCN, 100 mannitol, 40 HEPES, 0.1 MgSO₄·7H₂O, 8.16 LiN₃, 0.15 unlabelled nutrient, 0.01³H-nutrient. Osm 320; pH 7.4). After 5 s (lineal uptake zone previously determined. See Fig. 1) the reaction was stopped by adding 1 mL of cold stop buffer (in mM: 300 mannitol, 20 HEPES, 0.1 MgSO₄·7H₂O, 4.08 LiN₃. Osm 320; pH 7.4). We then rapidly passed 990 μL of the resulting mix under negative pressure through 0.22 μm cellulose nitrate filters (Millipore, Bedford MA) previously wet in cold stop buffer. Filters were washed with 10 mL of stop solution and dissolved in Filtron-X scintillation liquid (ITISA S.A, Spain). Samples were counted in a scintillation counter (Packard TRI-CARB 2100 TR). All measurements were done at 20ºC. Because of the hydrophobic properties of linoleic acid, it was complexed with albumin (following Tocher et al., 2002) before addition to the incubation buffer. Thus, an aliquot of ¹⁴C FA (0.75 μCi) was incubated for 10 min with a solution containing 0.428 mmols KOH · μCi⁻¹ and then for 45 min in the incubation buffer previously described containing 600 μg · μCi⁻¹ FA-free bovine serum albumin. We then mixed 40 μL of the resultant solution with 10 μL of vesicles and performed the analysis as previously described.

**Determination of vesicular volume using L-alanine:** The BBMV volume varies depending on the intestinal segment studied (Sala-Rabanal et al., 2004). Diet composition may also modify this parameter. Thus, we measured the vesicular volume of BBMV preparations following Sala-Rabanal et al. (2004). Briefly, L-alanine retained inside the vesicles in the steady state was measured by incubating 10 μL of BBMV preparation with 40 μL of the incubation buffer on ice. After 90 min the reaction was stopped and L-alanine was measured following the procedure described above.
The protein concentration of the individual BBMV preparations was measured following Bradford (1976) using the BIORAD\textsuperscript{R} protein assay reagent.

3.5. Histological observation

Samples conserved in buffered formol were embedded in paraffin, cut (5 μm), and stained with haematoxylin-eosin for observation under a light microscope.

3.6. Statistical analysis

All data presented a normal distribution (p>0.05 in Kolmogorov-Smirnov Test). The t-Student test was used to establish significant differences (p<0.05) between two groups. Differences between intestinal regions or dietary treatments were determined using one way ANOVA and Tukey test. The software used was SPSS 12.0 (SPSS Inc., US).

3.7. Chemicals

Radiolabelled nutrients were obtained from Amersham Pharmacia Biotech (Spain). The remaining reagents were purchased from Sigma-Aldrich (Spain), unless stated otherwise.

4. RESULTS

Total protease activity (TPA) was measured in PC and PI 5 h post-feeding (Fig. 1). The FO group showed maximal TPA activity in PI, with a TPA PI/PC ratio of 2.57. This pattern was maintained in the 33VO group, although the ratio decreased to 1.77. Fish in the 66VO and 100VO treatments showed higher TPA activity in PC than in PI (PI/PC ratio of 0.78 and 0.90, respectively). Moreover, the 100VO group showed a decrease in total alkaline TPA activity compared to the rest of the groups.

In the zymograms of PC and PI extracts, five bands (90, 60, 50, 30 and 25KDa) with proteolytic activity were detected (Fig. 2). The combination of the same extracts in polyacrylamide gels with specific inhibition solutions allowed identification of the specific activity of each band (Santigosa et al., 2008). Thus, the two highest molecular weight bands presented trypsin-like activity, while the three lower bands (50, 30 and 25KDa) showed chymotrypsin-like activity. The 60 KDa trypsin-like and 25 KDa chymotrypsin-like bands showed the greatest
activity, while a minor contribution was related to the highest molecular weight band (90KDa).

In PC zymograms (Fig. 2A), the increase in dietary VO resulted in a higher intensity and amplitude of trypsin- and chymotrypsin-like proteolytic bands. The progressive increase in the magnitude of all four PC proteolytic bands supports the results obtained using the spectrophotometric quantitative approach.

In PI extracts (Fig. 2B), although the 90 and 50 KDa bands were not modified in response to the replacement of the lipid source, a decrease in the intensity of 30 and 25-KDa bands was detected in the 66VO group and a decrease in the intensity of both 60 and 25 KDa bands and especially the 30 KDa band in the 100VO group.

α-Amylase activity in PC and PI samples 5h after manual feeding (Fig. 3) was not significantly modified by the replacement of FO by VOs. Neither was significant the difference found between the two intestinal regions of any of the VO-fed groups.

Lypase activity in PC samples (Fig. 4) was not significantly modified when FO was replaced by VOs (6.88 ± 2.80 and 4.81 ± 1.51 mU⁻¹ protein · min⁻¹ for FO and VO animals, respectively).

In order to study the effect of the diet on the capacity to absorb amino acids, D-glucose and linoleic acid, as well as the regionalization of this capacity along the intestinal tract, we also examined BBMVs from PC, PI and DI from fish on the 4 experimental diets. Vesicular preparations were checked in order to determine their purity (Table 2) and physiological orientation. The increase in alkaline phosphatase activity together with the decrease in Na⁺/K⁺ ATPase and citrate synthase activities in the final preparations when compared to the initial homogenates indicated that final suspensions were enriched in BBMVs and ruled out contamination with other cellular fractions. In all cases, vesicles presented a physiologic orientation (99.42 ± 0.25%; n=12). For all the nutrients examined, uptake was linear for almost 7 s for PC, PI and DI (Fig. 5). Vesicular volume was determined in BBMV preparations from the three vesicular types (Table 3). This parameter differed among intestinal regions and dietary treatments, being significantly higher in vesicular suspensions obtained from DI
than in PC and PI. Moreover, the volume of PI BBMVs was higher in the 66VO group. As a result of these changes we decided to normalize influx values, which are presented as intravesicular concentration after 5 s of incubation. We detected a regionalization of amino acid uptake capacity along the digestive tract, with a decrease in absorption capacity from PC to DI (Tables 4 and 5). In general, the replacement of FO by VO had no effect on amino-acid uptake. Similar values were observed for each intestinal segment in the 4 experimental groups. D-glucose uptake (Fig. 6A) was significantly higher in BBMVs from PI of the FO group. In VO groups, uptake decreased, whilst PC increased in response to 100% FO replacement. Linoleic acid uptake (Fig. 6B) was maximal in PC BBMVs obtained from the FO group. The uptake of this FA was lower in BBMVs obtained from the three intestinal segments in the VO groups. Histological preparations of PI from fish on the FO (Fig. 7A) and 100VO (Fig. 7B) diets showed no differences in fold length or intestinal layer integrity at the end of treatment. However, an increase in lipid droplet content, together with a decrease in the number of goblet cells, was detected in the 100VO group. These changes were progressive as the percentage of VO in the diet increased (images of fish on 33VO and 66 VO diets not shown).

5. DISCUSSION

The FO and FM traditionally used in aquafeeds are limited primary resources (SOFIA, 2008). Plant protein may provide a suitable substitute for the formulation of carnivorous fish diets. Digestive enzymatic activity is compromised when diets contain mid or high levels of plant protein sources (Santigosa et al., 2008). Here we fed gilthead sea bream with diets containing 75% of plant protein and graded levels (0, 33, 66 and 100%) of a blend of vegetable oils. Fish showed 5 proteolytic bands in PC and PI 5 h after feeding. These results correspond to the alkaline proteolytic bands reported by Alarcón et al. (1999) for this species. A 55KDa band with proteolytic activity detected in a previous trial (Santigosa et al., 2008) was not detected in the present study. This absence may be attributable to the distinct blends of VO used to replace FM in the studies. Furthermore, each plant
protein source has specific ANFs (Francis et al., 2001; Gatlin et al., 2007). Moreover, the modification of the dietary lipid sources may be the responsible of the quantitative modification of enzymatic digestive activities 5 h post-feeding in PC and PI of the 66VO and 100VO groups. In this regard, a significant decrease in apparent protein digestibility in Murray cod fed VO diets has been described (Francis et al., 2007). Similarly, lower trypsin activities were detected in Senegalese sole fed on Artemia enriched with soybean oil emulsion than those fed Artemia enriched with FO emulsion (Morais et al. 2006). Our data show a progressive variation of the TPA PI/PC ratio, which could be related to the inhibition of luminal proteases as a result of the presence of ANFs in the VO, as also reported when plant protein sources are used in carnivorous fish aquafeed (Moyano et al., 1999; Santigosa et al., 2008). However, the progressive increase in PC TPA respective to VO inclusion seems to contradict this hypothesis, and points to a decrease in transit rate as the most plausible explication. In this regard, the modification of dietary lipid sources could be responsible for modified emulsification of the dietary bolus and a differential micellar incorporation, which could lead to slower passage along the intestine, with a concomitant slower release of proteases in the lumen. Since proteases exert distinct functions in digestion (Guillaume and Choubert, 2001), the modifications described in the trypsin/chymotrypsin ratio may imply differential availability of luminal oligopeptides and amino acids, which may lead to an amino acid imbalance under these feeding conditions.

This prompted us to study possible modifications in absorptive phenomena along the intestinal tract. Our data show a regionalization of intestinal amino acid transport, with absorption being greater in PC and PI segments, which is consistent with the findings of other studies on nutrient uptake in carnivorous fish (Sala-Rabanal et al., 2004; Bakke-McKellep et al., 2000; Jutfelt et al., 2007). We detected no differences in the capacity of intestinal BBMVs to absorb the 3 essential amino acids (L-leucine, L-lysine and L-phenylalanine) and the 3 non-essential amino acids (L-alanine, L-glutamic and L-proline ) in response to dietary lipid source. This observation is in accordance with the satisfactory growth rates of marine species fed VO diets (Glencross et al., 2003; Mourente
et al., 2005). However, diet lipid composition modified the volume of the BBMVs in PI. This modification may be attributable to a variation in the lipid bilayer composition. In this regard, changes in the FA profile of intestinal mucosa polar lipids have been described in sea bream (Caballero et al., 2003; Sitjà-Bobadilla et al., 2005) in response to dietary plant lipids. The consideration of the vesicular volume in this experience may explain the divergence of our data from other existing works (Jutfelt et al., 2007; Geurden et al., 2009) in which VO inclusion modified intestinal uptake. Moreover, in mammals the modification of intestinal mucosa polar lipids alters passive properties across the membrane (Spector and Yorek, 1985). This observation could explain the decrease in linoleic acid uptake in the three intestinal segments observed in sea bream on 33VO, 66VO and 100VO diets, although no differences in intestinal lipase activity were found in this study. However, since enterocytes incorporate this FA at high concentrations by diffusion (Stremmel et al., 1985) as shown in this experiment, the increase in luminal linoleic availability in response to the progressive replacement of FO by VO (from 12.1% to 21.3% of total FAME in FO vs. 100VO groups, respectively; Benedito-Palos et al., 2007) should also be taken into account.

Our study indicates that the inclusion of VO in feed for juvenile sea bream does not alter amino acid absorption capacity. This finding contrasts to what occurs when FM is replaced by plant protein (data not shown). Our findings indicate good amino acid digestion and absorption efficiency of fish on FO, 33VO and 66VO diets and reinforce the previously stated hypothesis of decreased transit rate versus protease inactivation. This hypothesis is supported by the high SGR values obtained after 11 weeks of dietary treatment (Benedito-Palos et al., 2007). The lower growth rates recorded in the 100VO group after the 11-week trial could be associated with reduced feed intake (Benedito-Palos et al., 2007), or to the accumulation of lipid droplets in PI enterocytes, which has been previously related to impaired transport rate from intestinal mucosa to the blood (Salhi et al., 1999). Although VO inclusion in finfish diets has been associated with intestinal lipid accumulation (Caballero et al., 2003, Olsen et al., 2003), the histological study of the posterior intestine in sea bream on the same diets
showed no pathological lipid accumulation in the enterocytes (Benedito-Palos et al., 2008). These discrepancies may be due to sample origin (anterior versus posterior intestine), but may also be related to the supplementation of diets with phospholipids in the form of soya lecithin, as previously proposed by Benedito-Palos et al. (2008).

Our data showed that in fish fed the FO diet, D-glucose is absorbed mainly in the PI. This result is in agreement with those of other studies performed in sea bream (Sala-Rabanal et al., 2004). The three groups fed VO diets showed a decrease in monosaccharide uptake in this intestinal segment. This observation may indicate modifications in the density of specific transporters, as described in channel catfish (Houpe et al., 1997), or changes in the conformation of the binding sites of transport proteins, as described for rabbit SGLT1 and GLUT5 (Meddings et al., 1990). As expected, since carbohydratases are genetically programmed to be low (Cahu & Infante, 1995), fish on diets in which FM and FO were simultaneously replaced by plant protein and VO respectively did not have the capacity to modulate the activity of α-amylase in response to a decrease in glucose uptake.

In summary, the replacement of up to 66% of FO by a blend of VOs when 75% of FM is replaced by plant protein sources and slowed down protease digestion, increasing the trypsin/chymotrypsin ratio at 5 h post-feeding respective to VO-inclusion. No differences in the capacity of amino acid absorption were found. Total FO replacement resulted in decreased TPA and increased the accumulation of lipid droplets in the PI enterocytes. All these modifications could explain the lower growth performance in the fish on the diet with maximal simultaneous FO replacement (Benedito-Palos et al., 2007).

6. ACKNOWLEDGMENTS

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Table 1. Composition of the experimental diets.

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<th>FO</th>
<th>33VO</th>
<th>66VO</th>
<th>100VO</th>
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### Ingredient (g Kg⁻¹)

<table>
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<th>Ingredient</th>
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<td>CPSP 90</td>
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<td>Corn gluten meal</td>
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<td>Soybean</td>
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<td>Extruded wheat</td>
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<td>Linseed oil</td>
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<tr>
<td>Palm oil</td>
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</table>

### Proximate composition

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<th>PI</th>
<th>DI</th>
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<td>Protein (%)</td>
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<td>49.0</td>
</tr>
<tr>
<td>Lipids (%)</td>
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<td>22.3</td>
<td>22.1</td>
</tr>
<tr>
<td>Energy (MJ·Kg⁻¹ DM)</td>
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<td>24.7</td>
<td>24.7</td>
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Diets were supplemented with minerals and vitamins. For more details on the composition see Benedito-Palos et al. (2007).

### Table 2. BBMV marker enzyme enrichment factor.

<table>
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<tr>
<th>Intestinal segment</th>
<th>Phosphatase alkaline</th>
<th>Na⁺/K⁺ATPase</th>
<th>Citrate synthase</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>5.21 ± 0.45</td>
<td>0.42 ± 0.02</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>PI</td>
<td>3.39 ± 0.19</td>
<td>0.66 ± 0.02</td>
<td>0.12 ± 0.04</td>
</tr>
<tr>
<td>DI</td>
<td>5.15 ± 0.46</td>
<td>0.80 ± 0.01</td>
<td>0.09 ± 0.03</td>
</tr>
</tbody>
</table>
Enrichment factor is the ratio between the specific activity measured in the final BBMV preparations and the initial homogenate. Results are presented as the mean ± S.E.M. of the different treatments for each marker enzyme (n=16). No differences among treatment were found. Pyloric caeca (PC), proximal intestine (PI) and distal intestine (DI).

Table 3. Vesicular volume referred to protein content (μL · mg⁻¹ protein) of BBMVs obtained from intestinal segments of sea bream.

<table>
<thead>
<tr>
<th>Intestinal segment</th>
<th>FO</th>
<th>33VO</th>
<th>66VO</th>
<th>100VO</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>1.29 ± 0.13 ²</td>
<td>1.65 ± 0.18 ²</td>
<td>1.79 ± 0.40 ²</td>
<td>2.10 ±0.12 ²</td>
</tr>
<tr>
<td>PI</td>
<td>1.42 ± 0.04 b, ²</td>
<td>1.81 ± 0.50 b, ²</td>
<td>3.85 ± 0.63 a, ₁²</td>
<td>2.81 ± 0.29 ab, ²</td>
</tr>
<tr>
<td>DI</td>
<td>4.97 ± 0.34 ¹</td>
<td>6.48 ± 0.66 ¹</td>
<td>5.64 ± 0.94 ¹</td>
<td>4.70 ± 0.17 ¹</td>
</tr>
</tbody>
</table>

Results are presented as the mean ± S.E.M. of 8 measurements for each condition. Significant differences (p<0.05) between diets are shown by different letters; significant differences between intestinal segments are shown by different numbers. Pyloric caeca (PC), proximal intestine (PI) and distal intestine (DI).
Table 4. Intravesicular essential amino acid concentration (pmols mL\(^{-1}\)) in BBMVs obtained 5 h post-feeding.

<table>
<thead>
<tr>
<th>Intestinal segment</th>
<th>FO</th>
<th>33VO</th>
<th>66VO</th>
<th>100VO</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>1084.9 ± 127.4(^1)</td>
<td>1568.4 ± 124.9(^1)</td>
<td>1449.1 ± 250.5(^1)</td>
<td>1166.20 ± 29.74(^1)</td>
</tr>
<tr>
<td>L-Leu</td>
<td>1090.0 ± 56.2(^{ab,1})</td>
<td>1236.9 ± 97.6(^{a,1})</td>
<td>662.6 ± 120.5(^{b,2})</td>
<td>1092.30 ± 104.01(^{ab,1})</td>
</tr>
<tr>
<td>DI</td>
<td>298.4 ± 17.4(^{b,2})</td>
<td>473.2 ± 80.0(^{a,2})</td>
<td>486.8 ± 39.9(^{a,2})</td>
<td>455.92 ± 23.21(^{ab,2})</td>
</tr>
<tr>
<td>PC</td>
<td>2553.5 ± 314.8(^1)</td>
<td>2619.8 ± 234.0(^1)</td>
<td>2015.5 ± 359.1(^1)</td>
<td>2401.8 ± 24.1(^1)</td>
</tr>
<tr>
<td>L-Lys</td>
<td>1811.8 ± 283.7(^{ab,1})</td>
<td>2140.4 ± 437.2(^{a,1})</td>
<td>1530.6 ± 465.3(^{b,12})</td>
<td>1856.5 ± 98.1(^{ab,2})</td>
</tr>
<tr>
<td>DI</td>
<td>457.7 ± 12.5(^{b,2})</td>
<td>737.0 ± 69.0(^{a,2})</td>
<td>594.1 ± 42.8(^{ab,2})</td>
<td>736.6 ± 59.5(^{a,3})</td>
</tr>
<tr>
<td>PC</td>
<td>4059.2 ± 690.92(^1)</td>
<td>4965.2 ± 368.6(^1)</td>
<td>4003.7 ± 414.2(^1)</td>
<td>4272.34 ± 236.02(^1)</td>
</tr>
<tr>
<td>L-Phe</td>
<td>3565.8 ± 272.19(^{a,1})</td>
<td>3356.7 ± 138.4(^{a,2})</td>
<td>1000.0 ± 49.1(^{b,2})</td>
<td>3009.74 ± 192.87(^{a,2})</td>
</tr>
<tr>
<td>DI</td>
<td>936.2 ± 55.8(^{b,2})</td>
<td>1626.6 ± 255.5(^{a,3})</td>
<td>1214.9 ± 232.3(^{ab,2})</td>
<td>1002.22 ± 159.13(^{ab,3})</td>
</tr>
</tbody>
</table>

Results are presented as the mean ± S.E.M of 8 measurements. Significant differences (p<0.05) between diets are shown by different letters; significant differences between intestinal segments are shown by numbers. Pyloric caeca (PC), proximal intestine (PI) and distal intestine (DI).
Table 5. Intravesicular non-essential amino acid concentration (pmols mL⁻¹) in BBMVs obtained 5 h post-feeding.

<table>
<thead>
<tr>
<th>Intestinal segment</th>
<th>FO</th>
<th>33VO</th>
<th>66VO</th>
<th>100VO</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>147.3 ± 13.1 (^{b,2})</td>
<td>608.2 ± 268.0 (^{a})</td>
<td>734.6 ± 127.7 (^{ab,1})</td>
<td>684.42 ± 71.89 (^{ab})</td>
</tr>
<tr>
<td>L-Ala</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI</td>
<td>438.8 ± 53.1 (^{12})</td>
<td>1029.4 ± 245.0</td>
<td>826.9 ± 83.9 (^{1})</td>
<td>701.59 ± 152.02</td>
</tr>
<tr>
<td>DI</td>
<td>672.8 ± 113.6 (^{1})</td>
<td>424.8 ± 144.1</td>
<td>393.1 ± 37.2 (^{2})</td>
<td>487.33 ± 80.40</td>
</tr>
<tr>
<td>PC</td>
<td>233.5 ± 38.8 (^{1})</td>
<td>197.0 ± 7.5 (^{1})</td>
<td>218.6 ± 54.5 (^{1})</td>
<td>174.3 ± 5.6 (^{1})</td>
</tr>
<tr>
<td>L-Glu</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI</td>
<td>222.9 ± 5.4 (^{a,1})</td>
<td>189.6 ± 13.4 (^{ab,1})</td>
<td>164.4 ± 9.4 (^{b,12})</td>
<td>161.0 ± 4.2 (^{b,1})</td>
</tr>
<tr>
<td>DI</td>
<td>119.1 ± 8.6 (^{a,2})</td>
<td>110.0 ± 11.6 (^{ab,2})</td>
<td>90.4 ± 5.6 (^{ab,2})</td>
<td>80.3 ± 5.5 (^{b,2})</td>
</tr>
<tr>
<td>PC</td>
<td>436.954 ± 45.0 (^{1})</td>
<td>570.8 ± 34.6 (^{1})</td>
<td>451.4 ± 75.9 (^{1})</td>
<td>571.73 ± 48.43 (^{1})</td>
</tr>
<tr>
<td>L-Pro</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI</td>
<td>499.551 ± 8.5 (^{a,1})</td>
<td>491.8 ± 13.5 (^{a,2})</td>
<td>301.74 ± 32.5 (^{b,12})</td>
<td>421.98 ± 34.17 (^{a,2})</td>
</tr>
<tr>
<td>DI</td>
<td>147.851 ± 3.0 (^{2})</td>
<td>186.3 ± 8.0 (^{3})</td>
<td>185.9 ± 19.6 (^{2})</td>
<td>174.74 ± 11.59 (^{3})</td>
</tr>
</tbody>
</table>

Results are presented as the mean ± S.E.M of 8 measurements. Significant differences (p<0.05) between diets are shown by different letters; significant differences between intestinal segments are shown by numbers. Pyloric caeca (PC), proximal intestine (PI) and distal intestine (DI)
LEGEND OF FIGURES

Figure 1. Total protease activity at 5h post-feeding. Values are represented as the mean ± S.E.M of 12 fish. Significant differences (p<0.05) between diets are shown by different letters; significant differences (p<0.05) between intestinal segments are shown by different numbers. Pyloric caeca (white bars) and proximal intestine (grey bars). The FO diet contained fish oil as the only source of lipids; in the 33VO, 66VO and 100VO diets, 33, 66 and 100% of the fish oil was replaced by a blend of vegetable oils respectively.

Figure 2. Model zymograms of the alkaline proteolytic activity in pyloric caeca (A) and proximal intestine (B) extracts. Figure shows the molecular weight.

Figure 3. α-amylase activity at 5h post-feeding. Values are represented as the mean ± S.E.M of 12 fish. No significant differences (p<0.05) were found between diets or intestinal segments. Pyloric caeca (white bars) and proximal intestine (grey bars).

Figure 4. Lipase activity in pyloric caeca at 5h post-feeding. Values are represented as the mean ± S.E.M of 9 fish. No significant differences (p<0.05) were found between diets.

Figure 5. Time course (0 to 10 s) of nutrient uptake of BBMV obtained from pyloric caeca (black circles), proximal intestine (grey circles) and distal intestine (open circles) of sea bream: Values are represented as the mean ± S.E.M of 4 measurements. $r^2$ is shown for all the conditions.

Figure 6. Intravesicular D-glucose and linoleic acid concentration in BBMVs obtained from pyloric caeca (PC, white bars), proximal intestine (PI, grey bars) and distal intestine (DI, black bars) of sea bream 5h post-feeding. Results are presented as the mean ± S.E.M of 8 measurements. Significant differences (p<0.05) between diets are shown by different letters; significant differences between intestinal segments are shown by numbers.

Figure 7. Histological appearance (400X) of the proximal intestine obtained from sea bream after 11 weeks of dietary treatment. (A) Fish on the FO diet. (B) Fish on the 100VO diet. Lipid droplets (black arrows) and goblet cells (dotted arrows).
Figure 1

Total Protease Activity (U/mg protein/min.)

FO 33VO 66VO 100VO
Figure 3

α-amylase activity (mU mg\(^{-1}\) protein min\(^{-1}\))
Figure 4

[Bar graph showing lipase activity (mU·1 protein⁻¹·min⁻¹) for different conditions: FO, 33VO, 66VO, VO.]

FO 33VO 66VO VO
Figure 5

- **L-leucine**
  - $R^2 = 0.9606$
  - $R^2 = 0.9336$
  - $R^2 = 0.9746$

- **L-lysine**
  - $R^2 = 0.9914$
  - $R^2 = 0.9983$
  - $R^2 = 0.95$

- **L-phenylalanine**
  - $R^2 = 0.9412$
  - $R^2 = 0.9915$
  - $R^2 = 0.9663$

- **L-proline**
  - $R^2 = 0.9409$
  - $R^2 = 0.9161$
  - $R^2 = 0.9733$

- **L-lysine**
  - $R^2 = 0.9383$
  - $R^2 = 0.95$
  - $R^2 = 0.9214$

- **L-glutamic**
  - $R^2 = 0.9058$
  - $R^2 = 0.9338$
  - $R^2 = 0.9563$

- **L-linoleic**
  - $R^2 = 0.9467$
  - $R^2 = 0.9348$
  - $R^2 = 0.8421$

- **L-alanine**
  - $R^2 = 0.945$
  - $R^2 = 0.9474$
  - $R^2 = 0.9412$

- **L-leucine**
  - $R^2 = 0.9336$
  - $R^2 = 0.9746$
  - $R^2 = 0.9606$

- **D-glucose**
  - $R^2 = 0.9654$
  - $R^2 = 0.9064$
  - $R^2 = 0.91$

- **Linoleic acid**
  - $R^2 = 0.9654$
  - $R^2 = 0.9064$
  - $R^2 = 0.91$

**INFLUX (pmols mg prot⁻¹)**
Figure 6

A

D-glucose

Concentration (pmols mL⁻¹)

FO 33VO 66VO 100VO

Linoleic acid

Concentration (pmols mL⁻¹)

FO 33VO 66VO 100VO