Effects of partial substitution of dietary fish oil with blends of vegetable oils, on blood leukocyte fatty acid compositions, immune function and histology in European sea bass, (*Dicentrarchus labrax* L.)

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Within a decade or so insufficient fish oil (FO) will be available to meet the requirements for aquaculture growth. Consequently, alternative sources are being investigated to reduce reliance on wild fish as a source of FO. Vegetable oils (VO) are a feasible alternative to FO. However, it is important to establish that alternative dietary lipids are not only supplied in the correct quantities and balance for optimal growth, but can maintain immune function and prevent infection, since it is known that the nutritional state of the fish can influence their immune function and disease resistance. A way of maintaining immune function, while replacing dietary FO, is by using a blend of VOs rather than a single oil. In this study, juvenile European sea bass, *Dicentrarchus labrax,* were fed diets with a 60 % substitution of FO with a blend of rapeseed (RO), linseed (LO) and palm oils (PO). Two oil blends were used to achieve a fatty acid composition similar to FO, in terms of energy content and provide a similar balance of saturates, monounsaturates and polyunsaturated fatty acids. Fish were fed the diets for 64 weeks, after which time growth and fatty acid compositions of liver and blood leukocytes were monitored. The impact of the dietary blends on selected innate immune responses and histopathology were also assessed, together with levels of plasma prostaglandin E_2. The results suggest that potential exists for replacing FO with a VO blend farmed sea bass feeds without compromising growth, non-specific immune function or histology.

Global catches from the feed grade fisheries that provide fish oil (FO) and fish meal for aquafeed formulations have reached their sustainable limits[1] and it is likely that within a decade or so there may be insufficient FO to meet the quantities required for current aquaculture growth[2]. Consequently, there has been considerable interest in introducing sustainable alternatives to fish meal and FO that reduce reliance on marine raw materials[3,4] A number of recent studies suggest that dietary vegetable oil (VO) inclusion does not result in reduced growth performance or feed conversion in Atlantic salmon, *Salmo salar*[5,6], rainbow trout, *Oncorhynchus mykiss*[7] gilthead sea bream, *Sparus aurata*[8] or European sea bass, *Dicentrarchus labrax*[9]. However, at levels above 50% VO inclusion, significant accumulation of fatty acids derived from VO, especially 18:2*n*-6, and reduction of eicosapentaenoic (20:5*n*-3; EPA) and docosahexaenoic acids (22:6*n*-3; DHA) occurs in fish tissues[5,6,8,9].

The nutritional status of an organism, including fish, is known to influence immune functions[10] and the overall resistance of an organism to disease is therefore dependent on their nutritional status. The first review suggesting that fatty acids might be important in immune function was by Meade & Mertin[11] and more recent reviews have confirmed the importance of the polyunsaturated fatty acids (PUFA), of both the *n*-6 and *n*-3 series, as modulators of immune function[12,13]. Fatty acids are incorporated into the plasma membrane from dietary lipids, so that the fatty acid composition of cellular membranes reflects the composition of dietary lipids[14]. In fish, dietary fatty acids and tissue fatty acid compositions are closely correlated[15] and changes in the dietary *n*-3/*n*-6 ratio can influence the compositions of fish immune cells, including blood leukocytes.[16,17,18]

Fatty acids have diverse roles in all cells. They are important as a source of energy, as structural components of cell membranes and as signalling molecules. In mammalian studies, dietary fatty acids may be able to modulate the immune system through several mechanisms including reduction of lymphocyte proliferation, cytokine synthesis and phagocytic activity and also by modification of natural killer cell activity[19]. The main event in the modulation of immune function may be associated with changes in the cell membrane due to dietary fatty acid manipulation. It is likely that modulation of the overall immune system occurs as a result of alterations in membrane fluidity, lipid peroxidation, eicosanoid production or regulation of gene expression[20].

In the present study, triplicate groups of juvenile European sea bass were fed diets that were based on 60% substitution of FO with a blend of rapeseed (RO), linseed (LO) and palm oils (PO). The level of 60% substitution was chosen as this was the maximum level of VO inclusion that could be tolerated in marine fish without loss of growth performance.[8,9] The oils

were blended in two different formulations to achieve a fatty acid composition as similar to anchovy oil as possible, in terms of energy content and provide a similar balance of saturates, monounsaturates and PUFA to that found in FO, but without highly unsaturated fatty acids (HUFA). The fish were fed the diets for 64 weeks starting at an initial weight of approximately 5 g. Growth parameters and the fatty acid compositions of liver and peripheral blood leukocytes were monitored after 64 weeks. The impact of the dietary blends on selected aspects of the innate immune response (haematological parameters, serum lysozyme activity and macrophage respiratory burst activity) and histopathology were also assessed in the experimental fish at this time, together with levels of plasma prostaglandin E_2.

Materials and methods

Experimental fish and diets

European sea bass (*Dicentrarchus labrax* L.), 7.9 ±0.5 cm in length and 5.2 ±1.0 g in weight were purchased from MARESA, Huelva, Spain and transported to the marine aquarium facility at the University of Cádiz, Faculty of Marine and Environmental Sciences, Puerto Real (Cádiz). On arrival the fish were placed in nine 5000 l rectangular tanks at 600 fish per tank (approx. 0.6 kg/m^3), with salinity of 39‰, temperature of 20ºC and saturated with oxygen. Following 2 weeks acclimation (July 2002), triplicate groups of fish were fed to satiation, using mechanical belt automatic feeders with three iso-energetic and iso-nitrogenous experimental diets formulated to provide a constant lipid content of ~22 % (Nutreco ARC Stavanger, Norway). The diets contained ~47 % protein, primarily provided by fish meal, and 21.4 %, 24.1 % and 21.5 % lipid for diets of pellet size, 2, 3 and 5 mm, respectively. Two experimental diets contained 60 % of three VOs, LO, PO and RO, blended to provide a balance of saturates, monoenes and PUFA similar to that found in FO, but without HUFA. The control diet contained anchovy oil and the added oil combinations for the three experimental diets were: Diet A: 100 % anchovy oil (control); Diet B: 40 % anchovy oil, 35 % linseed oil, 15 % palm oil and 10 % rapeseed oil; Diet C: 40 % anchovy oil, 24 % linseed oil, 12 % palm oil and 24 % rapeseed oil. The formulation and proximate compositions of the experimental diets are shown in Table 1, while diet total lipid content and fatty acid compositions are shown in Table 2.

Sample collection and biometric measurements

After feeding the experimental diets for 64 weeks, thirty fish per tank (i.e. 90 fish per replicate) were sampled for length and live mass and condition factor K and specific growth rates

recorded. Ten fish per replicate (i.e. 30 fish per dietary treatment) were sampled and liver (live and dry mass), hepatosomatic index and flesh (live and dry mass) were recorded. Liver samples for fatty acid analyses were dissected from 4 fish per replicate (i.e. 12 fish per dietary treatment) and immediately frozen in liquid nitrogen and stored at -80ºC until analysed. Blood for eicosanoid analysis (2 ml) was collected in heparinised syringes from 6 fish per dietary treatment, and centrifuged at 12 000 x g for 2 min. The plasma was collected and acidified by the addition of 50 µl/ml 2 M formic acid and immediately frozen in liquid nitrogen for eicosanoid analysis. Heparinised blood samples were also used for haematological analyses. Live mass of the liver was determined by blotting the tissue on filter paper before weighing, and dry mass determined after heating to 60^{o}C for 24 h and cooling under vacuum before weighing. Hepatosomatic index (HSI) was calculated as liver live mass * 100/fish live mass. Fulton´s condition factor (K) = (W/L^3)*100, where W is the fish weight (g) and L the total length (cm). Specific growth rate (SGR) was calculated as % weight gain/day[21]. Non-specific mortality was measured at the end of the experiment and expressed as a percentage of surviving fish.

Proximate analysis of diets

Moisture content was determined by thermal drying to constant weight in an oven at 110ºC for 24 h. For the total protein content, the micro-Kjeldahl analysis method was followed, using a Digestion system 40–1006 Heating Unit and a KJELTEC AUTO 1030 Analyzer. To convert total nitrogen to total protein content, as a percentage of dry weight, the factor 6.25 (100/16) was used. Crude fat was determined by acid hydrolysis with a Soxtec System 1047 Hydrolyzing Unit, followed by Soxhlet extraction using a Soxtec System HT6. Ash content as % of dry weight was determined by dry ashing in porcelain crucibles in a muffle furnace, at 600ºC overnight[22].

Lipid analysis

Total lipid in samples was extracted after homogenisation, using an Ultraturrax tissue disrupter, in 10 volumes of chloroform/methanol (2:1, v/v) containing 0.01 % butylated hydroxytoluene (BHT) as antioxidant, basically according to Folch *et al.*[23] and essentially as described by Christie[24].

Fatty acid methyl esters (FAME) were prepared from aliquots of total lipids by acid-catalysed transmethylation for 16 h at 50^{o}C, using tricosanoic acid (23:0) as internal standard[24]. FAME were extracted and purified as described previously[25] and were separated

using a Hewlett-Packard 5890A Series II gas chromatograph equipped with a chemically bonded (PEG) Supelcowax-10 fused silica wall coated capillary column (30 m x 0.32 mm i.d., Supelco Inc., Bellefonte, USA), using an "on column" injection system and flame ionisation detection. Hydrogen was used as the carrier gas with an oven thermal gradient from an initial 50ºC to 180ºC at 25ºC/min and then to a final temperature of 235ºC at 3ºC/min, with the final temperature maintained for 10 min. Individual FAME were identified by comparison with known standards and quantified by means of a direct-linked PC and Hewlett-Packard ChemStation software.

Extraction and measurement of prostaglandins E_2 concentrations in plasma

The frozen acidified plasma samples were thawed and centrifuged at 12000 x g for 2 min to remove any precipitate. The supernatants were extracted using octadecyl silyl (ODS, C18) "Sep-Pak" mini-columns (Millipore) as described in detail by Bell *et al.*[26]. C18 "Sep-Pak" mini columns were pre-washed with 5 ml methanol and 10 ml of distilled water, plasma samples were charged on the mini-column, washed with a further 10 ml of distilled water and the eicosanoids eluted in 5 ml of ethyl acetate. Samples were dried under nitrogen and redissolved in immunoassay buffer. Quantification of prostaglandin E was performed using enzyme immunoassay (EIA) kits, according to the manufacturers protocol (SPI-Bio, Massy, France).

Measurement of cellular immune parameters

Eight fish per dietary treatment were sampled after 64 weeks feeding the experimental diets. Fish were anaesthetised with a lethal dose of tricaine methanesulphonate (MS-222, Sigma, UK). Blood samples were collected in heparinised vacuum tubes (vacutainer Becton Dickinson Vacutainer System, Oxford, UK) from the caudal vein.

Preparation of peripheral blood leucocytes

Peripheral blood leucocytes (PBL) were isolated from blood from three fish per dietary treatment using the lymphocyte separation medium, Histopaque® (Sigma, UK) and density gradient centrifugation. One ml of blood was diluted with 4 ml of L-15 medium and 3 ml of the diluted blood was layered onto 4 ml of Histopaque® and centrifuged at 400 x g for 45 minutes. The leucocyte band was collected using a Pasteur pipette and stored in 1 ml of chloroform:methanol (2:1 v/v) at –20°C until required for lipid extraction. If erythrocyte contamination of PBL was considered to be excessive (> 2%) then the PBL fraction was

centrifugation again on 4 ml of fresh Histopaque®.

Haematology

Blood was used immediately for haematological studies. Haematocrit values were obtained using heparinised micro-haematocrit tubes and centrifuging at 12,000 x g for 4 min (Microcentrifuge MH2, Sarstedt Ltd). Total erythrocyte and total leukocyte counts (including thrombocytes) were made using phosphate buffered saline (PBS) for dilution and an improved Neubauer haemocytometer (Hawksley, UK).

Serum lysozyme activity

An aliquot of blood was allowed to clot at 4°C overnight. Serum was separated by centrifugation at 4000 x g for 15 min and stored at -20°C until analysis. Serum lysozyme activity was assayed by a turbidimetric assay which measures the lytic activity of the seabass serum against *Micrococcus lysodeikticus*.[27,28] A suspension of 190 µl of bacteria (*Micrococcus lysodeikticus*, Sigma, UK) and 10 µl of serum sample was measured spectrophotometrically at 540nm in five replicate wells per serum sample after 1 and 5 min at 25°C, using a Dynatech MRX 1.2 ELISA reader (Dynatech Laboratories Limited, West Sussex, UK). The bacterial suspension (0.2 mg/ml) was prepared in sodium phosphate buffer (0.04 M, pH 5.8). The results are given as units (U)/ml/min (1U = the amount of sample causing a decrease in absorbance of $0.001 min^{-1}$).

Macrophage respiratory burst activity

The reduction of nitroblue tetrazolium salt (NBT) to formazan by oxygen radicals produced by head kidney macrophages during respiratory burst activity was measured spectrophotometrically as described by Chung & Secombes[29]. Isolation and culture of head kidney macrophages were performed as described by Secombes[30] however instead of placing the cell suspensions on Percoll to isolate the macrophages, 200 µl of each kidney suspension was added directly to four replicate wells of a 96 well microtitre plate. Plates were sealed and incubated for 3 h before washing them gently three times to remove non-adherent cells. Two hundred µl of L-15, containing 10 % foetal bovine serum, was added to all wells and cultures were incubated at 18° C for 2 to 3 days, after which the respiratory burst activity of the macrophages was determined by incubating the cells with 100 µl of NBT (1mg/ml)/phorbol myristate acetate (PMA, 1 µg/ml). This was added to three of the four wells and incubated at 18-20°C for 40 min. The assay was developed as described by the authors using a microplate

reader as described above to read the absorbance at 620 nm. The remaining well was used to determine the numbers of macrophages attached to the plate for individual kidney samples[30]. The results were expressed as "macrophage activity" by calculating the mean optical density for each of the triplicate cultures and dividing the mean OD by the number of cells/well to obtain an OD per 10^5 cells and multiplying by 100.

Histological examination of fish tissues

Samples were collected at 64 weeks to identify any effects of dietary treatment on the histology of the heart, liver or intestine. Samples of proximal, mid and distal intestine were collected from 6 fish from each dietary treatment, in addition to the heart and liver, for histopathological examination. Sections were fixed in 10 % buffered formalin at the time of dissection, embedded in paraffin wax and 5 µm sections were cut and stained with haematoxylin and eosin. Processed sections were examined "blind" to eliminate bias in interpretation. Stained sections of heart were assessed for signs of endocarditis and pericarditis. Liver sections were assessed on fat content, any indication of inflammation in the tissue, the degree of peri-vascular cuffing (PVC) and finally the presence of single cell necrosis (SCN). Intestinal sections were examined on the integrity of the intestinal mucosa, the appearance of the submucosa and lamina propria and the presence of any inflammatory response.

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Deleted: the number, size and variability of the absorptive vacuoles in the mucosal enterocytes,

Statistical analysis

Results are reported as means ± SD (n=3) unless otherwise stated. All statistical analyses were performed using the statistical computer package, Prism 4.0, GraphPad Software, Inc., San Diego, California, USA. The significance of treatment effects on biometry and growth rates, liver and leucocyte fatty acid compositions, haematology, serum lysozyme activity and macrophage respiratory burst activity were determined by one-way ANOVA followed by Tukey´s multiple comparison test where appropriate. Percentage data and data which were identified as non-homogeneous (Bartlett´s test) were subjected to either arcsine, square root or log transformation before analysis. Differences were reported as significant at $p < 0.05$[31]. Immune parameter results are reported as means ± SD (n=8).

Results

There were no significant differences in the total length of fish between dietary treatments, but fish fed Diet B showed significantly lower values for total live mass and liver mass than fish

fed Diets A (control) and C. Fish fed Diets A (control) and B presented significantly lower values for flesh dry mass (%) at the end of the 64 week feeding trial (Table 3).

The total lipid fatty acid compositions of livers from sea bass following 64 weeks of feeding the experimental diets are shown in Table 4. Total saturated fatty acids (primarily 16:0) were identical in all treatments. Total monounsaturated fatty acids, primarily oleic acid (OA), were significantly higher in liver total lipids from fish fed Diet C, followed by fish fed Diets B and A (control) due to the higher inclusion of OA in the VO blends. The proportion of linoleic acid (LA; 18:2*n*-6) in total lipid from liver was highest in fish fed the VO diets, due to the high inclusion of LA in RO, LO and PO, and was about 50 % lower in liver of fish fed Diet A (control). However, total n-6 PUFA values were not significantly different for all treatments. In contrast, arachidonic acid (20:4*n*-6, ARA) was highest in liver of control fish (Diet A) and fish fed Diet B, followed by fish fed Diet C. Total n-6 HUFA, primarily ARA, was highest in liver from fish that had been fed Diet A (control) followed by fish from treatments B and C that showed identical values. The percentage of linolenic acid (LNA; 18:3*n*-3) in liver total lipids was highest in fish fed Diets B and C (which contained the highest proportions of LO and, in consequence, the highest level of LNA), followed by fish fed diet A (control). Liver total lipid percentages of EPA, DHA and total *n*-3 PUFA were highest in fish fed Diet A (control) due to the highest content of these fatty acids in FO. The level of total PUFA was not significantly different in liver total lipids from all treatments (Table 4).

Inclusion of VO in the diets of sea bass modified the fatty acid composition of their PBLs. The fatty acid compositions of PBL from VO-fed fish were different from the corresponding PBL of fish fed FO, with the latter having more monounsaturated fatty acids and higher *n*-6 PUFA. PBLs of sea bass maintained on Diets B and C had significantly increased levels of 18:0, OA, LA, 20:2*n*-6 and LNA, and significantly reduced amounts of *n*-3 PUFA, ARA, EPA, DHA and others (Table 5). The overall ratio of *n*-3/*n*-6 was significantly reduced in sea bass fed the VO diets.

The effect of partial replacement of dietary FO with VO blends on the concentration of plasma PGE_2 in European sea bass after 64 weeks of feeding the diets is shown in Fig. 1a. The highest values were found in plasma of fish fed the control (FO) and C diets, with significantly lower values seen in fish fed diet B (46 % less). The source of dietary lipid did not affect any of the haematological parameters measured. No significant differences were found in haematocrit values (Fig. 1b) or the total number of leukocytes (Fig. 1c) and erythrocytes (Fig. 1d) between groups. The production of superoxide anion by head kidney macrophages, measured by the reduction of NBT, is presented in Fig. 1e. It appears that following PMA

triggering, the respiratory burst activity was significantly reduced in fish fed the VO based diets. Whether or not this change affects the innate immune response of the fish needs further investigation. It could be that the respiratory burst event takes place earlier or later than seen with macrophages from fish fed the FO diet and that this activity has not been measured at the optimal time for fish fed the VO diets. No effect of dietary VO was observed on sea bass serum lysozyme activity (Fig. 1f). Fish fed the FO diet showed the highest (1452.5 U/ml/min) (but not significantly different) value of lysozyme activity in serum compared to 1351.1 U/ml/min found for fish fed the Diet B and 1171.1 U/ml/min for the C diet.

Hearts examined from all three dietary groups showed no signs of pathological change. In livers, fat vacuoles were variable in size in many sections with some very large vacuoles present within some hepatocytes and relatively smaller vacuoles in other hepatocytes. Due to the level of vacuolation in some hepatocytes, there was some distortion of the cellular architecture and occasional breakdown of cells. Again, there were no differences between the three dietary groups. Small foci of inflammation were seen in some sections in all three groups, with a slightly higher incidence in sections from the Diet C group. PVC was not a feature in any of the dietary groups examined. With regard to the intestinal sections, mucus levels appeared very similar in all segments and in all dietary groups. Absorptive vacuoles were small and multiple in all sections. In the FO diet these were at relatively low levels in the proximal and mid segments and higher in the distal segments. In fish fed Diet B or Diet C, vacuolation in the proximal segments appeared to be much more pronounced, interestingly less so than in the mid sections. Some cellular infiltration was seen in the lamina propria of one fish in the FO diet (Fig. 2a) and two fish on Diet C (Fig. 2b). Sloughing of the mucosal membrane was not a feature in any of the sections examined. The major difference seen between these groups was the level of absorptive vacuolation in the proximal segment of fish fed Diet B or Diet C, compared with the FO diet.

Discussion

Considerable data has been accumulated on the effects of different dietary lipids on tissue fatty acid compositions of both mammals and fish, although the effects of dietary lipids on fish health and immune function are less well documented. Changes in dietary fatty acid composition have been shown to affect both innate[32,33,34,35] and adaptive immunity[16,34,36,37,38], as well as the resistance to infectious diseases[16,36,37,38,39]. However, the role of *n*-3 and *n*-6 fatty acids in fish immune response is unclear, and reports are not conclusive and are often contradictory.

The modulatory process is likely to occur at different cellular levels with the most obvious being a change in cell membrane phospholipid fatty acid composition, affecting the activity of membrane-bound enzymes, receptors and ion channels[40]. In addition, eicosanoids, a group of bioactive derivatives of ARA, EPA and dihomo-γ-linolenic acid (20:3*n*-6), which include prostaglandins (PG), thromboxanes, leukotrienes and lipoxins, act to regulate the immune response[13,41]. Other immune modulatory processes involving fatty acids include changes in intracellular signalling pathways[42] and direct interactions between fatty acids and nuclear transcription factors in cells of the immune system, such as the peroxisome proliferators activated receptors, that act to regulate immune cell function[20].

Fish tissues and cell membranes, including phagocytic cells (macrophages, neutrophils), contain relatively high concentrations of *n*-3 PUFA, and their compositions can be altered by changes in dietary lipid[18]. Specific macrophage functions may also be altered by lipids, mainly due to changes in membrane fluidity. If fluidity is altered by fatty acid composition, then potentially several aspects of phagocyte function may be affected including phagocytosis and eicosanoid production. Calder *et al.*[43] reported that unsaturated fatty acid incorporation is associated with an increase in the phagocytosis of zymosan particles.

Reduction in head kidney macrophage respiratory burst activity has been observed in sea bass and gilthead sea bream fed VO[9,44] Sea bass fed 60% RO, LO and olive oil had significantly reduced phagocytic capacity of head kidney macrophage to engulf yeast particles[9] while Montero *et al.*[44] found reduced macrophage activity in sea bream fed 60% RO. In addition, Sheldon & Blazer[32] found that channel catfish macrophage killing activity was positively correlated to the dietary content of *n*-3 PUFA. They found phagocytosis of live *Edwardsiella ictaluri* by catfish head kidney macrophages was not significantly affected by feeding soybean oil compared to fish fed menhaden oil or beef tallow. However, feeding soybean oil significantly reduced the ability of macrophages to kill engulfed bacteria compared to macrophages from those fed menhaden oil. Macrophages from the latter group also had a significantly higher killing index than macrophages from fish fed soybean oil. Waagbø *et al.*[34] showed that Atlantic salmon fed diets rich in *n*-3 PUFA significantly reduced the bacterial killing ability of macrophages at 12°C but not at 18°C indicating that temperature, perhaps related to membrane fluidity, also influences the activity of macrophages. In contrast, Thompson *et al.*[16] found no differences in phagocytosis and bactericidal activities of head kidney macrophages from Atlantic salmon fed diets enriched with either *n*-3 or *n*-6 PUFA.

In the present study, the concentration of circulating PGE_2 in plasma of sea bass fed the 60% VO blend (Diet B) was significantly lower than in fish fed FO. In addition, the fish fed

60% VO diets B and C also showed significantly reduced respiratory burst activity which coincided with a reduction in plasma PGE_2 levels. Since the production of PGE_2 was reduced in fish fed VO diets, it may be that the activity or expression of the cyclo-oxygenase enzymes is inhibited by dietary lipid. It is also possible that feeding VO for a long period may reduce the levels of ARA in plasma membranes and, thereby, compromise immune function. In support of the present study, a number of studies also showed a reduction in production of PGE_2 and leukotriene B_4 (LTB_4) by stimulated head kidney macrophages from salmon fed a diet containing LO compared to those fed diets containing sunflower oil or FO[17,18,45,46]. However, no differences in serum lysozyme activity were found in the present study, which was also reported in other studies with fish fed VO[18,44].

Montero *et al.*[44] found that seabream fed a FO diet had higher numbers of circulating erythrocytes compared to fish fed 60% LO or soybean oil diets, which may be related to a higher oxygen requirement due to higher peroxisomal β-oxidation activity induced by the VO diets[37]. Leray *et al.*[47] found that the fatty acid composition of erythrocyte membrane phospholipids from trout can be profoundly altered by dietary oils. Trout fed highly saturated coconut oil showed increased *n*-9 fatty acids in their phospholipids, and, consequently, their erythrocytes had a more shrunken appearance than fish fed FO. Perhaps the high levels of saturates in the diets caused reduced haematocrit levels, linked to a shrunken erythrocyte morphology causing a lower packed erythrocyte volume.

The lipid composition of monocytes, macrophages, lymphocytes and polymorpho nuclear cells (PMN's) reflect the fatty acid composition of dietary lipids in mammals[11,48]. Studies by Waagbbø *et al.*[35] Farndale *et al.*[49] and Montero *et al.*[44] reported that dietary oil determines the fatty acid profile of macrophages and immune cells in cod, sea bass and sea bream. Montero *et al.*[44] reported selective incorporation of certain fatty acids into head kidney macrophages of seabream. DHA was found to be preferentially incorporated and retained in this cell type. Generally, fish fed with a VO-containing diet had increased levels of oleic acid, LA, LNA and total *n*-6 PUFA in both their liver and their PBLs and decreased levels of EPA, DHA, total *n*-3 PUFA and a lower *n*-3/*n*-6 ratio than fish fed a FO diet. Fish fed a FO diet showed the highest *n*-3 HUFA in immune cells in the present study and in previous studies[35,44,49]. Evidence suggests that changing the fatty acid composition of immune cells can influence immune function by changing the physiology of the cell membrane but perhaps more importantly by influencing the production of modulatory prostaglandins and leukotrienes[20]. The production of eicosanoids is influenced, in part, by the availability of precursor fatty acids and, in particular, the EPA/ARA ratio. In a previous study with Atlantic salmon fed single VO

3-fold differences in the EPA/ARA ratio of immune cells were recorded[18] while in the present study with VO blends the difference in the EPA/ARA ratio, between the three dietary treatments, was only 13%. Perhaps the minor changes to the EPA/ARA ratio in the present study can partly explain lack of effect observed in innate immune function.

The overall histological appearance of sea bass sampled from all of the dietary treatments was normal with very few differences observed between the groups. The only difference was in the levels of absorptive vacuoles present in the proximal intestine. Sea bass fed the VO diets showed elevated numbers of absorptive vacuoles compared to fish fed the FO diet. The presence of increased absorptive vacuoles tends to suggest an "active" mucosa, however with increased mucosal vacuolation this could, in turn, leave the intestinal mucosal membrane more vulnerable to sloughing and breakdown. However, the vacuolization was minor and was still regarded as being within normal ranges for Atlantic salmon.

Results of this study suggest that potential exists for replacing FO with a blend of VO in the feeds of farmed sea bass without compromising growth, non-specific immune function and overall histological appearance. It is important to establish that alternative dietary lipids to FO are not only supplied in the correct quantities and balance for optimal growth and feed conversion, but can maintain optimal immune function and not increase susceptibility to infectious pathogens. This study suggests that normal immune function can be more successfully attained if dietary FO is replaced by a blend of VO, that provides a more physiologically balanced fatty acid composition, in comparison to replacement with a single VO[9,17,18,44].

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List of Figures

Fig. 1. Effects of feeding diets containing fish oil (FO, diet A), or two 60% vegetable oil blends (60% VO, diets B & C) on: (a) plasma PGE_2 concentration; (b) % haematocrit; (c) total circulating leukocytes; (d) total circulating erythrocytes; (e) head kidney macrophage activity (NBT reduction, measured as absorbance at 620 nm/10^5 cells x 100); (f) serum lysozyme activity. Values are mean ± SEM, n = 9. Columns assigned a different letter are significantly different ($P < 0.05$).

Fig. 2. Histopathology of sea bass fed (a) Fish oil, distal intestine showing slight cellular infiltration in the lamina propria and high levels of absorptive vacuoles (mag. x 175) (b) 60% VO diet C, distal intestine showing cellular infiltration but no sloughing of the mucosal folds (mag. x 430).

Table 1. Formulation and proximate composition of 5mm experimental diets (g/kg feed)

Diets	A	B	C
Components			
Fish meal [a]	400.0	400.0	400.0
Maize gluten [b]	262.7	262.7	262.7
Wheat [c]	152.3	152.3	152.3
Oil	160.0	160.0	160.0
Premixes [d]	25.0	25.0	25.0
Composition (%) of added oil			
Anchovy oil (FO) [e]	100	40	40
Rapeseed oil (RO) [f]	0	10	24
Linseed oil (LO) [g]	0	35	24
Palm oil (PO) [h]	0	15	12
Proximate composition (%)			
Crude protein	47.8	46.2	47.8
Crude lipid	22.2	24.3	21.2
Carbohydrate	13.1	13.3	15.4
Ash	6.7	6.3	6.2
Moisture	10.2	10.9	9.4

[a] Scandinavian LT-fish meal (Nordsildmel, Norway).

[b] Cargill, Staley, USA

[c] Statkorn, Oslo, Norway.

[d] Vitamin and mineral premix added exceed NRC (1993) recommendations.

[e] Anchovy oil (Denofa, Fredrikstad, Norway) supplemented with 200 ppm BHT.

[f] Crude rapeseed oil (Oelmühle Hamburg, Germany) no antioxidant added.

[g] Crude E.C.C. linseed oil (N.V. Oliefabriek Lictervelde, Belgium) supplemented with 500ppm Ronoxan A (Roche, Basel, Switzerland).

[h] Crude palm oil (Denofa, Norway).

Table 2. Total lipid content (% of dry mass) and fatty acid composition (weight % of total fatty acids) of the 5 mm experimental diets.

Dietary treatments	A	B	C
Total lipid (%)	20.2 ± 3.1	21.2 ± 4.3	21.1 ± 2.8
Fatty acid			
14:0	4.3 ± 0.1[a]	1.9 ± 0.2[b]	1.9 ± 0.2[b]
15:0	0.5 ± 0.0	0.3 ± 0.1	0.6 ± 0.0
16:0	14.0 ± 0.2[a]	13.3 ± 0.1[b]	12.5 ± 0.1[c]
18:0	3.0 ± 0.1[a]	3.1 ± 0.1[a]	2.7 ± 0.0[b]
Total saturated[1]	22.9 ± 0.1[a]	19.5 ± 0.2[b]	18.6 ± 0.3[c]
16:1*n*-9/*n*-7	13.6 ± 0.6[a]	11.4 ± 1.9[b]	10.2 ± 0.3[b]
18:1*n*-9	9.4 ± 0.2[c]	16.9 ± 0.4[b]	21.6 ± 0.4[a]
18:1*n*-7	2.2 ± 0.1[a]	1.8 ± 0.0[c]	2.0 ± 0.0[b]
20:1*n*-9	2.3 ± 0.0[a]	1.9 ± 0.0[c]	2.0 ± 0.0[b]
22:1*n*-11	2.0 ± 0.1[a]	1.7 ± 0.1[b]	1.7 ± 0.0[b]
Total monoenes[2]	30.7 ± 0.7[c]	34.6 ± 1.5[b]	38.1 ± 0.4[a]
18:2*n*-6	4.9 ± 0.0[c]	9.0 ± 0.7[b]	10.4 ± 0.2[a]
20:4*n*-6	0.6 ± 0.0[a]	0.3 ± 0.0[b]	0.3 ± 0.0[b]
Total *n*-6	7.4 ± 0.1[c]	10.7 ± 0.5[b]	11.8 ± 0.2[a]
18:3*n*-3	1.5 ± 0.0[c]	12.3 ± 0.7[a]	10.3 ± 0.3[b]
18:4*n*-3	2.0 ± 0.0[a]	1.1 ± 0.1[b]	1.0 ± 0.0[b]
20:4*n*-3	0.5 ± 0.0[a]	0.3 ± 0.0[b]	0.3 ± 0.0[b]
20:5*n*-3	9.8 ± 0.1[a]	5.3 ± 0.3[b]	5.2 ± 0.1[b]
22:5*n*-3	1.2 ± 0.0 [a]	0.7 ± 0.0 [b]	0.6 ± 0.0 [b]
22:6*n*-3	11.0 ± 0.3[a]	6.3 ± 0.5[b]	6.2 ± 0.1[b]
Total *n*-3	27.9 ± 0.5[a]	27.0 ± 1.7[ab]	24.5 ± 0.6[b]
Total PUFA[3]	35.4 ± 0.6	37.8 ± 2.1	36.3 ± 0.9
n-3/*n*-6	3.8 ± 0.5[a]	2.5 ± 0.5[b]	2.1 ± 0.5[b]

Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. [1]Includes 20:0 and 22:0. [2]Includes 18:1*n*-11, 20:1*n*-11, 20:1*n*-7 22:1*n*-9 and 24:1. [3]Includes 16:2, 16:3, 16:4, 20:2*n*-6, 20:3*n*-6 and 22:5*n*-6. Values bearing different superscript letter are significantly different (P<0.05). nd, not detected.

Table 3. Effect of partial replacement (60 %) of dietary fish oil (FO) with vegetable oils (rapeseed oil, RO; linseed oil, LO and palm oil, PO) on growth and performance of European sea bass (*Dicentrarchus labrax*, L.) fed experimental diets for 64 weeks

Dietary treatment	Initial	A*	B	C
64 weeks (final sampling point)				
Fish length (cm)	7.9 ± 0.5	24.9 ± 1.3	23.2 ± 1.4	24.1 ± 1.4
Fish live mass (g)	5.2 ± 1.0	176.2 ± 32.9 [a]	143.2 ± 29.4 [b]	159.8 ± 34.2[ab]
HSI[1]	1.4 ± 0.2	2.0 ± 0.3	1.9 ± 0.2	2.1 ± 0.3
Flesh dry mass (%)		22.9 ± 1.7 [b]	21.3 ± 1.5 [b]	26.0 ± 1.4 [a]
FCR (feed/gain)[2]		1.2 ± 0.3	1.6 ± 0.4	1.3 ± 0.3
Condition factor (K)[3]		1.14 ± 0.2	1.15 ± 0.3	1.14 ± 0.2
SGR[4]		0.8 ± 0.1	0.7 ± 0.1	0.8 ± 0.1

Data are mean ± SD (n = 90) for fish length and weight and (n = 30) for liver mass, HSI and flesh mass. Values in the same row assigned different superscript letters are significantly different ($P<0.05$). [1] Hepato Somatic Index. [2] FCR = Food Conversion Ratio. [3] Condition Factor, $K = (W/L^3)*100$. [4] Specific Growth Rate (SGR) = $(LnW_1-LnW_0) \times 100/t$. * Control diet where W = weight (g), L = length in cm, W_0 = initial weight in g, W_1 = final weight in g and t = time in days.

Table 4. Total lipid content (% of dry mass) and total lipid fatty acid composition (weight % of total fatty acids) of liver from European sea bass (*Dicentrarchus labrax*) fed the experimental diets for 64 weeks.

Dietary treatments	A*	B	C
Total lipid (%)	54.8 ± 7.6	52.9 ± 0.9	54.7 ± 5.8
Fatty acid (%)			
14:0	1.8 ± 0.3 [a]	1.3 ± 0.2 [ab]	1.0 ± 0.1 [b]
15:0	0.2 ± 0.0 [a]	0.2 ± 0.0 [a]	0.1 ± 0.0 [b]
16:0	16.6 ± 2.4	16.1 ± 2.1	17.3 ± 2.1
18:0	3.1 ± 0.7	3.7 ± 0.2	3.4 ± 0.7
Total saturated[1]	22.4 ± 3.1	21.8 ± 2.5	22.5 ± 2.9
16:1*n*-9/*n*-7	8.8 ± 1.0 [a]	6.0 ± 1.0 [b]	6.1 ± 0.2 [b]
18:1*n*-9	28.8 ± 2.0 [b]	34.1 ± 2.2 [ab]	39.2 ± 2.3 [a]
18:1*n*-7	3.4 ± 0.4 [a]	2.5 ± 0.2 [b]	2.7 ± 0.2 [ab]
20:1*n*-9	2.1 ± 0.1	2.0 ± 0.2	2.1 ± 0.3
22:1*n*-11	0.7 ± 0.0	0.6 ± 0.0	0.5 ± 0.1
Total monoenes[2]	44.7 ± 1.2 [b]	46.1 ± 1.5 [b]	51.3 ± 1.6 [a]
18:2*n*-6	3.3 ± 0.3 [b]	6.4 ± 1.5 [a]	6.2 ± 1.3 [a]
20:4*n*-6	0.5 ± 0.1 [a]	0.3 ± 0.1 [ab]	0.2 ± 0.0 [b]
Total *n*-6	4.9 ± 0.5	7.6 ± 1.4	7.2 ± 1.5
18:3*n*-3	0.8 ± 0.2 [b]	7.5 ± 2.8 [a]	5.1 ± 1.2 [a]
18:4*n*-3	1.0 ± 0.1 [a]	0.9 ± 0.1 [ab]	0.7 ± 0.1 [b]
20:4*n*-3	0.5 ± 0.0 [a]	0.3 ± 0.1 [ab]	0.2 ± 0.0 [b]
20:5*n*-3	6.2 ± 0.7 [a]	3.2 ± 1.0 [b]	2.5 ± 0.6 [b]
22:5*n*-3	1.2 ± 0.1 [a]	0.6 ± 0.2 [b]	0.4 ± 0.1 [b]
22:6*n*-3	10.2 ± 1.6 [a]	5.4 ± 1.8 [b]	4.2 ± 0.9 [b]
Total *n*-3	21.1 ± 2.5 [a]	19.3 ± 0.5 [ab]	13.8 ± 2.9 [b]
Total PUFA[3]	26.0 ± 2.9	27.0 ± 1.1	21.1 ± 4.4
n-3/*n*-6	4.3 ± 0.8[a]	2.5 ± 0.7[b]	1.9 ± 0.9[b]

Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. [1]Includes 20:0 and 22:0. [2]Includes 18:1*n*-11, 20:1*n*-11, 20:1*n*-7 22:1*n*-9 and 24:1. [3]Includes 16:2, 16:3, 16:4, 20:2*n*-6, 20:3*n*-6 and 22:5*n*-6. Values bearing different superscript letter are significantly different (P<0.05). nd, not detected. * Control diet.

Table 5. Total lipid fatty acid composition (weight % of total fatty acids) of peripheral blood leukocytes from European sea bass (*Dicentrarchus labrax*) fed the experimental diets for 64 weeks.

Dietary treatments/Fatty acid	A*	B	C
14:0	1.6 ± 0.0 [a]	1.0 ± 0.2 [b]	1.1 ± 0.1 [b]
15:0	0.5 ± 0.1 [a]	0.3 ± 0.0 [b]	0.3 ± 0.1 [b]
16:0	17.2 ± 1.4	16.8 ± 0.6	16.7 ± 0.8
18:0	3.9 ± 0.2 [a]	5.6 ± 0.5 [b]	4.7 ± 0.3 [c]
Total saturated[1]	23.5 ± 1.8	24.0 ± 0.7	23.1 ± 0.7
16:1*n*-9/*n*-7	3.4 ± 0.4 [a]	2.5 ± 0.5 [b]	2.6 ± 0.2 [b]
18:1*n*-9	10.5 ± 1.7 [a]	18.8 ± 0.4 [b]	20.4 ± 1.8 [b]
18:1*n*-7	2.0 ± 0.2	1.9 ± 0.2	1.9 ± 0.1
20:1*n*-9	1.7 ± 0.2	1.8 ± 0.2	1.8 ± 0.3
22:1*n*-11	0.9 ± 0.2	1.0 ± 0.3	1.0 ± 0.3
Total monoenes[2]	20.1 ± 2.7 [a]	27.6 ± 1.4 [b]	28.9 ± 2.6[b]
18:2*n*-6	2.6 ± 0.2 [a]	5.8 ± 0.4 [b]	6.1 ± 0.7 [b]
20:4*n*-6	1.2 ± 0.1 [a]	0.7 ± 0.1 [b]	0.7 ± 0.1 [b]
Total *n*-6	4.9 ± 0.4 [a]	7.5 ± 0.5 [b]	7.7 ± 0.7 [b]
18:3*n*-3	0.7 ± 0.2 [a]	5.9 ± 0.5 [b]	4.8 ± 0.6 [c]
18:4*n*-3	0.9 ± 0.2 [a]	0.7 ± 0.1 [ab]	0.6 ± 0.1 [b]
20:4*n*-3	0.3 ± 0.1 [a]	0.2 ± 0.2 [b]	0.2 ± 0.0 [b]
20:5*n*-3	13.4 ± 0.5 [a]	8.9 ± 0.2 [b]	8.3 ± 0.4 [b]
22:5*n*-3	1.2 ± 0.1 [a]	0.9 ± 0.2 [b]	0.8 ± 0.0 [b]
22:6*n*-3	27.6 ± 2.6 [a]	19.7 ± 1.0 [b]	20.6 ± 2.9 [b]
Total *n*-3	44.1 ± 2.6 [a]	36.4 ± 1.5 [b]	35.3 ± 2.6 [b]
Total PUFA[3]	49.1 ± 2.4 [a]	43.9 ± 1.9 [b]	43.1 ± 2.2 [b]
n-3/*n*-6	9.0 ± 1.2 [a]	4.8 ± 0.2 [b]	4.6 ± 0.7 [b]

Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. [1]Includes 20:0 and 22:0. [2]Includes 18:1*n*-11, 20:1*n*-11, 20:1*n*-7 22:1*n*-9 and 24:1. [3]Includes 16:2, 16:3, 16:4, 20:2*n*-6, 20:3*n*-6 and 22:5*n*-6. Values bearing different superscript letter are significantly different (P<0.05). nd, not detected. * Control diet.

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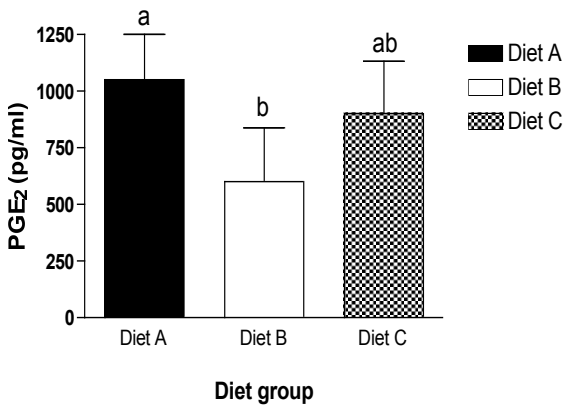

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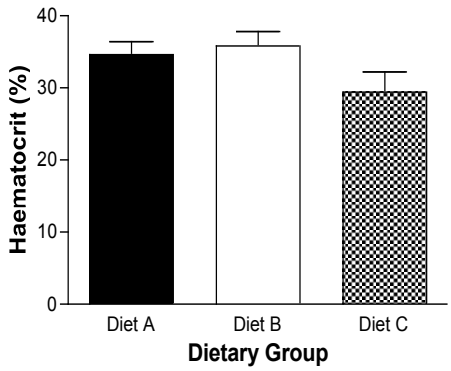

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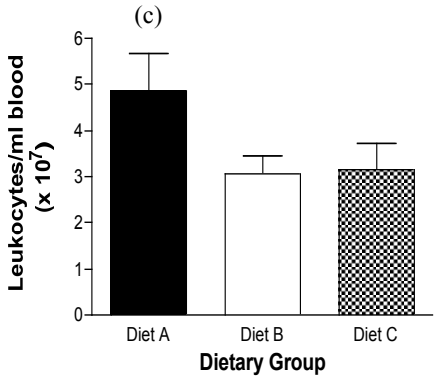

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4 Fig. 1

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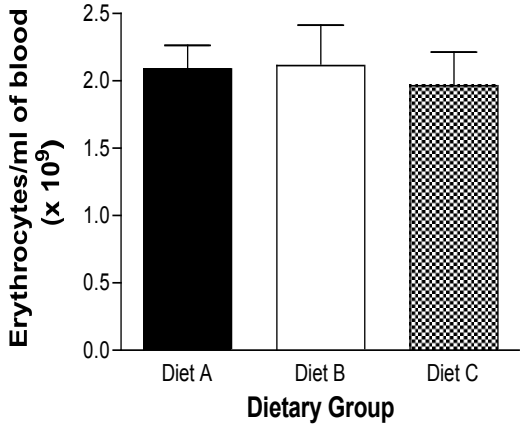

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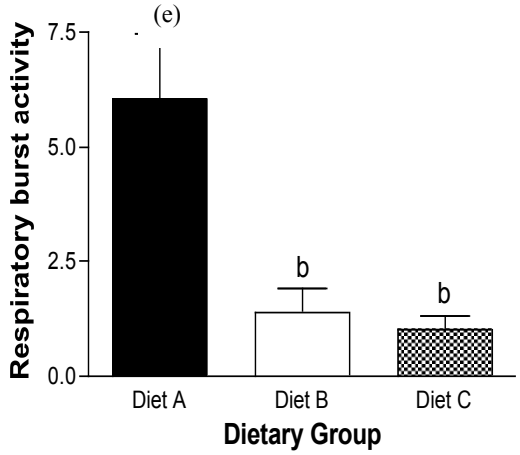

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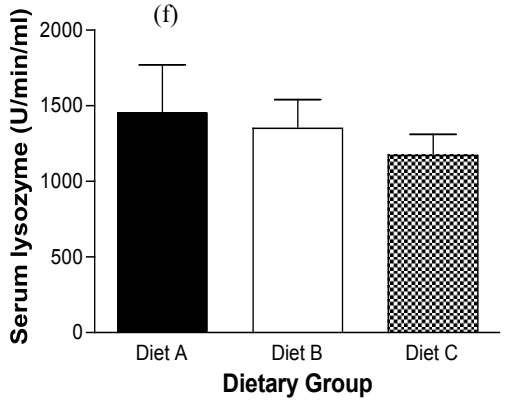

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4 Fig. 1

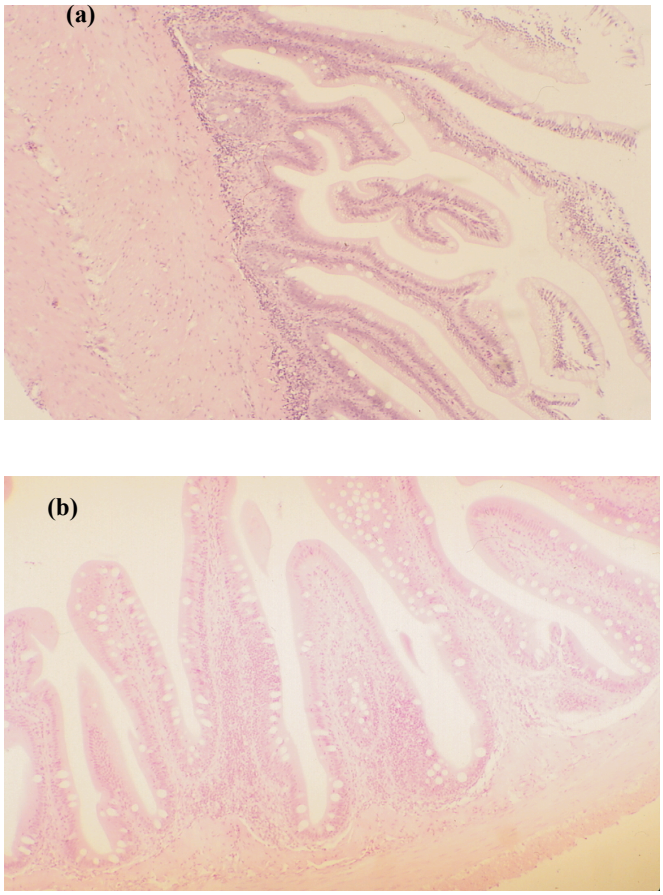

Fig. 2