EFFECTS OF DIETARY ARACHIDONIC ACID IN EUROPEAN SEABASS (*DICENTRARCHUS LABRAX*) DISTAL INTESTINE LIPID CLASSES AND GUT HEALTH

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ABSTRACT

The use of low fishmeal/fish oil in marine fish diets affects dietary essential fatty acids (EFAs) composition and concentration and subsequently, may produce a marginal deficiency of those fatty acids with a direct impact on the fish intestinal physiology. Supplementation of essential fatty acids is necessary to cover the requirements of the different EFAs, including the ones belonging to the n-6 series, such as arachidonic acid (ARA). ARA, besides its structural role in the configuration of the lipid classes of intestine, plays an important role on the functionality of the gut associated immune tissue (GALT).

The present study aimed to test five levels of dietary ARA (ARA0.5 (0.5%), ARA1 (1%), ARA2 (2%), ARA4 (4%) and ARA6 (6%) for European seabass (*Dicentrarchus labrax*) juveniles in order to: (a) determine its effect in selected distal intestine (DI) lipid classes composition; and (b) how these changes affected gut bacterial translocation rates and selected GALT-related genes expression pre and post challenge.

No differences were found between distal intestines of fish fed the graded ARA levels in total neutral lipids and total polar lipids. However, DI of fish fed the ARA6 diet presented higher ($P<0.05$) level of phosphatidylethanolamine (PE) and sphingomyelin (SM) than those DI of fish fed the ARA0.5 diet. In general terms, fatty acid profiles of DI lipid classes mirrored those of the diet dietary. Nevertheless, a selective retention of

ARA could be observed in glycerophospholipids when dietary levels are low (Diet ARA0.5), as reflected in the higher glycerophospholipids-ARA/dietary-ARA ratio for those animals. Increased ARA dietary supplementation was inversely correlated with eicosapentaenoic acid (EPA) content in lipid classes, when data from fish fed the diets with the same basal composition (Diets ARA1 to ARA6). ARA supplementation did not affect intestinal morphometry, goblet cells number or fish survival, in terms of gut bacterial translocation, along the challenge test. However, after the experimental infection with *Vibrio anguillarum*, the relative expression of *cox-2* and *il-1β* were up-regulated ($P<0.05$) in DI of fish fed the diets ARA0.5 and ARA2 compared to fish fed the rest of the experimental diets. Although dietary ARA did not affect fish survival, it altered the fatty acids composition of glycerophospholipids and the expression of pro-inflammatory genes after infection when included at the lowest concentration, which could be compromising the physical and the immune functionality of the DI, denoting the importance of ARA supplementation when low FO diets are used for marine fish.

Keywords

Aquaculture. *Dicentrarchus labrax*. Arachidonic acid. Gut Polar lipids. Distal intestine. Gut health.

1. INTRODUCTION

Nowadays, due to economic and environmental reasons aquafeeds include important levels of vegetable oil (VO), rich in 18:C polyunsaturated fatty acids (PUFAs) (Hardy et al. 2010). In marine finfish, contrarily to freshwater species, in some cases these substitutions are critical, since they have a limited capacity of elongate and desaturate PUFAs into their long chain families (Tocher 2003). Thus, presenting dietary requirements of long chain PUFA (LC-PUFAs), in particular for eicosapentaenoic acid (EPA, 20:5 n-3), docosahexaenoic acid (DHA, 22:6 n-3), and arachidonic acid (ARA, 20:4 n-6) (Tocher 2015), due to their important role into growth performance, nervous system or immune system development and functioning, for what they are recognized as essential fatty acids (EFA) for marine fish (Tocher et al. 2008).

LC-PUFAs are selectively esterified into cell surface glycerophospholipids (GPs) by fatty acyltransferase enzymes, affecting signaling processes as regulation of nuclear receptors and transcription (Crowder et al. 2017), membrane stability and

fluidity, and, eventually, cell functions (Tocher 2003; Fernandez and West 2005; Yaqoob and Calder 2007). These functions can be exerted directly by GPs as phosphatidylcholine (PC) and phosphatidylserine (PS) which are activators of protein kinase C (Tocher et al. 2008), or through derivates as phosphoinositides, diacylglycerol, lysophosphatidic acid or oxidized PC, to bind and activate receptors as, for instance, peroxisome proliferator activated receptor (Davies et al. 2001). Similarly, GPs constitute a reservoir of fatty acids (FA) that are released by phospholipase A2 (Pla2) to be used by cyclooxygenase (Cox) and lipoxygenase (Lox) enzymes for eicosanoid production (Tocher 2003) as prostaglandins (PGs), thromboxanes or leukotrienes, among others. Eicosanoids are a group of highly active hormone-like molecules that exert their biological effects in a paracrine manner in many physiological processes as the inflammatory response (Tocher 2003; Yaqoob and Calder 2007).

Given the fact that dietary oils and fats affect FA profile in fish tissues, especially in marine species (Tocher 2015), the organ function will be also influenced by dietary lipids (Tocher 2003). For instance, reductions of dietary EFA for gilthead seabream (*Sparus aurata*) together with changes on other FAs by the different dietary lipid sources are responsible for alterations in the morphology of intestine (Caballero et al. 2003 and 2004). The digestive tract of teleosts is one of the main entrances for pathogens (Zapata & Cooper, 1990), and particularly the gut-associated immune system (GALT) has a great importance in maintaining its health status (Rombout et al., 2011; Torrecillas et al. 2012). Fish gut houses a regional immune specialization and it is considered an important place for antigen uptaking, playing a key role achieving oral immune-protection (Rombout et al. 2011). In distal intestine (DI), lymphocytes, granulocytes and leukocytes, are spread on the epithelium and constitute the GALT, a local immune system that reacts to disturbances of homeostasis as those that occur during an infectious process or inclusion of terrestrial sources in diet (Torrecillas et al. 2014; Salinas 2015). These immune cells can produce eicosanoids to induce immune-cell proliferation, cytokine-release or to chemo-attract other immune cells (Zou and Secombes 2016). Hence, dietary imbalances of EFAs can lead to modifications on cell membranes composition and, therefore, alter gut morphology, growth performance and fish health (Tocher 2003; Montero et al. 2001, 2003, 2005, 2008, 2010).

Recent studies are demonstrating that ARA plays an important role on fish growth performance (Bessonart et al. 1999; Carrier et al. 2011; Koven et al. 2003; Lund et al.

2007; Bae et al. 2010; Luo et al. 2012; Torrecillas et al. 2018a), lipid metabolism (Luo et al. 2012; Xu et al. 2018), or fish health and disease resistance (Xu et al. 2010; Torrecillas et al. 2017c), among others. Besides, the essential role of ARA and its relative low levels compared to n-3 LC-PUFAs in the marine environment and in fish tissues, have probably led to the strong preference of enzymes involved in eicosanoid synthesis, at the expense of EPA (Liu et al. 2006; Yaqoob and Calder 2007; Furne et al. 2013). Indeed, the ratio ARA/EPA on the target organ, affects the synthesis of eicosanoids (Ganga et al. 2005,2006; Xu et al. 2018). Similarly, ARA-derived eicosanoids compete with those from EPA for the same cell membrane receptors (Sargent et al. 1999a; Ganga et al., 2005; Adam et al., 2017; Tian et al., 2017) although those originated from ARA seem to be more biologically active (Leslie 2004). Beyond eicosanoid production, the ARA role on immunity covers a great number of other mechanisms in cells as the activation of the NADPH oxidase enzyme in leukocytes to trigger the respiratory burst (Brash et al. 2001).

Farmed European seabass presents reduced ARA tissue levels when compared with wild specimens (Alasalvar et al. 2002; Bell et al. 2007; Fuentes et al. 2010 Lenas et al. 2011) indicating a necessary increase of dietary ARA. Indeed, studies of optimum levels of ARA have been made in larval stages of these species (Koven 2001, 2003; Atalah et al. 2011; Montero et al. 2015c) but scarce information exists in juveniles regarding ARA content in GPs and its influence in the intestinal immune response (Torrecillas et al. 2017c,d).

Therefore, an experiment was conducted out using graded levels of dietary ARA- for European seabass juveniles to determine the influence and the content of this EFA in lipid classes of DI and the related effects on gut morphology, expression of intestinal immune-related genes, survival and resistance to intestinal infection.

2. MATERIAL AND METHODS

2.1. Experimental diets

Five isolipidic and isoproteic experimental dry pelleted diets based on a commercial formulation were prepared to contain graded levels of ARA (total FA in diet, %) as follows: ARA0.5 (0.5%), ARA1 (1%), ARA2 (2%), ARA4 (4%) and ARA6 (6%). Diet ingredients, proximate composition, and FA) profiles are reported in Table 1

and 2. This basal diet was supplemented to achieve desired ARA content in diets ARA2, ARA4, and ARA6 with increasing quantities of Vevodar® (DSM Food Specialties, the Netherlands), a commercial fungal-oil rich in ARA obtained from *Mortierella alpine* (authorized in European Union by Commission Decision 2008/968/CE). Diet ARA0.5, was formulated with defatted fish meal (FM) and without fish oil (FO) to reduce the presence of ARA and supplemented with vegetable oils to reach requirements. When necessary, supplementation of DHA and EPA was done using DHA50 and EPA50 (CRODA, East Yorkshire, UK).

2.2. Fish and experimental conditions

For this feeding trial, eight hundred and forty European seabass juveniles reared in a commercial farm were maintained in quarantine in the facilities of Marine Science-Technology Park (PCTM) of University of Las Palmas de Gran Canaria (ULPGC), for 4 weeks before the experience, and fed a commercial diet. Tanks were supplied with seawater at a natural temperature of 22.8–24.9 ºC in a flow-through system and kept at a natural photoperiod (12L:12D). Dissolved oxygen ranged between 5-8 ppm. Fish were fed the experimental diets for 70 days and, at the end of this feeding trial, fish were submitted to a challenge test against *Vibrio anguillarum* via intestinal inoculation.

All animal manipulation in this trial complied European Union Council guidelines (86/609/EU) and Spanish legislation (RD 53/2013) and had been approved by Bioethical Committee of the ULPGC (Ref. 007/2012 CEBA ULPGC).

2.3. Feeding trial

With an average weight and length of 13.4 ± 0.3 g and 9.9 ± 0.1 cm respectively (mean ± SD), animals were randomly allocated in 15 fiberglass 200 L tanks (55 fish/tank; 4 kg m^{-3} of stocking density). Diets were assayed in triplicate and animals were fed by hand for 70 days until apparent satiation, three times a day, 6 days a week. After 70 days, samples of DI were taken for biochemical, histologial and gene-expression analyses. Survival was recorded during the whole period of the feeding trial.

2.4. Challenge trial

After 70 days of experiment, fish were transferred to the Biosecurity Facilities of ULPGC in PCTM (Telde, Las Palmas, Canary Island, Spain). After 2 weeks of adaptation to the new experimental conditions, fish were inoculated with a sublethal dose (10^7 CFU ml^{-1} per fish) of *V. anguillarum* using the method of anal cannulation assayed previously in similar experimental conditions (Torrecillas et al. 2007). Fish were fed their corresponding experimental diets for 7 days, as frequent than before. At 2 days after the infection, samples of DI were taken for immune-related genes analyses. Survival was recorded along this trial.

2.5. Lipid class and fatty acid content of selected glycerophospholipids of distal intestine

At day 70, eight fish per tank (N= 24 fish/diet), were used for biochemical analysis. The intestine was extracted out for analysis and distal section was separated as previously described by Torrecillas et al. (2013). Fish tissues were kept at -80º C until the analysis. Biochemical composition of distal intestine and diets were conducted following standard procedures from Association of Official Analytical Chemists (AOAC, 2016). The analysis of lipid class and fatty acid composition of selected glycerophospholipids (GPs) was conducted in the Institute of Aquaculture, Stirling University (UK). Separation of main lipid classes was realized in 10 × 10 cm plates (VWR, Lutterworth, UK) by double development high-performance thin-layer chromatography (HPTLC) using the technics described by Tocher and Harvie (1988), and Olsen and Henderson (1989). Firstly, plates were pre-run in diethyl ether and then activated at 120º C for 1 hour. The lipid classes were visualized after spraying with 3% (w/v) copper acetate, containing 8% (v/v) phosphoric acid by charring at 160º C for 20 min. Quantification was made by densitometry using a CAMAG-3 TLC scanner (Version Firmware 1.14.16; CAMAG, Muttenz, Switzerland) with winCATS Planar Chromatography Manager. Samples and authentic standards run alongside, in the same conditions, on high-performance thin layer chromatography (HPTLC) plates, as the way to determine the identities of individual lipid classes by contrasting Rf values. Total GPs, including PC, PS, phosphatidylethanolamine (PE), and phosphatidylinositol (PI) were isolated from HPTLC plates and subjected to acid-catalyzed transesterification according to the method of Tocher and Harvie (1988). Afterwards, extraction and purification were performed as described by Christie (1982). To separate and quantify fatty acid methyl esters (FAMEs) of selected GPs, a gas-liquid chromatography was

executed using a Fisons GC-8160 (Thermo Scientific, Milan, Italy) with the conditions determined previously (Izquierdo et al. 1992).

2.6. Histological studies

Samples from DI (N= 6 fish/diet) obtained after 70 days of feeding and taken as described by Torrecillas et al., (2013) were fixed in neutral-buffered formalin (4%). After 48 hours, tissues were dehydrated with an increased graded series of ethanol, submerged in xylene and embedded in paraffin blocks. Sections of 4µm were cut and stained with hematoxylin and eosin (H&E) and Alcian Blue-PAS (pH= 2.5) (Martoja and Martoja-Pierson 1970), for optical examinations and to differentiate mucus-secreting cells, respectively. Micrographs analyzed were obtained with a Nikon Microphot- FXA microscope (objective lens 20X plus eyepiece 10X) equipped with an Olympus DP50 camera. Cell count and measures of DI were made according to Torrecillas et al. (2007), using Image-Pro Plus v5 software (Media Cybernetics Inc., Rockville, MD, USA). Structural measures of DI were studied with a light microscope (N=72; 12 sections per fish × 6 fish per tank × 3 tanks per diet) and using individual fish weight as co-variable. Following measures were calculated: fold area, FA; fold perimeter, FP; fold length, FL; fold width, FW; submucosa width, SW. To estimate mucus production, the number of mucus-secreting cells by unit of area was counted (N= 288; 48 folds per fish × 2 fish per tank × 3 tanks per diet).

2.7. RNA extraction, cDNA synthesis and Quantitative Real-Time PCR analysis

After 70 days of feeding and during challenge trial (2 days), DI (N= 9 fish/diet) samples were collected in order to realize real time (RT) qPCR analyses. Tissues were submerged into Invitrogen™ RNAlater™ Stabilization Solution (Thermo Fisher Scientific Inc., USA) and conserved at -20^{o}C. Then, using TRI-Reagent (Sigma-Aldrich, Saint Louis, MO, USA) and RNeasy® mini Kit (QUIAGEN, Germany), total RNA was extracted from 100 mg of pooled tissues, (N=3 fish/tank). RNA was quantified by spectrophotometry using Nanodrop 1000 (Thermo Fisher Scientific Inc., USA) and integrity was evaluated on a 1.4% agarose gel with Gel Red™ (Biotium Inc., Hayward, CA). The synthesis of cDNA was realized from 1µg RNA with iScript™ cDNA Synthesis Kit (Bio-Rad Hercules, California) in 20µl final volume. Selected genes related to GALT functioning and eicosanoid production were as follows and

respectively: interleukin 10 (*il-10*), interleukin-1beta (*il-1ß*), tumor necrosis factor alpha (*tnfα*), and cyclooxygenase 2 (*cox-2*). RT-qPCR reactions were performed by triplicate and conditions were 1X (95^{o}C, 10min), 35x (95^{o}C, 45s/corresponding annealing temperature, 45s/72^{o}C, 45s) 1X (72^{o}C, 30s). Conditions, sequences and references are registered in Table 3. Two genes, elongation factor 1 (*ef-1*) and *β-actin*, were tested as housekeeping but *ef-1* was found to be more stable to make calculations. Reactions were performed in an iCycler Optical Module (Bio-Rad, USA), the final volume used was 15µl, containing 2µl of cDNA (diluted 1/10), 0.6µl of each primer (10 mM) and 7.5µl of Brilliant SYBR Green QPCR Master Mix (Bio-Rad Hercules, CA, USA). Blank samples, with 2µl of water replacing cDNA, were included in each assay as a contamination control. The Livak & Schmittgen (2001) method was used to calculate relative expression of each gene.

2.8. Statistical analysis

All statistical analyses were performed using SPSS 21 software package for Windows (IBM, Chicago, IL, USA). All data, presented as mean ± SD, were tested for normality and homoscedasticity. Statistical analyses followed methods outlined by Sokal and Rolf (1995). Data were submitted to a One-way analysis of variance (ANOVA). When F values showed significance, individual means were compared using post hoc tests for multiple means comparison. When data were not normally distributed, data analysis was made by non-parametric test (Kruskal-Wallis and U Mann-Whitney). When Levene's test showed $P<0.05$, but ANOVA and Wells test showed $P<0.05$, post hoc test used was Games-Howell. Pearson coefficient was used for correlations and statistical significance was set at $P<0.05$. Survival curves were performed and analyzed using the method described by Kaplan-Meier (Kaplan and Meier 1958).

3. RESULTS

3.1 Growth parameters

The growth study has been previously reported (Torrecillas et al., 2018a) but it is important to point out that fish growth presented differences at the end of feeding trial. Briefly, fish fed the lowest dietary ARA levels showed significantly lower ($P<0.05$) weight (g) (ARA0.5 =33.0 ± 1.1) than those from the other diets, that are those diets in which ARA was supplemented on the same base diet (ARA1= 44.4 ± 1.1; ARA2= 43.8

± 1.0; ARA4= 43.9 ± 3.7; ARA6= 42.8 ± 2.5) (mean ± SD). Dietary ARA levels did not affect (P>0.05) cumulative survival percentages for European sea bass fed the experimental diets for 70 days (over 95% for all diets).

3.2 Lipid class composition of distal intestine

No differences were found between diets in the Σ neutral lipids or the Σ polar lipids of DI (Table 4). Regarding polar lipids, PC, followed by PE, were in higher proportion than the rest of lipid class (Table 4). Lysophosphatidylcholine (LPC) presented the lowest proportion (Table 4). Among polar lipids, SM and PE were the only lipid class affected by dietary ARA (P=0.041 and P=0.049; respectively) (Table 4). Fish fed diet ARA6 had significant (P<0.05) higher level of PE than control diet (ARA0.5) (Table 4). Similarly, SM was more abundant in ARA6 than in ARA0.5, ARA1 and ARA2 (Table 4). Besides, significant correlations between dietary ARA and lipid classes in DI were found for PE (0.743/P=0.001), PC (0.640/P=0.010) and SM (0.700/P=0.004), (Pearson coefficient/P value).

3.3 Fatty acid composition of selected glycerophospholipids in distal intestine.

The FA composition of four main GPs (PC, PE, PS and PI) was analyzed in DI (Table 5 a, b, c & d). Increasing dietary ARA levels mirrored in the content of ARA in GPs (GPsARA). However, the lowest dietary ARA level (ARA0.5) induced a selective incorporation of ARA in all the GPs, reflected in the content of ARA (P<0.05; Tables 5a to 5d). The higher GPsARA/dietary ARA ratio (P<0.05) found for PC, PE and PS in fish fed ARA0.5 diet in comparison to the values obtained for the animals feeding either of the rest of the diets, was also reflecting the selective incorporation of ARA (Tables 5a to 5c). For PI, no differences (P>0.05) were found in the GPsARA/dietary ARA ratio between fish fed ARA0.5 and ARA1 diets (Table 5d). The GPsARA/dietary ARA ratio in all GPs analyzed in DI, reflected that content of ARA was higher than dietary ARA. Significant (P<0.05) correlations were found in DI between dietary ARA levels and the GPsARA in all analyzed polar lipids: PC (0.992/P<0.001), PS (0.872/P<0.001), PE (0.969/P<0.001), PI (0.750/P=0.001) (Pearson coefficient/P value) (Tables 5a to 5d).

Fish fed to ARA 0.5 diet presented high content of Σn-6 PUFA and Σn-3 PUFA due to the higher content of 18:2n-6 and 18:3n-3 from the diet, respectively. For the rest of the experimental diets, where ARA was supplemented on the same basal diet from diet

ARA1 to ARA6), all GPs analyzed in DI, increasing dietary ARA induced an accumulation of Σn-6 PUFA ($P<0.05$), mainly due to the increased GPsARA in the different GPs, (Tables 5a to 5d). Moreover, in PC, PE and PS, dietary ARA induced a significant ($P<0.05$) reduction of Σn-3 PUFA (Tables 5a to 5c). The increment of dietary levels of ARA was inversely correlated with the EPA content in GPs, although negative correlations were not significant ($P>0.05$), except for PE (data not shown), due to reduced dietary EPA level in diet ARA0.5 compared to the other diets (Table 2). Negative and significant ($P<0.05$) correlations between dietary ARA level and EPA content were found for all GPs when ARA0.5 diet was excluded from the statistical analysis: PC (-0.904/$P<0.001$), PS (-0.777/$P=0.003$), PE (-0.941/$P<0.001$), and PI (-0.807/$P=0.002$) (Pearson coefficient/P value) (Tables 5a to 5d). Besides, differences of Σ saturated and Σ PUFA were found in PC, with the higher ($P< 0.05$) Σ PUFA level and the lower ($P<0.05$) level of Σ saturated in those fish fed ARA0.5 diet, due to significant increases of oleic, linoleic and alpha-linolenic acids, (Table 5a). Differences in DHA content were found in PS and PE among fish fed the different dietary treatments (Tables 5b and 5c). In PS, lower ($P<0.05$) level of DHA was found in fish fed ARA0.5 diet than ARA1, ARA2, and ARA4 (Table 5b). In PE, lower ($P<0.05$) level of DHA was found in fish fed ARA0.5 and ARA6 diets when compared with the rest of experimental diets (Table 5c).

3.4 Histological studies

Morphometric analysis of DI showed no significant ($P>0.05$) differences in any intestinal measure (Table 6) when related to fish real weight. Similarly, no effect of dietary ARA was observed in the density of goblet cells by unit of area in relation to the real fish weight (Table 6).

3.5 Relative expression of selected genes after feeding trial and challenge test against *Vibrio anguillarum*.

The cumulative mortality after challenge test against *V. anguillarum* was not affected by dietary ARA ($P>0.05$). Despite the differences in the survival percentages were not significant, there was a trend to lower mortality in fish fed diet ARA6, which did not present mortality along the experimental intestinal infection, whereas the

survival percentage of fish fed the experimental diets ranged between 76.5 and 88.2%, for diets ARA0.5 and ARA4 respectively).

The relative expression of immune related genes, including *il-1β, tnfα, il-10* and *cox-2*, were analyzed in DI at both basal and 2 days post infection (Fig.1). No effect was found on *tnfα* relative gene expression (Fig. 1a). After the feeding period (basal level), increased expression of pro-inflammatory *il-1β* ($P=0.030$) was found in fish fed ARA0.5 diet in comparison to fish fed ARA1 and ARA2 (Fig.1b). After 2 days post infection, there was an up-regulation of *il-1β* relative gene expression in fish fed ARA0.5 and ARA2 diets when compared with those fish fed the rest of the diets ($P<0.001$) (Fig.1b). An increment of *il-10* relative expression was found in fish fed ARA1 and ARA6 ($P=0.002$) at basal level compared to fish fed the other diets, whereas after infection a reduction was found in fish fed ARA2 compared to those fed the rest of the diets ($P<0.001$) (Fig.1c). No differences ($P>0.05$) were found at basal level for *cox-2* relative expression (Fig.1d). At 2 DPI, *cox-2* gene expression was up-regulated ($P<0.05$) in fish fed ARA2 (Fig.1d) when comparing to fish fed the rest of the dietary treatments.

4. DISCUSSION

Fish have dietary requirements of GPs for normal growth, homeostasis maintenance, survival, or immune system function (Tocher et al. 2008; Adam et al., 2017; Tian et al., 2017). Among other functions, GPs are related with lipid transport and plasticity of the cell membranes (Tocher et al. 2008). Besides, GPs, act as precursors of metabolism mediators as diacylglycerol or phosphoinositides, these last related with cell polarity to keep cytoarchitecture, which is determinant in epithelial barrier and transport functions allocated in the enterocyte-mucose layer (Shewan et al. 2011). GPs have described to be affected by the dietary fatty acid profile, both the amount of each GP and also the fatty acid composition of each lipid class (Olsen et al. 2003).

In this study, levels of dietary ARA were correlated with the concentration of the different lipid class levels in DI of European seabass. Although increased dietary ARA seemed to be related with increased the concentration of PE and SM in DI, with values higher in the diets supplemented with high (ARA4 or ARA6) content of ARA, it was also correlated to PC level, a lipid class that is required for SM synthesis (Patel and Witt

2017) and is related to PE through remodeling pathways (Tocher et al. 2008). Previous studies have demonstrated the importance of SM in epithelial barriers of fish and other vertebrates, despite the structural differences between marine and terrestrial epithelia (Feingold 2007; Pullmannová et al. 2014; Cheng et al. 2018). In fact, this polar lipid, disposed in the outer leaflet of the cell membrane with another choline-container lipid as PC (Tocher et al. 2008), is more abundant in membranes of temperate-water fish suggesting its role in the membrane fluidity (Storelli et al. 1998; Palmerini et al. 2009). In Atlantic salmon, reductions in dietary EPA and DHA increased skin SM levels, denoting alterations of the barrier function of the skin with reductions of these EFAs (Cheng et al. 2018). Besides, SM has been linked with the regulation of the release of ARA, by the inhibition of the c-Pla2α bind to the GPs (Nakamura and Murayama 2014). In the present experiment, SM in DI increased when ARA increased in diet, with the subsequent decrease of the n-3 LC-PUFA/ARA ratio. The increase of SM in the gut of fish fed high dietary ARA could be ameliorating a possible increase of cPla2 activity induced by the high amount of ARA in the GPs of those fish fed the higher levels of ARA in diet.

It is known that high LC-PUFA content induces the decarboxylation of PS to PE at membrane level of different organelles as mitochondria or Golgi (Kainu et al. 2013). In the present study, PE levels in DI were increased by dietary ARA, with the highest level corresponding to those fish fed the highest dietary ARA level. This could be related to the fact that the generation of PE through the PS decarboxylation pathway generated preferentially PE species with a PUFA at the sn-2 position (Bleijerveld et al. 2007). However, the synthesis of PE through decarboxylation of PS has been shown to be promoted by DHA and not by ARA (Ikemoto et al. 1999), and thus, other metabolic pathways different than PS decarboxylation cannot be rejected to explain the increases of PE in the DI of the fish fed high ARA in diet.

Dietary ARA also influenced fatty acid profiles of lipid classes in the distal section of the intestine. Olsen et al. (2003) showed that the effect of the type of dietary lipid is reflected in the fatty acid profile of the intestine and it is dependent of the section of intestine studied. In this study, correlations were found between dietary ARA and content of ARA for the four GPs studied in DI.

As described for other species, PI was the lipid class with the highest content of ARA (Bell and Sargent 2003). Moreover, due to the abundance of PC and PE in the

tissue studied, higher ARA content was found in those GPs in agreement with previous studies (Bell et al. 1995). Besides, the increased content of ARA in studied GPs with respect to the dietary level occurred in all diets and GPs analyzed, although with more intensity in fish fed the lowest ARA level as reflected in the higher ratio GPs-ARA/dietary for those animals. This selective retention can be considered as a way to keep functionality during EFA deficiencies (Skalli et al. 2006) as negative effects of EFA deficiencies can be magnified at chronic stressful situations. Indeed, ARA reductions were found in liver polar lipids when gilthead sea bream were subjected to high stocking densities probably due to its selective utilization in that stressful situation (Montero et al. 2001). Moreover, DHA concentration was also higher than dietary DHA levels in all studied GPs, particularly in PE and PS, although it must be taken into account that DHA is preferentially esterified to PE and PS (Kim et al., 2004), and thus DHA concentration in polar lipids depends not only on the DHA level in diets but also on the esterification within those lipid classes. The relatively high levels of ARA and/or DHA despite their dietary inclusion were in agreement to their preferential incorporation previously found by other authors in European sea bass tissues (Farndale et al. 1999; Eroldoğan et al. 2013; Torrecillas et al. 2015a) including in polar lipids (Torrecillas et al. 2013) and in other species (Bell et al. 2001; Montero et al. 2001, 2003; Fountoulaki et al. 2003; Dantagnan et al. 2017). Furthermore, results from the present study indicate that inclusion of EPA in GPs was negatively correlated by the supplementation of ARA in diet (excluding from this correlation the results from diet 0.5 formulated with different ingredients and different fatty acid profile), suggesting competition between EPA and ARA during phospholipid esterification, in agreement with previous studies (Bell et al. 1991, Bessonart et al. 1999; Fountoulaki et al. 2003; Atalah et al. 2011). Competition between both fatty acids as substrate for different enzymes is of especial relevance during eicosanoid synthesis, as both fatty acids are substrates for eicosanoid production, affecting different fish functions, including immune system (Bell et al. 1996b, Montero et al., 2015c; Adam et al., 2017).

The graded dietary levels of ARA used in the present study did not affect survival, in agreement with previous studies using graded dietary ARA levels in European sea bass larvae (Atalah et al. 2011) or in other marine species such as gilthead seabream, Senegal sole (*Solea senegalensis*) or Japanese sea bass (*Lateolabrax japonicus*) (Fountoulaki et al. 2003; Villalta et al. 2005; Xu et al. 2010). Other studies in

gilthead seabream have found positive effects (Bessonart et al. 1999) related to stress resistance (Koven et al. 2001; Willey et al. 2003). Besides, low or too high dietary ARA has been described to induce a reduction of fish survival during a bacterial challenge in Atlantic salmon (*Salmo salar*) (Dantagnan et al. 2017). In the present experiment, the graded levels of dietary ARA did not affect survival after challenge test, but induced changes in the expression of GALT-related genes, as described for other species such as Atlantic salmon (Dantagnan et al. 2017) or guppy (*Poecilia reticulata*) (Khozing-Goldberg et al. 2006). Indeed, a previous study has related dietary ARA with mechanisms of protection against damage in the intestine (Tarnawski et al. 1989). In this sense, intestine is an organ subjected to injury, intestinal barrier being highly compromised and subsequently acting as one of the main entrances for pathogens (Ellis 2001; Campos-Pérez et al. 2000).

The relation between intestine and eicosanoid synthesis has been widely studied in different fish species (Sargent et al. 1999a; Tocher 2003; Calduch-Giner et al. 2016). Although ARA and EPA are substrates for COX and LOX enzymes to produce eicosanoids (Bell & Sargent 2003, Tocher et al. 2008), these enzymes seem to have stronger preference for released-ARA than for EPA at least in freshwater fish and salmonids (Bell and Sargent 2003; Tocher et al. 2008; Furne et al. 2013). In this trial, the supplementation of dietary ARA did not influence directly basal levels of *cox-2* relative expression in gut, suggesting no effect on PGE2 production in intestine as described for other vertebrates (Tateishi et al. 2014) which is also supported by the absence of significant differences in PI levels, the main pool of ARA for eicosanoids production (Yaqoob and Calder 2007). However, after infection with *V. anguillarum*, in the present study European seabass juveniles fed 2% of ARA in diet increased *cox-2* relative expression, which has been related with protection to gastric mucosal defenses including stimulation of mucus secretion and maintenance of mucosal blood flow (Wallace and Devchan 2005). The gastro-protective properties of Cox-2-derived PGs have been demonstrated in eel (*Anguilla anguilla*) gastric mucosa (Faggio et al. 2000), and *cox-2* expression in the intestine has been also associated to a response of Atlantic salmon to acute stress, mainly in DI (Oxley et al. 2010).

The up-regulation of *cox-2* levels found in the present study after bacterial infection was coincident with the increased *il-1β* gene relative expression. The Cox-2 enzyme and pro-inflammatory cytokines such as Il-1β seems to be linked through the

p38 mitogen-activated protein kinase (P38 mapk) (Camacho-Barquero et al. 2007), which is known to be present in fish (Ribeiro et al. 2010; Yang et al. 2014b). The Mapk can be activated by ARA metabolites in a dose-dependent manner (Alexander et al. 2001), which in turn can activate *cox-2* expression (Sui et al. 2014). Besides, Mapk constitutes a signaling pathway involved in regulation of multiple cell functions including autophagy, a cell process of self-degradation to maintain homeostasis in which proinflammatory cytokines are implicated (Sui et al. 2014). PE plays an important role in autophagy because it is utilized by proteins required for the formation of autophagosomes to attach to cell membranes (Ichimura et al. 2000; Iula et al. 2018), and, besides, these autophagic vesicles are utilized for secretion of cytosolic Il-1β (Iula et al. 2018). At the same time, Il-1β has been suggested to be involved in the PE synthesis via Mapk (Sluzalska et al. 2017). In this way, the modification of PE levels in DI can be related with the secretion of Il-1β. In the present study, increased *il-1β* relative expression at basal time in diet ARA0.5 could be related to PE reduction in that diet, although other factors influencing the PE reduction cannot be rejected, as this diet had lower amount of DHA and EPA. Besides, other authors have shown that increased levels of Il-1β can reduce SM synthesis without affecting other choline-GPs as PC (Kronqvist et al. 1999). In this experiment, and when considering only the diets with same basal composition and graded ARA (diets from ARA1 to ARA6), the reduced levels found in SM levels could be related to increments in ARA release in those fish fed lower ARA level or to regulation of its synthesis, both mechanisms affected by Il-1β release.

In conclusion, ARA is selectively retained in the GPs of DI of European seabass, supporting its important physiological role in this tissue. This ARA selective retention is especially evident when low dietary ARA levels are fed (Diet ARA0.5), as reflected in the higher glycerophospholipids-ARA/dietary-ARA ratio found. However, these variations were not enough to alter DI morphology or/and bacterial translocation rates, regardless of the ARA-deficiency related up-regulation of DI pro-inflammatory genes. Altogether pointing to a long-term compromised physical barrier integrity and immune functionality of the DI, denoting the importance of ARA supplementation when low FO diets are used for marine fish.

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Captions to figures

Figure 1. RT-qPCR of immune-related genes in distal intestine of D. labrax juveniles, at basal time and at 2 days post infection: (a) *tnfα*; (b) *il-1β*; (c) *il-10*; (d) *cox-2*. N= 9 fish/diet. All values of relative expression are represented as mean ± SD. Differences were significant when P<0.05, after One-way ANOVA. Significant (p<0.05) differences among diets within same sampling point indicate with letters: lowercase for Basal and uppercase for 2DPI.

910 Table 1. Ingredients and biochemical composition analyzed for the different experimental diets containing
911 graded levels of ARA (% of dry matter).

912

	DIETS				
	ARA0.6	ARA1	ARA2	ARA4	ARA6
Fish Meal[1]	--	52.50	52.50	52.50	52.50
Fish oil[1]	--	14.50	12.60	11.40	10.10
Defatted Fish Meal[2]	46.50	--	--	--	--
Corn Meal[3]	7.00	6.00	6.00	6.00	6.00
Soy 44 Meal[3]	10.00	10.00	10.00	10.00	10.00
Wheat Meal[3]	5.50	5.50	5.50	5.50	5.50
Wheat Gluten[3]	7.00	7.00	7.00	7.00	7.00
Vegetable fats and oils	14.50	--	--	--	--
Vitamins Mix [4]	2.00	2.00	2.00	2.00	2.00
Mineral Mix[5]	2.00	2.00	2.00	2.00	2.00
CMC [6]	0.50	0.50	0.50	0.50	0.50
ARA[7]	--	--	0.50	1.50	2.50
DHA & EPA[8]	5.00	--	1.40	1.60	1.90
Analyzed Proximate composition (g·kg^{-1}; d.w.)					
Crude Lipids	20.77	21.33	20.87	21.12	22.02
Crude Protein	43.71	43.32	44.93	44.61	45.14
Ash	9.75	10.51	10.47	10.39	10.49
Moisture	8.94	6.57	7.63	7.25	7.39

913 1.
914 2. Fish meal and oil, South American origin, (65% protein, 12% lipid).
915 3. Defatted soymeal (GIA-ECOAQUA laboratory, produced by 3 x chloroform extraction; 73% protein, 2%
916 lipid).
917 4. Vegetable ingredients locally found (SBM:46% protein, 3% lipid).
918 5. Vitamin premix contains (mg kg−1 or IU/kg of dry diet): thiamine 40 mg, riboflavin 50 mg, pyridoxine
919 40 mg, calcium pantothenate 117 mg, nicotinic acid 200 mg, biotin 1 mg, folic acid 10 mg, cyanocobalamin,
920 0.5 mg, choline chloride 2700 mg, Myo-inositol 2000 mg, ascorbic acid 5000 mg, menadione 20 mg,
921 cholecalciferol 2000 IU, ethoxyquin 100 mg, retinol acetate 5000 IU.
922 6. Mineral premix contains (g/kg of dry diet): calcium orthophosphate 1.60 g, calcium carbonate 4 g, ferrous
923 sulphate 1.5 g, magnesium sulphate 1.6 g, potassium phosphate 2.8 g, sodium phosphate 1 g, aluminum
924 sulphate 0.02 g, zinc sulphate 0.24 g, copper sulphate 0.20 g, manganese sulphate 0.08 g, potassium iodate
925 0.02 g.
926 7. Carboxymethyl cellulose (sodium salt, Sigma-Aldrich, Munich, Germany).
927 8. Vevodar®, DSM Food Specialties, Netherlands.
928 9. DHA50 and EPA50, CRODA, East Yorkshire, UK.

929

930 Table 2. Fatty acid composition (% of total identified FA) of total lipids in experimental diets.

931

	Diets				
	ARA0.5	ARA1	ARA2	ARA4	ARA6
14:0	0.19	4.53	4.99	4.76	4.29
15:0	0.04	0.53	0.56	0.53	0.48
16:0	6.15	16.55	18.10	17.92	16.66
17:0	0.01	0.55	0.50	0.48	0.48
18:0	3.19	3.92	4.57	4.89	5.04
20:0	0.25	0.29	0.35	0.38	0.40
Σ Saturates	9.83	26.36	29.07	28.97	27.35
16:1 n-7	0.28	5.78	6.04	5.73	5.19
18:1 n-9	17.31	17.36	18.31	18.02	17.09
18:1 n-7	0.89	3.00	3.15	3.01	2.75
20:1 n-9	0.11	0.33	0.34	0.31	0.28
20: 1n-7	0.73	2.54	2.58	2.43	2.16
22:1 n-11	0.19	2.27	2.12	1.98	1.71
22:1 n-9	0.28	0.44	0.43	0.41	0.36
Σ Monoenes	19.92	32.61	33.91	32.80	30.35
18:2 n-6	13.94	5.86	5.98	6.06	6.12
18: 3n-6	0.06	0.33	0.42	0.55	0.73
20:2 n-6	0.15	0.37	0.40	0.41	0.42
20:3 n-6	0.05	0.15	0.23	0.42	0.61
20:4 n-6	0.59	1.03	2.03	4.03	6.35
22:4 n-6	0.14	0.17	0.18	0.19	0.20
22:5 n-6	0.52	0.46	0.50	0.49	0.53
Σ n-6	15.45	8.39	9.75	12.16	14.96
16:4n-3	0.03	0.80	0.67	0.64	0.66
18:3 n-3	42.28	1.61	1.59	1.36	1.27
18:4 n-3	0.08	1.39	1.11	1.02	1.02
20: 3n-3	0.24	0.18	0.17	0.16	0.15
20:4 n-3	0.14	0.66	0.56	0.53	0.52
20:5 n-3	2.06	9.65	7.68	7.32	7.61
22:5 n-3	0.57	1.82	1.40	1.33	1.40
22:6 n-3	9.14	14.65	12.19	11.89	13.01
Σ n-3	54.57	30.93	25.54	24.42	25.79
Σn-3LC-PUFA	12.15	26.97	22.00	21.22	22.69
ARA/EPA	0.29	0.11	0.26	0.55	0.84
DHA/EPA	4.43	1.52	1.59	1.62	1.71
DHA/ARA	15.51	14.23	6.02	2.95	2.05
n-3/n-6	3.53	3.69	2.62	2.01	1.72

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Table 3. References, annealing temperatures, sequences and sources of primers for RT-qPCR.

Genes	Genbank reference	Annealing temperature	Primers sequence 5'-3'	From
IL-10	AM268529	52°C	F'ACCCCGTTCGCTTGCCA R'CATCTGGTGACATCACTC	Buonocore *et al.,* 2007.
IL1-ß	AJ311925	58°C	F'GGTGGACAAAGCCAGTC R'CCGAGCCTTCAACATCG	Picchietti *et al.,* 2009
TNF-α	DQ070246.1	58°C	F'ACAGCGGATATGGACGGTG R'GCCAAGCAAACAGCAGGAC	Román *et al.,* 2013
COX-2	AJ630649	52°C	F'CATTCTTTGCCCAGCACTTCACC R'AGCTTGCCATCCTTGAAGAGTC	Picchietti *et al.,* 2009
EF-1	AJ866727	60°C	F'GCTTCGAGGAAATCACCAAG R'CAACCTTCCATCCCTTGAAC	Geay *et al.,* 2011

Table 4. Lipid class composition (% of lipid classes detected) in distal intestine of *D. labrax*. All results are expressed as mean±SD. Letters denote significant differences (P<0.05) after ANOVA analysis.

	Diets				
	ARA0.5	ARA1	ARA2	ARA4	ARA6
TAG (1)	55.20±9.36	55.77±11.30	56.40±9.06	51.27±10.47	53.87±2.81
FFA (2)	10.20±4.79	7.50±5.20	7.97±4.10	9.07±4.04	6.57±0.74
Cholesterol/sterols	8.97±1.03	9.83±0.65	9.13±0.31	9.47±0.32	10.37±0.83
Unknown neutral lipid	4.40±2.43	3.87±2.11	4.03±1.97	4.03±1.83	3.07±0.23
Σ neutral lipids	78.77±1.56	76.97±5.31	77.53±3.54	73.83±4.42	73.87±2.05
PA/PGl/CL (3)	1.20±0.82	1.13±0.55	1.03±0.51	1.13±0.67	0.97±0.15
PtdCho	6.03±0.42	6.63±1.50	5.87±0.23	7.07±0.59	7.70±0.17
PtdSer	2.07±0.31	3.27±2.80	3.63±2.50	4.60±2.78	2.70±0.95
PtdEtn	3.90±0.46a	4.57±0.98ab	4.47±0.47ab	5.10±0.40ab	5.77±0.67b
PtdIns	1.83±1.07	1.80±0.75	1.83±0.74	2.17±0.76	1.97±0.38
SPM (4)	2.23±0.32a	2.03±0.51a	1.97±0.64a	2.60±0.40ab	3.17±0.32b
LSC (5)	0.43±0.15	0.73±0.40	0.57±0.21	0.40±0.17	0.47±0.31
Pigmented material	3.53±0.64	2.87±0.45	3.10±0.61	3.10±0.85	3.40±0.53
Σ polar lipids	21.23±1.56	23.03±5.31	22.47±3.54	26.17±4.42	26.13±2.05

(1) Triacylglycerols, (2) Free fatty acids, (3) Phosphatidic acid/Phosphatidylglycerol/cardiolipin, (4) sphingomyelin (5) Lysophosphatidylcholine

Table 5. Selected fatty acids composition (% fatty acid identified) of the different glycerophospholipids (GPs). a: Phospatidylcholine (PC); b: Phophatidylserine (PS); c: Phosphatidyletanolamine (PE); d: Phosphatidylinositol (PI) analyzed in distal intestine of European sea bass fed graded levels of ARA in diet. GPsARA/ARA diet: Ratio between ARA in GPs and ARA in diet. Diferent letters within the same row denote significant ($p<0.05$) differences.

a. **phosphatidylcholine (PC)**	DIETS				
	ARA0.5	ARA1	ARA2	ARA4	ARA6
16:0	19.86±1.09a	26.98±0.54b	27.37±0.80b	27.91±1.56b	25.93±0.59b
18:0	9.25±0.40	9.26±0.73	8.95±0.91	8.85±0.55	9.27±0.88
Σ saturated	30.88±1.17a	39.55±0.41b	40.32±1.17b	40.38±1.68b	38.90±1.49b
18:1 n-9	13.36±0.36b	10.51±1.04a	11.08±0.37ab	10.84±1.72ab	10.60±1.13ab
Σ monoenes	18.95±0.78	18.12±1.08	19.13±0.52	17.95±2.02	18.04±1.09
18:2 n-6	6.71±0.42c	2.51±0.18b	2.01±0.07a	1.87±0.04a	1.67±0.13a
20:4 n-6	3.10±0.08a	3.08±0.19a	5.26±0.13b	8.56±0.39c	11.28±0.12d
Σ n-6 PUFA	13.09±0.57d	7.10±0.16a	8.90±0.20b	12.05±0.38c	14.62±0.28e
18:3 n-3	11.82±1.06c	0.34±0.05b	0.29±0.04ab	0.26±0.02ab	0.23±0.05a
20:5 n-3	5.25±0.37a	8.20±0.79c	7.21±0.19bc	6.09±0.09ab	5.47±0.74a
22:6 n-3	18.89±2.23	25.17±1.70	22.82±0.73	22.13±3.47	21.65±1.29
Σ n-3 PUFA	37.09±1.35c	35.22±1.33bc	31.65±1.07abc	29.60±3.58ab	28.41±2.23a
Σ PUFA	50.17±0.81b	42.32±1.45a	40.55±1.26a	41.67±3.45a	43.06±2.56a
∑ n-3/n-6	2.84±0.22a	4.96±0.13c	3.56±0.05a	2.46±0.34ab	1.94±0.12b
∑ n-3 LC-PUFA	24.15±2.44a	33.37±1.21b	30.02±0.91ab	28.22±3.56ab	27.12±2.03ab
GPsARA/ARA diet	5.26±0.14d	2.99±0.19c	2.60±0.07b	2.12±0.10a	1.78±0.02a

b. **phosphatidylserine (PS)**	DIETS				
	ARA0.5	ARA1	ARA2	ARA4	ARA6
16:0	7.72±1.63	7.74±1.40	6.54±0.37	6.08±0.81	7.82±2.57
18:0	34.04±1.43	32.64±1.14	34.29±0.84	34.74±2.41	32.57±2.95
Σ saturated	44.90±0.73	43.54±2.78	43.89±1.12	43.89±1.26	43.87±2.17
18:1 n-9	7.39±1.61	5.38±0.49	4.60±0.65	4.69±0.44	7.22±2.35
Σ monoenes	12.90±1.58	10.19±0.84	9.45±1.06	9.36±1.06	12.33±2.91
18:2 n-6	2.11±0.39b	1.05±0.24a	0.71±0.15a	0.62±0.04a	0.77±0.11a
20:4 n-6	1.31±0.22a	1.82±0.50a	2.44±0.25ab	4.03±1.07bc	4.27±0.75c
Σ n-6 PUFA	8.10±0.22b	5.48±0.55a	6.16±0.33ab	7.72±1.33b	8.03±0.87b
18:3 n-3	2.69±0.30a	0.25±0.20b	0.16±0.03b	0.13±0.03b	0.16±0.04b
20:5 n-3	1.06±0.13a	1.92±0.35b	1.39±0.09ab	1.06±0.11a	1.00±0.27a
22:6 n-3	29.15±2.33a	36.39±2.65b	37.06±0.61b	35.87±1.99b	32.93±3.46ab
Σ n-3 PUFA	34.05±1.87a	40.73±3.01b	40.47±0.72ab	38.97±1.64ab	35.75±3.88ab
Σ PUFA	42.20±1.59	46.27±3.47	46.66±1.01	46.75±2.26	43.80±4.80

∑ n-3/n-6	4.21±0.35a	7.44±0.25b	6.58±0.24b	5.15±0.96ab	4.45±0.02a
∑ n-3 LC-PUFA	30.21±2.21a	38.31±3.01b	38.44±0.53b	36.94±1.90b	33.93±3.60ab
GPsARA/ARA diet	2.22±0.38d	1.77±0.49c	1.21±0.13bc	1.00±0.27ab	0.67±0.12a

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c. **phosphatidyletanolamine (PE)**	DIETS				
	ARA0.5	ARA1	ARA2	ARA4	ARA6
16:0	6.97±0.68	10.02±1.86	9.73±0.99	9.26±1.02	8.27±1.00
18:0	16.69±0.96	17.30±1.66	17.47±0.79	17.38±2.13	16.88±1.38
Σ saturated	25.78±1.68	30.12±1.87	29.84±2.01	29.15±3.11	27.34±0.36
18:1 n-9	6.66±0.13a	3.92±0.60b	4.05±0.11b	4.14±0.84ab	3.53±0.42b
Σ monoenes	14.87±0.45	12.72±0.80	11.79±1.52	12.22±1.17	12.77±1.44
18:2 n-6	2.72±0.33a	0.98±0.14b	0.80±0.14b	0.84±0.19b	0.96±0.68ab
20:4 n-6	6.43±0.78a	5.96±0.48a	9.27±0.57b	13.13±1.29c	16.13±1.13d
Σ n-6 PUFA	14.17±1.20bc	8.71±0.60a	12.24±0.85b	16.18±1.37c	19.50±0.54d
18:3 n-3	3.70±0.41a	0.18±0.01b	0.16±0.01b	0.19±0.03b	0.22±0.11b
20:5 n-3	5.01±0.27b	7.46±0.34d	6.00±0.41c	4.46±0.47ab	3.68±0.17a
22:6 n-3	35.10±0.86a	38.98±2.33b	38.40±2.09b	36.29±0.26a	35.07±1.61a
Σ n-3 PUFA	45.14±0.52bc	48.41±2.10c	46.02±2.59bc	42.38±0.70a	40.39±1.76a
Σ PUFA	59.34±1.51	57.16±2.48	58.36±3.53	58.64±1.95	59.89±1.33
∑ n-3/n-6	3.20±0.25bc	5.57±0.32d	3.76±0.05c	2.63±0.19ab	2.07±0.14a
∑ n-3 LC-PUFA	40.11±0.76a	46.44±2.13b	44.41±2.50b	40.75±0.64a	38.75±1.75a
GPsARA/ARA diet	10.91±1.32d	5.79±0.47c	4.58±0.28c	3.26±0.32ab	2.54±0.18a

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d. **phosphatidylinositol (PI)**	DIETS				
	ARA0.5	ARA1	ARA2	ARA4	ARA6
16:0	10.52±5.29	10.11±2.06	10.89±2.05	9.43±1.15	9.29±1.70
18:0	22.42±2.40	26.35±1.22	24.50±1.51	24.39±2.09	25.01±1.18
Σ saturated	37.00±6.13	39.52±1.59	42.44±3.96	41.05±4.24	40.22±3.94
18:1 n-9	10.29±1.47b	6.06±0.65a	5.69±0.41a	7.00±2.63a	6.13±1.00a
Σ monoenes	16.13±3.18	11.14±1.29	11.42±1.19	12.27±4.26	11.48±2.20
18:2 n-6	2.20±0.38b	1.12±0.15a	0.85±0.14a	0.93±0.22a	0.87±0.15a
20:4 n-6	14.48±1.86a	17.90±1.66ab	17.39±3.05ab	21.50±0.51b	21.13±0.62b
Σ n-6 PUFA	19.16±2.22a	20.51±1.77ab	19.75±3.07a	23.69±0.63b	23.50±0.54b
18:3 n-3	3.49±0.90b	0.22±0.05a	0.21±0.03a	0.21±0.14a	0.32±0.31a
20:5 n-3	2.37±0.49a	3.86±0.82b	2.87±0.21ab	2.00±0.22a	1.92±0.26a
22:6 n-3	20.71±4.14	22.90±3.23	22.04±3.45	19.57±1.96	21.13±2.70
Σ n-3 PUFA	27.52±4.88	28.73±2.64	26.37±3.61	22.94±1.68	24.60±3.02
Σ PUFA	46.86±7.10	49.34±2.59	46.14±4.98	46.68±1.40	48.30±3.50
∑ n-3/n-6	1.43±0.12c	1.41±0.20c	1.35±0.27bc	0.97±0.09a	1.05±0.11ab
∑ n-3 LC-PUFA	23.08±4.56	26.76±2.62	24.91±3.58	21.57±1.81	23.06±2.90
GPsARA/ARA diet	24.57±3.16e	17.39±1.61d	8.59±1.50c	5.33±0.13b	3.33±0.10a

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963 **Table 6.** Morphometric analysis and number of goblet cells in distal intestine of European sea bass
964 fed graded levels of ARA in diet. All measures considering individual fish weight (g) as co-variable. All
965 results are expressed as mean±SD. FA =fold area (µm^2/g), FP=fold perimeter (µm/g), FL= fold length
966 (µm/g), FW= fold width (µm/g), SW= submucosa width ([µm/g] * 100). GC= goblet cells/area (arbitrary
967 units * 10^4)

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	DIETS				
	ARA0.5	ARA1	ARA2	ARA4	ARA6
FA	252.99±21.12	198.76±22.27	215.94±36.01	222.36±26.11	225.45±32.28
FP	12.26±2.90	14.17±3.36	13.67±2.08	13.29±1.36	12.84±1.40
FL	3.90±0.85	3.74±0.51	4.04±0.51	4.09±0.20	4.08±0.46
FW	1.38±0.43	1.25±0.16	1.29±0.11	1.38±0.12	1.35±0.07
SW	68.24±7.56	50.38±4.55	60.04±13.58	50.54±6.18	50.85±6.04
GC	34.84±9.22	29.69±1.16	33.24±2.09	32.21±1.54	29.53±0.86

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