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Gilthead sea bream (*Sparus aurata*) metabolic and immunological responses to different dietary Arachidonic acid, Eicosapentaenoic acid and, Docosahexaenoic acid levels

Rui Pedro Moreira de Magalhães

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Rui Pedro Moreira de Magalhães
Tese de Doutoramento apresentada à
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Orientador

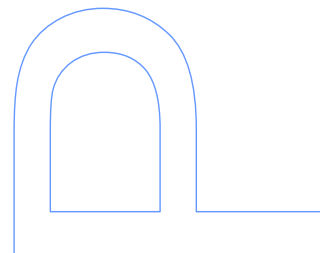
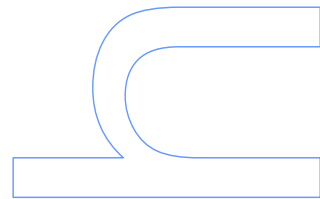
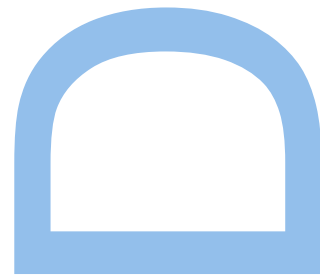
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Nota prévia

Dissertação apresentada à Faculdade de Ciências da Universidade do Porto para a obtenção do grau de Doutor em Biologia, no âmbito do programa doutoral em Biologia.

Esta tese foi escrita ao abrigo do número do Artigo 4º do Regulamento Geral dos Terceiros Ciclos de Estudos da Universidade do Porto e do Artigo 31º do D.L. 74/2006, de 24 de Março, com a nova redação introduzida pelo D.L. 230/2009, de 14 de Setembro. Na sua composição esta tese apresenta um conjunto coerente de trabalhos de investigação, já publicados, submetidos ou em vias de serem submetidos para publicação em revistas com comissões de seleção de mérito internacional. Uma vez que os artigos foram feitos em colaboração com outros autores, o candidato clarifica que, em todos eles participou ativamente no desenho, trabalho experimental, obtenção, análise e discussão de dados, assim como na preparação e publicação dos artigos.

Esta tese teve a colaboração do grupo de Nutrição de Peixes e Immunobiologia (NUTRIMU) do Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), da Faculdade de Ciências da Universidade do Porto, do Departamento de Biologia da Universidade Norueguesa de Ciências e tecnologia (NTNU, Noruega) e do Departamento de Biologia Celular e Histologia da Faculdade de Biologia da Universidade de Múrcia (Espanha). Esta tese foi financiada pela Fundação para a Ciência e Tecnologia (FCT) através da atribuição de uma bolsa de doutoramento (SFRH/BD/115870/2016) e do projeto Eicobream (PTDC/MAR-BIO/1949/2014) e Fundo Social Europeu (FSE) e parcialmente apoiada pelo financiamento estratégico UIDB/04423/2020 e UIDP/04423/2020 através de fundos nacionais fornecidos pela FCT.

Em todas as publicações que resultaram deste trabalho é devidamente referido que as instituições de origem do doutorando Rui Pedro Moreira de Magalhães são:

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- Magalhães, R., Martins, N., Fontinha, F., Moutinho, S., Olsen, R.E., Peres, H., Oliva-Teles, A., 2021b. Effects of dietary arachidonic acid and docosahexaenoic acid at different carbohydrates levels on gilthead sea bream growth performance and intermediary metabolism. *Aquaculture*. 545, 737233.
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Aos meus pais e avó

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Abstract

Over the past years, the reduced availability and increased prices of fish meal and fish oil (FO) has boosted the replacement of these feedstuffs in aquafeeds by more eco-friendly ingredients such as protein-rich plant ingredients and vegetable oils (VO). These alternative ingredients are usually rich in carbohydrates and C18 polyunsaturated fatty acids (C-18 PUFA) and devoid of long-chain polyunsaturated fatty acids (LC-PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Thus, their inclusion into aquafeeds for marine carnivorous species is limited because these species are adapted to diets deprived of carbohydrates and have limited capacities to produce LC-PUFA from C18-PUFA precursors. Therefore, FO inclusion in aquafeeds is still indispensable to meet the essential fatty acids (EFA) requirements of marine carnivorous specie.

Growing evidence shows that dietary LC-PUFA impacts fish muscle fatty acid profile, lipid and carbohydrate metabolism, oxidative stress, and immune and inflammatory responses. In this context, the present work aimed to evaluate the effect of different levels of the dietary n-6 and n-3 LC-PUFA, namely arachidonic acid (ARA), EPA, and DHA, and their potential interaction with dietary carbohydrates in plant-based diets for gilthead sea bream (*Sparus aurata*). A holistic approach was utilized, including the evaluation on fish performance, body composition, oxidative stress, lipid and carbohydrates intermediary metabolism, tissue histomorphology, intestinal microbiota diversity, response to a bacterial challenge, immunological status, and inflammatory response.

In chapter 2, four isoproteic (47% crude protein) and isolipidic (18% crude lipids) diets were formulated using a vegetable oil blend (20:50:30; rapeseed: linseed: palm oil) as basal lipid source and including ARA/EPA/DHA (% of diet) ratios of 2.0/0.2/0.1 (Diet A); 1.0/0.4/0.4 (Diet B); 0/0.6/0.6 (Diet C) and 0/0.3/1.5 (Diet D). All diets were fed for 56 days to triplicate groups of gilthead sea bream juveniles (15 g initial mean body weight (IBW)).

Growth performance and whole-body composition were not affected by the different dietary EFA ratios, but feed and protein utilization were higher with diet B than diets C or D. Muscle EFA profiles followed that of the diets, except for ARA which was reduced in fish fed diets A and B. Plasma triglycerides were higher with diets B and C than A. Plasma cholesterol was reduced in fish fed the DHA-rich diet. Hepatic elongation of very

long-chain fatty acids 5 protein (ELOVL₅) and phospholipase A₂ were upregulated in fish fed diet C compared to diet A. In the intestine, ELOVL₅ levels were down-regulated in fish fed with diets A and D compared to fish fed diet B. Overall, results showed that besides EPA and DHA, adequate dietary ARA level needs to be provided to optimize feed utilization efficiency of gilthead sea bream juveniles.

In chapter 3, the effect of the diets mentioned in chapter 2 in liver and intestine oxidative status, intestinal histomorphology, and gut microbiota of gilthead sea bream juveniles (IBW: 15 g) was assessed at the end of the growth trial.

Lipid peroxidation (LPO) was higher in fish fed diets C and D (higher n-3 LC-PUFA content) while no differences were observed between diets regarding total, oxidized (GSSG) and reduced glutathione (GSH), and oxidative stress index (OSI). Glutathione reductase (GR) activity was higher in fish fed diet A than diets C and D. No histological alterations were observed between groups in the distal intestine. Microbiota diversity was lower in the intestinal mucosa of fish fed diets C than A, while diets C and D seemed to inhibit the presence of pathogenic species like *Edwardsiella tarda*. Overall, results suggest that a balance between dietary ARA/EPA+DHA promotes gilthead sea bream juveniles' health. However, the high dietary content of n-3 LC-PUFA might contribute to limiting the presence of microbial pathogens in the intestinal mucosa.

In chapter 4, the effect in haematological profile, humoral innate immune parameters, and expression of immune-related genes in the intestine of fish fed the diets tested in chapter 2 was assessed at the end of the growth trial. Results showed that fish fed diet B (with a balanced ARA/EPA+DHA ratio) had increased monocyte numbers, alternative complement activity, and bactericidal activity against *Photobacterium damsela* when compared to fish fed the other diets. In conclusion, a diet with a balanced n-6 (ARA) and n-3 (EPA+DHA) LC-PUFA ratio seems to beneficially modulate the fish's innate immune response.

In chapter 5, fish fed the diets mentioned in chapter 2, were intraperitoneally injected (i.p.) with formalin-killed *Photobacterium damsela* subsp. *Piscicida* (*Phdp*) after 56 days of growth trial and the immunomodulatory response was assessed. The haematological profile, plasma humoral innate immune parameters, and the expression of immune-related genes in the intestine were quantified at 4 and 24 hours after the challenge. Results showed that fish fed diet D (high DHA content) had increased complement activity and higher intestinal expression of *cox₂* and *il-1 β* at 4h and 24h after i.p., as well as increased intestinal *tnf α* expression at 4 h. On the other hand, fish fed diet A (high

ARA and low EPA and DHA contents) had higher plasma bactericidal activity against *Phdp* than fish fed diet D (high DHA). These results suggest a possible positive modulatory effect of DHA in response to a bacterial challenge. Further studies are needed on the modulatory effects of different dietary ARA, EPA, and DHA levels used in the presented study in the immune response of gilthead sea bream juveniles after a bacterial challenge.

In chapter 6, the potential interaction between dietary LC-PUFA and carbohydrates was assessed. For that purpose, four plant-based isoproteic (47% crude protein) and isolipidic (18% crude lipids) diets were formulated to include low or high gelatinized starch (5 and 20 %; diets LS and HS, respectively) and different dietary ARA/DHA ratios (2.3/0.3 and 0/2.6; diets ARA and DHA, respectively). The diets were fed to gilthead sea bream juveniles (IBW: 47.5 g) for 84 days. Varying dietary ARA or DHA content did not affect growth performance, feed utilization, or whole-body composition. Higher levels of dietary starch increased feed efficiency, protein efficiency ratio, whole-body dry matter, total lipids, and gross energy content, and decreased protein content. Independently of the dietary starch level, DHA-rich diets increased plasma total protein, total lipids, triglycerides, and very-low-density lipoprotein cholesterol (VLDL) and decreased high-density lipoprotein cholesterol (HDL) and low-density lipoprotein cholesterol (LDL). On the other hand, in fish fed the LS diets, total cholesterol levels were lower with the DHA than the ARA diet. The HS diets increased plasma glucose, total protein, total lipids, triglycerides, and VLDL values, and decreased HDL. The HS diets increased carbohydrate intermediary metabolism and lipogenic enzymes activity. Dietary ARA and HS increased hepatic fatty acid desaturase 2 (FADS₂) expression and decreased ELOVL₅ expression, while HS decreased ELOVL₅ expression in the intestine. ARA, EPA, and DHA muscle content increased in fish fed the LS diets but, generally, muscle fatty acid contents followed the dietary fatty acid profile. In conclusion, there were few interactions between LC-PUFA and dietary digestible carbohydrates. The results indicate that in the presence of high dietary ARA levels, the n-3 LC-PUFA requirements of gilthead sea bream juveniles were fulfilled with 0.5% EPA + DHA, and that fish tolerate well diets with 20% starch.

In chapter 7, the effects on liver and intestine oxidative status and histomorphology, and intestinal microbiota were assessed in fish fed the diets tested in chapter 6. ARA-rich diets reduced liver and intestine LPO and increased liver glutathione redox status (total,

GSH, and GSSG) but only in fish fed the HS diets. Independently of dietary starch level, dietary ARA also reduced GSSG content and OSI in the intestine. Fish fed the DHA diets had increased hepatic superoxide dismutase (SOD) and GR activities. However, in the intestine dietary ARA or DHA did not affect the antioxidant enzymatic response. Overall, the lower LPO values found in fish-fed ARA diets seem to be associated with a lower dietary unsaturation index rather than stimulation of the antioxidant defense mechanisms. Dietary starch improved liver OSI, increased intestinal GSSG, and decreased intestinal LPO values in fish fed the DHA diets. Fish fed the HS diets presented increased hepatic glucose-6-phosphate dehydrogenase (G6PDH), GR, and glutathione peroxidase (GPX, only in DHA diets) activities but decreased GR and SOD activities in the intestine. Diet composition did not induce major histomorphological alterations in the distal intestine. Nevertheless, the HS diets, especially when combined with DHA, promoted hepatocyte hypertrophy and alterations in nuclei position. Compared to fish fed the DHA diets, fish fed ARA diets had a reduced number of operational taxonomic units, richness, and diversity, and higher similarity between bacterial communities in the intestinal mucosa.

In conclusion, fish fed high dietary ARA had reduced hepatic and intestinal LPO and fewer alterations in hepatocyte nuclei position (particularly in high starch diets) than fish fed high DHA diets. Further, dietary CHO and DHA seem to have an interactive effect against oxidative stress by reducing the intestine LPO and lowering liver GSH content

In chapter 8, the immunomodulatory effects of the diets mentioned in chapter 6 were tested at the end of the growth trial and following a bacterial challenge by intraperitoneal injection with formalin-killed *Photobacterium damsela* subsp. *Piscicida* (*Phdp*).

High dietary DHA level seems to beneficially modulate the immune status of unchallenged gilthead sea bream juveniles by increasing red blood cell (RBC) numbers, hemoglobin, plasmatic nitric oxide (NO) content, antiproteases, and alternative complement activity. ARA diets improve the immune system response to a bacterial challenge by increasing RBC, WBC, and thrombocytes numbers, peroxidase activity, total immunoglobulins, and plasmatic bactericidal activity against *Vibrio anguillarum*. Further research is essential to determine the ideal dietary ARA/DHA ratio for the optimized immune response of gilthead sea bream juveniles and clarify the potential effects of dietary carbohydrates.

In conclusion, this study indicates that gilthead sea bream juveniles can perform well when fed diets with low n-3 LC-PUFA content (EPA+DHA = 0.3-0.5 %) provided in diets that have adequate ARA and carbohydrate levels. However, a balanced dietary ARA/EPA+DHA ratio seems important to optimize feed efficiency. The present work also showed that dietary n-6 and n-3 LC-PUFA and carbohydrates levels contribute to regulating the expression of the genes related to LC-PUFA biosynthesis, immune response, and gut microbiota diversity.

Keywords: Gilthead sea bream, Fish nutrition, Lipids, Long-chain polyunsaturated fatty acids, Growth performance, Intermediary metabolism, Oxidative stress, Immunological status.

Resumo

Ao longo dos anos, a redução da disponibilidade e o aumento de preços da farinha e óleo de peixe (OP) aumentaram a substituição destes ingredientes em rações de aquacultura por ingredientes mais sustentáveis do ponto de vista ambiental como por exemplo fontes proteicas e óleos de origem vegetal. Estes ingredientes alternativos são geralmente ricos em hidratos de carbono e ácidos gordos polinsaturados com 18 átomos de carbono (C-18 AGP) e são desprovidos de ácidos gordos polinsaturados de cadeia longa (AGPICL). Assim, a sua inclusão em rações de aquacultura para espécies marinhas carnívoras pode ser limitada uma vez que estas espécies estão adaptadas a dietas desprovidas de hidratos de carbono e possuem limitações na expressão de dessaturases e elongases, sendo incapazes de produzir AGPICL a partir dos seus precursores C-18 AGP. Por consequência, a inclusão de OP em rações de aquacultura é ainda indispensável para suprimir as necessidades de ácidos gordos essenciais (AGE) das espécies marinhas carnívoras uma vez que a sua carência pode reduzir a performance de crescimento.

Existem cada vez mais evidências que denotam o impacto dos AGPICL presentes nas rações de peixes no perfil de ácidos gordos do músculo, metabolismo lipídico e dos hidratos de carbono, stress oxidativo e na resposta imune e inflamatória. Neste contexto, o presente trabalho pretendeu avaliar o efeito de diferentes conteúdos na dieta de n-6 e n-3 AGPICL, nomeadamente o ácido araquidónico (ARA), o ácido eicosapentaenóico (EPA), e o ácido docosahexaenóico (DHA) e a sua potencial interação com os hidratos de carbono em dietas elaboradas com ingredientes de origem vegetal para uma das principais espécies produzidas em aquacultura no sul da Europa, a dourada (*Sparus aurata*). Com o intuito de avaliar os efeitos de diferentes conteúdos de n-6 e n-3 AGPICL nas dietas e a sua potencial interação com os hidratos de carbono, foi utilizada uma abordagem holística, na avaliação do desempenho do crescimento dos peixes e na sua composição corporal, no stress oxidativo, no metabolismo intermediário dos lípidos e dos hidratos de carbono, na histologia dos tecidos, na diversidade da microbiota intestinal, na resposta a um desafio bacteriano, no seu estado imunológico e na resposta inflamatória.

No capítulo 2, 4 dietas com um conteúdo igual de proteína (47%) e lípidos (18%) foram formuladas usando uma mistura de óleos vegetais (20:50:30; óleos de colza:linhaça:palma, respetivamente) como fonte lipídica e incluindo os seguintes rácios de ARA/EPA/DHA, 2.0/0.2/0.1 (Dieta A); 1.0/0.4/0.4 (Dieta B); 0/0.6/0.6 (Dieta C); e

0/0.3/1.5 (Dieta D). As dietas foram fornecidas durante 56 dias a grupos triplicados de douradas juvenis (15 g de peso medio inicial (PMI)).

O crescimento e a composição corporal não foram afetados pelos diferentes rácios de AGE, mas a eficiência alimentar e da utilização da proteína foram superiores nas dietas B do que nas dietas C ou D. O perfil de AGE no musculo foi similar ao das dietas com a exceção do conteúdo em ARA que decresceu nas dietas A e B. Os triglicerídeos plasmáticos foram superiores nas dietas B e C do que na A. O colesterol no plasma foi reduzido nos peixes alimentados com a dieta mais rica em DHA. A expressão da elongase 5 e da fosfolipase A₂ foram aumentadas no fígado dos peixes alimentados com a dieta C em relação aos peixes alimentados com a dieta A. No intestino, a expressão da elongase 5 foi reduzida em peixes alimentados com as dietas A e D quando comparados com peixes alimentados com a dieta B. Em suma, os resultados mostraram que além do EPA e do DHA, um adequado conteúdo de ARA na dieta é necessário para otimizar a eficiência da utilização da ração nos juvenis de dourada.

No capítulo 3, os efeitos das dietas mencionadas no capítulo 2 foram avaliados no estado oxidativo do fígado e intestino, na histologia do intestino e na microbiota intestinal dos juvenis de dourada (15 g de PMI) no final do ensaio de crescimento.

A peroxidação lipídica (PL) foi superior nos peixes alimentados com as dietas C e D (maior conteúdo de n-3 AGPICL) mas não foram verificadas diferenças entre dietas no que diz respeito ao conteúdo de glutathione total, oxidada (GSSG) e reduzida (GSH) e índice do stress oxidativo (ISO). A atividade da glutathione redutase (GR) foi superior na dieta A em relação às dietas C e D. Não foram verificadas alterações histológicas do intestino distal entre os grupos. A diversidade da microbiota foi menor na mucosa intestinal dos peixes alimentados com a dieta C do que com a A, enquanto as dietas C e D parecem inibir a presença de bactérias patogénicas como a *Edwardsiella tarda*. No geral, os resultados sugerem que um balanço entre os conteúdos de ARA/EPA+DHA da dieta promovem a saúde dos juvenis de dourada. Contudo, um conteúdo superior de n-3 AGPICL pode contribuir para limitar a presença de agentes microbianos patogénicos na mucosa intestinal.

No capítulo 4, o efeito das dietas mencionadas no capítulo 2 foi avaliado no perfil hematológico, nos parâmetros humorais da imunidade inata e na expressão de genes relacionados com a imunidade no intestino após o ensaio de crescimento. Os resultados mostraram que os peixes alimentados com a dieta B (com um rácio equilibrado de ARA/EPA+DHA) aumentaram o número de monócitos, a atividade do sistema

complemento e a atividade bactericida contra *Photobacterium damsela* comparado com os valores encontrados nos peixes alimentados com outras dietas. Em conclusão, a dieta com um rácio equilibrado de n-6 (ARA) e n-3 (EPA+DHA) AGPICL parece modular benéficamente a resposta imune inata dos peixes.

No capítulo 5, os peixes alimentados com as dietas mencionadas no capítulo 2, foram injetados intraperitonealmente (i.p.) com *Photobacterium damsela* subsp. *Piscicida* (*Phdp*) morta com formalina após os 56 dias do ensaio de crescimento e a sua resposta imune foi avaliada. O perfil hematológico, os parâmetros da imunidade inata humoral no plasma, e a expressão no intestino de genes relacionados com a resposta imune foram quantificados 4 e 24 horas depois do desafio bacteriano. Os resultados mostraram que os peixes alimentados com a dieta D (alto teor de DHA) tiveram um aumento da atividade do complemento, e também da expressão no intestino dos genes *cox2* e *il-1 β* às 4 e 24 horas após i.p., assim como um aumento na expressão de *tnf α* no intestino às 4 horas. Por outro lado, os peixes alimentados com a dieta A (alto teor de ARA e baixo teor em EPA e DHA) denotaram maior atividade bactericida contra *Phdp* no plasma do que os peixes alimentados com a dieta D (alto teor em DHA). Estes resultados sugerem uma possível modulação positiva do DHA na resposta a um desafio bacteriano. No entanto, são necessários mais estudos, para aferir a importância dos AGPICL na resposta imune dos juvenis de dourada, particularmente após um desafio bacteriano.

No capítulo 6, a potencial interação entre os AGPICL e os hidratos de carbono da dieta foi avaliada. Com esse objetivo, 4 dietas com igual teor de proteína (47%) e de lípidos (18%) foram formuladas para incluir baixos ou altos níveis de amido gelatinizado (5 e 20%; dietas LS e HS, respetivamente) e diferentes rácios de ARA/DHA (2.3/0.3 e 0/2.6; dietas ARA e DHA, respetivamente). Juvenis de dourada (PMI: 47.5 g) foram alimentados com estas dietas durante 84 dias e os resultados mostraram que o conteúdo em ARA ou DHA não afetou o crescimento, utilização alimentar, composição corporal, mas os altos teores de amido aumentaram a eficiência alimentar e o rácio de eficiência proteica, o conteúdo corporal em matéria seca, lípidos e energia bruta e diminuiu o conteúdo proteico. Independentemente do nível de amido, as dietas ricas em DHA aumentaram, no plasma, o conteúdo em proteínas totais, lípidos, triglicerídeos e as lipoproteínas de muito baixa densidade (VLDL) e diminuiu o conteúdo plasmático de lipoproteínas de elevada densidade (HDL) e de baixa densidade (LDL). Por outro lado, nos peixes alimentados com dietas com baixo teor de amido, o teor de colesterol plasmático foi inferior nos peixes alimentados com as dietas ricas em DHA do que com

as dietas ricas em ARA. As dietas ricas em amido aumentaram, no plasma, a glucose, as proteínas e lípidos totais, triglicerídeos e VLDL e diminuíram as HDL. As dietas ricas em amido aumentaram o metabolismo intermediário dos hidratos de carbono e a atividade das enzimas lipogénicas. As dietas ricas em ARA, mas também as dietas ricas em amido aumentaram a expressão da dessaturase de ácidos gordos 2 (FADS₂) e diminuíram a expressão da elongase 5 (ELOVL₅) no fígado, enquanto apenas as dietas com elevado nível de amido diminuíram a expressão da ELOVL₅ no intestino. O conteúdo de ARA, EPA e DHA no músculo foi aumentado nos peixes alimentados com dietas com baixo teor de amido, mas na generalidade, o perfil de ácidos gordos do musculo refletiu o perfil de ácidos gordos das dietas. Em conclusão, poucas interações foram encontradas entre os AGPICL e os hidratos de carbono da dieta. Os resultados indicam que na presença de altos níveis de ARA na dieta, as necessidades de n-3 AGPICL podem ser satisfeitas com 0.5 % de EPA+DHA e que os juvenis de dourada toleram bem 20 % de amido gelatinizado nas dietas.

No capítulo 7, foram avaliados os efeitos das dietas testadas no capítulo 6 no estado oxidativo do fígado e intestino, na sua histologia e na microbiota intestinal dos peixes mencionados nesse capítulo. As dietas ricas em ARA reduziram a PL no fígado e intestino e aumentaram, apenas nas dietas com alto teor de amido, o conteúdo de glutathione total, GSH e GSSG no fígado. As atividades das enzimas superóxido dismutase (SOD) e GR foram aumentadas em peixes alimentados com as dietas ricas em DHA. Contudo, no intestino, o conteúdo da dieta em ARA ou DHA não induziu alterações na resposta das enzimas antioxidantes. Em geral, a reduzida PL encontrada nos peixes alimentados com dietas ricas em ARA parece estar associada a um índice de insaturação inferior destas dietas em vez de uma estimulação dos mecanismos de defesa antioxidante. O nível mais elevado de amido melhorou o ISO no fígado, aumentou a concentração de GSSG no intestino e para além disso, diminuiu os valores de PL nos peixes alimentados com as dietas ricas em DHA. Os peixes alimentados com as dietas HS tiveram um aumento da atividade da glucose-6-fosfato desidrogenase (G6PDH), GR e glutathione peroxidase (GPX, apenas nas dietas DHA) no fígado e um decréscimo da atividade da GR e SOD no intestino. A composição da dieta não induziu alterações histológicas no intestino distal. No entanto, as dietas HS, especialmente quando combinadas com DHA, promoveram uma hipertrofia dos hepatócitos e alterações na posição do seu núcleo. Comparando com os peixes alimentados com as dietas DHA, os peixes alimentados com as dietas ARA tiveram um menor número de

unidades taxonómicas operacionais, riqueza e diversidade e uma maior similaridade entre comunidades bacteriológicas na mucosa intestinal.

Em conclusão, os peixes alimentados com alto teor em ARA apresentaram menor PL no fígado e intestino e menores alterações na morfologia dos hepatócitos do que os peixes alimentados com alto teor de DHA. Além disso, o teor de amido e de DHA da dieta parecem ter um efeito interactivo contra o stress oxidativo uma vez que reduziram a suscetibilidade à PL no intestino e o conteúdo de GSH no fígado.

No capítulo 8, os efeitos imunomodelatórios das dietas mencionadas no capítulo 6 foram testadas no final do ensaio de crescimento assim como após um desafio bacteriano efetuado através da injeção intraperitoneal (i.p.) da bactéria *Photobacterium damsela* subsp. *Piscicida* (*Phdp*) morta com formalina.

Dietas com elevado teor em DHA parecem modelar beneficamente o estado imune do juvenis de dourada uma vez que aumentaram o número de hemácias no sangue, hemoglobina, o conteúdo de oxido nítrico (NO) no plasma e a atividade das antiproteases e do complemento. No entanto, as dietas ricas em ARA melhoraram a resposta do sistema imune após o desafio bacteriano através do aumento do número de hemácias, leucócitos e trombócitos, da atividade da peroxidase, das imunoglobulinas totais, e da atividade bactericida contra o *Vibrio anguillarum* no plasma. No entanto, são necessários mais estudos para determinar o rácio ideal de ARA/DHA na dieta e clarificar o potencial efeito dos hidratos de carbono para otimizar a resposta imune nos juvenis de dourada.

Em conclusão, este estudo indica que os juvenis de dourada crescem de um modo satisfatório quando alimentados com dietas com baixo teor de AGPICL n-3 fornecidos em dietas (EPA+DHA = 0.3-0.5 %) com um adequado conteúdo de ARA e de hidratos de carbono. No entanto, um rácio equilibrado de ARA/EPA+DHA na dieta parece ser importante para otimizar a eficiência alimentar. O presente trabalho também mostrou que os níveis de n-6 e n-3 AGPICL e de hidratos de carbono contribuíram para regular a expressão de genes relacionados com a biossíntese de AGPICL, a resposta imune e ainda a diversidade da mucosa intestinal.

Palavras-chave: Dourada, Nutrição de peixes, Lípidos, Ácidos gordos polinsaturados de cadeia longa, Crescimento, Metabolismo intermediário, Stress oxidativo, estado imunológico.

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List of Abbreviations

ALA - α -Linolenic acid

ARA - Arachidonic acid

Ca²⁺ - Calcium ions

Casp3 - Caspase 3

CAT – Catalase

CHO - Carbohydrate

COX – cyclooxygenase

CYP - Cytochrome P450 monooxygenase

DE - Digestible energy

DHA - Docosahexaenoic acid

DNA - Deoxyribonucleic acid

DP - Digestible protein

EAA - Essential amino acid

EFA - Essential fatty acids

ELOV - elongation of very long-chain fatty acid

ELOVL₂ - Elongation of very long-chain fatty acids protein 2

ELOVL₅ - Elongation of very long-chain fatty acids protein 5

EPA - Eicosapentaenoic acid

EU - European Union

FADS - Fatty acid desaturases

FADS₂ - Fatty Acid Desaturase 2

FAO - Food and Agriculture Organization of the United Nations

FAs - Fatty acids

Fe²⁺ - Ferrous ion

FM - Fish meal

FO - Fish oil

g – Gram

G6PDH - Glucose-6-phosphate dehydrogenase

GPX - Glutathione peroxidase

GR - Glutathione reductase

GSH - Reduced glutathione

GSSG - Oxidized glutathione

GST - Glutathione-S-transferase

H₂O₂ - H₂O₂

IBW - Initial body weight

IG – Immunoglobulins

LC-PUFAs - Long-chain polyunsaturated fatty acids

LA - Linoleic acid

LOX – Lipoxygenases

LTs – Leukotrienes

MUFAs - Monounsaturated fatty acids

NADPH - Nicotinamide adenine dinucleotide phosphate

NF_κB - Nuclear factor-kappa B

NO - Nitric oxide

NO₂ - Nitrogen dioxide

NSPs - Non-starch polysaccharides

O₂^{•-} - Superoxide anion radicals

OA - Oleic acid

OH[•] - Hydroxyl radical

ONOO[•] - Peroxynitrite

PGs – Prostaglandins

Phdp - *Photobacterium damsela* subsp. *Piscicida*

PPAR - Proliferator-activated receptor

ppar-γ - Proliferator-activated receptor γ

PUFA - Polyunsaturated fatty acids

R[•] - Lipid radical

RBC - Red blood cell

RNS - Reactive nitrogen species

ROO[•] - Lipid peroxy radical

ROOH - Lipid hydroperoxide

ROS - Reactive oxygen species

SDGs - Sustainable developing goals

SFAs - Saturated fatty acids

SOD - Superoxide dismutase

SPMs - Specialized pro-resolving mediators

SREBP - Sterol regulatory element-binding proteins

TX - Thromboxanes

VO - Vegetable oils

°C - Degrees Celsius

Δ⁹ FADS - Δ⁹ desaturase enzyme

¹O₂ - Singlet oxygen

5-LOX - 5-lipoxygenase

Chapter 1

General introduction

1.1. Aquaculture production in an overpopulated world

The world human population has surpassed 7.7 billion and is expected to reach 9.7 billion in 2050 (United Nations, 2019). The exponential populational growth will defy our capacity to produce an adequate supply of nutritionally rich foods meaning an inevitable increment of agriculture, fisheries, and aquaculture production. Furthermore, the human population is increasing their fish consumption. In fact, fish is increasingly considered a superfood that should be included in diversified and healthy diets due to its high-quality proteins, essential fatty acids (e.g n-3 long-chain polyunsaturated fatty acids), vitamins (A, B, and D), and minerals such as iron, calcium, zinc, and selenium.

The annual growth rate of total fish consumption between 1961 and 2017 was 3.1 %, outpacing the annual population growth rate (1.6%)(FAO, 2020). During this period, the annual growth rate of fish consumption also surpassed that of all other animal proteins produced except poultry (FAO, 2020). In 2017, fish accounted for about 17 % of total animal protein consumed worldwide (FAO, 2020). Thus, it is not surprising that the share of fish captured and produced destined for human consumption continues to grow, reaching 89 percent by 2030. The trend of increased fish consumption has continued steadily since 1961 (9 kg per capita). By 2018 the consumption reached 20.5 kg per capita. The Food and Agriculture Organization of the United Nations (FAO) predicts that fish consumption will reach 21.5 kg per capita in 2030. In the European Union (EU), the per capita fish consumption was 24.36 kg in 2018 (EUMOFA, 2020). However, there is high variability in fish consumption within the EU, ranging from 6 kg in the Czech Republic and Hungary to 86 and 61 kg in Malta and Portugal, respectively (EUMOFA, 2020).

Aquaculture is one of the fastest, if not the fastest, growing food production sector (FAO, 2020). The global aquaculture production more than tripled from 34 million tonnes of live weight in 1997 to 114.5 million tonnes in 2018 (FAO, 2020; Naylor et al., 2021). In 2018, fish aquaculture production accounted for 46 % of the total fish produced worldwide, and of the total world fish production destined for human consumption, 52% was produced by the aquaculture sector (Figure 1; FAO (2020)). The total share of fish produced by the aquaculture sector for human consumption is now higher than the share of the capture fisheries and is estimated amount to 59 % by 2030. The main reason for that is the expected increment of the aquaculture contribution to the total fish production (53 %) in 2030 (Figure 1).

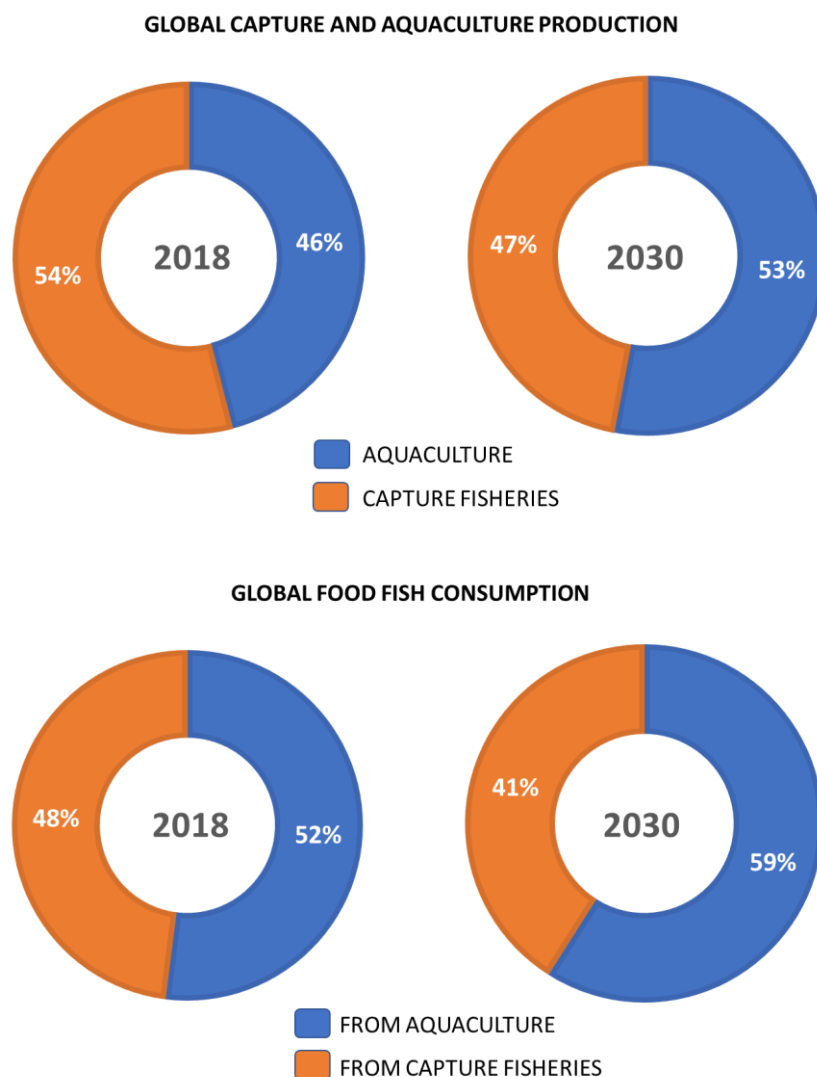


Figure 1: Relative percentage of Aquaculture or capture fisheries in global fish production and consumption. Source: FAO (2020).

Furthermore, most wild fish stocks are exploited at or beyond their sustainable capacity, which has stabilized between 90 to 95 million tonnes over the past 20 years (Figure 2. A). Further, an increase of captured fish for direct human consumption is unlikely. The expected increase of aquatic animals production to 204 million tonnes in 2030 will therefore mainly be through increased aquaculture production (Figure. 2. B). Thus, aquaculture production of aquatic animals is expected to reach 109 million tonnes in 2030, an increase of over 32 % compared to 2018 production (FAO, 2020).

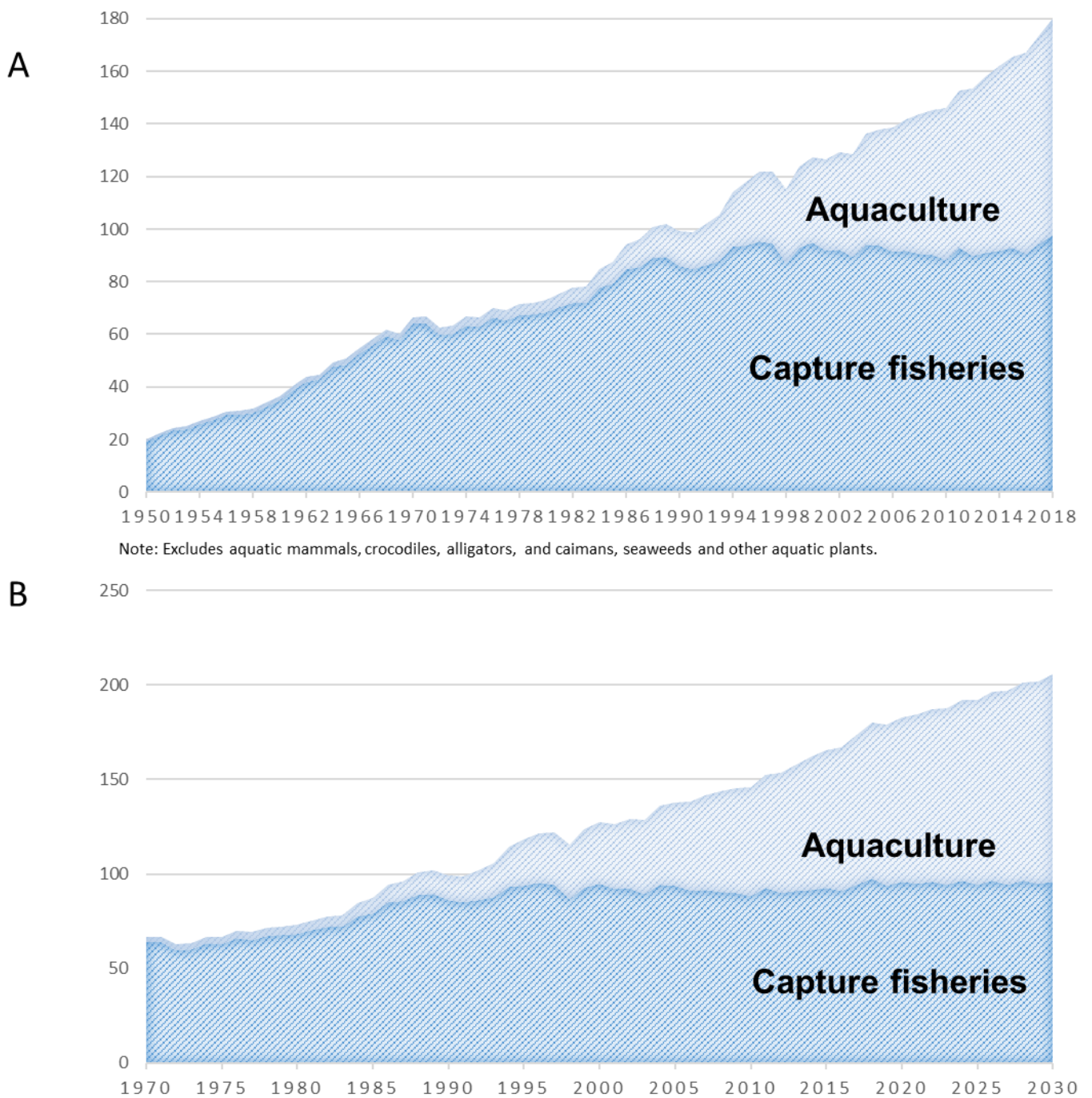


Figure 2: World capture fisheries and aquaculture production in 2018 (A) and a prevision for 2030 (B). Source: FAO (2020).

In September 2015, the United Nations launched the 2030 Agenda for Sustainable development and the sustainable developing goals (SDGs), denoting an incredible commitment of countries to shift the world onto a more sustainable path. Thus, the aquaculture sector has much to contribute to feeding the increasing human population in a world with limited resources and a deep economic crisis securing the core of SDG

14, which consists of conserving and sustainable use of oceans, seas, and marine life resources (FAO, 2020).

1.2. World fish meal and fish oil production

Fed aquaculture species production (57 million tonnes) is exceeding the non-fed (25 million tonnes), accounting for over 69 % of the total production (FAO, 2020). The aquafeed production sector increases up to 30 % every year according to Colombo et al. (2020) and is expected to increase in the following years due to the increasing fed aquaculture production.

Fish oil (FO) and fish meal (FM) are still important ingredients in fish feeds, especially for marine fish species, since the first is an excellent lipid source and crucial to assure essential fatty acids (EFA) coverage, and the second is considered the most adequate protein source for fish, due to its high digestibility, amino acids profile, vitamins, and minerals content, as well as palatability (Oliva-Teles et al., 2015). FM is a protein-rich ingredient obtained after milling and drying fish, and FO is produced through the pressing of cooked fish and subsequent centrifugation of the resulting liquid. Both can be obtained from whole fish, trimmings, or fish by-products. The fisheries, mainly small pelagic species, destined for FM production has been reduced as they are viewed as ecologically unsustainable and cause devastating impacts on wild fish stocks and marine ecosystems. There has also been a drive towards increasing direct human consumption of species previously utilized for FM and FO. In 1994, 30 million tonnes of world fish capture was utilized to produce FM and FO. In 2018 this had been reduced to 18 million tonnes (about 11.5 % of the world's fish production) (FAO, 2020). Furthermore, a growing share of FM and FO is produced from fish trimmings and by-products previously discarded due to increasing demand driven by a fast-growing aquaculture industry.

The total amount of FO and FM produced decreased during the past decade (Naylor et al., 2021). The resultant of higher demand and reduced availability has led to increased prices and higher utilization of the plant ingredients particularly since 2012 (Figure 3). The aquaculture industry consequently responded to this shortage and increased costs by lowering the incorporation level of FM and FO in aquafeeds. FM and FO are still important ingredients of aquafeeds, but the inclusion levels are lower particularly in grow-out and finishing aquafeeds. However, the aquaculture industry still uses 69 and 75 % of the global FM and FO production, respectively. If this trend continues, the prices will

continue to increase, driving the necessity for more innovations in the aquafeeds industry (Naylor et al., 2021).

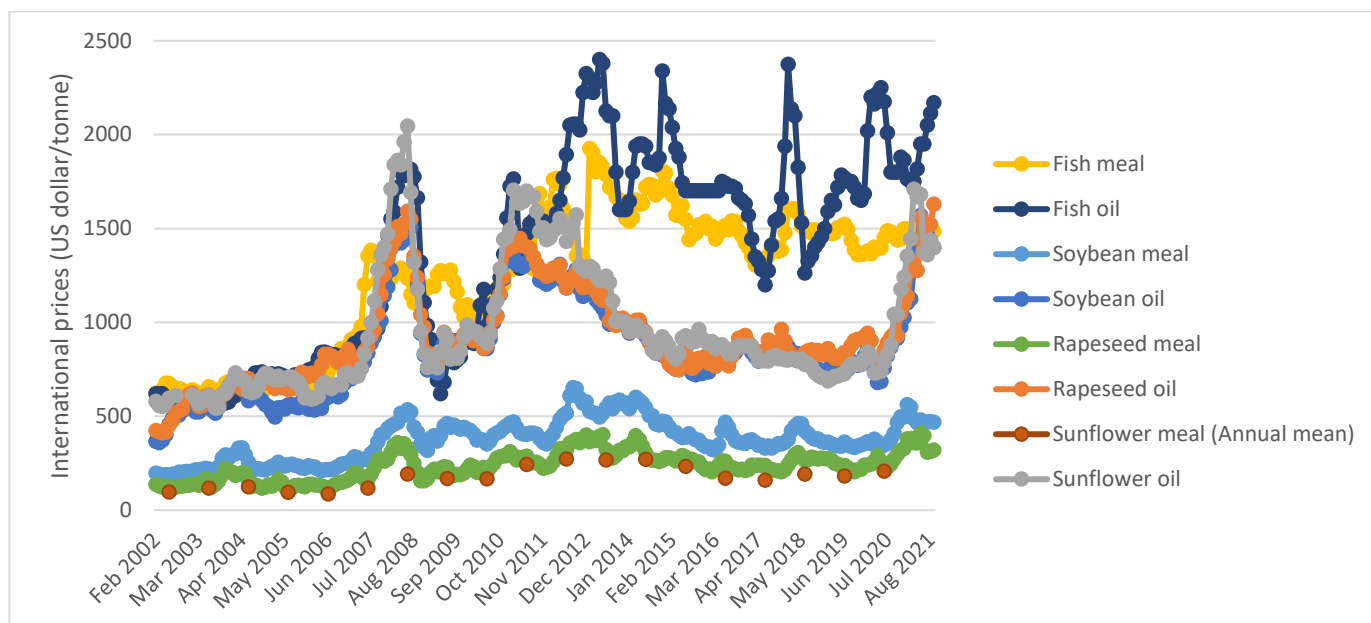


Figure 3: Prices of fishmeal and fish oil versus plant-based meals and oils. Sources: Indexmundi (2021), FPMA Toll (available at <https://fpma.apps.fao.org/giews/food-prices/tool/public/#/dataset/international>), National Sunflower Association (2021).

1.3. Alternative lipid sources in aquafeeds

The major aquaculture production cost is aquafeeds, which represent between 50 to 80 % of total operational cost (NRC, 2011). The increase in aquaculture and aquafeeds production has made it necessary to find alternative protein and lipid sources that are readily available, sustainable, and cost-effective. Therefore, the use of plant ingredients and animal by-products in aquafeeds has increased steadily, creating conditions for more sustainable aquaculture production.

Several factors have contributed to the expanding utilization of such ingredients: improved knowledge of nutritional requirements; improved processing minimizing ingredients antinutritional factors; and selective breeding programs to improve fish growth, feed efficiency, and health (Naylor et al., 2021). Nevertheless, the percentage of inclusion of these alternative ingredients in aquafeeds may be limited by the fish species considered, as carnivorous fish have more limitations in digesting carbohydrates than herbivorous or omnivorous fish and are more susceptible to plant antinutritional factors. Furthermore, marine fish species have an absolute requirement for long-chain polyunsaturated fatty acids (LC-PUFA) (Kamalam et al., 2017; Oliva-Teles et al., 2015).

Carnivorous farmed fish species are usually fed energy-dense diets formulated with high lipid levels (Oliva-Teles et al., 2015). Vegetable oils (VO) and animal fat are now commonly included in aquafeeds to replace FO and may be used together as the only lipid sources for freshwater fish because VO provides all the EFA required by them. VO are the principal choice to replace FO in aquafeeds due to environmental sustainability and product consistency. Also, the market availability of several vegetable oils continues to increase, with palm oil, soybean oil, rapeseed/canola oil, sunflower oil, and linseed oil as the most important sources for aquafeeds (Turchini et al., 2009).

Soybean oil contains high levels of linoleic acid (LA, 18:2n-6; 48-58%) and oleic acid (OA; 18:1 n-9; 19-30%) but is a poor source of α -linolenic acid (ALA, 18:3n-3; 4-10 %) (Brown and Hart, 2010).

Palm oil is one of the most economical vegetable oils being very rich in antioxidants and saturated fatty acids (SFAs) but has a low content of C18 PUFA (Colombo et al., 2020). Consequently, palm oil is less prone to oxidation than more unsaturated oils. Palm oil is a potential candidate for inclusion into aquafeeds, mainly in VO blends, due to its low LA and high 16:0 and 18:1n-9 levels which have been reported to be preferred substrates for mitochondrial energy production in fish (Mancini et al., 2015). Still, as with the other VO, high inclusion levels result in a lower concentration of LC-PUFA content in fish fillets if they are used to replace FO.

Rapeseed oil is very rich in monounsaturated fatty acids (MUFAs), amounting to 55 to 72 % of the total fatty acids (FA) (Turchini and Mailer, 2010). Among MUFAs, OA and 22:1n-9 are the predominant FA. MUFAs are easy to digest, are good energy sources, are liquid to room temperature, and are not prone to lipid peroxidation (Colombo et al., 2020). Thus, MUFAs-rich VO are among the most suitable candidates to replace FO in aquafeeds.

Linseed oil is another major candidate to replace FO in aquafeeds due to its high content of C18 n-3 polyunsaturated fatty acids (PUFAs) levels, particularly ALA (> 50 %; Lewinska et al. (2015)). Along with camelina oil, few other VO are richer in ALA than in LA and with an n-6/n-3 ratio lower than 1. Nevertheless, as with the other VO, the lack of dietary n-3 LC-PUFA (e.g. EPA and DHA), will lead to reduced levels of these fatty acids in fish muscle when linseed oil replaces FO in aquafeeds (Benedito-Palos et al., 2011).

Animal fats are little used for human consumption making them less expensive than FO and VO and a potential source of lipids for aquafeeds. The disadvantage of using animal fats lies in their FA profile. The majority are rich in SFAs but, depending on the source, may also be rich in MUFAs and PUFAs (Colombo et al., 2020). Poultry and swine fats are a moderate sources of LA and may provide the n-6 PUFA requirements for freshwater species (Oliva-Teles et al., 2015). High contents of SFA will increase the melting point and reduce lipid digestibility. Consequently, the content of SFA in fish diets must be thoroughly monitored and controlled. Reduced digestibility will lower energy availability, particularly at lower temperatures (Turchini et al., 2009). Therefore, in aquafeeds, lipids utilization by fish may be compromised when the level of terrestrial animal fat is higher than 30 of the total lipids (Colombo et al., 2020).

Compared to FO, VO and animal fats are devoid of n-3 LC-PUFA (Table 1). The requirements for n-3 LC-PUFA in marine fish species are generally higher than that of freshwater species. Furthermore, the n-3 LC-PUFA requirements are lower as the species trophic level decreases (Turchini et al., 2009). Thus, contrarily to freshwater, for marine fish species, FO inclusions into diets is essential to ensure that EFA requirements are met but even though 60 to 75 % can be substituted by alternative oils (Turchini et al., 2009). The selection of alternative oils to substitute FO in aquafeeds must always guarantee good energy availability, avoid excessive deposition of LA, sufficient n-3 and n-6 PUFA levels to meet the target fish species' EFA requirements and the possibility of the nutritional quality for human consumers be quickly restored by FO finishing diets.

Table 1: The essential fatty acid content (%) of potential fats and oils used in aquafeeds. Adapted from: Colombo et al. (2020).

Source	LA	ALA	ARA	EPA	DHA	SFA*	MUFA*	PUFA*	n-3/n-6
Fish oil (herring)	1.1	0.6	0.3	8.4	4.9	20.0	56.4	23.6	12.7
Fish oil (anchovy)	1.2	0.8	0.1	17.0	8.8	28.8	24.9	43.6	24.0
Fish oil (menhaden)	1.3	0.3	0.2	11.0	9.1	30.5	24.5	45.0	16.7
Vegetable oils									
Palm	9.1	0.2	0.0	0.0	0.0	47.8	36.9	84.7	0.02
Soybean	51.0	6.8	0.0	0.0	0.0	14.2	30.0	55.8	0.13
Rapeseed/Canola	20.2	12.0	0.0	0.0	0.0	4.5	60.0	35.5	0.59
Linseed	12.7	53.5	0.0	0.0	0.0	9.4	20.2	70.4	4.2
Camelina	23.8	29.3	0.0	0.0	0.0	9.6	28.9	61.5	1.18
Animal fats									
Poultry fat	19.5	1.0	0.0	0.0	0.0	28.5	43.1	28.4	0.05
Beef tallow	3.1	0.6	0.0	0.0	0.0	47.5	40.5	12.0	0.19
Pork fat	10.2	1.0	0.0	0.0	0.0	38.6	44.9	16.5	0.1
Novel oils									
Schizochytrium	1.0	1.0	0.0	1–16	18–44	16–38	1–30	32–83	—
<i>P. tricornutum</i>	—	—	—	24.8	10.3	21.5	27.9	50.6	—
<i>P. tricornutum</i>	—	—	—	16.1	10.0	24.8	36.5	38.7	—
Camelina (high EPA)	15.0	5.0	3.0	31.0	0.0	22.1	5.0	72.9	2.1
Camelina (high DHA)	17.0	7.0	1.0	7.0	14.0	21.0	9.0	70.0	2.0
Camelina (high DHA)	8.4	29.4	—	3.3	12.4	12.7	18.8	68.5	6.2
Canola (EPA + DHA)	13.5	9.3	0.3	0.5	3.3	7.1	58.3	65.4	0.9
Soybean (SDA)	19.1	11.0	0.0	0.0	0.0	17.7	16.7	65.6	1.5
<i>Y. lipolytica</i>	22.9	2.3	—	30.2	—	19.4	11.4	69.2	1.25

The EFA requirements of fresh water and diadromous fish species can generally be met by C18 PUFA, at around 1 % of the diet for the juvenile stage of life (Tocher, 2010). In salmonids, EFA requirements are met with PUFAs, particularly by ALA (Tocher, 2010). Despite salmonids do not require LC-PUFAs since they have all the genes/enzymes to biosynthesize them, in the majority of FO substitution studies performed in Atlantic salmon, the diets provided a minimum of 1.6 % of EPA and DHA of the total dietary FAs (Liland et al., 2013; Turchini et al., 2009; Yildiz et al., 2018). Freshwater species such as Nile tilapia (*Oreochromis niloticus*) require mainly LA as EFA but other species such as channel catfish (*Ictalurus punctatus*) and common carp (*Cyprinus carpio* L.) require significant levels of both ALA and LA (Tocher, 2010). Dietary C18 PUFA are usually effective in fulfilling EFA requirements in freshwater and diadromous fish species but LC-PUFA may satisfy these requirements at lower levels and promote growth performance over that obtained with C18 PUFA alone (Sargent et al., 1989; Tocher, 2015). Atlantic

salmon fed diets with different ALA and LA levels and with no EPA and DHA had lower final weight than those fed a reference diet (rich in n-3 LC-PUFA) (Sprague et al., 2019). The inclusion of dietary ARA or n-3 LC-PUFA also significantly improved growth performance in other diadromous or freshwater species such as Japanese eel (*Anguilla japonica*), channel catfish, and gibel carp (*Carassius auratus*) (Chen et al., 2011; Santha and Gatlin, 1991; Shahkar et al., 2016)

Due to the low/absence of enzymatic activity of desaturases and/or elongases, LC-PUFAs are essential dietary nutrients for marine fish species and to date, the only competitive commercial source of these EFA is FO (Turchini et al., 2009). Alternative oil sources rich in these FA, such as krill and microalgae oils, still have unbearable prices for the aquafeeds industry (Oliva-Teles et al., 2015).

For that reason, in marine fish diets, 5 to 10 % of FO inclusion should be considered sufficient to meet the EFA requirements (Oliva-Teles et al., 2015). In gilthead sea bream juveniles, 66 % FO replacement by VOs (soybean, rapeseed, and linseed oil) in diets with 20 % FM was possible without negative effects on growth performance (Benedito-Palos et al., 2008). Similarly, the total substitution of FO by a VOs blend without negative effects on gilthead sea bream growth performance was achieved with high inclusions of FM (65 %; providing 6.6 % of FO), suggesting that EFA requirements are met (Castro et al., 2016b). Nevertheless, lower growth was reported in European sea bass (*Dicentrarchus labrax*) fed with 38% FM diets (providing approximately 3.8 % of FO) and 80 and 60 % of FO substitution by linseed oil and rapeseed oil, respectively, but 60 % FO substitution by soybean or linseed oil did not produce such effect (Montero et al., 2005). Substitution of 69 % of FO by palm oil decreased growth performance and feed utilization of gilthead sea bream compared to soybean and rapeseed oil in diets with low FM (15%; approximately 1.5 % of FO), revealing that palm oils should not be used as the only FO replacer for marine fish species (Fountoulaki et al., 2009). The large variability of results recorded in several studies with carnivorous marine fish species such as gilthead sea bream and European sea bass may be attributed to different LC-PUFA content of the different FO and the level of FM used in the studies that may or may not fulfill the species EFA requirements. Even though, FO replacement is still not entirely understood and seems to be more challenging in carnivorous marine fish than in other species.

Reducing the level of dietary n-3 LC-PUFAs will lead to reductions of DHA and EPA in fish fillets and compromise the nutritional value and health benefits associated with fish

consumption by humans (Bell et al., 2010; Benedito-Palos et al., 2008; Montero et al., 2005). Fish fillet FA composition has high plasticity and tends to reflect that of the diet (Oliva-Teles et al., 2015). Thus, lowering the dietary LC-PUFA content by increasing VOs and animal oils inclusions will reduce fillet levels of LC-PUFA and increase PUFA, and SFA content (Oliva-Teles et al., 2015). In fact, in marine fish species such as gilthead sea bream, a linear correlation between dietary and muscle FA profile was reported for monoenes, C18 PUFA, and LC-PUFA (Benedito-Palos et al., 2011). Particular attention must be given to VO with high levels of LA due to the high incorporation of this FA in phospholipids and storage fats, particularly in marine fish species (Rosenlund et al., 2010). FO finishing diets have been used after a grow-out period where fish were fed with VO-based diets as a strategy to restore the FA profile (particularly by increasing the EPA and DHA content) of the fillet close to that of fish fed the whole production cycle with FO-based aquafeeds (Oliva-Teles et al., 2015). The objective is however difficult as LA is particularly difficult to reduce with this strategy (Oliva-Teles et al., 2015). Generally, this period must allow, at least, a duplication of fish weight to be efficient (Turchini et al., 2009). The length of the finishing period is also highly dependent on the type of FO used as FA composition varies according to the species that FO is produced from and also differs for specific FA (Rosenlund et al., 2010). For instance, DHA levels are recovered quickly (60 days) than EPA (more than 90 days) in gilthead sea bream but a reduction of LA to similar values of FO fed fish may not be achieved even after a long finishing period (Rosenlund et al., 2010).

Finally, FO replacement by VO will also modify the dietary n-6/n-3 ratio, and this may also impact the cardiovascular system, immune status, intestinal mucosa morphology, and intestinal microbiota, ultimately affecting fish health and welfare as will be further discussed in the following chapters of this thesis (Colombo et al., 2020; Oliva-Teles et al., 2015).

1.4. Lipid biosynthesis in marine fish species

In fish, as in all animals, SFA like 16:0 and 18:0 can be desaturated into 16:1n-7 and 18:1n-9, respectively, by Δ^9 desaturase (Δ^9 FADS) (Colombo et al., 2020). Fish do however lack the Δ^{12} desaturase that is needed for the conversion of OA to LA, and the Δ^{15} desaturase for the conversion of LA to ALA (Torstensen and Tocher, 2010). Thus, fish, like all vertebrates, cannot synthesize PUFA *de novo* and so ALA and LA are considered EFA (Sargent et al., 2002). In general, many freshwater fish have a greater

ability to convert C18 PUFA to LC-PUFA than marine fish (Tocher, 2010). However, as early mentioned, ALA and LA are considered EFA for freshwater fish and salmonids (Tocher, 2010). The marine fish natural diet contains high levels of EPA and DHA, so they do not need to synthesize EPA and DHA from their C18 precursors evolving with insufficient elongases and desaturases activity to biosynthesize these EFA.

Elongases and desaturases enzymes perform an essential role in biosynthesizing longer and more unsaturated FA. In some fish species, ALA and LA can be converted into LC-PUFA (ARA, EPA, and DHA) by the actions of fatty acid desaturases (FADS) and elongation of very long-chain fatty acid (ELOV) enzymes as depicted in figure 4 (Tocher, 2010). Thus, the ability of fish to synthesize LC-PUFA from ALA and LA is directly related to the activity of these enzymes (Tocher, 2003).

Teleost fish appear to have lost *FADS₁* but the exact number of *FADS₂* genes varies among species (Xie et al., 2021). Nevertheless, as in mammals, the substrate specificity of *FADS₁* and *FADS₂* enzymes in lesser-spotted dogfish (*Scylliorhinus canicula*) revealed they were $\Delta 5$ and $\Delta 6$ desaturases, respectively (Castro et al., 2016d). In many teleost fish species, *FADS₂* are $\Delta 6$ desaturases showing similar substrate selectivity to the mammalian *FADS₂* (Monroig et al., 2018). Nevertheless, *FADS₂* with dual $\Delta 6/\Delta 5$ desaturase activities, and able to introduce double bonds into separate and distinct carbons in the FA chain, has been described among teleost fish including marine species (Monroig et al., 2018). In teleost fish, the conversion of C18 PUFA into the important LC-PUFA (ARA, EPA, and DHA) may be facilitated by the existence of *FADS₂* enzymes with $\Delta 6$ and $\Delta 5$ activities enabling all the desaturation reactions required (Castro et al., 2016d). However, in marine species, the LC-PUFA biosynthesis pathway is limited at the $\Delta 5$ desaturase level due to the lack of *FADS₁* gene in the teleost genome, contrasting with the mammalian LC-PUFA pathway where $\Delta 6$ desaturation is regarded as rate-limiting (Tocher, 2015).

LC-PUFA biosynthesis from C18 precursors may start with an elongation of ALA and LA to 20:3n-3 and 20:2n-6, respectively, being an alternative pathway to the $\Delta 6$ desaturation route (Figure 4). Both products of elongation can be reincorporated into the LC-PUFA pathway through their desaturation at the $\Delta 8$ positions, producing 20:4n-3 and 20:3n-6, termed as “ $\Delta 8$ pathway” (Xie et al., 2021). *FADS₂* desaturases have already demonstrated $\Delta 8$ activities towards 20:3n-3 and 20:2n-6 in freshwater and marine fish species (Kabeya et al., 2017; Lopes-Marques et al., 2017; Monroig et al., 2011). Surprisingly, functional analyses reveal that marine fish *FADS₂* generally exhibit higher

$\Delta 8$ capability compared to those of freshwater species (Monroig et al., 2018). The advantages of higher $\Delta 8$ desaturation capacity in marine fish species remain unclear since they have high levels of preformed EPA and DHA in their natural diets and the absence or low activity of $\Delta 5$ desaturases is a major LC-PUFA synthesis pathway limitation (Monroig et al., 2018).

One of the most interesting findings regarding the LC-PUFA pathway in teleost marine fish has been the discovery of FADS₂ with $\Delta 4$ desaturation capabilities (Castro et al., 2016d). This capability enabled the production of DHA directly from docosapentaenoic acid (22:5n-3), resulting from a direct elongation of EPA, by a $\Delta 4$ desaturation and termed as “ $\Delta 4$ pathway” (Figure 4; Xie et al. (2021)).

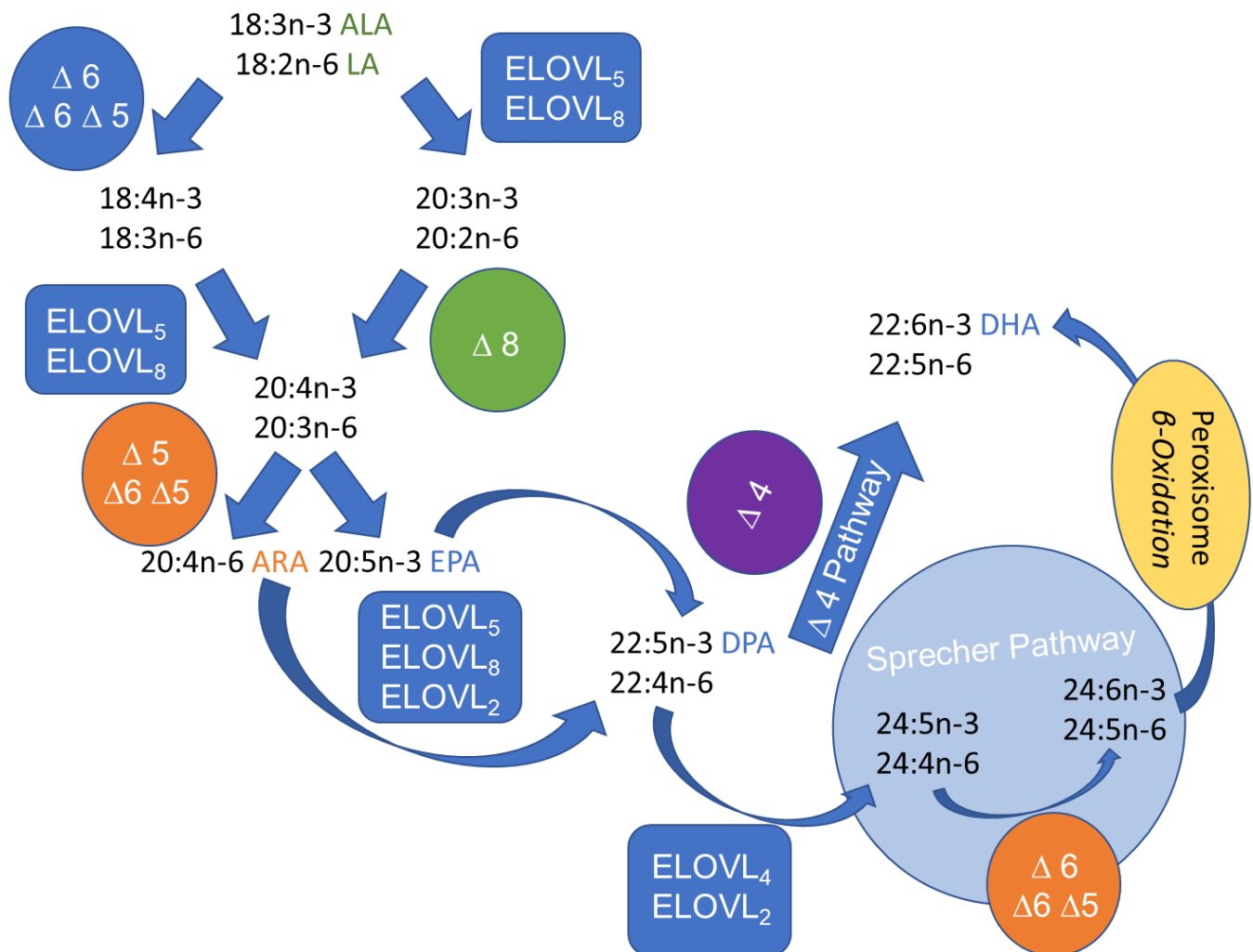


Figure 4: Biosynthetic pathways of long-chain polyunsaturated fatty acids in teleost fish. Desaturation reactions are catalyzed by fatty acid desaturases and highlighted with “ Δ ” that indicates the carbon position at which the inserted double bond locates within the carboxylic group end of the fatty acid chain. Adapted from: Xie et al. (2021).

ELOVL₅ is the most investigated elongase in fish and its functional characterization showed great ability in elongating C18 and C20 PUFAs and its elongation efficiency seems to be drastically reduced with C22 PUFAs (Castro et al., 2016d). Another important elongase in LC-PUFA biosynthesis is ELOVL₂. ELOVL₂ prefers C20 and C22 LC-PUFAs as substrates while C18 PUFAs are elongated to a lesser extent (Monroig et al., 2018). Thus, despite both enzymes sharing some substrate preference, the ability of ELOVL₂ to elongate C22 to C24 LC-PUFA is the main reason by which ELOVL₂ is considered a critical enzyme for DHA synthesis via the “Sprecher pathway” (22:5n-3 elongation to 24:5n-3 which is the substrate of FADS₂ to produce 24:6n-3 that is β -oxidized to produce DHA)(Xie et al., 2021). However, the large majority of marine fish species studied so far have lost the ELOVL₂ gene. This, along with the apparent lack of Δ 5 desaturase activity, are the main molecular mechanisms by which marine fish have low LC-PUFA biosynthesis activity compared to freshwater or salmonid species (Castro et al., 2016d; Torstensen and Tocher, 2010).

FO substitution by VO has been showing that desaturases and elongases genes are subjected to nutritional regulation. For instance, Atlantic salmon (*Salmo Salar*) have complete pathways to produce LC-PUFA, and the replacement of FO with VO or the decrease of n-3 LC-PUFA in diets upregulated desaturases and elongases expression (Betancor et al., 2014; Hixson et al., 2017; Huyben et al., 2021). This upregulation of desaturases and elongases expression enhanced LC-PUFA biosynthesis in the rainbow trout (*Oncorhynchus mykiss*) hepatocytes (Torstensen and Tocher, 2010). In carnivorous marine fish species such as European sea bass (*Dicentrarchus labrax*), gilthead sea bream, and black sea bream (*Acanthopagrus schlegelii*), FO substitution by VO diets also increased FADS₂ and ELOVL₅ expression (Betancor M, 2016; Castro et al., 2015a; Castro et al., 2016b; Houston et al., 2017; Jin et al., 2017b; Seilliez et al., 2003; Torres et al., 2020). The up-regulation of these enzymes in fish fed low FO or n-3 LC-PUFA diets may be due to higher substrate concentration (ALA or LA) provided by dietary VO inclusion or to the relieving of gene expression suppression by LC-PUFA, particularly DHA (Tocher, 2015). Nevertheless, dietary PUFAs are likely to induce effects through their role as ligands to key transcription factors such as sterol regulatory element-binding proteins (SREBP) (Carmona-Antoñanzas et al., 2014; Tocher, 2015). Accordingly, increased srebp-1, proliferator-activated receptor γ (ppar- γ), FADS₂, and ELOVL₄ mRNA levels were found in gilthead sea bream fed low dietary LC-PUFA levels suggesting that the transcription factors ppar- γ and srebp-1 may play a role in LC-PUFA biosynthesis regulation (Betancor M, 2016; Houston et al., 2017). In this species, despite

its ability to perform $\Delta 6$ and $\Delta 8$ desaturations, and PUFA elongations through ELOVL₅ and ELOVL₄, LC-PUFA biosynthesis capacity is low due to the apparent inability to perform $\Delta 5$ desaturations (Xie et al., 2021).

In short, regardless of the regulation of LC-PUFA biosynthesis mechanisms in different fish species, it does not compensate for the dietary reduction of EFA resulting from FO substitution being insufficient to maintain tissue EPA and DHA content (Tocher, 2015; Turchini et al., 2009). Thus, the fatty acid composition of the fish fillet is mainly a reflection of the dietary FA profile since the endogenous pathways of ARA, EPA, and DHA biosynthesis has only a limited ability to modify it (Torstensen and Tocher, 2010).

1.5. Carbohydrates and their interaction with lipids

Plant ingredients have natural constraints that limit their inclusion level in aquafeeds. They are usually rich in carbohydrates (CHO), either in the form of structural polysaccharides (fiber) or energy reserves (mainly starch) (Kamalam et al., 2017). Increasing the non-digestible fraction in aquafeeds with structural non-starch polysaccharides (NSPs) will be an energy sink where only a lower portion of the diet can be utilized. This reduces feed utilization, growth performance and may negatively affect fish physiology (Table 2) (NRC, 2011).

From an economical point of view, increasing the digestible CHO fraction (sugar and starches) in aquafeeds is advantageous as CHO are a cheaper energy source than proteins and lipids. Fish do not have CHO requirements because of their ability to biosynthesize glucose from amino acids, pyruvate, and lactate (NRC, 2011). However, optimal inclusions may spare dietary protein for growth rather than being used as an energy source (sparing effect), which also reduces nitrogen load into the water (environmental safeguard) (Kamalam et al., 2017).

The ability of fish to use CHO as an energy source is generally lower than in livestock and is highly variable depending on fish feeding habits and anatomical and physiological features (Kamalam et al., 2017). Nevertheless, fish use starch as an energy source since they possess the major enzymes involved in its digestion and glucose metabolism, several glucose transporters, and glucose sensing mechanisms in their tissues (Enes et al., 2009; Kamalam et al., 2017; Kroghdal et al., 2005).

Carnivorous fish tend to be less efficient users of CHO than herbivorous or omnivorous species since they usually have a lower CHO digestive enzymes activity, lower rate of glucose clearance from the bloodstream, and delivery to tissues (NRC, 2011). There are

several reasons for the prolonged hyperglycemia in fish, such as the lower capacity of glucose to stimulate insulin secretion compared with amino acids, insulin secretion inhibition by other hormones, lack of control in endogenous production of glucose, weak hepatic lipogenesis from glucose, and low numbers of insulin receptors and insulin binding capacity than higher vertebrates particularly in carnivorous species (Kamalam et al., 2017).

The expression of genes associated with glucose and lipid metabolism can be nutritionally regulated by both lipid and carbohydrate (Bou et al., 2014; Taj et al., 2020), and glucose and FAs have a strong interaction affecting glucose and lipid utilization (Hemre et al., 2002; Menoyo et al., 2006). Surplus of glucose can be deposited as storage lipids in fish tissues following stimulation of the *de novo* lipogenesis (Hemre et al., 2002). Dietary lipids can also stimulate gluconeogenesis to increase plasma glucose levels (Panserat et al., 2002). Normally high dietary CHO (glucose) that can be used as an energy source should inhibit fatty acid β -oxidation (Bou et al., 2014; Jin et al., 2014). However, some data from rainbow trout suggest opposite effects (Kamalam et al., 2013a). The effects of dietary CHO (glucose) in β -oxidation are therefore partly unresolved.

In general dietary CHO appears to induce FA synthesis by upregulating desaturases and elongases (FADS₂, ELOVL₂, and ELOVL₅) at the transcriptional level in salmonoids (Kamalam et al., 2013a; Seiliez et al., 2001). In marine fish, the effect seems to be species-dependent. For instance, while no effect was observed in European sea bass following CHO-rich diets feeding (Castro et al., 2015b), an upregulation of FADS₂ was reported in gilthead sea bream and black sea bream (Castro et al., 2016b; Taj et al., 2020). The reasons for these differences between species are mainly unknown but Kamalam et al. (2013a) suggested that the potentiation of lipogenesis by dietary CHO through the supply of reducing equivalents and the consequent increment of substrates may also improve fatty acid bioconversion.

Table 2: Mean carbohydrate content and worldwide production of common ingredients used in aquafeeds. Source: Kamalam et al. (2017).

Ingredient		Starch and sugars (g/kg DM) ¹	Total carbohydrate (g/kg DM) ²
Wheat	Whole grain	723	839
	Screenings	617	780
	Germ meal	373	561
	Bran	303	732
	Distillers grain ³	146	553
	Starch	877	-
Maize	Whole grain ³	741	843
	Bran	378	777
	Germ meal ³	330	660
	Gluten feed ³	221	689
	Gluten meal	181	278
	Starch	950	-
Oat	Whole grain ³	524	800
	Bran	499	696
Rice	Whole grain	642	837
	Bran ³	284	651
Rye	Whole grain	658	863
	Bran	359	756
Triticale	Whole grain	713	847
Barley	Whole grain ³	618	824
Pea	Whole seed	562	714
	Starch	954	-
Sorghum	Whole grain	759	837
Rapeseed	Whole seed	92	288
	Meal ³	99	469
Soybean	Whole seed	151	333
	Meal ³	98	376
Groundnut	Decorticated seed	136	199
	Meal ³	98	347
Cottonseed	Whole seed	42	541
Linseed	Whole seed	81	319
Faba bean	Whole seed ³	478	647
Sunflower	Whole seed	40	340
Lupin	Whole seed ³	110	520
Cassava	Tuber	808	938
	Starch	943	-
Jatropha	Kernel meal	130	196

DM: Dry matter.

¹ Starch and total sugars.

² Calculated as 100 – (crude protein + ether extract + ash), on DM basis.

³ Mean of different sources or types.

Digestible CHO may also impair intestinal lipid uptake and transport, as corroborated by the reduced intestinal expression of key chylomicron assembly proteins and plasmatic triglycerides levels found in gilthead sea bream 6 h after feeding (Castro et al., 2016a). Nevertheless, 12 hours post-feeding, dietary CHO promoted an increase of circulating

phospholipid and triglycerides probably due to induced lipogenesis (Castro et al., 2016a). Similarly, dietary CHO also increased plasma cholesterol and phospholipids in European sea bass 18 hours after the last meal (Castro et al., 2015b). These studies suggested that the inclusion of digestible CHO in aquafeeds for marine carnivorous species induced alterations in lipid utilization and metabolism. In certain situations, triglycerides and lipoprotein synthesis impairment may result in fatty liver and deficient lipid transportation to peripheral tissues and cholesterol clearance (Torstensen and Tocher, 2010). Thus, further studies are needed to clarify how these impacts on fish health may be attenuated.

1.6. The role of LC-PUFA on oxidative stress

A major factor for oxidative cell injury is free radicals, which are highly reactive species that oxidize and impair the functionality of lipids, carbohydrates, proteins, and DNA (Sánchez-Nuño et al., 2019). Reactive oxygen species and non-radicals species levels, collectively known as ROS are naturally generated in biological processes such as the mitochondrial electron-transfer chain, leukocytes respiratory burst, and oxidative enzymatic activities. Mitochondria are the main source of intracellular free radicals due to the electron transport chain that consumes 85 to 90 % of the oxygen utilized by the cell (Sánchez-Nuño et al., 2019). Thus, while passing through the electron transport chain, up to 2 % of total oxygen consumed suffers a one-electron reduction to generate superoxide anion radicals ($O_2^{\cdot-}$) that is catalyzed by superoxide dismutase to hydrogen peroxide (H_2O_2). Consequently, these $O_2^{\cdot-}$ and H_2O_2 may generate the extremely reactive hydroxyl radical ($OH^{\cdot}$). Singlet oxygen ($^1O_2$) may also be generated non enzymatically, following photosensitization in the presence of light and oxygen or by enzymatic processes (Sargent et al., 2002). Singlet oxygen overcomes spin restrictions and can directly react with organic molecules generating a peroxide. Additionally, several environmental physical, and chemical factors are known to increase ROS (Sánchez-Nuño et al., 2019).

Reactive nitrogen species (RNS) are oxidants naturally generated in aerobic biological systems due to the nitric oxide (NO) reaction with $O_2^{\cdot-}$ and peroxy radicals producing peroxynitrite ($ONOO^{\cdot}$) and nitrogen dioxide (NO_2), respectively (Ayla and Metin, 2015).

Under standard physiological conditions, ROS and RNS concentrations are kept under control by the antioxidant enzymatic and non-enzymatic defenses of fish. Oxidative stress is a condition that occurs when there is an imbalance between oxidants and

antioxidants defenses leading to several uncontrolled free radical chain reactions. Such reactions will potentially lead to damage of cell membrane integrity, impairment of cell metabolism, or even apoptosis (Ayla and Metin, 2015; Sánchez-Nuño et al., 2019; Sargent et al., 2002). Nevertheless, controlled ROS and RNS processes are important mediators of physiological functions such as cell signaling, vasodilatation, immune and inflammatory response, fertilization, and growth (Ayla and Metin, 2015; Lugrin et al., 2014; Sánchez-Nuño et al., 2019).

Fish are considered LC-PUFA rich organisms, being a unique source of EPA and DHA for human consumption. Fish lipids are very different from those of mammalian, including up to 40 % of long-chain FA, which are highly unsaturated (Secchi and Parisi, 2016). These LC-PUFA are essential constituents of the cell membrane structure, ensuring proper function and fluidity, but are very prone to attack by free radicals (Sargent et al., 2002). The main reason is the bis-allylic methylene groups that have lower dissociation energy compared to single double bonds or saturated fatty acids (Mostafa Abd El-Aal, 2012; Repetto et al., 2012). This carbon is more prone to lose a hydrogen resulting in a free radical. Consequently, the susceptibility to peroxidation of a particular LC-PUFA increases with the numbers of bis-allylic methylene groups in the lipidic chain (Mostafa Abd El-Aal, 2012). Thus, DHA seems to be more susceptible to lipid peroxidation due to its chemical structure than ARA and EPA. The attack on membrane phospholipids LC-PUFAs will result in deleterious consequences for cell membrane structure, fluidity, and function leading to pathological effects on cells and tissues (Sargent et al., 2002).

LC-PUFA peroxidation can occur by two different pathways: non-enzymatic and enzymatic oxidation (Repetto et al., 2012). Non-enzymatic lipid peroxidation and the production of lipid peroxides are started by the presence of ROS (Figure 5) and are facilitated by Fe^{2+} ions (Repetto et al., 2012). The enzymatic pathway is catalyzed by cyclooxygenase (COX), 5-lipoxygenase (5-LOX), and cytochrome P450 monooxygenase (CYP), which are enzymes that oxygenate DHA, EPA, or ARA generating eicosanoids, $^1O_2$, and peroxy radicals (Anderson and Taylor, 2012; Repetto et al., 2012; Sargent et al., 2002). Furthermore, ARA participates in the NADPH oxidase assembling, which is a key producer of ROS ($O_2^{\cdot-}$) and a vital enzyme for the immune system, by direct binding to S100 proteins (Hii and Ferrante, 2007).

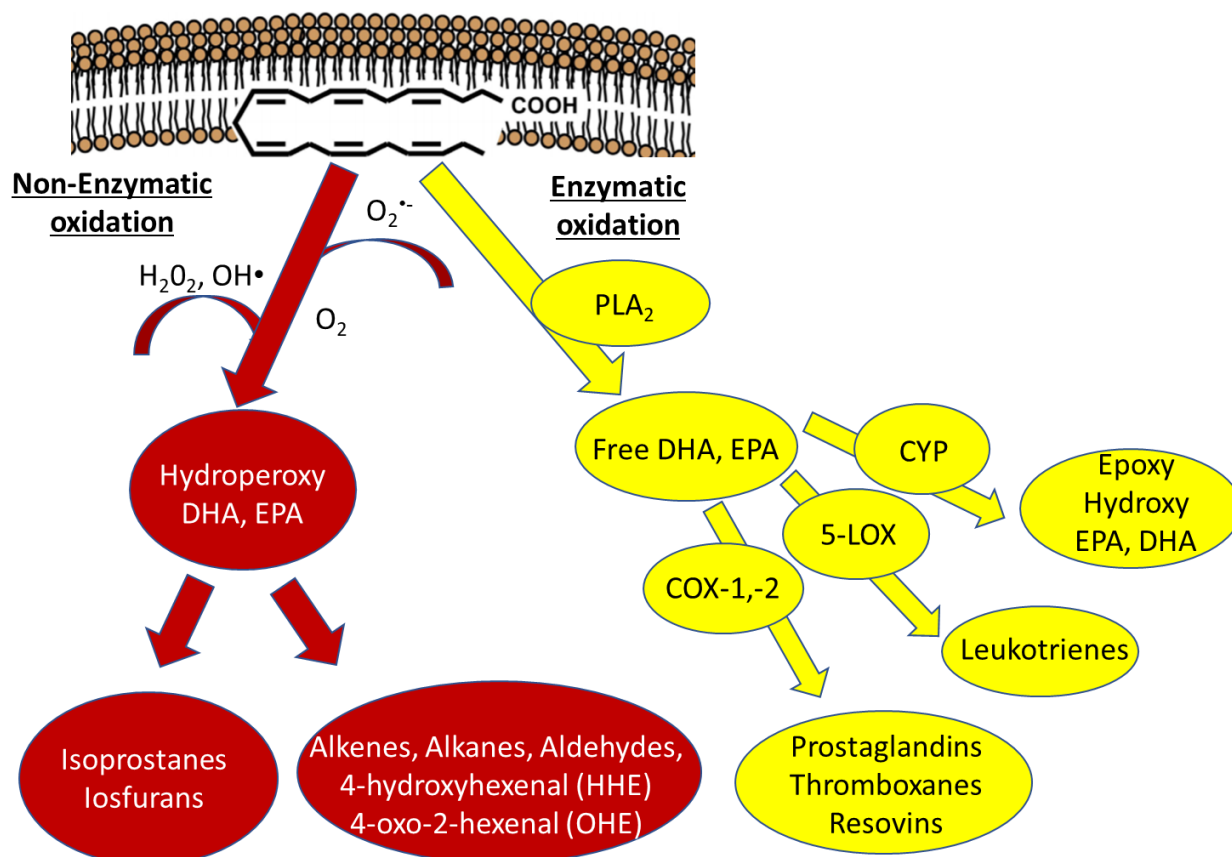


Figure 5: Pathways of enzymatic and non-enzymatic pathways of n-3 LC-PUFA oxidation. PLA₂, Phospholipase A₂; CYP, Cytochrome P450 monooxygenase. Adapted from: Anderson and Taylor (2012).

The LC-PUFA non-enzymatic oxidative deterioration involves three different steps: initiation, propagation, and termination. The first step requires the activation of oxygen to a high-energy state. Thus, oxygen must be transformed into high-energy state molecules like $O_2^{\cdot-}$, $OH^{\cdot}$, and H_2O_2 (Sargent et al., 2002). LC-PUFA oxidation begins with the removal of a hydrogen atom from a methylene group by a powerful ROS, leaving a carbon-centered lipid radical ($R^{\cdot}$). In the propagation phase, the latter one is rearranged to form a conjugated diene, and a quick reaction with oxygen forms a lipid peroxy radical ($ROO^{\cdot}$). The peroxy radical is capable of removing another hydrogen from another FA or other molecules such as DNA or proteins to produce a lipid hydroperoxide ($ROOH$) and, consequently, another lipid radical. The recently formed lipid radical propagates the radical chain. The reactions will proceed unless termination happens by the reaction of two radicals to form a nonradical product or by the intervention of antioxidant species such as tocopherols, carotenoids, or ascorbic acid, which will trap the damaging radicals after donating a hydrogen atom to the lipid peroxy radical. Finally, the lipid hydroperoxides can be decomposed to alkoxy and hydroxyl radicals, and the first one can be converted to secondary products such as aldehydes (malondialdehyde), ketones,

and alcohols which are usually quantified as end products of lipid peroxidation (Mostafa Abd El-Aal, 2012).

In the presence of FE^{2+} or CU^{+} copper, lipid hydroperoxides will participate in a Fenton–Haber–Weiss reaction leading to the formation of reactive hydroxyl radicals (Mostafa Abd El-Aal, 2012; Sargent et al., 2002). Thus, in the presence of iron and copper, the chain reaction will be amplified.

Lipid hydroperoxides present in the cell membrane disrupt its function due to the modification of its fluidity and allow ions such as Ca^{2+} to leak across the membrane. Consequently, phospholipases may be activated, the plasma membrane may form a bulge, or eventually the membrane may rupture (Storey, 1996).

Lipid peroxidation may also be generated from intermediates of the NO metabolism (Repetto et al., 2012). Thus, NO-derived species may pass across cell membranes, and their concentration in the hydrophobic side of membranes and lipoproteins will originate fast reactions with FA and lipid peroxy radicals ($ROO\cdot$) during the lipid oxidation process. The result is the production of oxidized and nitrated products of free and esterified fatty acids that may react with NO and NO-derived radicals originating nitroalkenes and nitroalcohols (Repetto et al., 2012).

Fish are particularly susceptible to oxidative damage because they contain high levels of LC-PUFA. However, aerobic animal cells evolved a very efficient antioxidant system with several levels of protection, guaranteeing relative safety against the adverse effects of living in an oxygen-rich environment (Figure 6).

Fish, like high vertebrates, possess two antioxidant protection mechanisms: enzymatic and non-enzymatic (Mostafa Abd El-Aal, 2012). The enzymatic system is composed of cytosolic and mitochondrial superoxide dismutase (SODs; EC 1.15.11), catalase (CAT; EC 1.11.1.6), glutathione peroxidase (GPX; EC 1.11.1.9), glutathione reductase (GR; EC 1.6.4.2), glutathione-S-transferase (GST), and glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) (Fogaça and Santana, 2009).

SOD represents a group of enzymes that catalyze the dismutation of $O_2^{\cdot-}$ and the formation of H_2O_2 and an oxygen molecule. CAT and GPX convert H_2O_2 into water but GPX requires the presence of reduced glutathione (GSH) and generates oxidized glutathione (GSSG). CAT and GPX have the same action on H_2O_2 , but GPX seems to be more efficient with high ROS levels and CAT with low H_2O_2 concentrations. GPX is also a major player involved in the detoxification of lipoperoxides. GR is important to

reduce the oxidized glutathione (GSSG) back to GSH in the presence of NADPH generated by G6PDH in the pentose phosphate pathway (Mostafa Abd El-Aal, 2012).

The non-enzymatic antioxidants include vitamins A (retinol), C (ascorbic acid) and E (tocopherols), flavonoids and carotenoids, thiols (GSH, bilirubin, ferritin), and minerals (iron, copper, zinc, and selenium) that may act as an enzymatic cofactor (Fogaça and Santana, 2009). Vitamin E is the main soluble lipid antioxidant in animals, acting as a radical scavenger (Martínez-Álvarez et al., 2005). Vitamin C is an important antioxidant molecule but also reduces vitamin E radical regenerating its activity (Babinszky et al., 2019). Minerals such as iron, manganese, copper, selenium, and zinc are also involved in the prevention of lipid peroxidation and are indispensable as cofactors for the proper function of several antioxidant enzymes (Martínez-Álvarez et al., 2005).

Moreover, aerobic animals have a secondary defense mechanism which is mainly a repair system to cope with the damage done by oxidative stress once it has occurred. There are multiple enzymes systems involved in DNA repair and lipolytic as well as proteolytic enzymes with restituting or replenishing functions (Sies, 1993). These enzymes' functions are associated with repairing damaged DNA, membrane damage due to phospholipid oxidation products, or damaged proteins (Sies, 1993).

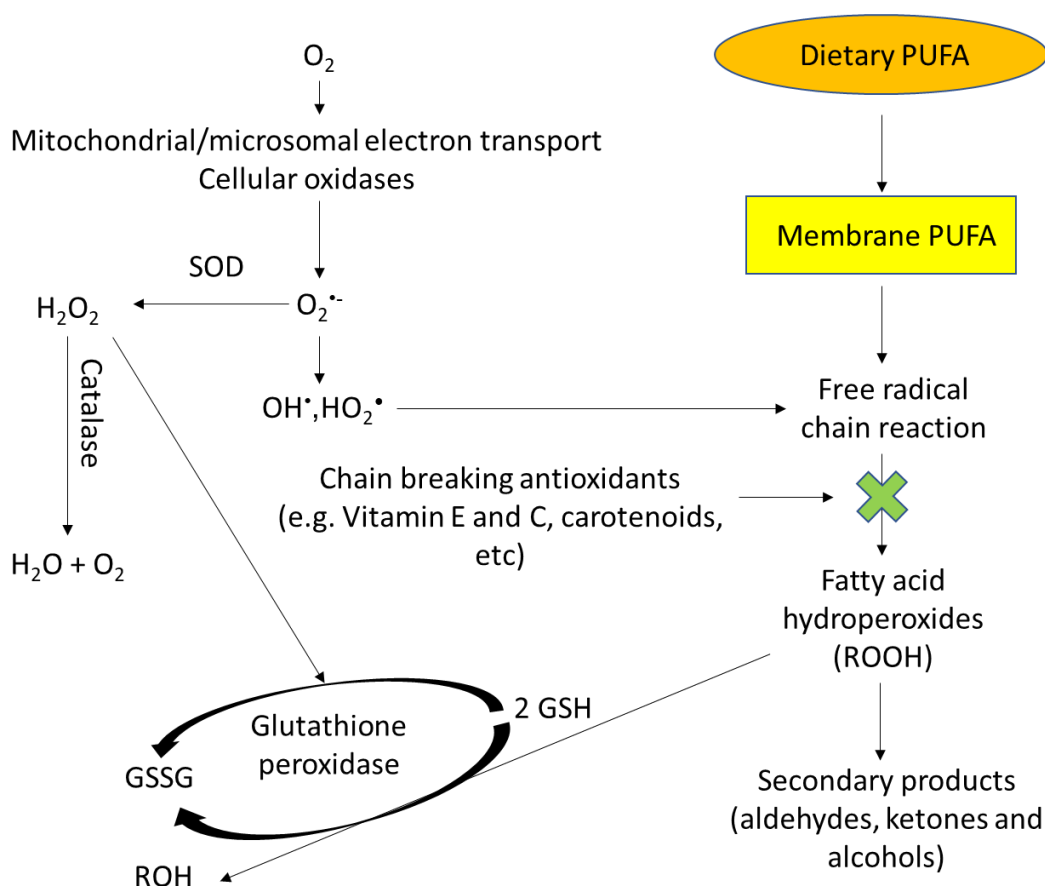


Figure 6: Representation of the interaction between cellular PUFA, reactive oxygen species, and antioxidant protective mechanisms. $HO_2^{\cdot}$: hydroperoxyl radicals. Adapted from: Sargent et al. (2002)

The fish antioxidant protective mechanism may become under stress with dietary deficiencies of essential antioxidant nutrients but also by excessive dietary content of high oxidable nutrients such as LC-PUFA, which will increase the vulnerability of tissues and cells to oxidative stress (Sargent et al., 2002).

VO and animal inclusion in aquafeeds to substitute FO will increase the dietary content of less oxidative FA such as n-6 PUFA and SFA and decrease the highly oxidative n-3 LC-PUFA impacting tissues FA composition in the same way. Therefore, it is important to study how the fish tissues' susceptibility to ROS and antioxidant protective mechanism reacts as a result of modifications in dietary n-6/n-3 ratio and LC-PUFA level due to FO substitution in aquafeeds. This is an important study subject because lipid peroxidation ultimately leads to histological lesions in fish, such as myopathy of skeletal muscle, lipid liver degeneration, and accumulation of ceroid pigments (Sargent et al., 2002). Furthermore, ROS production is important to modulate a proper organism defense system (Biller and Takahashi, 2018) and physiological processes (Babinszky et al., 2019). Previously, the replacement of dietary fish oil by a vegetable oil blend (20%

rapeseed, 50% linseed, and 30% palm oils) and consequent increment of the n-6/n-3 ratio and lower LC-PUFA levels reduced the hepatic and intestinal LPO in gilthead sea bream and intestinal LPO in European sea bass together with higher activity of several antioxidant enzymes such as GPX and GR (Castro et al., 2016c; Castro et al., 2015c).

Also, several studies in different fish species have reported a marked increment of lipid peroxidation levels associated with graded dietary levels of LC-PUFAs (increasing the dietary unsaturation level gradually), and, in some cases, the antioxidant enzymes activity increases in parallel as a measure to attenuate oxidative stress (Jin et al., 2017b; Ma et al., 2018; Mozanzadeh et al., 2015; Shahkar et al., 2016).

1.7. LC-PUFA and immune function

LC-PUFA or their derivatives have important specific functional roles as mediators of immune function. The regulatory role of LC-PUFA can be intracellular as ligands of transcription factors impacting genes expression or extracellular at tissues level through their derivatives, namely by eicosanoids (prostaglandins, leukotrienes, lipoxins, resolvins, and protectins) (Tocher, 2015).

The important regulatory effects of n-6 or n-3 LC-PUFA in the fish immune system reinforce the need to maintain a balanced dietary intake of the two series of LC-PUFA when FO is substituted by alternative oils in aquafeeds (Tocher, 2015).

Furthermore, membranes of immune system cells have a high content of LC-PUFA in their composition (Farndale et al., 1999; Mourente et al., 2007). LC-PUFA of the n-6 or n-3 series have different biological roles in immune cells that impact immune and inflammatory processes, and the modification of cell membrane EFA composition can modify immune cells' response. Thus, imbalanced n-6/n-3 LC-PUFA content may influence immune cells' maturation and function, leading to a potentially long-lasting effect on immune competence and a higher risk of diseases related to immune dysfunction (Miles et al., 2021).

LC-PUFA content of fish immune cells generally reflects the dietary FA profile (Farndale et al., 1999; Mourente et al., 2007). DHA is usually the most abundant LC-PUFA representing circa 20 % of the total FA of fish blood leucocytes even when FO is substituted by VO (Farndale et al., 1999; Mourente et al., 2007). EPA content is also significant, between 6 to 13 % of total FA, while ARA comprises 0.7 to 6.2 % of total FA (Farndale et al., 1999; Mourente et al., 2007) but still is one of the most abundant of n-6 PUFA.

Their highly unsaturated nature makes LC-PUFA able to influence cells' membrane fluidity and membrane proteins function, including LC-PUFA's ability to move within membranes to form "lipid rafts" which are signaling platforms (Calder, 2015). Consequently, LC-PUFA actively modulates intracellular signaling pathways of immune cells affecting transcription factors activations and gene expression (Figure 7) (Calder, 2015). ARA has important functions in cell signaling, and its role as an inflammation promoter is probably due to the activation of the nuclear factor kappa B (NF_κB), which regulates the expression of several genes critical for immune-inflammatory reactions (Camandola et al., 1996). Contrarily, n-3 LC-PUFA, particularly EPA, seems to impact inflammatory gene expression via inhibition of transcription factor NF_κB (Calder, 2006). LC-PUFA may also regulate a second transcription factor family, the proliferator-activated receptor (PPAR), which has anti-inflammatory properties (Korbecki et al., 2019). PPAR family activation causes inhibition of NF_κB activation by creating an interaction between them (Calder, 2010). Thus, NF_κB and PPAR activation may be regulated by different LC-PUFA depending on their concentration (Korbecki et al., 2019).

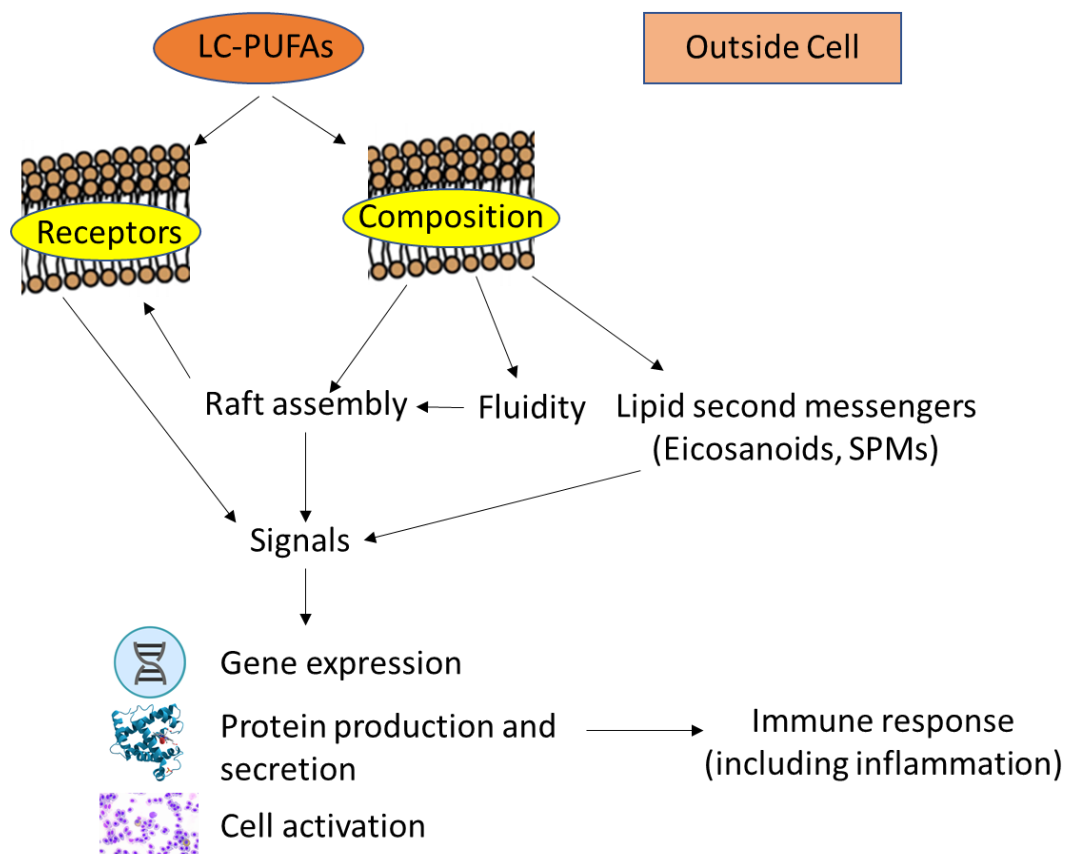


Figure 7: The mechanism of LC-PUFA action on immune cells. SPM: Specialized pro-resolving mediator. Adapted from: Miles et al. (2021).

LC-PUFAs have been demonstrated to affect the regulation of several immune cell types such as lymphocytes, monocytes, macrophages, neutrophils, and endothelial cells (Calder, 2010). Nevertheless, the best-known function of LC-PUFA regarding immune function is their role as substrates for the production of eicosanoids (bioactive lipid mediators). Eicosanoids are produced in every body tissue and have a range of physiological effects such as immune and inflammatory responses, blood clotting, cardiovascular tone, reproduction, and renal and neural functions (Torstensen and Tocher, 2010). These hormone-like compounds have a short half-life and are generated by cells to act in their proximity by affecting the production of mediators like cytokines (Torstensen and Tocher, 2010).

Eicosanoid production depends on the LC-PUFA within phospholipid located in cell membranes, usually at the sn-2 position, and can be released upon cell stimulation by phospholipase A2 (Miles et al., 2021). Once released, ARA, EPA and DHA can enter the COX or lipoxygenases (LOX) pathways (Figure 8) (Tocher, 2015). In fish, as in mammals, ARA is the main eicosanoid precursor, although usually there is a larger amount of EPA and DHA in tissues (Torstensen and Tocher, 2010). Nevertheless, n-3 LC-PUFAs, particularly EPA compete with ARA as an eicosanoid precursor in fish (Bell et al., 1994). Thus, EPA and DHA decreased the production of ARA-derived eicosanoids, which, when excessively produced, may suppress immune competence (Miles et al., 2021). ARA derivatives eicosanoids have mostly pro-inflammatory characteristics, while eicosanoids produced from EPA and DHA have less inflammatory effects or specialized functions that bring about resolution of the inflammatory response and return to homeostasis (Miles et al., 2021; Tocher, 2015).

From ARA, the COX pathway originates 2-series prostaglandins (PGs), and thromboxanes (TX), and the 5-LOX pathway produces 4-series leukotrienes (LTs) all with pro-inflammatory effects (Calder, 2010; Miles et al., 2021). ARA may also originate lipoxins which are lipoxygenase-derived eicosanoids with anti-inflammatory actions to prevent severe inflammatory reactions (Serhan, 2008). If the production of lipoxins is not sufficient to control the inflammatory response, EPA is utilized by the COX pathway to produce 3-series PGs and thromboxanes and by the 5-LOX pathway to produce 5-series LTs which have lower pro-inflammatory properties compared to eicosanoids formed from ARA (Calder, 2010; Miles et al., 2021). In recent years, it was found that EPA, and DHA, are precursors of other lipid mediators generated by the COX and LOX pathways (Tocher, 2015). These mediators are named specialized pro-resolving

mediators (SPMs), namely resolvins, maresins, and protectins, and help to end the ongoing inflammation (Miles et al., 2021; Tocher, 2015).

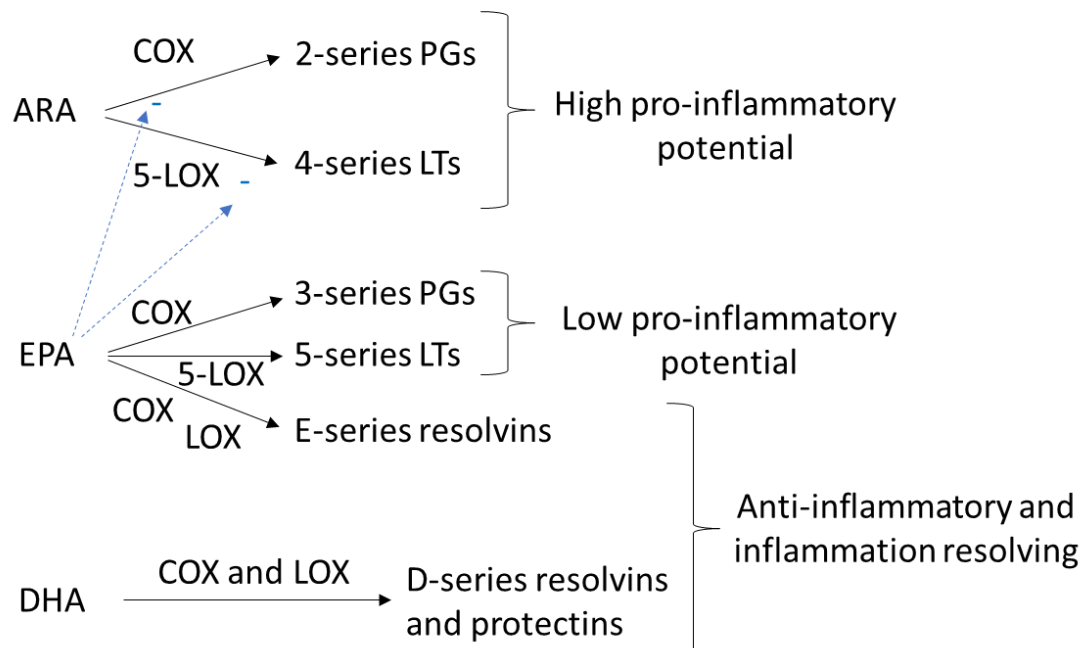


Figure 8: Lipid mediators produced from ARA, EPA, and DHA and their principal action. COX: cyclooxygenase; LOX: lipoxygenase; LT: Leukotriene; PG: prostaglandin. Adapted from: Calder (2010).

Consequently, the dietary and tissue content of the ARA, EPA, and DHA is extremely important to modulate inflammatory responses. The substitution of FO in aquafeeds will decrease EPA and DHA levels in fish tissues and may increase the n-6/n-3 LC-PUFA ratio. This is likely the main reason for eicosanoid metabolism modifications induced by alternative oil sources (Torstensen and Tocher, 2010). Modifications of dietary ARA, EPA, and DHA content may lead to imbalances in eicosanoid production and, consequently, negatively impact inflammatory processes by reducing the overall response to stress, injury, or infection, or even overshoot the inflammatory response causing a life-threatening situation (Torstensen and Tocher, 2010).

The relationship between dietary and tissue ARA level and eicosanoid productions has been barely studied in fish. Available data suggested a direct relationship between dietary and tissue ARA content and the ability of cells to produce its associated eicosanoids (Bell et al., 1994; Mourente et al., 2007; Torrecillas et al., 2017a). Despite the important role in fish metabolism, the EPA and DHA-derived eicosanoids have been little studied due to their short life *in vivo* and the complexity of their correct quantification (Montero and Izquierdo, 2010). In Atlantic salmon kidney macrophages, the ratio between produced LTB₄:LTB₅ and PGE₂:PGE₃ (ARA: EPA products) was higher in fish

fed with soybean oil-based diets compared to those of fish fed with FO and linseed oil-based diets (Bell et al., 1996a; Bell et al., 1996b).

Dietary ARA role as eicosanoids precursor has implications in immune, inflammatory, and stress responses in fish. An appropriate level of dietary ARA is effective to modulate the innate immune responses of fish species such as Japanese sea bass (*Lateolabrax japonicus*), Japanese eel, European sea bass, and rabbitfish (*Siganus rivulatus*) (Nayak et al., 2017; Shahkar et al., 2016; Torrecillas et al., 2017a; Xu et al., 2010).

Despite EPA role as eicosanoids precursor being associated with eicosanoids with less inflammatory and along with DHA with inflammation resolution properties, they may be essential to control inflammatory and immune response severity. Thus, adequate dietary levels of n-3 LC-PUFA were shown to be necessary to improve the innate immune response of fish species such as gilthead sea bream, silvery-black porgy (*Sparidentex hasta*), and yellow croaker (*Larmichthys crocea*), Japanese flounder (*Paralichthys olivaceus*), and *Sebastiscus marmoratus* (Montero et al., 2004; Mozanzadeh et al., 2015; Peng et al., 2014; Wang et al., 2006; Zuo et al., 2012b). In fish, DHA seems to play a more important role in immune function than EPA. In juvenile grouper, *Epinephelus malabaricus*, high dietary DHA/EPA ratios stimulated leucocytes functions than lower ones (Wu et al., 2003). Accordingly, DHA/EPA dietary ratios higher than 1:1 also enhanced innate immune responses in Japanese sea bass, large yellow croaker, and golden pompano (*Trachinotus ovatus*) (Xu et al., 2016a; Zhang et al., 2019; Zuo et al., 2012a).

The fish immune system is directly affected by dietary n-6 and n-3 LC-PUFA content, probably due to their interactions. Thus, it is imperative to gain further knowledge on the effects of different n-6/n-3 dietary ratios on fish immune systems particularly due to increasing FO substitution by alternative oils in the aquafeeds industry.

1.8. Gilthead Sea bream

Gilthead sea bream (*Sparus aurata* Linnaeus 1759, Sparidae) inhabits the eastern Atlantic Ocean coast from Cape Verde to the United Kingdom and around the Canary Islands. This species is very common in the western Mediterranean sea being less frequent in the eastern Mediterranean and the Black seas (FAO, 2005-2021). Gilthead sea bream body color is silvery-gray with a large black blotch at the beginning of the lateral line that extends to the upper margin of the operculum (Figure 9), and below that blotch, a reddish area may appear (FAO, 2005-2021). The dorsal fin presents 11 spines

and 13 to 14 soft rays and a dark band. Caudal fin fork and tips are edged with black. In mature individuals, a golden frontal band between the eyes is edged by two dark areas.

It is a protandrous hermaphrodite species, with most individuals starting (20-30 cm) as mature males in the first or second year of their life and turning into females after then (33-40 cm). The spawning season occurs between December and April (Basurco et al., 2011).

It is a benthopelagic species, found in many different coastal environments from seagrass beds and sandy bottoms to rocky ones until depths of about 30 to 150 m deep and is also a solitary and sedentary species, occasionally forming small aggregations (Basurco et al., 2011).

Gilthead sea bream is mainly carnivorous and an accessory herbivorous, with a trophic level of 3.7 according to Fish Base. In the wild, gilthead sea bream feed on molluscs, mainly mussels, and oysters that can easily crush, but also crustaceans and fish (Basurco et al., 2011).

It is a euryhaline species that tolerates a wide range of salinities and is often encountered in brackish waters (FAO, 2005-2021). It tolerates temperatures between 15 to 30°C, but a range between 18-26°C appears to optimize feed efficiency, activity, and metabolism (EFSA, 2008; FAO, 2005-2021). Temperatures below 15°C increase the risk for winter disease episodes (Ibarz et al., 2010). Nevertheless, gametogenesis is optimally induced between 15-17°C but may happen with temperatures between 13 to 20 °C (EFSA, 2008).



Figure 9: Gilthead sea bream juvenile.

Gilthead sea bream is one of the most relevant aquaculture species in the Mediterranean and ranks second in the European Union, together with the European sea bass, in terms of value, only after Atlantic salmon (Llorente et al., 2020). In 2019, around 259 thousand tonnes of gilthead sea bream were produced in aquaculture worldwide (FAO, 2021). Gilthead sea bream is mainly produced in the Mediterranean sea with Turkey and Greece as the leading producers followed by Egypt, Tunisia, Spain, Italy, and Croatia (FAO, 2021).

1.8.1. Nutritional requirements

Dietary protein requirement for gilthead sea bream juveniles (initial body weight (IBW) of 2.6 g) were initially reported to be 40 % using a casein-based semi-purified diet but the most favorable feed conversion ratio was found fish fed with a 60 % protein diet (Sabaut and Luquet, 1973). More recently, using practical diets, the protein requirement for fry (IBW of 0.8 g) was estimated to be 55% (Vergara et al., 1996b). For larger fish (IBW 9.8 and 5.6 g), protein requirement was established at 45-46%, reflecting a reduction of protein requirement in bigger fish (Santinha et al., 1996; Vergara et al., 1996a).

An estimation of essential amino acid (EAA) requirements of gilthead sea bream juveniles was done based on dose-response studies and is depicted in table 3. Quantitative data is available only for arginine, lysine, methionine and cysteine, and

tryptophane (Luquet and Sabaut, 1974; Marcouli et al., 2005). Kaushik (1998) estimated the EAA using the ideal protein approach, based on whole-body EAA composition and lysine requirements. Using the amino acid deletion method, the ideal EAA profile was more recently estimated (table 3; Peres and Oliva-Teles (2009).

Table 3: Amino acids requirements (g/16 g N) of gilthead sea bream.

Method	Dose-response	Ideal protein	Amino acid deletion
Amino acid			
Arginine	<2.6	5.4	5.55
Histidine	-	1.7	1.89
Isoleucine	-	2.6	2.55
Leucine	-	4.5	4.75
Lysine	4.9 - 5	5.0	5.13
Methionine	-	-	2.60
Methionine + Cystine	2.8 - 4	2.4	-
Phenylalanine + Tyrosine	-	2.9	5.76
Threonine	-	2.8	2.98
Tryptophan	0.6	0.6	0.75
Valine	-	3.0	3.21
Reference	(Luquet and Sabaut, 1974; Marcouli et al., 2005)	(Kaushik, 1998)	(Peres and Oliva-Teles, 2009)

Early studies reported an optimum dietary lipid level of 9 %, which was the lowest value tested (9 to 16 %) (Marais and Kissil, 1979). Posteriorly, the optimum dietary lipid level was revised and established around 15-16 % (Vergara and Jauncey, 1993; Vergara et al., 1996a). More recently, it was shown that increasing the dietary lipid level from 15 % to 22 % increased weight gain in diets with a good quality fish meal (Norse LT) as a protein source but with a standard quality fish meal, a further increase of dietary lipid level to 28 % was required for maximum growth and dietary protein sparing (Vergara et al., 1999). However, the authors discouraged using such high lipid levels for extended periods since hepatocyte abnormalities were reported. On the other hand, Vergara et al. (1999) did not report any dietary lipid level effect on feed intake or feed conversion but Santinha et al. (1999) observed that the increment of dietary lipid level from 15 to 21 % increased feed efficiency and protein retention but had no impact on growth performance.

Nowadays the trend in carnivorous fish aquafeeds is to use energy-dense diets with high digestible protein/ digestible energy (DP/DE) ratios (Oliva-Teles et al., 2015) and commercial aquafeeds for this species are usually extruded pellets with a protein content between 43 to 50 % and about 20% of lipids (Basurco et al., 2011; Pelusio et al., 2021). Despite being the main conventional sources of energy in gilthead sea bream aquafeeds, lipids are also the source of LC-PUFA, which are the EFA for this species, since this species is unable to synthesize n-3 and n-6 LC-PUFA from their precursors (Turchini et al., 2009).

FO is the only competitive commercial source of EFA and the total replacement of FO in aquafeeds by alternative lipidic sources, particularly in the context of dietary FM reduction, may not fulfill EFA requirements for gilthead sea bream during the on-growing phase. The EFA requirements for 1 or 42 g juveniles were estimated to be 0.9 or 1.9 % of LC-PUFA in the diets with 11-13 % of lipids and a dietary DHA/EPA ratio of 1 or 0.5, respectively (Ibeas et al., 1994; Kalogeropoulos et al., 1992). A dietary DHA/EPA ratio of 0.5 was estimated to be the most suitable for gilthead sea bream juveniles as growth performance decreased with increasing dietary DHA/EPA ratios of 1 and 2 (Ibeas et al., 1997).

In another study, using diets with a DHA/EPA ratio of 0.5, the n-3 LC-PUFA requirements for gilthead sea bream with 11-30 g were estimated to be 1 % (Ibeas et al., 1996). Izquierdo (2005) further suggested a minimum dietary level of DHA and EPA of 0.6 and 0.7 %, respectively, for gilthead sea bream juveniles. Nevertheless, EFA requirements may differ with fish size or with dietary lipid levels, since in 85-460 g fish fed diets with 22 % of lipid level, a dietary n-3 LC-PUFA of 1.8 % was not sufficient to maintain the best growth over 6 months (Izquierdo et al., 2005). Thus, the authors recommended that, during the on-growing period, over 2.5 % of n-3 LC-PUFA should be available in diets with high lipidic content (Izquierdo, 2005; Izquierdo et al., 2005).

Though n-6 LC-PUFAs are essential for marine fish, requirements of ARA have not yet been established for gilthead sea bream juveniles. In fish with 1.4-18 g and fed diets with 15 % of lipids and graded levels of ARA ranging from 0.03 to 1.7 %, no differences in growth or feed efficiency were found (Fountoulaki et al., 2003).

FM substitution by alternative plant feedstuffs rich in CHO may be a nutritional challenge for gilthead sea bream. Like most carnivorous fish species, gilthead sea bream does not use dietary CHO as efficiently as farm animals or omnivorous fish species (Oliva-Teles et al., 2011). Accordingly, Fernandez et al. (2007) also observed that dietary inclusion of

18 to 26 % of gelatinized maize starch spared dietary protein, enhanced growth performance, and reduced nitrogen losses compared to diets including 5 % of starch. Moreover, Enes et al. (2008) found no differences in growth performance, feed efficiency, and protein efficiency ratio in gilthead sea bream juveniles fed diets with 10 or 20 % of native or waxy maize starch. Overall, gilthead sea bream seems to tolerate diets with up to 30 % of gelatinized starch while increasing levels of native starch decrease fish performance and feed conversion ratio (Georgopoulos and Conides, 1999). However, in a per-feeding trial, it was shown that dietary incorporation of 10 or 20 % of dietary gelatinized starch was efficiently used for growth, feed efficiency, and protein-sparing effect while 30 % dietary gelatinized starch decreased fish performance (Couto et al., 2008). Thus, in general, 20% of digestible CHO may be incorporated in gilthead sea bream diets without detrimental effects on growth performance and feed efficiency.

Data on gilthead sea bream vitamin requirements are still limited. Yet, the requirements of thiamin (vitamin B₁) were established to be 10 mg kg⁻¹ with lipid-rich diets, while 0.5 mg kg⁻¹ was considered adequate when given carbohydrate-rich diets (Morris and Davies, 1995b). The same authors estimated that the nicotinic acid (vitamin B₃) requirements for fingerlings were between 63-83 mg kg⁻¹ (Morris and Davies, 1995a) and Kissil et al. (1981) estimated pyridoxine (vitamin B₆) requirements to be 1.97 mg kg⁻¹. Despite Vitamin C requirement was not estimated, it was observed that while graded levels of vitamin C in the diet did not affect growth performance, with the highest supplementation level (200 mg kg⁻¹) feed efficiency and protein efficiency ratio were improved compared to fish fed the non-supplemented diet (Henrique et al., 1998).

Data on minerals requirements are even more scarce. Until now, only phosphorus requirement was estimated to be 0.75 % of the diet for maximum growth (Pimentel Rodrigues and Oliva-Teles, 2001).

1.9. Objectives

This thesis aims to increase knowledge regarding FA and carbohydrate effects in the inflammatory and immune response of fish. Gilthead sea bream (*Sparus aurata*) was chosen as the model fish species to conduct this study as it is the most produced marine fish species in Mediterranean aquaculture, assuming great commercial importance.

Gilthead sea bream, as marine fish species in general, have limited capacity to express elongases and desaturases and is unable to produce LC-PUFA from C-18 PUFA.

Dietary manipulation of n-6/n-3 LC-PUFA ratio, EFA, and CHO levels due to the dietary replacement of fisheries ingredients by plant feedstuffs and vegetable oils may compromise zootechnical performance, affect lipid and glucose metabolism, inflammatory and immune processes, and oxidative status, thus affecting fish performance and health.

Furthermore, dietary n-6/n-3 LC-PUFAs ratio and level may impact eicosanoids production and the expression of inflammatory genes and low or excessive CHO dietary level may suppress the innate immune response in fish. Therefore, the possible effects of dietary modification of the n-6/n-3 LC-PUFA ratio and level and dietary inclusion of carbohydrate-rich plant feedstuffs cannot be overlooked.

A multidisciplinary approach was applied to provide an integrated evaluation of diet manipulation at biochemical and physiological levels focusing particularly on the hepatic and intestinal response.

Thus, four isoproteic (47% crude protein) and isolipidic (18% crude lipids) diets were formulated using a vegetable oil blend (20:50:30; rapeseed: linseed: palm oil) and the effects of dietary ARA/EPA/DHA ratios of 2.0/0.2/0.1 (Diet A); 1.0/0.4/0.4 (Diet B); 0/0.6/0.6 (Diet C); and 0/0.3/1.5 (Diet D) were evaluated after 56 days on gilthead sea bream juveniles growth performance and feed utilization, whole-body composition, muscle lipid content and fatty acid profile, liver and intestine enzymatic activity and expression of genes related to lipid and carbohydrate metabolism and LC-PUFA biosynthesis (chapter 2), liver and intestine oxidative stress, distal intestine histomorphology, and gut microbiota (chapter 3), hematological profile, plasma innate immune response, and intestinal expression of different immune-related genes (chapter 4). Additionally, the effects of the above diets in the same selected immune parameters evaluated in chapter 4 were assessed 4 and 24 hours after an inflammatory challenge with killed *Photobacterium damsela* subsp. *Piscicida* made immediately after the growth trial (chapter 5).

In a different trial, four plant-based isoproteic (47% crude protein) and isolipidic (18% crude lipids; 20:50:30; rapeseed: linseed: palm oil) diets were formulated and the effects of low or high gelatinized starch (5 and 20 %; diets LS and HS, respectively) and different dietary ARA/DHA ratios (2.3/0.3 and 0/2.6; diets ARA and DHA, respectively) were evaluated after 84 days in gilthead sea bream juveniles growth performance, feed utilization, body composition, plasma metabolites, carbohydrate and lipid metabolism, and LC-PUFA biosynthesis (chapter 6), liver and intestine oxidative stress and

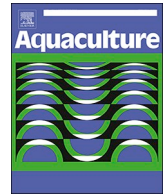
histomorphology and gut microbiota (chapter 7). Finally, at the end of the growth trial, fish were challenged with killed *Photobacterium damsela* subsp. *Piscicida* and after 4 and 24 hours the effects of the above diets before and after challenge in the hematological profile, plasmatic immune parameters, and immune-related gene expression were assessed (chapter 8).

Chapter 2

Effect of dietary ARA/EPA/DHA ratios on growth performance and intermediary metabolism of gilthead sea bream (*Sparus aurata*) juveniles

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Effect of dietary ARA/EPA/DHA ratios on growth performance and intermediary metabolism of gilthead sea bream (*Sparus aurata*) juveniles

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ABSTRACT

Vegetable oils (VO) are generally accepted as valuable alternatives to fish oils but are devoid of n-3 LC-PUFA (Long-Chain-Polyunsaturated Fatty Acids) and rich in C18-PUFA. Marine fish have limited capacity to express $\Delta 6/\Delta 5$ desaturases (FADS1 and FADS2) and elongases of very-long-chain fatty acids (ELOVL), which severely limits their capacity to produce LC-PUFA from C18-PUFA. This study aimed to evaluate the effects of dietary essential fatty acids (EFA) ratios, arachidonic (ARA), eicosapentaenoic (EPA), and docosahexaenoic (DHA) acids on growth performance, feed utilization, whole-body composition, hepatic and intestinal activity of key-enzymes of lipid and carbohydrate metabolism, and expression of lipid metabolism-related genes. Diets were formulated to include ARA/EPA/DHA ratios of 2.0/0.2/0.1 (Diet A); 1.0/0.4/0.4 (Diet B); 0/0.6/0.6 (Diet C); and 0/0.3/1.5 (Diet D) and were fed to triplicate groups of gilthead sea bream juveniles for 56 days.

Dietary EFA ratios did not affect growth performance and whole-body composition. Nevertheless, feed and protein utilization were higher with diet B than diets C or D. Except for ARA, muscle EFA profile followed that of the diets. Plasma triglycerides were higher with diet B and C than A, and plasma cholesterol decreased in fish fed the higher DHA diet. Hepatic ELOVL₅ and phospholipase A₂ were upregulated in fish fed diet C compared to diet A. In the intestine, ELOVL₅ levels were down-regulated in fish fed diet A and D compared to fish fed diet B.

Overall, results showed that besides EPA and DHA, adequate dietary ARA level needs to be provided to optimize feed utilization efficiency of gilthead sea bream juveniles.

1. Introduction

Aquaculture growth has been so high in the last decades that from 2014 onwards human population consumed more fish from aquaculture than wild-caught fish (FAO, 2016). This increase has been paralleled with the increased use of aquafeeds where fish meal and fish oil (FO) still are major dietary ingredients, particularly for carnivorous fish species (NRC, 2011). As the use of fisheries-based feedstuffs in aquafeeds is not environmentally or economically sustainable, for a steady increase of aquaculture it is, therefore, necessary to find sustainable alternatives to these fisheries-based ingredients.

Marine fish evolved in an environment where natural diets were rich in long-chain polyunsaturated fatty acids (LC-PUFA). Over the

years, marine fish lost or only has limited capacity to elongate and desaturate their precursors, linoleic (LA, 18:2n-6) and linolenic acid (ALA, 18:3n-3) to LC-PUFA (Jin et al., 2017; NRC, 2011; Torrecillas et al., 2018; Xue et al., 2014). Thus, arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3), and docosahexaenoic acid (DHA, 22:6n-3) are essential fatty acids (EFA) for marine fish. Therefore, marine fish rely on dietary sources of LC-PUFA for normal growth, development, and survival (Izquierdo, 2005; Jin et al., 2017; Rombenso et al., 2016; Xu et al., 2018). Further, LC-PUFA are also important for physiological functions related to lipid metabolism (Jin et al., 2017; Torrecillas et al., 2018), antioxidant status (Jin et al., 2017; Luo et al., 2012), skeletogenesis (Boglino et al., 2012; Lie et al., 2016), pigmentation (Lund et al., 2010; Villalta et al., 2008), stress tolerance (Liu

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et al., 2002; Rezek et al., 2010), and resistance to disease (Xu et al., 2016; Zuo et al., 2012a).

Over the last years, new sources of LC-PUFA, especially from microalgae and genetically modified *Camelina sativa* and yeast, reached the market giving the possibility to include the necessary EFA dietary content to optimize marine fish health and welfare (Tocher et al., 2019). Moreover, besides providing adequate levels of each EFA to meet requirements, it is also important to optimize the dietary balance of EFA. For instance, while no differences were observed in growth performance of juvenile black sea bream (*Acanthopagrus schlegelii*) fed diets with DHA/EPA ratios between 0.65 and 2.67, the highest ratios (2.03 and 2.67) decreased LC-PUFA biosynthesis and lipid anabolic pathway while increasing catabolic pathway and oxidative stress (Jin et al., 2017). In large yellow croaker (*Larimichthys crocea*), fed diets with similar lipid and LC-PUFA content, high dietary DHA/EPA ratios of 2.17–3.88 resulted in higher growth rates, nonspecific immunity, and immune-related gene expression than a dietary DHA/EPA ratio of 0.61 (Zuo et al., 2012b). In Atlantic salmon (*Salmo salar*), performance was improved with dietary inclusion of ARA/EPA at 0.7 ratio than with diets richer in ARA or EPA (ARA/EPA ratio of 2.4 and 0.1) (Norambuena et al., 2016). Also, in freshwater species, such as yellow catfish (*Pelteobagrus fulvidraco*), higher growth performance was found with moderate dietary inclusion levels of ARA (0.6 and 0.9%) when compared to fish fed low ARA (0.03 and 0.3%) levels (Ma et al., 2018). On the other hand, high dietary ARA levels (1.2 and 1.5%) increased liver oxidative stress. The diverse physiological responses to altered dietary n-6/n-3 ratios among species highlight the need to optimize these ratios for each species.

Gilthead sea bream (*Sparus aurata*) is the main farmed marine fish species in the Mediterranean region (Eurostat, 2019). Despite this, current knowledge on the EFA requirements and optimal dietary n-3/n-6 ratio for this species is limited. For juveniles, EPA and DHA requirements were estimated to be 0.7 and 0.6% of dry matter (DM), respectively (Izquierdo, 2005), while total n-3 LC-PUFA requirement was estimated to be around 0.9 or 1.9% of DM, depending on the dietary DHA/EPA ratio being 1 or 0.5, respectively (Houston et al., 2017; Ibeas et al., 1994; Kalogeropoulos et al., 1992; Tocher, 2010). Regarding ARA, no differences were observed in growth performance or feed utilization of sea bream juveniles with dietary ARA inclusion ranging from 0.2% to 11.2% of total fatty acids (FA) (Fountoulaki et al., 2003). However, for larvae fed diets with dietary EPA/DHA levels of 0.7/1.3% (Izquierdo, 2005) or 4/5% (Atalah et al., 2011), the inclusion of dietary ARA at levels of 1–1.2% increased growth and survival compared to fish fed ARA devoid diets.

Therefore, it is important to better understand the comparative effects of n-6 and n-3 EFA in marine fish species. Thus, the aim of this study was to evaluate the effect of different dietary ARA, EPA, and DHA ratios on growth performance, whole-body composition, muscle lipid content and FA profile, and relevant plasmatic, hepatic, and intestine parameters related to lipid metabolism in gilthead sea bream juveniles. For that purpose, four diets were tested with distinct n6 LC-PUFA/n-3 LC-PUFA ratios. The first diet was rich in ARA and had very low EPA + DHA content; the second diet was assumed to meet the EFA (n6 and n3 FA) requirements of gilthead seabream juveniles, the third diet meet n3-EFA requirements (with EPA and DHA ratio of 1:1) but was devoid of ARA; and the fourth diet also the meet n3-EFA requirements, but contained mainly DHA (EPA and DHA ratio of 0.2:1).

2. Material and methods

2.1. Diets composition

Four isoproteic (47% crude protein) and isolipidic (18% crude lipids) diets were formulated containing fish meal (FM) and vegetable feedstuffs (VF) as protein sources (26% protein from FM and 74% from VF) and a vegetable oil (VO) blend as lipid source (20:50:30 ratios from

rapeseed, linseed, and palm oils). Diets were supplemented with purified sources of EFA to obtain LC-PUFA ratios (% DM) of 2.0 ARA: 0.2 EPA: 0.1 DHA (Diet A); 1.0 ARA: 0.4 EPA: 0.4 DHA (Diet B); 0 ARA: 0.6 EPA: 0.6 DHA (Diet C); and 0 ARA: 0.3 EPA: 1.5 DHA (Diet D). ARA levels were achieved with a commercially available ARA oil obtained from *Mortierella alpina* (Vevodar®, DSM Food Specialties, the Netherlands); EPA levels with krill oil (*Euphausia superba*; SuperbaKrill™ Oil, Solchem®) and DHA with tuna oil (70% DHA; BrudyTechnology®). All dietary ingredients were thoroughly mixed and dry pelleted in a laboratory pellet mill (California Pellet Mill, CPM Crawfordsville, IN, USA) through a 3.0 mm die. All diets were supplemented with lysine, taurine, and dicalcium phosphate to avoid essential amino acid and phosphorus deficiencies. Pellets were dried in an oven at 40 °C for 48 h and then stored in airtight bags in a freezer until use. Ingredients and proximate composition of the experimental diets are presented in the additional file 1 - Table S1, and dietary FA composition is presented in the additional file 1 - Table S2.

2.2. Growth trial

The trial was performed in CIIMAR, Matosinhos, Portugal. Gilthead sea bream (*Sparus aurata*) juveniles were acquired from a commercial fish farm (Maresa S.A., Ayamonte, Huelva, Spain). The quarantine period lasted 1 month, and after that fish were moved to the experimental system and allowed to adapt to the experimental conditions for 15 days. The experimental system consisted of a recirculating water system equipped with 12 cylindrical fiberglass tanks of 100 L water capacity and thermo-regulated to 23.0 ± 1.0 °C. Tanks were supplied with a continuous flow of filtered seawater ($2.5\text{--}3.5$ L min⁻¹; salinity 35 ± 1 g L⁻¹; dissolved oxygen: 7 ± 1 mg L⁻¹) and controlled photoperiod of 12 h light and 12 h dark. At the beginning of the trial, 240 gilthead sea bream juveniles with an initial mean body weight of 15 g were grouped into 12 tanks. Each experimental diet was tested in triplicate and fish were fed by hand, twice daily, 6 days a week, until apparent visual satiation for 8 weeks. Utmost care was taken to avoid feed losses.

2.3. Sampling

At the end of the trial, fish in each tank were slightly anesthetized with 0.3 ml L⁻¹ of 2-phenoxyethanol and bulk-weighed after one day of feed deprivation. For whole-body composition analyses, ten fish from the initial stock population and three fish from each tank at the end of the trial were randomly sampled, euthanized by excess anesthesia (0.6 ml L⁻¹), and stored at -20 °C until analysis. Fish, liver, and viscera weights were recorded for measurement of hepatosomatic and visceral indices. The rest of the fish were then fed for two more days to allow recovery from final weighing manipulation stress. Thereafter, 4 h after the morning meal, three fish from each tank were randomly sampled for blood collection using heparinized syringes. Blood was collected from the caudal vein and immediately centrifuged at $10\,000 \times g$ for 10 min. The supernatant plasma was stored in aliquots and frozen at -80 °C for metabolites analysis. Thereafter, fish were euthanized and intestines of two and liver of three fish were dissected on ice-cold trays and stored for analysis of key intermediate metabolism enzymes. Two other fish were euthanized and dissected, the digestive tract discharged from adjacent adipose and connective tissues, and a small portion of the intestine and liver were sampled for gene expression analysis. The remaining liver was stored for quantification of liver glycogen and lipid content. The muscle of these fish was also sampled for total lipids and fatty acids profile quantification. All tissue samples were stored at -80 °C until used.

2.4. Chemical analysis

Chemical analysis of whole-fish and diets was performed following

the Association of Official Analytical Chemists methods (AOAC, 2000). Dietary starch was determined according to Beutler (1984), and hepatic glycogen as described by Plummer (1972). Hepatic and muscle lipid content was determined by the method of Folch et al. (1957) using dichloromethane instead of chloroform for muscle lipid content determination.

Fatty acid methyl esters were prepared by adding sodium methylate reagent and sequentially esterification with boron trifluoride in methanol according to Bondia-Pons et al. (2007) and analyzed in a Shimadzu GC-2010 gas chromatograph (Kyoto, Japan) equipped with a FID and a Shimadzu AOC-20i autoinjector.

2.5. Plasma metabolites analysis

Plasma cholesterol, glucose, triglycerides (TAG), and total protein were analyzed using enzymatic colorimetric kits from Spinreact, Girona, Spain (cholesterol kit, code 1001091; glucose kit, code 1001191; triglycerides kit, code 1001312; total proteins kit, code 1001291) as described in Magalhães et al. (2017).

2.6. Enzymatic activities

Liver and intestinal samples were homogenized (dilution 1:7 and 1:5, respectively) in ice-cold 100 mM Tris-HCl buffer containing 0.1 mM EDTA and 0.1% (v/v) Triton X-100, pH 7.8. The resulting homogenates were centrifuged at 30 000 × g for 30 min at 4 °C, and aliquots of the supernatants were separated and stored at −80 °C. All enzymatic assays were performed at 37 °C in a Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, Nanjing, China). To ensure maximal activity for each enzyme, the optimal substrate and protein concentrations were accessed by preliminary assays. The molar extinction coefficient used for NADPH was 6.22 mM^{−1} cm^{−1}. Glucokinase (GK, EC 2.7.1.2), hexokinase (HK, EC 2.7.1.1), L-type pyruvate kinase (PK, EC 2.7.1.40), fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11), fatty acid synthetase (FAS; EC 2.3.1.38) multienzymes complex activities, glucose 6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) and malic enzyme (ME, EC 1.1.1.40) were determined according to Guerreiro et al. (2014). β-hydroxyacyl-CoA dehydrogenase (HOAD, EC 1.1.1.35) activity was determined according to Pérez-Jiménez et al. (2009). Protein concentration in the homogenates was quantified following the Bradford method using Bio-Rad Protein Assay Dye Reagent (ref. 5000006) with bovine serum albumin as standard. Enzyme activity was expressed as milliunits per mg of hepatic or intestinal soluble protein.

2.7. Gene expression

Analyses of mRNA levels were performed on liver and intestine samples (two fish per tank). Total RNA was extracted using TRIzol reagent (Direct-zol™ RNA Miniprep, Zymo Research) according to manufacturer recommendations, and RNA quality and quantity were assessed by 1% agarose gel electrophoresis and spectrophotometry (μDrop™ plate, ThermoScientific). The resulting total RNA concentration was adjusted to 0.5 μg/8 μl H₂O. cDNA was produced using the NZY First-Strand cDNA Synthesis Kit (NZYTech, MB12501, Lisbon, Portugal). Gene expression was determined by real-time quantitative PCR (q-PCR) using the Bio-Rad, California, USA, CFX Connect Real-Time System. Analysis was carried out using 0.4 μl diluted cDNA (1:3 for liver samples and 1:2 for intestine samples) mixed with 0.2 μl of each primer (10 μM), 5 μl of SsoAdvanced™ Universal SYBR® Green Supermix, Bio-Rad Laboratories®, and 4.2 μl DNase/RNase/Protease-free water (Sigma-Aldrich), in a total volume of 10 μl. Primers were obtained from literature (see additional file 1 - Table S3). The slope of a standard curve using serial dilutions of cDNA measured PCR primers efficiency. Thermal cycling was initiated with incubation at 95 °C for 30s for hot-start iTaq™ DNA polymerase activation. A total of forty

PCR cycles were then performed, each one consisting of heating at 95 °C for 15 s for denaturing, and at 60 °C for 30 s for annealing and extension. Following the final PCR cycle, melting curves were systematically monitored (65 °C temperature 0.5 °C 10 s^{−1} from 65 to 95). Each PCR run included duplicates of reverse transcription for each sample and negative controls. The PCR run for the reference gene included duplicates for each sample and negative controls. Quantification of the target gene transcripts was performed using elongation factor 1α (EF1α) gene expression as a reference, as previously used for gilthead sea bream by Castro et al. (2016a). Relative quantification of the target gene transcript with EF1α reference gene transcript was performed using the mathematical model described by Pfaffl (2001). The relative expression ratio of a target gene was calculated based on real-time PCR efficiency and the cycle quantification value (CT) deviation of the unknown sample compared with the EF1α reference gene.

2.8. Statistical analysis

Data are presented as mean and pooled standard error of the mean. Normality and homogeneity of variances were tested by the Shapiro-Wilk and Levene tests, respectively, and normalized when appropriate (ln or log transformation). Statistical evaluation of the data was done by one-way ANOVA. When p-values were significant (p < 0.05), means were compared with Tukey's multiple range test. All statistical analyses were performed using SPSS 24.0 software package for Windows (IBM® SPSS® Statistics, New York, USA).

3. Results

Fish promptly accepted the experimental diets and survival was high and not affected by dietary treatment (Table 1). Growth performance and feed intake were not affected by diet composition, but feed efficiency and protein efficiency ratio were higher in fish fed diet B than diets C and D. However, nitrogen and energy retention (% intake) were also not affected by diet composition (Table 1).

Whole-body composition, muscle lipid content, hepatosomatic

Table 1

Growth performance, feed utilization and nitrogen and energy retention of gilthead sea bream fed the experimental diets.

Diets	A	B	C	D	SEM
(ARA/EPA/DHA ratio)	2.0/0.2/0.1	1.0/0.4/0.4	0/0.6/0.6	0/0.3/1.5	
Initial body weight (g)	15.0	15.0	15.0	15.0	0.0
Final body weight (g)	45.5	50.0	51.2	45.4	1.2
Weight gain (g kg ABW ^{−1} day ^{−1})	18.0	19.2	19.5	17.9	0.3
Daily growth index ^a	2.0	2.2	2.2	2.0	0.1
Feed intake (g kg ABW ^{−1} day ^{−1})	31.7	30.8	38.4	35.9	1.3
Feed efficiency ^b	0.57 ^{ab}	0.64 ^b	0.52 ^a	0.50 ^a	0.2
Protein efficiency ratio ^c	1.2 ^{ab}	1.4 ^b	1.1 ^a	1.1 ^a	0.0
Survival (%)	96.7	96.7	91.7	95.0	1.1
Nitrogen retention (% NI) ^d	21.0	23.4	19.0	19.2	0.8
Energy retention (% EI) ^e	26.3	28.1	24.8	23.9	0.9

Values presented as means (n = 3) and pooled standard error of the mean (SEM). Means in the same row with different superscript letters differ significantly (P < 0.05).

IBN/FBN or IBE/FBE: initial and final body nitrogen or energy content; NI: Nitrogen intake; EI: Energy intake; IBW and FBW: initial and final body weight. ABW: average body weight = ((initial body weight + final body weight)/2).

^a DGI: daily growth index = ((final body weight^{1/3} − initial body weight^{1/3})/time in days) × 100.

^b FE: feed efficiency = wet weight gain/dry feed intake.

^c PER: protein efficiency ratio = wet weight gain/crude protein intake.

^d Nitrogen retention = ((FBWxIBN − IBW x IBN)/NI) × 100.

^e Energy retention = ((FBWxFBE − IBW x IBE)/EI) × 100.

Table 2

Whole-body, liver, and muscle composition (% wet weight), hepatosomatic (HSI) and Visceral Somatic Index (VSI) of gilthead sea bream juveniles fed the experimental diets.

Diets	Initial	A	B	C	D	SEM
(ARA/EPA/DHA ratio)		2.0/0.2/0.1	1.0/0.4/0.4	0/0.6/0.6	0/0.3/1.5	
Whole-body composition						
Dry matter (%)	25.0	30.5	30.0	31.0	30.7	0.5
Protein	14.3	16.3	16.5	16.6	17.0	0.2
Lipids	6.1	10.5	10.0	11.5	10.0	0.4
Ash	5.5	4.3	4.0	3.8	4.2	0.2
Gross energy (kJ g ⁻¹)	5.9	8.6	8.3	9.0	8.7	0.1
Liver composition						
Lipid (%)	–	7.6 ^a	8.8 ^{ab}	10.9 ^b	11.2 ^b	0.5
Glycogen (%)	–	8.2	8.3	7.7	7.8	0.2
Muscle Lipids (%)	–	6.5	4.4	4.6	4.5	0.3
HSI^a (%)	–	1.3	1.3	1.3	1.2	0.3
VSI^b (%)	–	7.6	7.4	7.6	7.3	0.4

Values presented as means (n = 3 for body composition; n = 6 for liver composition and muscle lipids) and pooled standard error of the mean (SEM). Means in the same row with different superscript letters differ significantly (P < 0.05).

^a Hepatosomatic index: (liver weight/body weight) × 100.

^b Visceral Somatic Index: (Viscera weight/body weight) × 100.

index (HSI) and visceral somatic index (VSI) were not affected by dietary treatment (Table 2). Hepatic lipid content was higher in fish fed diets C and D than diet A, while hepatic glycogen content was not affected by diet composition (Table 2).

Muscle FA composition is presented in Table 3. Fish fed diet D had the highest ALA level while fish fed diet C had the lowest LA level. EPA and DHA levels varied according to the dietary inclusion level; the higher values were observed in fish fed diets C and D, respectively. Saturated fatty acids (SFA) content was lowest in fish fed diet D, while monounsaturated fatty acids (MUFA) content was higher with diet A, followed by diets B, C, and D, and this trend was mainly related to muscle C22:1 n-9 concentration. Fish fed diet D had the highest polyunsaturated fatty acids (PUFA) content, followed by fish fed diets C, B, and A, and this trend followed that of DHA content. Muscle ARA content was very low in all groups, but it was higher in fish fed the low ARA diets. Total n-3 FA content was highest in fish fed diet D, followed by C, B, and A while total n-6 FA content was higher in fish fed diets A and B than in fish fed diet C.

No differences in the activity of intermediary metabolism enzymes were observed in the intestine (Table 4), while in the liver, FAS activity was lower in fish fed diet D and HOAD activity was lower in fish fed diet C than diet A and B (Table 5).

No differences were found in plasma glucose and total protein content (Table 6). However, plasma triglycerides and cholesterol were higher in fish fed diets B and C than diets A and D, respectively.

Hepatic expression of Elov1₅ was higher in fish fed diets C than diet A, while expression of PLA₂ was higher in fish fed diet C than diet A (Table 7). No further differences were observed in the hepatic expression of other genes measured in relation to diet composition. In the intestine, only the expression of Elov1₅ was affected by dietary treatments, being higher in fish fed diet B than diets A and D (Table 8).

4. Discussion

Dietary EFA requirements of gilthead sea bream seem to be related both to dietary lipid level and EPA/DHA/ARA ratios. Thus, n-3 LC-PUFA requirements of juveniles were estimated to be 0.9% when EPA/DHA ratio was 1:1 and dietary lipid level was 13% (Kalogeropoulou et al., 1992), while with EPA/DHA ratio of 2:1 and 8% dietary lipids, n-

Table 3

Muscle fatty acid composition (% of total fatty acids) of gilthead sea bream fed the experimental diets.

Fatty acid	A 2.0/0.2/0.1	B 1.0/0.4/0.4	C 0/0.6/0.6	D 0/0.3/1.5	SEM
SFA					
C14:0	1.25 ^b	1.55 ^c	2.19 ^d	1.08 ^a	0.09
C15:0	0.14 ^a	0.15 ^{ab}	0.17 ^b	0.13 ^a	0.00
C16:0	15.0 ^a	16.2 ^b	17.3 ^c	15.2 ^a	0.22
C17:0	0.17 ^b	0.17 ^{ab}	0.15 ^{ab}	0.13 ^a	0.01
C18:0	4.49	4.43	4.37	4.09	0.08
C20:0	0.82 ^c	0.45 ^b	0.21 ^a	0.15 ^a	0.06
C22:0	1.11 ^c	0.69 ^b	0.22 ^a	0.16 ^a	0.08
C24:0	0.38 ^a	0.32 ^a	0.07 ^b	0.06 ^b	0.03
MUFA					
C16:1 n-7	1.93 ^a	2.23 ^b	2.75 ^c	1.89 ^a	0.08
C17:1 n-7	0.08 ^{ab}	0.09 ^{cb}	0.10 ^c	0.06 ^a	0.00
C18:1 n-9 c	29.7 ^a	30.0 ^a	31.6 ^b	30.5 ^a	0.19
C20:1n-9	0.72	0.71	0.74	0.78	0.02
C22:1 n-9	8.35 ^c	4.83 ^b	0.30 ^a	0.24 ^a	0.71
C24:1 n-9	0.24	0.25	0.25	0.22	0.01
PUFA					
C18:2 n-6 c (LA)	16.9 ^b	16.8 ^{ab}	16.3 ^a	16.9 ^b	0.08
C18:3 n-3 (ALA)	14.1 ^a	14.2 ^a	13.9 ^a	16.0 ^b	0.24
C18:3 n-6	0.26 ^c	0.25 ^{bc}	0.23 ^{ab}	0.20 ^a	0.01
C20:2 n-6	0.34	0.37	0.35	0.35	0.01
C20:3 n-3	0.27 ^a	0.36 ^{ab}	0.36 ^{ab}	0.47 ^b	0.02
C20:3 n-6	0.27 ^c	0.22 ^b	0.12 ^a	0.12 ^a	0.02
C20:4 n-6 (ARA)	0.10 ^a	0.10 ^{ab}	0.17 ^c	0.14 ^{bc}	0.01
C20:5 n-3 (EPA)	1.00 ^a	1.63 ^b	2.62 ^c	1.55 ^b	0.13
C22:6 n-3 (DHA)	2.30 ^a	3.92 ^b	5.52 ^c	9.65 ^d	0.58
ΣSFA	23.4 ^b	24.0 ^b	24.6 ^b	20.9 ^a	0.33
ΣMUFA	41.0 ^d	38.1 ^c	35.7 ^b	33.7 ^a	0.58
ΣPUFA	35.6 ^a	37.9 ^b	39.6 ^c	45.4 ^d	0.77
Σn3	17.7 ^a	20.1 ^b	22.4 ^c	27.7 ^d	0.78
Σn6	17.9 ^b	17.8 ^b	17.2 ^a	17.7 ^{ab}	0.09

Values presented as means (n = 6) and pooled standard error of the mean (SEM). Means in the same row with different superscript letters differ significantly (P < 0.05). FA: fatty acid; LA: Linoleic acid; ALA: α-Linolenic acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid.

Table 4

Specific activities of intestine intermediary metabolism related enzymes (mU/mg protein) in gilthead sea bream fed the experimental diets.

Diets	A 2.0/0.2/0.1	B 1.0/0.4/0.4	C 0/0.6/0.6	D 0/0.3/1.5	SEM
(ARA/EPA/DHA ratio)					
ME	12.1	11.0	13.4	11.0	0.80
FAS	–	–	–	–	–
HOAD	–	–	–	–	–
HK	17.1	14.0	14.4	14.5	0.83
GK	23.8	19.7	18.5	14.3	1.41
FBPASE	25.0	19.6	31.4	20.2	2.45
PK	26.1	20.1	21.6	22.1	2.13

Values presented as means (n = 6) and pooled standard error of the mean (SEM). Absence of superscript letters indicate no significant differences between dietary treatments (P > 0.05); – undetected enzymatic activity.

3 LC-PUFA requirement was estimated to be 1.9% (Ibeas et al., 1994). Recently, Houston et al. (2017) reported a dietary n-3 LC-PUFA requirement of circa 3% in diets with EPA: DHA ratio of 1:1 and 22% lipids. This is in line with the recommendation of Izquierdo (2005) that dietary n-3 LC-PUFA should be over 2.5% in actual commercial diets, which have high lipid contents. The effect of ARA on marine fish is less certain. When ARA:EPA ratios were changed from 0.04 to 1.7 in diets with a constant level of 2.7% n-3 LC-PUFA, there was no effect on

Table 5

Specific activities of liver intermediary metabolism related enzymes (mU/mg protein) in gilthead sea bream fed the experimental diets.

Diets	A	B	C	D	SEM
(ARA/EPA/DHA ratio)	2.0/0.2/0.1	1.0/0.4/0.4	0/0.6/0.6	0/0.3/1.5	
ME	5.3	5.0	4.5	3.9	0.25
FAS	5.4 ^b	5.2 ^b	5.4 ^b	2.9 ^a	0.31
HOAD	25.6 ^c	20.5 ^b	15.9 ^a	19.2 ^{ab}	0.77
HK	3.9	3.5	3.2	3.1	0.17
GK	4.2	3.5	3.3	3.5	0.15
FBPASE	23.9	20.7	19.8	20.6	0.64
PK	19.7	17.4	16.3	18.8	0.74

Values presented as means (n = 9) and pooled standard error of the mean (SEM). Means in the same row with different superscript letters differ significantly (P < 0.05).

Table 6

Plasma metabolites of gilthead sea bream juveniles fed the experimental diets.

Diets	A	B	C	D	SEM
(ARA/EPA/DHA ratio)	2.0/0.2/0.1	1.0/0.4/0.4	0/0.6/0.6	0/0.3/1.5	
Glucose (mg dl ⁻¹)	66.0	75.1	67.5	63.7	2.29
Total protein (g dl ⁻¹)	3.2	3.6	3.5	3.2	0.54
Triglycerides (mg dl ⁻¹)	317.7 ^a	662.7 ^b	681.6 ^b	492.5 ^{ab}	49.63
Total cholesterol (mg dl ⁻¹)	149.2 ^{ab}	154.2 ^b	154.5 ^b	128.4 ^a	3.50

Values presented as means (n = 9) and pooled standard error of the mean (SEM). Means in the same row with different superscript letters differ significantly (P < 0.05).

Table 7

Hepatic expression of genes involved in LC-PUFA biosynthesis (Elovl5, FADS2), lipoprotein assembly (ApoA1), transcription factor (Srebp1), lipolysis (LPL), TAG (DGAT), lipogenesis (FAS), lipid digestion (PLA2), lipid transport (FABP2), and cholesterol transport (ABGC5) in gilthead sea bream fed the experimental diets.

Diets	A	B	C	D	SEM
(ARA/EPA/DHA ratio)	2.0/0.2/0.1	1.0/0.4/0.4	0/0.6/0.6	0/0.3/1.5	
Elovl5	0.54 ^a	1.33 ^{ab}	2.02 ^b	1.79 ^{ab}	0.22
FADS2	1.41	1.26	1.28	1.12	0.21
ApoA1	539.5	503.2	788.7	458.7	74.1
Srebp1	0.26	0.43	0.81	0.52	0.10
LPL	1.61	2.28	3.09	1.77	0.43
DGAT	6.23	6.58	11.1	14.3	2.06
FAS	2.22	2.53	2.46	1.14	0.54
PLA2	13.3 ^a	20.6 ^{ab}	65.4 ^b	34.3 ^{ab}	8.17
FABP2	0.01	0.02	0.02	0.02	0.00
ABGC5	0.04	0.03	0.06	0.04	0.01

Expression were normalized by elongation factor 1-alpha (EF1α) expressed transcripts. Values presented as means (n = 6) and pooled standard error of the mean (SEM); for each gene, different letters indicate significant differences.

juvenile growth performance (Fountoulaki et al., 2003). However, Ibeas et al., (1997) found that higher dietary levels of EPA (1%) than DHA (0.5%) were required for maximum growth of juvenile sea bream, while Betancor et al. (2016) found reduced growth when juveniles were fed diets with high EPA:DHA ratios (2.4 and 0.75% DM, respectively; 18% of dietary lipidic content).

Diets used in this study were formulated to have very different n6/n3 EFA ratios and total n-3 LC-PUFA content. While diet A was rich in ARA (2%) and deficient in EPA:DHA (0.3%), diet B included ARA, EPA,

Table 8

Intestine expression of genes involved in LC-PUFA biosynthesis (Elovl5, FADS2), lipoprotein assembly (ApoA1), transcription factor (Srebp1), lipolysis (LPL), TAG (DGAT), lipogenesis (FAS), lipid digestion (PLA2), lipid transport (FABP2) and cholesterol transport (ABGC5) in gilthead sea bream fed the experimental diets.

Diets	A	B	C	D	SEM
(ARA/EPA/DHA ratio)	2.0/0.2/0.1	1.0/0.4/0.4	0/0.6/0.6	0/0.3/1.5	
Elovl5	0.039 ^a	0.071 ^b	0.051 ^{ab}	0.043 ^a	0.05
FADS2	0.014	0.009	0.024	0.011	0.00
ApoA1	0.103	0.082	0.084	0.119	0.04
Srebp1	0.038	0.026	0.048	0.031	0.01
LPL	0.019	0.024	0.025	0.027	0.00
DGAT	0.022	0.017	0.028	0.043	0.01
FAS	0.007	0.007	0.008	0.006	0.00
PLA2	0.002	0.001	0.002	0.001	0.00
FABP2	0.104	0.063	0.116	0.043	0.04
ABGC5	0.007	0.009	0.008	0.005	0.00

Expression were normalized by elongation factor 1-alpha (EF1α) expressed transcripts. Values presented as means (n = 6) and pooled standard error of the mean (SEM); for each gene, different letters indicate significant differences.

and DHA at levels which at least meet the minimum requirements, and diet C was almost devoid of ARA but had 1.2% EPA:DHA in a 1:1 ratio. Diet D was designed to have a 1.8% LC-PUFA level with a ratio of EPA:DHA of 1:5 and being almost devoid of ARA. The oils used in the presented study had their LC-PUFA attached to TAG and phospholipids (PL) mainly at the sn-2 position of glycerol. However, the position of the LC-PUFA was not the aim of the study and was not further analyzed.

Despite these differences in dietary ARA/EPA/DHA ratios and overall LC-PUFA content, the growth performance of gilthead sea bream juveniles was not affected by diet composition. This was unexpected as in the studies mentioned above, fish fed EFA deficient diets for periods similar to that of the present study had reduced performance. Although, according to Izquierdo (2005) juveniles may be able to survive for months on a diet with very low EFA content, their performance is expected to be affected, while in the present study sea bream performance was within or better than that obtained in other studies (Bandarra et al., 2011; Castro et al., 2016b; Guerreiro et al., 2015; Dias et al., 2009).

Thus, diet B seemed to meet EFA requirements of gilthead sea bream juveniles, while including only 0.73% DM of n-3 LC-PUFA in 18% lipid diet. This n-3 LC-PUFA content is slightly lower than that previously considered necessary to meet the n-3 LC-PUFA requirements of juveniles of this species (Kalogeropoulos et al., 1992) and well below recommendations of Izquierdo (2005) for high dietary lipid diets.

Despite the lack of effect on growth performance, this study showed that a balanced dietary n-6:n-3 LC-PUFA ratio was required to maximize feed utilization. Thus, higher FE and PER were observed in fish fed diet B, with ARA:EPA: DHA ratio of 1:0.4:0.4, than with diet C with ARA:EPA:DHA ratio of 0:0.6:0.6. Thus, FE and PER are affected by dietary n-6:n-3 LC-PUFA ratios.

The importance of appropriate dietary levels of ARA to improve feed utilization as demonstrated in this study for gilthead sea bream, has previously been highlighted for Japanese sea bass (*Lateolabrax japonicus*) (Xu et al., 2010), grass carp (*Ctenopharyngodon idellus*) (Tian et al., 2014), and Japanese eel (*Anguilla japonica*) (Shahkar et al., 2016).

Fish whole-body composition showed no differences between dietary treatments. This was expected, as previous studies in this and other species showed no major effects of dietary LC-PUFA ratios on whole-body composition (Jin et al., 2017; Norambuena et al., 2016; Fountoulaki et al., 2003). On the contrary, increasing dietary DHA and EPA levels decreased whole-body lipid content in Japanese sea bass (Xu et al., 2016). Further, in other fish species, it was also reported that whole-body lipids decreased in ARA supplemented diets (Norambuena

et al., 2016; Tian et al., 2014; Shahkar et al., 2016), though an excessive ARA supplementation resulted in lipid accumulation (Xu et al., 2010). Similarly, in the present study liver lipid content was also decreased in ARA supplemented diets.

It is known that dietary FA composition strongly influences muscle FA composition (Benedito-Palos et al., 2008; Izquierdo et al., 2003). In gilthead sea bream, when anchovy oil was replaced by soybean, rapeseed or linseed oil, muscle and liver content of EPA, DHA, and total n-3 LC-PUFA were accordingly reduced (Izquierdo et al., 2003). In the same way, FO substitution at 33%, 66%, or 100% by a VO blend (rapeseed, linseed, and palm oil) promoted a sequential reduction of muscle EPA and DHA content (Benedito-Palos et al., 2008).

Similarly, in this study muscle fatty acid n-3-PUFA content, namely EPA and DHA content, strongly reflected dietary FA composition. On the contrary, no such effect was observed for muscle n-6-PUFA content, namely for ARA content, which was very low and independent of dietary content. This was unexpected and contradicts previous results in this and other species (Fountoulaki et al., 2003; Shahkar et al., 2016; Torrecillas et al., 2018). Interestingly, muscle concentration of 22:1n-9 was correlated to that of dietary ARA. An unexpected result that needs to be further evaluated. In fact, fish and other vertebrates can produce 18:1n-9 from C18 FA by the action of stearoyl-CoA desaturase in response to n-3 LC-PUFA deficiency (Geay et al., 2011). Indeed, the presence of n-9-PUFA, particularly 18:1n-9 and 20:1n-9, were suggested to be a good indicator of EFA insufficiency in gilthead sea bream and grouper (*Epinephelus malabaricus*) (Rodriguez et al., 1993; Wu et al., 2002). Further, endogenous production of 22:1n-9 from 18:1n-9 was also observed in Atlantic salmon (Sissener et al., 2018). Accordingly, in the present study, high 22:1n-9 muscle content, 20:1n-9 product of elongation, was to be expected in fish fed diet A, which was almost devoid of EPA and DHA, but not in fish fed diet B, which seemed to include sufficient amounts of n-3 LC-PUFA.

Lipogenesis and lipolysis pathways are known to be regulated by dietary EFA (Castro et al., 2016a; Stubhaug et al., 2007). In the present study, dietary FA composition did not affect lipid metabolism at the intestine level, only at hepatic level. Indeed, high dietary ARA levels seemed to increase hepatic HOAD activity, a key enzyme involved in β -oxidation of FA (Wu, 2018). FA catabolism occurs in two organelles, mitochondria, and peroxisomes (Tocher, 2003). In mammals, these two biological pathways have different purposes, with mitochondrial β -oxidation being related to ATP production and peroxisome β -oxidation being involved in chain-shortening of long-chain FA that will be further oxidized in the mitochondria (Wu, 2018). Fish, however, appear to utilize peroxisome β -oxidation system at a significant level, including short-chain fatty acids (Stubhaug et al., 2007). Thus, the observed increase of hepatic HOAD may be related to increased catabolism of ARA and may explain the low ARA levels deposited in the muscle.

In the present study, high dietary DHA content in diet D promoted a reduction of hepatic FAS activity. Similarly, in mammals, DHA is known to be a potent inhibitor of FAS expression (Huang et al., 2017). Also in rainbow trout (*Oncorhynchus mykiss*) hepatocytes, n-3 LC-PUFA inhibited lipogenesis, but while EPA strongly inhibited FAS activity, DHA exerted a stronger effect on inhibiting acetyl-CoA carboxylase and G6PD activities (Alvarez et al., 2000).

Plasma glucose and total protein levels were within the reference values for gilthead sea bream (Peres et al., 2013) and were unaffected by dietary composition. Total plasma cholesterol was reduced in fish fed the high DHA diet, but no correlation was found with hepatic transcript levels of ABCG5, a protein involved in cholesterol transport. High dietary n-3 LC-PUFA level has been associated with hypocholesterolemia in both fish and humans (Hong et al., 2018; Pizzini et al., 2017). In fish, low-density lipoprotein (LDL) receptors are responsible for LDL clearance from plasma, and dietary FA modulates activity and expression of these receptors (Richard et al., 2006; Tocher, 2003). In rainbow trout, FO based diets increased LDL receptors gene expression in the liver, but also increased plasmatic cholesterol and LDL content

(Richard et al., 2006). However, the authors suggested that membrane fluidity increment by PUFA enrichment can increase LDL binding capacity leading to lower plasmatic cholesterol content, as observed in the present work.

ApoA₁ is the main apolipoprotein of plasmatic HDL, being the preferential receptor for free and remaining cholesterol (Xu et al., 2018). In the present study, there was a trend for hepatic ApoA₁ expression to be lower in fish fed the high DHA diet, though differences in ApoA₁ expression were not statistically different among groups. Previously, Xu et al. (2018) described in Japanese seabass a down-regulation of ApoA₁ transcription level with low (0.05–0.22%) and high (1.38–2.22%) dietary ARA content, and an upregulation with an intermediary (0.8%) dietary ARA content. However, no such ARA effect was observed in the present study.

Plasma TAG levels were lower in fish fed the highest ARA diet. Accordingly, liver expression levels of Srebp₁, LPL, and DGAT followed a similar trend. In Japanese seabass, liver downregulation of Srebp₁ and DGAT expression was previously associated with increased dietary ARA levels, but no effect on LPL expression level was reported (Xu et al., 2018). However, in yellow catfish, high dietary ARA (1.2 and 1.5%) levels increased SREBP₁ but not LPL gene expression (Ma et al., 2018). Contrary to present results, increasing DHA/EPA ratios in diets for juvenile black sea bream led to a decrease of SREBP₁ expression level while, similarly to present results, had no effect on LPL expression (Jin et al., 2017). Thus, present knowledge on the effect of dietary ARA in the regulation of Srebp₁, LPL, and DGAT is still very scarce and contradictory and needs to be further investigated.

As FAS is a target of Srebp₁, regulation of energy storage as TAG is mediated by this lipid-sensing transcription factor. DGAT is the most important enzyme involved in TAG synthesis and LPL hydrolyzes TAG in plasma lipoproteins providing FA for storage in adipose tissues (Betancor et al., 2016; Castro et al., 2016a; Minghetti et al., 2011). Accordingly, liver lipid deposition was also lower in fish fed the high ARA diet. Previously, upregulation of Srebp₁ and LPL in the liver, but not in the intestine, of gilthead sea bream fed VO based diets and supplemented with EPA and DHA compared to FO diets was already associated with more pronounced lipid deposition in the liver (Betancor et al., 2016). Similar results were observed in different fish species fed low n-3 LC-PUFA diets (Carmona-Antoñanzas et al., 2014; Limtipsuntorn et al., 2014; Peng et al., 2014).

Hepatic FABP₂ expression, a gene involved in lipid transportation and uptake, showed no differences between dietary treatments. In agreement, Castro et al. (2016a) also found no differences in FABP₂ expression in gilthead sea bream juveniles fed FO or VO-based diets. However, an upregulation of hepatic FABP₂ expression in gilthead sea bream fed high EPA: DHA diets was reported (Betancor et al., 2016).

Recent studies have already confirmed that gilthead sea bream expresses FADS₂, typically a $\Delta 6$ desaturase, as well as Elovl₅, an elongase highly efficient in elongation of C18 and C20 PUFA (Betancor et al., 2016; Castro et al., 2016b; Houston et al., 2017).

Present results indicate that high dietary ARA downregulated hepatic ELOVL₅, a result similar to that observed in grass carp and yellow catfish (Ma et al., 2018; Tian et al., 2014). High DHA/EPA ratio also decreased ELOVL₅, but this effect was only significant in the intestine. Similarly, in juvenile black seabream, increasing dietary DHA/EPA ratio decreased hepatic ELOVL₅ expression (Jin et al., 2017). This seems to indicate that maintaining an optimum dietary ratio of LC-PUFA is important for promoting FA elongation, and this will be of relevance in the new VO-rich aquafeeds.

In the present study, PLA₂ expression was downregulated in the liver of fish fed diets with high ARA level. A drastic reduction of PLA₂ expression was previously observed in gilthead sea bream larvae fed diets with ARA content above 0.4% (Alves Martins et al., 2012). In addition, salmon fed diets with high dietary n-6 PUFA and low n-3 PUFA levels also had reduced PLA₂ activity in the gills (Bell et al., 1996). ARA is primarily stored in the membrane PL and PLA₂

preferentially hydrolyzes ARA-containing PL, hydrolyzing the sn-2 ester bond of the cellular PL and generating free ARA (Gijón and Leslie, 1999). Thereby, the downregulation of PLA₂ expression in fish fed high ARA diets may be a negative feedback mechanism to maintain free ARA levels and its eicosanoids metabolites within normal physiological values. Moreover, fish fed low ARA and high EPA and DHA diets may present a higher cell-wall content of LC-PUFA and thus a higher vulnerability to peroxidation, leading to higher PLA₂ activity, as it is activated by peroxidized FA (Bell et al., 1996).

5. Conclusions

Overall, the results of this study showed that besides EPA and DHA, dietary ARA and ARA:EPA:DHA ratios need to be optimized for improved feed utilization efficiency of gilthead sea bream juveniles and for upregulating the expression of FA elongases. This is of high relevance when considering using low FO aquafeeds for marine fish, and such effect should be further evaluated in future studies.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

VO	vegetable oil
FO	fish oil
FM	fish meal
VF	vegetable feedstuffs
LC-PUFA	long chain polyunsaturated fatty acid
SFA	saturated fatty acid
MUFA	monounsaturated fatty acid
FA	fatty acid
EFA	essential fatty acid
ARA	arachidonic acid
EPA	eicosapentaenoic acid
DHA	docosahexaenoic acid
LA	Linoleic acid
ALA	α -Linolenic acid
DM	dry matter
ORBEA	Órgão responsável pelo bem-estar animal
TAG	triglycerides
FID	Flame ionization detector
EDTA	Ethylenediamine tetraacetic acid
GK	glucokinase
HK	hexokinase
PK	pyruvate kinase
FBPase	fructose-1,6-bisphosphatase
G6PD	glucose 6-phosphate dehydrogenase
ME	malic enzyme
HOAD	β -hydroxyacyl-CoA dehydrogenase
SEM	standard error of the mean
HSI	Hepatosomatic index
VSI	Visceral Somatic Index
ABW	average body weight
DGI	daily growth index
FE	feed efficiency

PER	protein efficiency ratio
IBN/FBN or IBE/FBE	initial and final body nitrogen or energy content
NI	Nitrogen intake
EI	Energy intake
IBW and FBW	initial and final body weight
LDL	low-density lipoprotein
PLA2	phospholipase A2
FABP2	FA binding protein
DGAT	Diacylglycerol acyltransferase
ApoA1	Apolipoprotein A1
LPL	Lipoprotein lipase
FAS	Fatty acid synthase
FADS1	Fatty acid desaturase 1
FADS2	Fatty acid desaturase 2
ELOVL	Elongation of very long chain fatty acids protein
Elovl5	Elongation of very long chain fatty acids protein 5
Srebp1	Sterol regulatory element-binding transcription factor 1
ABGC5	ATP binding cassette G5
EF1 α	elongation factor 1-alpha

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aquaculture.2019.734644>.

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Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions

R. E. O., H. P., and A.O.T. designed the study, R. M. and I. G. conducted the growth trial, R. M., H. P., F. C., S.M., S. S., C. D. M., and V.F.D. performed the experimental analysis and analyzed the data. R. M., H. P., and A. O. T. wrote the paper. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

The experiment was approved by CIIMAR ethical committee for Managing Animal Welfare (ORBEA), in compliance with the European Union directive 2010/63/EU and the Portuguese Law (DL 113/2013).

Consent for publication

Not applicable.

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Additional file 1

EFFECT OF DIETARY ARA/EPA/DHA RATIOS ON GROWTH PERFORMANCE AND INTERMEDIARY METABOLISM OF GILTHEAD SEA BREAM (*Sparus aurata*) JUVENILES

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Table S1. Ingredient and proximate analysis of experimental diets.

Diets (ARA/EPA/DHA ratio)	A 2.0/0.2/0.1	B 1.0/0.4/0.4	C 0/0.6/0.6	D 0/0.3/1.5
<i>Ingredients (% DM)</i>				
Fish meal ¹	17.5	17.5	17.5	17.5
Corn gluten meal ²	17.0	17.0	17.0	17.0
Soybean meal ³	17.5	17.5	17.5	17.5
Wheat gluten meal ⁴	10.5	10.5	10.5	10.5
Wheat meal ⁵	15.4	15.4	15.4	15.4
Taurine ⁷	0.3	0.3	0.3	0.3
Lysine ⁸	0.3	0.3	0.3	0.3
Vegetable oil blend ⁹	10.6	11.0	10.7	12.7
Choline chloride (50%)	0.5	0.5	0.5	0.5
Mineral premix ¹⁰	1.0	1.0	1.0	1.0
Vitamin premix ¹¹	1.0	1.0	1.0	1.0
Binder ¹²	1.0	1.0	1.0	1.0
CaHPO ₄	3.3	3.3	3.3	3.3
VEVODAR ¹³	4.2	2.1	—	—
EFA mix ¹⁴	—	1.7	4.1	—
DHA 70% Algatrium ¹⁵	—	—	—	2.1
<i>Proximate analyses (% dry weight)</i>				
Dry matter (%)	89.6	90.6	87.6	88.4
Crude Protein	46.5	46.9	47.4	47.8
Crude Lipid	18.7	17.8	18.3	19.1
Ash	7.4	8.0	7.6	7.7
Starch	13.1	10.3	10.3	11.2
Gross energy (kJ g ⁻¹)	21.9	21.5	21.5	21.4

Table S1. Ingredient and proximate analysis of experimental diets.

CP: Crude protein; CL: Crude lipids; DM: Dry matter.

¹Steam Dried LT-FM, Pesquera Centinela, Chile (CP: 69.7%; CL 7.2%).

²Sorgal, S.A. Ovar, Portugal (CP: 70.7%; CL: 3.6%).

³Sorgal, S.A. Ovar, Portugal (CP: 51.2%; CL: 3.7%).

⁴Sorgal, S.A. Ovar, Portugal (CP: 86.2%; CL: 3%).

⁶Sorgal, S.A. Ovar, Portugal. (CP: 14.5%; CL: 2.4%).

⁷Feed-grade taurine, Sorgal, S.A. Ovar, Portugal.

⁸Feed-grade lysine, Sorgal, S.A. Ovar, Portugal.

⁹Vegetable oil blend: 20% rapeseed oil + 50% linseed oil + 30% palm oil

¹⁰Minerals (mg kg⁻¹ diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g kg⁻¹ diet); potassium chloride, 1.15 (g kg⁻¹ diet); sodium chloride, 0.4 (g kg⁻¹ diet).

¹¹Vitamins (mg kg⁻¹ diet): retinol, 18000 (IU kg⁻¹ diet); calciferol, 2000 (IU kg⁻¹ diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.

¹²Carboxymethylcellulose sodium. Sigma-Aldrich Química, Portugal

¹³DSM Nutritional Products (ARA: 47.4 %FA; EPA: 0.11 %FA; DHA: 0.04 %FA)

¹⁴85% Krill oil (SuperbaKrill™ Oil, Solchem; ARA:0.78 %FA; EPA:21.1 %FA; DHA: 11 %FA) + 15% DHA

¹⁵BrudyTechnology (Tuna oil; ARA: 0 %FA; EPA: 10.2%FA; DHA: 89.5 %FA)

Table S2. Fatty acid composition of the experimental diets (% of total fatty acids).

Diets (ARA/EPA/DHA ratio)	A 2.0/0.2/0.1	B 1.0/0.4/0.4	C 0/0.6/0.6	D 0/0.3/1.5
SFA				
10:0	0.04	0.05	0.05	0.04
12:0	0.08	0.08	0.10	0.07
14:0	0.84	1.45	2.48	0.79
15:0	0.09	0.10	0.14	0.07
16:0	15.0	16.3	18.1	15.7
17:0	0.15	0.14	0.13	0.12
18:0	3.99	3.51	3.06	3.04
20:0	0.43	0.35	0.28	0.28
22:0	0.85	0.53	0.21	0.18
24:0	2.66	1.44	0.16	0.15
MUFA				
16:1i	0.06	0.07	0.09	0.06
16:1n-7	0.80	1.17	1.77	0.77
18:1n-9	23.2	24.5	25.7	26.2
18:1n-7	1.11	1.50	2.11	1.19
20:1n-11	0.05	0.05	0.05	0.03
20:1n-9	0.42	0.40	0.40	0.33
22:1n-11	0.19	0.19	0.21	0.17
22:1n-9	0.05	0.08	0.12	0.03
24:1n-9	0.19	0.17	0.16	0.12
PUFA				
16:2n-4	0.07	0.10	0.15	0.07
16:3n-4	0.06	0.07	0.08	0.06
16:4n-1	0.12	0.16	0.22	0.12
18:2n-6 (LA)	16.1	16.27	15.8	16.5
18:3n-6	0.52	0.28	0.04	0.01
18:3n-3 (ALA)	17.0	18.1	17.5	20.5
18:4n-3	0.12	0.28	0.48	0.11
20:2n-6	0.12	0.08	0.05	0.04
20:3n-6	0.94	0.49	0.04	0.02
20:4n-6 (ARA)	10.6	5.43	0.18	0.11
20:4n-3	0.05	0.07	0.10	0.05
20:5n-3 (EPA)	0.96	2.02	3.24	1.70
21:5n-3	0.04	0.12	0.21	0.50
22:4n-6	0.08	0.05	0.02	0.01
22:5n-3	0.12	0.29	0.49	1.33
22:6n-3 (DHA)	0.70	2.06	3.52	7.71
ΣSFA	24.2	24.1	24.9	20.6
Σ MUFA	26.2	28.2	30.8	28.9
Σ PUFA	47.7	46.0	42.2	48.8
Σn-6	28.4	22.6	16.2	16.6
Σn-3	19.0	23.0	25.5	31.9
n-6/n-3	1.50	0.98	0.63	0.52

FA: fatty acid; LA: Linoleic acid; ALA: α-Linolenic acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid.

Table S3. Primer pairs used for determination of the transcript level of several gilthead sea bream genes.

Gene	5'3' Forward primer	5'3' Reverse primer	Primer efficiency		Accession number	Reference
			Liver	Intestine		
PLA₂	CCAGACCATCTTCACCATCC	CACCCAATCCACAGGAGTTC	1.93	2.10	AM972037	Castro et al., 2016 ³⁷
FABP₂	CGAGCACATTCCGCACCAAAG	CCCACGCACCCGAGACTTC	1.95	1.94	AM957164	Castro et al., 2016 ³⁷
DGAT	GAGCCAATCACTGTGCCAAA	TGAGGAGGGTCTGAAGATGC	1.91	1.96	AM957740	Castro et al., 2016 ³⁷
ApoA₁	GAATACAAGGAGCAGATGAAGCAGATG	TGGTGACGGAGGCAGCGATG	1.90	2.04	AF013120	Castro et al., 2016 ³⁷
LPL	CGTTGCCAAGTTTGTGACCTG	AGGGTGTTCTGGTTGTCTGC	1.84	1.91	AY495672	Castro et al., 2016 ³⁷
FAS	TGAAACTGAAGCCCTGTGTG	TCTCGGCTGATGTTCTTGTG	1.99	2.1	AM952430	Castro et al., 2016 ⁴⁴
FADS₂	GCAGGCGGAGAGCGACGGTCTGTTCC	AGCAGGATGTGACCCAGGTGGAGGCAGAAG	1.91	2.06	AY055749	Houston et., 2017 ²²
Elovl₅	TGCCAGGACACTCACAGTGC	GGACGAAGCTGTTTAGGGAGG	1.95	2.11	AY660879	Castro et al., 2016 ⁴⁴
Srebp₁	AGGGCTGACCACAACGTCTCCTCTCC	GCTGTACGTGGGATGTGATGGTTTGGG	1.91	2.05	JQ277709	Houston et.,2017 ²²
ABGC₅	CCATCAGATGCCAGAGGTCT	GGGCAGGGAGGAAAGAGTAG	2.06	2.06	AM973022	Castro et al.,2016 ⁴⁴
EF1α	CTGTCAAGGAAATCCGTCGT	TGACCTGAGCGTTGAAGTTG	2.15	2.05	AF184170	Cerezuela et al., 2012 ⁶⁹

PLA₂, phospholipase A2; FABP₂, FA binding protein; DGAT, Diacylglycerol acyltransferase; ApoA1, Apolipoprotein A1; LPL, Lipoprotein lipase; FAS, Fatty acid synthase; FADS₂, Fatty acid desaturase 2; Elovl₅, Elongation of very long chain fatty acids protein 5; Srebp1, Sterol regulatory element-binding transcription factor 1; ABGC5, ATP binding cassette G5; EF1 α , elongation factor 1-alpha

Chapter 3

Oxidative status and intestinal health of gilthead sea bream (*Sparus aurata*) juveniles fed diets with different ARA/EPA/DHA ratios.

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Oxidative status and intestinal health of gilthead sea bream (*Sparus aurata*) juveniles fed diets with different ARA/EPA/DHA ratios

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The present work assessed the effects of dietary ratios of essential fatty acids, arachidonic (ARA), eicosapentaenoic (EPA) and docosahexaenoic acid (DHA), on liver and intestine oxidative status, intestinal histomorphology and gut microbiota of gilthead sea bream. Four isoproteic and isolipidic plant-based diets were formulated containing a vegetable oil blend as the main lipid source. Diets were supplemented with ARA/EPA/DHA levels (%DM) equivalent to: 2%:0.2%:0.1% (Diet A); 1.0%:0.4%:0.4% (Diet B); 0%:0.6%:0.6% (Diet C); 0%:0.3%:1.5% (Diet D) and tested in triplicate groups for 56 days. Lipid peroxidation was higher in fish fed diets C and D while no differences were reported between diets regarding total, oxidized, and reduced glutathione, and oxidative stress index. Glutathione reductase was higher in fish fed diet A than diets C and D. No histological alterations were observed in the distal intestine. Lower microbiota diversity was observed in intestinal mucosa of fish fed diet C than A, while diets C and D enabled the proliferation of health-promoting bacteria from Bacteroidetes phylum (*Asinibacterium* sp.) and the absence of pathogenic species like *Edwardsiella tarda*. Overall, results suggest that a balance between dietary ARA/EPA + DHA promotes gilthead sea bream juveniles' health however higher dietary content of n-3 LC-PUFA might limited the presence of microbial pathogens in intestinal mucosa.

In marine fish species, dietary supply of linoleic (LA; 18:2n-6) and α -linolenic (ALA; 18:3n-3) acids cannot meet essential fatty acids (EFA) requirements due to the evolutionary loss or low capacity of long-chain polyunsaturated fatty acids (LC-PUFA) biosynthesis^{1–3}. In gilthead sea bream (*Sparus aurata*), the leading finfish species in Mediterranean aquaculture, the limited capacity to synthesize LC-PUFAs from C-18 precursors is related to low activities of the $\Delta 5$ fatty acid desaturase and elongase^{2,4}. Thus, dietary supply of the main LC-PUFA, arachidonic acid (ARA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3), and docosahexaenoic acid (DHA; 20:6n-3) is necessary to fulfill the physiological requirements of EFA of this species^{3,5}.

For gilthead sea bream juveniles, total dietary EPA + DHA requirements vary with lipid level and DHA/EPA ratio and were estimated to be 0.9 or 1.9% with a DHA/EPA ratio of 1 or 0.5, respectively^{6–8}. However individual EPA and DHA requirements have been reported to be 0.7 and 0.6% of dry matter (DM), respectively (reviewed by⁹). As for ARA, the only available study indicates that increasing the dietary levels (0, 0.6, 1.14, 1.7%DM) has no effect on growth performance¹⁰. However, in European sea bass juveniles, dietary ARA levels below 0.2% reduced growth performance¹¹.

In marine aquafeeds, the main source of LC-PUFA has been fish oil (FO) but, due to its stagnant availability in the world market, its dietary replacement by alternative lipid sources is required for promoting further growth of the marine aquaculture industry.

Thus, in recent years, research focused on FO replacement by more available and sustainable oil sources, such as vegetable oils (VO)^{12–15}, land animal oils^{13,16–18}, or microalgae oils^{19–21}. However, most of the commercially

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available alternative oil sources, namely VO and animal oils, are deficient in LC-PUFA and its dietary inclusion may jeopardize normal fish growth, health, intestinal histomorphology, and microbiota community^{5,22–24}.

LC-PUFAs are highly susceptible to peroxidation, generating reactive oxygen species (ROS) causing tissue oxidative damage^{14,25,26}.

ROS induce damage to lipids, proteins, carbohydrates, and nucleic acids, with the potential to induce cellular death and compromising tissue functionality and palatability of the final product^{14,25}. LC-PUFA oxidation can compromise membrane structure, by decreasing its fluidity and increasing its permeability to harmful substances, with potential to be pathological to the cell or tissue²⁷. The balance between generation and clearance of ROS is maintained through the action of the antioxidant system, involving radical scavenging enzymes (catalase, CAT; superoxide dismutase, SOD; glutathione reductase, GR; and glutathione peroxidase, GPX) and non-enzymatic antioxidants (Vitamin A, C, and E; glutathione, GSH; bilirubin and flavonoids)^{25,28}. However, this balance may be highly affected by diet composition, including the level of LC-PUFA. For instance, increasing dietary levels of n-3 LC-PUFA were associated with increased liver and plasma oxidative stress of silvery black porgy (*Sparidentex hasta*)²⁹. Also, high dietary content of n-3 LC-PUFA increased oxidative stress in the muscle of rainbow trout, *Oncorhynchus mykiss*³⁰, the intestine of European sea bass (*Dicentrarchus labrax*)³¹ and liver and intestine of gilthead sea bream¹⁴. Increasing levels of ARA were also shown to increase liver oxidative stress in *Synechogobius hasta* juveniles³² and Japanese eel serum (*Anguilla japonica*)³³.

Dietary DHA/EPA ratio also seems to affect oxidative stress, apparently in a species-specific manner. For instance, higher ratios caused increased oxidative damage in serum of black sea bream (*Acanthopagrus schlegelii*) while the opposite was observed in the plasma of silvery-black porgy juveniles^{2,34}. In an in vitro study, it was shown that increasing concentrations of EPA, but not of DHA, increased lipid peroxidation levels in yellow croaker (*Larimichthys crocea*) macrophages³⁵. Further, while EPA increased superoxide anion production the opposite was observed for DHA. Thus, EPA and DHA seem to have different capacities for modulating redox mechanisms.

Intestinal microbiota is vital to promote fish physiological balance and health, and its effects on fish feeding, digestion, metabolism, immunity, and disease resistance have been highlighted^{24,36,37}. Fish intestinal microbiota is modulated by several factors including host genetic background, living environment, section of the gastrointestinal tract, stress, and diet^{24,36,37}. Intestinal microbiota seems to be particularly sensitive to diet composition^{12,24,38,39}. However, the impact of dietary PUFA, particularly dietary LC-PUFAs, on gut microbiota has been poorly explored both in humans and aquatic species^{12,24,40}.

Previously, Magalhães et al.⁴¹ showed that a diet with a balanced ARA/EPA/DHA (1/0.4/0.4) ratio that closely matches the estimated EFA requirements of gilthead seabream increased feed efficiency and protein efficiency ratio compared to diets with unbalanced ARA/EPA/DHA ratios (0/0.6/0.6 and 0/0.3/1.5). Dietary ARA/EPA/DHA ratios also affected lipid metabolism, with increased β -oxidation activity in fish fed high ARA/EPA/DHA (2.0/0.2/0.1) and decreased lipogenesis in fish fed high DHA (0/0.3/1.5). Furthermore, liver lipid content was higher in fish fed high dietary EPA and DHA levels.

The objective of this study was to further evaluate dietary ARA/EPA/DHA ratios of the fore mentioned diets on oxidative stress in target organs (liver and intestine), distal intestine histomorphology, and gut microbiota composition of gilthead sea bream juveniles. Therefore, the importance of dietary ARA or n-3 LC-PUFA on these indicators of wellbeing was compared to the EFA balanced diet.

Results

The results of the feeding trial are presented elsewhere⁴¹ as it was not the aim of this study.

Oxidative stress. Oxidative stress indicators, namely liver and intestine enzymatic activities and glutathione responded similarly to the diet treatments, as no organ $\times$ diet interaction in any parameter analyzed was observed (Tables 1 and 2).

Liver and intestinal glutathione and oxidative stress index (OSI) were not affected by dietary treatments (Table 1). Oxidized glutathione (GSSG) and OSI values were higher while reduced glutathione (GSH) was lower in the intestine compared to liver. However, total glutathione (tGSH) was similar in both tissues. Further, LPO was higher in the intestine than in the liver and in fish fed diets C and D than diets A and B (Table 1).

Regarding antioxidant enzymes, no organ $\times$ diet interaction was observed (Table 2). G6PDH and GPX activities were higher in the liver than in the intestine, while the opposite was true for CAT, GR, and SOD activities. Regarding dietary effects,

GR activity was higher in fish fed diet A than diets C and D, whereas G6PDH, SOD, and GPX activities were unaffected by dietary treatments. For CAT, although Anova indicates a significant dietary effect, that effect was not detected by the Tukey's multiple range test.

Distal intestine histological evaluation. Distal intestine histomorphology parameters analyzed, namely enterocyte vacuolization, height of mucosal folds, width of lamina propria and leukocyte infiltration of the lamina propria and submucosa were not affected by dietary treatments (Table 3; Fig. 1).

Intestinal microbiota. Number of OTU's, species richness, in both digesta and mucosa, and digesta diversity were not affected by dietary treatments, except for species diversity in the intestinal mucosa of fish fed diet C which was lower than in fish fed diet A (Table 4).

The Bray–Curtis dendrogram showed that for digesta samples the three dietary replicates failed to cluster (Fig. 2A). For mucosa samples, dendrogram showed that diets with and without ARA supplementation cluster separately. A clearer visualization of this result can be seen in the MDS plot of DGGE bands (Fig. 2B).

Organ	Liver					Intestine				
	A	B	C	D		A	B	C	D	
Diets	2.0/0.2/0.1	1.0/0.4/0.4	0/0.6/0.6	0/0.3/1.5	SEM	2.0/0.2/0.1	1.0/0.4/0.4	0/0.6/0.6	0/0.3/1.5	SEM
tGSH	1017	935	928	739	51.8	947	797	884	788	42.8
GSSG	39	29	36	28	3.8	111	114	135	133	7.5
GSH	977	906	893	711	49.0	837	675	748	655	42.3
OSI ¹	7.19	6.12	7.25	7.00	0.5	23.7	27.7	33.9	34.3	2.2
LPO	13.5	14.9	18.7	19.5	0.8	69.6	62.6	90.6	79.2	3.0
Variance source					Diets					
	Diet	Organ	Interaction	A	B	C	D			
Two-way Anova										
tGSH	Ns	Ns	ns	–	–	–	–			
GSSG	Ns	0.000	ns	–	–	–	–			
GSH	Ns	0.043	ns	–	–	–	–			
OSI ¹	Ns	0.000	ns	–	–	–	–			
LPO	0.000	0.000	ns	a	a	b	b			

Table 1. Liver and intestine total glutathione (tGSH), oxidized glutathione (GSSG), reduced glutathione (GSH), oxidative stress index (OSI) and lipid peroxidation (LPO) levels of gilthead sea bream fed the experimental diets. Values presented as means (n = 9 for liver and n = 6 for intestine) and pooled standard error of the mean (SEM). LPO values expressed as nmols MDA g⁻¹ tissue and GSH, tGSH, and GSSG as nmol g⁻¹ tissue Two-way ANOVA: ns: non-significant ($P > 0.05$). ¹OSI = $100 \times (2 \times \text{GSSG}/\text{tGSH})$.

Organ	Liver					Intestine				
	A	B	C	D		A	B	C	D	
Diets	2.0/0.2/0.1	1.0/0.4/0.4	0/0.6/0.6	0/0.3/1.5	SEM	2.0/0.2/0.1	1.0/0.4/0.4	0/0.6/0.6	0/0.3/1.5	SEM
CAT	56.7	51.8	42.1	48.5	1.68	153.8	92.4	76.6	158.6	16.0
G6PDH	136.6	132.1	127.8	110.0	5.25	24.6	25.8	31.0	20.2	1.66
GR	8.8	7.3	7.0	6.6	0.28	20.7	15.9	14.9	16.1	0.96
SOD	106.9	126.9	110.3	108.5	6.59	1,341.4	1,220.3	1,232.5	929.0	63.7
GPX	54.2	36.4	38.5	35.5	3.22	10.2	9.6	9.6	9.0	0.64
Variance source					Diets					
	Diet	Organ	Interaction	A	B	C	D			
Two-way Anova										
CAT	0.022	0.000	ns	–	–	–	–			
G6PDH	Ns	0.000	ns	–	–	–	–			
GR	0.008	0.000	ns	b	ab	a	a			
SOD	Ns	0.000	ns	–	–	–	–			
GPX	Ns	0.000	ns	–	–	–	–			

Table 2. Liver and intestine antioxidant enzymes activity of gilthead sea bream fed the experimental diets. Values presented as means (n = 9 for liver and n = 6 for intestine) and pooled standard error of the mean (SEM). Enzyme activities expressed as mU mg protein⁻¹ for G6PDH, GR, and GPX and as U mg protein⁻¹ for CAT and SOD Two-way ANOVA: ns: non-significant ($P \geq 0.05$).

	A	B	C	D	
Diets	2.0/0.2/0.1	1.0/0.4/0.4	0/0.6/0.6	0/0.3/1.5	SEM
Fold height	2.7	1.8	2.3	2.5	0.13
Lamina propria	2.5	2.2	1.7	2.7	0.17
Submucosa	1.0	1.0	1.0	1.0	0.00
Intraepithelial leucocytes	1.8	2.2	2.7	2.3	0.17
Enterocytes	1.5	2.0	1.7	2.8	0.26
Mean score	1.9	1.8	1.9	2.3	0.08

Table 3. Score-based histological evaluation of the distal intestine in gilthead sea bream juveniles fed the experimental diets Values presented as means (n = 6) and pooled standard error of the mean (SEM). Absence of superscript letters indicates no significant differences between dietary treatments ($P > 0.05$).

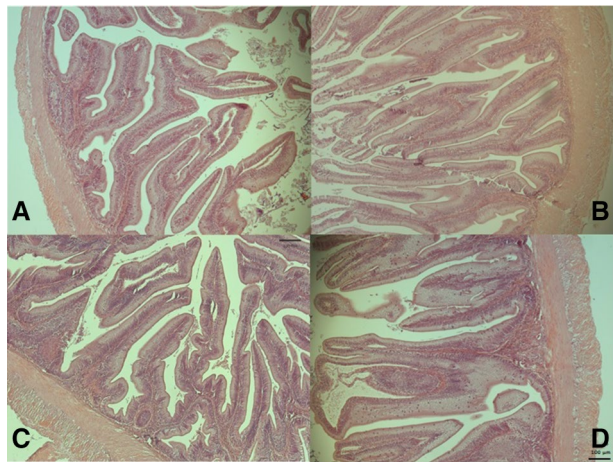


Figure 1. Histomorphology features of distal intestine of gilthead sea bream juveniles fed the experimental diets (A–D) recorded with Zen software (Blue edition). Normal height of mucosal folds, normal width lamina propria with normal leukocyte infiltration of the lamina propria and submucosa. No signs of inflammation. H-E staining.

Diets	Digesta					Mucosa				
	A	B	C	D	SEM	A	B	C	D	SEM
	2.0/0.2/0.1	1.0/0.4/0.4	0/0.6/0.6	0/0.3/1.5		2.0/0.2/0.1	1.0/0.4/0.4	0/0.6/0.6	0/0.3/1.5	
OTUs ¹	21.0	22.0	17.7	20.7	0.64	28.7	25.0	22.0	24.0	1.07
Richness ²	1.2	1.3	1.0	1.2	0.04	1.7	1.5	1.3	1.4	0.06
Diversity ³	2.9	3.0	2.8	2.9	0.03	3.3 ^b	3.2 ^{ab}	3.0 ^a	3.1 ^{ab}	0.05
Similarity (%) ⁴	80.7	85.8	77.8	85.4	1.37	83.9	76.6	73.2	83.5	2.31

Table 4. Ecological parameters obtained from PCR-DGGE fingerprints of the intestinal allochthonous (digesta) and autochthonous (mucosa) microbiota of gilthead sea bream fed with the experimental diets. Values presented as means (n = 3) and pooled standard error of the mean (SEM). Different superscript letters indicate significant differences between dietary treatments ($P < 0.05$). ¹OTUs: Average number of operational taxonomic units. ²Margalef species richness: $d = (S - 1) / \log(N)$ where S is the number of species, and N is the total number of individuals in the sample. ³Shannons diversity index: $H' = - \sum (p_i \ln p_i)$ where p_i is the proportion of individuals belonging to the i th species present in the sample. ⁴SIMPER, similarity percentage within group replicates.

Identification of the selected DGGE bands from the digesta and mucosa microbiota is shown in Table 5. Sequence analysis of 35 DGGE bands showed 20 sequences below 97% of sequence similarity, indicating a poor resolution for species identification. However, at the genus level identification, sequence analysis revealed that the dominant allochthonous (digesta) bacterial genus belonged to the Proteobacteria phylum, with three bands of *Klebsiella* genus detected, two bands of *Pantoea*, and one of *Nitrospira*. Three bands of *Lactobacillus* (phylum Firmicutes) were also identified.

Autochthonous (mucosa) bacteria analysis revealed one genus of the phylum Bacteroidetes (*Asinibacterium* sp.) and 4 bands of the phylum Firmicutes (1 *Lactobacillus* and 3 bands of the genus *Bacillus*). Nevertheless, the predominant phylum present in the mucosa was Proteobacteria with 15 bands identified belonging to the *Pseudomonas*, *Thalassomonas*, *Klebsiella*, *Janthinobacterium*, *Thiohalophilus*, *Massila*, *Edwardsiella* and *Ralstonia* genera (Table 5).

Discussion

The diets in the presented study had different n6/n3 EFA ratios as well as total n-6 and n-3 LC-PUFA content. Thus, Diet A was rich in ARA and had low EPA and DHA levels; diet B had balanced ARA, EPA and DHA levels; diet C had no ARA and was balanced in EPA and DHA; Diet D had no ARA and had high levels of DHA than EPA.

It is well known that susceptibility of fatty acids (FA) to peroxidation increases with unsaturation, as the weak carbon-hydrogen bond of the bis-allylic methylene groups is particularly prone to oxidation²⁵. In the present study, liver and intestine TBARS levels, a well-accepted physiological index of tissue LPO, were inversely related to the dietary n-6/n-3 ratio (diets C and D) and this may be related to increased levels of n-3 LC-PUFA in these tissues. Indeed, a positive correlation between LC-PUFA content in different tissues and lipid peroxidation was already demonstrated in several fish species^{14,29,31,42}. Even though liver and intestine FA profiles were

not determined in the present study, muscle EPA and particularly DHA contents were higher in fish fed diets C and D than the other diets, while ARA content showed only slight variations⁴¹ and it is expected that the FA concentrations in liver and intestine should follow a similar pattern. Therefore, a lower concentration of EPA and DHA might explain the lower LPO values in ARA rich diets. Previously it was already observed that the degree of unsaturation of dietary FA correlates with the FA unsaturation in tissues^{43,44} as well as its susceptibility to oxidation^{14,45}.

The higher LPO content appeared to be related to elevated contents of DHA in diets C and D. One reason for this may relate to the tendency of DHA to accumulate in mitochondrial cardiolipin that would impair electron transport efficiency and increasing ROS production⁴⁶. The fact that ARA and EPA are not incorporated into mitochondrial cardiolipin may explain the lower LPO content in fish fed diets A and B⁴⁶.

The primary defense against oxidative stress includes antioxidant enzymes such as GR, SOD, CAT, and GPX²⁵. GR is essential for maintenance of GSH level by reducing GSSG. The activity was higher in fish fed the high ARA, diet A, followed by diet B, while CAT, SOD and GPX activities were not affected in this experiment. This suggests that the glutathione system was important in the modulation of the redox environment of fish in relation to the dietary EFA composition. This is also supported by tGSH and GSH levels, which were higher (though not statistically higher), and the LPO levels, which were lower, in fish fed diets A and B than the other diets. Similar to the present results, other authors have also reported that higher dietary ARA levels improved the response of antioxidant enzymes in fish^{32,33,47}.

As already reported in gilthead sea bream juveniles^{48,49} the antioxidant defense mechanism responded differently in liver and intestine. CAT, GR, SOD, and GSSG were higher in the intestine, while G6PDH and GPX activities were higher in the liver. Overall, LPO values were also considerably higher in the intestine than in the liver, which agrees with previously published data⁴⁸. Higher intestine LPO and OSI values were expected due to the high enterocyte turnover rate that increases susceptibility to oxidation.

In the present study, the dietary effect on intestine histomorphology was accessed in the distal section as previous studies showed that this intestine portion is more sensitive to dietary treatments than the anterior and mid-intestine^{50,51}. However, distal intestine histomorphology was not affected by the different dietary n-6/n-3 LC-PUFA levels used in the experimental diets. This confirms previous studies on mid- or distal- intestines of European seabass fed diets with 60–70% of VO blends^{52,53}. Also, gilthead sea bream juveniles and Atlantic salmon fed diets with wild type or genetically modified Camelina oil (richer in ARA, EPA, and DHA) showed no differences in distal intestine morphology^{54–56}. On the contrary, an accumulation of enterocytes lipid droplets with dietary VO incorporation, leading to lower dietary content of ARA, EPA, and DHA was reported in gilthead sea bream juveniles⁵⁷.

The dietary ARA, DHA, and EPA ratios used in this study, altered gilthead sea bream intestinal microbiota at the mucosal level. The MDS plot and the Bray–Curtis dendrogram representation of mucosal data showed a higher similarity between microbial communities of fish fed diets with elevated levels of EPA/DHA (diets C and D) than within fish fed diets with high ARA content (diets A and B). This shows the potential of dietary fatty acids to select for bacterial species affecting attachment sites of the intestinal mucosa resulting in establishment of different microbial communities²⁴. In fact, the isolation of the genus *Thalassomonas* spp., *Janthinobacterium* spp., *Thiohalophilus* spp. and *Massilia* spp. were only isolated in diets A and B. Also, diet C contained 1.2% of EPA + DHA showed lower microbial diversity (Shannon index) at the intestinal mucosa than the other diets. Previous studies reported that 58% and 84% substitution of dietary FO by VO lowered Shannon's diversity index in gilthead sea bream juveniles⁵⁹. Similarly, other PUFA, such as 2.5% of LA (18:2n-6), were also shown to reduce the culturable intestinal microbiota diversity of Arctic charr (*Salvelinus alpinus*)⁶⁰. On the other hand, the dietary inclusion of 0.5% 1-monoglycerides of short- and medium-chain FA (from C3 to C12) did not influence the same diversity index in gilthead sea bream juveniles⁶¹. The mechanism for dietary PUFA to reduce fish gut microbiota is not fully understood. It is however interesting to note that reduced bacterial diversity also seems to occur in humans with increase intake of n-3 LC-PUFA⁴⁰. Here the reduction of gut microbial diversity was accompanied by an increase of potential butyrate-producing bacterial species (e.g., *Asinibacterium* sp.), a short-chain fatty acid (SCFA) known to promote gastrointestinal health and host immunity⁴⁰. Accordingly, in Arctic charr fed casein-based diets supplemented with LA, ALA (18:3n-3), or EPA and DHA, the frequency of lactic acid bacteria (LAB, commonly used as probiotics and associated with benefits to host), was higher in diets supplemented with 7% of ALA or 4% of EPA + DHA⁵⁸. Despite this, in our study, the main SCFA-producing species such as *Bacillus* spp. and *Lactobacillus* spp. (LAB)⁶² were present in both digesta and mucosa samples without significant changes between diets.

As reported for other fish species³⁶, Proteobacteria and Firmicutes were the dominant phyla in both digesta and mucosa microbiota of gilthead sea bream juveniles in this study, and appear to be predominant in many fish species regardless of dietary treatment^{59,61,63–65}. While digesta microbial community is considered transient, mucosal community is assumed to be more stable³⁶. The colonization of gut mucosal epithelium is essential for establishing health-promoting bacterial species as autochthonous microbiota and, as consequence, reduce the establishment of opportunistic bacteria^{36,37}. The presence of pathogenic species such as *Klebsiella* spp.⁶⁶, despite the different intensity of the bands, was equally distributed among all dietary treatments, and previously isolated from different fish species, suggesting that these microorganisms are part of normal fish microbiota^{67–69} or an opportunistic pathogen attacking stressed animals⁷⁰.

The isolation of one bacterial species from Bacteroidetes phylum (*Asinibacterium* sp.) in diets C and D might highlight the influence of n-3 LC-PUFA in autochthonous microbiota. Although *Asinibacterium* sp. is a recently described genus⁷¹ whose metabolic potential is not yet totally known, bacteria from Bacteroidetes phylum are also (adding to the Firmicutes, in particular *Bacillus* spp. and *Lactobacillus* spp.⁶²), related to the fermentation of dietary non-starch polysaccharides, starch, and sugars into SCFAs as acetate and propionate⁷². These SCFA are absorbed and used as energy sources by the enterocytes and have anti-inflammatory and bactericidal activities⁶¹.

In fact, the isolation of a fish-pathogen such as *Edwardsiella tarda*, causative agent of edwardsiellosis with severe losses in aquaculture⁷³, in fish fed with lower levels of n-3 LC-PUFA (diets A and B) might be another indication of the positive effect n-3 LCPUFA had on gilthead sea bream mucosal microbiota.

Increasing bactericidal activity was already associated with long carbon chain length and degree of unsaturation of dietary FA^{58,74}. Thus, the higher content of n-3 LC-PUFA might have limited the presence of pathogens such as *E. tarda* in the mucosal microbial community of fish fed diets C and D. Accordingly, a very recent study with golden pompano (*Trachinotus ovatus*) showed that FO substitution by VO increased the abundance of intestinal pathogenic bacteria such as *Mycoplasma* and *Vibrio*⁷⁵. As recently reviewed⁷⁶, gut microbial community, host immune system, and dietary n3-PUFA are interdependent pieces that together control intestinal wall integrity, and thus pathogen proliferation. Furthermore, the host-derived ROS was previously associated with bactericidal activity against pathogens such as *Salmonella* in mice⁷⁷. Also, ROS was associated with gut epithelial response to microbial signals and stimulating immune responses against bacteria⁷⁸. Thus, more studies are required to confirm if increasing ROS production may impair pathogenic bacterial proliferation as it was reported in the present study for fish fed diets C and D.

We report significant modifications in the oxidative status and mucosa microbiota with different dietary n-6/n-3 LC-PUFA content, revealing the importance of correct EFA ratios for gilthead sea bream health. This is especially important with increasing VO incorporation in aquafeeds. The impairment of mucosa colonization by pathogenic bacteria reduces the risk of fish infection and consequently the need for antibiotics utilization, leading to a better image of fish produced in aquaculture to the consumers.

Material and methods

Diets composition. Four plant-based diets were formulated as described in Magalhães et al.⁴¹. Shortly, the experimental diets were isoproteic (47% crude protein; 74% protein from plant feedstuffs and 26% protein from fish meal) and isolipidic (18% crude lipids) with a vegetable oil blend (20:50:30 rapeseed, linseed, and palm oils) as main lipid source. *Mortierella alpine* oil (Vevodar, DSM Food Specialties, the Netherlands); krill oil (*Euphausia superba*; SuperbaKrill Oil, Solchem); and tuna oil (70% DHA; BrudyTechnology) were used to adjust the ARA, EPA, and DHA levels of the diets, to obtain a final ARA/EPA/DHA level of 2%:0.2%:0.1% (Diet A); 1.0%:0.4%:0.4% (Diet B); 0%:0.6%:0.6% (Diet C); 0%:0.3%:1.5% (Diet D). The fatty acids profile of the oils was confirmed before diets were formulated. Ingredients, proximate composition and fatty acids composition of the experimental diets are presented in supplementary file 1—Table S1, and dietary FA composition is presented in additional file 1—Table S2.

Experimental trial

The growth trial was conducted in CIIMAR, Matosinhos, Portugal. Gilthead sea bream (*Sparus aurata*) juveniles were obtained from a commercial fish farm (Maresa S.A., Ayamonte, Huelva, Spain). Fish were moved to the experimental system after a quarantine period of 1 month and allowed to adapt to the experimental conditions for 15 days. The trial was conducted in a thermo-regulated recirculating marine water system (23.0 ± 1.0 °C; 35 ± 1 g L⁻¹ salinity; 7 mg L⁻¹ oxygen) equipped with 12 cylindrical fiberglass tanks. Tanks (100 L water capacity) were supplied with filtered seawater (flow of 2.5–3.5 L min⁻¹) and kept under controlled photoperiod (12:12 h of light:dark). At the beginning of the experiment, a total of 240 gilthead sea bream juveniles with an initial mean body weight of 15 g were utilized and groups of 20 fish were distributed into the 12 experimental tanks. Each experimental diet was randomly assigned to triplicate groups. The trial was carried on during 56 days and fish were fed by hand, until apparent visual satiation, two times a day, 6 days a week. Extreme care was taken to minimize feed waste.

Sampling. At the end of the growth trial, fish were randomly sampled 4 h after the morning meal, euthanized by decapitation and dissected on chilled trays. The adjacent adipose and connective tissues of the intestine was removed from 2 fish per tank, and a small portion of the distal intestine (DI, differentiated by a darker mucosa and enlarged diameter from the mid intestine) was collected for histomorphology assessment. Therefore, DI samples were cleaned in phosphate-buffered saline (PBS), carefully absorbed with a paper towel and promptly fixed in phosphate-buffered formalin (4%, pH 7.4) for 24 h and immediately changed to ethanol (70%) until further processing according to Couto et al.⁵¹. The rest of the intestine was stored at -80 °C until quantification of oxidative stress enzymatic and non-enzymatic indicators and lipid peroxidation levels. Liver from 3 fish was also sampled for the same purpose. Two other fish per tank were sampled under aseptic conditions (working with an open flame using sterilized solutions, collecting tubes and tools) for allochthonous (digesta) and autochthonous (mucosa) microbiota characterization. Mucosa samples were collected by scraping the internal intestinal mucosal surface, after opening the intestine in its longitudinal axis. Digesta samples were obtained by squeezing the entire intestinal content into a sterile tube. Samples were instantly frozen with liquid nitrogen and stored at -80 °C until analyzed.

Chemical analysis. The Association of Official Analytical Chemists methods AOAC⁷⁹ were used to perform the chemical analysis of experimental diets. Dietary starch was quantified as described by Beutler⁸⁰; The Folch et al.⁸¹ method was utilized for total lipids determination using dichloromethane instead of chloroform. FA methyl esters of oil ingredients and diets were prepared by transmethylation of total lipids extract and analyzed by chromatography using Shimadzu GC-2010 Plus gas chromatograph (Shimadzu Europe GmbH, Germany) equipped with a flame-ionization detector (GC-FID) and an Omegawax 250 capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness; Supelco, Bellefonte, USA). FA were identified by comparison with known standard mixtures as described by Magalhães et al.⁴¹.

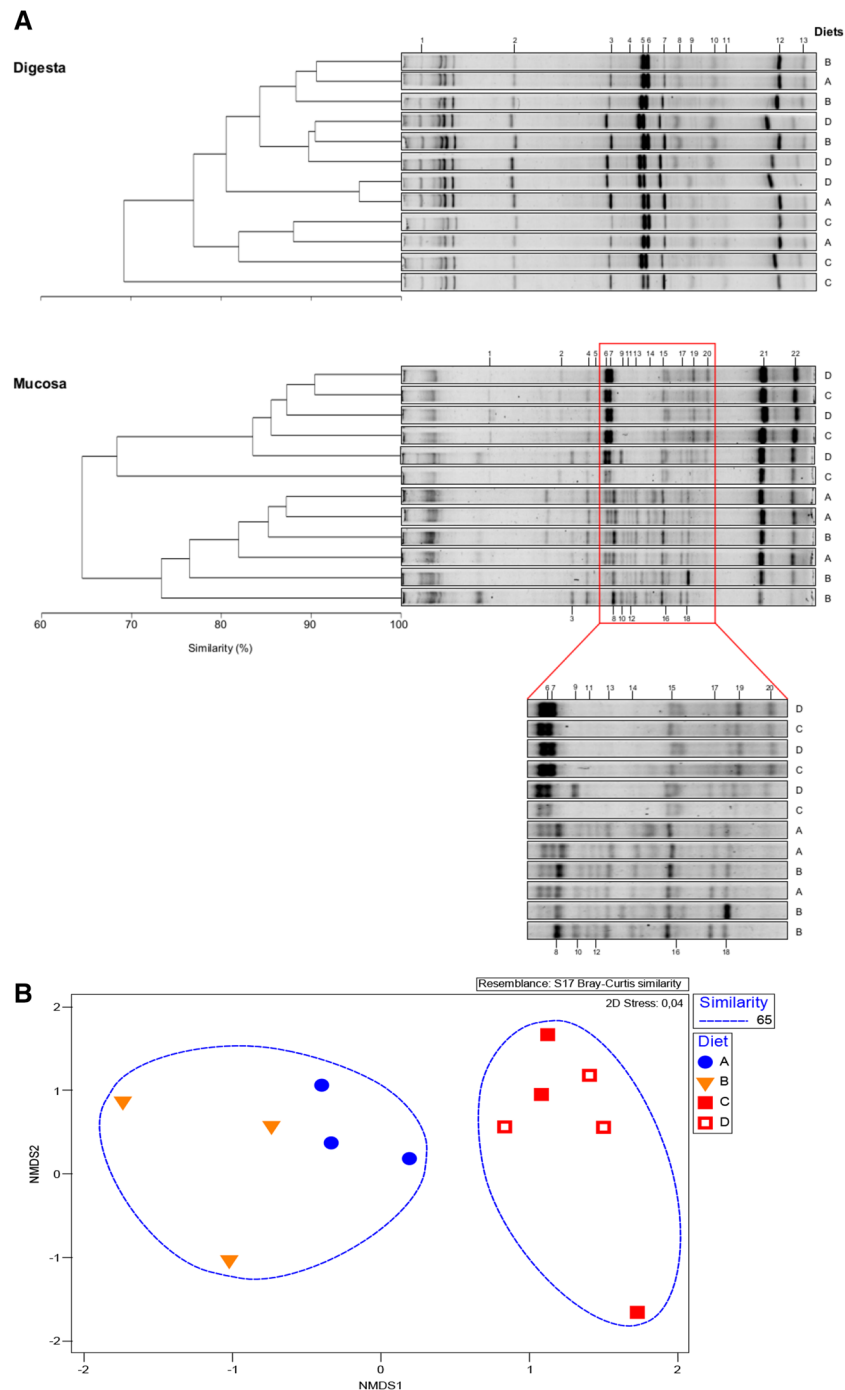


Figure 2. **A** Dendrograms and PCR-DGGE fingerprints of the allochthonous (digesta) and autochthonous (mucosa) intestinal microbiota of gilthead sea bream fed the experimental diets. Numbers (1 to 13 on digesta; 1 to 22 on mucosa) indicate bands excised for sequence analysis, identified on Table 5. **B** Multidimensional scaling (MDS) plot of DGGE bands presence and abundances in intestinal mucosa samples from gilthead sea bream juveniles fed the same experimental diets depicted in A: Diet A (●); Diet B (▼); Diet C (■); Diet D (■). Dotted lines represent 65% similarity between samples.

Enzyme activity. Liver and intestine samples were homogenized (dilution 1:7 and 1:5, respectively) in ice-cold 100 mM Tris-HCl buffer, containing 0.1 mM EDTA and 0.1% (v/v) Triton X-100, pH 7.8. All the procedures were performed on ice. Homogenates were centrifuged at 30,000×g for 30 min at 4 °C and supernatants were divided into aliquots and stored at – 80 °C until use. All assays were carried out at 37 °C in a Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, Nanjing, China). The specific assay conditions for each enzyme were as follows:

Band	Closest known species (BLAST)	Phylum	ID (%)	Accession nr
Digesta				
1	<i>Lactobacillus delbrueckii</i>	Firmicutes	89	MF784102.1
2	<i>Lactobacillus aviarius</i>	Firmicutes	99	LC071826.1
3	<i>Lactobacillus helveticus</i>	Firmicutes	99	KX951719.1
4	<i>Pantoea</i> sp.	Proteobacteria	86	KR029059.1
5	<i>Klebsiella pneumoniae</i>	Proteobacteria	100	MG923524.1
6	<i>Klebsiella</i> sp.	Proteobacteria	98	KT301470.1
7	Uncultured bacterium	n/a	97	LC031369.1
8	Uncultured bacterium clone OTU5449	n/a	84	KT788917.1
9	<i>Pantoea</i> sp.	Proteobacteria	90	KR029059.1
10	<i>Klebsiella pneumoniae</i>	Proteobacteria	89	MG923524.1
11	<i>Nitrospira</i> sp.	Proteobacteria	79	AY293079.1
12	Uncultured bacterium clone ac-25 16S rRNA	n/a	92	KY857639.1
13	<i>Klebsiella pneumoniae</i>	Proteobacteria	96	MH266241.1
Mucosa				
1	Uncultured bacterium clone SHFH766	n/a	94	KT981153.1
2	<i>Asinibacterium</i> sp.	Bacteroidetes	96	KP301113.1
3	<i>Lactobacillus helveticus</i>	Firmicutes	98	KX951719.1
4	<i>Pseudomonas veronii</i>	Proteobacteria	96	Kt302366.1
5	<i>Thalassomonas</i> sp.	Proteobacteria	90	MG819691.1
6	<i>Klebsiella pneumoniae</i>	Proteobacteria	99	MH150802.1
7	<i>Klebsiella pneumoniae</i>	Proteobacteria	97	KU550763.1
8	<i>Janthinobacterium</i> sp.	Proteobacteria	91	AB196254.1
9	<i>Bacillus</i> sp.	Firmicutes	98	FJ195793.1
10	<i>Thiohalophilus</i> sp.	Proteobacteria	81	KX599116.1
11	<i>Massilia</i> sp.	Proteobacteria	97	KY912083.1
12	<i>Massilia</i> sp.	Proteobacteria	93	FN386762.1
13	<i>Edwardsiella tarda</i>	Proteobacteria	96	MF034724.1
14	<i>Bacillus</i> sp.	Firmicutes	78	JX191913.1
15	<i>Ralstonia</i> sp.	Proteobacteria	95	MG238553.1
16	<i>Klebsiella</i> sp.	Proteobacteria	86	KT300944.1
17	<i>Planktothricoides</i> sp.	Cyanobacteria	95	JX628815.1
18	<i>Bacillus halodurans</i>	Firmicutes	98	MH299852.1
19	<i>Klebsiella pneumoniae</i>	Proteobacteria	99	MH266241.1
20	<i>Klebsiella pneumoniae</i>	Proteobacteria	98	MH266241.1
21	<i>Klebsiella pneumoniae</i>	Proteobacteria	100	CP029226.1
22	<i>Klebsiella variicola</i>	Proteobacteria	100	KR092086.1

Table 5. Closest relatives (BLAST) to the sequenced PCR-DGGE gel bands (from Fig. 2) of the intestinal communities of gilthead sea bream fed the experimental diets.

Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activity was analyzed by measuring the reduction of NADP⁺⁸².

Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured by the ferricytochrome method using xanthine/xanthine oxidase as the source of superoxide radicals⁸³. One unit of activity was defined as the amount of enzyme necessary to produce a 50% inhibition of the ferricytochrome c reduction rate.

Catalase (CAT; EC 1.11.1.6) activity was assessed by measuring the decrease of hydrogen peroxide concentration⁸⁴.

Glutathione peroxidase (GPX; EC 1.11.1.9) activity was determined by measuring the NADPH consumption rate generated by the oxidized glutathione (GSSG) produced by GPX activity and reduced by glutathione reductase (GR)⁸⁵.

Glutathione reductase (GR; EC 1.6.4.2) activity was analyzed by measuring the oxidation of NADPH, according to Morales et al.⁸⁶.

Soluble protein concentration was determined according to Bradford⁸⁷ using Sigma-Aldrich protein assay kit and bovine serum albumin as standard.

Except for SOD and CAT which are expressed as units per mg of soluble protein, the activities of the other enzymes are expressed as milliunits per mg of soluble protein. One unit of the enzyme was defined as the amount of enzyme required to transform 1 mmol of substrate per min under the assay conditions.

Lipid peroxidation. Malondialdehyde (MDA) concentration was used as a marker of lipid peroxidation (LPO) level in the liver and intestine following the methodology described by Buege and Aust⁸⁸. In the presence of thiobarbituric acid, MDA reacts producing colored thiobarbituric acid reacting substances (TBARS) that were measured using a spectrophotometer at 535 nm. Results were calculated from an MDA calibration curve.

Total and oxidized glutathione. Liver and intestine samples were homogenized (1:10 and 1:5, respectively) in ice-cold solution containing 1.3% 5-sulfosalicylic acid (w/v) and 10 mM HCl, and the whole procedure was done in ice to avoid glutathione oxidation. Homogenates were centrifuged at 14,000g for 10 min at 4 °C and the supernatants stored at −80 °C. Total glutathione (tGSH) and oxidized glutathione (GSSG) were determined accordingly Griffith⁸⁹ and Vandeputte et al.⁹⁰ with some modifications according to Castro et al.¹⁴. Standard curves of reduced glutathione (GSH) and GSSG were used for tGSH and GSSG calculations, respectively. GSH level was calculated by subtracting GSSG from tGSH values.

Histological processing and morphological evaluation. Distal intestine samples were processed and sectioned using standard histological techniques and stained with hematoxylin and eosin. Blind evaluation of histological preparations was performed, analyzing mucosal folds height, width, and cellularity of the lamina propria and submucosa, number of intraepithelial lymphocytes, nucleus position size, and variation of enterocyte vacuolization^{91,92}. A scale scoring system ranging from 1 (normal) to 5 (highly modified) was used as described in Penn et al.⁹³. The overall score of histomorphology alterations was calculated by averaging scores of all the parameters. Images were acquired with Zen software (Blue edition; Zeiss, Jena, Germany).

Microbial diversity. Samples of 2 fish per tank were pooled to reduce variability. The extraction of bacterial DNA from fish intestinal digesta and mucosa was done by weighting around 300 mg of sample to a 2 mL bead-beater (Sigma-Aldrich, Buchs, Switzerland) tube previously prepared with 500 µL STE buffer (0.1 M NaCl, 10 mM Tris, 1 mM EDTA, pH 8) and 0.4 g of glass beads (Sigma-Aldrich G8772). Samples were then homogenized twice for 30 s in the BeadBug bead-beater (Benchmark Scientific, Edison, NJ, USA) at 2500 speed with an interval of at least 30 s on ice. Following 15 min incubation at 75 °C, with gentle agitation every 5 min, tubes were centrifuged for 1 min at 13,000g and 500 µL of supernatant was transferred to new sterile 2 mL micro-centrifuge tubes. From this point, the protocol used for bacterial DNA extraction was based on the method of Pitcher et al.⁹⁴.

Bacterial 16S rRNA gene fragments were amplified using a touchdown PCR on a T100Thermal Cycler (Bio-Rad Laboratories Lda., Amadora, Portugal), using oligonucleotide primers 16S-358F (which contained a GC clamp at the 5' end) and 16S-517R⁹⁵. 300 ng of each PCR product was resolved on 8% polyacrylamide gel composed by a denaturing gradient of 40–60% 7 M urea/40% formamide. DGGE was performed using a DCode universal mutation detection system (Bio-Rad Laboratories Lda.) during 16 h at 60 °C, 65 V in 1 × TAE buffer. Gels were stained for 1 h with SYBR-Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific, Waltham, Massachusetts, EUA) and imaged on a Gel Doc EZ System (Bio-Rad Laboratories Lda., Amadora, Portugal). Distinct bands were excised from the gel and eluted in 20 µL ultrapure water prior to DNA re-amplification using the same oligonucleotide primers as above, but without the GC clamp⁹⁵. Amplicons were sequenced to identify microbiota OTUs (Operational Taxonomic Units). Phylogenetic analysis, to identify the closest known species, was done by comparison with sequences in the GenBank non-redundant nucleotide database using BLAST (<https://www.ncbi.nlm.nih.gov>) (Macrogen Europe, Amsterdam, The Netherlands). Only sequences higher than 100 bp reads and 80–100% query coverage were considered valid identification.

Statistical analysis. Data are presented as the mean and pooled standard error. Normality and homogeneity of variances were tested by the Shapiro–Wilk and Levene tests, respectively, and normalized when appropriate. Statistical evaluation of the data was done by one-way or two-way ANOVA. When *p* values were significant (*p* < 0.05), the means were compared with Tukey's multiple range test. Histological data were analysed by the Kruskal–Wallis non-parametric test because the data were neither normal nor homogeneous and could not be normalized. All statistical analyses were performed using SPSS 24.0 software package for Windows (IBM SPSS Statistics, New York, USA).

Microbiota data analysis was done according to Serra et al.⁶⁹ using the DGGE banding patterns, the band's intensity was measured with Quantity One 1-D Analysis Software v4.6.9 (Bio-Rad Laboratories Lda., Amadora, Portugal) and converted into absence/presence matrices. The calculation of the relative similarities between experimental groups and replicates was done with Primer software v7.0.5. 5 (PRIMER-E, Ivybridge, UK). Non-metric multidimensional scaling (MDS) was based on Bray–Curtis similarities using relative band abundances. Data representation from MDS was considered reliable considering the Kruskal stress value (< 0.2)⁹⁶. Species Richness was established with the use of Margalef's diversity index, while Shannon–Weaver index was utilized to establish species diversity. Similarity percentages (SIMPER) were utilized to represent the relative similarities between studied groups.

Ethics approval and consent to participate. The experiment was approved by CIIMAR ethical committee for Managing Animal Welfare (ORBEA), in compliance with the European Union directive 2010/63/EU and the Portuguese Law (DL 113/2013).

Data availability

The data generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

R. E. O., H. P., and A.O.T. designed the study, R. M. and I. G. conducted the growth trial and sampling, R. M., H. P., F. C., R.A.S., C.R.S., and A.C. performed the experimental analysis and analyzed the data. R. M., H. P., and A. O. T. wrote the paper. All authors reviewed and approved the final manuscript.

Competing interests

The authors declare no competing interests.

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Chapter 4

Effect of different dietary arachidonic, eicosapentaenoic, and docosahexaenoic acid content on selected immune parameters in gilthead sea bream (*Sparus aurata*).

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Effect of different dietary arachidonic, eicosapentaenoic, and docosahexaenoic acid content on selected immune parameters in gilthead sea bream (*Sparus aurata*)

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ABSTRACT

The increasing replacement of fish oil (FO) by vegetable oils (VO) in aquafeeds for marine carnivorous species may lead to modifications of the dietary arachidonic (ARA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) levels and ratios that may compromise the fish immune status. Therefore, this study aimed to assess how different dietary ratios of ARA, EPA, and DHA modulate the immune response of gilthead sea bream juveniles. For that purpose, four isoproteic and isolipidic plant-based diets were formulated to include different dietary Long-Chain-Polyunsaturated Fatty Acids (LC-PUFA) ratios (%DM): Diet A (2.0% ARA: 0.2% EPA: 0.1% DHA); Diet B (1.0% ARA: 0.4% EPA: 0.4% DHA); Diet C (0% ARA: 0.6% EPA: 0.6% DHA); Diet D (0% ARA: 0.3% EPA: 1.5% DHA). Fish were fed the experimental diets for 56 days and then the haematological profile, humoral innate immune parameters, and expression of immune-related genes in the gut were determined. Our results showed that fish fed diet B exhibited an increase in the monocyte numbers, alternative complement activity, and bactericidal activity against *Photobacterium damsela* compared to the values found in fish fed other diets. Contrarily, peroxidase, antiproteases, and proteases activity and nitric oxide levels were not affected by the dietary treatments. Immune-related gene expression in the distal intestine did not show differences between dietary treatments. Overall, a diet with a balanced n-6 (ARA) and n-3 (EPA+DHA) LC-PUFA ratio seemed to better modulate the fish innate immune response, thus better preparing the fish innate immune system against potential immunological insults.

1. Introduction

Vegetable oils (VO) have been increasingly used as a more sustainable source of energy for aquafeeds [1] and are regarded as the major alternative for fish oil (FO) replacement in the diets due to their steady availability and lower price. However, in contrast to FOs, which are rich in LC-PUFA, namely eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids, VOs are devoid of Long-Chain-Polyunsaturated Fatty Acids (LC-PUFA) [2, 3]. LC-PUFA are essential for marine fish since they have limited expression of $\Delta 6$ and 5 desaturases, which are necessary to synthesize LC-PUFA from their

C18-PUFA precursors [4]. This difficult meeting the essential fatty acids (EFA) requirements of marine fish species using VO as the major dietary lipid source [5]. Nevertheless, different VO blends have been used in the diets to replace FO at different levels in several marine fish species without loss of growth performance, proving that the EFA requirements are met [6]. For gilthead sea bream (*Sparus aurata*), 66 to 69 % of FO substitution by VO blend, soybean oil, or rapeseed oil has been achieved without significant effects on growth performance [7, 8].

The total n-3 LC-PUFA requirement for gilthead sea bream is still not adequately estimated and requirements of 0.9 or 1.9 % were reported for 42 g and 1 g fish, respectively [9, 10]. Further, the dietary DHA/EPA

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ratio of the diets was 1 and 0.5, which further complicates defining requirements as the optimum dietary n-3/n-6 ratio is still not defined. ARA requirement for gilthead sea bream juveniles was not yet established, but no variations in growth performance and feed utilization seem to occur with dietary ARA levels ranging from 0 to 1.7 % [11].

Besides growth, variations in the dietary n-3/n-6 fatty acids (FA) ratio due to the use of VO may have a significant impact on fish health, since many physiological functions are directly dependent on the dietary balance of these FA [4, 12]. For instance, the FA composition of cell membranes is affected by dietary lipids and can affect the immune cell FA composition [13–15]. Therefore, it can modulate the immune response in fish [14, 16] by modulating the physical properties of cell membranes and membrane-associated enzymes and receptor sites [17], immune cells functionality (i.e. phagocytosis and respiratory burst activity) [13, 18–20], humoral defenses (i.e. lysozyme and alternative complement activity) and eicosanoid production [18, 19, 21, 22].

However, the mechanisms by which EFA modulates fish immune response are still not fully understood. Arachidonic acid content in the cell membrane is a substrate for cyclooxygenase-2 (Cox₂) and 5-lipoxygenase (5-LOX) enzymes, that synthesize eicosanoids such as prostaglandin E₂ (PGE₂) and 4-series leukotrienes that promotes leucocytes chemotaxis, reactive oxygen species formation, and other pro-inflammatory effects [23]. Contrarily, n-3 LC-PUFA are known to compete with ARA as a substrate for the mentioned enzymes to produce less inflammatory eicosanoids such as PGE₃ and 5-series leukotrienes and reducing the pro-inflammatory ARA derivatives [24, 25]. Furthermore, ARA, EPA, and DHA may also regulate immune-related transcription factors such as nuclear factor kappa B (NF κ B) which is a potent inducer of proinflammatory cytokine production [26]. Therefore, alterations in the cell membrane EFA profile of different immune cell types in circulation or specific tissues such as the distal intestine, which is characterized by a diffused presence of leucocytes in lamina propria and epithelium [27], may result in an inadequate immune response or humoral protection.

For gilthead sea bream, it has already been shown that the dietary FA profile has a significant impact on the humoral immune response [17, 21]. For instance, 1.5 % of dietary n-3 LC-PUFA was found to be necessary for the maintenance of alternative complement activity, especially at high stocking density [21], while a dietary decrease from 2 to 0.5 % of n-3 LC-PUFA reduced the alternative complement activity and the number of lymphocytes [17]. Further, the high dietary n-6 FA levels due to the replacement of FO by soybean oil induced a reduction of the serum bactericidal capacity against *Flavobacterium psychrophilum* [28]. Given the increased use of VO in aquafeeds, the purpose of this study was to assess the modulation of dietary n-6 /n-3 LC-PUFA ratio on the humoral innate immune and distal intestine inflammatory responses of gilthead sea bream juveniles.

2. Material and methods

2.1. Diets composition

Four practical diets were formulated to be isoproteic (47 % crude protein) and isolipidic (18 % crude lipids) containing fish meal (FM) and plant feedstuffs (PF) (26:74 protein from FM: PF) as main protein sources and a VO blend (20:50:30 rapeseed, linseed, and palm oils) as lipid source. The diets were supplemented EFA to obtain n6/n3 LC-PUFA ratios (%DM) of 2.0 ARA: 0.2 EPA: 0.1 DHA (Diet A); 1.0 ARA: 0.4 EPA: 0.4 DHA (Diet B); 0 ARA: 0.6 EPA: 0.6 DHA (Diet C); 0 ARA: 0.3 EPA: 1.5 DHA (Diet D). The commercial products used as EFA sources were the following: for ARA, Vevodar® (DSM Food Specialties, the Netherlands); for EPA, SuperbaKrill™ Oil, Solchem®; and for DHA tuna oil (70 % DHA; BrudyTechnology®).

All diet ingredients were thoroughly mixed and dry pelleted in a laboratory pellet mill (California Pellet Mill, CPM Crawfordsville, IN, USA) through 3.0 mm die. Pellets were dried in an oven at 40°C for 48 h

and kept in a freezer in airtight bags until use. Proximate composition of the ingredients and experimental diets and fatty acid profiles are presented in Magalhães et al. [29] and dietary EFA profiles are summarized in Table 1.

2.2. Experimental design

The experiment was conducted in CIIMAR, Matosinhos, Portugal, with gilthead sea bream juveniles (15 g) obtained from a commercial fish farm (Maresa S.A., Ayamonte, Huelva, Spain). The trial was performed in a recirculating aquatic system (RAS) thermo-regulated to 23.0 $\pm$ 1.0°C and equipped with 12 cylindrical fiberglass tanks of 100 L water capacity. Tanks were maintained with a continuous flow of filtered seawater (2.5–3.5 L min⁻¹), salinity (35 $\pm$ 1 g L⁻¹) and dissolved oxygen near saturation (7 mg L⁻¹). A photoperiod of 12 hours light and 12 hours of darkness was established. Fish were transferred to the experimental system after a quarantine period of one month and adapted to the experimental conditions for 15 days and fed with a commercial diet (NEO, Aquasoja). Then, twenty fish were distributed to each tank, and the experimental diets aleatory assigned to triplicate groups. Fish were fed by hand, twice daily, 6 days a week, until apparent visual satiation for 56 days. Care was taken to avoid waste of feed. The experiment was approved by CIIMAR ethical committee for Managing Animal Welfare (ORBEA), in compliance with the European Union directive 2010/63/EU and the Portuguese Law (DL 113/2013).

2.3. Sampling

Three fish from each tank were randomly collected 4 hours after the morning meal for blood. Blood samples were withdrawn from the caudal vein with heparinized syringes and utilized for total erythrocytes and leucocytes counts. Then blood smears were prepared. The remaining blood was immediately centrifuged (10,000 $\times$ g for 10 min, room temperature) and plasma collected and stored at -80°C until further analyses. Following blood collection, the fish were euthanized by decapitation and the intestine was dissected on chilled trays. The digestive tract was freed from adjacent adipose and connective tissues and a portion of the distal intestine (DI; distinguished from the mid intestine by an enlarged diameter and darker mucosa) from two fish per tank were sampled and then stored for gene expression analysis.

2.4. Chemical analysis

Chemical analysis of the diets was performed according to the Association of Official Analytical Chemists methods AOAC [30]. Briefly, the dry matter was assessed by drying the samples in an oven at 105°C until constant weight and ash content by incineration in a muffle furnace at 450°C for 16 h. The Kjeldahl method was used for the determination of protein content (N $\times$ 6.25) using a Kjeltac digestion and distillation units (Tecator Systems; models 1015 and 1026, respectively). Crude lipids were determined using petroleum ether as a solvent and a Soxtec HT System (Höganäs); dietary starch was determined according to Beutler [31]. Gross energy was quantified by direct combustion in an adiabatic bomb calorimeter (PARR model 6200; Parr Instruments). FA quantification was done according to Magalhães et al. [29].

Table 1
Essential fatty acid composition and n-6/n-3 ratio of the experimental diets (% of total fatty acids).

Diets (ARA/EPA/DHA ratio)	A	B	C	D
20:4n-6 (ARA)	10.6	5.43	0.18	0.11
20:5n-3 (EPA)	0.96	2.02	3.24	1.70
22:6n-3 (DHA)	0.70	2.06	3.52	7.71
n-6/n-3	1.50	0.98	0.63	0.52

ARA: Arachidonic; EPA: eicosapentaenoic; DHA: docosahexaenoic

2.5. Blood parameters

Blood parameters were accessed according to Peres *et al.* [32]. Briefly, total white (WBC) and red (RBC) blood cell count was made from blood dilutions using a hemocytometer. Fresh heparinized blood was used for haematocrit (HT) and its value was determined by the micro centrifugation (10,000 x g for 10 min, at room temperature) of haematocrit tubes. Drabkin's solution was used for haemoglobin determination (HB; SPINREACT kit, ref. 1,001,230, Spain). The mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), and mean corpuscular haemoglobin (MCHC) were calculated as follows:

- $MCH \text{ (pg cell}^{-1}\text{)} = (HB/RBC) \times 10$
- $MCV \text{ (}\mu\text{m}^3\text{)} = (HT/RBC) \times 10$
- $MCHC \text{ (g 100 mL}^{-1}\text{)} = (HB/HT) \times 100$

Immediately after blood collection, blood smears were made from fresh blood, air-dried, fixed with formol-ethanol (3.7% formaldehyde in absolute ethanol), and stained with Wright's solution (Haemacolor; Merck). Neutrophils were detected by their peroxidase activity as described by Afonso *et al.* [33]. The slides were carefully analyzed under oil immersion (1,000x), and at least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes, and neutrophils. The absolute value ($\times 10^4 \mu\text{L}^{-1}$) of each cell type was calculated.

2.6. Humoral innate immune parameters

2.6.1. Protease activity

Protease activity was quantified using the azocasein hydrolysis according to Guardiola *et al.* [34] with some modifications. After adding 110 μL of 0.1 M phosphate buffer (pH 7.0) and 125 μL of 2 % azocasein (Sigma) to 10 μL of plasma, samples were incubated for 24 h at 22 °C and, following the addition of 250 μL of 10 % trichloroacetic acid (TCA), were incubated for 30 min at 22 °C. The mixture was then centrifuged at 10,000 x g for 5 min. Supernatants (100 μL) were transferred to 96-well plates containing 100 μL well⁻¹ of 1 N NaOH and the optical density (OD) was read at 450 nm using a microplate reader (Synergy HT). Plasma was replaced by trypsin (5 mg mL⁻¹, Sigma) as a reference sample (100% of protease activity), or by a buffer as blank (0% activity).

2.6.2. Antiprotease activity

Total antiprotease activity was determined by the ability of plasma to inhibit trypsin activity according to Machado *et al.* [35]. Briefly, 10 μL of each plasma sample was incubated with the same volume of standard trypsin solution (Sigma, 5 mg mL⁻¹) for 10 min at 22 °C. After adding 100 μL of 0.1 M phosphate buffer (pH 7.0) and 125 μL of 2 % azocasein (Sigma), samples were incubated for 1 h at 22 °C and, following the addition of 250 μL of 10 % TCA, were incubated for 30 min at 22 °C. The mixture was then centrifuged at 10,000 x g for 5 min. Supernatants (100 μL) were transferred to 96-well plates containing 100 μL well⁻¹ of 1 N NaOH, and the optical density was read at 450 nm using a microplate reader (Synergy HT). Phosphate buffer instead of plasma and trypsin served as blank whereas the reference sample was phosphate buffer instead of plasma. The percentage of inhibition of trypsin activity was calculated by comparison with the reference sample.

2.6.3. Peroxidase activity

The peroxidase activity was measured according to Quade and Roth [36]. Briefly, 5 μL of plasma was diluted with 40 μL of Hank's buffer (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. 100 μL of 10 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB, Sigma-Aldrich), and 0.015% H_2O_2 were used as substrates. The colorimetric reaction was concluded after 2 min by adding 50 μL of 2 M sulphuric acid (H_2SO_4) and the OD was read at 450 nm in a microplate reader (Synergy HT). HBSS instead of plasma was used as blank. The peroxidase activity was calculated by defining 1 OD absorbance change

as corresponding to 1 unit of peroxidase activity (units mL⁻¹ of plasma).

2.6.4. Alternative complement pathway activity

Alternative complement pathway (ACP) activity was measured according to Sunyer and Tort [37] with slight modifications. Several buffers were used: EDTA (ethylene glycol tetraacetic acid)-GVB (Isotonic veronal buffered saline), pH 7.3, containing 0.1% gelatin, 20 mM EDTA, 5 mM Sodium barbiturate, 0.13 mM NaCl; and Mg^{+2} -EGTA-GVB, which is GVB with 10 mM Mg^{+2} and 10 mM EGTA instead of EDTA. Rabbit red blood cells (RaRBC; Probiologica Lda, Portugal) were used for natural haemolytic complement determination. RaRBC were washed several times in 0.9 % NaCl and resuspended to a concentration of 2.8×10^8 cells mL⁻¹ in 0.9 % NaCl. 20 μL of RaRBC suspension was then added to 20 μL of serially diluted plasma in Mg-EGTA-GVB buffer. Maximum (100%) and minimum (spontaneous) haemolysis values were obtained by adding 40 μL of distilled water or Mg-EGTA-GVB buffer to 20 μL RaRBC suspension. Samples were incubated at room temperature for 100 min with shaking every 20 min. Subsequently, 150 μL of cold EDTA-GVB buffer was used to stop the reaction. Samples were centrifuged (400 x g, 2.5 min, 22 °C) and the degree of haemolysis was quantified by measuring the OD of the supernatant at 414 nm in a microplate reader (Synergy HT). The degree of haemolysis (Y) was estimated plotting $Y(1-Y)^{-1}$ on a log-log scale graph against the lysis curve for each specimen. The volume of plasma producing 50% hemolysis (ACH₅₀) was estimated and the number of ACH₅₀ units mL⁻¹ was obtained for each sample.

2.6.5. Nitric oxide (NO)

Plasmatic total nitrite plus nitrate content was determined using a Nitrate/Nitrite colorimetric kit (Roche Diagnostics GmbH, Mannheim, Germany, ref: 11,746,081,001) adjusted for 96-well microplates. Briefly, 10 μL of defrosted plasma and 90 μL of distilled water were added to a microplate well. Thereafter, the nitrate reduction into nitrite was accomplished by adding 50 μL of reduced NADPH and 4 μL of the enzyme nitrate reductase and incubating the microplates for 30 min at 25 °C. Subsequently, 50 μL of sulfanilamide and 50 μL of N-(1-naphthyl)-ethylene-diamine dihydrochloride were added and the incubation continued for more 10 min at 25 °C. Then, the OD was measured at 540 nm in a Synergy HT microplate reader. The blank was made using water instead of plasma. Nitrite and nitrate concentrations were determined by comparison with sodium nitrite and potassium nitrate standard curve. As both compounds are oxidative metabolites of endogenously produced NO, they were utilized to quantify NO content in plasma [38].

2.7. Bactericidal activity

Two opportunist marine pathogenic bacteria [*Vibrio anguillarum* and *Photobacterium damsela* subsp. *piscicida* strain PP3 (*Phdp*)] were used in the bactericidal activity assay. Bacteria were cultured for 48 h at 25 °C on tryptic soy agar (TSA) in agar plates and then inoculated in tryptic soy broth (TSB, Sigma), both media were supplemented with 1 % NaCl (w/v), and cultured overnight at the same temperature with continuous shaking (100 rpm). Then, bacteria were collected by centrifugation at 3500 x g for 30 min and resuspended in sterile HBSS and adjusted to 1×10^6 colony forming units (cfu) mL⁻¹ according to Costas *et al.* [39]. Plasma bactericidal activity was assessed according to Graham *et al.* [40], with slight modifications. Briefly, 20 μL of plasma and 20 μL of both bacteria (1×10^6 cfu mL⁻¹) were added to duplicate wells of U-shaped 96-well plates. Two wells with 20 μL of the bacterial solution and 20 μL of Hank's Balanced Salt Solution (HBSS) served as positive control and two wells with 40 μL of HBSS were used as a negative control. The plates were incubated for 3 h at 25 °C. Then, 25 μL of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (1 mg mL⁻¹; Sigma) was added to each well and incubated for 10 min at 25 °C to enable the formation of formazan. Then, plates were centrifuged (2000 x g for 10 min) and the precipitate was dissolved in 200 μL of

dimethyl sulfoxide (Sigma). Then, 100 μ L of that solution was added to flat-bottomed 96-well plates and the OD measured at 490 nm in a microplate reader (Synergy HT). Bactericidal activity is expressed as the percentage of bacteria that survived relatively to the number of bacteria from the positive controls (100%).

2.8. Gene expression

Analyses of mRNA levels were performed in DI samples (6 fish per treatment). Total RNA was extracted using TRIzol reagent (Direct-zol™ RNA Miniprep, Zymo Research) according to manufacturer recommendations, and RNA quality and quantity were assessed by electrophoresis in 1% agarose gel and spectrophotometry (μ Drop™ plate, Thermo-Scientific). The resulting total RNA concentration was adjusted to 0.5 μ g/8 μ L H₂O to complementary DNA synthesis, using the NZY First-Strand cDNA Synthesis Kit (NZYTech, MB12501, Lisbon, Portugal). Gene expression was determined by real-time quantitative PCR (q-PCR) using the Bio-Rad CFX Connect Real-Time System (California, USA). The analysis was carried out using 0.4 μ L diluted cDNA (1:2) mixed with 0.2 μ L of each primer (10 μ M), 5 μ L of SsoAdvanced™ Universal SYBR® Green Supermix, Bio-Rad Laboratories®, and 4.2 μ L DNase/RNase/Protease-free water (Sigma-Aldrich), in a total volume of 10 μ L. Primers were obtained from the literature and are shown in Table 2. The efficiency of PCR primers was measured by the slope of a standard curve using serial dilutions of cDNA. Thermal-cycling was initiated with incubation at 95°C for 30 s for hot-start iTaq™ DNA polymerase activation. A total of forty steps of PCR were then performed, each one consisting of heating at 95°C for 15 s for denaturing followed by 30 s at 60°C for annealing and extension. Following the final PCR cycle, melting curves were systematically monitored (65°C temperature 0.5°C 10 s⁻¹ from 65 to 95). Each PCR run included duplicates of reverse transcription for each sample and negative controls. The PCR run for the reference gene included duplicates for each sample and negative controls. Quantification of the target gene transcripts was performed using elongation factor 1 α (*ef1a*) as the reference gene. Relative quantification of the target gene transcript with *ef1a* reference gene transcript was performed using the mathematical model described by Pfaffl [41]. For each mRNA, gene expression was normalized by *ef1a* content in each sample.

2.9. Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM) or standard error (SE). Outliers were removed and normality and homogeneity of data were accessed by the Shapiro-Wilk and Levene tests, respectively, and normalized when appropriate. Statistical evaluation of the data was done by one-way ANOVA. Differences were considered statistically significant at $P < 0.05$. When p -values were significant, differences between means were evaluated with the Tukey's test. All statistical analysis was performed using SPSS 24.

Table 2

Sequences of the primer pairs used for determination of the transcript level of immune-related genes in the distal intestine in gilthead sea bream.

Gene	Gene abbreviation	Primer sequences (5'–3')	Primer efficiency	Accession number
Major histocompatibility complex II	<i>mhc-IIa</i>	F:CTGGACCAAGAACGGAAGA R:CATGGACTCTGAGTAGCGGA	2.00	DQ019401
Tumor necrosis factor α	<i>tnfa</i>	F:TCGTTTCAGAGTCTCCTGCAG R:CATGGACTCTGAGTAGCGGA	2.02	AJ413189
Immunoglobulin M heavy chain	<i>igm</i>	F:CAGCCTCGAGAAGTGGAAC R:GAGGTTGACCAGGTTGGTGT	2.04	AM493677
Cyclooxygenase 2	<i>cox2</i>	F:GAGTACTGGAAGCCGAGCAC R:GATATCACTGCCGCTGAGT	1.92	AM296029
Interleukin 1 β	<i>il-1β</i>	F:GGGCTGAACAACAGCACTCTC R:TTAACACTCTCCACCCTCCA	2.05	AJ277166
Proliferating cell nuclear antigen	<i>pcna</i>	F:GATGTGGAGCAGCTGGGTAT R:TGTCTACGTTGCTGCTCTGG	1.94	FG263675
Elongation factor 1-alpha	<i>ef1a</i>	F:CTGTCAAGGAAATCCGTCGT R:TGACCTGAGCGTTGAAGTTG	2.05	AF184170

3. Results

3.1. Haematological profile, RBC, WBC, and differential cell counts

After the 56 days of the feeding trial period, dietary treatments did not affect, final weight, weight gain and daily growth index, but diet B lead to the highest feed efficiency and protein efficiency ratio and diet A and D modulated liver lipid content, β -oxidation and lipogenesis (results presented in [29]). Dietary ARA/EPA/DHA ratios did not affect the RBC and WBC numbers, haematocrit (HT), haemoglobin (HB), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) (Table 3). In the case of differential cell counts, no variations in the differential WBC numbers were observed, except for monocytes numbers which were higher in fish fed diet B than fish fed diet C whilst no variations were found respect to values found in the fish fed other experimental diets (A and D) (Table 3).

3.2. Humoral immune parameters

Innate immune parameters evaluated, namely peroxidase, anti-protease, protease, and nitric oxide, were not affected by any dietary treatment as is summarized in Table 4. Contrarily, the haemolytic complement activity increased in the plasma of fish fed diet B compared to values found in fish fed diet D, whereas no significant differences were

Table 3

Haematological profile in gilthead sea bream fed the experimental diets (A, B, C, D) after 56 days.

Parameters	Dietary treatments (ARA/EPA/DHA ratio)				SEM
	A	B	C	D	
	2.0/0.2/ 0.1	1.0/0.4/ 0.4	0/0.6/ 0.6	0/0.3/ 1.5	
Haemoglobin (g dL)	3.7	4.1	3.6	3.4	0.1
Haematocrit (%)	29.1	30.9	28.4	29.8	0.5
MCV (μ m ³) ¹	200.6	220.6	198.9	209.5	6.2
MCH (pg cell ⁻¹) ²	25.9	29.2	24.5	23.8	1.2
MCHC (g 100 mL ⁻¹) ³	12.9	13.4	13.1	11.0	0.5
RBC (x 10 ⁶ μ L ⁻¹)	1.5	1.4	1.4	1.5	0.0
WBC (x 10 ⁴ μ L ⁻¹)	6.9	6.7	7.1	7.5	0.2
Neutrophils (10 ⁴ μ L ⁻¹)	0.38	0.50	0.36	0.48	0.0
Monocytes (10 ⁴ μ L ⁻¹)	0.25 ^{ab}	0.29 ^b	0.14 ^a	0.15 ^{ab}	0.0
Lymphocytes (10 ⁴ μ L ⁻¹)	0.60	0.69	0.84	0.92	0.1
Thrombocytes (10 ⁴ μ L ⁻¹)	5.4	5.2	5.7	6.0	0.2

Values presented as means (n=9) and standard error of the mean (SEM). Superscript letters indicate significant differences between dietary treatments ($P < 0.05$). MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; MCV: mean corpuscular volume; RBC: red blood cells; WBC: white blood cells.

Table 4

Immune humoral parameters measured in gilthead sea bream juveniles plasma fed the experimental diets (A, B, C, D) after 56 days.

Parameters	Dietary treatments (ARA/EPA/DHA ratio)				SEM
	A	B	C	D	
	2.0/ 0.2/0.1	1.0/ 0.4/0.4	0/0.6/ 0.6	0/0.3/ 1.5	
Peroxidase activity (U mL ⁻¹)	33.2	57.8	58.6	81.7	8.4
Nitric oxide (μM)	986.6	1000.4	1107.3	1069.8	24.2
Antiprotease activity (%)	86.3	86.8	86.7	86.3	0.1
Protease activity (%)	7.3	7.3	7.2	7.0	0.1
Alternative complement pathway activity (ACH ₅₀ U mL ⁻¹)	12.6 ^{ab}	15.6 ^b	13.4 ^{ab}	9.9 ^a	0.6

Values are presented as means (n=9) and standard error of the mean (SEM). Superscript letters indicate significant differences between dietary treatments (P < 0.05).

observed compared to values registered in the other experimental groups (Table 4).

3.3. Bactericidal activity

Bactericidal activity against two pathogenic bacteria was measured in the plasma of gilthead sea bream fed experimental diets (Fig. 1). In the case of plasma bactericidal activity against *Phdp*, the values increased in fish fed diet B compared to fish from group A, whilst the activity not varied in fish fed diets C and D (Fig. 1). Contrarily, the bactericidal activity against *V. anguillarum* was not affected in the plasma of fish from any dietary treatment (Fig. 1).

3.4. Immune-related gene expression in the intestine

At the end of the feeding trial, no significant differences were observed in the gene expression of major histocompatibility complex II (*mhc-IIα*), tumor necrosis factor α (*tnfα*), immunoglobulin M heavy chain (*igm*) and proliferating cell nuclear antigen (*pcna*) in the intestine of fish fed experimental diets (Fig. 2). Similarly, in the case of expression of cyclooxygenase 2 (*cox2*) and interleukin 1β (*il-1β*) genes, both related to inflammatory processes, no significant variations were observed in the intestine of any experimental group (Fig. 2).

4. Discussion

The n-3 LC-PUFA requirements for juvenile gilthead sea bream (average body weight of 42 g) were estimated to be 0.9 % DM with an optimum DHA/EPA ratio of 1 [9]. The ARA requirements for gilthead

sea bream have not yet been determined. Accordingly, Diet B was designed to match the minimum requirements of ARA, EPA, and DHA for gilthead sea bream juveniles and represents a balance diet of the study with similar ARA and EPA+DHA levels dietary content (ARA, 1% DM= EPA, 0.4%DM + DHA, 0.4%DM; DHA/EPA=1). Diet A was formulated with an extreme ARA level (2% DM) and almost devoid of EPA (0.2%DM) and DHA (0.1%DM). Diet C was formulated to be devoid of ARA and rich in EPA +DHA (0.6+0.6 % DM; 1:1 ratio) and diet D was formulated with high levels of n-3 LC-PUFA (1.8%DM) but with an unbalance DHA:EPA ratio of 5:1 (DHA, 1.5% DM; EPA, 0.3% DM) and without ARA.

The dietary EFA composition also affects the lipid composition of RBC and WBC and cell function by impacting fluidity [42] and fragility [17]. In the present study, after 56 days of feeding the gilthead seabream juveniles with the experimental diets, no differences were detected in the numbers of RBC and WBC in the blood of fish. These results are in agreement with previous data from gilthead sea bream where low dietary n-3 LC-PUFA (0.42 %) level did not affect the values of RBC numbers and HT at low stocking densities, while at high stock densities, the number of RBC was reduced compared to fish fed high n-3 LC-PUFA (1.5 %) diet [21]. Results are however inconsistent and in a more recent study with gilthead sea bream, the RBC numbers were increased in fish fed a diet low in n-3 LC-PUFA (0.5 %) compared to groups fed the control diet with 2% n-3 LC-PUFA [17]. Similarly, n-3 LC-PUFA deficient diets also did not affect the RBC numbers, HB, and HT in rockfish (*Sebastes schlegelii*) [43] and Japanese eel (*Anguilla japonica*) fed increasing dietary ARA levels from 0 to 1.65% [44]. Contrarily, for other species, reduction of dietary n-3 LC-PUFA levels through the replacement of FO by a VO reduced the values of HT and HB in adult cod (*Gadus morhua*) [45] and RBC numbers in turbot (*Scophthalmus maximus*) [46]. Moreover, contrary to present results, reduction of dietary DHA/EPA ratio (0.9) decreased the RBC numbers and HT values and consequently increased MCV and MCH values in silvery-black porgy (*Sparidentex hasta*) [47].

In the case of leucocytes, the WBC numbers were also not affected by dietary ARA or n-3 LC-PUFA levels in Japanese eel [44], silvery-black porgy [48], channel catfish, (*Ictalurus punctatus*) [49], Atlantic salmon (*Salmo salar*) [50], and black seabream (*Acanthopagrus schlegelii*) [51], while in Indian major carp (*Catla catla*), the increase of dietary n-3 LC-PUFA decreased the WBC numbers [52]. Concerning differential counts of WBC, the number of monocytes decreased in fish fed diets C and D. Interestingly, these diets, had not been supplemented with ARA. This could suggest that adequate dietary ARA content is necessary for proper monocytes recruitment. The lack of effect of diet A which only had 0.3% n-3 LC-PUFA contrasts that of Montero *et al.* [17] who found reduced lymphocytes numbers in gilthead sea bream when fed 0.5 % n-3

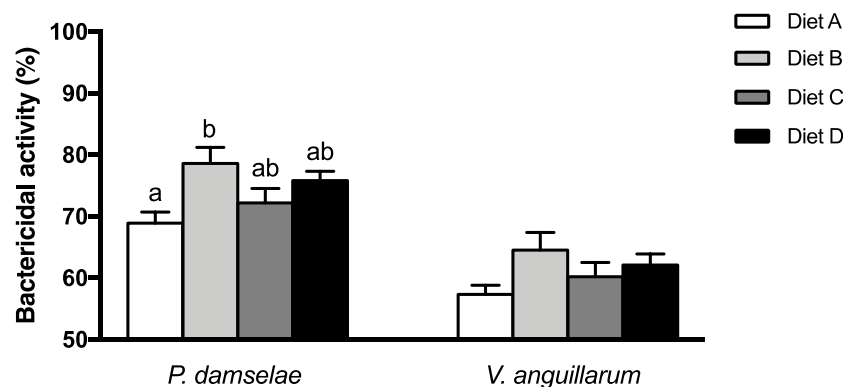


Fig. 1. Bactericidal activity (%) against *Photobacterium damsela* subsp. *piscicida* and *Vibrio anguillarum* in the plasma of gilthead sea bream juveniles fed the experimental diets (A, B, C, D) after 56 days. Data represent the mean ± standard error (SE) (n=9). Different letters denote significant differences between experimental groups (P < 0.05).

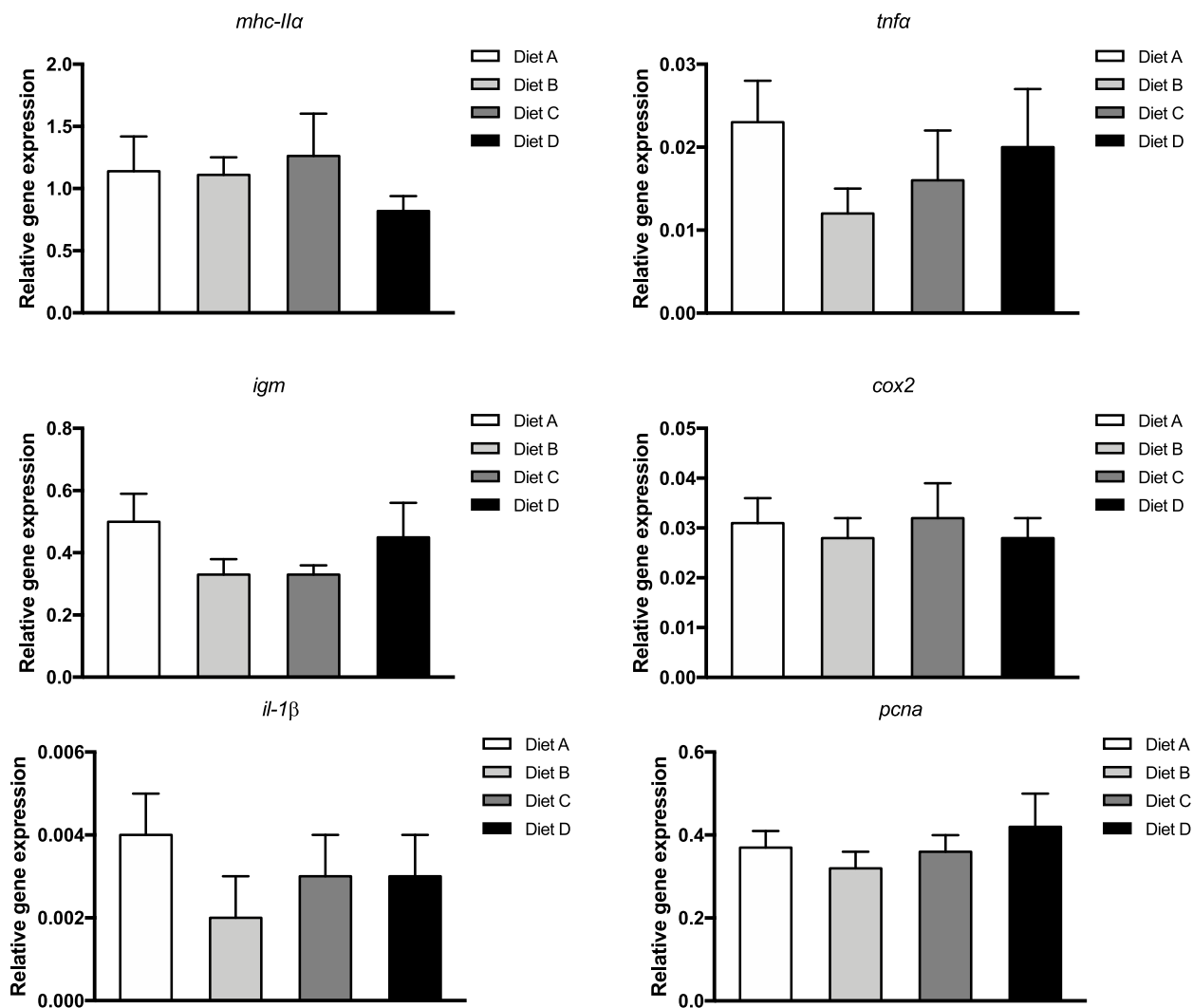


Fig. 2. Gene expression in the distal intestine of gilthead sea bream fed the experimental diets (A, B, C, D) after 56 days. Immune-related gene expression detected by rt-PCR and normalized to housekeeping gene elongation factor 1-alpha (*ef1a*). Data represent the mean $\pm$ standard error (SE) (n=6).

LC-PUFA content. This could indicate that elevated ARA levels may compensate for some of the n-3 LC-PUFA deficiency. Further, in Atlantic salmon and channel catfish, diets with high n-3 LC-PUFA levels increased the thrombocytes numbers [49, 50]. This was not seen in the present experiment, but there was a small but notable increase in thrombocytes in fish fed high n-3 LC-PUFA (diets C and D).

Previously, it was been shown that deficiency of n-3 LC-PUFA leads to depletion of several innate immune responses including haemolytic complement activity in gilthead sea bream [17, 21]. On the other hand, also in gilthead sea bream, elevated levels of n-6 FA along with reduced levels of EPA and DHA have also been shown to decrease serum bactericidal activity [53]. In the present study, the results clearly suggest that balanced ARA, EPA, and DHA levels are required for optimum immune capacity in gilthead sea bream. Compared to the other diets, diet B (1.0/0.4/0.4, ARA/EPA/DHA, respectively) had elevated haemolytic complement activity and bactericidal response against *Phdp* compared to the values found in fish fed other experimental diets [54]. As is known, bactericidal activity measures the capability of plasma to kill a bacteria and is considered one of the most practical immune parameters to assess the general health status of the fish as it includes the activity of several plasmatic compounds [55]. However, in the present study, deficiency in the n-3 LC-PUFA (Diet A) clearly impaired the bactericidal activity against *Phdp* but no diet influenced the response against

V. anguillarum. This fact could be related to the different virulence mechanisms of each pathogenic bacterium. The virulence of *Phdp* is associated with a polysaccharide capsular layer [56] while the virulence of *V. anguillarum* is linked with exopolysaccharides secreted into the surrounding environment that increase resistance to host lysozyme and antimicrobial peptide activities [57] and that may protect more effectively the bacterial cell wall.

Despite numerous studies on the potential influence of dietary EFA on fish immunity the underlying mechanisms on how ARA, EPA, or DHA affects these responses remain unclear [16, 44, 58–65]. An imbalance of eicosanoid production is probably central in innate immune responses [16]. Indeed, the balance of eicosanoids production with anti- or pro-inflammatory properties is largely determined by the ARA and EPA ratio in cellular membranes [66]. ARA is the precursor of pro-inflammatory and EPA is a precursor of anti or less-inflammatory prostaglandins and leukotrienes [25]. In recent years, several studies have attributed the immunostimulant effect of dietary n-3 LC-PUFA to DHA, and high DHA/EPA ratios have been shown to promote lysozyme and haemolytic complement activity [61], phagocytic and respiratory burst activities in head-kidney leucocyte (HKLs) [65], serum IgM levels and lysozyme activity [58, 64], that was not observed in the present study probably due to the extremely high DHA/EPA ratio (5:1) compared to the ratio used in the mentioned studies or absence of

inflammatory response.

While it is generally given more relevance to n-3 LC-PUFA as EFA in marine fish, as growth performance is mainly depressed when they are included at low levels in the diets, an adequate dietary ARA level is also relevant when the immune status is considered, as shown in the present study. The importance of dietary ARA was also shown in European seabass, as increasing dietary levels from 0.5 to 1% improved HKLs phagocytic capacity [16]. Furthermore, in Japanese eel, giving 1.06 % ARA increased the serum lysozyme activity compared to those fed 0 or 0.33 % ARA diets [44]. Also, in juvenile Japanese seabass (*Lateolabrax japonicus*), modest dietary ARA levels of 0.36 and 0.56 % enhanced serum lysozyme and haemolytic complement activity compared with fish fed 0.08 % diet [60]. Furthermore, though not statistically different, increasing dietary ARA (0.05–0.65%) content improved immune parameters, HKLs respiratory burst, and serum lysozyme activity in Malabar red snapper (*Lutjanus malabaricus*) [67].

The intestine is one of the main entrances for pathogens if its integrity is compromised [27], and this may occur by using alternative-feedstuffs based diets. However, in the present trial, distal intestine histomorphology was not compromised by the dietary treatments (results presented in [68]). Also, the DI cellular content of ARA, EPA, and DHA determined the pattern of eicosanoids produced by cyclooxygenases and lipoxygenases [69]. ARA derived eicosanoids are, generally, associated with a potent inflammatory effect via PGE₂ due to the production and release of proinflammatory cytokines such as TNF α and IL-1 β [70]. Contrary, eicosanoids produced from EPA such as leukotriene 5 (LTB₅), and PGE₃ are less potent and frequently associated with anti-inflammatory properties by decreasing the expression of inflammatory cytokines [24]. However, the different dietary EFA ratios of the present study did not affect cytokines gene expression, *tnfa*, and *il-1 β* , which are proteins produced by activated immune-related cells such as monocytes and lymphocytes, and modulate the immune response [71]. The lack of effects in immune-related genes expression in the DI reveal significant robustness of the intestine of gilthead sea bream and a possible lack of inflammatory response. Previously in gilthead sea bream, it was also observed that replacement of FO by soybean or linseed oils did not affect the intestinal expression of *tnfa* and *il-1 β* genes whilst in the HK was observed an up-regulation of *tnfa* gene expression [28], suggesting different responses according to the tissues.

Contrary to the present results, 0.5 and 1 % dietary ARA was associated with changes in the expression of GALT-related genes in European seabass, increasing the gene expression of *il-1 β* and the anti-inflammatory cytokine *il-10* [72]. However, 2 days post-infection with *V. anguillarum*, 2 % of ARA increased the expression of *il-1 β* and *cox2* genes and 4 % increased *il-10* expression [72]. Furthermore, in the anterior intestine of gilthead sea bream, dietary inclusion of modified *Camelina sativa* oil with 2.4 % of EPA and DHA down-regulated the expression of the *il-8* gene compared to the control diet [73]. In the current work, different dietary n6/n3 ratios did not affect the expression of the *cox2* gene in the DI implying no effect on PGE₂ production, as described recently in European seabass by Rivero-Ramírez et al. [72]. However, a preference for released ARA instead of EPA by the COX2 enzyme as substrate was already described in brook trout (*Salvelinus fontinalis*) [74] and Atlantic cod (*Gadus morhua*) [75]. The authors suggested an evolutionary adaptation of the enzyme to the scarce amounts of ARA in the marine system leading to a strong preference for this EFA compared to EPA or other n-3 fatty acids available [74]. The lack of dietary effect on the expression of the *cox2* gene of DI may, at least in part, be attributed to relatively low ARA levels of intestine tissue, irrespective of the dietary ARA levels. Indeed, in the present study, the muscle ARA levels of gilthead sea bream fed the experimental diets, were maintained relatively constant despite the increase of dietary ARA level [29], and a similar intestinal FA profile is expected.

For Senegalese sole (*Solea senegalensis*), Montero et al. [76] also showed that FO substitution by linseed oil did not affect the intestinal *cox2* gene expression levels whereas with soybean oil the expression

levels increased. The result can not be explained by the ARA/EPA ratio of intestine FA composition because it was similar [76]. Instead, the explanation of the results may be associated with the n-3/n-6 ratio that was lower in fish fed soybean oil diet compared to the control diet mainly due to the higher linoleic acid (18:2n-6) content in linseed oil [76]. It has also been suggested, for gilthead sea bream, that ARA mediated effects could not always be attributed to an increase in prostaglandin synthesis by cyclooxygenase. Indeed, in gilthead sea bream juveniles submitted to stress, it was observed higher plasma cortisol levels in fish fed high ARA dietary content and acetylsalicylic acid (ASA), an irreversible blocker of cyclooxygenase, than fish fed high ARA diets without ASA [77]. Nonetheless, in Atlantic cod HKLs, the *cox2* mRNA expression seems to be correlated with PGE₂ production but not with PGE₃ [75]. Furthermore, increasing concentrations of EPA and ARA in equal amounts showing the preference of *cox2* by ARA as substrate but similar to the presented results, the addition of ARA alone increased PGE₂ secretion but did not induce *cox2* transcription [75].

The dietary EFA ratio also did not modulate the gene expression in the distal intestine of major histocompatibility complex (*mhc-IIa*), which has a pivotal role in immune response activation and cellular markers produced by B-cells (*igm*). Furthermore, some shreds of evidence suggested that EPA and DHA content of fish oil might decrease *mhc-IIa* expression consequently decreasing the acquired immunity to a pathogen [78]. The presented results suggested a lack of acquired immunity response. The *mhc-IIa* is a key molecule to initiate the T cell-mediated adaptive response through the action of antigen-presenting cells like macrophages or B-cells [79, 80]. Similarly, also in gilthead sea bream, intestinal *igm* expression was not affected by the dietary substitution of 66 % FO by a blend of VOs [81]. Also with dietary inclusion of microalgae, which are rich in EFA, namely EPA, minor dietary influence on the expression of *mhc-IIa* and *igm* genes was reported in HK and intestine of gilthead sea bream [82, 83].

In conclusion, results of the present study indicate that diets with balanced dietary ARA, EPA, and DHA (1/04/0.4% DM) level increased circulating monocytes numbers, haemolytic complement and bactericidal activity against *Phdp*, thus improving the immunological status of gilthead sea bream juveniles under unchallenged conditions. Furthermore, no differences in the immune-related genes evaluated in the distal intestine were found between different ARA/EPA/DHA levels. However, further studies will be necessary to further evaluate the effects of the dietary EFA ratios under challenging conditions.

Data availability

The data generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter 5

Immunomodulatory effect of different dietary ARA/EPA/DHA ratios after infection with *Photobacterium damsela* subsp. *Piscicida* in gilthead sea bream (*Sparus aurata*) juveniles

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Immunomodulatory effect of different dietary ARA/EPA/DHA ratios in gilthead sea bream (*Sparus aurata*) juveniles after infection with *Photobacterium damsela* subsp. *piscicida*

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Abstract

The immunomodulatory effect of different dietary arachidonic acid (ARA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) ratios was evaluated in gilthead sea bream juveniles after intraperitoneal injection (i.p.) with formalin-killed *Photobacterium damsela* subsp. *piscicida* (*Phdp*). Fish were fed with four isoproteic (47% crude protein) and isolipidic (18% crude lipids) diets with the following long-chain polyunsaturated fatty acids (LC-PUFA) ratios: Diet A (2.0 %ARA: 0.2%EPA: 0.1%DHA); Diet B (1.0%ARA: 0.4%EPA: 0.4%DHA); Diet C (0%ARA: 0.6% EPA: 0.6%DHA); Diet D (0%ARA: 0.3%EPA: 1.5%DHA). After being fed for 56 days with the experimental diets, 24 fish from each dietary treatment were randomly selected and i.p. injected with 0.2 mL of killed *Phdp* (1×10^8 CFU/ mL⁻¹) or phosphate-buffered saline (control group). Four and twenty-four hours after the i.p., the hematological profile, plasma humoral innate immune parameters, and the intestine expression of immune-related genes were assessed. Results showed that fish fed diet D (high DHA content) had increased complement activity and higher intestinal expression of cyclooxygenase 2 (*cox2*) and interleukin 1 β (*il-1\beta*) at 4h and 24h after i.p., as well as increased intestinal tumor necrosis

factor α (*tnfa*) expression at 4 h. On the other hand, fish fed with diet A (high ARA content) showed higher plasma bactericidal activity against *Phdp* than fish fed with diet D. Overall, the results of this study suggest a positive modulatory effect of DHA in response to the pathogenic agent. Further studies are needed on the modulatory effects of different dietary ARA, EPA, and DHA ratios used in the presented study in the immune response of gilthead sea bream juveniles after a bacterial challenge.

Key Words

Arachidonic acid; Eicosapentaenoic acid; Docosahexaenoic acid; Immune response; gilthead sea bream; Bacterial challenge

1. Introduction

Aquaculture is currently responsible for half of the worldwide fish produced for human consumption and this contribution is expected to increase in the upcoming years [1]. Fishery products and by-products, such as fishmeal and fish oils (FO), have been the preferred protein and lipid sources for marine carnivorous feeds due to their nutritional quality and, up to this point, steady availability. However, given the stagnating capture fisheries, concerns about environmental issues, and future availability, new nutritional strategies are required to reduce the industry's dependence on these commodities.

Vegetable ingredients arise as a more sustainable and cost-effective alternative to fishmeal and fish oils. While fishmeal replacement by vegetable protein sources has been successfully achieved, replacement of FO by vegetable oils (VO) is more complicated. VO are rich in C₁₈ fatty acids (FA) such as linoleic acid (LA; 18:2n-6), α -linolenic acid (ALA; 18:3n-3), and oleic acid (OA; 18:1n-9), but are devoid of long-chain polyunsaturated fatty acids (LC-PUFA) such as arachidonic acid (ARA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) [2]. This precludes the use of VO as the sole lipid source in marine fish diets since these LC-PUFA are essential for marine fish as they have limited gene expression of delta 6 and 5 desaturases, necessary for the synthesis of LC-PUFA from their C₁₈-PUFA precursors [3]. Nevertheless, the use of VO as a replacement for FO in aquafeeds has been increasing [3], and partial replacement of FO with VO has been successfully achieved for important marine fish species including gilthead sea bream (*Sparus aurata*) [4, 5] and European sea bass (*Dicentrarchus labrax*) [6, 7]. Provided that the essential fatty acids (EFA) requirements are met, in high lipid diets circa 70 % FO can be replaced by VO without affecting growth performance [4, 8].

Besides being crucial for optimal fish growth, dietary EFA are also important mediators of several biological functions, including the immune response. Dietary EFA can affect the structure and functionality of immune cell membranes by modification of the membrane phospholipid composition and through a modulatory action on membrane-bound enzyme activities (i.e., phagocytosis, macrophage respiratory burst activity) [7, 9-12]. EFA also affect the production of eicosanoids, which are biochemical mediators of inflammatory and immunological responses [7, 13]. EFA, namely ARA and EPA, are precursors of eicosanoids with antagonistic properties and compete for cyclooxygenase (COX) and lipoxygenase (LOX) active sites. ARA-derived eicosanoids, such as 2-series prostanoids and 4-series leukotrienes, being generally associated with pro-inflammatory functions and having high biological activity [14]. On the other hand, EPA and DHA produce less active eicosanoids such as 3-series prostanoids and 5-series leukotrienes, which are less pro-inflammatory and have anti-inflammatory properties and control the inflammatory response [14-16]. Substrates for COX and LOX are EFA released from the plasmatic membrane phospholipids by phospholipase A₂ [14]. Therefore, eicosanoid actions are dependent on the cell membrane content of ARA, EPA, and DHA, which is determined by the dietary intake of these EFA [14]. The ratio of eicosanoids produced from ARA or EPA will further stimulate macrophages and leucocytes with bactericide properties and control the severity of the inflammatory response [11, 17]. Indeed, the alteration of dietary EFA profile through the modification of the dietary lipid sources has been shown to affect different non-specific immune response parameters in fish such as phagocytic activity and serum alternative complement pathway activity [5, 10, 18, 19], respiratory burst activity of head-kidney leucocytes [7, 20], lysozyme activity [10], immune cells FA composition [11], and eicosanoid production [13].

In aquaculture, fish are exposed to several environmental stress factors, including infectious diseases, which can be responsible for high mortalities and loss in profitability [21]. A balanced dietary n-3/n-6 FA ratio is essential for the maintenance of the health and immune status of farmed fish and with the dietary inclusion of VO this dietary balance can be altered, compromising fish's ability to cope with these challenges. In previous studies, it was shown that low dietary n-3/n-6 FA ratios induced imbalances in the immune response of gilthead sea bream, following a bacterial challenge with *Photobacterium damsela* subsp. *piscicida* (*Phdp*) [19] and of Atlantic salmon (*Salmo salar*), after a bacterial challenge with *Aeromonas salmonicida* and *Vibrio anguillarum* [21]. On the other hand, high levels of n-3 PUFA compromised the immune response of *Labeo rohita* after a challenge with *Aeromonas hydrophila* [22], and in dark barbel catfish (*Pelteobagrus vachelli*) the dietary inclusion of linseed oil at 4% (2% of α -linolenic acid content in the diet), improved fish resistance to

bacterial challenge with *Edwardsiella ictaluri* after an ammonia induced stress compared to fish fed diets with 0% linseed oil (0% of α -linolenic acid content in the diet) [23].

Gilthead sea bream is one of the most produced marine species in the European aquaculture [24]. However, the FA requirements and the optimal n3/n6 ratio for this species have not yet been well established. Optimal dietary n-3 LC-PUFA levels were reported to be 0.9 or 1.9 % of the diet with DHA/EPA ratios of 1 and 0.5, respectively. Fish in these studies had 42g and 1 g, respectively, and this confounding effect further difficult to establish the dietary n-3 LC-PUFA requirements [25, 26]. Studies evaluating dietary ARA requirements for fish are very scarce. In gilthead sea bream juveniles, no differences in growth performance and feed utilization were found in fish fed with dietary ARA ranging from 0.03 to 1.7 % [27].

In a recent study, Magalhães et al. [28] found that after a 56 days growth trial, fish fed an EFA balanced diet (1.0 ARA: 0.4 EPA: 0.4 DHA, %DM) had increased monocyte numbers, alternative complement activity, and plasma bactericidal activity against *Phdp* compared to fish fed ARA rich diet (2.0 ARA: 0.2 EPA: 0.1 DHA, %DM) or n-3 LC-PUFA rich diets (0 ARA: 0.6 EPA: 0.6DHA; 0 ARA: 0.3 EPA: 1.5 DHA, %DM). In that study, the modulatory action of dietary EFA ratio on immune response and the capacity of gilthead sea bream to cope with pathogenic outbreaks was not fully understood. Given this, the present work aimed to evaluate how different dietary EFA ratios affect the immune response of gilthead sea bream when subjected to an inflammatory challenge with killed *Phdp*.

2. Material and Methods

2.1 Diets composition

Four isoproteic (47% crude protein) and isolipidic (18% crude lipids) diets were formulated. Dietary protein was provided by plant feedstuffs and fish meal (74 and 26% of the total protein, respectively), and dietary lipids were provided by a blend of vegetable oil (20:50:30, rapeseed: linseed: palm). Purified sources of EFA were used to adjust the dietary ARA:EPA:DHA (%DM) ratio accordingly to: Diet A (2.0 %: 0.2%: 0.1%); Diet B (1.0%: 0.4%: 0.4%); Diet C (0%: 0.6%: 0.6%); Diet D (0%: 0.3%: 1.5%). Commercial ARA oil obtained from *Mortierella alpina* (Vevodar®, DSM Food Specialties, the Netherlands), krill oil (*Euphausia superba*; SuperbaKrill™ Oil, Solchem®) and tuna oil (70 % DHA; BrudyTechnology®) were used as ARA, EPA, and DHA sources. All diet ingredients were carefully mixed and dry pelleted in a laboratory pellet mill (California Pellet Mill, CPM Crawfordsville, IN, USA) through a 3.0 mm die. Pellets were dried in an oven at 40 °C for 48h and kept in a freezer in airtight

bags until use. Proximate composition of the ingredients, experimental diets, and dietary fatty acid profiles are presented in Magalhães et al. [29], and dietary EFA profiles are summarized in table 1.

2.2 Fish and experimental conditions

The experiment was approved by CIIMAR ethical committee for Managing Animal Welfare (ORBEA; reference ORBEA_CIIMAR_30_2019), in compliance with the European Union directive 2010/63/EU and the Portuguese Law (DL 113/2013).

Fish and experimental conditions are described in detail in Magalhães et al.,[29]. Gilthead sea bream (*Sparus aurata*) juveniles were obtained from a commercial fish farm (Maresa S.A., Ayamonte, Huelva, Spain). Fish were moved to the experimental system after a quarantine period of 1 month and allowed to adapt to the experimental conditions for 15 days. Thereafter, 12 groups of 20 fish (initial body weight of 15 g) were distributed into a thermo-regulated recirculating aquatic system (Temperature: 23.0 ± 1.0 °C; Salinity: 35 ± 1 g L⁻¹; Dissolved oxygen: 7 mg L⁻¹; Seawater flow: 2.5-3.5 L min⁻¹) equipped with 12 fiberglass tanks of 100 L capacity. Fish were fed 6 days a week, twice a day, by hand until apparent visual satiation for 56 days.

2.3 Bacterial Challenge

Formalin-killed aqueous bacterin was prepared with the virulent strain Lg_{H41/01} of *P. damselae* subsp. *piscicida* (*Phdp*) according to Díaz-Rosales et al.[30, 31]. Initially, bacteria were cultured on tryptic soy agar supplemented with 1.5 % NaCl (w/v) (TSAs) for 48 h at 22°C. Then, one colony was transferred to tubes containing 5 mL of tryptic soy broth supplemented with 1.5 % NaCl (TSBs) and incubated for 18 h at 22° C. Next day, 50 µL aliquot of the culture were inoculated in flasks with 50 mL TSBs and incubated at 22° C for 24h with continuous shaking. The bacterial culture was then adjusted to 1×10^8 CFU mL⁻¹ and killed with formaldehyde (1% final concentration) during 24 h. The formalin-killed bacterin was collected by centrifugation at 6 000 x g for 30 min at 4°C, washed 3 times in phosphate-buffered saline (PBS), resuspended in the same volume of PBS (50 mL). Efficacy of formalin-treatment was confirmed by plating on TSAs plates and incubating for 2 days at 22° C.

After the 56 days of feeding trial, 24 fish per dietary treatment (48 g of mean body weight) were intraperitoneally injected (i.p.) with either 0.2 mL of bacterin *Phdp* or with 0.2 mL PBS (sham group) and reallocated into the experimental system tanks according to dietary treatment and stimuli.

2.4 Sampling

Following 4 and 24 h after i.p. injection, blood from six fish/treatment injected with killed *Phdp* and six fish/treatment injected with PBS was collected from the caudal vein with heparinized syringes to access haematological parameters and the remaining blood was immediately centrifuged at $6800 \times g$ for 10 min. Plasma was collected and frozen at -80°C until humoral immune status analyses. Then, fish were euthanized by decapitation, the digestive tract was freed from adjacent adipose and connective tissues on chilled trays, and a portion of the distal intestine (DI, distinguished from the mid intestine by an enlarged diameter and darker mucosa) was sampled for gene expression analysis. DI samples were kept in RNeasy lysis buffer (25 mL of 1 M Sodium Citrate; 40 mL of 0.5 M EDTA; 700 g ammonium sulfate; 935 Mili-Q water; pH 5.2; 1:10 w/v) at 4°C for 24 h and then stored at -80°C until analysis.

2.5 Haematological procedures

Blood parameters were determined as described by Peres et al.[32]. Total white (WBC) and red (RBC) blood cell count was made from blood dilutions using a hemocytometer. Fresh heparinized blood was used for haematocrit (Ht) and its value was determined by the haematocrit tubes microcentrifugation ($10\,000 \times g$ for 10 min, at room temperature). Drabkin's solution was utilized for hemoglobin determination (HB; SPINREACT kit, ref. 1001230, Spain). The mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCHC) were calculated as follows:

- $\text{MCH (pg cell}^{-1}\text{)} = (\text{HB/RBC}) \times 10$
- $\text{MCV (}\mu\text{m}^3\text{)} = (\text{Ht/RBC}) \times 10$
- $\text{MCHC (g }100\text{ mL}^{-1}\text{)} = (\text{HB/Ht}) \times 100$

Blood smears were made from fresh blood immediately after blood collection, air-dried, fixated with formol-ethanol (3.7% formaldehyde in absolute ethanol), and stained using Wright's stain (Haemacolor; Merck). Detection of peroxidase activity was used for neutrophils identification, as described by Afonso et al. [33]. The glass slides were then examined ($1\,000 \times$), under oil immersion, and at least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes, and neutrophils. Each cell type absolute value ($\times 10^4 \mu\text{L}^{-1}$) was subsequently calculated.

2.6 Humoral innate immune response parameters

The ability of plasma to inhibit trypsin activity was used to determine total antiprotease activity, according to Guardiola et al., [34].

Total peroxidase activity was quantified as described by Quade and Roth [35]. One unit of peroxidase activity was defined as the concentration inducing an absorbance change of 1 OD (units mL⁻¹ of plasma).

Total plasma nitric oxide (NO) content was quantified using a Nitrate/Nitrite colorimetric kit (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions. As both compounds are oxidative metabolites of endogenously produced NO, they were utilized to quantify NO content in plasma [36].

Alternative complement pathway activity (ACH50) was measured as described in Magalhães et al. [28]. The volume of plasma necessary to induce 50 % haemolysis was quantified and the number of ACH₅₀ units mL⁻¹ was calculated for each experimental fish.

Plasma bactericidal activity against *Vibrio anguillarum* and *Photobacterium damsela* subsp. *piscicida* strain PP3 (*Phdp*) was assessed as described by Graham et al. [37] with 25 µL of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (1 mg mL⁻¹; Sigma) instead of 3-(4,5 dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide. The difference between the samples and the positive control (100% living bacteria) was used to calculate the total bactericidal activity as a percentage.

2.7. Gene Expression

Total RNA from distal intestine samples was extracted using TRIzol reagent (Direct-zol™ RNA Miniprep, Zymo Research) according to manufacturer recommendations, and RNA quality and quantity were assessed by electrophoresis in 1% agarose gel and spectrophotometry (µDrop™ plate, ThermoScientific). The resulting total RNA concentration was adjusted to 0.5 µg/8 µL H₂O to complementary DNA synthesis, using the NZY First-Strand cDNA Synthesis Kit (NZYTech, MB12501, Lisbon, Portugal). Gene expression was determined by real-time quantitative PCR (Q-PCR) using the Bio-Rad, California, USA, CFX Connect Real-Time System. The analysis was carried out using 0.4 µL diluted cDNA (1:2) mixed with 0.2 µL of each primer (10 µM), 5 µL of SsoAdvanced™ Universal SYBR® Green Supermix, Bio-Rad Laboratories®, and 4.2 µL DNase/RNase/Protease-free water (Sigma-Aldrich), in a total volume of 10 µL. The efficiency of PCR primers was measured by the slope of a standard curve using serial dilutions of cDNA. Thermal cycling was initiated with incubation at 95°C for 30 s for hot-start iTaq™ DNA polymerase activation. A total of forty steps of PCR were then performed, each one consisting of heating at 95°C for 15 s for denaturing, and at 60°C for 30 s for annealing and extension. Following the final PCR cycle, melting curves were systematically monitored (temperature increment of 0.5°C 10 s⁻¹ from 65

to 95 °C). Each PCR run included duplicated reverse transcription for each sample and negative controls. The PCR run for the reference gene included duplicates for each sample and negative controls. Quantification of the target gene transcripts was performed using elongation factor 1 α (*ef1 α*) gene expression as a reference, as previously used for gilthead sea bream by Castro et al. [38]. Relative quantification of the target gene transcript with *ef1 α* reference gene transcript was performed using the mathematical model described by Pfaffl [39]. The relative expression ratio of a target gene was calculated based on real-time PCR efficiency and the CT deviation of the unknown sample compared with the *ef1 α* reference gene. The primers used are shown in Table 2.

2.8 Statistical analysis

Data are presented as fold change relative to the PBS injected fish (defined as 1) at the same sampling time (mean and standard error of the mean (SEM) for tables and standard error (SE) for figures). Normality and homogeneity of variances were tested by the Shapiro-Wilk and Levene tests, respectively, and normalized when appropriate. Statistical evaluation of the data was done by two-way ANOVA and if the interaction was significant, one-way ANOVA was performed for dietary treatment in each time of sampling and a t-test was used to access the effect of time in each dietary treatment. Differences were considered statistically significant at $p < 0.05$. When p -values were significant, means were compared with Tukey's test.

3. Results

The immunological response (haematological, humoral, and DI immune-related gene expression) of gilthead sea bream juveniles after 56 days of feeding the experimental diets was presented in Magalhães, et al. [28]. Here we present results of the same group of fish 4 and 24h after being challenged with killed *Phdp*.

3.1 Haematological response

Compared to the other diets, in fish fed with diet D, the RBC numbers and Ht increased while MCH and MCHC decreased at 4 h after injection with killed *Phdp* (Table 3). Regardless of the time, MCV decreased in fish fed with diet D compared to diet A. WBC numbers were not affected by dietary treatments at 4h but at 24 h were higher in fish fed diet C compared to diet D. Fish fed diet B had the highest lymphocytes and thrombocytes numbers at 4h whereas at 24 h the highest thrombocytes numbers were observed in fish fed diet C and diet A and the highest lymphocytes numbers were observed in fish fed diet C. Compared to 4h, at 24 h the WBC, lymphocytes, and thrombocytes numbers were reduced in fish fed diet B, while

RBC was increased in fish fed diets A and C and decreased in fish fed diet D. Neutrophils and monocytes numbers were neither affected by diet nor time post-challenge.

3.2 Humoral immune parameters

Plasma peroxidase activity showed higher activity at 24 than at 4 h after challenge in fish fed diets A and D, while the opposite was observed in fish fed diet C and no significant differences with time were observed in fish fed diet B. For plasma antiproteases activity, although a trend for increased values at 24h was noticed it was not detected by Tukey's multiple range test. NO activity was not affected by sampling time or dietary treatments. The ACP activity was not affected by sampling time, but it was higher in fish fed with diet D than with diets B and C (Fig. 1).

Plasma bactericidal activity was not affected by sampling time. Comparing infectious agents, plasma bactericidal activity against *V. anguillarum* did not show differences between dietary treatments while against *Phdp* it was higher in fish fed diet A compared to diet D (Fig. 2).

3.3 Immunity-related gene expression

Expression of major histocompatibility complex II (*mhc-II*) was not affected by diet composition at 4h after challenge, while at 24 h was higher in fish fed with diet C than with diets B and D. In fish fed diet B, but not in the other experimental groups, the expression of *mhc-II* decreased from 4 to 24 h after challenge.

Immunoglobulin M heavy chain (*igm*) expression was not affected by time after challenge but was higher in fish fed with diet C than in fish fed diets B and D.

Tumor necrosis factor α (*tnfa*) expression at 4 h after the challenge was higher in fish fed with diet D than the other dietary treatments, while at 24 h post-challenge was higher in fish fed with diet C than with diets A and B. Within the same dietary group, *tnfa* expression increased with time in fish fed diets A and C, while it was not significantly affected with time in fish fed the other diets.

Interleukin 1 β (*il-1 β*) gene expression was not affected with time post-challenge and was higher in fish fed with diet D than diets A and B.

Cyclooxygenase-2 (*cox₂*) gene expression was not affected with time post-challenge and was higher in fish fed with diet D than diet A.

Proliferating cell nuclear antigen (*pcna*) expression was not affected by diet composition at 4 h post-challenge and at 24h it was higher in fish fed with diet C than diet B. In fish fed with diet C, but not the other diets, *pcna* expression increased with time post-challenge.

4. Discussion

The objective of the present study was to compare the effects of diets with very different ARA, EPA, and DHA levels on the inflammatory response of gilthead sea bream juveniles challenged with a bacterial pathogen (killed *Phdp*). Diets were designed to have increasing n-3 LC-PUFA levels and decreasing n6/n3 ratios (diets A to C) while diet D was highly fortified with DHA.

Results showed several modulatory effects of dietary EFA profile on haematological parameters after inflammatory challenge with killed *Phdp*. Fish fed diet D, with high DHA content, presented at 4 h after challenge a higher haematocrit and RBC numbers and lower MCH, MCHC, and MCV than those fed the other diets, suggesting an enhanced oxygen delivery to the tissues [40]. However, 24 h post-injection these differences were not maintained. This seems to indicate a better physiological preparation of fish fed with diet D to the acute stress caused by the intraperitoneal injection, as previously observed in this species [41]. The importance of EPA and DHA for erythrocytes production in stressed gilthead sea bream was already demonstrated. Accordingly, fish fed a diet including 1.5 % n-3 LC-PUFA had higher RBC numbers than fish fed a 0.42 % diet when maintained under high-density stress but not under no stress conditions [18]. Contrarily, in unstressed gilthead sea bream, fish fed diets with a low level of n-3 LC-PUFA (0.5 %) had increased RBC count and lower MCV than fish fed diets with higher n-3 LC-PUFA content (2%;[12]). The authors suggested that a high saturated fatty acids content in the n-3 LC-PUFA deficient diet increased oxygen consumption resulting in an increment of RBC numbers. However, the authors also reported higher RBC fragility, measured as spontaneous haemolysis, in fish fed with low n-3 LC-PUFA content diets [12]. In the present work, the high RBC numbers 4 h after the challenge in fish fed diet D with a high DHA/EPA ratio (5:1) suggest that DHA may be more relevant than EPA for RBC production after a stressful event. Previously, after 56 days of growth trial, Magalhães, et al. [28] did not found any differences in RBC counts of unchallenged fish fed these same diets. Nevertheless, similar results regarding the importance of a high DHA/EPA ratio to increase RBC numbers were also reported in silvery-black porgy (*Sparidentex hasta*) [42]. The authors found that a high dietary DHA/EPA ratio

(3.3) resulted in increased RBC and Ht and decreased MCV and MCH values when compared to a 0.9 DHA/EPA ratio.

WBC are key elements in the immune response during inflammation [43]. In the present study, no differences in WBC numbers were detected at 4 h post-challenge, while at 24 h fish fed diet D, which had the highest DHA content, had lower WBC numbers compared to fish fed diet C, which had an equilibrated EPA: DHA ratio. Other studies reported no effect of dietary ARA, n-3 LC-PUFA, or DHA/EPA ratios on WBC numbers [42, 44-46]. These studies, however, were conducted with unchallenged fish, and this may explain the different responses observed in the present study.

The highest lymphocytes and thrombocytes numbers found in fish fed diet B and the highest lymphocyte numbers found in fish fed diet C at 4 h and 24 h post-challenge, respectively were, probably, the result of the n-3 LC-PUFA content and the DHA/EPA ratio of these diets. These two diets were designed to meet n-3 LC-PUFA requirements of gilthead sea bream juveniles with a balanced DHA/EPA ratio (1:1). In unchallenged gilthead sea bream juveniles, diet B increased the monocyte number indicating that adequate n-3 LC-PUFA dietary levels are important in the production of these WBC [28]. Accordingly, Montero et al., [12], showed, also in gilthead sea bream, that EFA deficient diets (0.5 % n-3 LC-PUFA; DHA/EPA = 1.6) reduced the circulating lymphocyte numbers compared to a control diet (2 % n-3 LC-PUFA; DHA/EPA = 0.9). In Atlantic salmon and channel catfish (*Ictalurus punctatus*), thrombocyte numbers increase with the adequate n-3 LC-PUFA dietary content provided by FO-based diets compared to fish fed diets with alternative lipidic sources to FO [47, 48]. The presented data suggested that adequate dietary n-3 LC-PUFA content with a DHA: EPA ratio of 1 may be important to lymphocytes and thrombocytes production during an immunological challenge.

The antiproteases and peroxidase activities were higher at 24h than at 4 h post-challenge, indicating an increased inflammatory response along the time. Although neutrophils are major producers of antiproteases and peroxidase in the bloodstream [43, 49], their number did not increase with the increase of antiproteases and peroxidase activities. A similar time response, 4, 24, and 48 h after injection with *Phdp* (5×10^3 cfu/fish) was also reported for several plasma immune parameters in European seabass, feeding graded levels of methionine as a result of an improved activation of phagocytic cells and a better development of an inflammatory response [50].

Different dietary EFA ratios did not affect antiproteases activity and NO production which is also in agreement with the absence of differences in neutrophils numbers between dietary treatments but, surprisingly, we found an increment of peroxidase 4 h post-challenge in fish fed diet C (similar DHA:EPA ratio) probably due to a quick activation of peroxidase production by neutrophils.

In the present study, differently from the bactericidal activity response against *Phdp*, no diet or time effect was observed on the bactericidal activity against *V. anguillarum*. This might be related to the distinct virulence mechanisms of the bacterial species [51, 52]. The highest bactericidal activity against *Phdp* was observed in fish fed diet A, with the higher dietary level of ARA and the lowest level of n-3 LC-PUFA. In the previous study by Magalhães, et al. [28], in fish under unchallenged conditions, it was observed that fish fed during 56 days a balanced ARA: EPA+DHA content (diet B), had higher bactericidal activity against *Phdp*. Therefore, under an immunological insult with killed *Phdp*, dietary extreme levels of ARA may be beneficial to boost the immunological response against this pathogenic. Previously, in gilthead sea bream submitted to a challenge with *Phdp*, it was found that the reduction of dietary n-3 LC-PUFA and increase n-6 FA level, through the replacement of 70% or 100% of FO by soybean oil decreased serum bactericidal capacity [19]. Nevertheless, the effect of dietary ARA as an immunomodulator was already shown with moderate levels (2.6% of total FA) by improving survival in challenged rabbitfish (*Siganus rivulatus*) with *Streptococcus iniae* [56]. Contrarily, in European seabass, the increasing dietary ARA levels (0.5 to 6 % of total FA) did not show such effects in the survival rate after an intestinal inoculation with *V. anguillarum* [57]. However, positive effects of a gradual increase of dietary ARA levels on immune response were reported in unchallenged European seabass, Japanese seabass (*Lateolabrax japonicus*), and Japanese eel (*Anguilla japonica*) [43, 54, 55]. Therefore, ARA supplementation in diets may be beneficial under unchallenged and challenged conditions but the level of supplementation may have to be adjusted to a specific pathogenic or stressful factor.

In the present study, the high dietary DHA levels and DHA/EPA ratio of diet D induced the highest ACP activity in post-challenge conditions. A different response was found when fish were fed the same diets under unchallenged conditions since diet B enhanced the ACP activity at that sampling point [28]. A similar response under challenging conditions (high stock density 12 kg per m³) was reported in gilthead sea bream once the reduction of dietary n-3 LC-PUFA level from 1.5 to 0.42% of the diet, reduced the ACP activity, while under no-stress conditions (3.3 kg per m³) that difference was not reported [18]. Albeit, in the presented

study rather than a total dietary n-3 LC-PUFA level effect the enhanced ACP activity with diet D in post-challenge conditions seems to be attributed more to DHA than EPA (DHA/EPA ratio= 5:1). Accordingly, in yellow croaker (*Larimichthys crocea*), after a natural infestation of the parasite *Cryptocaryon irritans*, high DHA/EPA ratios (3.04) improved nonspecific immunity through higher lysozyme activity (Zuo et al.,2012) compared to fish fed an 0.61 ratio. Also, in unchallenged fish (*Epinephelus malabaricus*), dietary DHA/EPA ratios of 3:1 and 2:1 enhanced nonspecific immunity parameters such as head-kidney leucocyte phagocytic and respiratory burst activity [20].

The integrity of the intestine is essential to prevent the entrance of pathogens, but it can be compromised by the use of alternative ingredients [53], which is the case of the experimental diets of the presented trial. Previously, at the end of a 56 days growth trial, it was observed that the present dietary treatments did not affect the intestinal expression of immune-related genes *mhc-IIa*, *tnfa*, *igm*, *cox₂*, *il-1β* and *pcna* in gilthead sea bream juveniles [28]. However, after the inflammatory insult with killed *Phdp* the expression of these genes was modulated by the diets, with an overall higher expression in fish fed diet C (0:0.6:0.6; ARA:EPA:DHA) and D (0:0.3:1.5; ARA:EPA:DHA). Different dietary effects on immune-related genes before and after a stressful or bacterial challenge were already reported in different fish species [54, 55].

Gilthead sea bream fed the highest dietary DHA (DHA/EPA ratio = 5:1) and the lowest ARA level (diet D) had the highest intestinal expression of *cox₂* and of two pro-inflammatory cytokines, *tnfa*, and *il-1β* which correlates well with the increased plasmatic ACP activity. Therefore, the results indicated that diet D may improve the non-specific immunity of gilthead sea bream after the killed *Phdp* challenge which seems to be more important in fish than in humans [56]. The higher *cox₂*, *tnfa*, and *il-1β* expression in fish fed low ARA diets was an unexpected result because high dietary ARA, have been associated with inflammatory response induction by pro-inflammatory eicosanoids such as 2-series prostanoids and 4-series leukotrienes through the production and release of proinflammatory cytokines such as *tnfa* and *il-1β* [14, 15]. On the other hand, EPA and DHA associated eicosanoids, the 3-series prostanoids, and 5-series leukotrienes are described as less potent and associated with anti-inflammatory properties by reducing the expression of inflammatory cytokines produced by activated immune-related cells and are also involved in the resolution of inflammation [14, 15, 57].

In the present study, the upregulation of *cox₂*, *tnfa*, and *il-1β* gene expression, in fish fed diet D, with the lowest ARA level, may be associated with the ARA content in the intestine.

Previously, it was observed that muscle ARA level of gilthead sea bream fed the same diets (A, B, C, and D) did not reflect the ARA content of the diet, being expected a similar pattern in the intestine tissue [29]. Moreover, the *cox₂* enzyme is not only activated by ARA and EPA, but they are preferential substrates [58]. DHA is also a precursor of other *cox₂* producing eicosanoid derivatives such as resolvins, maresins, and protectins with effects associated with the resolution of inflammation [15]. In fact, after a bacterial challenge with *V. anguillarum* in European seabass fed graded levels of ARA (0.5 to 6 % FA) the expression of *cox₂* and *il-1 β* in the DI was up-regulated only in diets with 0.5 and 2% ARA of total FA showing that the proinflammatory effect of ARA is not progressively correlated with the dietary content of this EFA [55]. In Senegalese sole (*Solea senegalensis*), submitted to a chasing-stress challenge, expression of EP₄ (low affinity prostaglandin E₂ receptor) increased in fish fed the FO and linseed oil diets and the expression of EP₂ (high affinity prostaglandin E₂ receptor) and phospholipase A₂ gene decreased in fish fed linseed oil diet, while a soybean oil diet did not modulate this gene expression, compared to the non-stress conditions [54]. These results suggested that fish fed FO and linseed oil-based diets, with higher levels of n-3 PUFA than soybean oil, were better prepared to deal with a stressful situation than those fed soybean oil diet (high n-6 PUFA content). The effects of dietary FA profile on the intestinal immune response after a challenge with killed *Phdp* may be time dependent. In gilthead sea bream juveniles, 24h after *Phdp* challenge, the proximal intestinal expression of *il-1 β* and *tnfa* were not affected by 70 or 100 % FO substitution by soybean or linseed oil [19]. The authors found overexpression of *il-1 β* and *tnfa* only 3 days after the challenge in fish fed diets with 100 % of FO substitution by soybean or linseed oil [19].

After 24h, and 4 and 24h post-challenge, the DI gene expression of two relevant genes of the adaptive immunity, *mhc-II* and *igm*, respectively, of fish fed diet C (0:0.6:0.6; ARA:EPA:DHA), was the highest among dietary treatments. However, *igm* expression in gilthead sea bream posterior intestine seems to be time-dependent, increasing with the time after infection with *Enteromyxum leei*, in fish fed with 66 % of VO diets compared to fish fed FO diets [59]. Therefore, the time of sampling of the presented study might be limited to further explore this result.

In the present study, 24 h post-challenge, PCNA expression results suggested that fish fed diet C (0:0.6:0.6; ARA:EPA:DHA) increased their cellular response against *Phdp* when compared to fish fed diet B (1:0.4:0.4; ARA:EPA:DHA). PCNA is a nuclear factor linked to DNA replication and it is widely accepted as a marker of cellular proliferation [60]. Furthermore, in brown trout (*Salmo trutta*), PCNA was used as a marker of the inflammatory

response [60]. Thus, the PCNA up-regulation 24 h after i.p. of fish fed diet C may be an indication of enterocytes or cells of the immune system proliferation but that hypothesis needs further investigation [60, 61].

The results of the presented study suggested that the dietary content of ARA, EPA, and DHA impacted the organism's response in post-challenge conditions. The fish response under a bacterial challenge to the dietary treatments was different than that observed in the non-challenged situation (Magalhães et al., 2021). Fish fed diet D (1.8% DM of EPA+DHA; DHA: EPA= 5:1) showed increased RBC counts 4 h after i.p., ACP activity, and upregulation of several immune-related genes in the DI. Therefore, the presented results indicate that in a bacterial challenge with killed *Phdp*, DHA rich diets can beneficially modulate the immunological response of gilthead sea bream juveniles. Nevertheless, fish fed diet A showed higher plasma bactericidal activity against *Phdp* than fish fed diet D, underlining the importance to further study the immunomodulatory effects of these two LC-PUFA in gilthead sea bream.

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Diets (ARA/EPA/DHA ratio)	A 2.0/0.2/0.1	B 1.0/0.4/0.4	C 0/0.6/0.6	D 0/0.3/1.5
% FA				
20:4n-6 (ARA)	10.6	5.43	0.18	0.11
20:5n-3 (EPA)	0.96	2.02	3.24	1.7
22:6n-3 (DHA)	0.7	2.06	3.52	7.71
n-6/n-3	1.5	0.98	0.63	0.52
% Diets				
20:4n-6 (ARA)	2.0	1.0	0.0	0.0
20:5n-3 (EPA)	0.2	0.4	0.6	0.3
22:6n-3 (DHA)	0.1	0.4	0.6	1.5
EPA+DHA	0.3	0.7	1.2	1.8
n-6/n-3	0.3	0.2	0.1	0.1

Table 1: Essential fatty acid composition and n-6/n-3 ratio of the experimental diets (% of total fatty acids). FA: fatty acid; ARA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid

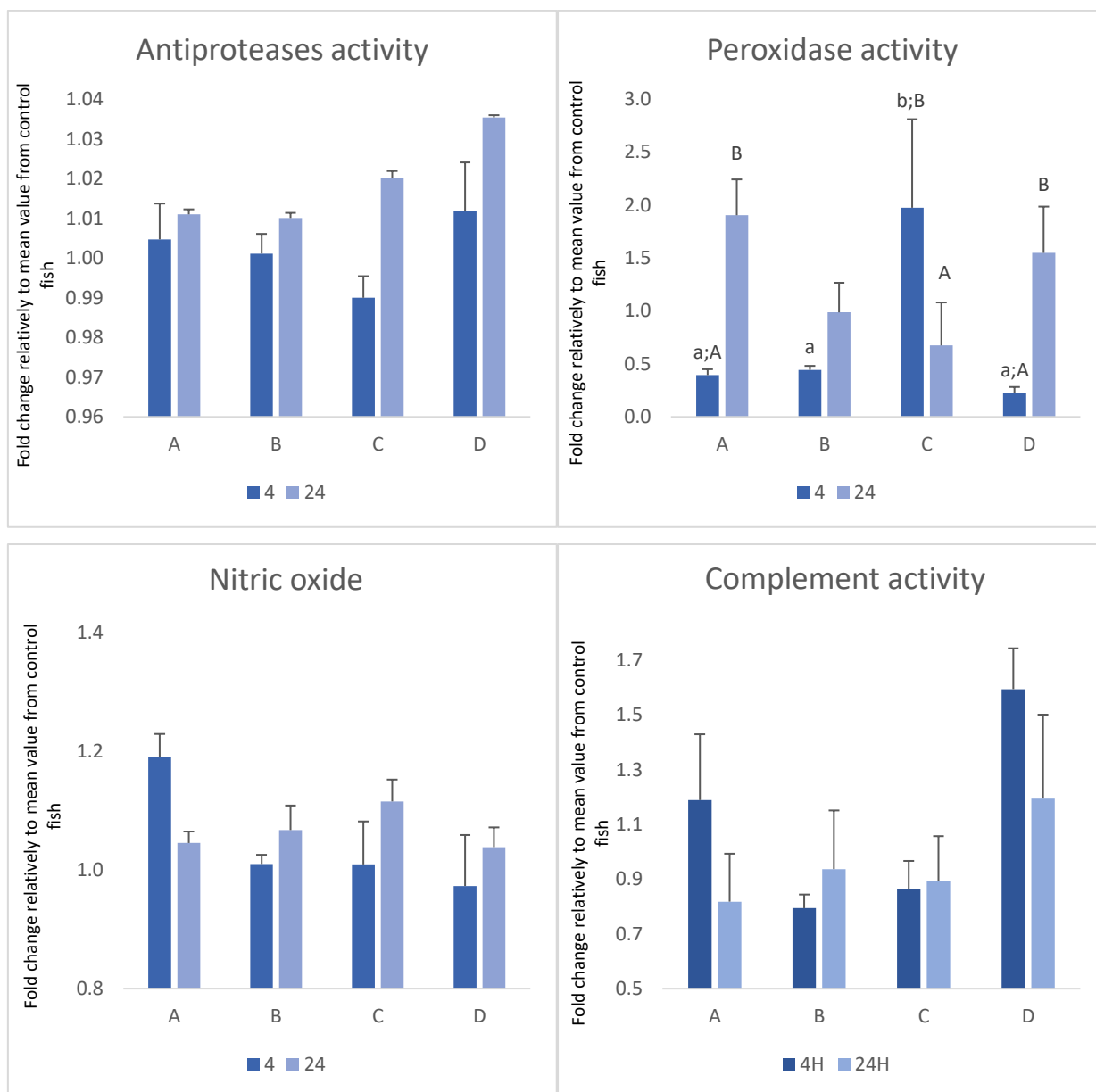
Gene	Gene abbreviation	Primer sequences (5'→3')	Primer efficiency	Accession number
Major histocompatibility complex II	<i>mhc-IIα</i>	F:CTGGACCAAGAACGGAAAGA R:CATGGACTCTGAGTAGCGCGA	2.00	DQ019401
Tumor necrosis factor α	<i>tnfa</i>	F:TCGTTTCAGAGTCTCCTGCAG R:CATGGACTCTGAGTAGCGCGA	2.02	AJ413189
Immunoglobulin M heavy chain	<i>igm</i>	F:CAGCCTCGAGAAGTGGAAAC R:GAGGTTGACCAGGTTGGTGT	2.04	AM493677
Cyclooxygenase 2	<i>cox2</i>	F:GAGTACTGGAAGCCGAGCAC R:GATATCACTGCCGCCTGAGT	1.92	AM296029
Interleukin 1β	<i>il-1β</i>	F:GGGCTGAACAACAGCACTCTC R:TTAACACTCTCCACCCTCCA	2.05	AJ277166
Proliferating cell nuclear antigen	<i>pcna</i>	F:GATGTGGAGCAGCTGGGTAT R:TGTCTACGTTGCTGGTCTGG	1.94	FG263675
Elongation factor 1-alpha	<i>ef1α</i>	F:CTGTCAAGGAAATCCGTCGT R:TGACCTGAGCGTTGAAGTTG	2.05	AF184170

Table 2. Sequences of the primer pairs used for determination of the transcript level of immune-related genes in the distal intestine in gilthead sea bream.

Dietary Treatments	A 2.0/0.2/0.1		B 1.0/0.4/0.4		C 0/0.6/0.6		D 0/0.3/1.5		SEM	
Parameters	4h	24h	4h	24h	4h	24h	4h	24h	4h	24h
RBC	^A 0.8 ^a	^B 1.0	0.8 ^a	1.1	^A 0.8 ^a	^B 1.1	^B 1.2 ^b	^A 0.9	0.05	0.04
Hemoglobin	0.9	1.1	1.0	1.1	0.9	1.0	0.8	0.9	0.03	0.04
Hematocrit	^A 1.0 ^a	^B 1.2	0.9 ^a	1.1	^A 0.9 ^a	^B 1.0	^B 1.5 ^b	^A 1.0	0.06	0.03
MCV	1.3	1.3	1.3	1.0	1.2	1.0	0.9	1.0	0.07	0.05
MCH	1.2 ^b	1.1	1.2 ^b	1.0	^B 1.3 ^b	^A 0.9	^A 0.7 ^a	^B 1.0	0.07	0.05
MCHC	0.8 ^b	0.8	1.0 ^b	1.0	^B 1.1 ^b	^A 0.9	^A 0.6 ^a	^B 0.8	0.04	0.04
WBC	1.1	1.1 ^{ab}	^B 1.3	^A 1.0 ^{ab}	1.1	1.2 ^b	1.0	1.0 ^a	0.05	0.03
Neutrophils	1.4	1.3	1.1	1.0	0.8	1.5	1.1	2.2	0.15	0.18
Monocytes	1.0	1.2	0.7	1.2	0.8	2.0	1.0	1.0	0.12	0.24
Lymphocytes	0.6 ^a	0.8 ^{ab}	^B 1.5 ^b	^A 0.6 ^a	^A 0.3 ^a	^B 1.5 ^b	0.3 ^a	0.9 ^{ab}	0.14	0.11
Thrombocytes	1.0 ^a	1.1 ^b	^B 1.5 ^b	^A 1.1 ^{ab}	1.2 ^{ab}	1.1 ^b	0.9 ^a	0.8 ^a	0.07	0.05

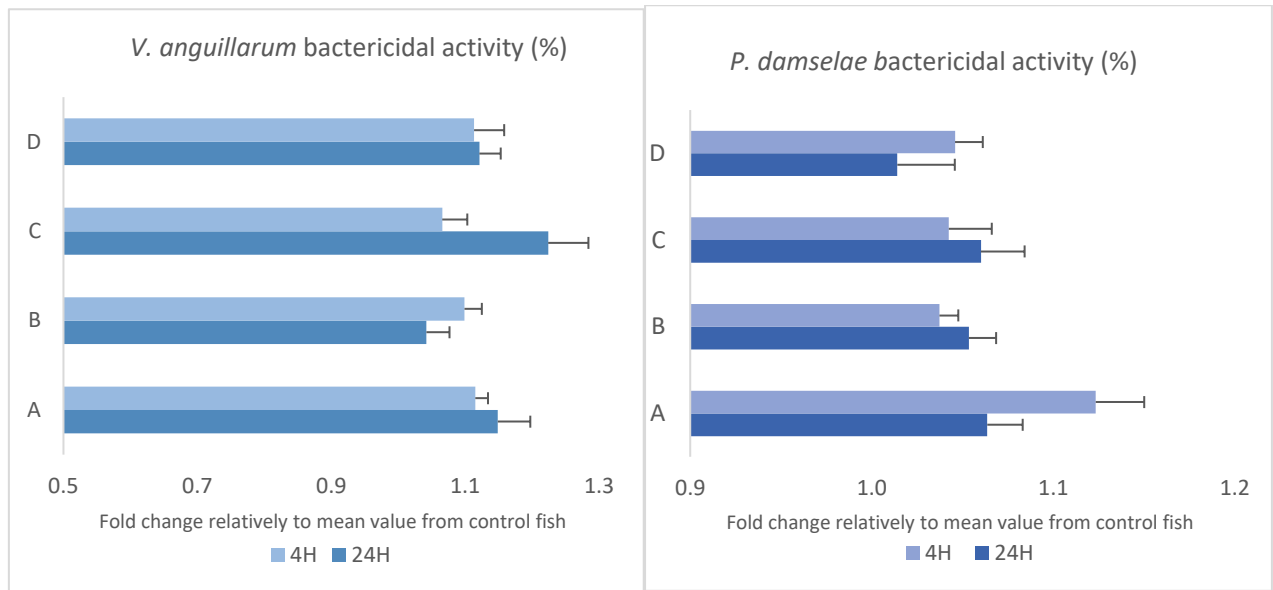
Two-way Anova				Diets			
Parameters	Diet	Time	Diet x Time	A	B	C	D
RBC	ns	0.023	0.000	-	-	-	-
Hemoglobin	ns	0.016	ns	-	-	-	-
Hematocrit	0.000	ns	0.000	-	-	-	-
MCV	0.031	ns	ns	b	ab	ab	a
MCH	0.019	ns	0.011	-	-	-	-
MCHC	0.000	ns	0.008	-	-	-	-
WBC	ns	ns	0.031	-	-	-	-
Neutrophils	ns	ns	ns	-	-	-	-
Monocytes	ns	ns	ns	-	-	-	-
Lymphocytes	ns	ns	0.000	-	-	-	-
Thrombocytes	0.000	ns	0.041	-	-	-	-

Table 3. Haematological profile of gilthead seabream fed the experimental diets at 4 and 24 hours after killed Phdp injection. Values presented as fold change relative to the PBS-injected fish (defined as 1).
MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; RBC: red blood cells; WBC: white blood cells.
Two-way ANOVA: ns: non-significant ($P > 0.05$); if interaction was significant, one-way ANOVA was performed in each time of sampling and a t-test was used to access the effect of time in each dietary treatment. Lowercase letters stand for significant differences among diets and uppercase letters for significant differences between sampling times.



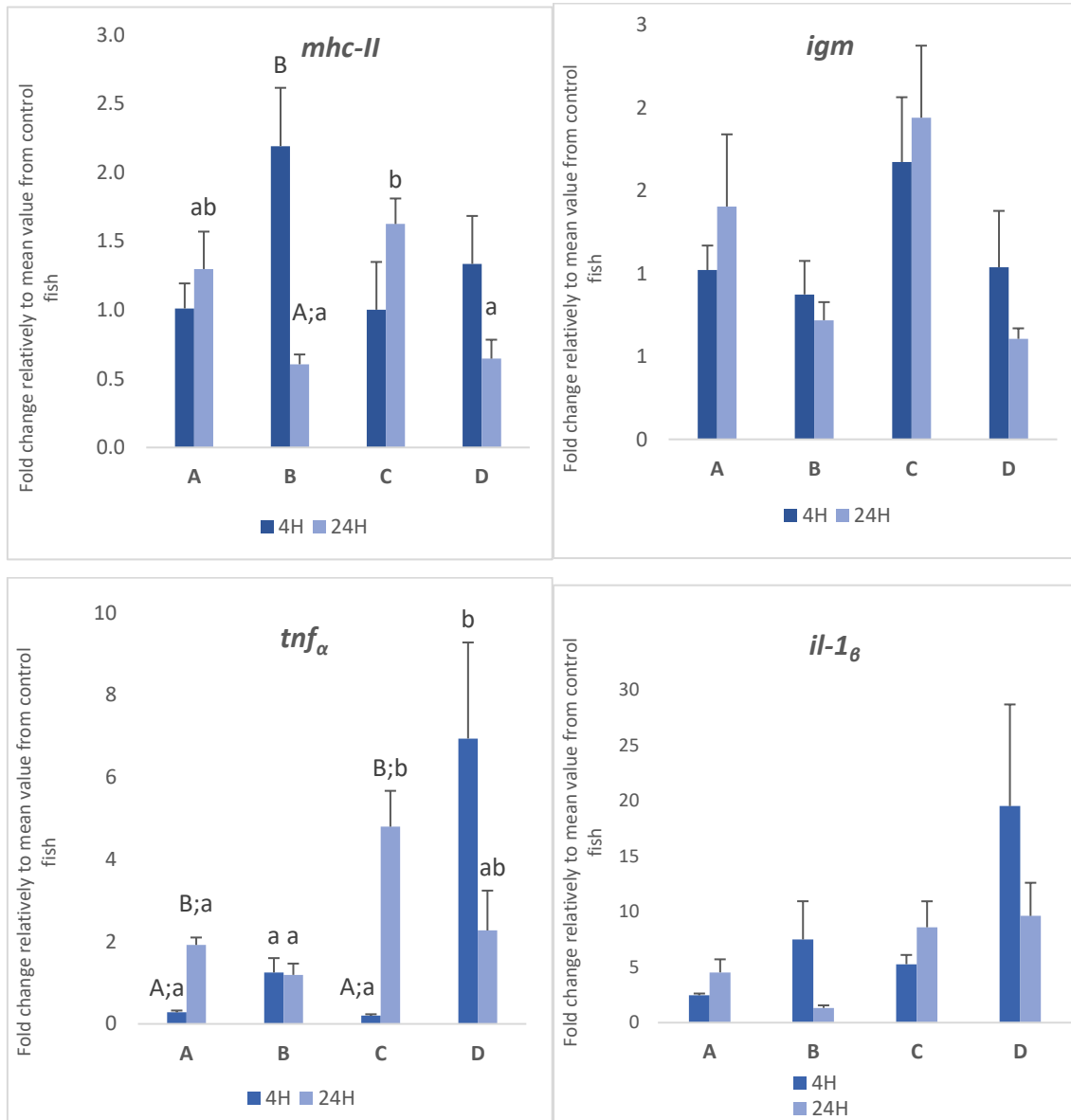
Two-way Anova	Diets (ARA/EPA/DHA ratio)						
	Diet	Time	Diet x Time	A	B	C	D
				2.0/0.2/0.1	1.0/0.4/0.4	0/0.6/0.6	0/0.3/1.5
Peroxidase activity	ns	0.025	0.000	-	-	-	-
Antiproteases activity	0.026	0.001	ns	-	-	-	-
Nitric oxide	ns	ns	ns	-	-	-	-
Complement activity	0.026	ns	ns	ab	a	a	b

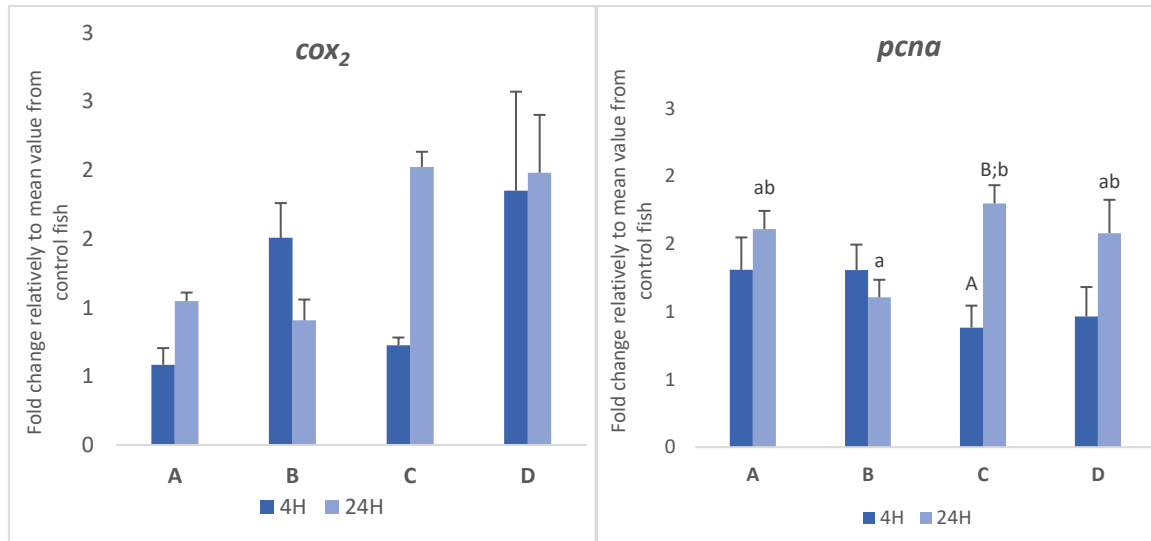
Fig 1. Plasma peroxidase, antiproteases, nitric oxide, and complement pathway activity in gilthead seabream juveniles fed the experimental diets at 4 and 24 hours after killed *Phdp* injection. Values (means $\pm$ SE) presented as fold change relatively to the PBS-injected fish (defined as 1) and analyzed using two-way ANOVA; if interaction was significant, one-way ANOVA was performed in each time of sampling and a t-test was used to access the effect of time in each dietary treatment. Lowercase letters stand for significant differences among diets and uppercase letters for significant differences between sampling times. Differences were considered statistically significant at $p < 0.05$. ns: non-significant.



Two-way Anova	Diets (ARA/EPA/DHA ratio)						
	Diet	Time	Diet x Time	A	B	C	D
				2.0/0.2/0.1	1.0/0.4/0.4	0/0.6/0.6	0/0.3/1.5
<i>V. anguillarum</i>	ns	ns	ns	-	-	-	-
<i>P. damsela</i>	0.039	ns	ns	b	ab	ab	a

Fig 2. Plasmatic bactericidal activity against *V. anguillarum* and *P. damsela* in gilthead seabream juveniles fed the experimental diets at 4 and 24 hours after killed *Phdp* injection. Values (means $\pm$ SE) presented as fold change relative to the PBS-injected fish (defined as 1) and analyzed using two-way ANOVA; if interaction was significant, one-way ANOVA was performed in each time of sampling and a t-test was used to access the effect of time in each dietary treatment. Lowercase letters stand for significant differences among diets and uppercase letters for significant differences between sampling times. Differences were considered statistically significant at $p < 0.05$. ns: non-significant.





Two-way Anova				Diets (ARA/EPA/DHA ratio)			
Genes	Diet	Time	Diet x Time	A	B	C	D
				2.0/0.2/0.1	1.0/0.4/0.4	0/0.6/0.6	0/0.3/1.5
<i>mhc-II</i>	ns	ns	0.008	-	-	-	-
<i>tnfa</i>	0.001	ns	0.000	-	-	-	-
<i>igm</i>	0.007	ns	ns	ab	a	b	a
<i>cox2</i>	0.019	ns	ns	a	ab	ab	b
<i>il-1β</i>	0.009	ns	ns	a	a	ab	b
<i>pcna</i>	ns	0.004	0.036	-	-	-	-

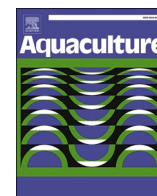
Fig. 3. Intestinal expression levels of Major histocompatibility complex II (MHC-II), Tumor necrosis factor α (TNF α), Immunoglobulin M heavy chain (IgM), Cyclooxygenase 2 (Cox2a), Interleukin 1 β (IL1 β) and Proliferating cell nuclear anti-gen (PCNA) of gilthead seabream juveniles fed the experimental diets at 4 and 24 hours after killed *Phdp* injection. Expression levels were normalized by housekeeping gene elongation factor 1- α (EF1 α). Values (means $\pm$ SE) presented as fold change of normalized expression level relative to the PBS-injected fish (defined as 1) and analyzed using two-way ANOVA; if interaction was significant, one-way ANOVA was performed in each time of sampling and a t-test was used to access the effect of time in each dietary treatment. Differences were considered statistically significant at $p < 0.05$. Lowercase letters stand for significant differences among diets and uppercase letters for significant differences between sampling times. ns: non-significant.

Chapter 6

Effects of dietary arachidonic acid and docosahexaenoic acid at different carbohydrates levels on gilthead sea bream growth performance and intermediary metabolism.

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Effects of dietary arachidonic acid and docosahexanoic acid at different carbohydrates levels on gilthead sea bream growth performance and intermediary metabolism

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ABSTRACT

A growth trial was performed to assess the interactive effects of high dietary levels of arachidonic acid (ARA) or docosahexanoic acid (DHA), and carbohydrates on growth performance, feed utilization, proximate composition, plasma metabolites, and intermediary metabolism. Four isoproteic (47% crude protein) and isolipidic (18% crude lipids) diets were formulated for gilthead sea bream juveniles (47.5 g of initial mean body weight) to include low or high gelatinized starch (5 and 20% starch; LS and HS diets, respectively) and different dietary ARA/DHA content (2.3/0.3 and 0/2.6; ARA and DHA diets, respectively). Dietary ARA/DHA content did not affect growth performance or feed utilization. However, dietary starch inclusion increased feed efficiency, protein efficiency ratio, and energy retention (% intake). Dietary ARA/DHA profile did not affect the whole-body composition, but dietary starch increased whole-body dry matter, lipid, and gross energy content and decreased protein content. Low dietary ARA/DHA ratio increased plasma total protein and total lipids, triglycerides, and very-low-density lipoprotein cholesterol (VLDL) and decreased high-density lipoprotein cholesterol (HDL) and low-density lipoprotein cholesterol (LDL), independently of the dietary starch level. On the other hand, in LS diets, total cholesterol levels were lower in fish fed the diet rich in DHA than those fed with ARA. HS diets increased glucose, total protein and lipids, triglycerides, and VLDL plasmatic values, and decreased HDL. HS diets increased the carbohydrates intermediary metabolism and lipogenic enzymes. ARA and HS increased fatty acid desaturase 2 (FADS₂) and decreased elongation of very long-chain fatty acid 5 protein (ELOVL₅) in the liver, and only HS decreased ELOVL₅ in the intestine. The muscle fatty acid content followed the dietary fatty acid profile, but ARA, EPA, and DHA content were increased in LS diets. In conclusion, few interactions between ARA or DHA and dietary digestible carbohydrates were found. The results indicate that at least in the presence of high ARA levels the n-3 LC-PUFA requirements were fulfilled with 0.5% EPA + DHA and gilthead sea bream tolerates well dietary inclusion of 20% starch.

1. Introduction

Gilthead sea bream is a highly important marine aquaculture fish in Europe, with a continuous increase in production reaching 91 thousand tonnes in 2019 (FAO, 2021; Llorente et al., 2020). Being a carnivorous species, gilthead sea bream does not tolerate high dietary carbohydrate levels (Enes et al., 2011). According to Couto et al. (2008) diets for sea

bream juveniles can contain up to 20% digestible CHO content without affecting growth performance or feed utilization.

The n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) requirements for gilthead sea bream juveniles are not clearly defined. Estimated requirement values range from 0.9 to 1.9% of the diet depending on the dietary DHA/EPA ratio (1:1 or 1:2, respectively) (Ibeas et al., 1994; Kalogeropoulos et al., 1992). Recently, Magalhães

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et al. (2020) tested diets with different ARA/EPA/DHA ratios and n-3 LC-PUFA levels ranging from 0.3 to 1.8% and concluded that the growth performance of gilthead sea bream juveniles was neither affected by the dietary essential fatty acid (EFA) ratio nor level. Nonetheless, the dietary EFA ratio needs to be optimized for improved feed efficiency. Of relevance, it was also shown that the dietary EFA ratio affected the expression of fatty acid elongases, with high dietary ARA or DHA/EPA ratios down-regulating ELOVL5 expression. Thus, optimization of dietary EFA ratio is of relevance when vegetable oils (VO), which are devoid of n-3 LC-PUFA, are included in the diets.

Up to now, a dietary ARA requirement was not established for gilthead sea bream. According to Fountoulaki et al. (2003), dietary ARA levels ranging from 0.2% to 11.2% of total FA did not affect growth performance and feed utilization of gilthead sea bream juveniles. Nonetheless, it was also shown that diets with high ARA or DHA levels (2 and 1.5%, respectively) modulated plasmatic triglycerides and cholesterol levels, liver lipid content, liver β -oxidation, lipogenesis, and ELOVL5 expression (Magalhães et al., 2020).

Although fish do not have a dietary carbohydrate (CHO) requirement, CHO are a cost-effective energy source that, within limits, will spare protein and lipid, improve pellets quality, and control feed costs (Krogdahl et al., 2005; N.R.C., 2011). The inclusion level of CHO in aquafeeds depends on the feeding habits of the fish, botanical source, molecular complexity, and nutrient interactions (Kamalam et al., 2017).

Fish, particularly carnivorous species, have a prolonged glycemia after being fed a CHO-rich meal (Hemre et al., 2002). This apparent lack of rapid glycemic regulation results from the conjugation of several factors, as an inefficient regulation of insulin/glucagon secretion, a low number of insulin receptors, inadequate regulation of glucose metabolism, and low glucose utilization in muscle and adipose tissues (Kamalam et al., 2017).

CHO are lipogenesis and glycogenesis enhancers (Castro et al., 2016b; Ekmann et al., 2013; Kamalam et al., 2012) and are known to interact with EFA synthesis by up-regulating desaturases, and elongases expression in salmonids (Kamalam et al., 2013; Seiliez et al., 2001) and gilthead sea bream (Castro et al., 2016b). Recently, dietary CHO were also shown to modulate circulating triglycerides and cholesterol levels, impacting lipid transport and fish oxidative status (Castro et al., 2016a; Castro et al., 2016b; Zhou et al., 2017).

Current aquafeeds for marine fish include increasing amounts of VO as an alternative to the traditional inclusion of fish oil (FO) (Colombo et al., 2020). However, while VO are rich in linoleic acid (LOA; 18:2n-6) and α -linolenic acid (ALA; 18:3n-3), they do not contain LC-PUFA, namely eicosapentaenoic acid (20:5 n-3, EPA), docosahexaenoic acid (22:6 n-3, DHA), and arachidonic acid (20:4 n-6, ARA), which are considered essential fatty acids (EFA) for marine fish (Monroig et al., 2018).

DHA has several structural and physiological functions, being essential for fish reproduction, growth, survival, and disease prevention (Izquierdo, 2005; Tocher, 2015). For instance, DHA is part of optic and neural tissues' structural composition, intervenes in the regulation of integrity and function of cell membranes, and is a precursor of cell-signaling compounds. Until recently, the importance of ARA has been neglected despite its potential effect on growth, survival, reproduction, lipid metabolism, stress resistance, and regulation of immune function (Bell and Sargent, 2003; Magalhães et al., 2020; Shahkar et al., 2016). Moreover, the balance between ARA and DHA, and EPA should also be considered. For instance, in California Yellowtail (*Seriola dorsalis*), a marine carnivorous species, it was shown that DHA and ARA are the primary drivers of FA essentiality (Rombenso et al., 2016). From a practical perspective is important to gain further knowledge on fish physiological functions and nutritional quality for consumers when CHO rich plant feedstuffs and VO are used to develop economic and environmentally friendly aquafeeds for marine fish. In fact, in gilthead sea bream juvenile's lipid digestibility decreased with dietary VO inclusion and interactions between dietary lipid source (VO or FO) and

carbohydrates level induced modifications in tissue lipid and glycogen content and cholesterol absorption and transport (Castro et al., 2016a; Castro et al., 2016b). These results, together with the effects of different dietary ARA/EPA/DHA dietary content on overall lipidic metabolism and muscle composition reported by Magalhães et al. (2020) may greatly influence the fish quality to consumers.

Thus, the objective of this study was to evaluate the dietary effect of ARA and DHA in diets with different CHO levels and potential interaction between them on growth performance, feed utilization, body composition, plasma metabolites, intermediary metabolism, and LC-PUFA biosynthesis.

2. Material and methods

2.1. Experimental diets

Four experimental diets were formulated to be isoproteic (47% crude protein) and isolipidic (18% crude lipids) and to include different dietary ARA/DHA content (2.3/0.3 and 0/2.6; ARA and DHA diets, respectively) combined with high (20%; HS) or low (5%; LS) gelatinized starch. A vegetable oil blend was used as the main lipid source (rapeseed: 20% + linseed: 50% + palm: 30%). The ingredients were carefully mixed and dry pelleted (California Pellet Mill, CPM Crawfordsville, IN, USA) through a 3 mm die. The pellets were dried in an oven at 40 °C for 48 h and stored at -4 °C until used. Ingredient and diet compositions are presented in Table 1, and dietary fatty acid composition is given in Table 2.

2.2. Growth trial

The experiment was approved by CIIMAR ethical committee for Managing Animal Welfare (ORBEA) in compliance with the European Union directive 2010/63/EU and the Portuguese Law (DL 113/2013).

Gilthead sea bream juveniles were purchased from a commercial fish farm (Sonrionansa, S.L., Cantabria, Spain). The trial was carried out in CIIMAR, Matosinhos, Portugal. Fish were kept in quarantine for a month and moved to the experimental system 15 days before the start of the trial for acclimatization to the experimental conditions. A recirculating water system with 12 cylindrical fiberglass tanks of 100 L capacity was used for the experiment trial. The system was thermo-regulated at 23 ± 1 °C and had a continuous flow of permanently filter seawater ($2.5\text{--}3.5$ L min⁻¹; salinity: 35 ± 1 ‰; dissolved oxygen: 7 ± 1 mg L⁻¹). The photoperiod was set to 12 h of light and 12 h of dark.

At the start of the trial, 20 fish per tank with 47.5 g of initial mean body weight were randomly assigned to 12 tanks (240 fish in total), and the experimental diets were also randomly assigned to triplicate tanks. During the trial, fish were fed 6 days a week by hand, until apparent visual satiation, for 12 weeks. Extreme care was taken to avoid feed losses.

2.3. Sampling

At the beginning of the trial, ten fish from the stock population were euthanized by anesthesia overdose with 2-phenoxyethanol (1 mL L⁻¹) and stored at -20 °C until whole-body composition analysis. At the end of the trial, fish were kept unfed for 24 h to reduce contamination of the digestive tract with diet leftovers and feces, lightly anesthetized (0.3 mL L⁻¹) and bulk weighed. Three fish from each tank were randomly sampled, euthanized with an overdose of anesthesia, weighted and liver, and viscera weights recorded for the determination of hepatosomatic and visceral indices and stored at -20 °C until whole-body composition analysis. The remaining fish were fed for two more days to recover from stress. Then, 3 fish from each tank were randomly sampled and blood was drawn from the caudal vein with heparinized syringes, 4 h after the morning meal to assure they were fully fed and in an absorptive and metabolic activity peak. The blood was immediately centrifuged at

Table 1

Ingredient and proximate analysis of experimental diets.

Diets	ARA/HS	ARA/LS	DHA/HS	DHA/LS
(ARA/DHA ratio)	2.3/0.3	2.3/0.3	0/2.6	0/2.5
Ingredients (% DM)				
Fish meal ^a	20.0	20.0	20.0	20.0
Corn gluten meal ^b	20.0	20.0	20.0	20.0
Wheat gluten meal ^c	18.8	18.8	18.8	18.8
Cellulose ^d	–	17.9	–	17.9
Gelatinized starch ^e	17.9	–	17.9	–
Taurine ^f	0.3	0.3	0.3	0.3
Lysine ^g	0.7	0.7	0.7	0.7
Vegetable oil blend ^h	10.0	10.0	11.8	11.8
Choline chloride (50%)	0.5	0.5	0.5	0.5
Mineral premix ⁱ	1.0	1.0	1.0	1.0
Vitamin premix ^j	1.0	1.0	1.0	1.0
Binder ^k	1.0	1.0	1.0	1.0
CaHPO ₄	3.7	3.7	3.7	3.7
VEVODAR ^l	5.3	5.3	–	–
DHA 70% AlgaTridium ^m	–	–	3.5	3.5
Proximate analyses (% DM)				
Dry matter (DM)	91.1	91.9	90.2	92.0
Crude Protein	46.5	46.7	46.9	46.1
Crude Lipid	17.6	17.7	18.7	17.7
Ash	8.6	9.2	8.9	8.7
Starch	19.6	4.6	20.0	5.4
Gross energy (kJ g ⁻¹)	23.4	23.0	23.1	23.1

CP: Crude protein; CL: Crude lipids; DM: Dry matter.

^a Steam Dried LT-FM, Pesquera Centinela, Chile (CP: 73.2%; CL: 9.2%).^b Sorgal, S.A. Ovar, Portugal (CP: 71.6%; CL: 1.6%).^c Sorgal, S.A. Ovar, Portugal (CP: 84.2%; CL: 2.3%).^d Sigma-Aldrich, Saint Louis, USA.^e C-Gel Instant – 12,016, Cerestar, Mechelen, Belgium.^f Feed-grade taurine, Sorgal, S.A. Ovar, Portugal.^g Feed-grade lysine, Sorgal, S.A. Ovar, Portugal.^h Vegetable oil blend: 20% rapeseed oil +50% linseed oil +30% palm oil.ⁱ Minerals (mg kg⁻¹ diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g kg⁻¹ diet); potassium chloride, 1.15 (g kg⁻¹ diet); sodium chloride, 0.4 (g kg⁻¹ diet).^j Vitamins (mg kg⁻¹ diet): retinol, 18,000 (IU kg⁻¹ diet); calciferol, 2000 (IU kg⁻¹ diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.^k Liptosa, Madrid, Spain.^l Vevodar, DSM Nutritional Products (ARA: 47.4%FA;EPA: 0.11%FA;DHA: 0.04%FA).^m BrudyTechnology (Tuna oil; ARA: 0%FA; EPA: 10.2%FA; DHA: 89.5%FA).

10000 xg for 10 min, the plasma was distributed in aliquots and frozen at –80 °C for metabolites analysis. The fish were then euthanized by decapitation and dissected in ice-cold trays. The liver was removed and stored at –80 °C until analysis of the activity of key intermediate metabolism enzymes. A portion of the lateral muscle was collected and pooled for total fatty acids quantification. A small portion of the liver and the distal intestine were collected, stored in RNAlater solution (1:10) for 24 h at 4 °C, and then frozen at –80 °C until gene expression analysis.

Two other fish per tank were sampled, the liver and lateral muscle were collected and stored at –80 °C until analysis of glycogen and lipid or glycogen, respectively.

2.4. Chemical analysis

The proximate composition of ingredients, diets, and whole-body composition was performed following Castro et al. (2016b). The liver lipid content was determined according to Folch et al. (1957). The starch content of the diets was quantified by the method of Beutler (1984) and hepatic and muscle glycogen content as described by Roehrig and Allred

Table 2

Fatty acid composition of the experimental diets (% of total fatty acids).

Diets	ARA/HS	ARA/LS	DHA/HS	DHA/LS
(ARA/DHA ratio)	2.3/0.3	2.3/0.3	0/2.6	0/2.5
SFA				
10:0	0.02	0.02	0.02	0.02
12:0	0.02	0.03	0.03	0.03
14:0	0.81	0.82	0.77	0.77
15:0	0.09	0.09	0.07	0.07
16:0	14.92	14.78	14.55	14.30
17:0	0.21	0.21	0.13	0.13
18:0	5.09	5.12	3.05	3.04
20:0	0.50	0.50	0.27	0.27
22:0	0.85	0.86	0.18	0.18
24:0	1.61	1.62	0.17	0.18
MUFA				
16:1n-7	0.75	0.75	0.71	0.72
16:1n-9	0.05	0.05	0.05	0.05
17:1n-7	0.01	0.01	0.01	0.01
18:1n-9	22.63	22.58	23.85	23.86
18:1n-7	1.04	1.03	1.07	1.07
20:1n-11	0.05	0.05	0.05	0.05
20:1n-9	0.52	0.51	0.41	0.41
20:1n-7	0.03	0.03	0.03	0.03
22:1n-11	0.34	0.33	0.36	0.36
22:1n-9	0.06	0.06	0.05	0.05
24:1n-9	0.21	0.21	0.16	0.17
PUFA				
16:2n-4	0.08	0.08	0.08	0.08
16:3n-4	0.08	0.08	0.08	0.08
16:4n-1	0.11	0.12	0.12	0.12
18:2n-6 (LA)	15.80	15.55	15.25	15.13
18:3n-6	0.68	0.69	0.02	0.02
18:3n-4	0.02	0.02	0.02	0.02
18:3n-3 (ALA)	14.88	14.98	17.87	17.95
18:4n-3	0.16	0.16	0.16	0.16
20:2n-6	0.23	0.23	0.04	0.04
20:3n-6	0.94	0.94	0.02	0.02
20:4n-6 (ARA)	13.11	13.24	0.17	0.14
20:3n-3	0.02	0.03	0.02	0.02
20:4n-3	0.06	0.06	0.08	0.08
20:5n-3 (EPA)	1.21	1.24	2.66	2.69
21:5n-3	0.05	0.05	0.86	0.87
22:2n-6	0.01	0.01	0.01	0.01
22:4n-6	0.10	0.10	0.02	0.01
22:5n-3	0.19	0.20	2.31	2.34
22:6n-3 (DHA)	1.53	1.60	13.67	13.90
Σ SFA	24.20	24.12	19.30	19.06
Σ MUFA	25.73	25.67	26.78	26.79
Σ PUFA	49.25	49.38	53.44	53.67
Σ n-6	30.86	30.76	15.53	15.37
Σ n-3	18.10	18.32	37.63	38.00
n-6/n-3	1.70	1.68	0.41	0.40

FA: fatty acid; LA: Linoleic acid; ALA: α-Linolenic acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid.

(1974). The fatty acid methyl esters (FAME) contained in the experimental diets and fish muscle were prepared by direct transesterification according to Lepage and Roy (1984) and analyzed by gas chromatography as described by Maia et al. (2019). The identification of fatty acids was made by comparison with known standard mixtures and quantified with the internal standard. Total fatty acids were determined by the sum of individual fatty acids.

2.5. Plasma metabolites analysis

Plasma glucose, total lipids, triglycerides, total cholesterol, cholesterol high-density lipoprotein (HDL), cholesterol low-density lipoprotein (LDL), cholesterol very-low-density lipoprotein (VLDL), and free fatty acids were analyzed according to standard clinical methods, with an auto-analyzer (Architect ci8200; Abbot Diagnostics, Saint-Laurent, Canada), by a clinical laboratory - certified by Bureau Veritas (NP EN

ISO 9001-2000). Plasma total proteins were analyzed using enzymatic colorimetric kits from Spinreact, Girona, Spain (total proteins kit, code 1001291), and measurements were taken utilizing a microplate reader (Multiskan Go Model 5111 9200; Thermo Scientific, Nanjing, China).

2.6. Enzymatic activity

Liver samples (dilution 1:6) were homogenized with the Precellys evolution apparatus (Bertin Instruments, France) at 4 °C and 7200 RPM (6 cycles of 20 s) using an ice-cold buffer, 100 mM Tris-HCl, 0.1 mM EDTA, and 0.1% (v/v) Triton X-100, pH 7.8. After homogenization, samples were centrifuged at 30000 xg for 30 min at 4 °C, and aliquots of the supernatants were carefully divided and stored at -80 °C. All enzymatic assays were performed at 37 °C by monitoring the modification in absorbance of NADPH at 340 nm using a microplate reader (Multiskan Go Model 5111 9200; Thermo Scientific, Nanjing, China), using 6.22 mM⁻¹ cm⁻¹ as the millimolar extinction coefficient for NADPH. The optimal substrate and protein concentrations were determined by preliminary assays to guarantee maximal activity for each enzyme. Glucokinase (GK, EC 2.7.1.2), hexokinase (HK, EC 2.7.1.1), pyruvate kinase (PK, EC 2.7.1.40), fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11), glucose 6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), fatty acid synthetase (FAS; EC 2.3.1.38) multienzymes complex activities, and malic enzyme (ME, EC 1.1.1.40) activities were determined as described by [Guerreiro et al. \(2014\)](#). 3-hydroxyacyl-CoA dehydrogenase (HOAD, EC 1.1.1.35) activity was quantified according to [Singer et al., 1990](#). The sample protein concentration was assessed following the [Bradford \(1976\)](#) method using Bio-Rad Protein Assay Dye Reagent (ref.5000006) and bovine serum albumin as standard. Specific enzyme activity was expressed as milliunits per mg of hepatic soluble protein.

2.7. Gene expression

Total RNA extraction in the liver and intestine samples was done using TRIzol reagent (Direct-zolTM RNA Miniprep, Zymo Research) following the manufacturer's recommendations. RNA integrity was verified by 1% agarose gel electrophoresis and the quality and quantity by spectrophotometry (μDropTM plate, ThermoScientific). The total RNA concentration was adjusted to 0.5 μg/8 μL H₂O, and cDNA synthesis was

performed using the NZY First-Strand cDNA Synthesis Kit (NZYTech, MB12501, Lisbon, Portugal).

Gene expression was quantified by real-time PCR (CFX ConnectTM Real-Time System, Bio-rad, California, USA). The primers were obtained in the literature ([Table 3](#)). Primer efficiency was determined according to [Pfaffl \(2001\)](#) using five serial two-fold dilutions of cDNA and calculated from the slope of the regression line of the quantification of the cycle (Ct) versus the relative concentration of cDNA.

The analyses of real-time PCR were carried out in a total volume of 10 μL using 0.5 μL of diluted cDNA (1:2) mixed with 0.5 μL of each primer (10 μM), 5 μL of SsoAdvancedTM Universal SYBR[®] Green Supermix, Bio-Rad Laboratories[®] and 3.5 μL DNase/RNase/Protease-free water (Sigma-Aldrich). The reaction was initiated by iTaqTM DNA polymerase activation with an incubation step (95 °C for the 30s). A total of forty cycles were completed, and each cycle comprised a denaturation (15 s at 95 °C), annealing, and extension (30 s at 56 °C) steps. After the final cycle, melting curves were systematically monitored (65 °C temperature 0.5 °C 10 s⁻¹ from 65 to 95 °C). Duplicates of reverse transcription for each sample and negative controls (reverse transcription free samples) were included in each PCR. The elongation factor 1 α (ef1α) and 18 s ribosomal RNA (18 s) were used as reference genes to normalize the target gene transcripts. Reference gene stability was checked by the BestKeeper method ([Pfaffl et al., 2004](#)). Expression levels are given as the ratio between copy numbers of the gene of interest and the geometric mean of copy numbers of reference genes ef1α and 18 s, following the method described by [Vandesompele et al. \(2002\)](#).

2.8. Statistical analysis

Data are presented as means ± standard deviation of the mean (SEM) for all the data except for gene expression which are presented as means ± standard error (SE). The normality and homogeneity of variances were checked and log-transformed when necessary. Data were analyzed by two-way ANOVA, with essential fatty acids or carbohydrate levels as fixed factors. In case of significant interactions, one-way ANOVA was performed for each factor. A significant level of 0.05 was used to reject the null hypothesis. All statistical analyses were conducted using SPSS 24.0 software package for Windows (IBM[®] SPSS[®] Statistics, New York, USA).

Table 3

Primer pairs sequences used for the transcript level determination of selected target and reference genes.

Gene	Gene abbreviation	Primer sequences (5' → 3')	Primer efficiency Liver	Primer efficiency Intestine	Accession number
Peroxisome proliferator-activated receptor alpha	<i>PPARα</i>	F: TCTCTTCAGCCCACCATCCC R: ATCCCAGCGTGTCTCTCC	2.04	2.07	AY590299
Peroxisome proliferator-activated receptor gamma	<i>PPARY</i>	F: CGCCGTGGACCTGTCAGAGC R: GGAATGGATGGAGGAGGAGGAGATGG	1.80	1.94	AY590304
Mechanistic target of rapamycin kinase	<i>mTOR</i>	F: CAGACTGACGAGGATGCTGA R: AGTTGAGCAGCGGGTCATAG	2.06	1.99	XM_030422395.1
Elongation of very long-chain fatty acids protein 4a	<i>Elovl4</i>	F: GCCCAAGTACATGAAGAACAGAG R: GGGGTCTGCTCTGAGTAGTCCA	1.98	1.87	MK610320
Carnitine palmitoyltransferase 1A	<i>CPT1A</i>	F: GTGCCTTCGTTTCCTCATGATC R: TGATGC TTATCTGCTGCTGTTTG	1.94	2.04	JQ308822
Fatty acid desaturase 2	<i>FADS₂</i>	F: GATGTGGAGCAGCTGGGTAT R: TGTCTACGTTGCTGGTCTGG	1.87	1.98	AY055749
Sterol regulatory element-binding protein 1	<i>Srebp₁</i>	F: AGGGCTGACCACAACGTCCTCTCC R: GCTGTACGTGGGATGTGATGGTTGGG	1.95	2.19	AF184170
Liver X receptor	<i>LXR</i>	F: GCACCTTCGCTCCAGGACAAG R: CAGTCTTCACACAGCCACATCAGG	1.86	1.82	FJ502320
Elongation of very long-chain fatty acid 5 protein	<i>Elovl₅</i>	F: TGCCAGGACACTCACAGTGC R: GGACGAAGCTGTTAGGGAGG	1.88	2.02	AY660879
Elongation factor 1α	<i>Ef1α</i>	F: CTGTCAAGGAAATCCGTCGT R: TGACCTGAGCGTTGAAGTTG	1.92	1.87	AF184170
Ribosomal protein S18	<i>18 s</i>	F: AGGGTGTGGCAGACGTTAC R: CTCTCGCTGTTGAGGAACC	2.09	1.95	AM490061

3. Results

Dietary ARA or DHA content did not affect growth performance or feed utilization, but HS diets promoted feed efficiency, protein efficiency ratio, and energy retention (% intake) (Table 4).

Dietary ARA or DHA level also did not affect the whole-body composition, while HS diets increased dry matter, lipid, and gross energy, and reduced protein content (Table 5). ARA diets decreased liver lipid content while HS diets increased liver glycogen and muscle fatty acids content, and the hepatosomatic and visceral somatic indexes (Table 5).

DHA diets increased plasma total protein, lipids, triglycerides, and VLDL, and reduced HDL and LDL (Table 6). The HS diets increased plasma glucose, total protein, total lipids, triglycerides, and VLDL and decreased HDL levels. An interaction between ARA or DHA and CHO dietary levels was found in plasma total cholesterol results. Plasma total cholesterol was lower in fish fed the DHA than the ARA, but only within the LS diets, and was lower in fish fed the HS than the LS diets only in fish fed the ARA diets (Table 6).

Dietary ARA or DHA level did not affect the activity of the hepatic metabolism enzymes assessed, except for HK activity where interaction with CHO level was found. Thus, HK activity was higher in fish fed the HS diet in the ARA group and in fish fed the DHA diet in the LS group (Table 7). The ME, FAS, G6PDH, GK, FBPASE, and PK activities were

Table 4

Growth performance, feed utilization, nitrogen, and energy retention of gilthead sea bream fed the experimental diets.

Diets	ARA/ HS	ARA/ LS	DHA/ HS	DHA/ LS	SEM
(ARA/DHA ratio)	2.3/0.3	2.3/0.3	0/2.6	0/2.5	
Initial body weight (g)	47.5	47.5	47.5	47.5	0.0
Final body weight (g)	134.7	128.6	140.1	135.4	1.9
Weight gain (g kg ABW ⁻¹ day ⁻¹)	11.4	11.0	11.7	11.4	0.1
Daily growth index ^a	1.79	1.70	1.87	1.80	0.0
Feed intake (g kg ABW ⁻¹ day ⁻¹)	14.0	14.5	14.6	15.0	0.2
Feed efficiency ^b	0.81	0.76	0.81	0.76	0.0
Protein efficiency ratio ^c	1.75	1.62	1.73	1.66	0.0
Survival (%)	100	100	96.7	100	0.6
Nitrogen retention (% NI) ^d	30.1	29.3	29.8	29.9	0.3
Energy retention (% EI) ^e	34.3	27.8	35.9	27.8	1.2

Two-way ANOVA

Variance source	EFA	CHO	Interaction
Initial body weight	NS	NS	NS
Final body weight	NS	NS	NS
Weight gain	NS	NS	NS
Daily growth index	NS	NS	NS
Feed intake	NS	NS	NS
Feed efficiency	NS	0.003	NS
Protein efficiency ratio	NS	0.006	NS
Survival	NS	NS	NS
Nitrogen retention	NS	NS	NS
Energy retention	NS	0.000	NS

Values presented as means ($n = 3$) and standard error of the mean (SEM).

ABW: average body weight = ((initial body weight + final body weight)/2).

^aDGI: daily growth index = ((final body weight^{1/3} - initial body weight^{1/3})/time in days) × 100.

^bFE: feed efficiency = wet weight gain/dry feed intake.

^cPER: protein efficiency ratio = wet weight gain/crude protein intake.

^dNitrogen retention = ((FBW × FBN - IBW × IBN)/NI) × 100.

^eEnergy retention = ((FBW × FBE - IBW × IBE)/EI) × 100;

IBN/FBN or IBE/FBE: initial and final body nitrogen or energy content; NI: Nitrogen intake; EI: Energy intake; IBW and FBW: initial and final body weight.

Two-way ANOVA: NS: non-significant ($P \geq 0.05$).

Table 5

Whole-body (% wet weight), liver, and muscle composition, hepatosomatic (HSI), and Visceral Somatic Index (VSI) of gilthead sea bream juveniles fed the experimental diets.

Diets	Initial	ARA/ HS	ARA/ LS	DHA/ HS	DHA/ LS	SEM
(ARA/DHA ratio)		2.3/0.3	2.3/ 0.3	0/2.6	0/2.5	
<i>Whole-body composition</i>						
Dry matter (%)	28.0	35.3	33.0	35.3	32.6	0.4
Protein	15.8	16.7	17.3	16.8	17.3	0.1
Lipids	8.7	14.6	11.9	14.4	10.9	0.5
Ash	4.5	4.2	4.2	4.4	4.3	0.1
Gross energy (kJ g ⁻¹)	6.9	8.8	7.9	9.2	7.9	0.2
<i>Liver composition</i>						
Lipid (mg/g)	–	113.8	119.9	207.0	168.5	10.7
Glycogen (mg/g)	–	109.1	73.1	100.6	58.8	5.0
<i>Muscle Composition</i>						
Total fatty acids (mg/g)	–	48.2	41.1	55.9	35.1	3.0
Glycogen (μg/g)	–	537.5	338.5	553.0	350.4	47.5
HSI ^a (%)	–	1.3	0.9	1.4	0.9	0.0
VSI ^b (%)	–	5.8	5.1	5.7	5.3	0.1

Two-way ANOVA

Variance source	EFA	CHO	Interaction
<i>Whole-body composition</i>			
Dry matter	NS	0.000	NS
Protein	NS	0.007	NS
Lipids	NS	0.000	NS
Ash	NS	NS	NS
Gross energy	NS	0.001	NS
<i>Liver composition</i>			
Lipid	0.000	NS	NS
Glycogen	NS	0.000	NS
<i>Muscle Composition</i>			
Total fatty acids	NS	0.014	NS
Glycogen	NS	NS	NS
HSI	NS	0.000	NS
VSI	NS	0.012	NS

Values presented as means ($n = 3$ for body composition and muscle total fatty acids; $n = 6$ for liver composition and muscle glycogen) and standard error of the mean (SEM) and were analyzed using two-way ANOVA. Differences were considered statistically significant at $p < 0.05$.

^aHepatosomatic index: (liver weight/body weight) × 100.

^bVisceral Somatic Index: (Viscera weight/body weight) × 100.

higher in the HS than in the LS groups.

The hepatic *FADS2* and *Srebp1* gene expression was higher in fish fed the ARA and HS diets than in the other groups, while the opposite was observed for *Elovl5* (Fig. 1). Liver X receptor (*LXR*) expression was higher in fish fed the LS than HS diets.

Intestinal *cpt1a* and *Elovl5* expression were higher in fish fed the LS diets (Fig. 2). *LXR* expression results showed an interaction between dietary ARA or DHA and CHO level resulting in higher expression in fish fed ARA than DHA but only within the LS diets, and in the LS than HS diet, within ARA diets.

In the muscle, saturated fatty acids (SFA) were generally higher in fish fed the ARA than DHA and HS than the LS diets (Table 8). Mono-unsaturated fatty acids (MUFA) were not affected by dietary EFA, except for 24:1n-9, which was higher in fish fed the ARA diets. 16:1n-7, 18:1n-9, and 22:1n-9 were higher in fish fed the HS diet. Regarding poly-unsaturated fatty acid (PUFA), DHA and n-3 PUFA were higher and n-6 PUFA was lower in fish fed the DHA diets, while the ARA content was higher in fish fed the ARA diets. The sum of PUFA was higher in fish fed the LS than HS diets.

Table 6

Plasma metabolites of gilthead sea bream juveniles fed the experimental diets.

Diets	ARA/ HS	ARA/ LS	DHA/ HS	DHA/ LS	SEM
(ARA/DHA ratio)	2.3/0.3	2.3/0.3	0/2.6	0/2.5	
Glucose (mg dL ⁻¹)	274.6	143.1	360.6	143.8	23.0
Total protein (g dL ⁻¹)	7.5	6.9	8.2	7.5	0.12
Total lipids (g dL ⁻¹)	2.3	1.7	3.3	2.4	0.16
Triglycerides (mg dL ⁻¹)	586.9	420.2	760.0	617.4	37.7
Total cholesterol (mg dL ⁻¹)	^A 261.3	^B 311.2 ^b	258.4	235.0 ^a	6.94
Cholesterol HDL (mg dL ⁻¹)	57.9	73.9	37.6	39.5	3.04
Cholesterol LDL (mg dL ⁻¹)	118.2	153.2	70.7	71.8	8.27
Cholesterol VLDL (mg dL ⁻¹)	102.0	84.0	152.0	123.7	6.70
Free Fatty Acids (μmol L ⁻¹)	1.1	1.1	1.2	1.5	0.10

Two-way ANOVA			
Variance source			
	EFA	CHO	Interaction
Glucose	NS	0.000	NS
Total protein	0.004	0.004	NS
Total lipids	0.002	0.006	NS
Triglycerides	0.009	0.026	NS
Total cholesterol	0.000	NS	0.002
Cholesterol HDL	0.000	0.018	NS
Cholesterol LDL	0.000	NS	NS
Cholesterol VLDL	0.000	0.046	NS
Free Fatty Acids	NS	NS	NS

Differences between treatments are presented as mean ($n = 9$) and standard error of the mean (SEM) and were analyzed using two-way ANOVA. If the interaction was significant, one-way ANOVA was performed for each factor. Differences were considered statistically significant at $p < 0.05$. The capital letters stand for differences between the digestible carbohydrate (CHO) levels within each essential fatty acid (EFA) level and subscript letters stand for differences between EFA within each CHO Level.

Table 7

Specific activities of liver intermediary metabolism related enzymes (mU/mg protein) in gilthead sea bream fed the experimental diets.

Diets	ARA/HS	ARA/LS	DHA/HS	DHA/LS	SEM
(ARA/DHA ratio)	2.3/0.3	2.3/0.3	0/2.6	0/2.5	
ME	7.0	4.0	9.1	4.1	0.5
FAS	7.4	4.0	7.1	2.2	0.4
G6PDH	171.4	81.5	166.0	94.0	9.3
HOAD	36.8	46.5	46.2	47.4	2.5
HK	^B 12.9	^A 6.9 ^a	11.3	10.8 ^b	0.7
GK	12.2	6.6	12.6	10.1	1.0
FBPASE	38.3	23.0	41.8	25.7	1.9
PK	55.3	29.0	45.7	31.1	3.1

Two-way ANOVA			
Variance source			
	EFA	CHO	Interaction
ME	NS	0.000	NS
FAS	NS	0.000	NS
G6PDH	NS	0.000	NS
HOAD	NS	NS	NS
HK	NS	0.009	0.024
GK	NS	0.0035	NS
FBPASE	NS	0.000	NS
PK	NS	0.000	NS

Values presented as means ($n = 9$) and standard error of the mean (SEM). Two-way ANOVA: ns: non-significant ($P \geq 0.05$). If the interaction was significant, one-way ANOVA was performed for each factor. The capital letters stand for differences between the digestible carbohydrate (CHO) levels within each essential fatty acid (EFA) level and subscript letters stand for differences between EFA within each CHO Level.

4. Discussion

In a previous study, Magalhães et al. (2020) showed no effects on the growth of gilthead sea bream juveniles fed diets with different ARA/EPA/DHA ratios. Nevertheless, besides adequate n-3 LC-PUFA (EPA and DHA) levels, diets should include an adequate dietary ARA level for feed efficiency optimization.

This study aimed to further assess the effects of dietary ARA and DHA ratios and potential interactions with dietary carbohydrate levels.

4.1. Effects of dietary essential fatty acid

The fact that DHA had no effect on the growth of sea bream concur with Magalhães et al. (2020) showing that the dietary n-3 LC-PUFA requirement of gilthead sea bream juveniles is met with 0.3–0.5% EPA + DHA. This is much lower than previous estimations, which have indicated EFA requirements of 0.9 or 1.9%, depending on the DHA/EPA ratio (Ibeas et al., 1994; Kalogeropoulos et al., 1992). Both in the present study and that of Magalhães et al. (2020), the dietary DHA/EPA ratio did not seem to affect EFA requirement.

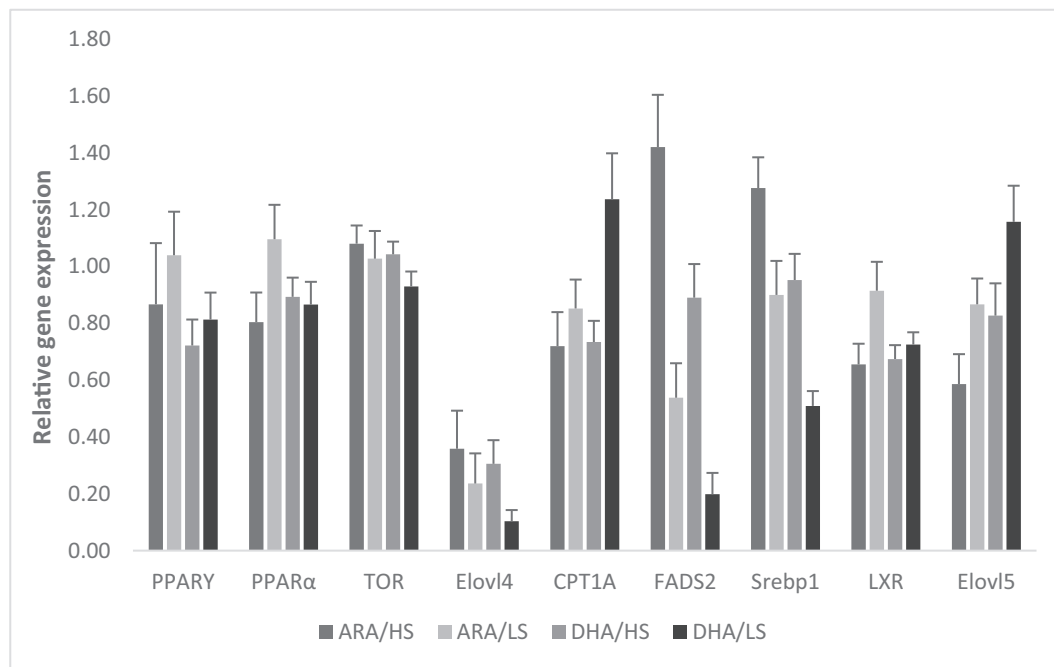
Furthermore, growth performance was not affected by the dietary ARA level used in the present study. This agrees with previous observations in gilthead sea bream (Fountoulaki et al., 2003) and Malabar red snapper, *Lutjanus malabaricus* (Chee et al., 2020). In other marine species, such as European seabass (*Dicentrarchus labrax*), turbot (*Scophthalmus maximus*), and California Yellowtail increasing dietary ARA content has been shown to improve growth performance (Rombenso et al., 2016; Torrecillas et al., 2018; Wei et al., 2021). However, was recently shown in Atlantic salmon post-smolt (*Salmo salar*) that high dietary ARA levels reduced the growth performance (Løvmo et al., 2021).

Fish fed the DHA diets had increased plasma total protein, total lipids, triglycerides, and VLDL compared to fish fed the ARA diets. In mammals, DHA in plasma can be transported bound to albumin and the increment of total protein observed in the present study may be related to higher plasma albumin levels associated with DHA transport (Chen et al., 2015). The increased plasmatic total lipids, triglycerides, and VLDL levels (the lipoprotein responsible for cellular triglycerides delivery) in fish fed the DHA diets seem to indicate a higher lipid transport and deposition capacity, as further suggested by the higher liver lipid content in this group compared to that of fish fed the ARA diets. In a previous study also in this species, fish fed high DHA/EPA diets also presented higher liver lipid content than fish fed the ARA diets (Magalhães et al., 2020).

Dietary n-3 LC-PUFA has been shown to decrease cholesterol and to have a protective effect on cardiovascular disease in both humans and fish (Hong et al., 2018; Mozanzadeh et al., 2015; Tocher, 2015). Accordingly, dietary DHA reduced plasma total cholesterol in fish fed the LS diets in agreement with our earlier observations (Magalhães et al., 2020). Further, plasma HDL and LDL concentrations were also lower in fish fed DHA diets, which seems to agree with a reduction of plasma cholesterol levels in fish fed these diets.

Dietary ARA and DHA did not affect the activity of key enzymes related to the intermediary metabolism of lipid and glucose. The expression of genes associated with β -oxidation, *PPAR α* , and *CPT1A*, was not affected by diet composition. This contrasts with an earlier study in gilthead sea bream where growth performance was reduced and hepatic expression of *PPAR α* and *CPT1A* increased when fish were fed low n-3 LC-PUFA levels (Houston et al., 2017). The difference appears to be due to EFA deficiency in the latter and that the requirements were met in the present study.

The expression of most genes involved in intermediary metabolism in the liver and intestine were also unaffected by diet composition. Nevertheless, hepatic *Srebp1* expression was increased in fish fed the ARA diets. *Srebp1* is involved in lipogenesis and lipid deposition in the liver (Carmona-Antoñanzas et al., 2014). In gilthead sea bream, *Srebp1*



Two-way ANOVA		Variance source	
Gene target	EFA	CHO	Interaction
PPARY	NS	NS	NS
PPARα	NS	NS	NS
TOR	NS	NS	NS
Elovl ₄	NS	NS	NS
CPT1A	NS	NS	NS
FADS ₂	0.001	0.000	NS
Srebp ₁	0.001	0.000	NS
LXR	NS	0.031	NS
Elovl ₅	0.022	0.009	NS

Values presented as means (n=9) and standard error (SE);

Fig. 1. Hepatic expression of peroxisome proliferator activated receptors (*ppary*, *ppara*), mechanistic target of rapamycin (*mtor*), genes involved in LC-PUFA biosynthesis (*Elovl₄*, *Elovl₅*, *FADS₂*), sterol regulatory element-binding protein 1 (*Srebp₁*), carnitine palmitoyl transferase I (*cpt1a*), liver X receptor (*LXR*). Values presented as means (n = 9) and standard error (SE).

expression increased when fed low dietary n-3 LC-PUFA levels (Houston et al., 2017). Though in this study *Srebp1* expression was also higher in the low n-3 LC-PUFA diets (ARA diets), liver lipid deposition was higher in fish fed the DHA diets. This response seems contradictory and needs to be further elucidated.

Liver *FADS2* expression was higher in fish fed the ARA than DHA diet while *ELOVL5* expression was higher in fish fed the DHA diets. In the intestine, however, dietary ARA or DHA did not affect the expression of these enzymes. Previously, also in gilthead sea bream juveniles, it was also shown that hepatic *FADS2* was induced by low n-3 LC-PUFA, while *ELOVL5* expression was unaffected by n-3 LC-PUFA level (Castro et al., 2016b). In the intestine, however, *ELOVL5* expression was higher in fish fed the low LC-PUFA diet (Castro et al., 2016b). Also recently, it was shown in gilthead sea bream post-larvae that low DHA diets increased visceral *FADS2* and *ELOVL5* expression (Torres et al., 2020). These results suggest that these enzymes are nutritionally regulated and induced by low levels of EFA.

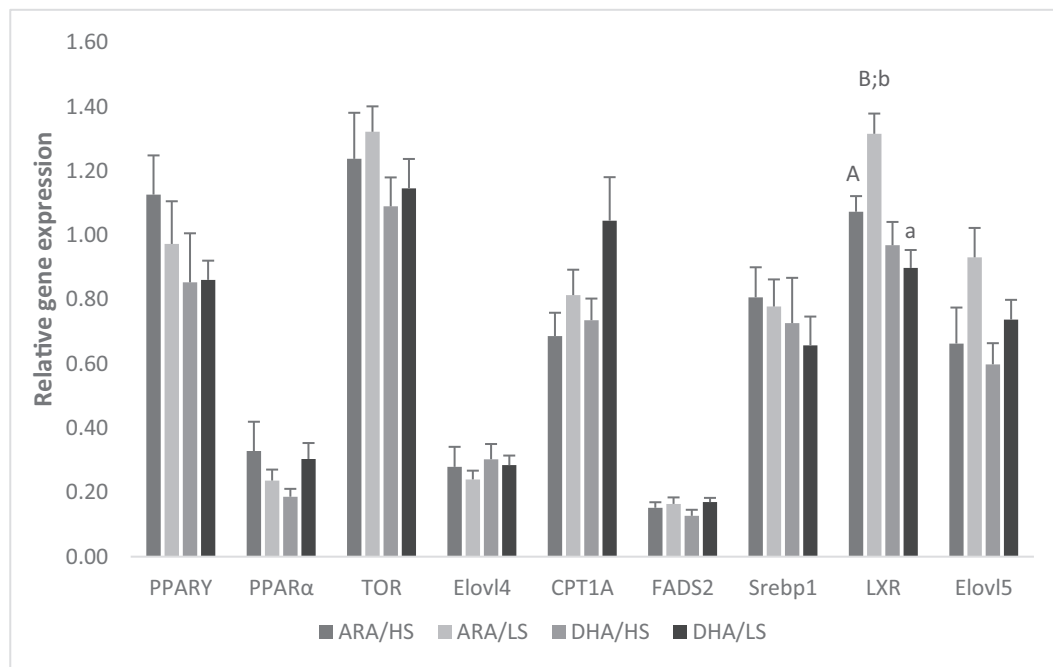
As expected, the muscle ARA, EPA, and DHA profile followed that of the diets, as previously observed in this species (Benedito-Palos et al., 2008). Further, the muscle fatty acid profile reflected the upregulation of *FADS2* expression in fish fed the ARA diets. In fact, *FADS2* desaturation of 18:2n-6 but also 18:3n-3 resulting in higher levels of the desaturation

products such as 18:3n-6 and 18:4n-3. Increased *FADS2* expression in sea bream fed low EFA diets has also been shown to lead to increased hepatic 18:2n-9 resulting from 18:1n-9 desaturation (Houston et al., 2017) despite the increment of 18:2n-9 was not seen in the presented study.

4.2. Effects of dietary carbohydrate level

As the diets were isoproteic and isolipidic, HS diets contained more digestible energy than LS diets. Given diets that meet nutritional requirements, fish usually eat to meet their digestible energy needs (Cho and Kaushik, 1990). In this study, feed intake and growth were not significantly affected by the dietary energy density. There was however a trend towards increased feed intake and reduced growth in fish fed the LS diets, leading to a significant reduction in feed efficiency. Accordingly, also in gilthead sea bream juveniles, a tendency for lower feed efficiency was reported in fish fed no starch diet compared to diets containing 20% starch (Enes et al., 2008).

The differences in dietary digestible energy were also reflected in body composition, with fish fed the HS diet having increased whole-body lipid and energy content and muscle fatty acid content. Moreover, VSI and HSI were also higher in fish fed the HS diets, reflecting an increased lipid and glycogen deposition, respectively.



Two-way ANOVA		Variance source	
Gene target	EFA	CHO	Interaction
PPARY	NS	NS	NS
PPARα	NS	NS	NS
TOR	NS	NS	NS
Elovl ₄	NS	NS	NS
CPT1A	NS	0.027	NS
FADS ₂	NS	NS	NS
Srebp ₁	NS	NS	NS
LXR	0.000	NS	0.016
Elovl ₅	NS	0.021	NS

Fig. 2. Intestine expression of peroxisome proliferator activated receptors (*ppary*, *ppara*), mechanistic target of rapamycin (*mTOR*), genes involved in LC-PUFA biosynthesis (*Elovl₄*, *Elovl₅*, *FADS₂*), sterol regulatory element-binding protein 1 (*Srebp₁*), carnitine palmitoyl transferase I (*cpt1a*), liver X receptor (*LXR*). Values presented as means ($n = 9$) and standard error (SE); Capital letters stand for differences between dietary starch levels within each essential fatty acid level. Lower case letters stand for differences between dietary essential fatty acids within each CHO Level.

The higher whole-body lipid content was, at least in part, promoted by the higher available glucose, as suggested by the increased activity of lipogenesis-related enzymes in fish fed the HS diets. This was in line with previous observations in sea bream (Castro et al., 2016b; Couto et al., 2008).

The increased plasma lipids, triglycerides, HDL, and VLDL levels in fish fed the HS diets seems to reflect increased hepatic lipid mobilization and transport leading to elevated muscle fatty acids accumulation.

The HS diets induced hypocholesterolemia in fish fed the ARA diet but not in those fed the DHA diet. Also, intestinal *LXR* gene expression, a molecular regulator involved in cholesterol homeostasis by transactivating the expression of cholesterol ester transfer proteins, apolipoproteins, and genes that control cholesterol efflux from the cells (Jakobsson et al., 2012) followed a similar response pattern. Furthermore, *LXR* expression was up-regulated in the liver of fish fed the LS diets and may contribute to explain the high plasma cholesterol observed in fish fed the ARA/LS diet. Thus, further investigation is necessary to understand the interaction between dietary ARA and CHO in cholesterol regulation.

The increased GK and PK activity in fish fed the HS diets confirms the capability of sea bream to control glycolysis following increased glucose intake (Couto et al., 2008; Enes et al., 2008). Increased glucose intake

also led to the accumulation of hepatic glycogen as noted earlier (Enes et al., 2009). However, as previously observed by other authors, the increased FBPASE activity in fish fed the HS diets confirm a limited capacity to regulating gluconeogenesis in sea bream (Couto et al., 2008; Enes et al., 2008) eventually leading to the observed hyperglycemia. Though it is considered that HK activity is not under nutritional control (Enes et al., 2011), our results show a major increase in HK activity but only in fish fed the ARA diet. This needs to be studied in detail in posterior studies.

mTOR activation is involved in the regulation of the mature form of *SREBP₁* and, consequently, the expression of its target genes, such as FAS (Porstmann et al., 2008). In the present study, although *mTOR* expression was not affected by dietary carbohydrate level, hepatic *SREBP₁* expression increased in fish fed the HS diets. Contrarily, in rainbow trout (*Oncorhynchus mykiss*) dietary carbohydrates failed to activate *SREBP₁* expression (Seilliez et al., 2011).

CPT1A is a marker of mitochondrial β -oxidation, and its up-regulation in the intestine of fish fed the LS diets suggests an increased utilization of lipids as an energy substrate in this organ when the availability of dietary glucose is limited. This was not confirmed by hepatic HOAD activity, which was not significantly affected by dietary carbohydrate level, though a trend for higher activity in fish fed the LS

Table 8

Muscle fatty acid composition (% of total fatty acids) of gilthead sea bream fed the experimental diets.

						Two-way ANOVA		
						Variance source		
Fatty acid	ARA/HS	ARA/LS	DHA/HS	DHA/LS	SEM	EFA	CHO	Interaction
(ARA/DHA ratio)	2.3/0.3	2.3/0.3	0/2.6	0/2.5				
SFA								
C14:0	1.50	1.46	1.39	1.28	0.03	0.018	NS	NS
C15:0	0.14	0.16	0.12	0.14	0.01	0.001	0.000	NS
C16:0	16.44	14.31	15.47	13.70	0.32	0.000	0.000	NS
C17:0	0.18	0.20	0.12	0.14	0.01	0.000	0.000	NS
C18:0	4.65	4.03	3.56	2.94	0.19	0.000	0.000	NS
C20:0	0.29	0.30	0.18	0.19	0.02	0.000	NS	NS
C22:0	0.36	0.36	0.12	0.11	0.04	0.000	NS	NS
C24:0	0.45	0.46	0.02	0.02	0.07	0.000	NS	NS
MUFA								
14:1n-5	0.04	0.04	0.04	0.04	0.00	NS	NS	NS
C16:1 n-7	2.45	2.25	2.77	2.20	0.08	NS	0.003	NS
C17:1 n-7	0.04	0.05	0.04	0.05	0.00	NS	0.015	NS
C18:1n-7	1.21	1.24	1.27	1.24	0.02	NS	NS	NS
C18:1 n-9	24.98	23.21	26.25	23.16	0.42	NS	0.000	NS
C20:1n-7	0.05	0.06	0.06	0.06	0.00	NS	NS	NS
C20:1n-9	1.20	1.33	1.19	1.13	0.04	NS	NS	NS
C20:1n-11	0.19	0.23	0.17	0.20	0.01	NS	NS	NS
C22:1 n-9	0.24	0.23	0.25	0.20	0.01	NS	0.043	NS
C22:1n-11	0.95	1.23	0.91	1.03	0.05	NS	NS	NS
C24:1 n-9	0.36	0.34	0.19	0.25	0.03	0.020	NS	NS
PUFA								
C16:3n-4	0.06	0.07	0.06	0.06	0.00	0.019	0.001	NS
C16:4n-1	0.09	0.11	0.09	0.10	0.00	NS	0.05	NS
C18:2 n-6 (LA)	12.30	13.46	11.83	13.01	0.20	0.018	0.000	NS
C18:3 n-3 (ALA)	10.54	11.40	12.28	13.10	0.30	0.000	0.004	NS
C18:4n-3	0.44	0.44	0.38	0.36	0.01	0.006	NS	NS
C18:3 n-6	0.59	0.60	0.08	0.04	0.08	0.000	NS	NS
C20:2 n-6	0.36	0.38	0.27	0.25	0.02	0.000	NS	NS
C20:3 n-3	0.31	0.30	0.43	0.42	0.02	0.001	NS	NS
C20:3 n-6	0.88	0.88	0.10	0.06	0.12	0.000	NS	NS
C20:4n-3	^B 0.30	^A 0.27 ^b	^B 0.31	^A 0.22 ^a	0.01	0.042	0.000	0.011
C20:4 n-6 (ARA)	^A 9.09 ^b	^B 10.46 ^b	0.20 ^a	0.28 ^a	1.45	0.000	0.002	0.004
C22:2n-6	0.06	0.05	0.05	0.05	0.00	0.021	0.001	NS
C22:4n-6	^B 1.59 ^b	^A 1.43 ^b	0.04 ^a	0.05 ^a	0.22	0.000	0.026	0.029
C20:5 n-3 (EPA)	1.32	1.52	2.06	2.30	0.12	0.000	0.001	NS
C21:5n-3	^A 0.09 ^a	^B 0.11 ^a	^A 0.63 ^b	^B 0.68 ^b	0.08	0.000	0.001	0.019
C22:5n-3	0.68	0.77	2.36	2.60	0.27	0.000	0.002	NS
C22:6 n-3 (DHA)	3.40 ^a	4.21 ^a	^A 12.99 ^b	^B 16.74 ^b	1.72	0.000	0.000	0.001
ΣSFA	24.20	21.51	21.15	18.72	0.59	0.000	0.000	NS
ΣMUFA	32.24	30.56	^B 33.56	^A 29.83	0.47	NS	0.000	0.025
ΣPUFA	42.19	46.55	44.25	50.42	0.94	0.000	0.000	NS
Σn3	^A 17.08 ^a	^B 19.02 ^a	^A 31.43 ^b	^B 36.43 ^b	2.46	0.000	0.000	0.001
Σn6	^A 24.87 ^b	^B 27.25 ^b	^A 12.59 ^a	^B 13.74 ^a	1.97	0.000	0.000	0.031

Values presented as means (n = 3) and standard error of the mean (SEM). Capital letters stand for differences between the dietary starch levels within each essential fatty acid (EFA) level and lower case letters stand for differences between EFA within each CHO Level. FA: fatty acid; LA: Linoleic acid; ALA: α-Linolenic acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid.

diets was noticed.

As also previously observed in this species, dietary carbohydrates modulated differently the expression of *FADS₂* and *ELOVL₅* in the liver and intestine (Castro et al., 2016b).

Liver *FADS₂* expression was higher in fish fed the HS diets while *ELOVL₅* expression was higher in fish fed the LS diets. Differently, in the intestine dietary carbohydrate did not affect the *FADS₂* expression though *ELOVL₅* expression was also higher in fish fed the LS diets. The increased expression of *ELOVL₅* in fish fed LS diets is in line with the higher PUFA content found in the muscle of fish fed those diets. As in the present study, Castro et al. (2016b) also observed in this species that hepatic *FADS₂* was induced by HS diets while *ELOVL₅* expression was induced by LS diets. In the intestine, however, *ELOVL₅* expression was higher in fish fed the HS diets within the VO group and *FADS₂* expression was not detected.

5. Conclusions

The lack of growth performance differences between diets seems to indicate that n-3 LC-PUFA requirements were fulfilled with 0.5% EPA + DHA, at least in the presence of high ARA levels. High dietary carbohydrates and ARA induced *FADS₂* expression while low carbohydrates and DHA induced *ELOVL₅*. These apparently contradictory effects need to be further evaluated as they may help to define strategies for diet formulation when alternative ingredients to fish meal and oil are considered.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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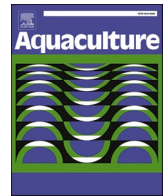
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Chapter 7

Effects of dietary ARA, DHA, and carbohydrates levels on gilthead sea bream liver and intestine oxidative stress, tissue histomorphology, and gut microbiota.

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Effects of dietary ARA, DHA, and carbohydrates levels on gilthead sea bream liver and intestine oxidative stress, tissue histomorphology, and gut microbiota

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ABSTRACT

The present study aims evaluate the oxidative status, histomorphology of liver and intestine, and intestinal microbiota modulation of gilthead sea bream juveniles (initial body weight = 47.5 g) fed four isoproteic and isolipidic diets (47% protein; 18% lipids) with high or low gelatinized starch levels (20% or 5%; diets HS or LS, respectively) and different dietary ARA or DHA content (2.3/0.3 or 0/2.6, diets ARA or DHA, respectively).

ARA-rich diets reduced liver and intestine lipid peroxidation (LPO) circa 12 and 66%, respectively. Dietary ARA also increased liver glutathione redox status (total, reduced (GSH) and oxidized glutathione (GSSG)) circa 35% but only in the HS diets. Independently of dietary starch level, dietary ARA also reduced GSSG content (80 and 63 nmol g⁻¹ tissue) and oxidative stress index (OSI) (8.7 and 7%) in the intestine. Fish fed DHA diets had increased hepatic superoxide dismutase (SOD) (749.1 and 489.1 U mg protein⁻¹) and glutathione reductase (GR) (3.7 to 1.9 mU mg protein⁻¹) activities. In the intestine, dietary ARA or DHA did not induce alterations in the activity of the antioxidant enzymes. Dietary starch improved liver OSI (1.5%), increased intestinal GSSG (80 and 128 nmol g⁻¹ tissue), and decreased intestinal LPO values (104 nmol MDA g⁻¹ tissue) in fish fed the DHA diets. Fish fed the HS diets had increased hepatic glucose-6-phosphate dehydrogenase (G6PDH) (171.4 and 166 mU mg protein⁻¹), GR (2 and 3.71 mU mg protein⁻¹), and glutathione peroxidase (GPX, only in DHA diets) (63.8 mU mg protein⁻¹) activities, but decreased GR (27.2 and 27.6 mU mg protein⁻¹) and SOD (2089.7 and 2158.8 U mg protein⁻¹) activities in the intestine.

Diet composition did not induce major histomorphological alterations in the distal intestine. Nevertheless, the HS diets, especially when combined with DHA, promoted hepatocyte hypertrophy and alterations in nuclei position.

Compared to the DHA diets, in fish fed the ARA diets, mucosa bacterial profile had a reduced number of operational taxonomic units (10.3 and 12.0), richness (0.6 and 0.7), and diversity (2.2 and 2.3), and higher similarity between bacterial communities (85.0 and 80.4%).

In conclusion, fish fed high dietary ARA presented lower hepatic and intestinal LPO and fewer hepatocyte morphological alterations than fish fed high DHA diets. Furthermore, interactive effects between dietary starch and DHA on liver and intestine GSH metabolism, antioxidant enzymes, LPO susceptibility, and liver histomorphology may be attenuated by a balanced ARA/DHA dietary ratio.

1. Introduction

Nowadays, aquafeeds incorporate high levels of plant ingredients,

and this leads to differences in the nutritional profiles such as increased dietary carbohydrate (CHO) level and lower n-3 long-chain polyunsaturated fatty acid (LC-PUFA) content (Oliva-Teles et al., 2015).

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Marine fish species have limited capacity to use carbohydrates (Enes et al., 2011; Kamalam et al., 2017) and to biosynthesize LC-PUFA, ARA (20:4n-6), EPA (20:5n-3), and DHA (22:6n-3), from the precursors linoleic (18:2n-6) and α -linolenic (18:3n-3) acids, due to the absence or low expression of elongases and desaturases (Monroig et al., 2018).

Gilthead sea bream (*Sparus aurata*) is an important marine aquaculture species in Europe (Llorente et al., 2020). The dietary n-3 LC-PUFA requirements of gilthead sea bream juveniles were estimated to be 0.9 or 1.9% of the diet when the DHA/EPA ratio was 1:1 or 1:2, respectively (Ibeas et al., 1994; Kalogeropoulos et al., 1992). This highlights the relevance of DHA as essential fatty acid (EFA) over EPA (Izquierdo and Koven, 2011; Rombenso et al., 2016). It has been suggested that a dietary DHA/EPA ratio of 0.5 (1:2) is most suitable for gilthead sea bream juveniles as growth performance decrease when the ratios increase to 1 and 2 (Ibeas et al., 1997).

Although ARA is also an EFA, up to now a dietary requirement has not been established for gilthead sea bream juveniles. Indeed, no differences in growth performance and feed utilization were found in fish fed with quite variable dietary ARA levels (ranging from 0.03 to 1.7%; Fountoulaki et al. (2003)).

The double bonds structures of LC-PUFA are highly susceptible to oxidation and the dietary reduction of LC-PUFA content resulting from FO substitution by alternative oils may induce modification in fish oxidative status (Mostafa Abd El-Aal, 2012). In several fish species, the increment of the dietary unsaturation index caused by LC-PUFA increased serum and liver lipid peroxidation as well as modulation of the antioxidant enzymes activities (Jin et al., 2017; Ma et al., 2018; Xu et al., 2018). In gilthead sea bream, it was already shown that due to the higher unsaturation level, diets with high dietary n-3 LC-PUFA content (EPA and DHA) increased liver and intestinal lipid peroxidation when compared to diets contained high levels of n-6 LC-PUFA content, especially ARA, although the glutathione cycle and antioxidant enzymes activities were little affected by dietary treatments (Magalhães et al., 2020).

The use of dietary vegetable oils (VO) to replace FO was shown to increase hepatic lipid intracytoplasmic vacuolization in Atlantic salmon (*Salmo salar*), gilthead sea bream, and European seabass (Betancor et al., 2017; Fountoulaki et al., 2009; Wassef et al., 2016). At the intestinal level, dietary VO increased enterocytes supranuclear vacuolization and hyperplasia of the basal nuclei in the distal intestine of European seabass and increased lipidic droplet content and goblet cells numbers in gilthead sea bream proximal intestine (Santigosa et al., 2011; Wassef et al., 2016).

Changing diet composition may also affect the gut microbiota, and consequently fish health and welfare (Egerton et al., 2018). For instance, in Atlantic charr (*Salvelinus alpinus* L.), the replacement of dietary FO by VO resulted in differences in aerobic gut microbial communities and improved fish resistance to pathogenic bacteria (Ringo et al., 2002). Recently, Magalhães et al. (2020) found that gilthead sea bream fed high dietary EPA and DHA levels had lower levels of pathogenic species, such as *Edwardsiella tarda* in the intestinal mucosa compared to fish fed high dietary ARA and low n-3 LC-PUFA. Thus, further research is necessary to explore the potential effects of dietary FA profile on fish gut microbiota composition.

Carbohydrates are abundant in plant feedstuffs (PF) and are incorporated in aquafeeds as a low-cost energy source (Krogdahl et al., 2005; NRC, 2011). Dietary CHO may interact with LC-PUFA biosynthesis, by up-regulating desaturases and elongases at the transcriptional level, as was described in salmonids (Kamalam et al., 2013; Seiliez et al., 2001) and gilthead sea bream (Castro et al., 2016a). Nevertheless, the potential interactions between dietary LC-PUFA profile and CHO on the physiological processes that impact oxidative stress in different tissues are poorly known. Previously, both in gilthead sea bream and European seabass (*Dicentrarchus labrax*) juveniles, dissimilarities in the liver and intestine LPO susceptibility with dietary CHO level and lipid source were observed (Castro et al., 2016b; Castro et al., 2015).

The role of dietary CHO on antioxidant defense mechanisms is still not clearly understood. In some studies, there is a reduction of hepatic antioxidant enzymes with dietary CHO (Wang et al., 2014; Zhou et al., 2017) while in other studies there is an increase (Castro et al., 2016b; Perez-Jimenez et al., 2017; Wu et al., 2016). However, liver oxidative damage seems to decrease with high dietary CHO levels. It is known that glucose can directly scavenge ROS and increase NADPH production via glucose-6-phosphate dehydrogenase (G6PDH) activity (Castro et al., 2016b), and NADPH is required to produce reduced glutathione (GSH) that in turn is necessary to reduce peroxides (Castro et al., 2016b). This, may, at least in part, explain the reduction of oxidative stress with increased dietary CHO levels.

Further, high dietary carbohydrates levels were also associated with negative effects on liver morphology due to glycogen and lipid accumulation (Kamalam et al., 2017). For instance, hepatocytes hypertrophy was reported in *Labeo rohita* fry, and lipid vacuolization was observed in yellow catfish (*Pelteobagrus fulvidraco*) juveniles (Mohapatra et al., 2003; Wang et al., 2014).

The objective of the present study was to evaluate the effect of diets with high ARA or DHA levels and the possible interaction with digestible carbohydrates in oxidative stress, liver and intestine histomorphology, and gut microbiota of gilthead sea bream juveniles.

2. Material and methods

2.1. Experimental diets

Four isoproteic and isolipidic diets (47% crude protein; 18% crude lipids) were formulated to include high dietary ARA or DHA content (2.3/0.3 or 0/2.6, ARA or DHA diets, respectively) combined with high (20%; HS) or low (5%; LS) starch. A vegetable oils blend was utilized as lipid source (rapeseed: 20% + linseed: 50% + palm: 30%). Diets were pelletized according to Magalhães et al. (2021). Detailed diet composition and FA profile are given elsewhere (Magalhães et al., 2021). Table 1 presents a summary of the most relevant data.

2.2. Growth trial

The experiment was approved by CIIMAR ethical committee for Managing Animal Welfare (ORBEA; reference ORBEA_CII-MAR_30_2019), in compliance with the European Union directive 2010/63/EU and the Portuguese Law (DL 113/2013). The growth trial was

Table 1

Proximate analysis (% dry weight) and essential fatty acid composition (% of total fatty acids) of the experimental diets.

Diets (ARA/DHA)*	ARA/HS	ARA/LS	DHA/HS	DHA/LS
	2.3/0.3	2.3/0.3	0/2.6	0/2.5
Dry matter (%)	91.1	91.9	90.2	92.0
Crude Protein	46.5	46.7	46.9	46.1
Crude Lipid	17.6	17.7	18.7	17.7
Ash	8.6	9.2	8.9	8.7
Starch	19.6	4.6	20.0	5.4
Gross energy (kJ g ⁻¹)	23.4	23.0	23.1	23.1
Fatty acids				
20:4n-6 (ARA)	13.1	13.2	0.17	0.14
20:5n-3 (EPA)	1.21	1.24	2.66	2.69
22:6n-3 (DHA)	1.53	1.60	13.7	13.9
Σ SFA	24.2	24.1	19.3	19.1
Σ MUFA	25.7	25.7	26.8	26.8
Σ PUFA	49.3	49.4	53.4	53.7
Σ n-6	30.9	30.8	15.5	15.4
Σ n-3	18.1	18.3	37.6	38.0
n-6/n-3	1.70	1.68	0.41	0.40

MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid.

* ARA/DHA expressed as % DM.

performed at CIIMAR, Matosinhos, Portugal. Gilthead sea bream juveniles were acquired from a certified fish farm (Sonrionansa, S.L., Cantabria, Spain). After transportation, fish were submitted to a 15 days quarantine period in a recirculation system with 1500 L of capacity and hand-fed with a commercial diet (NEO diet, Aquasoja; Sorgal, S.A., Portugal). Fish were then transferred to the experimental system 15 days before the start of the trial, for acclimatization to the experimental conditions. The experimental system consisted of a recirculating water system with 12 cylindrical fiberglass tanks with 100 L capacity each. During the trial, water quality was assessed weekly under postprandial conditions ($2.5\text{--}3.5\text{ L min}^{-1}$; salinity: $35 \pm 1\text{‰}$; dissolved oxygen: $7 \pm 1\text{ mg L}^{-1}$; nitrogenous compounds: $< 0.02\text{ mg L}^{-1}$), a constant water temperature was maintained ($23 \pm 1\text{ °C}$) and the photoperiod was controlled to 12 h of dark and 12 h of light. At the beginning of the trial, 20 fish with 47.5 g initial body weight were stocked to each tank and each experimental diet was randomly assigned to three tanks. The trial lasted for 12 weeks. Details about the growth trial were presented in Magalhães et al., 2021.

2.3. Sampling

At the end of the trial, 4 h after the morning meal, blood was collected, using heparinized syringes, from the caudal vein of 3 fish from each tank. The blood was immediately centrifuged at 6800 xg for 10 min, and plasma aliquots were frozen at -80 °C for cortisol analysis. Then, fish were euthanized by decapitation and dissected on chilled trays. The intestine connective and adjacent adipose tissues were removed, and a small portion of the distal intestine (DI, differentiated from the mid intestine by a darker mucosa and enlarged diameter) and the liver were collected for histomorphology assessment. Liver and DI samples were cleaned in phosphate-buffered saline (PBS), gently dried with a paper towel, fixed in phosphate-buffered formalin (4%, pH 7.4) for 24 h, and then stored in 70% ethanol until further processing as described by Couto et al. (2014). Liver and intestine from other 3 fish per tank were stored at -80 °C until quantification of lipid peroxidation and oxidative stress non-enzymatic and enzymatic indicators. For allochthonous (digesta) and autochthonous (mucosa) microbiota characterization, 2 fish per tank were sampled under aseptic conditions (sterilized solutions and work near an open flame). The intestinal content was squeezed into a sterile tube for digesta sample collection. Mucosa samples were obtained by opening the intestine on its longitudinal axis and scraping the mucosal surface. Samples were immediately frozen in liquid nitrogen and stored at -80 °C until analyzed.

2.4. Chemical analyses

The chemical analyses of ingredients and diets were performed as described in Magalhães et al. (2021). The experimental diets' fatty acid methyl esters (FAME) were prepared by direct transesterification according to Lepage and Roy (1984) and analyzed as described by Maia et al. (2019). Briefly, FAME were analyzed by gas chromatography using a Shimadzu GC-2010 Plus (Shimadzu Corporation, Kyoto, Japan) equipped with a capillary column (Omegawax 250, 30 m $\times$ 0.25 mm $\times$ 0.25 μm ; Supelco, Bellefonte, PA, USA), and a flame-ionization detector. Nonadecanoic acid (Matreya LLC, Pleasant Gap, PA, USA) was utilized as internal standard. The retention time of standards (Supelco 37 Component FAME Mix, BAME Mix, PUFA No.1, PUFA No.2, PUFA No.3, Sigma-Aldrich Co. LLC, St. Louis, MO; GLC-110 Mixture, Matreya LLC, Pleasant Gap, PA, USA) were compared with those of FA to identify them and the internal standard was used to FA quantification.

2.5. Plasma cortisol

Cortisol in plasma was analyzed according to standard methods, using an auto-analyzer (Architect ci8200; Abbot Diagnostics, Saint-Laurent, Canada), by a Clinical Laboratory-certified by Bureau Veritas

(NP EN ISO 9001-2000).

2.6. Enzyme activity

Liver and intestine samples were homogenized (dilution 1:6 and 1:5, respectively) in ice-cold 100 mM Tris-HCL buffer, with 0.1 mM EDTA and 0.1% (v/v) Triton X-100, pH 7.8. All procedures were done on ice to ensure maximal enzymatic activity preservation. After homogenization, samples were centrifuged at 30000 xg at 4 °C and the supernatants were divided into aliquots and stored at -80 °C until utilization. All enzymatic assays were performed in a Multiskan Go microplate reader (Model 5111 9200; Thermo Scientific, Nanjing, China) at 37 °C. The enzymatic reactions were started by the addition of tissue extract or substrate. Protein concentration of the homogenates was done according to Bradford (1976), using a Bio-Rad protein assay kit (ref. 5,000,006).

Oxidative stress enzymatic activities were measured as following: glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) and glutathione reductase (GR; EC 1.6.4.2) according to Morales et al. (1990), superoxide dismutase (SOD; EC 1.15.1.1) according to McCord and Fridovich (1969), catalase (CAT; EC 1.11.1.6) according to Aebi (1984), glutathione peroxidase (GPX; EC 1.11.1.9) according to Flohé and Günzler (1984).

CAT activity was expressed in units per mg of soluble protein. Except for SOD, the activity of the remaining enzymes was expressed as milli-units per mg of soluble protein. One unit of enzyme activity is defined as the quantity of enzyme needed to transform 1 μmol of substrate per minute under the assessed conditions. One unit of SOD activity is defined as the quantity of enzyme required to produce 50% inhibition of the ferricytochrome C and is expressed as units of SOD per milligram of soluble protein.

2.7. Lipid peroxidation

The concentration of thiobarbituric acid reacting substances (TBARS) in the liver and intestine was considered a marker of lipid peroxidation (LPO); it was determined according to the method described by Buege and Aust (1978) and values expressed as nmol MDA g^{-1} tissue.

2.8. Glutathione

Liver and intestine samples were homogenized (dilution 1:6) in ice-cold solution containing 1.3% 5-sulfosalicylic acid (w/v) and 10 mM HCL. To avoid glutathione oxidation, all procedures were done on ice. Samples were centrifuged at 14000 xg for 10 min at 4 °C and the supernatants were collected and stored at -80 °C until analysis. Total glutathione (tGSH) and oxidized glutathione (GSSG) were quantified according to Castro et al. (2016b). Reduced glutathione (GSH) was calculated by the difference between tGSH and GSSG values.

2.9. Histological analysis

Standard histological techniques were used to process liver and DI samples. Samples were stained with hematoxylin and eosin. All histological preparations were blindly evaluated. DI was evaluated by analyzing mucosal folds height, width, and cellularity of the lamina propria and submucosa, number of intraepithelial lymphocytes and goblet cells, and variation of enterocyte vacuolization and nucleus position (Couto et al., 2014). The liver was evaluated by paying special attention to hypertrophy and nuclei position of the hepatocytes (Betancor et al., 2017). Additionally, the ratio between peripancreatic adipose tissue and pancreatic tissue was calculated. For that, the pancreatic and peripancreatic adipose tissue areas were measured with Zen software (Blue edition; Zeiss, Jena, Germany) in four random areas of each liver sample. Images of the liver, DI, and pancreatic and peripancreatic adipose tissue areas were obtained with Zen software.

For the evaluation of the histomorphology modifications, a semi-quantitatively score system was used, ranging from 1 (normal) to 5 (highly modified) for the intestine and from 1 (normal) to 3 (highly modified) for the liver. The average of all individual parameter scores was considered the overall score of histomorphological alterations.

2.10. Microbial diversity

Extraction of digesta and mucosa bacterial DNA was accomplished using a commercial kit (DNeasy® PowerSoil® Pro Kit, QIAGEN) and following the manufacturer's recommendations. To reduce variability, samples of 2 fish per tank were pooled together.

A touchdown PCR on a T100Thermal Cycler (Biorad Laboratories Lda., Amadora, Portugal) was used to amplify bacterial 16S rRNA gene fragments using oligonucleotide primers 16S-358F (containing a GC clamp at the 5' end) and 16S-517R (Muyzer et al., 1993). Each PCR product (300 ng) was dissolved on 8% polyacrylamide gel constituted by a denaturing gradient of 40–60% 7 M urea/40% formamide. Denaturing Gradient Gel Electrophoresis (DGGE) was carried out in a DCode universal mutation detection system (Bio-Rad laboratories Lda.) for 16 h at 60 °C, 65 V in a 1 x TAE buffer. The gel was stained for 1 h with SYBR-Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and then visualized on a Gel Doc EZ System (Bio-Rad Laboratories Lda., Amadora, Portugal). Different bands of interest were excised from the gel and eluted in 20 µL ultrapure water before DNA re-amplification using the same oligonucleotide primers mentioned above, but without the GC clamp (Muyzer et al., 1993). The identification of microbiota OTUs (Operational Taxonomic Units) was made by sequencing the amplicons. The identification of the closest known species was made by comparison with sequences in the Genbank non-redundant nucleotide database using BLAST (<https://www.ncbi.nlm.nih.gov>) (Macrogen Europe, Amsterdam, The Netherlands). Only sequences with 80–100% query coverage and higher than 100 bp reads were considered valid identifications.

2.11. Statistical analysis

Data are presented as the mean and standard error of the mean (SEM). The normality and homogeneity of variances were evaluated, and data were log-transformed when necessary. Essential fatty acid or carbohydrate level was fixed as factors and data were analyzed by two-way ANOVA. If interactions were significant, one-way ANOVA was carried out for each factor. Histological data were neither normal nor homogeneous (except the ratio between the peripancreatic adipose tissue and pancreatic tissue area data) and were impossible to normalize, having been analyzed by the non-parametric test Kruskal-Wallis with multiple comparisons (all pairwise). SPSS 24.0 software package for Windows (IBM® SPSS® Statistics, New York, USA) was used to conduct the statistical analyses.

Microbiota data analysis was made following Serra et al. (2018) using the DGGE banding patterns. The band's intensity was quantified with Quantity One 1-D Analysis Software v4.6.9 (Bio-Rad Laboratories Lda., Amadora, Portugal) and converted into absence/presence matrices. Primer software v7.0.5.5 (Primer-E, Ivybridge, UK) was used to calculate the relative similarities between experimental groups and replicates. Non-metric multidimensional scaling (MDS) was based on Bray-Curtis similarities, using relative band abundances. MDS data representation was found reliable considering the Kruskal stress value <0.2 (Clarke et al., 2001). Species diversity was established by the Shannon-Weaver index, Margalef's diversity index was used to establish species richness, and the relative similarities between groups were represented by similarity percentages (SIMPER). Bray-Curtis dendrograms construction for clustering DGGE patterns was made using the Unweighted Pair Groups Method with Arithmetic Averages (UPGMA). *P* values inferior to 0.05 were considered significant.

3. Results

The growth performance and feed utilization of fish fed the experimental diets was not the aim of this study and data is presented elsewhere (Magalhães et al., 2021). Briefly, diet composition did not affect growth performance; however, dietary starch improved feed utilization efficiency. Further, although few interactions between dietary FA and carbohydrates were found, each factor differently affected intermediary metabolism.

Plasma cortisol concentration was unaffected by dietary treatments (Table 2). There was a significant interaction between dietary EFA and CHO level in liver glutathione content (Table 2). Within the HS diets, but not the LS diets, tGSH, GSSG, GSH levels were higher in fish fed the ARA than DHA diets. Within the DHA diets, tGSH, GSSG, GSH levels were higher in the LS than the HS diet.

Irrespective of the EFA ratio, the hepatic oxidative stress index (OSI) was lower in the HS than in the LS diets (Table 2). On the other hand, regardless of CHO level, the hepatic LPO was higher in the DHA diets.

Total and reduced glutathione levels were much lower in the intestine than in the liver, while the opposite was true for OSI and LPO levels (Table 3). In the intestine, tGSH and GSH were unaffected by diet compositions, while GSSG was higher in fish fed the DHA and HS diets. OSI and LPO were higher in fish fed the DHA diets. LPO was also higher in the LS than the HS diets, but this effect was only significant within the DHA diets.

In the liver, GR and SOD activities were higher in fish fed the DHA than the ARA diets while CAT, G6PDH, and GPX activity were unaffected by dietary EFA (Table 4). On the other hand, GR and G6PDH activity were higher in fish fed the HS than the LS diets while CAT and SOD activity were unaffected by dietary CHO level. GPX activity was also higher in fish fed the HS diet but only within the DHA diets.

In the intestine, dietary EFA did not affect the activity of any of the oxidative stress-related enzymes measured, while GR and SOD activity was higher in fish fed the LS diets (Table 5).

Dietary EFA or CHO dietary level did not affect the distal intestine histological morphology (Table 6; Fig. 1A). However, HS diets promoted hepatocyte hypertrophy and modified nucleus position, particularly in DHA diets (Table 6; Fig. 1B). The ratio between the area of

Table 2

Plasmatic cortisol concentration (ng ml⁻¹) and liver total glutathione (tGSH), oxidized glutathione (GSSG), reduced glutathione (GSH) (nmol g⁻¹ tissue), oxidative stress index (OSI) (%), and lipid peroxidation (LPO; nmol MDA g⁻¹ tissue) levels of gilthead sea bream fed the experimental diets.

Diets (ARA/DHA)	ARA/HS	ARA/LS	DHA/HS	DHA/LS	SEM
	2.3/0.3	2.3/0.3	0/2.6	0/2.5	
Cortisol	22.0	34.3	31.8	28.5	5.1
tGSH	4892 ^b	4857	^A 3665 ^a	^B 4749	154.1
GSSG	37 ^b	52	^A 27 ^a	^B 59	3.3
GSH	4855 ^b	4805	^A 3637 ^a	^B 4690	153.7
OSI ¹	1.5	1.9	1.5	2.0	0.1
LPO	18.6	18.2	18.9	22.9	0.6

Two-way ANOVA	Variance source		
	EFA	CHO	Interaction
Cortisol	NS	NS	NS
tGSH	0.018	NS	0.044
GSSG	NS	0.000	0.038
GSH	0.019	NS	0.048
OSI ¹	NS	0.021	NS
LPO	0.027	NS	NS

Values presented as means (*n* = 9) and standard error of the mean (SEM). Two-way ANOVA: NS: non-significant (*P* > 0.05). Different capital letters indicate differences between CHO level within each EFA; lower case letters indicate differences between EFA within each CHO level. ¹OSI (%) = 100 x (2 x GSSG/tGSH).

Table 3

Intestine total glutathione (tGSH), oxidized glutathione (GSSG), reduced glutathione (GSH) (nmol g⁻¹ tissue), oxidative stress index (OSI) (%), and lipid peroxidation (LPO) (nmol MDA g⁻¹ tissue) levels of gilthead sea bream fed the experimental diets.

Diets (ARA/DHA)	ARA/HS	ARA/LS	DHA/HS	DHA/LS	SEM
	2.3/0.3	2.3/0.3	0/2.6	0/2.5	
tGSH	1847	1995	2170	1606	102.1
GSSG	80.0	63.0	128	77	6.5
GSH	1767	1932	1878	1529	92.2
OSI ¹	8.7	7.0	11.8	10.3	0.6
LPO	55.2 ^a	65.0 ^a	^A 104.0 ^b	^B 244.9 ^b	17.0

Two-way ANOVA		Variance source	
	EFA	CHO	Interaction
tGSH	NS	NS	NS
GSSG	0.006	0.003	NS
GSH	NS	NS	NS
OSI ¹	0.006	NS	NS
LPO	0.000	0.000	0.009

Values presented as means (n = 9) and standard error of the mean (SEM). Two-way ANOVA: NS: non-significant (P > 0.05). Different capital letters indicate differences between CHO level within each EFA; lower case letters indicate a difference between EFA within each CHO level. ¹OSI (%) = 100 x (2 x GSSG/tGSH).

Table 4

Hepatic activities of glucose-6-phosphate dehydrogenase (G6PDH), glutathione peroxidase (GPX), glutathione reductase (GR) (mU mg protein⁻¹), catalase (CAT), and superoxide dismutase (SOD) (U mg protein⁻¹) of gilthead sea bream fed the experimental diets.

Diets (ARA/DHA)	ARA/HS	ARA/LS	DHA/HS	DHA/LS	SEM
	2.3/0.3	2.3/0.3	0/2.6	0/2.5	
CAT	857.6	787.6	1055.7	766.6	55.6
G6PDH	171.4	81.5	166.0	94.0	9.3
GR	2.00	1.59	3.71	1.90	0.2
SOD	444.3	370.2	749.1	489.1	53.6
GPX	45.2	49.8	^B 63.8	^A 41.9	3.1

Two-way ANOVA		Variance source	
	EFA	CHO	Interaction
CAT	NS	NS	NS
G6PDH	NS	0.000	NS
GR	0.006	0.001	NS
SOD	0.042	NS	NS
GPX	NS	NS	0.030

Values presented as means (n = 9) and standard error of the mean (SEM). Two-way ANOVA: NS: non-significant (P ≥ 0.05). Different capital letters indicate differences between CHO level within each EFA.

peripancreatic adipose tissue and pancreatic tissue in the liver was not affected by dietary treatment (Table 6; Fig 1B).

Dietary EFA or CHO level did not affect digesta microbiota ecological parameters (Table 7). However, in the mucosa, the number of OTU's, Margalef species richness, and Shannon diversity index were higher and the similarity percentage within replicates was lower in fish fed the DHA diets (Table 7). The Bray-Curtis dendrogram showed that digesta samples failed to cluster (Fig. 2A) but mucosa samples of fish fed ARA diets (except for one replicate) clustered together (Fig. 2B). The presence and abundances of DGGE bands represented in the non-metric multidimensional scaling (nMDS) plot enabled clear visualization of this result (Fig. 2B). The digesta and mucosa Bray-Curtis dendrogram and mucosa nMDS confirmed that HS diets did not affect gilthead sea bream gut allochthonous or autochthonous microbiota. Different bands of interest (identified with a red dot in Fig. 2) of the DGGE gels were sequenced but

Table 5

Intestinal activities of glucose-6-phosphate dehydrogenase (G6PDH), glutathione peroxidase (GPX), glutathione reductase (GR) (mU mg protein⁻¹), catalase (CAT), and superoxide dismutase (SOD) (U mg protein⁻¹) of gilthead sea bream fed the experimental diets.

Diets (ARA/DHA)	ARA/HS	ARA/LS	DHA/HS	DHA/LS	SEM
	2.3/0.3	2.3/0.3	0/2.6	0/2.5	
CAT	233.2	235.4	179.9	396.8	33.4
G6PDH	34.1	44.9	38.5	31.5	2.7
GR	27.2	35.2	27.6	31.7	1.3
SOD	2089.7	2748.4	2158.8	2555.3	114.3
GPX	15.0	17.9	15.6	15.6	1.0

Two-way ANOVA		Variance source	
	EFA	CHO	Interaction
CAT	NS	NS	NS
G6PDH	NS	NS	NS
GR	NS	0.023	NS
SOD	NS	0.022	NS
GPX	NS	NS	NS

Values presented as means (n = 9) and standard error of the mean (SEM). Two-way ANOVA: NS: non-significant (P ≥ 0.05).

Table 6

Histological evaluation of the distal intestine and liver of gilthead sea bream juveniles fed the experimental diets.

Dietary treatments					
Diets (ARA/DHA)	ARA/HS	ARA/LS	DHA/HS	DHA/LS	SEM
	2.3/0.3	2.3/0.3	0/2.6	0/2.5	
Intestine					
Fold height	2.2	2.8	2.3	2.5	0.1
Lamina propria	2.4	2.3	2.6	2.3	0.1
Submucosa	1.6	1.1	2.0	1.5	0.1
Intraepithelial leucocytes	3.2	3.0	2.9	2.8	0.1
Enterocytes	1.3	1.2	1.6	1.8	0.1
Goblet cells	2.3	2.1	1.3	1.9	0.2
Mean score	2.2	2.1	2.1	2.1	0.1
Liver					
Nuclei position	1.4 ^{ab}	1.0 ^a	2.1 ^b	1.3 ^{ab}	0.1
Hepatocyte hypertrophy	2.6 ^b	1.1 ^a	2.3 ^b	1.2 ^a	0.1
Mean score	2.0	1.1	2.2	1.3	0.1
Peripancreatic adipose tissue (mm ²)/Pancreatic tissue (mm ²)	0.8	0.6	0.4	0.6	0.1

Two-way ANOVA		Variance source		
		EFA	CHO	Interaction
Peripancreatic adipose tissue / Pancreatic tissue		NS	NS	NS

Values presented as means (n = 9) and standard error of the mean (SEM). The absence of superscript letters indicates no significant differences between dietary treatments (P > 0.05). Two-way ANOVA: NS: non-significant (P ≥ 0.05).

were identified as uncultured bacterial clones (> 95%).

4. Discussion

4.1. Dietary EFA effects

In the present study, we found no differences in plasmatic cortisol levels between fish fed the ARA or DHA-rich diets. Differently, using in vitro preparations of gilthead sea bream interrenal cells, Ganga et al. (2006) found that DHA stimulated cortisol production to a lower extent than ARA. Also in *Prochilodus lineatus* larvae, feeding DHA-rich artemia emulsions attenuated the cortisol response after air exposure compared

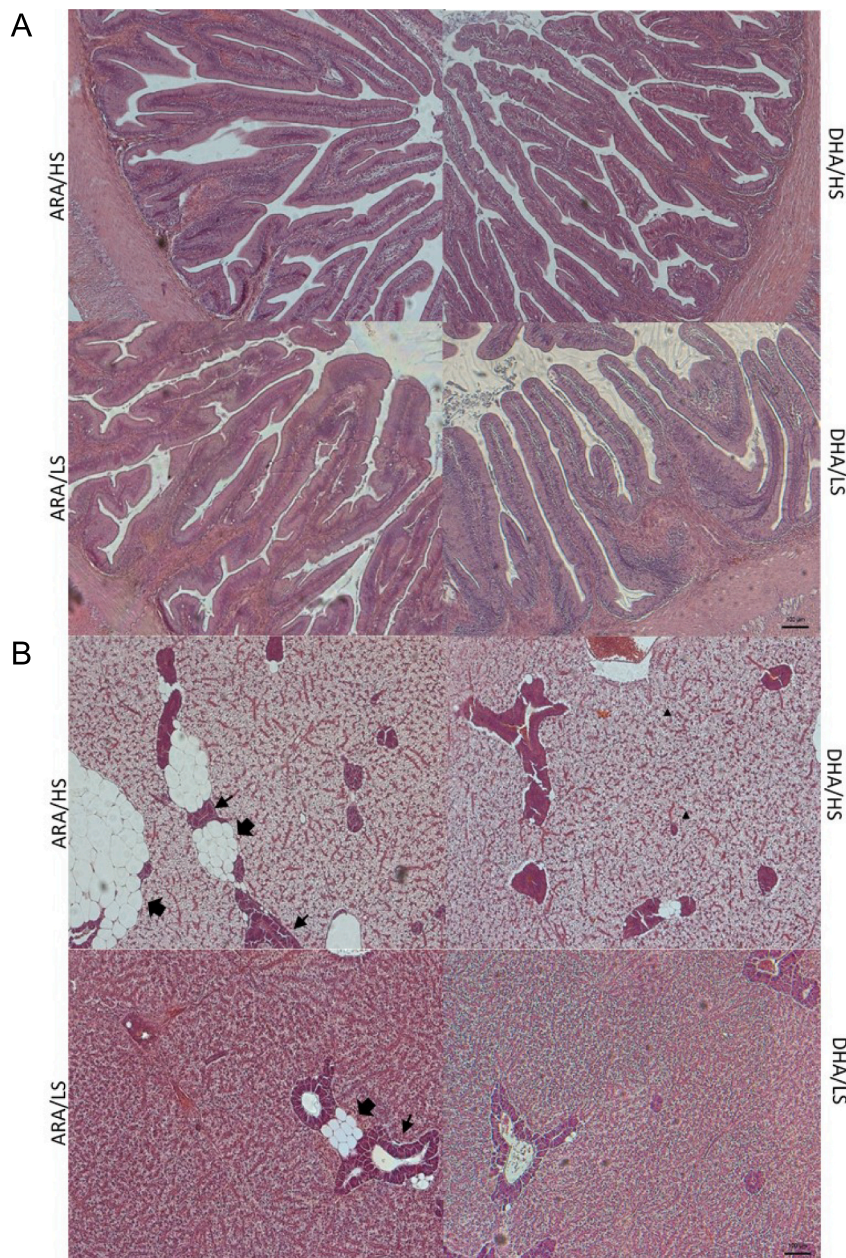


Fig. 1. Histomorphology features of the distal intestine (A) and liver (B) of gilthead sea bream juveniles fed the experimental diets recorded with Zen software (Blue edition). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

A: Intestine. Normal height of mucosal folds, normal width lamina propria with normal leukocyte infiltration of the lamina propria and submucosa. No signs of inflammation. Bar scale = 100 μ m B: Liver. ARA/HS and DHA/HS diets show increased hypertrophy compared to diets ARA/LS and DHA/LS diets. DHA/HS shows decentralized nuclei ($\blacktriangle$), peripancreatic adipose tissue: $\blacktriangleup$; Pancreatic tissue: $\blacktriangleright$ Bar scale = 100 μ m. H-E staining.

with fish fed ARA-rich emulsions (Araujo and Rosa, 2017). It should however be noted that in the present study the fish were under non-stressed conditions where cortisol levels should be low regardless of diet. Also in Senegalese sole (*Solea senegalensis*) post-larvae fed graded levels of ARA, no effect of dietary ARA on whole-body cortisol levels were observed before and after 3 h of air exposure for 2 min (Alves Martins et al., 2013).

The diets used in the present study were particularly rich in ARA or DHA, which are major substrates for lipid peroxidation due to the presence of several bis-allylic methylene groups. As lipidic susceptibility towards peroxidation increases with the number of unsaturated double bonds, it was expected that DHA-rich diets might induce higher LPO (Mostafa Abd El-Aal, 2012). This was indeed the case in the present study in both liver and intestine and is in agreement with results previously reported by Magalhães et al. (2020) who also found increased LPO in gilthead sea bream juveniles fed n-3 LC-PUFA rich diets (0.6%

EPA + 0.6%DHA and 0.3%EPA + 1.5%DHA) compared to fish fed ARA-rich diets (1 and 2%).

The effect of dietary n-3 LC-PUFA, especially DHA, on increased oxidative stress was also observed in European seabass larvae and in the liver and serum of juvenile black sea bream, *Acanthopagrus schlegelii* (Betancor et al., 2013; Jin et al., 2017). In golden pompano (*Trachinotus ovatus*) fed diets with similar EPA + DHA dietary content, a VO-based diet decreased LPO compared to a FO-based diet probably due to a lower dietary unsaturation index of the VO diet (You et al., 2021).

Besides its four double bonds, which make it prone to oxidation, ARA may also induce ROS production through direct binding to S100 proteins, which regulate and activate NADPH-oxidase, resulting in the production of superoxide ions (Chee et al., 2020; Hii and Ferrante, 2007). Previous studies in yellow catfish (*P. fulvidraco*), Japanese eel (*Anguilla japonica*), and *Synechogobius hasta* also showed that graded levels of dietary ARA induced increasing oxidative stress (Luo et al.,

Table 7

Ecological parameters were obtained from PCR-DGGE fingerprints of the intestinal allochthonous (digesta), and autochthonous (mucosa) microbiota of gilthead sea bream fed with the experimental diets.

Digesta						Mucosa				
Diets	ARA/HS	ARA/LS	DHA/HS	DHA/LS	SEM	ARA/HS	ARA/LS	DHA/HS	DHA/LS	SEM
OTUs ¹	10.3	12.0	14.0	14.3	0.7	13.7	12.0	15.7	15.3	0.6
Richness ²	0.6	0.7	0.8	0.8	0.0	0.8	0.7	0.9	0.9	0.0
Diversity ³	2.2	2.3	2.4	2.5	0.1	2.5	2.3	2.6	2.6	0.1
Similarity (%) ⁴	85.0	80.4	78.3	71.2	2.4	82.5	87.6	69.3	77.3	2.6

Two-way ANOVA	Digesta			Mucosa		
	EFA	CHO	Interaction	EFA	CHO	Interaction
OTUs	NS	NS	NS	0.034	NS	NS
Richness	NS	NS	NS	0.023	NS	NS
Diversity	NS	NS	NS	0.031	NS	NS
Similarity	NS	NS	NS	0.018	NS	NS

Values presented as means ($n = 3$) and standard error of the mean (SEM). Two-way ANOVA: NS: non-significant ($P \geq 0.05$).

¹ OTUs: Average number of operational taxonomic units.

² Margalef species richness: $d = (S-1)/\log(N)$ where S is the number of species, and N is the total number of individuals in the sample.

³ Shannons diversity index: $H' = -\sum(\pi_i \ln \pi_i)$ where π_i is the proportion of individuals belonging to the i th species present in the sample.

⁴ SIMPER, similarity percentage within-group replicates.

2012; Ma et al., 2018; Shahkar et al., 2016). Differently, in juvenile turbot (*Scophthalmus maximus* L.) fed plant feedstuffs based diets, dietary ARA supplementation (2.6, 7.4, and 11.8 g/kg ARA) decreased serum LPO to values similar to that of fish fed fish meal diets (Wei et al., 2021).

The high enterocyte turnover rates and direct contact with the external medium make the intestine highly susceptible to oxidative stress. However, the intestinal capacity to produce and accumulate glutathione seems limited, as indicated by the lower tGSH concentration in the intestine than in the liver.

The results of this study also indicate that in the intestine, but not in the liver, DHA diets induced a much higher oxidative stress than ARA diets as confirmed by the GSSG, OSI, and LPO values. Further, while dietary EFA did not affect the intestinal antioxidant enzyme activity, in the liver SOD and GR activities were higher in fish fed the DHA than the ARA diets. This increased antioxidant enzyme activity in the liver may have contributed to counterbalancing the LPO in fish fed the DHA diets.

On the other hand, SOD and GR activities were much higher in the intestine than in the liver, while the opposite was true for CAT and GPX activities. This suggests that major mechanisms of detoxification of the hydroperoxides formed might be different in the two tissues. Accordingly, in juvenile yellow catfish, it was shown that increasing dietary ARA levels led to increased hepatic LPO values but did not affect antioxidant enzyme activity (GPX, SOD, and CAT) (Ma et al., 2018). This partially agrees with the results of the present study and further suggests that different mechanisms might be activated to counterbalance oxidative stress caused by ARA and DHA.

Dietary ARA and DHA content did not significantly affect distal intestinal scores. Similar results have also been observed in gilthead sea bream fed with diets having different ARA/EPA/DHA ratios (Magalhães et al., 2020). Also in golden pompano, no major histological differences were observed in the anterior intestine between fish fed VO-based or FO-based diets with similar ARA and EPA + DHA contents (You et al., 2021). Nevertheless, dietary FO replacement by VO resulted in widening and increased cellularity of the intestinal lamina propria and accumulation of lipid droplets in enterocytes of gilthead sea bream (Caballero et al., 2003; Castro et al., 2019; Santigosa et al., 2011) and low dietary EPA (0 and 1%) increased vacuolization in Atlantic salmon enterocytes compared to DHA or 2% EPA + DHA diets (Bou et al., 2017).

Dietary fatty acids (FA) may impact intestinal membrane composition, function, and fluidity, thus affecting mucosa attachment sites for bacteria (Kankaanpää et al., 2001; Ringo et al., 1998). In the present study, DHA diets increased the intestinal mucosa bacterial diversity and

richness and decreased bacterial similarity. This may be of practical relevance as higher mucosa microbial richness and diversity might be beneficial in outcompeting invading opportunistic pathogens (Tarnecki et al., 2017). Although not observed in this study, it has previously been noted that the pathogenic bacteria *E. tarda* was not found in gilthead sea bream fed n-3 LC-PUFA rich diets, contrarily to fish fed ARA-rich diets (Magalhães et al., 2020). Also in golden pompano, FO-based diets increased intestinal microbial diversity and decreased the abundance of the genera *Vibrio* and *Mycoplasmata* compared to soybean and linseed oil-based diets, and a correlation analysis showed that EPA and DHA had a profound effect in increasing intestinal genera typically associated with probiosis, such as *Bacillus* and *Lactococcus* (You et al., 2019). Accordingly, no differences were found in intestinal diversity or richness indexes of golden pompano fed with vegetable oil-based diets supplemented with EPA or DHA when compared to fish fed with FO-based diets (You et al., 2021). Similarly, in Arctic charr, *S. alpinus*, it was also observed a higher frequency of lactic acid bacteria in the gut of fish fed an 18:3n-3 and LC-PUFA mix diet compared to fish fed an 18:2n-6 based diet (Ringo et al., 1998).

In the present study, the Bray-Curtis dendrogram showed that mucosa samples of fish fed ARA diets clustered together (except for one replicate). Previously, also in gilthead sea bream, the Bray-Curtis dendrogram showed that ARA or EPA + DHA rich diets originated two clear distinct mucosa microbial profiles (Magalhães et al., 2020). Thus, present results showed that changes in dietary n-3 LC-PUFA content affected the mucosa microbial profile, suggesting it may be of importance to prevent the proliferation of opportunistic pathogens.

4.2. Effects of dietary carbohydrate level

Dietary carbohydrates were previously associated with decreased LPO values in fish species such as gilthead sea bream, European seabass, and rainbow trout (Alvarez et al., 1999; Castro et al., 2016b; Castro et al., 2015). The cause for this reduction was associated with increased availability of glucose for NADPH production by G6PDH in the pentose phosphate pathway, which is required for the regeneration of GSH from GSSG by GR (Liemburg-Apers et al., 2015). Glucose may also function directly as a hydroxyl radicals' scavenger (Liemburg-Apers et al., 2015; Sagone Jr. et al., 1983) and this may contribute to explain the lower OSI values in the liver and LPO values in the intestine of fish fed the HS diets.

Present results also showed that carbohydrates interacted with EFA in the liver response to oxidative stress, as in fish fed the HS diets, the decrease of TGSH, GSH, and GSSG levels were observed with the DHA

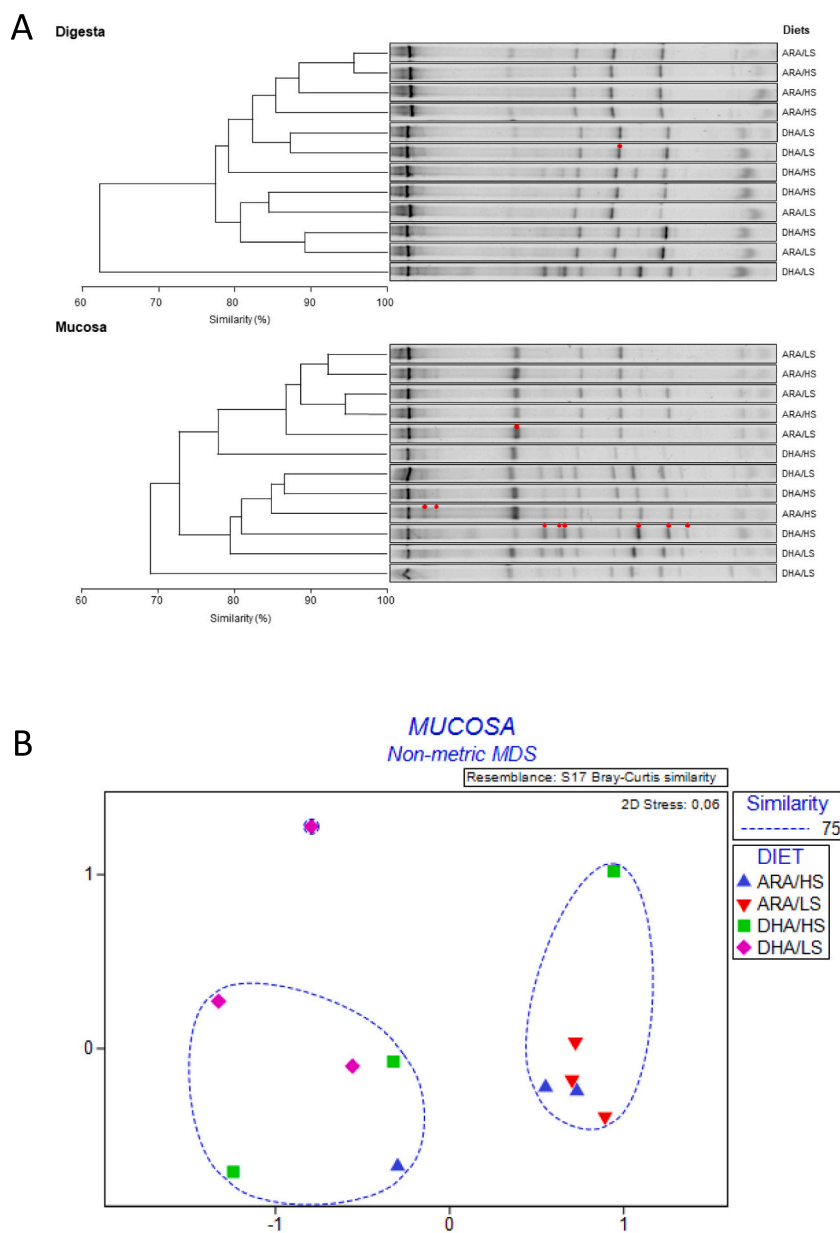


Fig. 2. (A) Dendrograms and PCR-DGGE fingerprints of the allochthonous (digesta) and autochthonous (mucosa) intestinal microbiota of gilthead sea bream fed the experimental diets. Red dots (●) indicate bands excised for sequence analysis. (B) Non-metric multidimensional scaling (MDS) plot of DGGE bands presence and abundances in intestinal mucosa samples from gilthead sea bream juveniles fed the same experimental diets depicted in A: ARA/HS (▲); ARA/LS (▼); DHA/HS (■); DHA/LS (◆). The dotted blue line represents 75% similarity between samples.

diet but not the ARA diet. Previously, [Castro et al. \(2016b\)](#) also reported a decrease of GSH in the intestine of gilthead sea bream fed carbohydrate-rich diets when the dietary lipid source was FO (high DHA diet) but not when it was a VO (low DHA diet).

An interactive effect between dietary carbohydrates and DHA was also observed in the liver GPX activity, which was higher in fish fed the HS diet than in the LS diet. GSH is required for GPX-mediated detoxification of hydrogen peroxide (H_2O_2) and lipoperoxides and is oxidized to GSSG. The increased hepatic GR and G6PDH activities in the HS diet are in agreement with the need to restore GSH required for GPX activity.

The liver histomorphology showed that dietary CHO promoted hepatocyte hypertrophy. The morphology of liver cells and size, along with the increased hepatic somatic index of fish fed the HS diets ([Magalhães et al., 2021](#)) agrees with the liver being a fast glycogen storage organ in gilthead sea bream as previously highlighted by [Enes et al. \(2011\)](#).

Despite that no specific staining was used to confirm the presence of glycogen in the hepatocytes, the increased hepatic glycogen content recorded in fish fed the HS diets (see [Magalhães et al. \(2021\)](#)) supports this statement. The hepatic histomorphology modifications were more evident in the DHA/HS group, denoting an interaction effect between dietary carbohydrate and EFA. The mild hepatic histological alterations observed suggest a reversible condition rather than irreversible and pathological liver damage, similar to what was previously reported for gilthead sea bream ([Castro et al., 2019](#)).

In the present study, dietary CHO did not modulate autochthonous or allochthonous intestinal microbiota. Previously, [Castro et al. \(2019\)](#) also did not found differences in the intestinal microbial profile of gilthead sea bream fed diets with 0 and 20% gelatinized starch. Nevertheless, despite PCR-DGGE is a very useful technique as a first approach to study the intestinal bacterial community, it is not powerful enough to detect

all the subtle changes in OTUs abundance and some differences between dietary treatments may have been undetected.

5. Conclusions

Overall, ARA-rich diets induced lower tissue LPO than DHA-rich diets. Also, high dietary ARA affected mucosa microbiota by decreasing diversity and richness which may have deleterious effects on preventing pathological episodes. The lower oxidative stress observed in fish fed the ARA diets may be related to the lower degree of unsaturation of this molecule as no induction of oxidative stress mechanisms was observed. Further, dietary CHO and DHA seem to have an interactive effect against oxidative stress by reducing the intestine LPO and lowering liver GSH content.

Credit author statement

R. E. O., H. P., and A.O.T. designed the study, R. M. conducted the growth trial, R. M., H. P., N.M, F.F., A.C, C.R.S, R.A.S. performed the experimental analysis and analyzed the data. R. M., H. P., R.E.O and A. O. T. wrote the paper. All authors reviewed and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter 8

Dietary effects of ARA/DHA ratio and carbohydrates level on the immune status of Gilthead sea bream.

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Dietary effects of ARA, DHA, and carbohydrates levels on the immune status of Gilthead sea bream

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Abstract

Aquafeeds industry is reducing n-3 LC-PUFA and increasing carbohydrate dietary content due to plant ingredients utilization. Thus, it is important to study how this dietary modification impacts the health status of marine fish such as gilthead sea bream during the growing out phase but also when challenged by a pathological agent. For that purpose, gilthead sea bream juveniles (initial body weight = 47.5 g) were fed four isoproteic (47% crude protein) and isolipidic (18% crude lipids) diets with high or low gelatinized starch (HS or LS diets) content and different dietary ARA/DHA ratios (2.3/0.3; ARA diets or 0/2.6; DHA diets). 9 fish per diet were sampled after 84 days of feeding the experimental diets whereas 24 fish from each dietary treatment were randomly selected and intraperitoneal injection (i.p.) with 0.6 mL of killed *P. damselae* subsp. *piscicida* (*Phdp*) (1×10^8 CFU/ mL⁻¹) or PBS (control group). Those fish were sampled 4 and 24 hours after i.p.

After the growth trial, DHA diets increased red blood cell (RBC) count, hemoglobin and plasmatic nitric oxide (NO) content, antiproteases activity, and alternative complement activity, and decreased proteases activity. ARA diets increased the prostaglandin E₂ receptor *ep*₃ expression in the distal intestine. In ARA groups, HS diets increased thrombocytes and plasmatic bactericidal activity against *Vibrio anguillarum*.

4 hours after i.p., dietary DHA increased RBC numbers within HS groups and overall hematocrit and thrombocytes numbers. ARA diets increased total immunoglobulins (IG), and plasmatic bactericidal activity against *Vibrio anguillarum*. Dietary starch increased prostaglandin E₂ receptor *ep*₂ and *ep*₄ expression in the distal intestine and within ARA-rich

diets, increased white blood cell (WBC) and lymphocytes numbers, and alternative complement activity.

24 hours after i.p. ARA diets increased RBC, WBC, and thrombocytes numbers, total IG, peroxidase activity, and *casp3* expression in the distal intestine. HS diets increased NO content and bactericidal activity against *Phdp* and decreased proteases, antiproteases activity, and bactericidal activity against *Vibrio anguillarum*.

In conclusion, high DHA dietary content seems to better modulate the immune status of gilthead sea bream juveniles in the growing out phase but ARA diets improve the immune system response to a bacterial challenge while the effects of dietary CHO were still not clarified. Further research is essential to determine the ideal dietary ARA/DHA ratio for the optimized immune response of gilthead sea bream juveniles.

Keywords

Essential fatty acids; Bacterial challenge; Hematology; Innate immune system; Gene expression

Introduction

The utilization of vegetable and animal oils to replace fish oil (FO) in commercial aquafeeds for carnivorous marine fish species received a great deal of attention because they are not able to bio convert linoleic and α -linolenic acid (18:2n-6 and 18:3n-3) into arachidonic acid (ARA; 20:4n-6) and docosahexaenoic acid (DHA; 22:6n-3) due to the absence of $\Delta 5$ -desaturase and very-long-chain fatty acid enzyme (Elovl₂) resulting in essential dietary requirements of these fatty acids that must be fulfilled [1]. Consequently, the modifications of the dietary essential fatty acid (EFA) profile could result in lower growth performance if the minimum requirements are not met [2, 3].

Carbohydrates (CHO) are present in plant ingredients and are a low-cost energy and carbon source for fish being incorporated in aquafeeds to maximize the use of protein for growth [4]. Nevertheless, carnivorous fish species have a limited ability to digest and utilize dietary CHO than herbivorous and omnivorous fish species [5]. Dietary CHO are described as glycogenesis and lipogenesis enhancers [6, 7] but they also up-regulated desaturases and elongases in salmonids [8, 9] and gilthead sea bream [6] that may modify the tissue fatty acid profiles ultimately leading to changes in the eicosanoid associated immune response. Additionally, dietary CHO may improve fish oxidative stress status [10] but also impair

intestinal lipid uptake and transportation and impact the plasmatic triglycerides and cholesterol levels [11, 12].

Gilthead sea bream is one of the prominent species for European aquaculture and its production is continuously increasing [13]. The dietary requirements of n-3 LC-PUFA for juveniles of this species were estimated to be 0.9 or 1.9 % if the DHA/EPA ratio was 1 or 0.5, respectively [14, 15]. ARA requirements are not fully understood. Previously, graded dietary ARA levels from 0.03% to 1.7%, did not affect growth performance and feed utilization [16].

Gilthead sea bream is considered a carnivorous species, however, seems to tolerate a dietary inclusion of 20% digestible CHO without negative effects on growth performance and feed utilization [17].

The importance of dietary ARA in the immune system was associated with its capacity to modify protein-encoding genes involved in lipid metabolism that directly impacted immune-related transcription factors such as nuclear factor kappa B (NF κ B)[18] but also to its participation in assembly, and activate NADPH oxidase, control respiratory burst as well as the function of infiltrating neutrophils and other leucocytes [19]. Previously, ARA preferential incorporation to other fatty acids in leucocytes of different fish species was described [20, 21]. Cell membrane ARA is also a substrate for the synthesis of eicosanoids such as PGE₂ and LTB₄ from cyclooxygenase-2 (Cox₂) and 5-lipoxygenase (5-LOX), respectively, that promote leucocytes chemotaxis, reactive oxygen species formation, and other pro-inflammatory effects such as vascular permeability and vasodilatation [18, 19, 22]. Consequently, the production of inflammatory cytokines like TNF- α , IL-1 β , and IL-6 was associated with ARA-related eicosanoids [22]. Indeed, the immune system modulations by dietary ARA were highlighted in different fish species [23-26].

Although several studies have shown that the modulation of the fish immune system might be induced by n-3 LC-PUFA the exact mechanism of their action is not clearly understood [27-30]. In the past, the major mechanism was thought to be the decreased production of ARA-derived eicosanoid mediators like PGE₂ [31]. N-3 LC-PUFA, especially EPA, are none to compete with ARA as a substrate for COX₂ and 5-LOX producing less inflammatory eicosanoids such as PGE₃ and 5-series LTs and reducing the pro-inflammatory ARA derivatives [31, 32]. Both EPA and DHA also originate resolvins and docosanoids (only DHA) through the action of COX₂ and lipoxygenase enzymes which are mediators with demonstrated anti-inflammatory, inflammation resolution, and immunomodulatory properties [31]. Additionally, n-3 LC-PUFA imparts their anti-inflammatory effects by decreasing the

activation of the NF κ B [22]. Therefore, NF κ B, as a potent inducer of proinflammatory cytokine production, can be regulated by EPA and DHA [33, 34].

However, in fish, DHA may be more relevant than EPA to enhancing immune functions as it was demonstrated in juvenile grouper, *Epinephelus malabaricus* [30]. Accordingly, dietary DHA/EPA ratios higher than 1 enhanced innate immune response in several marine fish species, reinforcing the role of DHA over EPA as an immunomodulator [29, 35, 36].

Different dietary EFA profiles distinctively affected the immunological status of gilthead sea bream in unchallenged and in challenging conditions. For instance, in unchallenged conditions a balanced dietary ARA:EPA+DHA ratio (1% ARA; 0.4% EPA; 0.4 % DHA) increased the monocyte numbers, the alternative complement activity, and the bactericidal activity against *Photobacterium damsela* subsp. *piscicida* (*Phdp*) compared to fish fed an ARA rich diet (2 %) or n-3 LC-PUFA rich diets (0.6%:0.6% EPA:DHA or 0.3 %:1.5% EPA:DHA) [37]. However, after gilthead sea bream juveniles being intraperitoneally injected with killed *Phdp* fish fed DHA-rich diets (1.5 % DHA) presented higher alternative complement activity and intestinal expression of pro-inflammatory genes while fish fed ARA-rich (2%) had higher plasma bactericidal activity against *Phdp* [38].

Studies in the implications of dietary CHO in fish health are still scarce but there is evidence that low or excessive CHO content in diets could suppress the innate immune defenses in carnivorous fish species [39-41].

Thus, the objective of this study was to evaluate the dietary effects of ARA, DHA, and digestible CHO levels on selected immune parameters such as hematological profile, plasmatic immune parameters, and immune-related gene expression before and after an immunological challenge.

2. Material and Methods

2.1. Experimental diets

The experimental diets were formulated as described in Magalhães et al [42]. Briefly, four diets were designed to be isoproteic and isolipidic (47% crude protein; 18% crude lipids) and to include approximately 2.4 % ARA or DHA of the diet with low (5%; LS) or high starch (20%; HS). A vegetable oil blend was utilized as lipidic source (rapeseed: 20% + linseed : 50% + palm: 30%). All the ingredients were thoroughly mixed and dry pelleted in a pellet mill (California Pellet Mill, CPM Crawfordsville, IN, USA). The 3 mm die pellets were dry in an oven at 40°C for 48 hours and stored at -4 °C until utilization. Ingredients and diets composition and dietary FA composition are present in Tables 1 and 2, respectively.

2.2. Growth trial

The experiment was approved by CIIMAR ethical committee for Managing Animal Welfare (ORBEA; reference ORBEA_CIIMAR_30_2019) in compliance with the European Union directive 2010/63/EU and the Portuguese Law (DL 113/2013). The growth trial was performed as described in Magalhães et al., [42]. The trial was carried out in CIIMAR, Matosinhos, Portugal. The gilthead sea bream juveniles were acquired from a commercial fish farm (Sonrionansa, S.L., Cantabria, Spain). Fish were subjected to a quarantine period of one month and posteriorly were moved to the experimental system and acclimatized for 15 days. A thermo-regulated system ($23 \pm 1^\circ \text{C}$) with 12 cylindrical fiberglass tanks of 100 L capacity was used for the experimental trial. The system had a continuous flow of constantly filtered seawater ($2.5\text{-}3.5 \text{ L min}^{-1}$; salinity: $35 \pm 1 \text{ ‰}$; dissolved oxygen: $7 \pm 1 \text{ mg L}^{-1}$). Initially, 12 groups of 20 fish with 47.5 of initial mean body weight were distributed in the experimental system. The experimental diets were tested in triplicate, and fish were fed for 12 weeks by hand until apparent visual satiation.

2.3. Bacterial challenge

Formalin-killed bacterin was prepared with the virulent strain Lg_{H41/01} of *P. damselae* subsp. *Piscicida* (*Phdp*) according to Diaz-Rosales, et al. [43], [44]. Initially, bacteria were cultured on tryptic soy agar supplemented with 1.5 % NaCl (w/v) (TSAs) for 48 h at 22°C . Then, one colony was transferred to tubes containing 5 mL of tryptic soy broth supplemented with 1.5 % NaCl (TSBs) and incubated for 18 h at 22°C . Next day, 50 μL aliquot of the culture were inoculated in flasks with 50 mL TSBs and incubated at 22°C for 24h with continuous shaking. The bacterial culture was then adjusted to $1 \times 10^8 \text{ CFU mL}^{-1}$ and killed with formaldehyde (1% final concentration) during 24 h. The formalin-killed bacterin was collected by centrifugation at $6\,000 \times g$ for 30 min at 4°C , washed 3 times in phosphate-buffered saline (PBS), resuspended in the same volume of PBS (50 mL). Efficacy of formalin-treatment was confirmed by plating on TSAs plates and incubating for 2 days at 22°C . After the feeding trial of 12 weeks, 24 fish per dietary treatment (135 g of mean body weight) were intraperitoneally injected (i.p.) with 0.6 mL of bacterin *Phdp* or with 0.6 mL PBS (sham group) and reallocated into the experimental system tanks according to dietary treatment and stimuli.

2.4. Sampling

After the 12 weeks of the growth trial, blood from 3 fish per tank was collected from the caudal vein with heparinized syringes. Following 4 and 24 h after intraperitoneal injection, blood from six fish injected with killed *Phdp* and six fish injected with PBS was collected from the same

vein. The blood was used to assess haematological parameters and the remaining blood was immediately centrifuged at 6800 x g for 10 min. Plasma was collected and frozen at -80 °C until immunological parameters analysis. Then, the same fish were euthanized by decapitation and a portion of the distal intestine (DI, distinguished from the mid intestine by an enlarged diameter and darker mucosa) was sampled for gene expression. DI samples were maintained in RNAlater solution (1:10) for 24 hours at 4 °C and then stored at -80 °C until be analyzed.

2.5. Haematological procedures

Blood parameters were assessed following Peres, et al. [45]. Total red (RBC) and white (WBC) blood cell count was performed from blood dilutions using a hemocytometer. Fresh heparinized blood was utilized for haematocrit (Ht) and its values were determined by the haematocrit tubes microcentrifugation (10 000 g for 10 min, at room temperature). Drabkin's solution was used for haemoglobin determination (HB; SPINREACT kit, ref. 1001230, Spain). The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were calculated:

- $MCV (\mu m^3) = (Ht/RBC) \times 10$
- $MCH (pg \text{ cell}^{-1}) = (HH/RBC) \times 10$
- $MCHC (g \text{ 100 mL}^{-1}) = (HB/Ht) \times 100$

Fresh blood was utilized to made blood smears immediately after collection. Then, they were air-dried, fixated with formol-ethanol (3.7% formaldehyde in absolute ethanol), and stained with Wright's stain (Haemacolor; Merck). Neutrophils identification was made by the detection of peroxidase activity according to Afonso, et al. [46]. Then, the glass slides were examined (1000x), under oil immersion, and at least 200 leucocytes were counted and classified as lymphocytes, thrombocytes, monocytes, and neutrophils. The absolute value ($\times 10^4 \mu L^{-1}$) of each cell type was subsequently calculated.

2.6. Plasmatic immune parameters

Protease activity was determined by the azocasein hydrolysis method and antiprotease activity was assessed by the ability of plasma to inhibit trypsin activity according to Machado, et al. [47].

The quantification of total peroxidase activity was assessed as described by Quade and Roth [48] and one unit of peroxidase activity was defined as that results in an absorbance change of 1 OD (units mL⁻¹ of plasma).

Plasmatic total nitrite plus nitrate content was quantified using a Nitrate/Nitrite colorimetric kit (Roche Diagnostics GmbH, Mannheim, Germany, ref: 11746081001) adjusted for 96-well microplates. As both compounds are oxidative metabolites of endogenously produced nitric oxide (NO), they were used to quantify NO content in plasma.

Plasma total immunoglobulins (Ig) were determined following Siwicki and Anderson [49]. The determination was made based on the measurement of plasmatic total protein content using the Pierce™ BCA Protein Assay Kit (Thermo Scientific™, ref: 23225, Rockford, U.S.A) before and after the precipitation of Ig molecules with the utilization of a 12% solution of polyethylene glycol. The total plasmatic Ig content was determined by the difference between the protein content.

Alternative pathway complement activity was measured according to Sunyer and Tort [50]. The haemolysis degree (Y) was estimated plotting $Y(1-Y)^{-1}$ on a log-log scale graph against the lysis curve for each specimen. The volume of plasma producing (50%) hemolysis (ACH₅₀) was estimated and the number of ACH₅₀ units mL⁻¹ was calculated for each sample.

Plasma bactericidal activity assay was made against two opportunist marine pathogenic bacteria [*Vibrio anguillarum* and *Photobacterium damsela* subsp. *piscicida* (*Phdp*)] according to Graham, et al. [51]. Total bactericidal activity is presented as the percentage of killed bacteria, calculated from the difference between the percentage of bacteria that survived and the positive control (100% living bacteria).

2.7. Gene expression

The evaluation of mRNA levels was measured on DI according to Magalhães et al [42]. Briefly, the RNA extraction was made using a TRIzol reagent (Direct-zol™ RNA Miniprep, Zymo Research) and for cDNA synthesis was used NZY First-Strand cDNA Synthesis Kit (NZYTech, MB12501, Lisbon, Portugal) following the manufacturer's recommendations. Gene expression was quantified by real-time PCR (CFX Connect™ Real-Time System, Bio-rad, California, USA). The primers were obtained in the literature except for the sequences for prostaglandin E2 receptor EP₂, EP₃, and EP₄ that were obtained from Genebank (Table 3). Primer efficiency was determined according to Pfaffl [52]. The normalization of the target gene was made using the elongation factor 1 α (*ef1 α*) and 18 s ribosomal RNA (18s) as a reference gene. Expression levels are given as relative quantification of the target gene

calculated by the ratio between copy numbers of the gene of interest and the geometric mean of copy numbers of reference genes *ef1α* and *18s* following the method described by Vandesompele, et al. [53].

2.8. Statistical analysis

Data of the hematological and immune status of gilthead sea bream juveniles after the growth trial are presented as means and data of bacterial challenge are presented as means of the fold change relative to the mean value of PBS injected fish (defined as 1) at the same sampling time $\pm$ standard error of the mean (SEM) or standard error (SE). The normality and homogeneity of variances were assessed and log-transformed when needed. Data were analyzed by two-way ANOVA, with EFA or CHO level as a fixed factor. If interactions were found a one-way ANOVA was performed for each factor. The null hypothesis was rejected with a significant level of 0.05.

3. Results

3.1. Immune status of gilthead sea bream juveniles after the growth trial

Fish fed DHA diets had higher red blood cells (RBC) numbers and hemoglobin (HB) content (Table 4). No dietary effect was found in haematocrit (Ht), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), white blood cells numbers (WBC), and neutrophils, monocytes, and lymphocytes numbers (Table 4). Dietary HS increased thrombocytes numbers but only in ARA diets.

NO, antiproteases and alternative complement activities were higher in fish fed DHA diets but proteases activity was lower in fish fed those diets (Table 5). Dietary HS increased plasmatic bactericidal activity against *Vibrio anguillarum* only in the ARA group (Table 5).

The expression in the intestine of lipoxygenase (*5-lox*), cyclooxygenase 2 (*cox₂*), interleukin 1 β (*il-1β*), tumor necrosis factor α (*tnfa*), immunoglobulin M heavy chain (*igm*), Major histocompatibility complex II (*mhc-II*), interleukin 10 (*il-10*), prostaglandin E2 receptor *ep₂* and *ep₄* was not affected by dietary treatment (Fig 4). Dietary HS induces caspase3 (*casp3*) expression but only in DHA groups (Fig. 1). ARA diets induce the expression of prostaglandin E2 receptor *ep₃*.

3.2. Immune status of gilthead sea bream juveniles 4 hours after the inoculation of killed *Phdp*

Dietary DHA increased the RBC numbers within HS groups (Table 6). HT and thrombocytes numbers were higher and MCHC was lower in fish fed DHA diets. WBC numbers were higher in fish fed DHA diet within LS groups, but dietary HS increased WBC and lymphocytes in fish fed ARA diets.

Dietary ARA increased total immunoglobulins (IG) and plasmatic bactericidal activity against *Vibrio anguillarum* (Table 8). In HS diets, ARA increased alternative complement activity but in LS diets decreased it. Alternative complement activity, within ARA diets, was high in fish fed HS diets.

Distal intestinal expression of *5-lox*, *cox2*, *il-1 β* , *tnfa*, *igm*, *mhc-II*, *il-10*, and *casp3* were not affected by dietary treatment (Fig. 2). However, *ep2* and *ep4* expression was higher in HS diets.

3.3. Immune status of gilthead sea bream juveniles 24 hours after the inoculation of killed *Phdp*

RBC, WBC, and thrombocyte numbers were higher in fish fed ARA diets (Table 8). HB and MCV values were increased in LS groups. HT was higher in DHA diets within LS groups. HS diet decreased HT values in DHA diets. ARA diet decreased MCHC in HS groups but increased it in LS groups. HS diets decreased MCHC within the ARA group but increased it within the DHA group (Table 8).

ARA diets increased total Ig production and peroxidase activity. NO, and bactericidal activity against *Phdp* were higher in HS diets (Table 9). On the other hand, proteases, antiproteases activity and bactericidal activity against *Vibrio anguillarum* were higher in the plasma of fish fed the LS diets (Table 9).

Distal intestinal *casp3* expression was higher in ARA diets (Fig 3). Despite the interaction between EFA ratio and starch level in *mhc-II* expression, no statistical differences were found between dietary treatments.

4. Discussion

4.1. Growth trial

The growth trial results were presented elsewhere [42]. Briefly, the growth performance was not affected by dietary treatments revealing that dietary n-3 LC-PUFA requirements were

fulfilled with 0.5% EPA+DHA at least in the presence of high levels of ARA. This value was much lower than the 0.9 or 1.9 %, depending on the DHA/EPA ratio, that were previously estimated [14, 15] but in line with the 0.3 % found by Magalhães, et al. [54]. Nevertheless, starch dietary incorporation promoted better feed efficiency and protein efficiency ratio.

4.1.1. Effects of dietary essential fatty acid

Hematological parameters may be used to provide substantial diagnostic information regarding fish nutritional, physiological, and health status [45]. In the present study, despite the absence of dietary effect on most hematological parameters after the growth trial, the RBC counts, and HB content were increased in fish fed DHA diets. Dietary DHA seems to improve RBC function probably due to its important role in RBC membrane fluidity [55]. However, in gilthead sea bream juveniles fed for 56 days with different ARA, EPA, DHA dietary levels no differences in RBC counts and HB concentrations were found between groups [37].

The role of specific fatty acids in modulate immune system process is not fully understood but different dietary FA profile, particularly variations on n-3 LC-PUFA and ARA content, affects the immune system mechanisms of cultured fish species [25-28, 36]. In this study, the decreased concentration of the NO and antiproteases and alternative complement activities in fish fed low DHA diets was probably due to the effect of n-3 LC-PUFA dietary deficiency. The depletion of alternative complement activity was already associated with n-3 LC-PUFA dietary deficiency in gilthead sea bream [27, 56]. Moreover, in different teleost species, the increment of dietary n-3 LC-PUFA content improved nonspecific immunological parameters [57-59].

Nevertheless, the increment of plasma proteases activity with high dietary ARA content suggests the importance of this EFA to stimulate plasma proteases basal activity in gilthead sea bream juveniles. Positive effects of moderate or high dietary ARA levels on other innate immune parameters were reported in different fish species [24, 25, 60]. The presented results showed that DHA diets better modulated the overall plasmatic immune parameters activities but ARA-rich diets positively modulated the proteases activity suggesting that those parameters may be enhanced by a balance dietary ARA/DHA ratio. Accordingly, in unchallenged gilthead sea bream, a balanced EFA diet with 1.0 % ARA, 0.4% EPA, and 0.4% DHA increased the plasmatic alternative complement activity and bactericidal activity against *Photobacterium damselae* compared to fish fed an ARA rich diet (2 %) or n-3 LC-PUFA rich diets (0.3 % EPA and 1.5 % DHA; [37]).

Despite the possible modulatory effect of dietary ARA or DHA on immune-related gene expression, in the presented study, after the growth trial, most of them were not under dietary regulation in the distal intestine. These results were in line with those obtained by Magalhães, et al. [37] where the distal intestine expression of immune-related genes of unchallenged gilthead sea bream juveniles fed different ARA, EPA, and DHA levels were not modulated by the dietary treatments. In the presented study, the exception was the upregulation of prostaglandin E2 receptor *ep₃* expression in fish fed ARA diets. This result may be an indication of increased conversion of ARA into PGE₂ in the distal intestine of gilthead sea bream juveniles despite *cox₂* expression being unaffected by dietary treatment. Prostanoids derived from LC-PUFA via cyclooxygenase enzymes were associated with gastrointestinal cytoprotection and the initiation of the gut inflammation process [61]. PGE₂ exerts its pleiotropic effects by binding to one or more of its four receptors (EP₁, EP₂, EP₃, and EP₄) and for that reason, the expression of three PGE₂ receptors was evaluated in the present work [62]. The upregulation of *ep₃* expression in ARA diets could be associated with an ARA increment on intestine cells FA composition following the dietary FA as previously reported by Magalhães et al. [42] for muscle FA composition. Previously, increasing dietary ARA levels were also associated with increasing plasmatic prostaglandins in European seabass juveniles [26]. Despite *ep₃* is one of the receptors with a higher affinity to PGE₂, the four receptors were associated with different roles in the regulation of the immune responses of gilthead sea bream, and further studies are needed to evaluate the effects of dietary EFA on their expressions and actions [63].

4.2. Challenge trial with killed *Phdp*

4.2.1. Effects of dietary essential fatty acid

The decreased RBC numbers in HS groups and HT, 4 hours after i.p., in fish fed ARA diets can be associated with a possible delayed response after a stressful event due to dietary n-3 LC-PUFA deficiency. Previously, in the same species, fish fed with n-3 LC-PUFA deficient diet and stressed by high stocking density had lower RBC counts [56]. Also in gilthead sea bream, 4 hours after a challenge with killed *Phdp*, DHA rich diets (1.5 %) increased RBC compared to ARA rich diets (2%) but that difference was not maintained at 24 hours after i.p. [38]. In the presented work, RBC counts were increased in ARA dietary groups, 24 hours after i.p.. Montero, et al. [27] reported that n-3 LC-PUFA dietary deficiency (0.5 %DM) increased RBC counts and hemoglobin of gilthead sea bream blood, and the authors associated this result with the high saturated fatty acid levels of this diet. The incorporation of dietary ARA in red blood cells was previously demonstrated in rabbitfish, *Siganus rivulatus*

[24], and our results suggest an improving effect in RBC numbers 24 hours after a stressful or pathological event that deserves further attention.

DHA increased the circulating WBC numbers in the LS group and thrombocytes 4 hours after i.p. but dietary ARA stimulates the production of both after 24 hours. The results indicate that fish fed ARA diets (low n-3 LC-PUFA content) slowly increased WBC numbers after the i.p. surpassing even the WBC counts of fish fed n-3 LC-PUFA rich diets. Previously, in challenged gilthead sea bream juveniles fed diets with a balanced DHA:EPA ratio (1.2 % EPA+DHA; DHA:EPA= 1:1) had higher WBC, lymphocytes, and thrombocytes numbers than fish fed ARA (2 % ARA; DHA:EPA = 1:2) or DHA rich diets (1.5 %; DHA:EPA ratio = 5:1; [38]). However, in European seabass juveniles subjected to a 1 minute air exposure, VO diets (low n-3 LC-PUFA content) did not affect circulating WBC count but decreased neutrophils count compared to the FO diets group [64]. These results need to be further clarified since studies on the dietary effects of ARA and DHA on hematological parameters after a stressful or bacteriological challenge are scarce. Dietary ARA, besides the slowly increased WBC count, improves innate immune parameters, such as plasmatic peroxidase activity, alternative complement activity in HS groups, and bactericidal activity against *Vibrio anguillarum*, at 4 and 24 hours after i.p.. Generally is assumed that the principal reason for the observed effects is the fact that ARA is an essential precursor of eicosanoids through the action of *Cox₂* and *5-Lox*, being directly responsible for the regulation and activation of biochemical cascades within the immune system [31]. However, in the presented study, neither the mentioned enzymes, PGE₂ receptors, or pro-inflammatory cytokines gene expression was upregulated in the distal intestine of gilthead sea bream juveniles after challenge. Contrarily, Magalhães et al., [38] showed that in the distal intestine of gilthead sea bream, DHA-rich diets (1.5%) induced the expression of *cox₂* and *il-1 β* , 4 and 24 h, and of *tnfa*, 4 h after i.p. with *Phdp*. Nevertheless, the increment of bactericidal activity against *Vibrio anguillarum* was an important result to highlight the importance of high dietary ARA content in the immune function since the direct ability to kill bacteria is one of the most practical humoral immune parameters for fish farmers. Previously, also in challenged gilthead sea bream, ARA-rich diets (2%) increased the plasmatic bactericidal activity against *Phdp* compared to fish fed DHA-rich diets (1.5 %) but the latest group had the highest alternative complement activity [38]. In addition, high peroxidase activity was observed in ARA groups 24 hours after i.p. injection despite the absence of differences in neutrophils counts. Thus, being the neutrophils the main source of plasma peroxidase this result is a potential indication of a direct stimulatory effect of dietary ARA on neutrophil activity as previously suggested [19]. The role of ARA as an immunomodulator

was previously highlighted by other authors with similar significant improvement of non-specific immune parameters associated with ARA supplementation in fish diets [24-26]. Furthermore, the ARA diets had a significant impact on total immunoglobulins compared to DHA diets suggesting the improved capacity of gilthead sea bream juveniles to assemble adaptative immune responses. Similarly, rabbitfish juveniles fed during to 2 months with ARA supplemented diets (2.6 and 4.7 % total FA) had higher total immunoglobulin levels compared to fish fed the control diet (0.6% total FA) [24]. Normally, elevated immunoglobulin levels could suggest an underlying state of infection, whereas low levels, as it was seen in DHA groups, may serve as a sign of immunodeficiency [65]. Nevertheless, increasing systemic immune competence might also be potentiated by dietary n-3 LC-PUFA since silvery black porgy juveniles fed diets with 4.2 % had the highest total plasma Ig levels compared to fish fed 0.1 to 1.9 % [57]. In addition, diets with a balance DHA:EPA ratio (0.6 % EPA; 0.6% DHA) increased the expression of *mhc-II* and *igm* in gilthead sea bream distal intestine after a challenge with killed *Phdp* compared to fish fed high DHA:EPA ratio (5:1; 0.3 % EPA; 1.5 % DHA) suggesting that this ratio impacts the adaptative immune responses genes expression [38]. However, in the presented study, ARA diets demonstrated an overall improved innate and adaptative immune response after i.p.

In the presented study, 24 hours after the challenge, the induction of *casp3* expression in the distal intestine of fish fed ARA diets, suggests an immediate response of the host immune defense system to the i.p. that enhanced *Phdp* clearance through apoptosis. This hypothesis is supported by Kumaresan, et al. [66] who found increased *casp3* gene expression in striped murrel (*Channa striatus*) tissues 24 hours after inoculation with *Aeromonas hydrophila* and by Long and Sun [67] that also described *Casp3* upregulation in head kidney and spleen of tongue sole (*Cynoglossus semilaevis*) 12 hours following *Edwardsiella tarda* infection.

4.2.2. Effects of carbohydrate level

The effect of carbohydrate level as a fish health modulator was previously highlighted [40, 41]. The dietary effects on hematological and innate immune parameters in this study were found mainly after the i.p. challenge.

4 hours after the challenge the RBC counts were unaffected by the dietary starch level. 24 hours after i.p. the RBC numbers were increased and the HB content and MCV value decreased. These results could be associated with a higher production of immature and smaller RBC to enhanced tissue oxygen delivery to react to the infection as a result of higher digestible energy available in those diets. However, in European seabass fed VO or FO

based diets with different CHO levels and stressed by one minute of air exposure the number of RBC and other hematological parameters were little affected [64].

In fish fed ARA diets, 4 hours after injection, circulating WBC and lymphocytes numbers were increased in HS compared to LS diets and could indicate a direct effect of carbohydrates in lymphocytes activity in low n-3 LC-PUFA diets which may result in the same increment of alternative complement activity. Furthermore, the distal intestine expression of *ep₂* and *ep₄*, the PGE₂ receptors, was also upregulated in fish fed HS diets at this sampling point possibly indicating an inflammatory response. Moreover, the lack of differences in the expression of other tested pro-inflammatory genes in the presented study contrasts with the finds of Wu, et al. [41] that found higher expression of those with adequate levels of dietary CHO in the blood and liver of black carp (*Mylopharyngodon piceus*). The authors also highlighted that deficiency or excess of carbohydrates decreased plasmatic immune parameters ultimately leading to diminished resistance after infection with *Aeromonas hydrophilla* [41]. Accordingly, in juvenile golden pompano (*Trachinotus ovatus*), 0 to 16.8% of dietary CHO enhance the plasmatic non-specific immune parameters but further inclusion (up to 28%) leading to the opposite effect, reinforcing the importance to the fish immune system of an adequate level of dietary CHO [40]. Results of the presented study did not clear the role of dietary CHO in gilthead sea bream juveniles immunity. The dietary CHO had a higher impact in plasmatic immune parameters 24 hours after p.i however HS diets increased NO and bactericidal activity against *Phdp* but decreased the bactericidal activity against V.A., proteases, and antiproteases activity. The same apparent unclear effect of dietary CHO levels in VO or FO based diets on immunes responses was also reported in European seabass with contradictory results regarding plasmatic NO, antiproteases activity, peroxidase, and bactericidal activity against *Phdp* [64].

5. Conclusions

Nowadays, the aquafeeds industry is decreasing the dietary content of n-3 LC-PUFA and increasing carbohydrate-rich plant ingredients which may impact gilthead sea bream hematology and immune system as shown in the presented study. The results reinforcing the need to provide more than growth performance as an accurate view of dietary requirements. The global vision of fish health status pointed out dietary effects that can only become apparent under stressful situations such as infection challenges. Despite the dietary DHA importance to better modulate the innate system in the growing out phase, the presented study highlighted the role of ARA as a strong immune modulator after a bacterial challenge with killed *Phdp*. The results showed the necessity of further research on the ideal ARA/DHA

ratio to optimize disease resistance in gilthead sea bream juveniles. Finally, the small number of interactions between dietary EFA and starch level did not emphasize the capacity of starch to deteriorate the immune response in diets with low n-3 LC-PUFA content had even, in some specific parameters, improved it.

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Table 1. Ingredient and proximate analysis of experimental diets.

Diets (ARA/DHA ratio)	ARA/HS 2.3/0.3	ARA/LS 2.3/0.3	DHA/HS 0/2.6	DHA/LS 0/2.5
<i>Ingredients (% DM)</i>				
Fish meal ¹	20.0	20.0	20.0	20.0
Corn gluten meal ²	20.0	20.0	20.0	20.0
Wheat gluten meal ³	18.8	18.8	18.8	18.8
Cellulose ⁴	—	17.9	—	17.9
Gelatinized starch ⁵	17.9	—	17.9	—
Taurine ⁶	0.3	0.3	0.3	0.3
Lysine ⁷	0.7	0.7	0.7	0.7
Vegetable oil blend ⁸	10.0	10.0	11.8	11.8
Choline chloride (50%)	0.5	0.5	0.5	0.5
Mineral premix ⁹	1.0	1.0	1.0	1.0
Vitamin premix ¹⁰	1.0	1.0	1.0	1.0
Binder ¹¹	1.0	1.0	1.0	1.0
CaHPO ₄	3.7	3.7	3.7	3.7
VEVODAR ¹²	5.3	5.3	—	—
DHA 70% Algatrium ¹³	—	—	3.5	3.5
<i>Proximate analyses (% DM)</i>				
Dry matter (DM)	91.1	91.9	90.2	92.0
Crude Protein	46.5	46.7	46.9	46.1
Crude Lipid	17.6	17.7	18.7	17.7
Ash	8.6	9.2	8.9	8.7
Starch	19.6	4.6	20.0	5.4
Gross energy (kJ g ⁻¹)	23.4	23.0	23.1	23.1

CP: Crude protein; CL: Crude lipids; DM: Dry matter.

¹Steam Dried LT-FM, Pesquera Centinela, Chile (CP: 73.2%; CL: 9.2%).

²Sorgal, S.A. Ovar, Portugal (CP: 71.6%; CL: 1.6%).

³Sorgal, S.A. Ovar, Portugal (CP: 84.2%; CL: 2.3%).

⁴Sigma-Aldrich, Saint Louis, USA

⁵C-Gel Instant – 12016, Cerestar, Mechelen, Belgium.

⁶Feed-grade taurine, Sorgal, S.A. Ovar, Portugal.

⁷Feed-grade lysine, Sorgal, S.A. Ovar, Portugal.

⁸Vegetable oil blend: 20% rapeseed oil + 50% linseed oil + 30% palm oil

⁹Minerals (mg kg⁻¹ diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g kg⁻¹ diet); potassium chloride, 1.15 (g kg⁻¹ diet); sodium chloride, 0.4 (g kg⁻¹ diet).

¹⁰Vitamins (mg kg⁻¹ diet): retinol, 18000 (IU kg⁻¹ diet); calciferol, 2000 (IU kg⁻¹ diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.

¹¹Liptosa, Madrid, Spain

¹²Vevodar, DSM Nutritional Products (ARA: 47.4 %FA; EPA: 0.11 %FA; DHA: 0.04 %FA)

¹³BrudyTechnology (Tuna oil; ARA: 0 %FA; EPA: 10.2%FA; DHA: 89.5 %FA)

Table 2. Fatty acid composition of the experimental diets (% of total fatty acids).

Diets (ARA/DHA ratio)	ARA/HS 2.3/0.3	ARA/LS 2.3/0.3	DHA/HS 0/2.6	DHA/LS 0/2.5
SFA				
10:0	0.02	0.02	0.02	0.02
12:0	0.02	0.03	0.03	0.03
14:0	0.81	0.82	0.77	0.77
15:0	0.09	0.09	0.07	0.07
16:0	14.92	14.78	14.55	14.30
17:0	0.21	0.21	0.13	0.13
18:0	5.09	5.12	3.05	3.04
20:0	0.50	0.50	0.27	0.27
22:0	0.85	0.86	0.18	0.18
24:0	1.61	1.62	0.17	0.18
MUFA				
16:1n-7	0.75	0.75	0.71	0.72
16:1n-9	0.05	0.05	0.05	0.05
17:1n-7	0.01	0.01	0.01	0.01
18:1n-9	22.63	22.58	23.85	23.86
18:1n-7	1.04	1.03	1.07	1.07
20:1n-11	0.05	0.05	0.05	0.05
20:1n-9	0.52	0.51	0.41	0.41
20:1n-7	0.03	0.03	0.03	0.03
22:1n-11	0.34	0.33	0.36	0.36
22:1n-9	0.06	0.06	0.05	0.05
24:1n-9	0.21	0.21	0.16	0.17
PUFA				
16:2n-4	0.08	0.08	0.08	0.08
16:3n-4	0.08	0.08	0.08	0.08
16:4n-1	0.11	0.12	0.12	0.12
18:2n-6 (LA)	15.80	15.55	15.25	15.13
18:3n-6	0.68	0.69	0.02	0.02
18:3n-4	0.02	0.02	0.02	0.02
18:3n-3 (ALA)	14.88	14.98	17.87	17.95
18:4n-3	0.16	0.16	0.16	0.16
20:2n-6	0.23	0.23	0.04	0.04
20:3n-6	0.94	0.94	0.02	0.02
20:4n-6 (ARA)	13.11	13.24	0.17	0.14
20:3n-3	0.02	0.03	0.02	0.02
20:4n-3	0.06	0.06	0.08	0.08
20:5n-3 (EPA)	1.21	1.24	2.66	2.69
21:5n-3	0.05	0.05	0.86	0.87
22:2n-6	0.01	0.01	0.01	0.01
22:4n-6	0.10	0.10	0.02	0.01
22:5n-3	0.19	0.20	2.31	2.34
22:6n-3 (DHA)	1.53	1.60	13.67	13.90
ΣSFA	24.20	24.12	19.30	19.06
Σ MUFA	25.73	25.67	26.78	26.79
Σ PUFA	49.25	49.38	53.44	53.67
Σn-6	30.86	30.76	15.53	15.37
Σn-3	18.10	18.32	37.63	38.00
n-6/n-3	1.70	1.68	0.41	0.40

FA: fatty acid; LA: Linoleic acid; ALA: α-Linolenic acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid.

Table 3. Primer pairs sequences used for the transcript level determination of selected target and reference genes.

Gene	Gene abbreviation	Primer sequences (5'→3')	Primer efficiency	Accession number
Arachidonate 5-lipoxygenase	<i>5-Lox</i>	F: CCTGGCAGATGTGAACTTGA R: CGTTCTCCTGATACTGGCTGA	1.88	FP334124
Caspase 3	<i>Casp3</i>	F: CCAGTCAGTCGAGCAGATGA R: GAACACACCCTCGTCTCCAT	1.98	EU722334
Interleukin 1-β	<i>IL1β</i>	F: GGGCTGAACAACAGCACTCTC R: TTAACACTCTCCACCCTCCA	2.14	AJ277166
Immunoglobulin M	<i>IGM</i>	F: CAGCCTCGAGAAGTGGAAC R: GAGGTTGACCAGGTTGGTGT	2.10	AM493677
Cyclooxygenase 2	<i>Cox2</i>	F: GAGTACTGGAAGCCGAGCAC R: GATATCACTGCCGCCTGAGT	1.96	AM296029
Tumor necrosis factor-α	<i>TNFα</i>	F: TCGTTCAGAGTCTCCTGCAG R: CATGGACTCTGAGTAGCGCGA	2.02	AJ413189
Major Histocompatibility Complex class IIa	<i>MHCII</i>	F: CTGGACCAAGAACGGAAGA R: CATCCAGATCCTGGTCAGT	1.94	DQ019401
Interleukin 10	<i>IL-10</i>	F: TGGAGGGCTTTCCTGTCAGA R: TGCTTCGTAGAAGTCTCGGATGT	1.94	FG261948
Prostaglandin E2 receptor EP2	<i>EP2</i>	F: ACACGTTGGACAGAGCAAGT R: TAGTGGCACGTGTCGTTCTC	2.12	XM_030410284.1
Prostaglandin E2 receptor EP3	<i>EP3</i>	F: ACCCATGAAACCACTGTCC R: CCCTGGGCGGATACACAAAT	1.80	XM_030434233.1
Prostaglandin E2 receptor EP4	<i>EP4</i>	F: GCGGACAGACTCTCTGGTTC R: TCTCAGTGCTCAACGACACC	1.99	XM_030415999.1
Elongation factor 1α	<i>Ef1α</i>	F: CTGTCAAGGAAATCCGTCGT R: TGACCTGAGCGTTGAAGTTG	1.87	AF184170
Ribosomal protein S18	<i>18s</i>	F: AGGGTGTGGCAGACGTTAC R: CTCTGCCTGTTGAGGAACC	1.95	AM490061

Table 4. Haematological profile in gilthead seabream fed the experimental diets after 84 days.

Diets (ARA/DHA ratio)	ARA/HS 2.3/0.3	ARA/LS 2.3/0.3	DHA/HS 0/2.6	DHA/LS 0/2.5	SEM
RBC ($\times 10^6 \mu\text{L}^{-1}$)	2.7	3.1	3.1	3.3	0.07
Hemoglobin (g dL ⁻¹)	3.5	3.8	4.1	4.2	0.12
Hematocrit (%)	39.6	40.1	42.1	40.1	1.55
MCV (μm^3)	146.2	132.9	133.4	122.4	3.44
MCH (pg cell ⁻¹)	13.1	12.7	13.2	12.0	0.35
MCHC (g 100 mL ⁻¹)	9.0	9.6	9.9	9.8	0.26
WBC ($\times 10^4 \mu\text{L}^{-1}$)	2.9	1.5	2.7	2.7	0.18
Neutrophils ($10^4 \mu\text{L}^{-1}$)	0.2	0.1	0.2	0.2	0.03
Monocytes ($10^4 \mu\text{L}^{-1}$)	0.1	0.1	0.2	0.1	0.01
Lymphocytes ($10^4 \mu\text{L}^{-1}$)	0.7	0.3	0.6	0.6	0.06
Thrombocytes ($10^4 \mu\text{L}^{-1}$)	1.8 ^B	1.0 ^A	1.7	1.7	0.11
Two-way ANOVA					
Parameters	EFA	CHO	INT		
RBC	0.039	NS	NS		
Hemoglobin	0.049	NS	NS		
Hematocrit	NS	NS	NS		
MCV	NS	NS	NS		
MCH	NS	NS	NS		
MCHC	NS	NS	NS		
WBC	NS	NS	NS		
Neutrophils	NS	NS	NS		
Monocytes	NS	NS	NS		
Lymphocytes	NS	NS	NS		
Thrombocytes	NS	NS	0.036		

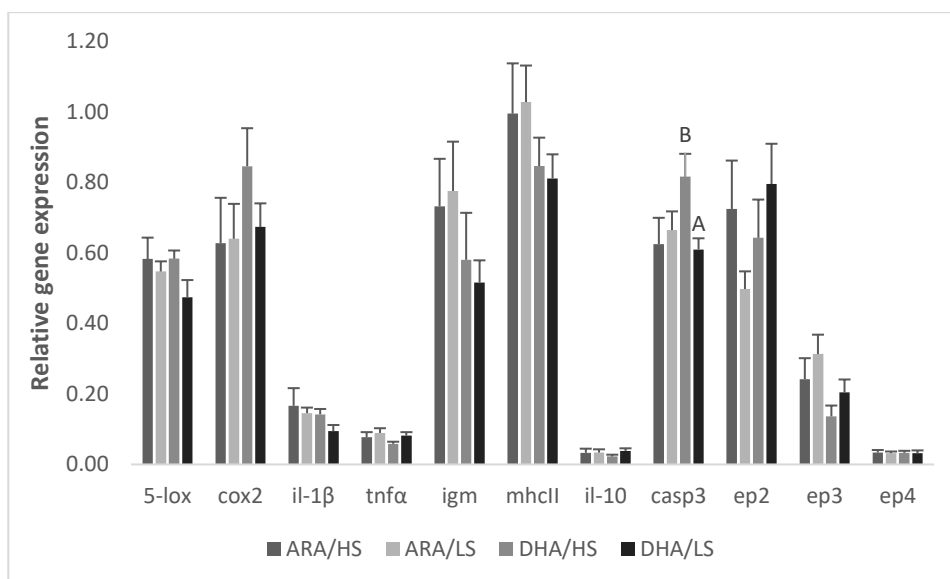
Values presented as means (n=9) and standard error of the mean (SEM). Two-way ANOVA: ns: non-significant ($P \geq 0.05$). If the interaction was significant, one-way ANOVA was performed for each factor. The capital letter means differences between the CHO level in each EFA and subscript letters between EFA in each CHO Level. MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; MCV: mean corpuscular volume; RBC: red blood cells; WBC: white blood cells.

Table 5. Immune humoral parameters measured in gilthead sea bream juveniles plasma fed the experimental diets after 84 days.

Diets (ARA/DHA ratio)	ARA/HS 2.3/0.3	ARA/LS 2.3/0.3	DHA/HS 0/2.6	DHA/LS 0/2.5	SEM
NO (μM)	662.4	688.2	735.1	767.9	18.4
Total Ig (mg ml^{-1})	18.3	16.5	17.2	18.5	0.46
Peroxidase (units ml^{-1})	22.5	22.3	32.2	22.7	1.74
Proteases (%)	5.7	5.6	5.1	5.3	0.09
Antiproteases (%)	82.7	81.3	84.4	83.3	0.39
Alternative complement pathway activity ($\text{ACH}_{50} \text{ U mL}^{-1}$)	2.48	1.81	4.43	4.46	0.35
Bactericidal activity against <i>Phdp</i> (%)	19.1	37.8	26.1	24.1	2.72
Bactericidal activity against <i>Vibrio</i> <i>anguillarum</i> (%)	^B 64.4	^A 51.6	60.8	61.5	1.62
Two-way ANOVA					
Parameters	EFA	CHO	INT		
NO	0.040	NS	NS		
Total Ig	NS	NS	NS		
Peroxidase	NS	NS	NS		
Proteases	0.004	NS	NS		
Antiproteases	0.015	NS	NS		
ACH_{50}	0.001	NS	NS		
Bactericidal activity against <i>Phdp</i>	NS	NS	NS		
Bactericidal activity against <i>Vibrio</i> <i>anguillarum</i>	NS	0.047	0.028		

Values are presented as means (n=9) and standard error of the mean (SEM). Two-way ANOVA: ns: non-significant ($P \geq 0.05$). If the interaction was significant, one-way ANOVA was performed for each factor. The capital letter means differences between the CHO level in each EFA and subscript letters between EFA in each CHO Level.

Figure. 1. Intestinal expression levels of Lipoxygenase (*5-lox*), Cyclooxygenase 2 (*cox2*), Interleukin 1 β (*il-1 β*), Tumor necrosis factor α (*tnf α*), Immunoglobulin M heavy chain (*igm*), Major histocompatibility complex II (*mhcII*), Interleukin 10 (*il-10*), caspase 3 (*casp3*) and Prostaglandin E2 receptor *ep2*, *ep3*, and *ep4* of gilthead seabream juveniles fed the experimental diets for 12 weeks.



Two-way ANOVA		Variance source	
Gene target	EFA	CHO	Interaction
<i>5-lox</i>	NS	NS	NS
<i>cox2</i>	NS	NS	NS
<i>il-1β</i>	NS	NS	NS
<i>tnfa</i>	NS	NS	NS
<i>igm</i>	NS	NS	NS
<i>mhcII</i>	NS	NS	NS
<i>il-10</i>	NS	NS	NS
<i>casp3</i>	NS	NS	0.039
<i>ep2</i>	NS	NS	NS
<i>ep3</i>	0.027	NS	NS
<i>ep4</i>	NS	NS	NS

Samples expressions were calibrated with the sample with a higher expression value and then normalized with elongation factor 1-alpha (*EF1 α*) and ribosomal RNA 18S (*18S*) expressed transcripts. Values presented as means (n=9) and pooled standard error (SE); Two-way ANOVA: NS: non-significant ($P \geq 0.05$). The capital letter means differences between the CHO level in each EFA and subscript letters between EFA in each CHO Level.

Table 6. Hematological profile of gilthead seabream fed the experimental diets at 4 hours after killed *Phdp* injection. Values presented as fold change relative to the PBS-injected fish (defined as 1).

Diets (ARA/DHA ratio)	ARA/HS 2.3/0.3	ARA/LS 2.3/0.3	DHA/HS 0/2.6	DHA/LS 0/2.5	SEM
RBC	0.77 ^a	0.86	1.16 ^b	0.86	0.05
Hemoglobin	1.00	1.07	1.06	0.94	0.03
Hematocrit	0.85	0.95	1.11	1.01	0.03
MCV	1.08	1.11	0.93	1.11	0.03
MCH	1.19	1.26	0.97	0.96	0.07
MCHC	1.05	1.07	0.95	0.93	0.02
WBC	^B 1.25	^A 0.86 ^a	1.35	1.60 ^b	0.09
Neutrophils	1.19	1.05	1.35	2.06	0.14
Monocytes	1.62	0.90	0.78	0.72	0.15
Lymphocytes	^B 1.24	^A 0.67	0.78	0.94	0.08
Thrombocytes	1.26	0.81	1.34	1.60	0.11
Two-way ANOVA					
Parameters	EFA	CHO	INT		
RBC	0.024	NS	0.018		
Hemoglobin	NS	NS	NS		
Hematocrit	0.002	NS	NS		
MCV	NS	NS	NS		
MCH	NS	NS	NS		
MCHC	0.019	NS	NS		
WBC	0.007	NS	0.040		
Neutrophils	NS	NS	NS		
Monocytes	NS	NS	NS		
Lymphocytes	NS	NS	0.040		
Thrombocytes	0.033	NS	NS		

Values presented as fold change relative to the PBS-injected fish (defined as 1).

MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; RBC: red blood cells; WBC: white blood cells.

Two-way ANOVA: ns: non-significant ($P \geq 0.05$). If the interaction was significant, one-way ANOVA was performed for each factor. The capital letter means differences between the CHO level in each EFA and subscript letters between EFA in each CHO Level.

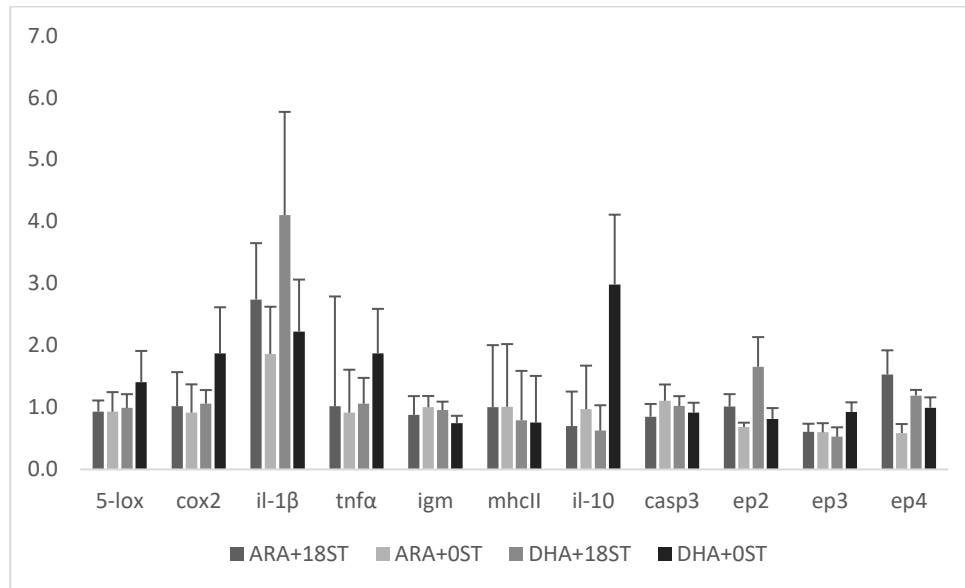
Table 7. Immune humoral parameters in plasma of gilthead seabream juveniles fed the experimental diets at 4 hours after killed *Phdp* injection.

Diets (ARA/DHA ratio)	ARA/HS 2.3/0.3	ARA/LS 2.3/0.3	DHA/HS 0/2.6	DHA/LS 0/2.5	SEM
NO	1.06	1.02	1.05	0.88	0.04
Total Ig	1.46	1.92	0.92	0.96	0.16
Peroxidase	1.65	0.53	1.54	1.50	0.24
Proteases	0.98	0.95	0.98	0.99	0.03
Antiproteases	0.99	1.03	1.00	0.99	0.01
ACH ₅₀	^B 1.40 ^b	^A 0.29 ^a	0.92 ^a	1.02 ^b	0.13
Bactericidal activity against <i>Phdp</i>	0.64	0.54	0.89	0.46	0.10
Bactericidal activity against <i>vibrio anguillarum</i>	1.26	1.11	0.97	1.01	0.04

Two-way ANOVA			
Parameters	EFA	CHO	INT
NO	NS	NS	NS
Total Ig	0.034	NS	NS
Peroxidase	NS	NS	NS
Proteases	NS	NS	NS
Antiproteases	NS	NS	NS
ACH ₅₀	NS	0.013	0.005
Bactericidal activity against <i>Phdp</i>	NS	NS	NS
Bactericidal activity against <i>Vibrio anguillarum</i>	0.027	NS	NS

Values (means ± SEM) presented as fold change relative to the PBS-injected fish (defined as 1). Two-way ANOVA: ns: non-significant ($P \geq 0.05$). If the interaction was significant, one-way ANOVA was performed for each factor. The capital letter means differences between the CHO level in each EFA and subscript letters between EFA in each CHO Level.

Figure. 2. Intestinal expression levels of Lipoxxygenase (*5-lox*), Cyclooxygenase 2 (*cox2*), Interleukin 1 β (*il-1 β*), Tumor necrosis factor α (*tnf α*), Immunoglobulin M heavy chain (*igm*), Major histocompatibility complex II (*mhcII*), Interleukin 10 (*il-10*), caspase 3 (*casp3*) and Prostaglandin E2 receptor *ep2*, *ep3*, and *ep4* of gilthead seabream juveniles fed the experimental diets at 4 hours after killed *Phdp* injection.



Gene target	Two-way Anova		
	EFA	CHO	Interaction
<i>5-lox</i>	NS	NS	NS
<i>cox2</i>	NS	NS	NS
<i>il-1β</i>	NS	NS	NS
<i>tnfa</i>	NS	NS	NS
<i>igm</i>	NS	NS	NS
<i>mhcll</i>	NS	NS	NS
<i>il-10</i>	NS	NS	NS
<i>casp3</i>	NS	NS	NS
<i>ep2</i>	NS	0.029	NS
<i>ep3</i>	NS	NS	NS
<i>ep4</i>	NS	0.017	NS

Samples expressions were calibrated with the sample with a higher expression value and then normalized with elongation factor 1-alpha (*EF1 α*) and ribosomal RNA 18S (*18S*) expressed transcripts. Values (means $\pm$ SE) presented as fold change of normalized expression level relative to the PBS-injected fish (defined as 1). Two-way ANOVA: NS: non-significant ($P \geq 0.05$).

Table 8. Hematological profile of gilthead seabream fed the experimental diets at 24 hours after killed *Phdp* injection. Values presented as fold change relative to the PBS-injected fish (defined as 1).

Diets (ARA/DHA ratio)	ARA/HS 2.3/0.3	ARA/LS 2.3/0.3	DHA/HS 0/2.6	DHA/LS 0/2.5	SEM
RBC	0.93	0.88	0.92	0.75	0.03
Hemoglobin	0.89	1.03	0.96	0.96	0.02
Hematocrit	0.91	0.91 ^a	^A 0.89	^B 1.10 ^b	0.03
MCV	0.96	1.06	0.93	1.35	0.05
MCH	0.94	1.24	0.84	1.05	0.06
MCHC	^A 0.97 ^a	^B 1.16 ^b	^B 1.11 ^b	^A 0.86 ^a	0.03
WBC	1.48	1.49	0.96	0.90	0.10
Neutrophils	2.11	2.03	2.42	1.98	0.21
Monocytes	1.56	1.39	1.39	0.86	0.28
Lymphocytes	0.96	0.74	1.06	0.93	0.12
Thrombocytes	1.40	1.42	0.80	0.97	0.09

Two-way ANOVA

Parameters	EFA	CHO	INT
RBC	0.041	0.031	NS
Hemoglobin	NS	0.044	NS
Hematocrit	NS	0.032	0.032
MCV	NS	0.004	NS
MCH	NS	NS	NS
MCHC	NS	NS	0.000
WBC	0.004	NS	NS
Neutrophils	NS	NS	NS
Monocytes	NS	NS	NS
Lymphocytes	NS	NS	NS
Thrombocytes	0.004	NS	NS

Values presented as fold change relative to the PBS-injected fish (defined as 1).

MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; RBC: red blood cells; WBC: white blood cells.

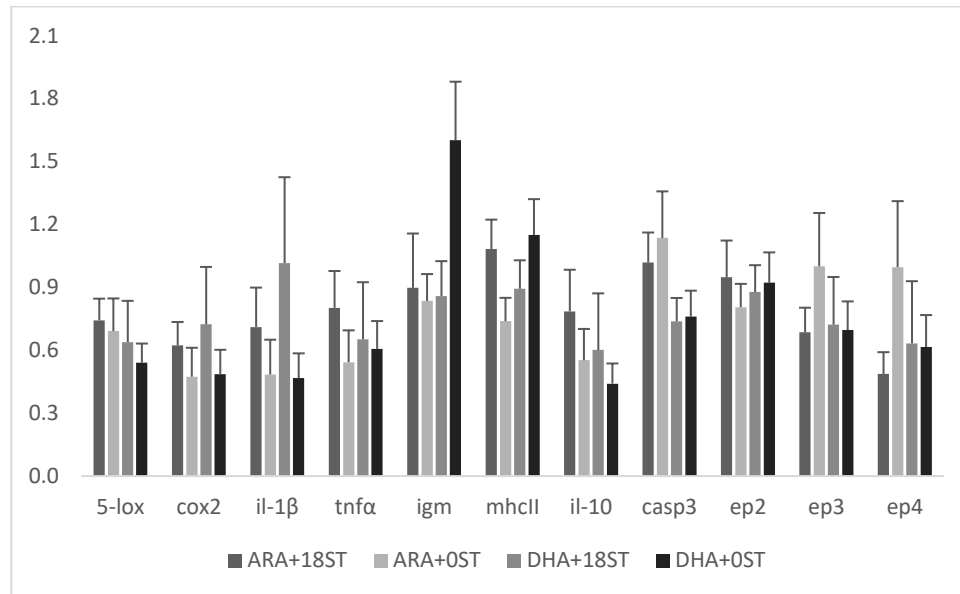
Two-way ANOVA: ns: non-significant ($P \geq 0.05$). If the interaction was significant, one-way ANOVA was performed for each factor. The capital letter means differences between the CHO level in each EFA and subscript letters between EFA in each CHO Level.

Table 9. Immune humoral parameters in plasma of gilthead seabream juveniles fed the experimental diets at 24 hours after killed *Phdp* injection.

Diets (ARA/DHA ratio)	ARA/HS 2.3/0.3	ARA/LS 2.3/0.3	DHA/HS 0/2.6	DHA/LS 0/2.5	SEM
NO	1.26	0.97	1.11	0.92	0.05
Total Ig	2.22	1.17	0.77	0.62	0.18
Peroxidase	2.17	2.27	0.86	0.86	0.27
Proteases	0.89	1.05	0.94	0.98	0.02
Antiproteases	0.99	1.01	0.99	1.00	0.00
ACH₅₀	1.12	0.87	0.64	0.56	0.10
Bactericidal activity against <i>Phdp</i>	1.03	0.56	1.05	0.66	0.08
Bactericidal activity against <i>vibrio anguillarum</i>	0.81	0.99	0.82	1.01	0.03
<i>Two-way ANOVA</i>					
Parameters	EFA	CHO	INT		
NO	NS	0.017	NS		
Total Ig	0.002	NS	NS		
Peroxidase	0.012	NS	NS		
Proteases	NS	0.020	NS		
Antiproteases	NS	0.006	NS		
ACH₅₀	NS	NS	NS		
Bactericidal activity against <i>Phdp</i>	NS	0.010	NS		
Bactericidal activity against <i>Vibrio anguillarum</i>	NS	0.000	NS		

Values (means $\pm$ SEM) presented as fold change relative to the PBS-injected fish (defined as 1). Two-way ANOVA: ns: non-significant ($P \geq 0.05$). If the interaction was significant, one-way ANOVA was performed for each factor. The capital letter means differences between the CHO level in each EFA and subscript letters between EFA in each CHO Level.

Figure. 3. Intestinal expression levels of Lipoxygenase (*5-lox*), Cyclooxygenase 2 (*cox2*), Interleukin 1 β (*il-1 β*), Tumor necrosis factor α (*tnf α*), Immunoglobulin M heavy chain (*igm*), Major histocompatibility complex II (*mhc-II*), Interleukin 10 (*il-10*), caspase 3 (*casp3*) and Prostaglandin E2 receptor *ep2*, *ep3*, and *ep4* of gilthead seabream juveniles fed the experimental diets at 24 hours after killed *Phdp* injection.



Two-way ANOVA		Variance source	
Gene target	EFA	CHO	Interaction
<i>5-lox</i>	NS	NS	NS
<i>cox2</i>	NS	NS	NS
<i>il-1β</i>	NS	NS	NS
<i>tnfa</i>	NS	NS	NS
<i>igm</i>	NS	NS	NS
<i>mhcII</i>	NS	NS	0.046
<i>il-10</i>	NS	NS	NS
<i>casp3</i>	0.048	NS	NS
<i>ep2</i>	NS	NS	NS
<i>ep3</i>	NS	NS	NS
<i>ep4</i>	NS	NS	NS

Samples expressions were calibrated with the sample with a higher expression value and then normalized with elongation factor 1-alpha (*EF1 α*) and ribosomal RNA 18S (*18S*) expressed transcripts. Values (means $\pm$ SE) presented as fold change of normalized expression level relative to the PBS-injected fish (defined as 1). Two-way ANOVA: NS: non-significant ($P \geq 0.05$). The capital letter means differences between the CHO level in each EFA and subscript letters between EFA in each CHO Level.

Chapter 9

General conclusions and final considerations

9.1. General conclusions

The following conclusion can be extracted from the results presented in this thesis:

- Different dietary ARA/EPA/DHA ratios did not affect the growth performance of gilthead sea bream juveniles.
- In ARA-rich diets, 0.3 to 0.5 % of EPA+DHA seems to fulfill the n-3 LC-PUFA requirements of gilthead sea bream juveniles.
- A balanced dietary ARA/EPA+DHA ratio contributed to improving feed utilization.
- Dietary ARA/EPA+DHA ratio needs to be optimized for the upregulating of fatty acids elongases.
- Fish muscle fatty acids profile generally followed the dietary fatty acids profile.
- Different dietary n-6/n-3 LC-PUFA content induced modifications in the oxidative status since n-3 LC-PUFA rich diets promoted higher hepatic and intestinal lipidic peroxidation without affecting antioxidant enzymes activities or glutathione metabolism.
- The mucosa microbiota was affected by the dietary n-6/n-3 LC-PUFA ratio. Dietary ARA and n-3 LC-PUFA content originated two distinct bacterial profiles in the intestinal mucosa. Diets rich in n-3 LC-PUFA showed to beneficially modulate the intestinal mucosa microbiota by reducing the colonization of pathogenic bacteria such as *Edwardsiella tarda*.
- Dietary n-6/n-3 LC-PUFA ratio does not induce histological structural damages in the liver and distal intestine.
- A balanced dietary ARA/EPA+DHA ratio (1/0.4+0.4) increased monocytes number, haemolytic complement, and bactericidal activity against *Photobacterium damsela* subsp. *Piscicida* (*Phdp*) thus beneficially modulates selected immune parameters of unchallenged gilthead sea bream juveniles.
- Under challenging conditions with killed *Phdp* DHA-rich diets increased RBC numbers,

complement activity, and upregulation of several immune-related genes in the distal intestine. ARA-rich diets induced higher plasma bactericidal activity against *Phdp*.

- Dietary LC-PUFA and carbohydrates had low interactive effects on lipid and carbohydrate metabolism.
- High dietary starch and ARA induced desaturases expression while low dietary starch and high dietary DHA induced elongases expression.
- Diets with a high ARA/DHA ratio induced less oxidative stress than diets with low ARA/DHA ratio, probably related to the lower unsaturation degree of ARA compared to DHA without affecting antioxidant enzymes activities or the GSH cycle.
- Dietary starch and DHA seem to have an interactive effect against tissue oxidative stress. In DHA-rich diets, but not in ARA-rich diets, starch inclusion reduced intestinal lipid peroxidation and improved liver GPX activity.
- The increased activation of hepatic G6PDH and GR activity in starch-rich diets ensures the high reducing power essential to GR restore GSH required for GPX activity.
- Dietary high starch level induced alterations in liver histomorphology by promoting hepatocyte hypertrophy and modified nucleus position, particularly in DHA-rich diets.
- DHA-rich diets increased intestinal mucosa microbiota diversity and richness indices.
- DHA-rich diets compared to ARA-rich diets, increased red blood cell (RBC) count, hemoglobin and plasmatic nitric oxide (NO) content, antiproteases activity, and alternative complement activity in unchallenged gilthead sea bream juveniles.
- ARA-rich diets had a stimulatory effect on total immunoglobulins (IG), and plasmatic bactericidal activity against *Vibrio anguillarum*, peroxidase activity, and *casp3* expression in the distal intestine, after an immunological challenge with killed *Phdp*.
- Dietary starch (20 %) did not negatively affect the gilthead sea bream juveniles immune parameters measured, and improved plasmatic bactericidal activity against *Vibrio*

anguillarum in ARA rich-diets. 20 % of dietary starch improved the alternative complement activity in ARA rich-diets as well as plasmatic NO content and bactericidal activity against *Phdp* after an immunological challenge with killed *Phdp*.

Overall, the presented results seem to indicate that diets for gilthead sea bream juveniles should contain around 1 % of ARA and 1 % of EPA+DHA, and 20% of digestible carbohydrates to improve feed efficiency with similar growth performance and maintain lipid peroxidation at lower levels than those found with low n-6/n-3 LC-PUFA ratios. However, in challenging situations, such as an infection with a bacterial pathogen, diets with high EPA+DHA or ARA levels (approximately 2 %), may be beneficial to improve gilthead sea bream juveniles' immune system response.

9.2. Final considerations

FO is still the most economical and viable source of LC-PUFA, particularly EPA and DHA, in aquafeeds, but the aquaculture industry faces a dilemma. FO-based aquafeeds ensure the consumers' expectations in terms of LC-PUFA content in fish fillets and meet fish FA requirements. Still, the industry stays at the mercy of FO increasing prices, and aquaculture sustainability and ecological awareness are questioned by the consumer.

Fish tissue composition is strongly influenced by dietary FA intake. Thus, for marine and salmonids species, partial FO replacement in aquafeeds, may not decrease growth performance, but will negatively affect the nutritional value of the resulting seafood by the decrease of LC-PUFA content. In the aquafeeds industry, dietary LC-PUFA demand is defined not only as the quantities needed to support optimal growth performance and prevent nutritional pathologies but also to maintain desired levels of LC-PUFA in producing seafood (Rombenso et al., 2021). Fish nutritionists are aware of this problem and have delineated several approaches to using less FO in aquafeeds but ensuring adequate EPA and DHA levels in fish fillets.

The most obvious solution to this apparent deadlock is the utilization of supplements rich in n-3 and n-6 LC-PUFA derived from algal, fungal, and other microbial taxa or also from other marine oils such as krill that proved to be good sources of LC-PUFA. These supplements have high levels of ARA, EPA, and/or DHA depending on the source and have been used as targeted nutritional amendments to aquafeeds (Trushenski and Bowzer, 2013). However, a few constraints limit their utilization at a larger scale in aquafeeds, such as their sustainability and economic and logistical viability (Colombo et al., 2020; Trushenski and Bowzer, 2013). Genetic engineered oilseed crops to produce LC-PUFA have raised interest in the potential

application of these products in aquafeeds. Transgenic camelina oil seems to be a promising lipid source to replace FO in feeds for salmonids and marine fish (Betancor M, 2016; Betancor et al., 2015; Betancor et al., 2016). However, the utilization of genetically modified organisms faces several regulatory constraints and a negative perspective from the public sphere point of view (Rombenso et al., 2021).

FO inclusion in aquafeeds is being used only to ensure fish EFA requirements and this reduction of incorporation decrease the EPA and DHA content of the resulting fish fillet. Finishing or wash-out diets are used to tailor fish fillet composition because the FA profile of fish tissues generally reflects the dietary FA composition. This strategy uses FO-rich aquafeeds to increase fillet LC-PUFA content of fish produced with low FO diets during the growing-out phase. FO rich finishing diets have been used in fish with predetermined age or sub-marketable size with some success in restoring muscle EPA and DHA levels but the initial concentration of lipids in the tissue directly affects the time required for its FA composition to be influenced by a dietary change (Turchini et al., 2009). Thus, the savings achieved during the grow-out phase with low FO aquafeeds is eroded by the quantity of FO used during the finishing period (Rombenso et al., 2021).

Another strategy used by the fish nutritionists was to potentiate the biosynthesis of EPA and DHA via the direct provision in the diets of their precursors namely, 18:3n-3 or 18:4n-3, but despite it may nominally increase the n-3 LC-PUFA biosynthesis among species capable of these transformations that increment was not high enough as necessary to enrich fillets with the required EPA and DHA levels found in wild fish or fish fed with FO based diets (Trushenski and Bowzer, 2013).

A different strategy to conserve LC-PUFA content in fish muscle may be the dietary inclusion of SFA (Rombenso et al., 2021). C-18 PUFA, specifically LA, can displace LC-PUFA and become significantly represented in the cell membrane structure but most SFA and MUFA are directed to catabolic pathways (Rombenso et al., 2021; Sissener et al., 2020). The abundance of a respective fatty acid, chain length, and degree of unsaturation influence the proportion of their catabolism favoring SFA over LC-PUFA (Rombenso et al., 2021). This may reveal benefits to including SFA sources such as animal sources instead of LA-rich vegetable oils in aquafeeds to spare dietary LC-PUFA content catabolism and ensure their deposition in the polar lipid fraction of cells. Thus, SFA may contribute to the LC-PUFA sparing effect in aquafeeds with low FO inclusion, but the underlying mechanisms need to be further explored.

Finally, another method to decrease FO utilization and maintain the LC-PUFA tissue content is the farming of genetically modified fish with higher biosynthesis capacity, but the consumers do not favorably accept this approach. On the other hand, epigenetic may be a helpful approach and deserves further attention. Recently, two studies showed that epigenetic mechanisms were present in the nutritional regulation of LC-PUFA biosynthesis in gilthead sea bream (Perera et al., 2020; Turkmen et al., 2019). The authors showed that the promoter of two desaturases, stearoyl-CoA desaturase 1a and FADS₂, respond to broodstock nutrition, and parents fed with VO-based diets and their offspring shared increased DNA-methylation. These studies suggest the possibility of nutritional programming gilthead sea bream for better use of VO-based diets.

This thesis showed that different LC-PUFA dietary ratios influence feed utilization, lipid metabolism, and LC-PUFA biosynthesis. This approach of evaluating how different n-6 and n-3 LC-PUFA interact and how they affect the efficiency of n-3 LC-PUFA utilization, synthesis, and storage may provide clues for the importance of their inclusion levels in aquafeeds for gilthead sea bream juveniles. In this sense, we demonstrated that n-3 LC-PUFA requirements (EPA+DHA) for juvenile gilthead sea bream may be achieved with 0.3 to 0.5 % of the diet in ARA-rich diets. This n-3 LC-PUFA content is lower than that previously pointed out (Ibeas et al., 1994; Kalogeropoulos et al., 1992) and well below that recommended by Izquierdo (2005).

The applications of the different approaches pointed out above to overcome FA tissue modification that results from FO replacement by alternative oils appear to be unfeasible in the nearest times. Therefore, nutritional, and epigenetic strategies to enhance LC-PUFA retention and biosynthesis should be thoroughly evaluated as these seem to have more potential to be successfully applied soon.

In this thesis, we showed that the dietary ARA/EPA+DHA ratio needed to be optimized for the nutritional regulation of elongases. Furthermore, we showed a positive effect of dietary carbohydrates and ARA-rich diets upregulating FADS₂ expression, and of DHA-rich diets in upregulating ELOVL₅ expression. Nevertheless, a negative effect of dietary carbohydrates in the ELOVL₅ expression was found. In fact, the upregulation of FADS₂ by high digestible carbohydrates seemed to be not as relevant as the downregulation of ELOVL₅ in the biosynthesis of LC-PUFA since ARA, EPA, and DHA muscle content was higher in the muscle of fish-fed diets with low digestible carbohydrates content. These apparently contradictory results need to be further studied and it would be interesting to test the combined effect of a similar n-6/n-3 LC-PUFA ratio and carbohydrates levels in low FO diets

in gilthead sea bream juveniles with higher ability to utilize VO-based diets enhanced through broodstock nutrition and epigenetic mechanisms.

This thesis also showed that the impact of dietary LC-PUFA on mucosa microbiota was significant denoting very different profiles in fish fed diets rich in n-6 or n-3 LC-PUFA. In fact, n-3 LC-PUFA rich diets seem even to decrease the intestinal mucosa colonization of pathogenic bacteria such as *Edwardsiella tarda*. This result highlights the importance of n-3 LC-PUFA as intestinal microbiota modulators and further studies are important to elucidate the exact mechanisms by which LC-PUFA impacted mucosa intestinal microbiota. It would be interesting to test if different n-6/n-3 LC-PUFA dietary content impact in a similar way other high-value aquaculture species like the marine carnivorous, European sea bass, meagre (*Argyrosomus regius*), and turbot (*Scophthalmus maximus*) as these results may provide new insights on the importance of n-3 LC-PUFA in preventing epizootic outbreaks caused by bacterial infections.

Our data showed that LC-PUFA modulates the immune system of gilthead sea bream as it was previously demonstrated in several fish species (Mozanzadeh et al., 2015; Shahkar et al., 2016; Torrecillas et al., 2017a; Zuo et al., 2012b). Nevertheless, the present study showed that different dietary LC-PUFA ratios seem to differently modulate the immune system of gilthead sea bream under challenging and unchallenging conditions. Although a diet with a balanced ARA/EPA+DHA ratio appears to be adequate under unchallenging conditions, after an immunological challenge, ARA or DHA-rich diets seem to better prepare the fish immune system to respond to the assault. The dietary inclusion of carbohydrates revealed no detrimental effects on fish immune status and even improved some immune parameters. This is particularly important in diets with low n-3 LC-PUFA content. In the future, it would be valuable to test the effects of different diets with high levels of ARA and DHA (1:1), only ARA and only DHA after an immunological challenge with live pathogenic bacteria to assess if there is a synergetic or an inhibitory response by these n-6 and n-3 LC-PUFA to the assault.

Chapter 10

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