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Em todas as publicações que resultaram deste trabalho é devidamente referido que as instituições de origem da doutoranda Sara Patrícia Pinto Moutinho são:

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List of published papers

Moutinho, S., Oliva-Teles, A., Martínez-Llorens, S., Monroig, Ó., Peres, H., 2022. Total fishmeal replacement by defatted *Hermetia illucens* larvae meal in diets for gilthead seabream (*Sparus aurata*) juveniles. Journal of Insects as Food and Feed 8 (12): 1455-1468. https://doi.org/10.3920/JIFF2021.0195.

Moutinho, S., Oliva-Teles, A., Pulido-Rodríguez, L., Magalhães, R., Monroig, Ó., Parisi, G., Peres, H., 2023. Black soldier fly larvae oil as an alternative lipid source in diets for gilthead seabream (*Sparus aurata*) juveniles. Aquaculture 574. https://doi.org/10.1016/j.aquaculture.2023.739705.

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Moutinho, S., Peres, H., Martins, N., Serra, C., Santos, R.A., Monroig, Ó., Oliva-Teles, A., 2024. Use of black soldier fly (*Hermetia illucens*) larvae meal in diets for gilthead seabream juveniles: Effects on growth-related gene expression, intermediary metabolism, digestive enzymes, and gut microbiota modulation. Aquaculture 580: 740357. https://doi.org/10.1016/j.aquaculture.2023.740357.

List of manuscripts submitted

Moutinho, S., Oliva-Teles, A., Fontinha, F., Martins, N., Monroig, Ó., Peres, H. Black soldier fly larvae meal as a potential modulator of immune, inflammatory, and antioxidant status in gilthead seabream juveniles. Submitted to Comparative Biochemistry and Physiology, Part B: Biochemical and Molecular Biology

Moutinho, S., Peres, H.., Fontinha, F., Estevão, T., Monroig, Ó., Magalhães, R., Pulido-Rodrigues, L., Parisi, G., Oliva-Teles, A. Effects of black soldier fly larvae oil on the immune, inflammatory, and oxidative status of gilthead seabream juveniles. Submitted to Fish and Shellfish Immunology

Moutinho, S., Monroig, Ó., Peres, H., Villena-Rodríguez, Magalhães, R., Pulido-Rodrigues, L., Parisi, G., Oliva-Teles, A., Black soldier fly larvae oil as an alternative lipid source for gilthead seabream juveniles: effects on lipid metabolism, liver fatty acid composition and plasma metabolites profile. Submitted to Aquaculture

À minha família,

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Resumo

O papel da aquacultura na garantia da segurança alimentar global tem vindo a crescer de forma significativa. Atualmente, é responsável por cerca de metade do consumo mundial de pescado, uma percentagem que é espera que aumente nos próximos anos. Para sustentar o previsto crescimento da produção aquícola intensiva, torna-se imperativo adotar práticas alimentares mais sustentáveis. Ingredientes de origem marinha, como a farinha de peixe (FM) e o óleo de peixe, juntamente com os óleos vegetais (VO), já são amplamente utilizados em formulações de rações comerciais. Contudo, preocupações com o fornecimento instável, aumento de preços, competição com outras indústrias e questões de sustentabilidade estão a impulsionar a procura de ingredientes alternativos para a alimentação animal. Nos últimos anos, o interesse por insetos para alimentação humana e animal tem aumentado, principalmente devido à sua elevada qualidade nutricional e baixo impacto ambiental. Entre as espécies de insetos atualmente autorizadas para utilização em rações para peixes, a mosca soldado-negro (*Hermetia illucens*, HI) surge como uma das mais promissoras, devido ao seu elevado teor em proteínas, lípidos, vitaminas e minerais.

O objetivo desta tese é avaliar o potencial da inclusão de farinha de larvas de HI (HM) e óleo de larvas de HI (HIO) em dietas para a dourada (*Sparus aurata*), uma espécie de elevada relevância económica na aquacultura mediterrânica. Para tal, foi utilizada uma abordagem multidisciplinar, abrangendo o desempenho zootécnico, impacto económico, digestibilidade de nutrientes, atividade de enzimas relacionadas com o estado oxidativo, digestão e metabolismo intermediário, bem como a expressão de genes relacionados ao crescimento muscular, metabolismo lipídico, estado inflamatório e imunológico, modulação da microbiota intestinal e características da qualidade do filete.

O potencial da HM como fonte proteica alternativa para juvenis de dourada está representada nos capítulos 2, 3 e 4. Foram formuladas quatro dietas experimentais contendo níveis crescentes de HM desengordurada: 0% (dieta controlo; HM0), 15% (dieta HM15), 30% (dieta HM30) e 45% (dieta HM45), substituindo a proteína da FM em 0, 22, 66 e 100%, respetivamente.

No Capítulo 2 foram avaliados os efeitos no crescimento, utilização de alimento, digestibilidade, sustentabilidade e índices económicos em juvenis de dourada (peso inicial de 32 g). Não foram observadas diferenças no crescimento, eficiência alimentar (FE), digestibilidade de proteínas e lípidos, embora a digestibilidade da matéria seca e

da energia tenha aumentado nas dietas contendo HM. A inclusão de HM melhorou também o índice “fish in: fish out” (FIFO) mas diminui os índices de eficiência económica.

No Capítulo 3 foram avaliados os efeitos na expressão de genes relacionados ao crescimento, metabolitos do plasma, atividade de enzimas do metabolismo intermediário e modulação da microbiota intestinal foram também avaliados. No músculo, foi observada uma regulação negativa na expressão do “fator de determinação miogénica muscular 2” (*myod2*) e uma regulação positiva na expressão de “miostatina” (*mstn*) com o aumento de HM nas dietas. No entanto, a expressão dos genes “recetores da hormona de crescimento” (*ghr1* e *ghr2*), “fatores de crescimento semelhantes à insulina” (*igf1* e *igf2*), “catepsinas” (*cts-a* e *cts-b*) e o “fator de determinação miogénica 1” (*myod1*) não foram afetadas. No fígado, as atividades das enzimas alanina aminotransferase (ALT) e glutamato desidrogenase (GDH) aumentaram com a inclusão de HM, enquanto as atividades da glucose-6-fosfato desidrogenase (G6PDH), da enzima málica (ME) e da sintase de ácidos gordos (FAS) diminuíram. Além disso, a atividade da β-hidroxiacil-CoA desidrogenase (HOAD) aumentou nos peixes alimentados com a dieta HM15 em comparação com a controlo. As atividades da amilase, tripsina e quimiotripsina foram maiores no intestino posterior dos peixes alimentados com a dieta HM15 em comparação com outras dietas. Observou-se também um aumento no número de unidades taxonómicas operacionais (OTUs), riqueza microbiana e diversidade na digesta dos peixes alimentados com as dietas com HM, enquanto esses índices foram reduzidos na mucosa. A inclusão de HM aumentou a abundância de *Oceanobacillus*, *Lederbergia*, *Bacillus* e *Corynebacterium*, enquanto reduziu a abundância de *Lactobacillus* e *Caldalkalibacillus* na digesta.

No Capítulo 4, foram avaliados o perfil hematológico, parâmetros imunes, estado inflamatório intestinal e a resposta antioxidante nos peixes alimentados com as dietas experimentais anteriores. Não foram observadas diferenças nos parâmetros imuno-humorais ou no perfil hematológico, exceto uma diminuição da hemoglobina e do hematócrito com o aumento da inclusão de HM. No fígado, as atividades de G6PDH e glutationa peroxidase (GPX) foram reduzidas nos peixes alimentados com a dieta HM45 em comparação com a controlo. As atividades de glutationa redutase (GR) e superóxido dismutase (SOD) foram, também, reduzidas nos peixes alimentados com dieta HM30 e HM15, respetivamente, comparativamente com controlo. No intestino, a peroxidação lipídica foi menor nos peixes alimentados com a dieta HM30 em comparação com a dieta controlo. A expressão dos genes pró-inflamatórios “interleucina-1-beta” (*il-1β*) e “ciclooxigenase-2” (*cox2*) foi reduzida com a inclusão de HM na dieta, enquanto a

expressão do gene anti-inflamatório “fator de crescimento transformador” (*tgfβ*) aumentou nos peixes alimentados com a dieta HM30 comparativamente à controlo e HM45.

A avaliação de HIO como fonte alternativa lipídica está representada nos capítulos 5, 6, 7 e 8. Foram formuladas quatro dietas experimentais para substituir uma mistura de VO (50% de óleo de linhaça, 30% de óleo de palma e 20% de óleo de colza) por HIO em diferentes proporções: 0% (dieta HIO0), 42% (dieta HIO42), 84% (dieta HIO84) e 100% (dieta HIO100) correspondendo a um nível de inclusão de HIO de 0%, 4%, 7,9% e 9,5%, respetivamente.

No Capítulo 5, os efeitos destas dietas no desempenho de crescimento, utilização do alimento, digestibilidade e atividade das enzimas digestivas foram avaliados (dourada com peso inicial de 63 g). Após 10 semanas de alimentação, não foram observadas diferenças no desempenho de crescimento, enquanto a FE e taxa de eficiência proteica aumentaram com o aumento do teor de HIO. A digestibilidade das proteínas, energia e ácidos gordos saturados (SFA) aumentou, enquanto a digestibilidade de ácidos gordos insaturados diminuiu com a inclusão de HIO.

No Capítulo 6, os efeitos do HIO na qualidade e nas características nutricionais do filete foram também avaliados. No final do ensaio, não foram observadas diferenças nos parâmetros morfométricos e somatométricos nem na cor da pele e do filete do peixe. O perfil de ácidos gordos do filete foi significativamente modelado pelas dietas experimentais, aumentando o seu teor de SFA, particularmente em ácido láurico, e diminuindo o teor em ácidos gordos monoinsaturados e ácidos gordos polinsaturados (PUFA) n-3 e n-6, enquanto a quantidade dos ácidos eicosapentaenóico (EPA) e docosahexaenóico (DHA) não foi afetada pela inclusão de HIO na dieta. Os índices de qualidade de ácidos gordos (PUFA/SFA, rácio n-3/n-6 PUFA, rácio hipocolesterolémico/hipercolesterolémico, índices de trombogenicidade e aterogenicidade) foram também modelados pela inclusão de HIO na dieta. No filete, foi observada redução no pH e na capacidade de retenção de água, bem como uma diminuição na peroxidação lipídica com a inclusão de HIO na dieta, apesar de um aumento nos dienos conjugados.

No Capítulo 7, foram avaliados os efeitos da inclusão de HIO no estado imunológico, inflamatório e oxidativo da dourada. Não foram observados efeitos nos parâmetros imuno-humorais, exceto um aumento linear na atividade da peroxidase. No fígado, a peroxidação lipídica não foi afetada, enquanto as atividades das enzimas GPX e catalase aumentaram linearmente com a inclusão de HIO. A atividade das enzimas

G6PDH e GR aumentaram também linearmente, sendo superior nos peixes alimentados com dieta HIO100 em comparação à controlo. No intestino, a peroxidação lipídica diminuiu com a inclusão de HIO, sendo que a atividade das enzimas antioxidantes não foi afetada. Também no intestino, a expressão do "fator de necrose tumoral alfa" (*tnfα*) e da "interleucina-10" (*il-10*) foi regulada positivamente pela inclusão de HIO nas dietas, sendo maior nos peixes alimentados com a dieta HIO100 do que nas restantes.

No Capítulo 8, foram avaliados os efeitos no metabolismo lipídico, perfil de ácidos gordos do fígado e no perfil de metabolitos plasmáticos nos peixes alimentados com as dietas contendo HIO. No plasma, houve uma redução linear no teor em lípidos e triglicerídeos, enquanto os níveis de HDL aumentaram com a inclusão de HIO na dieta. No fígado, houve aumento no teor de SFA, principalmente ácido láurico, e de ácidos gordos monosaturados, como o ácido oleico. No entanto, o teor hepático em C16:0 e C18:0 (em % de AG total) foi maior do que o das dietas correspondentes, enquanto o conteúdo em PUFA n-3 e n-6 foi reduzido e as concentrações de EPA e DHA não foram afetadas. A inclusão de HIO teve pouco efeito na expressão relativa de genes relacionados com a síntese de ácidos gordos, transporte e β-oxidação. No entanto, foi observada uma regulação negativa da "elongase dos ácidos gordos de cadeia muito longa 6" e "elongase dos ácidos gordos de cadeia muito longa 1b" (*elovl6* e *elovl1b*) e da sintase dos ácidos gordos (*fas*) com o aumento do teor de HIO.

Em conclusão, este estudo demonstrou que a HM e o HIO são bem tolerados pela dourada, podendo ser utilizados como fontes de proteína e lípidos alternativas. A substituição total da FM da dieta por HM desengordurada não comprometeu o crescimento, a digestibilidade ou a utilização de nutrientes. Para além disso, o estado antioxidante e inflamatório dos peixes foi melhorado e a microbiota intestinal foi modelada positivamente. A inclusão HM apresentou, ainda benefícios ambientais. Do mesmo modo, a inclusão de HIO foi possível sem comprometer o crescimento, melhorando a eficiência alimentar, a utilização de nutrientes e a digestibilidade de nutrientes e ácidos gordos. A inclusão de HIO também teve efeitos positivos na resposta antioxidante e no estado inflamatório da dourada, com efeitos reduzidos na expressão de genes-chave relacionados ao metabolismo lipídico. Por outro lado, embora o perfil de ácidos gordos dos filetes se tenha alterado e refletisse aquela das dietas experimentais, o teor de EPA e DHA dos filetes não foi afetado. No entanto, ambas as alternativas ainda enfrentam desafios económicos para competir com ingredientes convencionais em larga escala.

No geral, os resultados deste estudo consolidam a ideia de que os insetos podem ser usados como ingredientes alternativos em rações para peixes, contribuindo assim para um o desenvolvimento mais sustentável da aquacultura. Novos estudos dever-se-ão focar na modulação e otimização do perfil nutricional dos insetos de forma a otimizar a qualidade nutricional do produto final para consumo humano. No caso da dourada, em particular, a utilização de HM sem ser desengordurada poderá ser considerada como forma de reduzir os custos de processamento associados ao desengorduramento e aumentar a rentabilidade.

Palavras-chave: aquacultura, nutrição de peixes, ingredientes alternativos, sustentabilidade, farinha de inseto, óleo de inseto, saúde dos peixes, crescimento dos peixes

Abstract

Aquaculture has an increasingly important role in providing global food security. It is currently responsible for almost half of the total worldwide fish consumption, a percentage expected to increase in the coming years. To support the predicted growth of intensive aquaculture production, more sustainable feed practices are required. Marine-derived ingredients such as fish meal (FM) and fish oil (FO), and vegetable oils (VO) are already commonly used in commercial feed formulations. However, concerns regarding unstable supply, prices, competition with other industries, and sustainability issues have prompted the search for alternative feed ingredients. In recent years, the interest in insects as food and feed has increased, mostly due to their high nutritional value and low environmental impact of their production. Among the insect species authorized for use in aquafeeds in the European Union, the black soldier fly (*Hermetia illucens*, HI) is one of the most promising because of its high content of proteins, lipids, vitamins, and minerals.

The objective of this thesis was to evaluate the potential of the inclusion of HI larvae meal (HM) and HI larvae oil (HIO) in diets for a highly economically important species in Mediterranean aquaculture – the gilthead seabream (*Sparus aurata*). A multidisciplinary approach was used to address the effects of dietary HM and HIO on zootechnical performance, nutrient digestibility, the activity of enzymes related to oxidative stress, digestion, and intermediary metabolism, as well as the expression of genes related to muscle growth, lipid metabolism, inflammatory and immunological status, gut microbiota modulation, and fillet quality traits.

The evaluation of the potential of HM as an alternative protein source for gilthead seabream juveniles is presented in Chapters 2, 3, and 4. Four experimental diets were formulated to contain increasing levels of defatted HM: 0% (control diet; HM0), 15% (diet HM15), 30% (diet HM30), and 45% (diet HM45), replacing FM protein at 0, 22, 66, and 100%, respectively.

In Chapter 2, the effects on growth performance, feed utilization, digestibility, sustainability, and economic efficiency indexes of gilthead seabream juveniles (initial body weight of 32 g) were evaluated. Growth performance, feed efficiency (FE), and protein and lipid digestibility were not affected, while dry matter and energy digestibility were improved in the HM-containing diets. The Fish in:Fish out (FIFO) ratio was improved by dietary HM inclusion, while the economic efficiency indexes decreased.

In Chapter 3, the effects of the experimental diets with varying levels of HM on the expression of growth-related genes, plasma metabolites, activity of intermediary metabolic enzymes, and gut microbiota modulation were assessed. A downregulation of muscle myogenic determination factor 2 (*myod2*) and upregulation of myostatin (*mstn*) expressions were observed with dietary HM inclusion, while the expression of growth hormone receptors (*ghr1* and *ghr2*), insulin-like growth factors (*igf1* and *igf2*), cathepsins (*cts-a* and *cts-b*), and myogenic determination factor 1 (*myod1*) were not affected. Liver alanine aminotransferase (ALT) and glutamate dehydrogenase (GDH) activities increased while glucose-6-phosphate dehydrogenase (G6PDH), malic enzyme (ME), and fatty acid synthase (FAS) activities decreased with the dietary inclusion of HM. Also, the activity of β-hydroxy acyl-CoA dehydrogenase (HOAD) was higher in fish fed diet HM15 compared to the control. Amylase, trypsin, and chymotrypsin activities were higher in the posterior intestine of fish fed with diet HM15 compared to the other diets. There was an increase in the number of operational taxonomic units (OTUs), microbial richness, and diversity in the digesta of fish fed the HM-containing diets, while a reduction of these indexes was observed in the mucosa. Including HM increased the abundance of *Oceanobacillus*, *Lederbergia*, *Bacillus*, and *Corynebacterium* in the digesta, while Lactobacillus and *Caldalkalibacillus* abundances were reduced.

In Chapter 4, the hematological profile, immune parameters, intestinal inflammatory status, and antioxidant response were evaluated in fish fed the experimental diets containing varying levels of HM. No differences were observed in the plasma immune humoral parameters or the hematological profile, except for a decrease in hemoglobin and hematocrit with dietary HM inclusion. In the liver, G6PDH and glutathione peroxidase (GPX) activities were lower in fish fed diet HM45 compared to the control. Compared to the control, lower activity of glutathione reductase (GR) and superoxide dismutase (SOD) activities were observed in fish fed with the diets HM30 and HM15, respectively. In the intestine, lipid peroxidation (LPO) was lower in fish fed with the diet HM30 compared to the control. The expression of the pro-inflammatory genes interleukin-1-beta (*il-1β*) and cyclooxygenase-2 (*cox2*) were reduced by dietary HM inclusion, while the expression of the anti-inflammatory gene transforming growth factor (*tgfβ*) was higher in fish fed the diet HM30 than in the control and the HM45 diets.

The evaluation of the potential of HIO as an alternative lipid source is presented in Chapters 5, 6, 7, and 8. Four experimental diets were formulated to replace a VO blend (50% linseed, 30% palm, and 20% rapeseed oils) with HIO at different concentrations:

0% (diet HIO0), 42% (diet HIO42), 84% (diet HIO84) and 100% (diet HIO100), corresponding to a dietary inclusion level of 0%, 4%, 7.9% and 9.5% of HIO, respectively.

In Chapter 5, the effects of these diets with varying levels of HIO on growth performance, feed utilization, digestibility, and digestive enzyme activity of gilthead seabream (initial body weight of 63 g) were assessed. After 10 weeks of feeding, there were no differences in growth performance, while FE and protein efficiency ratio improved with the increasing content of HIO. The digestibility of protein, energy, and saturated fatty acids (SFA) was improved, while the digestibility of unsaturated fatty acids decreased with the increasing inclusion of dietary HIO.

In Chapter 6, the effects of dietary HIO on fillet quality and nutritional traits were evaluated. At the end of the feeding trial, no differences were observed in the morphometric and somatometric parameters or skin and fillet color of gilthead seabream juveniles. Fillet's fatty acid (FA) profile was modulated by the experimental diets, showing increased SFA content, particularly lauric acid, and decreased monosaturated FA and n-3 and n-6 polyunsaturated fatty acids (PUFA) with the dietary inclusion of HIO. In contrast, the contents of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), physiologically essential FA for *S. aurata*, were unaffected by dietary HIO inclusion. FA quality indexes (PUFA/SFA, n-3/n-6 PUFA ratio, hypocholesterolemic/hypercholesterolemic ratio, thrombogenicity, and atherogenicity indexes) were also impacted by dietary HIO inclusion. A reduction in fillets' pH and water-holding capacity was also observed. There was also a decrease in fillet lipid peroxidation with dietary HIO inclusion despite an increase in conjugated dienes.

In Chapter 7, the effects of dietary HIO inclusion on the immune, inflammatory, and oxidative status were also assessed. No effects were observed in the plasma immune humoral parameters, except for a linear increase in plasma peroxidase activity. Liver LPO was unaffected, while GPX and catalase activities linearly increased with HIO inclusion. Moreover, liver G6PDH and GR also increased linearly, with higher levels in fish fed with the diet HIO100 compared to the control. In the intestine, LPO was decreased by including HIO, while the activity of the antioxidant enzymes was not affected. Also, in the intestine, the expression of tumor necrosis factor-alpha (*tnfα*) and interleukin-10 (*il-10*) were upregulated by dietary HIO inclusion, being higher in fish fed with the diet HIO100 than in the other diets.

In Chapter 8, the effects of the HIO-containing diets on lipid metabolism, liver FA composition, and plasma metabolite profiles were also evaluated. There was a linear

decrease in plasma lipids and triglyceride contents, while HDL levels were increased with the dietary inclusion of HIO. In the liver, there was an increase in SFA content, particularly lauric acid, and in monosaturated FAs, like oleic acid. However, the percentual levels of C16:0 and C18:0 in liver were higher than those in the corresponding diets, while the percentual levels of n-3 and n-6 PUFA were reduced, and EPA and DHA contents were unaffected. The dietary inclusion of HIO had little effect on the relative expression of genes related to FA synthesis, transport, and β-oxidation. However, a downregulation of elongation of very long-chain fatty acids proteins 6 and 1b (*elovl6* and *elovl1b*) and fatty acid synthase (*fas*) was observed with increasing content of HIO.

In conclusion, this study shows that HM and HIO are well tolerated by gilthead seabream juveniles and can be used as alternative protein and lipid sources in modern aquafeed formulations. Total dietary FM replacement by defatted HM was achievable without compromising growth, diet digestibility or nutrient utilization. Further, the antioxidant and inflammatory status of fish was enhanced, and gut microbiota positively modulated. The dietary inclusion of HM ad a positive environmental impact (through the improvement of the FIFO ratio); however, with the actual prices of this feedstuff, the use of HM in practical diets for gilthead seabream is still not economically competitive. Likewise, the dietary inclusion of HIO was achievable without compromising growth, improving feed efficiency, nutrient utilization, or nutrient and FA digestibility. Dietary HIO inclusion also had positive effects on the antioxidant response and inflammatory status of the fish and had little effect on the expression of key genes related to lipid metabolism. On the other hand, while the fillets' FA profile was modulated and reflected that of the experimental diets, the fillets' EPA and DHA content was not affected.

Overall, results from this study reinforce the concept that insect products can be effectively used as alternative ingredients in aquafeeds, thus contributing to the sustainable development of the aquaculture industry. Further studies should focus on modulating and improving the insects' nutritional profile to optimize the nutritional quality of the final product for human consumption. For gilthead seabream, in particular, the use of full-fat HM should also be addressed as a way to reduce processing costs associated with defatting and boost the profitability of aquafeed production.

Keywords: aquaculture, fish nutrition, alternative ingredients, sustainability, insect meal, insect oil, fish health, fish growth

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Abbreviation list

AA – Amino acid
ADC_{DM} - Dry matter apparent digestibility coefficients
ADC_E - energy apparent digestibility coefficients
ADC_L - Lipids apparent digestibility coefficients
ADC_P - Protein apparent digestibility coefficients
ARA – Arachidonic acid
CAT – Catalase
CHO - Cholesterol
CL – Crude lipid
CP – Crude protein
cpt-1 - Carnitine palmitoyltransferase-1
DHA - Docosahexaenoic acid
DM – Dry matter
EAA – Essential amino acids
ECR - Economic conversion ratio
EFA – Essential fatty acids
EPA - Eicosapentaenoic acid
EPI - Economic profit index
EU – European Union
FA – Fatty acid
FBW – Final body weight
FCR – Feed conversion ratio
FF – Full fat
FI – Feed intake
FM – Fish meal
FO – Fish oil
G6PDH - Glucose-6-phosphate dehydrogenase
gh - Growth hormone
GPX - Glutathione peroxidase
GR - Glutathione reductase
HDL - High-density lipoprotein
HI – *Hermetia illucens*
HIO – *Hermetia illucens* larvae oil
HM – *Hermetia illucens* larvae meal
HP - Hematological parameters
igf1 - Insulin-like growth factor 1
igf2 - Insulin-like growth factor 2
il-10 - Interleukin 10
il-1β - Interleukin 1 beta
LA – Lauric acid
LCFA – Long-chain fatty acids
LC-PUFA - Long-chain polyunsaturated fatty acid
LDL – low-density lipoprotein
M – Million
MCFA – Medium chain fatty acid
MD – *Musca domestica*
MDA – Malondialdehyde
mstnb - Myostatin b
MUFA – Monounsaturated fatty acid
N - Nitrogen
NFE – Nitrogen-free extract
PAP – Processed animal proteins
PDF – Partially defatted
PER – Protein efficiency ratio
PF – Plant feedstuffs
pparα – Peroxisome proliferator-activated receptor alpha
PUFA - Polyunsaturated fatty acid
SFA – Saturated fatty acids
SGR - Specific growth rate
SOD -Superoxide dismutase
TG – Triglycerides

tgfβ - Transforming growth factor beta

TM – *Tenebrio molitor*

tnfα - Tumour necrosis factor-alpha

TP - Total protein

VO – Vegetable oil

WG – Weight gain

Chapter 1: General Introduction

1. Aquaculture: current trends and prospects

Global fisheries and aquaculture production reached an all-time high record of 214 million tonnes in 2020, comprising 178 million (M) tonnes of aquatic animals (FAO 2022). This increase was largely due to increasing aquaculture production, particularly in Asian countries. According to FAO, global aquaculture production reached a record of 126 M tonnes in 2021 (**Figure 1.1**), with the production of aquatic animals accounting for 90.9 M tonnes and worth 281.1 billion USD (FAO 2023). Indeed, aquaculture is considered to be one of the fastest-growing food production sectors worldwide, with an average growth rate of 6.7% per year. In 2020, global farmed finfish production was estimated at 57.5 M tonnes and was dominated (in volume) by freshwater species, such as grass carp, silver carp, and Nile tilapia, mostly produced in Asian countries. In Europe, however, production was mostly associated with marine fish species, with salmon, seabream, and seabass being the main farmed species (FEAP 2021; FAO 2022).

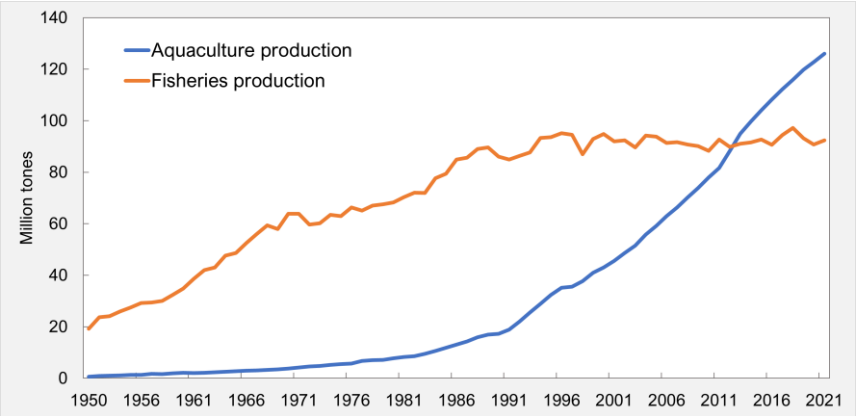

Figure 1.1: Aquaculture production and capture fisheries (1950-2021). Source: FishstatJ (FAO 2023)

Global consumption of aquatic foods (excluding algae) has had an average annual increase of 3% since 1961, compared to the 1.6% population growth rate. The amount destined for human consumption was 20.2 kg per capita in 2020, more than double the average of 9.9 kg per capita in the 1960s and represented 7% of all animal protein consumed (FAO 2022). Aquaculture is currently responsible for 49.2% of the total fish consumed worldwide, a percentage expected to increase in the coming years. This increase is necessary due to the increasing demand for fish products, which is not only associated with the increasing human population and the consequent need for food and protein, but also with income growth and changes in dietary habits, declines in the availability of wild fish, and competitive product prices (Naylor et al. 2021; FAO 2022). Given that fisheries production has stagnated at 90 M tonnes over the last four decades,

aquaculture has an increasingly important role in providing global food security and nutrition for the future.

Fish is considered a high-quality source of protein, lipids, and other highly bioavailable vitamins, such as vitamin D, as well as minerals, including phosphorus, selenium, iron, and iodine (Tacon 2022). The consumption of fish is especially beneficial for human health as it contains high levels of omega-3 (or n-3) long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA), namely EPA (eicosapentaenoic acid; C20:5n-3) and DHA (docosahexaenoic acid, C22:6n-3). Intake of n-3 LC-PUFA has been shown to provide several health benefits, such as improved cardiovascular function, anti-inflammatory properties, improved fetal development and immune response in infants, and improved cognitive function in adults, with positive effects on neurodegenerative diseases like Alzheimer's (Deckelbaum and Torrejon 2012; Swanson et al. 2012).

2. Aquaculture challenges: fish meal and fish oil use in aquafeeds

Intensive aquaculture (based on fed aquaculture species) has grown substantially and has emerged as the most promising and practical way to supply the global demand for cultured species (Sharven and Loh 2022). Currently, around 70% of the total aquaculture production consists of fed species, which must be supported by increased aquafeed production (Liland et al. 2021). Over the last 20 years, aquafeed production has increased by 600%, reaching approximately 55 M tonnes globally in 2020 (Glencross et al. 2023).

One of the major challenges in aquaculture is to formulate feeds that support not only the growth and health of fish but also the economic and environmental sustainability of aquaculture production, as feed represents one of the highest production costs (up to 70% or more of the variable costs) and can account for 90% of the environmental impact of fed aquaculture production (Naylor et al. 2021).

Finfish production, particularly of carnivorous fish species, requires an input of high-quality protein and lipids, which vary among species and according to life stage. Marine-derived ingredients, such as fish meal (FM) and fish oil (FO) have been regarded, as ideal protein and lipid sources, respectively, in diets for carnivorous fish. FM is high in protein, with a balanced amino acid (AA) profile, high nutrient digestibility, high palatability, low carbohydrate content, and lacks antinutrients. FM also has varying levels of n-3 LC-PUFA-rich lipids, as well as minerals and other micronutrients. Moreover, FO is rich in n-3 LC-PUFAs, like EPA and DHA, which are essential for the growth, health,

and reproduction of marine fish (Tocher 2003; Turchini et al. 2009), as they lack or have limited capability for the biosynthesis of these molecules (Monroig et al. 2018; Turchini et al. 2022)

In 2020, over 20 M tonnes (11%) of global fisheries production were directed toward non-food purposes, including the production of FM and FO (FAO 2022). The majority of FM and FO are derived from whole fish (mainly through the catch of small pelagic fish such as Peruvian anchoveta), but they are also produced from fish trimmings and other fish processing by-products, albeit at a lower level (FAO 2022). As the largest portion of FM and FO production derives from wild-caught fisheries, overfishing and global events such as El Niño (FAO 2018; Costello et al. 2020) have affected supply and increased prices, which more than doubled since 2000 (Naylor et al. 2021). This, along with increasing competition from the food sector and other industries (pet food and pharmaceutical industries, for instance), is putting into question the supply of these commodities for the aquaculture sector in the future.

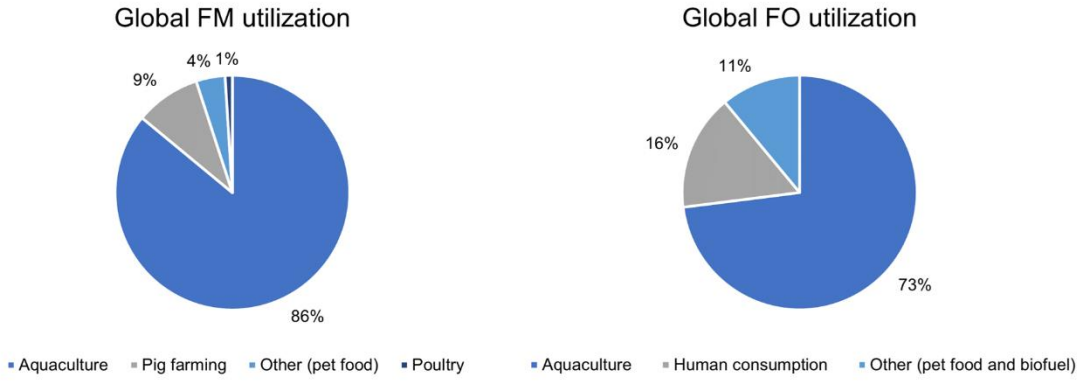

Figure 1.2: Global fish meal (FM) and fish oil (FO) utilization in 2020. Source: FAO (2022)

In response to this paradigm, over the last two decades, the aquaculture industry has shifted towards using FM and FO more strategically in all life stages, especially during the grow-out, broodstock, and finishing stages of production (Naylor et al. 2021). Nevertheless, aquaculture still uses the majority of FM (86%) and FO (73%) produced globally (**Figure 1.2**). Thus, the increase of aquaculture production, and consequently the aquafeed industry, must rely upon increasing the use of alternative ingredients to FM and FO to supply the essential nutrients for the growth and wellbeing of farmed fish, but also promote the sustainable development of aquaculture, addressing the Sustainable Development Goals established by the United Nations.

3. Alternative ingredients

The recognition that the limited supply of FM and FO traditionally used in aquafeeds can limit the expansion of aquaculture has led to a global effort to move toward the use of alternative ingredients to support the growing aquafeed demand and provide adequate nutrition for the fish (Glencross et al. 2023). Several alternative ingredients for aquafeeds have been evaluated. These include protein and oil sources from terrestrial plant feedstuffs and by-products, terrestrial animal by-products, ingredients from bacterial origin, algae, and others (Oliva-Teles et al. 2015; Gasco et al. 2018; Hodar et al. 2020). In the following paragraphs, an overview of the most used and relevant alternative ingredients for aquafeeds will be discussed.

Currently, the main ingredients in most aquafeeds are from terrestrial sources, such as plant feedstuffs (PF), and plant protein concentrates, such as those derived from soy, wheat, corn, peas, and other grains or oilseeds (Hardy 2010; Ytrestøyl et al. 2015; Liland et al. 2021). These ingredients are easily accessible, have a relatively constant nutrient composition, can be produced in large quantities, and are economically competitive. PF such as rapeseed, corn, wheat, and soybean meals, have been used as FM replacements at various levels (Oliva-Teles et al. 2015, 2022; Kaiser et al. 2022). However, plant proteins also have some limitations, such as palatability issues, low protein content, and suboptimal AA profiles (Oliva-Teles et al. 2022) and the presence of starch, fiber, and non-soluble carbohydrates that results in low digestibility and growth (Gatlin et al. 2007; Oliva-Teles 2012). Also, the presence of anti-nutritional factors, such as trypsin inhibitors, haemagglutinin, and antivitamins (Francis et al. 2001), interfere with digestion, health, and immune function, and cause changes in the gut microbiome, gut morphology and inflammatory status (Naylor et al. 2021; Oliva-Teles 2012). Plant protein concentrates that are now widely available are high in protein and have been suggested to minimize some of the detrimental effects of PF caused by antinutrients (Hardy 2010). However, plant ingredients have sustainability challenges to address, such as biodiversity loss and deforestation, higher carbon footprint than FM, excessive land occupation and freshwater use, food-feed competition, and competition with other industries (livestock and agriculture sectors, biofuel) (Naylor et al. 2021). PF are also highly vulnerable to the effects of climate change that can disrupt supply with high costs for aquaculture feeds (Glencross et al. 2023).

Terrestrial processed animal proteins (PAP), such as poultry meal, feather meal, blood meal, and meat and bone meal, have also been used as alternative ingredients for aquafeeds (Moutinho et al. 2017; Fontinha et al. 2021). PAP are rich in proteins and

lipids, are readily available, economically competitive, and lack anti-nutritional compounds (Gasco et al. 2018). However, their nutritional quality is highly variable and largely depends on the sub-product source and processing technology. PAP are also usually high in saturated fats and have negligible levels of LC-PUFA, and their use is limited in some regions by regulations related to perceived disease risk and social objections (Glencross et al. 2023).

Novel potential alternative ingredients from insects, microbial biomass, single-cell organisms, and macro and microalgae, are being increasingly advocated as sustainable alternatives for aquafeeds (Walker and Berlinsky 2011; Sánchez-Muros et al. 2014; Wan et al. 2019; Carvalho et al. 2023; Woolley et al. 2023). They have a good nutritional profile, and some have significantly low greenhouse gas emissions. However, high production and processing costs limit, for now, their large-scale use in aquafeeds (Gamboa-Delgado and Márquez-Reyes 2018).

Besides protein, the search for alternative lipid sources to FO is also essential to support the development of the aquaculture industry. Vegetable oils (VO) obtained from rapeseed, soybean, and palm, and animal fats, such as lard and poultry fat, are now commonly used in commercial aquafeeds (Turchini et al. 2022). It has been demonstrated that, as long as essential fatty acid (EFA) requirements are satisfied, it is possible to partially or completely replace FO with VO in diets for marine fish without adversely affecting fish growth or health (Turchini et al. 2009). However, VO are typically rich in n-6 PUFA like linoleic acid (C18:2n-6) and lack n-3 LC-PUFA such as EPA and DHA, which will eventually cause a shift in the fish FA profile, compromising fillet quality for human consumption (Sprague et al. 2020). The use of LC-PUFA-rich oils from algae or genetically modified oilseed crops can alleviate the pressure on FO demand while maintaining the health benefits for consumers, but their use could be economically challenging and, in the case of transgenic oilseed crops, have issues associated with customer acceptance. Thus, the use of FO in feeds for marine fish is still considered necessary to fulfil their EFA requirements and to guarantee the deposition of EPA and DHA in the fillets, offering health benefits for consumers.

4. Insects as novel feed ingredients

Insects are the most diverse group of animals, with approximately one million species worldwide (Nogales-Mérida et al. 2018). Insects have been part of the human diet for more than 2000 years (Alfiko et al. 2021), especially in many parts of Asia, Latin America,

and Africa (Makkar et al. 2014), and have been well-documented as part of the natural diet of many fish species (Nogales-Mérida et al. 2018). In recent years, due to the rising cost of protein, food and feed insecurity, environmental pressures, population growth, and demand for protein, the interest in insects as food and feed has remarkably increased (Henry et al. 2015). Insects are easy to bread and reproduce, have fast growth rates, and have high feed conversion efficiency (e.g., 1 kg of insect biomass can be produced from as little as 1.7 kg of feed biomass) (van Huis 2013; Makkar et al. 2014; Tran et al. 2015). Insect production is usually performed in warehouses, without the need for large areas as they can be farmed vertically and require low freshwater input (Sánchez-Muros et al. 2014). Moreover, insects can be farmed locally, improving economic security in those communities, and reducing carbon footprint and the dependency on imports from other countries, which can be affected by social and political issues, disrupting the supply and production chain.

Insects have a potentially great economic role from a circular economy perspective due to their ability to convert other industries' waste and by-products with low value into highly valuable and nutritious products (van Huis 2013; Madau et al. 2020). This is an interesting characteristic, considering that 1.4 billion tons of organic by-products are generated globally each year (Bhatia et al. 2023). Species such as the black soldier fly (*Hermetica illucens,* HI), the common housefly (*Musca domestica*, MD), and the yellow mealworm (*Tenebrio molitor*, TM) have been recognized by their efficiency in bioconverting organic waste (van Huis 2013). The earliest published research on the use of insects to convert animal waste into high-quality protein appeared in the 1970s (Hale 1973; Newton et al. 1977), and since then, many studies have addressed other potential subtracts for rearing insects (Hussein et al. 2017; Spranghers et al. 2017; Ewald et al. 2020; Hopkins et al. 2021). Thus, using insects as feed or food would environmentally, socially, and economically improve the performance of agri-food systems (Madau et al. 2020).

4.1 Production and processing

Insect farming is done practically on every continent (Hawkey et al. 2021). In Europe, at least 42 enterprises were active in producing insect meals in 2019 (Mancuso et al. 2019), representing roughly 6000 tonnes of insect meal per year (Tran et al. 2022). Despite the great progress in recent years, insect production is still not sufficient to provide the required volumes of protein in aquafeeds (Liland et al. 2017), resulting in higher and less competitive prices of insect meals compared to other widely available feed commodities

(Mancuso et al. 2019). Nevertheless, both insect production and the use of insect products in aquafeeds are predicted to rise in the upcoming years as the EU is pushing for insect production within Europe with significant investments made in the last few years. Insect meal production is expected to increase to 500.000 tonnes by 2030, with 40% destined for aquafeeds (Tran et al. 2022). It will require significant investment, research, and development to mature insect production into a viable competitive commodity for commercial aquafeed production (Alfiko et al. 2021). Improved infrastructures, automation, and constant composition will be necessary for large-scale manufacturing so that prices become competitive (Sánchez-Muros et al. 2014; Tran et al. 2022). To a larger extent, the wider adoption of insect utilization in aquafeeds will likely depend on aquafeed and aquaculture producers and consumer acceptance (Maulu et al. 2022). Most of the studies conducted in the EU have shown favorable responses to the consumption of fish fed with insects, associated with low risk and improved sustainability of fish production (Sogari et al. 2019; Baldi et al. 2021; Rumbos et al. 2021).

4.2 Insects in aquafeeds

Aiming to reduce the dependency on FM and FO, and to achieve a more sustainable aquaculture production, the EU authorized in 2017 the use of seven insect species for use in aquafeeds (Regulation (EU) 2017/893): black soldier fly (*Hermetia illucens*, HI), common housefly (*Musca domestica*, MD), yellow mealworm (*Tenebrio molitor,* TM), lesser mealworm (*Alphitobius diaperinus*), house cricket (*Acheta domesticus*), banded cricket (*Gryllodes sigillatus*) and field cricket (*Gryllus assimilis*). As of 2021, the EU added silkworm (*Bombyx mori*) to the list of authorized insect species (Regulation (EU) 2021/1925) (**Figure 1.3**).

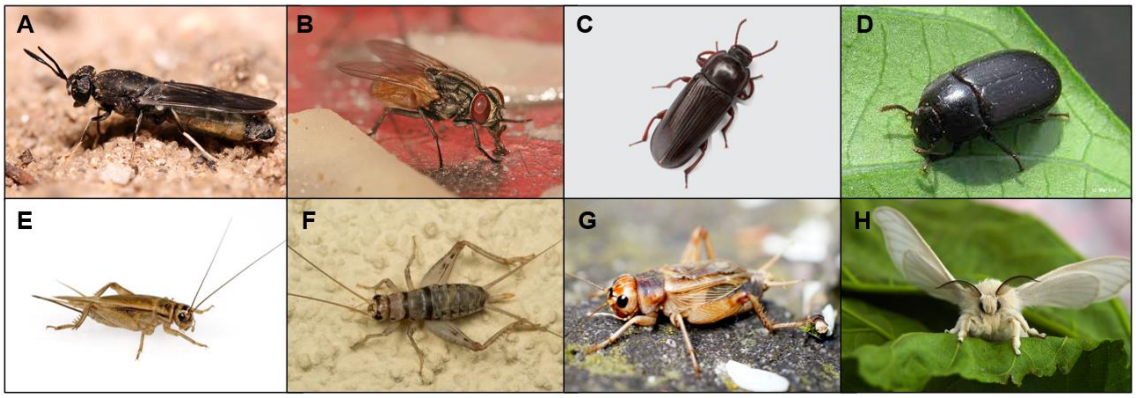

Figure 1.3: Insect species authorized for use in aquafeeds. **A.** black soldier fly, **B.** common housefly, **C.** yellow mealworm, **D**. lesser mealworm, **E.** house cricket, **F.** banded cricket, **G**. field cricket, **H.** silkworm.

In the last decade, the number of studies that evaluated the potential of insect meals in fish diets has increased exponentially, as demonstrated by the increasing number of meta-analyses and reviews published on the topic in the last years (Hua 2021; Liland et al. 2021; Weththasinghe et al. 2021a; Maulu et al. 2022; Tran et al. 2022). These studies have demonstrated that insect meal can be used as a protein source to partially or completely replace FM in the diets of many fish species of high economic interest such as Atlantic salmon (*Salmo salar*) (Belghit et al. 2019; Fisher et al. 2020), rainbow trout (*Oncorhynchus mykiss*) (Belforti et al. 2015; Gasco et al. 2016; Renna et al. 2017; Caimi et al. 2021; Biasato et al. 2022), European seabass (*Dicentrarchus labrax*) (Gasco et al. 2016; Magalhães et al. 2017; Abdel-Tawwab et al. 2020; Mastoraki et al. 2020), and gilthead seabream (*Sparus aurata*) (Cusimano et al. 2017). However, a high degree of variation regarding the maximum dietary inclusion levels of insect meals is observed. Recent meta-analyses by Liland et al. (2021) and Hua (2021) showed that the optimum inclusion levels of insect meal were about 25-30%, as higher levels compromised, among other parameters, growth or feed efficiency. However, some studies have reported that higher dietary inclusion levels did not affect growth performance (Renna et al. 2017; Stejskal et al. 2020), while other studies reported low tolerance for insect meal inclusion in the diets (Dumas et al. 2018; Guerreiro et al. 2020; Reyes et al. 2020; Karapanagiotidis et al. 2023). These discrepancies can be attributed to several factors, such as the studied fish species, growth stage, feed formulation, insect species used, and meal processing method, making it difficult to extrapolate results and compare studies (Liland et al. 2021).

Unlike the use of insects as a protein source, the use of insect oil as an alternative lipid source in aquafeeds has only recently begun to be evaluated. Defatting has become a standard practice in insect meal production to reduce nutrient variation, the risk of lipid oxidation and improve the protein content, yielding better outcomes in many fish species (Dumas et al. 2018; Wang et al. 2019). This process results in high amounts of insect oil, which is currently an underexploited resource. Li et al. (2016) were the first to show the potential of including HI oil in diets for Jian carp (*Cyprinus carpio* var. Jian), including up to 25% without compromising growth and nutrient utilization while reducing lipid deposition. Later, Dumas et al. (2018) and Fawole et al. (2021) included 10% and 16% of HI larvae oil (HIO) in rainbow trout diets without adverse effects on growth. Recently, Xu et al. (2020) suggested that HIO is a better lipid source for mirror carp (*C. carpio* var. specularis) when compared to yellow mealworm and silkworm oils. However, totoaba (*Totoaba macdonaldi*) and red hybrid tilapia (*Oreochromis* spp.) did not tolerate more

than 2% of dietary HIO inclusion without significantly reducing growth performance (Bakar et al. 2021; Maldonado-Othón et al. 2022).

4.3 Insect nutritional composition

Insects are an interesting feed resource for aquafeeds as they are rich in proteins, lipids, vitamins, minerals, and other bioactive compounds. Based on extensive reviews published in recent years, the nutritional composition of insect meals was shown to be highly variable, affected by several factors, including insect species, life stage, rearing substrates, rearing conditions, and processing techniques (**Table 1.1**) (Barroso et al. 2014; Nogales-Mérida et al. 2018; Maulu et al. 2022). Nevertheless, insect meals are high in protein, with a crude protein (CP) content usually ranging between 42 and 63% dry matter (DM) (Tran et al. 2015; Alfiko et al. 2021; Maulu et al. 2022) but can go as high as 74% when the meal is highly defatted (Alfiko et al. 2021). However, in the review by Nogales-Mérida et al. (2018), the authors reported significant variability in the nutritional composition among insect species. For example, the CP content of TM larvae varied from 8.3 to 59.8%, while for HI larvae, it varied from 30.7% to 58.8% and from 28.6% to 70.4% for MD.

Table 1.1: Nutritional composition of different insect species (% DM) used in aquafeeds.

Insect species	CP[1]	CL[2]	NFE (CHO)[3]	Ash	Source
Black soldier fly (*Hermetia illucens*)	30.7-58.8	11.3-40.7	0.8	3.5-9.8	Nogales-Mérida et al. (2018) Koutsos et al. (2019)
Yellow mealworm (*Tenebrio molitor*)	8.3-59.8	16.6-40.3	2.7	3.5-4.8	Barroso et al. (2014) Nogales-Mérida et al. (2018)
Common housefly (*Musca domestica*)	28.8-70.4	7.1-25.3	6.3	6.2-7.1	Barroso et al. (2014) Nogales-Mérida et al. (2018) Hussein et al. (2017)
Lesser mealworm (*Alphitobius diaperinus*)	58.0-65.0	13.9-29.0	3.6-5.1	3.6-4.3	Rumbos et al. (2019) Kurečka et al. (2021)
House cricket (*Acheta domesticus*)	8.8-64.1	7.9-24.0	5.4	5.6	Barroso et al. (2014) Nogales-Mérida et al. (2018)
Banded cricket (*Gryllodes sigillatus*)	65.3-70.0	18.2-23.5	-	4.2	Zielińska et al. (2018) Ribeiro et al. (2019)
Field cricket (*Gryllus assimilis*)	55.6-65.5	11.9-29.5	8.6	4.1	Araújo et al. (2019) Mlček et al. (2018)
Silkworm (*Bombyx mori*)	34.4-82.9	19.2-57.6	1.8-28.2	0.9-10.6	Tassoni et al. (2022)

[1]CP: Crude protein; [2]CL: Crude Lipid; [3]NFE: Nitrogen free extract (carbohydrates).

In general, insects are also rich in essential amino acids (EAA) that are present in high levels in FM but not in plant feedstuffs protein sources, such as lysine, methionine, and leucine (Barroso et al. 2014; Sánchez-Muros et al. 2014). However, there is also great variability in EAA content among insect meal products depending on the species. For example, HI, MD, and silkworms contain more lysine than crickets and TM (Alfiko et al. 2021), and tryptophan levels are typically higher in silkworms and MD than in other insect species (Sánchez-Muros et al. 2014; Henry et al. 2015). Among the different classes of insects, those that belong to Diptera (i.e., HI and MD) seem to have an EAA profile more similar to that of FM (Barroso et al. 2014).

Insects also have a high lipid content that is affected by the development stage, culture conditions, and feed (Nogales-Mérida et al. 2018; Alfiko et al. 2021; Hossain et al. 2023). Insects accumulate more fat in immature stages of development, but lipid contents decrease during pupation and adult stages as it is used as an energy source (Tran et al. 2015; Oonincx and Finke 2021). Nogales-Mérida et al. (2018) reported that the crude lipid (CL) content of HI varied between 11.3% and 40.7%, while for TM it varied from 16.6% to 40.3%. Lower CL content, but also with high variation, was observed in house cricket and MD, which varied from 7.9% to 24% and from 7.1% to 25.3%, respectively (Nogales-Mérida et al. 2018). FA profiles are also very distinct among insect species. HI larvae are rich in saturated FA (SFA), mostly composed of lauric acid (C12:0), while MUFAs such as oleic acid (C18:1n-9), and PUFAs such as α-linolenic acid (C18:3n-3) and linoleic acid (C18:2n-6), predominate in silkworm pupae and TM, respectively (Nogales-Mérida et al. 2018; Hossain et al. 2023). Lipids from insects contain negligible amounts of EPA and DHA and higher levels of C_{18} PUFAs (C18:3n-3 and C18:2n-6) compared to FM (Nogales-Mérida et al. 2018). This is a limiting factor in the inclusion of insect products in aquafeeds for marine fish since n-3 LC-PUFAs are essential nutrients, playing important functions in vertebrates. With regard to nutritional value, the FA profile of the fillet of fish fed with insect oil-containing diets tends to reflect that of the insect products and may compromise the nutritional value of fish for human consumption due to the resulting low n-3-LC-PUFA contents. Nevertheless, the FA profile of insects can be improved to some extent, as it has been shown to partially reflect the FA composition of the diet used for their production (St-Hilaire et al. 2007a; Oonincx and Finke 2021). Some reports have demonstrated high plasticity in the insects' larvae FA composition, and it was shown that FA profile could have increased n-3 LC-PUFA content by feeding larvae with n-3 LC-PUFA enriched substrates such as fish offal and seaweeds (St-Hilaire et al. 2007a; Sealey et al. 2011; Liland et al. 2017; Truzzi et al. 2020; Hossain et al. 2023).

Besides proteins and lipids, insects are also important sources of vitamins and minerals. They contain vitamin D, vitamin E, and B-complex vitamins such as riboflavin (vitamin B2), niacin, pantothenic acid, pyridoxine (vitamin B6), biotin, folic acid, and cyanocobalamin (vitamin B12) (Oonincx and Finke 2021). They are also rich in macro minerals such as calcium, phosphorous, magnesium and potassium, as well as trace minerals such as iron, zinc, copper, manganese and selenium (Alfiko et al. 2021; Oonincx and Finke 2021; Maulu et al. 2022). While carbohydrates (calculated as Nitrogen Free Extract) are limited in the insect body (1-7%), they contain significant amounts of chitin and sclerotized chitin, which are the main components of the insect exoskeleton (Oonincx and Finke 2021).

5. Insects as a source of bioactive compounds

5.1 Chitin

Chitin is a structural polysaccharide composed of β-(1-4) N-acetyl-D-glucosamine units (**Figure 1.4**) that is found in the exoskeleton of arthropods, including crabs, shrimps, and insects, and is the second most abundant polysaccharide in nature after cellulose (Finke 2007; Ringø et al. 2012; Ngo and Kim 2014). In the insects' exoskeleton, chitin is linked with other components, such as proteins, lipids, and minerals, to form a complex structural matrix (Finke 2007; Rangel et al. 2022). Chitin content varies according to insect species and development stage (Gasco et al. 2019). For example, chitin content was reported to range within 2.7-16.6 % in HI meal, 2.8-22.6% in TM meal, and 9.2-16.7% in silkworm meal (Liland et al. 2021), being usually higher in the prepupae stages compared to the larvae and adult stages (Wang et al. 2020).

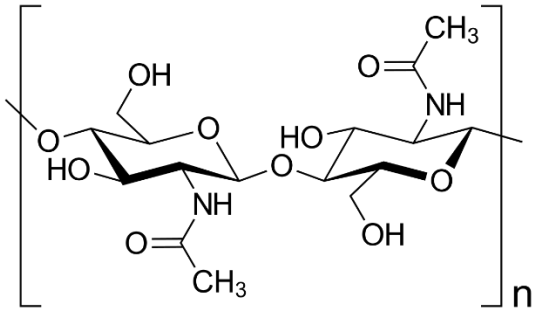

Figure 1.4: Structure of chitin molecule with two units of β-(1-4) N-acetyl-D-glucosamine.

As in other monogastric animals, chitin is considered to be indigestible in fish, due to the lack of chitinolytic enzymes in most species (Rust 2003). High levels of chitin in insect

meals have been linked to reduced nutrient digestibility, growth and feed efficiency in many species, including rainbow trout (Renna et al. 2017), turbot (*Psetta maxima*) (Kroeckel et al. 2012), Siberian sturgeon (*Acipenser baerii*) (Caimi et al. 2020b), and brown trout (*Salmo trutta*) (Mikolajczak et al. 2022). This is attributed to chitin's ability to bind with proteins and lipids, reducing their availability for hydrolysis, thus lowering digestion and absorption (Muzzarelli 1996; Ringø et al. 2012). Moreover, a negative correlation between chitin content and protein digestibility was found in Atlantic salmon (Karlsen et al. 2015) and in *in vitro* studies (Marono et al. 2016). Thus, it is generally accepted that chitin is a limiting factor in insect meals, restricting their inclusion in aquafeeds (Sánchez-Muros et al. 2014). Nevertheless, chitinase activity has been detected in a few fish species, such as rainbow trout (Lindsay et al. 1984), Atlantic cod (*Gadus morhua*) (Danulat and Kausch 1984; Lindsay and Gooda 1985), Nile tilapia (*Oreochromis niloticus*) (Eggink et al. 2022), and cobia (*Rachycentron canadum*) (Fines and Holt 2010), but not in Atlantic salmon (Olsen et al. 2006; Karlsen et al. 2015) or meagre (*Argyrosomus regius*) (Coutinho et al. 2021). Whether this chitinase activity is of endogenous origin or produced by gut microbiota still needs to be confirmed.

Despite the perceived adverse effects on nutrient digestibility, moderate levels of chitin and its derivatives, such as chitosan, have been shown to have beneficial effects on the antioxidant, immune and inflammatory status of fish, as well as provide antitumoral and antibacterial properties (Ringø et al. 2012; Ngo and Kim 2014; Tran et al. 2015; Abdel-Ghany and Salem 2019). By acting as an immunostimulant, chitin can protect against pathogens when supplemented in fish feeds (Harikrishnan et al. 2012; Ringø et al. 2012; Henry et al. 2015; Kumar et al. 2019). For instance, the innate immune system of gilthead seabream (Esteban et al. 2001) and macrophage activity of rainbow trout (Sakai 1992) were improved by chitin supplementation, while chitosan enhanced the innate immune system and survivability of common carp (*C. carpio*) (Gopalakannan and Arul 2006). When fed insect meals, improvements in the immune response were also observed in species such as red seabream (*Pagrus major*) (Ido et al. 2015), mandarin fish (*Siniperca scherzer*) (Sankian et al. 2018), European seabass (Henry et al. 2018), and yellow catfish (*Pelteobagrus fulvidraco*) (Su et al. 2017).

Chitin also has prebiotic potential, improving gastrointestinal health by shaping the gut microbial community (Ringø et al. 2012; Zhou et al. 2013; Bruni et al. 2018; Terova et al. 2019; Lopez-Santamarina et al. 2020). The inclusion of insect meals in fish diets was shown to increase gut microbiota diversity and richness (Bruni et al. 2018; Antonopoulou et al. 2019; Huyben et al. 2019; Terova et al. 2019; Gaudioso et al. 2021) and enhance the proliferation of bacteria with probiotic potential (Rimoldi et al. 2019; Terova et al.

2019; Gaudioso et al. 2021; Li et al. 2021; Rangel et al. 2022). Due to the free radical scavenging potential of chitin (Ngo and Kim 2014; Guerreiro et al. 2023), the inclusion of insect meals in fish feeds has shown increased antioxidant potential or decreased oxidative damage in several fish species including channel catfish (*Ictalurus punctatus*) (Fan et al. 2023), hybrid catfish (Fawole et al. 2023), mandarin fish (Sankian et al. 2018), mirror carp (Xu et al. 2020; Li et al. 2022), rainbow trout (Henry et al. 2018), yellow catfish (Su et al. 2017), and Siberian sturgeon (Caimi et al. 2020a).

Therefore, due to the presence of chitin, insect meals not only have the potential to be used in fish feeds as an ingredient that provides nutrients for growth and metabolism but can also have functional properties.

5.2 Lauric acid

Insect meals can also be a source of FA with functional properties. Lauric acid (**Figure 1.5**) is a medium chain (C_{12}-C_{18}) fatty acid (MCFA) with 12 carbons found in high quantities in HIO. In terrestrial VO, lauric acid is commonly found in coconut oil and palm kernel oil (Franco et al. 2021). MCFAs have been widely studied in animal and human nutrition as a readily available energy source, due to their rapid absorption and oxidation rate, without increasing lipid deposition (Marten et al. 2006; Belghit et al. 2018; Sudha et al. 2022). Since MCFA are less dependent on chylomicron and lipoproteins for transport, they can pass directly from the intestine to the liver via the portal vein, being more efficiently absorbed and utilized than longer chain FAs (Marten et al. 2006; Baltić et al. 2017; Belghit et al. 2018). Also, MCFAs do not require prior modifications by carnitine palmitoyltransferase-1 (Cpt-1) for crossing the mitochondrial membrane and are therefore not dependent on this rate-limiting step of FA β-oxidation (Baltić et al. 2017).

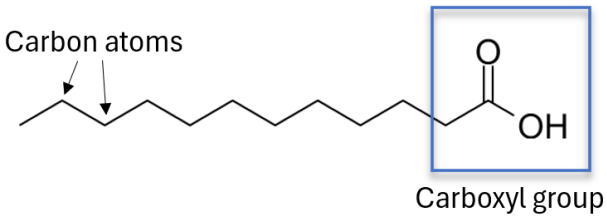

Figure 1.5: Skeletal formula of lauric acid.

The positive effects of dietary inclusion of MCFAs and lauric acid have already been described in fish (Nordrum et al. 2000; Williams et al. 2006). For instance, in black seabream (*Acanthopagrus schlegelii*), growth, and feed efficiency were improved by

dietary lauric acid supplementation (Ullah et al. 2022). Moreover, lauric acid was shown to be a good energy source for striped catfish (*Pangasianodon hypophthalmus*) with lower deposition of this FA in the body (Sudha et al. 2022). Likewise, dietary medium chain triglycerides/MCFA supplementation improved nutrient digestibility and protein retention in Atlantic salmon (Nordrum et al. 2000, 2003), growth in gilthead seabream (Simó-Mirabet et al. 2017) and suppressed lipid deposition in red drum (*Sciaenops ocellatus*) (Davis et al. 1999) and Atlantic salmon (Røsjø et al. 2000). Due to their preferred use for β-oxidation, the inclusion of lauric acid in diets may also have a sparing effect on other FA, namely n-3 LC-PUFA, allowing them to be used for other biological purposes (Marten et al. 2006; Li et al. 2016; Maldonado-Othón et al. 2022). The utilization of MCFAs increases malonyl-CoA concentration and decreases Cpt-1 activity, resulting in reduced intramitochondrial transport and oxidation of longer-chain FAs (Marten et al. 2006).

Besides their role as an energy source, MCFAs have been reported to stimulate animal immune and antioxidant responses (Hinton and Ingram 2006; Dayrit 2015). For example, MCFA supplementation improved the immune response in growing pigs (Zhang et al. 2019) and antioxidant status in weanling pigs (Li et al. 2015). Dietary HIO inclusion increased lysozyme activity in rainbow trout compared to dietary soybean oil (Kumar et al. 2021), improved oxidative status, and reduced the expression of pro-inflammatory genes in mirror carp (Xu et al. 2020). Due to their antibacterial, anti-viral, and anti-fungal properties, MCFAs can be also used as an alternative to in-feed antibiotics for controlling microbial infections in aquaculture (Franco et al. 2021; Ullah et al. 2022).

6. Black soldier fly (*Hermetia illucens*, HI)

6.1 Biology, life cycle, and production

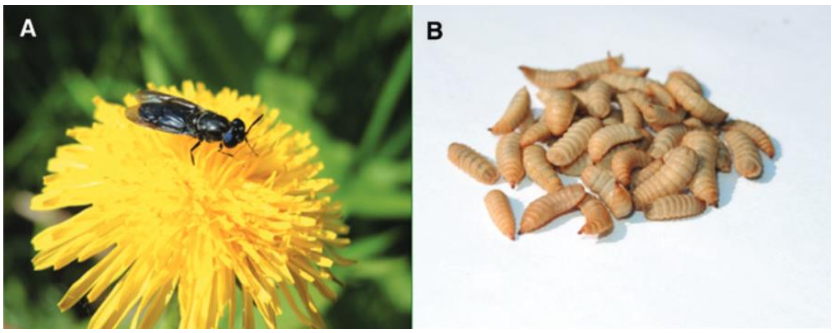

Figure 1.6: Adult (A) and larvae (B) of black soldier fly (*Hermetia illucens*). Source: Müller et al. (2017)

The black soldier fly (*Hermetia illucens* Linnaeus 1758; HI) is a fly of the class Diptera, family Stratiomyidae (**Figure 1.6**). It is native to tropical, subtropical, and warm temperate zones of America, but has now been naturalized in many regions of the world (Makkar et al. 2014). The adult fly is black, wasp-like shaped, reaching a length of 15–20 mm. The cream-colored larvae can attain a length of up to 27 mm long and a width of 6 mm, weighing up to 220 mg in their final larval stage (Diclaro and Kaufman 2009).

The life cycle of HI (**Figure 1.7**) can be divided into four phases: egg, larvae, prepupae/pupae, and adult fly, with a lifecycle of about 45 days (Sheppard et al. 2002). After hatching (up to 4 days after laying), the larvae undergo five instar stages, and feed quickly and abundantly, from 25 to 500 mg of fresh matter/larva/day, on a wide range of decaying organic materials (Diener et al. 2011; van Huis 2013). The growth of prepupae/larvae can be differentiated by color, as they turn dark brown to black on the dorsal surface (Sheppard et al. 2002). At the final larval stage (prepupae), the larva empties its digestive tract, stops feeding, and migrates to higher ground for the next stage – pupation - which usually lasts 2-3 weeks but can go as high as 5 months under unfavorable conditions (Dortmans et al. 2017). Production takes advantage of this instinctive migration pattern as it eliminates the labor-intensive step of harvesting (Sheppard et al. 2002; Liu et al. 2017). After 2 days of emerging, the females mate and deposit the eggs (from 400 to 800) into dry cracks and crevices adjacent to a feed source (Diener et al. 2011; Dortmans et al. 2017). After emerging, the adult fly lives for about 1 week. As they do not possess mouths, they do not require feeding and rely solely on the fats stored during the larval stage (Diclaro and Kaufman 2009; Liu et al. 2017). The HI larvae and adults are not vectors for any known diseases, are extremely resilient, and are capable of dealing with drought, food shortage and oxygen deficiencies (Diener et al. 2011).

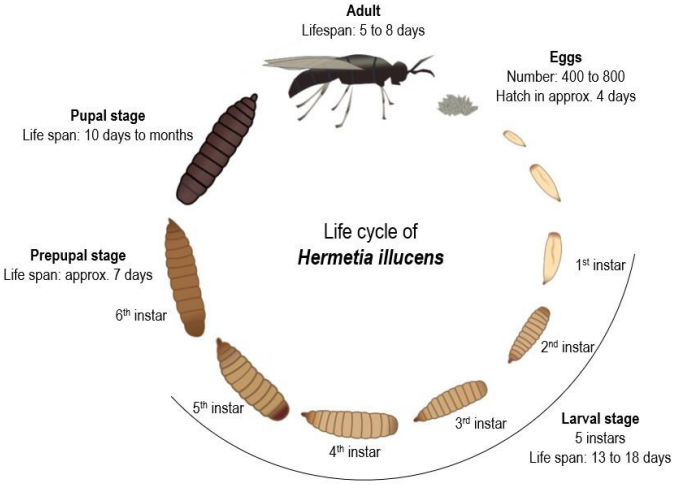

Figure 1.7: Life cycle of black soldier fly (*Hermetia illucens*). Adapted from Lievens et al. (2021)

As mentioned previously, HI larvae are recognized as efficient bio-converters, transforming large volumes of low-valued waste into highly valuable biomass, thus sustaining a circular bioeconomy (Barragan-Fonseca et al. 2017; Alfiko et al. 2021; Mohan et al. 2022). Various substrates have been evaluated as feed for HI larvae, including agriculture waste, animal manure, brewery by-products, restaurant waste, and sewage sludge (Ewald et al. 2020; Franco et al. 2021; Kaczor et al. 2022). This makes HI one of the most promising species to be farmed at a larger scale and for use in aquafeeds as a sustainable alternative ingredient, currently being the most widely cultured insect species (Hossain et al. 2023).

6.2 HI larvae as a protein source in aquafeeds

6.2.1 Nutritional value of HI protein for aquafeeds

Among the authorized insect species to be used in aquafeeds in the EU, HI appears to be the most promising, mostly due to its nutritional quality. The average CP of HI meals is high and usually varies from 40 to 45% DM but can increase up to 63% when the meal is defatted (Hua 2021). HI larvae possess a balanced EAA profile, most similar to that of FM (Spranghers et al. 2017; Wang et al. 2020). HI larvae are particularly rich in leucine (7.9 % CP), alanine (7.7% CP), and lysine (6-8% of CP) (Makkar et al. 2014; Tran et al. 2015), and contain higher levels of the EAA alanine, methionine, histidine, and tryptophan than soybean meal (Barragan-Fonseca et al. 2017).

HI larvae ash content varies from 5 to 28% (Hua 2021) and is a good source of minerals important for nutrition (Liu et al. 2017). They contain 3 to 10 times more magnesium than other insects (Finke 2013; Spranghers et al. 2017) and are rich in calcium and other minerals including phosphorus, potassium, sodium, iron, manganese, zinc, and copper (Makkar et al. 2014; Liland et al. 2017; Oonincx and Finke 2021; Mohan et al. 2022). The mineral composition can be affected by the diet insects are fed on but also by chitin, as minerals like calcium and magnesium can bond and form a complex with chitin (Oonincx and Finke 2021).

6.2.2 Studies with HI as a protein source in aquafeeds

Over the last few years, the number of studies evaluating HI meal as a protein source alternative to FM in aquafeeds has increased exponentially, with approximately 60% of the studies being published since 2020. These studies have shown that HI meal has great potential to partially or entirely replace FM in aquafeeds. However, the ideal dietary

HI inclusion level or FM replacement level varies significantly among studies. While some studies report that HI meals can be included in diets up to 40-45% without negative effects on fish growth and feed efficiency (Renna et al. 2017), other studies report that more than 10-13% inclusion impairs growth performance (Dumas et al. 2018; Guerreiro et al. 2020). Even for the same fish species, results vary among studies. For example, for rainbow trout, Renna et al. (2017) were able to include 40% HI meal while in another study, more than 13% inclusion in the diets reduced final body and weight gain (Dumas et al. 2018). Likewise, for Siberian sturgeon, Rawski et al. (2020) were able to include up to 30% HI meal while more than 19% inclusion compromised growth performance in a previous study (Caimi et al. 2020b). As mentioned previously, this apparent discrepancy in results between studies can be attributed to several factors, such as different fish species, life stages, trial duration, and experimental conditions, but also to the quality and characteristics of the HI meals used in each feeding trial (i.e., defatted or full-fat, rearing subtracts, etc.). Nevertheless, studies also showed that the digestibility of HI meal-containing diets depends on fish species and the characteristics of the meal. For example, for gilthead seabream (Gai et al. 2023) and rainbow trout (Caimi et al. 2021; Biasato et al. 2022), nutrient digestibility was not affected by dietary HI meal inclusion, while for pikeperch (*Sander lucioperca*) (Stejskal et al. 2022), Siberian sturgeon (Caimi et al. 2020b) and yellow catfish (Hu et al. 2017), a decrease in nutrient digestibility was observed with HI meal inclusion.

Besides the effects on fish growth and digestibility, the dietary inclusion of HI meal has also been shown to have functional proprieties, with beneficial effects on the health and well-being of fish. Reduction in plasma cholesterol or triglycerides was reported for snakehead (Siddaiah et al. 2023), meagre (Guerreiro et al. 2020), Jian carp (Li et al. 2017), European seabass (Magalhães et al. 2017), and yellow catfish (Hu et al. 2017). Likewise, positive effects on antioxidant capacity (Zhou et al. 2018; Fawole et al. 2020; Moutinho et al. 2021; Hidalgo et al. 2022), and immune and inflammatory responses (Xiao et al. 2018; Zarantoniello et al. 2020a; Abdel-Latif et al. 2021; Kishawy et al. 2022) were reported in several studies. A compilation of published studies where FM was replaced by HI meal in fish diets and the main effects observed can be found in the following table (**Table 1.2**).

Table 1.2: Published studies where fishmeal (FM) was replaced by HI meal and the main effects observed regarding growth and feed efficiency, digestibility, digestive enzymes and intermediary metabolism enzymes, plasma metabolites, oxidative stress, inflammatory and immune response, and economic efficiency.

Species	HI meal used[a]	% Inclusion in the diet (% FM substitution)[b]	Main results[c] (% HM inclusion), [tissue]	Reference
African catfish (*Clarias gariepinus*)	PDF	4 (25), **8 (50),** 15 (100)	↓ FBW, FI, PER, and ↑ FCR (15%) = Haem. Param.	Adeoye et al. (2020)
	PDF	6 (25), 12 (50), **18 (75)**	↑ FBW, WG, PER, and ↓ FCR (18%) = Haem. Param.; ↓ plasma TG, glucose ↓ SOD, ↑ CAT, = MDA [plasma]	Fawole et al. (2020)
Atlantic salmon (*Salmo salar*)	PDF*	5 (25), 10 (50), **25 (100)**	↓ FBW (25%), ↓ FCR (≥ 10%)	Lock et al. (2016)
		5 (25), 25 (100)	↓ FBW, ↑ FCR (25%)	
	PDF	5 (33), 10 (66), **15 (100)**	= FBW, FI, FCR ↓ plasma glucose (10%) = ADC_P, $ADC_{L;}$ = trypsin	Belghit et al. (2019)
	FF	8 (7), **16 (14),** 32 (29)	↓ FBW, PER (32%), ↓ FCR (16%) ↓ ADC_L (≥ 16%), = ADC_P, ADC_E	Weththasinghe et al. (2021b)
			= *il-1β* expression [intestine]	Weththasinghe et al. (2021c)
Barramundi (*Lates calcarifer*)	PDF	15 (25), **31 (50)**, 46 (75), 62 (100)	↓ WG (≥ 46%), ↑ FCR (62%)	Katya et al. (2017)
	PDF	**22 (30%)**	= FBW, WG, FCR = plasma metabolites ↑ *il-10*, *il-1β* expression [liver]	(Hender et al. (2021)
Brown trout (*Salmo trutta* m. fario)	FF	5 (7), 10 (14), **20 (29)**	= FBW, WG, FCR, PER ↑ plasma cholesterol (≥ 10%) ↓ ADC_P, ADC_L (20%)	Mikolajczak et al. (2022)
Climbing perch (*Anabas testudineus*)	DF*	15 (50), **29 (100)**	= FBW, WG, FCR, ↑ PER (29%)	Vongvichith et al. (2019)
Dusky Kob (*Argyrosomus japonicus*)	PDF	5 (7), 10 (15), **20 (29)**	= FBW, FI, FCR = HP; ↑ plasma TG	Madibana et al. (2020)

Table 1.2 (cont.): Published studies where fishmeal (FM) was replaced by HI meal and the main effects observed regarding growth and feed efficiency, digestibility, digestive enzymes and intermediary metabolism enzymes, plasma metabolites, oxidative stress, inflammatory and immune response, and economic efficiency.

Species	HI meal used[a]	% Inclusion (% FM substitution)[b]	Main results[c] (% HM inclusion), [tissue]	Reference
Eurasian Perch (*Perca fluviatilis*)	PDF	20 (20), **41 (40)**, 62 (60)	↓ FBW, WG (62%) = HP; = ECR, EPI (< 40%)	Stejskal et al. (2020)
European seabass (*Dicentrarchus labrax*)	DF*	7 (15), 13 (30), **20 (45)**	= FBW, WG, FE; ↓ PER (20%) = ADC_{DM}, ADC_P, ADC_L, ADC_E ↓ plasma CHO (20%)	Magalhães et al. (2017)
	PDF	7 (25), 10 (35), **15 (50)**	= FBW; WG, FCR = HP; = plasma metabolites ↓ Feeding costs	Abdel-Tawwab et al. (2020)
	PDF	7 (25), 10 (35), **15 (50)**	↑ MDA, CAT, SOD, GPX ↑ *il-10*, *il-1β* expression [liver]	Abdel-Latif et al. (2021)
	FF and DF	20 (20)	↓ ADC_{DM}, ADC_P, ADC_L, ADC_E	Basto et al. (2020)
	DF	7 (15), 13 (30), **20 (45)**	↓ SOD, CAT, MDA [liver]	Moutinho et al. (2021)
Gilthead seabream (*Sparus aurata*)	FF*	10 (10), 19 (20), **28 (30)**	↓ FBW, WG, FI; = FCR, PER	Karapanagiotidis et al. (2014)
	DF	9 (25), 18 (50), **28 (75)**	= WG, FCR, PER, FI	Cusimano et al. (2017)
	DF	15 (22), 30 (60), **45 (100)**	= FBW, WG, FE; ↑ $ADC_{DM,}$ ADC_E ↓ ECR, = EPI (< 30%)	Moutinho et al. (2022) (Present thesis)
			↓ *myod2*, ↑ *mstn* [muscle]; ↑ ALT, ↓ G6PDH (>45%); ↑ HOAD (15%) [liver] ↑ trypsin, chymotrypsin, amylase (15%) [intestine]	Moutinho et al. (2024b) (Present thesis)
	DF	**5 (33),** 10 (66)	↓ FBW (10%), = FCR, PER	Carvalho et al. (2023)

Table 1.2 (cont.): Published studies where fishmeal (FM) was replaced by HI meal and the main effects observed regarding growth and feed efficiency, digestibility, digestive enzymes and intermediary metabolism enzymes, plasma metabolites, oxidative stress, inflammatory and immune response, and economic efficiency.

Species	HI meal used[a]	% Inclusion (% FM substitution)[b]	Main results[c] (% HM inclusion), [tissue]	Reference
Gilthead seabream (*Sparus aurata*)	DF	8 (25), **11 (35),** 16 (50)	= FBW, FCR, PER = plasma glucose, TP	Di Rosa et al. (2023)
	DF	9 (25), **18 (50),** 28 (75)	= WG, ↓ FCR, PER (28%) = ADC_{DM}, ADC_P, ADC_L, ADC_E = proteases, amylase, lipase	Gai et al. (2023)
	FF*	10 (9), 19 (17), 28 (25)	↓ FBW, WG; = FCR, PER	Karapanagiotidis et al. (2023)
	DF*	5.8 (10), **11.6 (20),** 17.4 (30)	↓ FBW, WG, PER and ↑ FCR (17%)	
Japanese seabass (*Lateolabrax japonicus*)	DF	5 (16), 10 (32), 14 (48), **19 (64)**	= FBW, WG, FCR, ↑ (> 10%) ↓ CHO, TG, HDL, MDA [plasma] ↑ lipase (> 14%)	Wang et al. (2019)
Jian carp (*Cyprinus carpio* var. Jian)	DF	3 (25), **5 (50),** 8 (75), 11 (100)	= FBW, WG, FCR, PER; ↓ plasma CHO ↑ CAT (≥ 8%), = MDA, SOD [plasma]	Li et al. (2017)
	n.d.	3.5 (25), 7 (50), 10.5 (75), **14 (100)**	= FBW, WG, FCR ↓ MDA, ↑ antioxidant capacity [plasma]	Zhou et al. (2018)
Largemouth bass (*Micropterus salmoides*)	FF	**12 (67)**	↓ FBW, WG, FCR, = FI =*igf1*, *gh* expression [liver]	Fischer et al. (2022)
	FF*	9.8 (67)	↓ FBW, WG, FCR, FI =*igf1*, *gh* and ↓ *tgfβ* expression [liver]	
Lemon fin barb hybrid (*Hypsibarbus wetmorei* ♂ x *Barbodes gonionotus* ♀)	DF*	2.5 (25), 5 (50), **8 (75),** 10 (100)	↓ FBW, WG (10%); = FCR, FI	Kamarudin et al. (2021)
Meagre (*Argyrosomus regius*)	PDF	**10 (17)**, 20 (35), 30 (52)	↓ FBW (30%); ↓ PER, FE ↓ N retention; ↓ plasma total lipids, TG; ↓ HOAD	Guerreiro et al. (2020)
			= SOD, CAT, GPX, G6PDH, GR [intestine]	Guerreiro et al. (2022)

Table 1.2 (cont.): Published studies where fishmeal (FM) was replaced by HI meal and the main effects observed regarding growth and feed efficiency, digestibility, digestive enzymes and intermediary metabolism enzymes, plasma metabolites, oxidative stress, inflammatory and immune response, and economic efficiency.

Species	HI meal used[a]	% Inclusion (% FM substitution)[b]	Main results[c] (% HM inclusion), [tissue]	Reference
Nile tilapia (*Oreochromis niloticus*)	PDF	3 (20), 5 (50), **8 (70)**	= FBW, WG, FCR, PER	Devic et al. (2018)
	PDF	2.5 (25), 5 (50), **10 (100)**	= FBW, WG, FCR, PER ↑ lysozyme (10%), NO (≥5%) ↓ *il-1β*, *tnfα*, ↑ *il-10*, *tgfβ* expression [spleen] ↑ Economic efficiency	Kishawy et al. (2022)
Pearl gentian grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂)	FF	7 (10), 14 (20), 21 (30)	↓ FBW, WG; ↑ FCR ↓ trypsin (21%); ↑ *il-10* expression [intestine]	Huang et al. (2022)
Pikeperch (*Sander lucioperca*)	DF	**9 (25),** 18 (50), 36 (100)	= SOD, CAT, ↓ GPX (36%) [liver] = SOD, GPX, ↑ CAT (9%) [intestine]	Tran et al. (2021)
	DF	9 (25), **18 (50),** 36 (100)	↓ FBW, WG, PER, FI and ↑ FCR (36%) ↓ ADC_P, ↓ADC_{DM} (≥ 18%), ↓ ADC_E (36%) ↑ ECR, ↓ EPI (36%)	Stejskal et al. (2022)
Red drum (*Sciaenops ocellatus*)	FF	15 (65)**	HM A: ↓ WG, FE HM B: = WG, ↓ FE	Yamamoto et al. (2022)
Red hybrid tilapia (*Oreochromis* spp.)	FF	**30 (66)**	↑ FBW, WG; ↓ FCR ↑ ADC_{DM}, ADC_P, ADC_E	Muin and Taufek (2022)
Rainbow trout (*Oncorhynchus mykiss*)	FF*	**15 (25),** 30 (50)	↓ FBW, WG and ↑ FCR (30%)	St-Hilaire et al. (2007b)
	n.d.*	16 (25), 33 (50)	↓ WG, = FCR	Sealey et al. (2011)
	n.d.*	18 (25), **36 (50)****	= WG, FCR	
	n.d.*	**n.d. (50),** n.d. (75)	↓ FBW, WG, PER, and ↑ FCR (75%)	Stamer et al. (2014)
	DF	**28 (46)**	= FBW, WG, FCR; ↓ PER	Stadtlander et al. (2017)

Table 1.2 (cont.): Published studies where fishmeal (FM) was replaced by HI meal and the main effects observed regarding growth and feed efficiency, digestibility, digestive enzymes and intermediary metabolism enzymes, plasma metabolites, oxidative stress, inflammatory and immune response, and economic efficiency.

Species	HI meal used[a]	% Inclusion (% FM substitution)[b]	Main results[c] (% HM inclusion), [tissue]	Reference
Rainbow trout (*Oncorhynchus mykiss*)	PDF	20 (25), **40 (50)**	= FBW, WG, FCR, PER = ADC_{DM}, ADC_P, ADC_L, ADC_E	Renna et al. (2017)
	PDF	7 (25), **13 (50)**, 26 (100)	↓ FBW, WG (26%), ↑ FCR, ↓ PER ↓ ADC_L	Dumas et al. (2018)
	PDF	**20 (25),** 40 (50)	↓ GPX, = MDA, CAT, SOD, GR [liver]	Elia et al. (2018)
	FF	20 (100)	↓ FBW, = FCR; ↓ ADC_{DM}, ADC_P	Cui (2019)
	FF	6 (15), **11 (30)**	= FBW, FCR, PER; =ADC_P; ↑ amylase (11%) ↓ MDA (6%) [liver]; ↓ anti-protease (6%) [plasma]	Melenchón et al. (2020)
	PDF	8 (25), 16 (50), **32 (100)**	= FBW, WG, FCR, PER = ADC_{DM}, ADC_P, ADC_L, ADC_E	Biasato et al. (2022)
	PDF	3 (10), 6 (20), 9 (30), 12 (40), **15 (50)**	=FBW, WG, FCR, PER = ADC_{DM}, ADC_P, ADC_L, ADC_E	Caimi et al. (2021)
	FF*	30 (75) and 30 (75)**	↓ FBW, WG, = FCR, PER	Hoc et al. (2021)
	FF	18 (50)	= FBW, PER, ↑ FCR ↓ ADC_P, = digestive enzymes ↓ GPX = CAT, SOD, MDA, GR [liver]	Melenchón et al. (2022)
Rice Field eel (*Monopterus albus*)	FF	5 (2.5), 11 (5), **16 (7.5)**	↑ FBW, WG and ↓ FCR (≤ 11%) ↑ lipase (16%); ↑ CAT; MDA (16%) [plasma]	Hu et al. (2020)
Siberian sturgeon (*Acipenser baerii* Brandt)	DF	**19 (25),** 38 (50), 75 (100)	↓ FBW, WG (38%); = FCR, PER ↓ ADC_{DM} (38%), ↓ ADC_P	Caimi et al. (2020b)
		19 (25), 38 (50)	↑ SOD, GPX, GR (38%) [liver]	Caimi et al. (2020a)

Table 1.2 (cont.): Published studies where fishmeal (FM) was replaced by HI meal and the main effects observed regarding growth and feed efficiency, digestibility, digestive enzymes and intermediary metabolism enzymes, plasma metabolites, oxidative stress, inflammatory and immune response, and economic efficiency.

Species	HI meal used[a]	% Inclusion (% FM substitution)[b]	Main results[c] (% HM inclusion), [tissue]	Reference
Siberian sturgeon (*Acipenser baerii* Brandt)	FF*	5 (10), 10 (20), 15 (30), 20 (41), 25 (51), **30 (61)**	↑ FBW, WG, PER; ↓ FCR = ADC_P, ADC_P	Rawski et al. (2020)
			↓ ECR, ↑ EPI	Rawski et al. (2021)
Snakehead (*Channa striata*)	PDF	10 (25), **20 (50),** 30 (75), 37 (100)	↓ FBW, WG, PER and ↑ FCR (>30%) ↓ proteases, ADC_{DM}, ADC_P (>30%) ↑ SOD, CAT [liver]; ↑ SOD, CAT, GPX [plasma] ↓ CHO (>30%)	Siddaiah et al. 2023)
Tench (*Tinca tinca*)	FF	6 (15), **11 (30)**	= FBW, FCR ↓ SOD, ↓ MDA (11%) [intestine]; ↓ GPT, ↓ GOT (6%) [intestine] = Immune and HP [plasma]	Hidalgo et al. (2022)
Turbot (*Psetta maxima*)	PDF*	17 (20), **33 (39),** 49 (56), 64 (74), 76 (88)	↓ FBW; ↓ FI, PER and ↑ FCR (≥ 49%)	Kroeckel et al. (2012)
Yellow catfish (*Pelteobagrus fulvidraco*)	FF	6 (10), 9 (15), **11 (20),** 14 (25), 17 (30)	↓ FBW, WG and ↑ FCR (≤ 14%) ↓ ADC_{DM} (11%), ADC_P (≥ 9%), ADC_L (≥ 14%), ADC_E (9%); ↓ plasma CHO, NO (17%)	Hu et al. (2017)
	PDF*	5.5 (13), 10.8 (25), 16.5 (37), **22.3 (48),** 34.3 (68), 46.2 (85), 58.5 (100)	↓ WG (≥ 34.3), ↑ FCR (≥ 46.2%) ↑ lysozyme, ↑ SOD (10.8%) [plasma]	Xiao et al. (2018)
Zebrafish (*Danio rerio*)	FF*	10.5 (25), **21 (50)**	↑ FBW (21%) ↑ *igf1*, *igf2, mstnb* expression (21%) ↑ *tnfa,* (21%)	Zarantoniello et al. 2018)
			↑ *igf1*, *igf2* (21%)	Zarantoniello et al. (2019)

Table 1.2 (cont.): Published studies where fishmeal (FM) was replaced by HI meal and the main effects observed regarding growth and feed efficiency, digestibility, digestive enzymes and intermediary metabolism enzymes, plasma metabolites, oxidative stress, inflammatory and immune response, and economic efficiency.

Species	HI meal used[a]	% Inclusion (% FM substitution)[b]	Main results[c] (% HM inclusion), [tissue]	Reference
Zebrafish (*Danio rerio*)	FF*	15 (25), **<u>28 (50),</u>** 35 (75), 46 (100)	↑ SGR (≥ 28%) ↑ *igf1*, *igf2* expression (≥ 35%) ↑ *mstnb*, *il-1b*, *1l-10*, *tnfα* expression (≥ 28%)	Zarantoniello et al. (2020a)
	DF or DF*	50 (100)	↑ FBW, WG (*) ↑ *igf1*, *igf2* and ↑ *mstnb* (*) expression [muscle] ↑ *il-1β*, *tnfα* [liver]	Lanes et al. (2021)
	FF*	12 (25), 34 (50), 35 (75), **<u>46 (100)</u>**	= *igf1*, *igf2, mstn, il-1β, il-10* expression ↓ *tnfα* (≥ 35%)	Zarantoniello et al. (2021)

[a] DF: defatted meal (crude lipid, CL, content ≤ 10%); PDF: partially defatted meal (CL content between 11 and 25%); FF: full-fat meal (CL content > 26%); n.d.: not defined; *prepupae larvae meal; ** HM Reared 2 different substrates

[b] % HI meal inclusion in the diet (% FM substitution). The recommended inclusion levels by the authors are underlined and marked in bold. If not mentioned by the authors, the level with similar growth performance to the control diet was considered.

[c] Symbols represent increased (↑), reduced (↓), or no effect (=) relative to the control diet. ADC_{DM}: dry matter apparent digestibility coefficients; ADC_E: energy apparent digestibility coefficients; ADC_L: lipids apparent digestibility coefficients; ADC_P: protein apparent digestibility coefficients; ALT: alanine aminotransferase; CAT: catalase; CHO: cholesterol; ECR: economic conversion ratio; EPI: economic profit index; FAS: fatty acid synthase; FBW: final body weight; FCR: feed conversion ratio; FI: feed intake; G6PDH: glucose-6-phosphate dehydrogenase; GDH: glutamate dehydrogenase; *gh*: growth hormone; GPX: glutathione peroxidase; GR: glutathione reductase; HOAD: β-hydroxy acyl-CoA dehydrogenase; HP: hematological parameters; HDL: High-density lipoprotein; *igf1*: insulin-like growth factor 1; *igf2*: insulin-like growth factor 2; *il-10*: interleukin 10; *il-1β*: interleukin 1 beta; MDA: malondialdehyde; ME: malic enzyme; *mstn*: myostatin b; *myod2*: myogenic determination factor 2; N: nitrogen; OTUs: operational, taxonomic units: PER: protein efficiency ratio; SGR: specific growth rate; SOD: Superoxide dismutase; TG: triglycerides; *tgfβ*: transforming growth factor beta; *tnfa*: tumour necrosis factor-alpha; TP: total protein; WG: weight gain.

6.3 HI larvae oil (HIO) as a lipid source in aquafeeds

6.3.1 Nutritional value of HO

HI larvae have a high lipid content, averaging 30-35% DM, though it can reach up to 40-45% DM (Liland et al. 2017; Nogales-Mérida et al. 2018; Benzertiha et al. 2020). It is mostly composed of triglycerols, although cholesterol, mono- and di-acylglycerols, free FA, phospholipids, and wax esters are also present (Hossain et al. 2023). Regarding FA profiles, HI larvae are characterized by high levels of SFAs, representing 58-72% of total FA content, followed by MUFAs (12-26% total FA) and PUFAs (5-26% total FA) (Benzertiha et al. 2020). Among SFA, lauric acid (C12:0) is the most abundant component, representing up to 52% of total FA content (Liland et al. 2017; Ewald et al. 2020; Oonincx and Finke 2021). Other SFA like myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0), are also found in HI larvae, although at lower quantities (Surendra et al. 2016; Ewald et al. 2020; Franco et al. 2021). HI larvae also contain significant amounts of the MUFA oleic acid (C18:1n-9), up to 17%, as well as the PUFAs linoleic acid (C18:2n-6) and linolenic acid (C18:3n-3), in concentrations similar to those of soybean oil and other vegetable oils (Hossain et al. 2023). Like other insect species, levels of n-3 LC-PUFA, including EPA and DHA, are very low in HI larvae (Ewald et al. 2020). Nevertheless, as stated above, it is possible to manipulate the FA profile of HI larvae, thus improving its nutritional value for aquafeeds. Increased contents of n-3 LC-PUFA in HIO have been achieved by rearing HI larvae on substrates supplemented with biomass from *Schizochytrium* sp. (Truzzi et al. 2020; Zarantoniello et al. 2020b; Xu et al. 2021), the seaweed *Ascophyllum nodosum* (Liland et al. 2017), fish offal (St-Hilaire et al. 2007a; Sealey et al. 2011), salmon oil (Erbland et al. 2020), squid liver (Tirtawijaya and Choi 2021), mussels (Ewald et al. 2020), and fish waste (Barroso et al. 2019), among others.

6.3.2 Studies with HIO in aquafeeds

The evaluation of HIO in aquafeeds is still in the initial stages, and to the best of our knowledge, only 12 studies have been published so far addressing the effects of dietary HIO inclusion (**Table 1.3**). Based on the published literature, results suggest that HIO has great potential to replace FO and VO in diets for fish, as shown in Jian carp (Li et al. 2016), rainbow trout (Dumas et al. 2018; Fawole et al. 2021), and mirror carp (Xu et al. 2021). In these studies, no detrimental effects on growth performance and feed efficiency

were observed in fish fed with diets including HIO. HIO has also shown high lipid digestibility for Atlantic salmon (Belghit et al. 2018).

Some studies also suggest that HIO has the potential to improve the antioxidant status (Fawole et al. 2021; Gou et al. 2023) and reduce fat deposition and plasma glucose content in several fish species (**Table 1.3**) (Li et al. 2016, Dumas et al. 2018; Fawole et al. 2021, Gou et al. 2023). It was also shown that dietary HIO modulates the muscle FA profile of fish, increasing its content in SFA, lauric acid, and n-6 PUFA but reducing n-3 PUFA, EPA and DHA levels (Li et al. 2016; Fawole et al. 2021; Maldonado-Othón et al. 2022).

Table 1.3: Published studies with the inclusion of HIO and the main effects observed regarding growth and feed efficiency, digestibility, digestive enzymes and intermediary metabolism enzymes, plasma metabolites, oxidative stress, and inflammatory and immune response.

Species	Ingredient replaced[b]	Inclusion % (Substitution %)[c]	Main results (% HIO inclusion) [tissue][d]	Reference
Atlantic salmon (*Salmo salar*)	Rapeseed oil	**12 (100)** or **4.8***	= FBW; = ADC_{EPA}, ADC_{DHA}, ADC_{PUFA} ↓ cholesterol [plasma and liver]	Belghit et al. (2018)
Barramundi (*Lates calcarifer*)	FO	**10.5 (30)**	= FBW, EG, FCR = plasma glucose, cholesterol ↑ *il-1β* expression [liver] = SFA, n-3/n-6 PUFA, EPA, DHA; ↑ LA [muscle]	Hender et al. (2021)
Gilthead seabream (*Sparus aurata*)	VO mix	4 (42), 7.9 (84), **9.5 (100)**	= FBW, WG; ↑ FE, PER =ADC_L, ADC_{EPA}, ADC_{DHA}; ↑ADC_E, ADC_{SFA}, ADC_{LA} ↑ Total proteases	Moutinho et al. (2023) Present thesis
			↑ SFA, lauric acid, ↓ MUFA, PUFA, = EPA, DHA [muscle] ↓ lipid peroxidation [muscle]	Moutinho et al. (2024a) Present thesis
Jian carp (*Cyprinus carpio* var. Jian)	SBO	6.25 (25), 12.5 (50), 18.8 (75), **25 (100)**	= FBW, WG, FCR ↑ SFA, LA, DHA; = EPA [muscle] = plasma metabolites ↑ *pparα* expression [intraperitoneal fat] ↓ fat deposition	Li et al. (2016)
Mirror carp (*Cyprinus carpio* var. specularis)	**	2.5 (-)	↑ FBW, WG; ↓ FCR ↓ intraperitoneal fat; ↑ *pparα* [adipose tissue] ↑ SOD, = CAT [liver]; ↑ *il-10* expression [liver]	Xu et al. (2020)
	SBO	6.25 (25), 12.5 (50), 18.8 (75), **25 (100)*** **	↑ FBW, WG; ↓FCR (> 12.5%) = SFA, LA; ↑ EPA, DHA, n-3PUFA; ↓ n-6PUFA [muscle] ↑ Protein, TG, LDL [plasma]; ↓ lipids [liver] ↑ *sod*, *pparα*, *cpt1* expression (> 12.5%) [liver] ↑ *il-1β*, *tnfα*; ↓ *il-10* expression [head kidney]	Xu et al. (2021)
Onychostoma macrolepis	FO	2.25 (25), **4.5 (50)**, 9 (100)	↓ FBW, WG; = FCR (9%) ↑ *pparα, cpt1* expression [liver] ↓ fat deposition (9%) ↑ SOD, ↓ MDA [liver]	Gou et al. (2023)

Table 1.3 (cont.): Published studies with the inclusion of HIO and the main effects observed regarding growth and feed efficiency, digestibility, digestive enzymes and intermediary metabolism enzymes, plasma metabolites, oxidative stress, and inflammatory and immune response.

Species	Ingredient replaced[a]	Inclusion % (Substitution %)[b]	Main results[c]	Reference
Rainbow trout (*Oncorhynchus mykiss*)	FO	**16 (100)**	= FBW, WG, FCR ↑ SFA, n-6 PUFA, ↓ EPA, DHA, = n-3 PUFA [muscle] ↑Δ*5 desaturase*, *fabp*, *fas* expression [liver] ↑ SOD; = CAT, GPX [plasma] ↓ plasma glucose	Fawole et al. (2021)
	SBO	**16 (100)**	= FBW, WG, FCR ↑ SFA, n-6 PUFA; = EPA, DHA, n-3 PUFA [muscle] = Δ*5 desaturase*, *fabp*, *fas* expression [liver] ↑ SOD; = CAT, GPX [plasma]; = plasma glucose	
	FO	2.5 (25), 5 (50), **10 (100)**	= FBW, WG, FCR ↓ glucose [plasma]; = ADC_L	Dumas et al. (2018)
	FO or SBO	**16 (100)**	↑ *il-8, tnf* expression [kidney] ↑ serum peroxidase	Kumar et al. (2021)
Red hybrid tilapia (*Oreochromis* sp.)	FO	**1.9 (25)**, 3.8 (50), 5.7 (75), 7.6 (100)	↓ FBW, WG; = FCR ↓ ADC_L (7.6 %); ↑ plasma cholesterol	Bakar et al. (2021)
Striped Catfish (*Pangasianodon hypophthalmus*)	FO	**2.3 (100)**	= FBW, WG, FCR ↓ EPA, DHA, PUFA, MUFA; ↑SFA [whole-body] = digestive enzymes = plasma metabolites	Sudha et al. (2022)
Totoaba *(Totoaba macdonaldi)*	FO	**2 (30),** 4 (60)	↓ FBW, WG (4%) ↑ SFA, LA, n-6 PUFA; ↓ EPA, n-3 PUFA [muscle]	Maldonado-Othón et al. (2022)

[a]FO: fish oil, SBO: soybean oil, VO: Vegetable oil; [b] The recommended inclusion levels by the authors are underlined and marked in bold; [c] Symbols represent increased (↑), reduced (↓), or no effect (=) relative to control diet; ADC_{DHA}: DHA apparent digestibility coefficient; ADC_E: energy apparent digestibility coefficient, ADC_{EPA}: EPA apparent digestibility coefficient, ADC_L: lipid apparent digestibility coefficient, ADC_{LA}: lauric acid apparent digestibility coefficient, ADC_{PUFA}: PUFA apparent digestibility coefficient, ADC_{SFA}: SFA apparent digestibility coefficient, CAT: catalase, *cpt1*: carnitine palmitoyltransferase 1, DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid, *fabp*: fatty acid binding protein, *fas*: fatty acid synthase, FBW: final body weight, FCR: feed conversion ratio, FE: Feed efficiency, GPX: glutathione peroxidase, *il-10*: interleukin 10, *il-1β*: interleukin 1 beta, LA: lauric acid, LDL: low-density lipoprotein, MDA: malondialdehyde, MUFA: monounsaturated fatty acids, *myod*: myogenic determination factor, *myog*: myogenin, *mtsn*: myostatin, *ppara*: peroxisome proliferator-activated receptor alpha, PUFA: polyunsaturated fatty acids, SFA: saturated fatty acids, SOD: superoxide dismutase, TG: triglycerides, *tnfa*: tumor necrosis factor alpha, WG: weight gain.; *two different HIO sources, alone or in combination with HM, ** comparison with other insect oils, not replacing an ingredient; ***n-3 PUFA enriched HIO

7. Gilthead seabream (*Sparus aurata*)

7.1 Biology

The gilthead seabream (*Sparus aurata*) is a marine finfish species belonging to the class Actinopterygii, order Perciformes, and family Sparidae (**Figure 1.8**). It is naturally distributed in the Mediterranean Sea and along the Eastern Atlantic Sea, from the British Isles to Cape Verde, and around the Canary Islands. Being eurythermal (tolerating 2-32°C) and euryhaline (3‰ to full strength sea water) species, juveniles can be found along the coastal inshore waters, seagrass beds, estuaries, and sandy bottoms, with depths reaching up to 150 m. The gilthead seabream is a solitary fish although it can form small schools. Spawning occurs in the open sea from October to December. Being a protandrous hermaphrodite species, fish function as males during the first and second years of life, transitioning to females in the third year.

Figure 1.8: Gilthead seabream (*Sparus aurata*). Source: FAO

7.2 Production and consumption

Most of the gilthead seabream available commercially is derived from aquaculture (97%), with wild catches accounting for only 3% of global production (EUMOFA 2022). In 2021, global aquaculture production of gilthead seabream was estimated at 319.2 thousand tonnes, with Turkey, Greece, and Egypt being the main producers (FAO 2023) (**Figure 1.9**). The gilthead seabream is one of the main farmed finfish species within the EU, with a total volume of 93,131 tonnes in 2020, corresponding to a value of 432.49 million euros (APROMAR, 2022). It was the 4th most-produced finfish species in marine and coastal aquaculture worldwide (FAO 2022), and Portugal was responsible for 2000 tonnes produced in 2020, representing 2% of the total EU production of gilthead seabream (FEAP 2021).

Gilthead seabream is a highly appreciated fish species in EU markets. In 2019, apparent consumption was estimated at 111,780 tonnes, with Italy, Spain, Portugal, and France

being the main consumers (EUMOFA 2022). The average first sale price for seabream in the EU is around 5.5/6 €/kg but has been declining over the years.

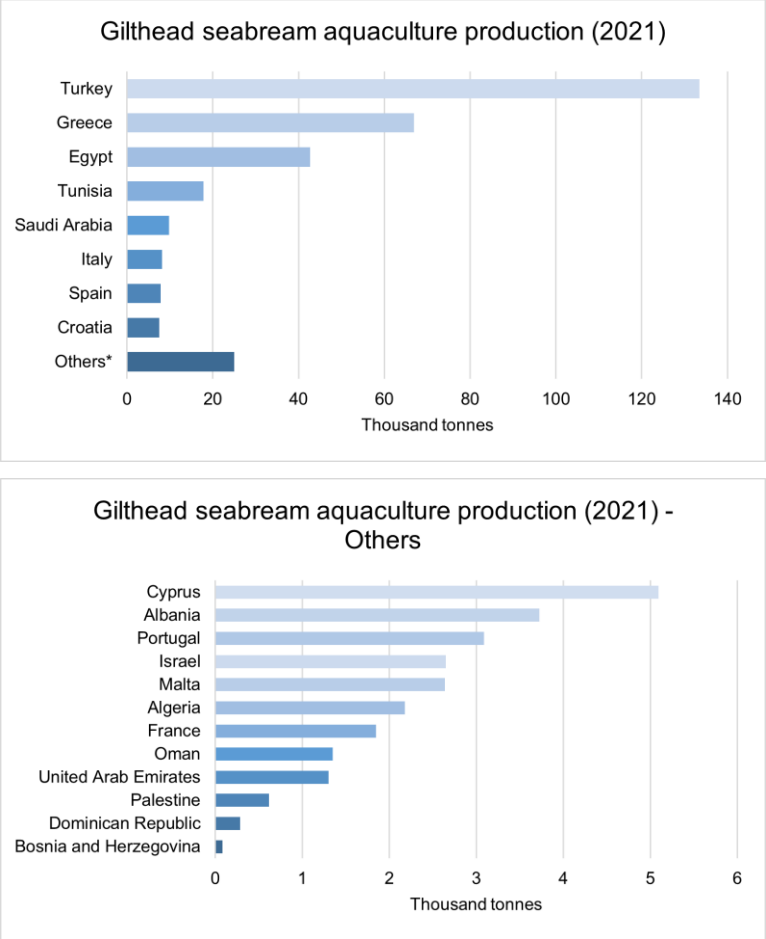

Figure 1.9: Gilthead seabream (*Sparus aurata*) global aquaculture production (2021). Source: FishstatJ (FAO 2023*)*

7.3 Nutritional requirements

In the wild, the gilthead seabream is mostly a carnivorous species, feeding on shellfish, gastropods and bivalves (Pita et al. 2002), thus requiring feeds with a high protein content (>40%) (Oliva-Teles 2000). However, this requirement varies depending on the life stage, with high dietary protein being needed during early life-cycle stages of development.

Fish do not have specific protein requirements, but instead require AA, which are the building blocks of proteins and play important physiological roles in growth and well-being. Gilthead seabream requires the same 10 EAA as other fish (Oliva-Teles 2000). Based on the “ideal protein” concept, the quantitative EAA for gilthead seabream was estimated at (g 16 g N^{-1}): 3.1-5.6 for arginine, 1.9-3.5 for histidine, 1.1-2.6 for isoleucine,

4.8-5.3 for leucine, 5.1 for lysine, 2.4-2.6 for methionine, 3.2-5.8 for phenylalanine + tyrosine, 1.4-3.0 for threonine, 0.8-0.9 for tryptophan, and 2.7-3.2 for valine (Kaushik 1998; Peres and Oliva-Teles 2009). Based on dose-response studies, gilthead seabream EAA requirement is 5 g/16 g N for lysine, 4 g/16 g N for methionine + cystine, 0.6 g/16 g N for tryptophan and 2.6 g/16 g N for arginine (Luquet and Sabaut 1974; Marcouli et al. 2006)

Lipids are also important components in gilthead seabream diets, as they not only supply energy but are also a source of EFA with an important role in several biological functions (Sargent et al. 1999). It has been estimated that the optimal dietary level for gilthead seabream increased with fish growth, ranging from 15-16% for fingerlings (Fountoulaki et al. 2005), 16-21% for juveniles (Santinha et al. 1999; Houston et al. 2017, 2022), and 22-28% for adult fish (Vergara et al. 1999). As for other marine fish species, gilthead seabream also requires n-3 LC-PUFAs in their diets (Tocher 2010; NRC 2011) since they have limited ability to synthesize them from their C_{18} precursors (Monroig et al. 2022). Requirements of n-3 LC-PUFA for gilthead seabream juveniles were estimated to range from 0.5 to 1.9% (Kalogeropoulos et al. 1992; Ibeas et al. 1997), but more recent studies conducted with current commercially relevant formulations suggested a slightly higher n-3 LC-PUFA requirements (2% EPA+DHA) (Houston et al. 2017, 2022).

Fish, like other vertebrates, do not have specific carbohydrate requirements. Carbohydrates are used in aquafeeds as a cheap and strategic energy source, allowing the sparing of more expensive macronutrients such as protein and lipids for other biological purposes. However, carnivorous fish species, like gilthead seabream, have limited capability to utilize dietary carbohydrates (NRC 2011; Oliva-Teles et al. 2015). Thus, it is suggested that dietary carbohydrate inclusion should be limited to 20%, as higher levels have been shown to impair growth and feed utilization (Fernández et al. 2007; Couto et al. 2008; Enes et al. 2011) and affect digestibility and absorption capacity (Enes et al. 2011; NRC 2011).

Data on vitamins and mineral requirements for gilthead seabream are scarce. Morris et al. (1995) described the importance of B-complex vitamins on the growth and feed efficiency of gilthead seabream and the pathologies associated with their dietary deficiencies. The authors recommended the inclusion of 5 g kg^{-1} of vitamin B complex in diets for juveniles, including 70 mg kg^{-1} of thiamine, 208 mg kg^{-1} of riboflavin, 49 mg kg^{-1} of pyridoxine, 800 mg kg^{-1} of niacin, 305 mg kg^{-1} of pantothenic acid, 300 mg kg^{-1} of biotin, and 17 mg kg^{-1} diet of folic acid. Ascorbic acid (vitamin C) requirements were recommended to be less than 25 mg kg^{-1} diet for juveniles (Henrique et al. 1998) and

more recently, Sivagurunathan et al. (2022) recommended the inclusion of 25 to 30 µg kg^{-1} of vitamin D3 to improve survival and reduce incidence of skeletal anomalies in gilthead seabream larvae. Regarding mineral requirements, they are generally less studied due to being inexpensive components of aquafeed formulations (NRC 2011). Nevertheless, for juvenile fish, phosphorus requirements were estimated at 0.75% (Pimentel-Rodrigues and Oliva-Teles 2001), while selenium and copper levels should be 0.94-1.1 and 5.5 mg kg^{-1}, respectively (Domínguez et al. 2019; Mechlaoui et al. 2019; Domínguez et al. 2020). Zinc inclusion should range between 60 and 300 mg kg^{-1} depending on the year season (Serra et al. 1996; Carpeme et al. 1999), and manganese up to 19 mg kg^{-1} (Dominguez et al. 2020).

8. Objectives and thesis overview

The use of novel feed ingredients from insects has emerged as a promising strategy to reduce the use of marine-derived ingredients in aquafeeds, thus supporting the long-term sustainability of the aquaculture industry. Among the authorized insect species, the black soldier fly (*Hermetia illucens*, HI) appears to be the most promising due to its nutritional quality but also the environmental benefits associated with its production. Despite the increasing number of studies with insect meals in aquafeeds, very little information is available regarding the use of HI meals in diets for gilthead seabream juveniles. Likewise, very little information is available regarding using HIO as an alternative lipid source for aquafeeds. Therefore, the main objective of this thesis was to assess the potential of HI meal and oil as alternative ingredients for gilthead seabream juveniles through a multidisciplinary approach.

Two main approaches were followed. Firstly, HI meal was evaluated as an alternative protein source to FM. A trial was conducted where dietary FM protein was gradually replaced by defatted HI meal (up to 100% substitution, corresponding to a dietary inclusion level of 45%). Results from this trial are described in Chapters 2, 3, and 4. More specifically, results regarding growth performance, feed efficiency, diet digestibility, and economic efficiency are presented in Chapter 2. The effects on muscle growth-related gene expression, intermediary metabolism, digestive enzyme activity, and gut microbiota modulation are presented in Chapter 3. The effects of HI meal inclusion on the immune, inflammatory, and antioxidant response are described in Chapter 4.

The second approach was to evaluate the potential of HIO as a dietary lipid source. To do so, a trial was conducted where a VO blend (20% rapeseed, 30% palm, and 50%

linseed oil) was replaced by increasing levels of HIO (up to 100% substitution, corresponding to a dietary inclusion level of 9.5%), and results from this trial are described in Chapters 5, 6, 7 and 8. Chapter 5 describes the effects on growth performance, feed efficiency, and diet digestibility. The effects on fillet quality and nutritional traits are presented in Chapter 6. The effects on lipid metabolism, liver fatty acid and plasma biochemical profile are presented in Chapter 7, whereas Chapter 8 describes the effects on the immune, inflammatory, oxidative status and microbiota modulation.

Finally, an integrated discussion and the main conclusions of this thesis are presented in Chapter 9.

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Chapter 2: Total fishmeal replacement by defatted *Hermetia illucens* larvae meal in diets for gilthead seabream (*Sparus aurata*) juveniles

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Total fishmeal replacement by defatted *Hermetia illucens* larvae meal in diets for gilthead seabream (*Sparus aurata*) juveniles

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Abstract

This work aimed to evaluate the digestibility of defatted *Hermetia illucens* larvae meal (HM), and its effects as fishmeal (FM) replacement on growth performance, feed and nutrient utilization, and whole-body composition of gilthead seabream (*Sparus aurata*) juveniles. To assess apparent digestibility coefficients (ADC) of defatted HM, a reference-diet (43% crude protein, CP; 17% crude lipid, CL) and a test-diet, in which 30% of the reference diet was replaced with defatted HM, were formulated. To assess the effect of dietary FM replacement, four isonitrogenous (43% CP) and isolipid (18% CL) diets were formulated to include defatted HM at 0 (HM0), 15 (HM15), 30 (HM30), and 45% (HM45), replacing FM at 0, 22, 60, and 100%, respectively. Triplicate groups of fish (initial body weight of 32g) were randomly assigned to the experimental diets and the growth trial lasted for 67 days. The digestibility trials were conducted in a digestibility system, and each diet was tested in triplicate. Protein, lipid, and energy digestibility of defatted HM were high (>74%). Replacement of FM by defatted HM did not compromise the ADC of protein and lipid, while ADC of dry matter and energy increased. Complete dietary replacement of FM by defatted HM had no negative effects on growth performance, feed efficiency, whole-body composition, and protein utilization of gilthead seabream juveniles, establishing the potential of defatted HM as an alternative ingredient for aquafeeds.

Keywords: Insect meal, Black soldier fly, alternative proteins, digestibility, growth

1. Introduction

Aquaculture has a crucial role in providing global food security, given the increasing human population, along with the increasing demand for fish and fish products associated with income growth and changes in dietary habits (Naylor et al., 2021). Between 2001 and 2018, aquaculture production of aquatic animals grew on average 5.3% per year, being one of the fastest-growing food production industries worldwide (FAO, 2020). However, as aquaculture production of fed species increases, the need for raw materials also increases. Fishmeal (FM) has been regarded as the ideal protein source for fish, particularly for carnivorous species, due to its high protein content and balanced amino acid profile, high quantities of minerals, and essential fatty acids (Oliva-Teles et al., 2015). However, due to increasing demand and environmental concerns regarding overexploitation of natural fish stocks, FM production will not be able to meet the expected growth of aquaculture for the upcoming years (Gasco et al., 2018). Therefore, research efforts have been shifted towards replacing marine-derived ingredients with more practical and sustainable protein alternatives.

Plant feedstuffs have been regarded as a FM alternative as they are widely available and have a steady production. However, their use in carnivorous fish diets is limited due to their overall lower protein content, essential amino acid imbalances, and high fiber content. The inclusion of plant feedstuffs has been linked to lower growth performance and feed efficiency, and intestinal inflammation (Oliva-Teles, 2000; Oliva-Teles et al., 2015). There is also concern about their environmental sustainability as the production of these commodities demands high land and water usage (Tschirner and Kloas, 2017), while also competing with human consumption.

Due to their low water footprint and low land requirements (Tschirner and Kloas, 2017), insects have been receiving attention as candidates to partially or completely replace FM in aquafeeds (Vargas-Abúndez et al., 2019; Wang et al., 2019). In 2017, the EU authorized processed insects in feeds for aquaculture animals (Regulation 2017/893/EC, 2017) and more recently, for pigs and poultry (Regulation EU 2021/1372). Insects are easy to breed and can efficiently convert biowastes such as animal manure, food waste, agri-food side streams, and other organic wastes, into high-value biomass, being relevant within the circular bioeconomy concept (Diener et al., 2009). Amongst the authorized insect species to be used in fish feeds, the black soldier fly (*Hermetia illucens*; HI) is one of the most promising candidates (van Huis et al., 2013). HI larvae meals (HM) are rich in protein (35-46% DM), with an amino acid profile similar to that of FM, and are also a good source of vitamins, minerals, and lipids (up to 30% DM) (Henry et al., 2015).

However, HM fatty acid (FA) profile usually lacks essential n-3 long-chain ($\geq C_{20}$) polyunsaturated FAs, which are required by marine fish, and is rich in saturated FAs (particularly in lauric acid, C12:0). Thus, the use of partially defatted HM (lipid content < 10%) as feedstuff has become standard practice, as it reduces the feedstuff's composition variation, lipid oxidation risk, and changes of fish fillet FA profile (Dumas et al., 2018; Stamer, 2015).

Chitin is the primary component of the exoskeleton of insects, and its quantity varies depending on the insect species and development stage (Finke, 2007). Chitin and derivate products obtained by hydrolyses, such as chitosan, glucosamine, and N-acetyl-glucosamine, have been shown to positively affect several biological mechanisms in fish, including immune and oxidative responses, gut health, and microbiota diversity (Huyben et al., 2019). However, since most monogastric animals cannot digest chitin, it has also been linked to reduced nutrient bioavailability, ultimately affecting feed utilization (Belforti et al., 2015; Dumas et al., 2018). Thus, it is generally accepted that chitin is one of the major factors limiting insect meal use in aquafeeds (Fischer et al., 2022; van Huis et al., 2015).

Previous studies have demonstrated the potential use of HI larvae or pre-pupae meals as an ingredient for several fish species, including European seabass (*Dicentrarchus labrax*) (Abdel-Tawwab et al., 2020; Magalhães et al., 2017), Atlantic salmon (*Salmo salar*) (Belghit et al., 2019; Bruni et al., 2020), rainbow trout (*Oncorhynchus mykiss*) (Dumas et al., 2018; Renna et al., 2017), Eurasian perch (*Perca fluviatilis*) (Stejskal et al., 2020), turbot (*Psetta maxima*) (Kroeckel et al., 2012), and Japanese seabass (*Lateolabrax japonicus*) (Wang et al., 2019). However, the results regarding the maximum inclusion level have been inconsistent.

For gilthead seabream (*Sparus aurata*), previous studies have evaluated mealworm (*Tenebrio molitor*) (Antonopoulou et al., 2019; Iaconisi et al., 2019; Piccolo et al., 2017) and full-fat HM and pre-pupae HI meals (Karapanagiotidis et al., 2014; Fabrikov et al. 2020) as a dietary ingredient. However, the effects of defatted HM as a dietary FM alternative are yet to be addressed. Therefore, this study aimed to evaluate the dietary FM replacement with defatted HM and its effects on growth performance, feed utilization, body composition, and nutrient digestibility.

2. Materials and methods

2.1 Ethics statement

All procedures were performed by accredited scientists (following FELASA category C recommendations). The trials were conducted per the European Union Directive (2010/63/EU) on animal protection for scientific purposes and approved by the Ethics Committee of CIIMAR (reference ORBEA_CIIMAR_27_2019).

2.2 Ingredients and experimental diets

Ingredients were supplied by Sorgal, S.A. (Ovar, Portugal), and defatted *H. illucens* larvae meal (HM) by Hermetia Baruth GmbH (Baruth/Mark, Germany). *H. illucens* larvae were reared in a substrate composed of a mixture of vegetable ingredients for 8 days, after which they were separated from the frass by sieving. After drying, the fat was separated by a mechanical process using a screw mill and the leftover mass was milled with a hammer mill.

To assess the digestibility of defatted HM (digestibility trial I), two diets were formulated: a reference diet containing 43% crude protein and 17% crude lipid, and a test diet in which 30% of the reference diet was replaced with defatted HM and to which chromium oxide was added as inert marker (Table 2.1).

To assess the potential replacement of FM by defatted HM, four experimental diets were formulated to be isonitrogenous (43% crude protein) and isolipidic (18% crude lipid), meeting gilthead seabream's nutritional requirements (NRC, 2011). The control diet (HM0) included 34% fishmeal (FM) and no defatted HM; three other diets were formulated to include 15, 30, and 45% defatted HM, replacing 22, 60, and 100% of FM protein (diets HM15, HM30, and HM45, respectively). Chromium oxide (Cr_2O_3) was used as a digestibility marker and incorporated into the diets at 0.5% (digestibility trial II). All ingredients were finely ground before mixing and dry pelleted in a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA) through a 3 mm matrix. Pellets were dried in an oven at 55 °C for 24 h and stored at −20 °C until use. Proximate analysis and dietary composition of the diets are presented in Table 2.1.

Table 2.1: Ingredients (% DM) and proximate composition (% DM) of the experimental diets used in the growth and digestibility trials.

	Digestibility trial I		Growth and digestibility trial II			
	Reference diet	**Test diet**	**HM0**	**HM15**	**HM30**	**HM45**
Defatted HM[1]	-	30.0	-	15.0	30.0	45.0
Fishmeal[2]	39.2	27.4	34.3	26.7	13.6	0.0
Hemoglobin[3]	4.0	2.8	4.0	4.0	4.0	4.0
Corn gluten[4]	9.8	6.9	11.8	7.7	7.7	7.7
Whole wheat[5]	28.9	20.2	30.2	26.1	21.9	17.9
Shrimp hydrolysate[6]	0	0	1.5	1.5	1.5	1.5
Fish oil[7]	13.6	9.55	13.7	13.9	14.5	15.1
Vitamins premix[8]	1.0	0.7	1.0	1.0	1.0	1.0
Minerals premix[9]	1.0	0.7	1.0	1.0	1.0	1.0
Choline	0.5	0.35	0.5	0.5	0.5	0.5
Binder[10]	0.5	0.35	0.5	0.5	0.5	0.5
Betaine	0.2	0.14	0.2	0.2	0.2	0.2
Soy lecithin	0.5	0.35	0.5	0.5	0.5	0.5
Calcium biphosphate[11]	-	-	-	0.5	1.6	2.7
Taurine	0.3	0.21	0.3	0.3	0.3	0.3
Arginine	-	-	-	-	0.4	0.8
Methionine	-	-	-	0.1	0.3	0.5
Lysine	-	-	-	-	-	0.3
Chromium oxide†	0.5	0.35	0.5	0.5	0.5	0.5
Dry matter	93.7	94.0	95.6	95.4	95.6	96.3
Total nitrogen (N)	6.79	7.70	6.88	6.96	6.94	7.28
Crude Protein (N x 6.25)	42.4	48.1	43.0	43.5	43.4	45.5
Crude Protein (N x 5.62)	-	43.3	-	39.1	39.0	40.9
Crude Lipid	16.9	10.8	17.8	17.8	18.1	18.0
Energy (kJ kg^{-1})	21.7	21.0	22.8	21.3	22.2	22.6
Ash	10.5	10.7	8.77	9.23	8.94	8.74
Chitin[12]	-	-	0	0.93	1.86	2.79

[1]Defatted HM used in trial I: 96.6 % DM, 63.2 % CP (N x 6.25), 55.5 % CP (N x 5.62); 4.7 % CL, 12.1 % Ash; defatted HM used in trial II: 93.3 % DM, 61.8 %CP, 6.8 %CL, 11.9 % Ash, 21.6 kJ g^{-1} Energy, 6.2 % chitin); Hermetia Bartuh GmbH, Germany

[2]Fishmeal used in trial I: 71 % CP, 9.3 % CL; Fishmeal used in trial II: 73 g kg^{-1} CP, 89 g kg^{-1} CL, 150 g kg^{-1} Ash, 197 kJ kg^{-1} Energy; Sorgal, S.A. Ovar, Portugal

[3]Hemoglobin powder AP310P; APC Europe S.A.

[4]Corn gluten meal (79.5 % CP, 29 % CL); Sorgal, S.A. Ovar, Portugal

[5]Whole wheat used in trial I: 14.5 % CP, 1.7 % CL; Whole wheat used in trial II: 11.4 % CP, 1.8 % CL; Sorgal, S.A. Ovar, Portugal

[6]Shrimp hydrolysate (*Litopenaeus vannamei* trimmings 73 % CP, 12.1 % CL; 13 % Ash); Sorgal, S.A. Ovar, Portugal

[7]Fish oil (from tuna, sardine, and mackerel trimmings); Sorgal, S.A. Ovar, Portugal

[8]Vitamin premix (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

[9]Mineral premix (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)

[10]Binder: Carboxymethylcellulose sodium. Sigma-Aldrich Química, Portugal

[11]Calcium biphosphate, Sorgal, S.A. Ovar, Portugal

[12]Chitin content of diets was estimated from the chitin content of defatted HM

†Chromium oxide was only incorporated in the diets used in the digestibility trials

2.3 Fish and growth trial

Gilthead seabream juveniles were provided by Sonríonansa S.L. (Pesués, Spain) and were quarantined for two weeks after arriving at the experimental facilities at CIIMAR. After the quarantine period, fish were transferred and acclimatized to the experimental systems for further two weeks. The experimental setup consisted of a thermo-regulated recirculation seawater system equipped with twelve 300 L fiberglass tanks, each supplied with continuous aeration and water flow. During the trial, the temperature averaged 23.0 ± 0.5 °C, salinity 35 ‰, dissolved oxygen was kept at 8 mg L^{-1}, and nitrogenous compounds were kept below 0.02 mg L^{-1}. Photoperiod was set to 12 h light: 12 h dark. At the beginning of the growth trial, five fish were collected and pooled for initial whole-body composition analysis. Then, gilthead seabream juveniles with an initial body weight of 32 g were homogeneously distributed into the tanks (13 fish/tank). Diets were randomly assigned to triplicate groups, and fish were hand-fed twice a day (9 am and 3 pm), six days a week, until apparent visual satiation for 67 days. All feed consumption was recorded for each tank and uttermost care was taken to avoid feed waste and to ensure all feed supplied was consumed.

At the end of the growth trial, fish were fasted for one day and then bulk weighed after mild anaesthesia (0.3 ml L^{-1} ethylene glycol monophenyl ether), and the total weight was recorded for each tank. Three fish per tank were euthanized by a sharp blow to the head and collected for whole body composition analysis. The liver and viscera were excised and weighed to calculate hepatosomatic and visceral indexes.

2.4 Digestibility trial I and trial II

Two digestibility trials were performed: one to determine the digestibility of defatted HM (trial I), and another to assess the diet's digestibility (trial II). Both trials were conducted in a thermo-regulated recirculation seawater system, equipped with 12 fiberglass tanks (60 L capacity), each with continuous aeration and water flow. Each tank was connected to a feces settling column, designed according to Cho et al. (1982), and water parameters were maintained as described above for the growth trial. Homogenous groups of 10 fish were allocated in each tank, and diets were tested in triplicate. Fish were hand-fed twice a day to apparent visual satiation. After five days of adaption to the experimental diets, the fecal collection was carried out as follows: approximately 30 min after the afternoon meal, tanks, pipes, and settling columns were thoroughly cleaned to assure no uneaten food or feces remained in the system. The next day, before the morning meal, feces were collected from the settling column, centrifugated at 4000 g for 10 min, and pooled for each tank. The fecal collection was performed daily for 21 days.

2.4 Chemical analysis

Ingredients, diets, whole-body, and feces were ground and homogenized before analysis. Whole-body samples were ground to approximately 400 microns using a commercial coffee grinder. All samples were analysed according to the AOAC (2000) procedures: dry matter by drying in an oven at 105 ºC until constant weight, ash by combustion in a muffle furnace at 450 ºC for 16 h, crude protein (N x 6.25 or N x 5.62, according to Janssen *et al.* 2017) by the Kjeldahl method following acid digestion, using the Kjeltec digestion and distillation units (Tecator Systems, Höganäs, Sweden; models 1015 and 1026, respectively), crude lipid by extraction with petroleum ether using a Soxtec system (Tecator Systems, Höganäs, Sweden; extraction unit model 1043 and service unit model 1046), and gross energy by direct combustion of samples in an adiabatic bomb calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261). Chromium oxide (Cr_2O_3) of diets and feces was determined according to Bolin *et al*. (1952). Chitin of defatted HM was determined as described by Han and Heinonen (2021). Chitin analysis was not performed on diets and feces due to the interference with carbohydrates (i.e., hexosamines and aldehydes) present in the plant ingredients (Han and Heinonen, 2021).

2.5 Calculations

Growth performance and feed efficiency were calculated as follows: Feed efficiency (FE) = wet weight gain / dry feed intake; Daily growth index (DGI) = ((final weight$^{1/3}$ − initial weight$^{1/3}$) / days) × 100; Protein efficiency ratio (PER) = wet weight gain / crude protein intake. Hepatosomatic and visceral index were evaluated using the following formulas: Hepatosomatic index (HSI) = (liver weight (g) / fish wet weight (g)) × 100; Visceral index (VSI) = (visceral weight (g) / fish wet weight (g)) × 100. Nitrogen and energy retention (%intake) was calculated as follows: Nitrogen (or energy) retention (g kg^{-1} day^{-1}) / nitrogen (or energy) intake (g kg^{-1} day^{-1}) x 100. The apparent digestibility coefficients (ADCs) of insect meal were calculated according to Bureau et al. (1999): $ADC_{test\ ingredient} = ADC_{test\ diet} + [(ADC_{test\ diet} - ADC_{ref.\ diet}) \times 0.7 \times D_{ref} / 0.3 \times D_{ingr})]$, where $D_{ref.diet}$ is the % nutrient (or kJ g^{-1}) of reference diet (dry matter basis) and $D_{ingr.}$ is the % nutrient (or kJ g^{-1}) of test ingredient (dry matter basis). The ADCs (%) of dry matter, protein, lipids, and energy of the experimental diets were calculated as follows: $ADC_{diet} = [1- ((diet\ Cr_2O_3 \times Y\ in\ feces) / (feces\ Cr_2O_3 \times Y\ in\ diets))] \times 100$, where Y is the nutrient or energy content. Fish in-fish out (FIFO) ratio was used as a practical method to determine dependence on wild capture fisheries of feeds. FIFO was calculated according to Jackson (2009), where FIFO = (level of fishmeal in the diet + level of fish oil in the diet) / (yield of fishmeal

derived from wild-caught fish + yield of fish oil derived from wild-caught fish) x FCR. It was considered a global average wet weight (whole fish) to fishmeal yield of 22.5% and wet weight to fish oil yield of 5% (Jackson 2009).

2.6 Economic analysis

The Economic Conversion Ratio (ECR) was calculated as follows:

$$ECR\ (€\ kg\ of\ fish^{-1}) = FCR\ (kg\ diet\ kg\ of\ fish^{-1}) \times diet\ price\ (€\ kg\ of\ diet^{-1}).$$

The price of each diet was calculated by multiplying the respective contributions of each ingredient by their respective cost per kg and summing all ingredients for each formulated diet. The prices of the major dietary constituents were the following: fishmeal 1.26€/kg, defatted HM 2€/kg (Llagostera *et al.* 2019), corn gluten 1.12€/kg, whole-wheat 0.3€/kg and fish oil 1.75€/kg. The Economic profit index (EPI) was calculated according to Moutinho *et al*. (2017) as follows:

$$EPI\ (€\ fish^{-1}) = [weight\ gain\ (kg) \times selling\ price] - [weight\ gain\ (kg) \times diet\ price\ (€\ kg\ of\ diet^{-1})],$$

where gilthead seabream sale price was calculated at 4.5€ kg^{-1}.

2.7 Statistical analysis

Data are presented as mean (n=3) and pooled standard error of the mean (SEM). All data were checked for normal distribution and homogeneity of variances and normalized when appropriate. One-way ANOVA was used to assess differences between treatments. Polynomial contrasts analysis was performed to determine if data followed a linear or quadratic response to dietary defatted HM inclusion. The probability level of 0.05 was used to reject the null hypothesis, and significant differences amongst means were determined by Tukey's multiple range test. All statistical analyses were performed using SPSS 25.0 software package (IBM Corp.) for Windows.

3. Results

3.1 Diets

All diets were well accepted by the fish and were comparable in terms of dry matter (DM), crude protein, crude lipids, and energy content, fulfilling the nutritional requirements of gilthead seabream. The chitin content of defatted HM was determined to be 6.2 % DM, corresponding to 0.93, 1.86 and, 2.79 % for diets HM15, HM30, and HM45, respectively.

3.2 Growth performance and feed efficiency

At the end of the growth trial, fish tripled their initial body weight, reaching an average of 96.5 g, without being significantly affected by dietary treatment (Table 2.2). Feed efficiency was unaffected by the experimental diets, as well as daily growth index and protein efficiency ratio, averaging 0.79, 2.11 and 1.78, respectively (Table 2.2). Feed intake, averaging 19.1 g DM kg^{-1} day^{-1}, was also unaffected by dietary treatment (ANOVA p-value > 0.05), but polynomial regression showed a quadratic trend, increasing in fish fed diet HM15 ($p < 0.05$, Table 2). Mortality during the trial was low (n=2) and only occurred in the control group due to a technical failure (Table 2.2).

Table 2.2: Growth performance, feed utilization efficiency, nitrogen and energy retention (% nitrogen or energy intake), and the hepatosomatic and visceral indexes of gilthead seabream fed the experimental diets.

						Polynomial contrasts		
	HM0	**HM15**	**HM30**	**HM45**	**SEM**	**ANOVA *P*-value**	**Linear *P*-value**	**Quadratic *P*-value**
IBW (g)[1]	32.0	32.0	32.1	32.0	0.02	ns	ns	ns
FBW (g)[2]	97.3	97.8	97.0	94.0	1.39	ns	ns	ns
WG (g kg^{-1} day^{-1})[3]	15.0	15.1	15.0	14.7	0.17	ns	ns	ns
FI (g DM kg^{-1} day^{-1})[4]	18.8	19.8	19.2	18.5	0.25	ns	ns	0.020
FE[5]	0.80	0.77	0.78	0.79	0.01	ns	ns	ns
DGI[6]	2.12	2.14	2.12	2.04	0.03	ns	ns	ns
PER[7]	1.87	1.82	1.80	1.79	0.03	ns	ns	ns
Survival (%)	93.4	100	100	100	1.12	ns	0.028	ns
N retention (% intake)	30.8	29.3	28.2	28.9	0.57	ns	ns	ns
E retention (% intake)	38.2	39.0	38.3	35.9	0.76	ns	ns	ns
HSI[8]	1.86	1.86	1.91	1.62	0.06	0.073	ns	ns
VSI[9]	7.48	7.43	7.44	7.03	0.13	ns	ns	ns

Values are presented as mean (n=3). SEM: Pooled standard error of the mean. ns: non-significant *P*-value ($p < 0.05$). [1]Initial body weight; [2]Final body weight; [3]Wight gain; [4]Feed intake; [5]Feed efficiency; [6]Daily growth index; [7]Protein efficiency ratio; [8]Hepatosomatic index, [9]Visceral index

3.3 Whole-body composition and nutrient retention

Nitrogen and energy retentions were unaffected by dietary composition, averaging 29.3, and 37.9 (% intake), respectively (Table 2.3). Similarly, the hepatosomatic (average of 1.8) and visceral (average of 7.3) indexes were not affected by the experimental diets (Table 2.3). Also, the inclusion of defatted HM had no effects on whole-body composition, with dry matter content averaging 65 %, crude protein 15.9 % (% fresh weight, FW), crude lipid 13.2 % (% FW), ash 3.7 % (% FW), and energy 9.2 kJ g^{-1} (% FW) (Table 2.3).

Table 2.3: Whole-body composition of gilthead seabream fed the experimental diets (% fresh weight).

						Polynomial contrasts		
	HM0	**HM15**	**HM30**	**HM45**	**SEM**	**ANOVA *P*-value**	**Linear *P*-value**	**Quadratic *P*-value**
Moisture	67.0	64.0	64.8	64.9	0.70	ns	ns	ns
Crude Protein	15.9	16.1	15.6	16.1	0.13	ns	ns	ns
Crude Lipid	13.7	13.4	13.4	12.3	0.27	ns	ns	ns
Ash	3.61	3.56	3.77	3.90	0.07	ns	ns	ns
Energy (kJ kg^{-1})	9.35	9.37	9.36	8.91	0.11	ns	ns	ns

Values are presented as mean (n=3). SEM: Pooled standard error of the mean. ns: non-significant *P*-value ($p < 0.05$)

3.4 Apparent digestibility coefficients

The apparent digestibility coefficients (ADCs) of protein and lipid of defatted HM were high (85 and 79 %, respectively), while that of dry matter and energy were slightly lower (70 and 74 %, respectively) (Table 2.4). The ADCs of protein and lipids of the experimental diets were unaffected by the inclusion of defatted HM, averaging 90 and 89%, respectively (Table 2.4). However, the ADC of energy followed a linear trend and significantly increased in the HM-containing diets (76, 78, and 78 %, for diets HM15, HM30, and HM45, respectively), compared to the control diet (66 %). The ADC of dry matter also followed a linear trend, increasing with the dietary inclusion of HM, being significantly higher in diets HM30 (70 %) and HM45 (72 %) than the control (57 %).

Table 2.4: Apparent digestibility coefficients (ADC, %) of nutrients and energy of defatted *Hermetia illucens* larvae meal (HM) and the experimental diets.

							Polynomial contrasts		
	Defatted HM*	**HM0**	**HM15**	**HM30**	**HM45**	**SEM**	**ANOVA *P*-value**	**Linear *P*-value**	**Quadratic *P*-value**
Dry matter	70.0 ± 2.3	57.0^{b}	65.9^{ab}	69.6^{a}	71.6^{a}	1.97	0.010	0.002	ns
Protein	84.4 ± 0.5	89.7	90.2	90.0	89.6	0.22	ns	ns	ns
Lipids	79.1 ± 4.6	89.0	89.9	88.8	89.7	0.34	ns	ns	ns
Energy	74.2 ± 0.9	66.6^{b}	76.6^{a}	78.1^{a}	78.5^{a}	1.65	0.004	0.002	0.026

Values for diets ADCs are presented as mean (n=3) and pooled standard error of the mean (SEM). Different superscript letters indicate significant differences between experimental diets (ANOVA *P*-value < 0.05). ns: non-significant *P*-value ($p < 0.05$). Values for ingredients ADC are presented as mean (n=3) ± standard error of the mean. *ADCs of defatted HM were not included in the statistical analysis.

3.5 Economic analysis and Fish in:Fish Out (FIFO)

Results from the economic analysis and FIFO ratio are presented in Table 2.5. The inclusion of defatted HM led to a significant increase in ECR compared to the control diet (1.43), being the highest with diet HM45 (2.13), followed by diets HM15 (1.66) and HM30 (1.87). Compared to the control diet, fish fed diets HM15 and HM30 had similar EPI values (corresponding to 0.23, 0.21, and 0.20, respectively), while fish fed diet HM45

had a significantly lower value (0.17) compared to the control diet. Additionally, FIFO ratio significantly decreased with the increase of defatted HM, corresponding to a reduction of 15% (diet HM15), 43% (diet HM30), and 70% (diet HM45) when compared to the control diet (HM0).

Table 2.5: Sustainability and economic analysis of gilthead seabream production at the end of the trial.

						Polynomial contrasts		
	HM0	**HM15**	**HM30**	**HM45**	**SEM**	**ANOVA *P*-value**	**Linear *P*-value**	**Quadratic *P*-value**
Diet price (€ kg^{-1})	1.09	1.26	1.47	1.68	-	-	-	-
ECR (€ kg^{-1})[1]	1.43^{c}	1.66^{b}	1.87^{b}	2.13^{a}	0.08	0.000	0.000	ns
EPI (€ fish^{-1})[2]	0.23^{a}	0.21^{ab}	0.20^{ab}	0.17^{b}	0.01	0.035	0.005	ns
FIFO[3]	2.29^{a}	1.95^{b}	1.30^{c}	0.70^{d}	0.19	0.000	0.000	ns

Values are presented as mean (n=3). SEM: Pooled standard error of the mean. ns: ns: non-significant *P*-value ($p < 0.05$).
[1]Economic Conversion Ratio; [2]Economic Profit Index; [3]Fish in:Fish Out ratio

4. Discussion

With the increasing demand for alternative feedstuffs and the environmental benefits associated with insect meal production, it is vital to understand its suitability as an alternative ingredient to FM in aquafeeds. Currently, in the European Union, there are 7 authorized insect species to be used in aquafeeds, such as Common Housefly (*Musca domestica*), Yellow Mealworm (*Tenebrio molitor*), House cricket (*Acheta domesticus*), and Black Soldier Fly (*Hermetia illucens*, HI) (EU Commission Regulation 2017/893). The latter, HI, is one of the most promising species due to the superior feed conversion ratios and the high protein and lipid content of its larvae (Wang and Shelomi, 2017). Up to now, at least 26 studies were published regarding FM replacement with HI larvae or pre-pupae meals in farmed fish, with approximately 85 % of them (22 studies) being published in the last four years. However, results regarding maximum inclusion levels have shown significant discrepancies (Table 6). Differences in dietary formulations, rearing conditions, species-specific physiological responses, and quality of insect meals, itself dependent upon the insect's life stage, rearing substrate, and processing techniques (e.g., full-fat or defatted), might account for the apparent discrepancies of these studies with the present study. Even for a given insect meal product, nutritional quality has been shown to vary between products and batches (Wang and Shelomi, 2017).

Maximum dietary HM inclusion levels range from as low as 5.3 % to as high as 40 % (Li et al. 2017; Stekjkal et al. 2020) (Table 5). For instance, in FM-based diets, it was shown that defatted HI meals could replace 45-50 % FM (19.5 - 40.0 % HM inclusion) without

affecting the growth performance of European seabass and rainbow trout (Magalhães et al., 2017; Renna et al., 2017). On the other hand, 25 % or 39 % FM replacement (18.5 % and 33 % inclusion) negatively affected the growth of Siberian sturgeon, *Acipenser baerii*, and juvenile turbot, respectively (Caimi et al. 2020; Kroeckel et al., 2012), and in meagre (*Argirosomus regius*), growth linearly decreased with dietary increasing levels of defatted HM (Guerreiro et al., 2020). On the other hand, high dietary FM replacement levels were successfully achieved in Japanese seabass where defatted HM replaced 64 % of FM (19.2 % inclusion) without affecting growth (Wang et al., 2019).

Regarding full-fat HI larvae or pre-pupae meals, moderate (15-16 %) dietary inclusion levels promoted a decrease in growth performance of rainbow trout (St-Hilaire et al., 2007) and Atlantic salmon (Weththasinghe et al., 2021), while 14.8 % inclusion (replacing 50% of dietary FM protein) had no effects on growth performance of European seabass (Abdel-Tawwab et al., 2020).

Results of the present study showed that total dietary replacement of FM (included at 34% in the control diet) by defatted HM did not compromise growth performance, feed intake, and feed efficiency of gilthead seabream juveniles. This corresponded to a dietary inclusion level of 45 % of HM, indicating that gilthead sea bream seems to tolerate well insect meal as a feed ingredient.

Previously, total FM substitution with HM has also been achieved for Jian carp (*Cyprinus carpio*) (Zhou et al., 2018) and Atlantic salmon (Belghit et al., 2019), although at lower dietary HM inclusion levels than in the present study. The HM inclusion level in the present study is, to the best of our knowledge, the highest to be achieved without compromising fish growth (Table 2.6). A high dietary HM inclusion level was also achieved for Eurasian perch, where 42 % of partially defatted HM (replacing 40% of FM) did not affect growth (Stejskal et al., 2020).

Partly contradicting our results, replacing up to 30 % of FM with full-fat HI meal or pre-pupae meal (corresponding to an 11 % and 27 % inclusion, respectively) reduced the growth of gilthead seabream juveniles, although feed efficiency was not affected (Fabrikov et al. 2020; Karapanagiotidis et al., 2014). Karapanagiotidis et al., (2014) attributed this growth performance impairment to a reduction in feed intake, indicating that palatability might have been an issue. On the other hand, diets used in Karapanagiotidis et al. (2014) were not isolipidic, so it is difficult to attribute the observed results to HM inclusion alone. Decreased feed acceptance was also reported for turbot juveniles (Kroeckel et al., 2012) and Siberian sturgeon (Caimi et al., 2020). In present study however, no differences were observed in gilthead seabream's feed intake,

indicating that the inclusion of defatted HM had no effects on diet's palatability, even at 100% FM substitution. In Atlantic salmon, feed intake was not also affected by the inclusion of HM at 100% FM substitution (Belghit et al., 2018), and it was reported that defatted HM could even improve feed palatability, increasing feed intake of Japanese seabass (Wang et al., 2019).

In the present study, dietary inclusion of defatted HM did not affect the whole-body proximate composition nor the hepatosomatic and visceral indexes of gilthead seabream juveniles. Similar results were also previously reported for rainbow trout (Devic et al., 2018) and European seabass (Abdel-Tawwab et al., 2020). In Japanese seabass, whole-body crude protein and lipid contents were also unaffected, although ash content decreased with dietary defatted HM inclusion (Wang et al., 2019). Reduced ash was also observed in meagre (Guerreiro et al., 2020) and rainbow trout muscle (Renna et al., 2017) without changes in protein content. On the contrary, total FM replacement with HM reduced rainbow trout's whole-body protein and lipid content, and compromised protein deposition and efficiency (Dumas et al., 2018).

In the present study, protein digestibility of defatted HM was higher, but lipid digestibility was lower than previously reported for European seabass (Basto et al., 2020) and Atlantic salmon (Fisher et al., 2020). It is generally accepted that insect meal chitin may be responsible for reducing nutrient digestibility, particularly that of protein and lipids fractions (Belforti et al., 2016; Caimi et al., 2020; Dumas et al., 2018; Weththasinghe et al., 2021). Due to its binding properties, chitin can decrease the accessibility of digestive enzymes to the substrates or, being undigestible, increase bulk and transit time, thus decreasing the time available for digestion (Kroeckel et al., 2012). This was not the case in the present study, as both protein and lipid digestibility of the experimental diets was not compromised by the dietary inclusion of defatted HM. This is similar to what was already reported for other species, such as European seabass (Basto et al., 2020; Magalhães et al., 2017) and Atlantic salmon (Belghit et al., 2019). On the other hand, Piccolo et al. (2017) reported a reduction in nutrients' digestibility when gilthead seabream juveniles were fed diets including full-fat *T. molitor* larvae meal.

Present results also showed an increase in dry matter and energy digestibility in the HM-diets. This may, however, be related to the decrease of dietary whole wheat inclusion level rather than the increased dietary inclusion of defatted HM. These results may also be, at least in part, attributed to the enzymatic hydrolysis of chitin resulting in an overall energy gain from the feed, as suggested by Gasco et al. (2016) evaluating digestibility of full-fat *T. molitor* larvae meal for European seabass. Chitinase activity, either through

endogenous production or exogenous production by gut bacteria, has been reported for several marine species (Danulat and Kausch, 1984; Fines and Holt, 2010; Gutowska et al., 2004), but it has not been detected in the intestine turbot fed defatted HI pre-pupae meal (Kroeckel et al., 2012). Crustaceans are part of gilthead seabream's natural diet, so some chitinase activity may be expected. However, no reports on the chitinase activity of gilthead seabream have been published to the author's knowledge and should be explored in further studies involving high-chitin ingredients such as insect meals. These effects on digestibility may also be partially attributed to the modulation of intestinal microbiota. Studies have reported improved gut microbiota diversity of fish when fed diets with chitin or insect meals (Askarian et al., 2012; Huyben et al., 2019; Rimoldi et al., 2019). In fact, chitin can act as a substrate for chitinase-producing bacteria not commonly found in the fish gut (Askarian et al., 2012; Bruni et al., 2018). Thus, diets with HI larvae and pre-pupae meal increased the proliferation of chitinolytic bacteria in rainbow trout (Huyben et al., 2019). Unfortunately, no reports on the effects of insect meals on gilthead seabream gut microbiota are yet available.

It is well known that the downward trend in the use of marine feed resources is essential for the sustainable growth of fed aquaculture production (Tacon et al., 2021). Insect products are widely recognized as more ecologically sustainable alternatives, since their production requires fewer resources and has a lower carbon footprint than conventional feedstuffs, such as soybean meal and other crops (Tacon et al., 2021; Tschirner and Kloas, 2017). The fish in-fish out (FIFO) ratio is a valuable indicator of the dependence on wild capture fisheries for aquaculture production, and a ratio of <1 indicates no net removal of fish from natural stocks (Rawski et al., 2021). Our study reported FIFO values of the HM-containing diets of 1.9, 1.3, and 0.7, suggesting that inclusion of defatted HM in diets for gilthead seabream juveniles arises as a promising strategy to enhance sustainability without affecting growth performance and feed utilization parameters. Indeed, a reduction of 70 % of the FIFO ratio was achieved with total FM substitution. In order words, with total FM substitution with defatted HM, it would be possible to produce 1 kg of fish with just 0.7 kg of wild-caught fish. These results are also supported by Stejskal *et al*. (2020), which reported a remarkable decrease in FIFO production in Eurasian perch fed partially defatted HM, and even lower ratios were obtained by Rawski et al. (2021) for Siberian sturgeon using full-fat HM. Along replacing FM as explored in the present study, it is also important to note that further studies are required to investigate the combined effects of reducing dietary fish oil, not only to improve sustainability targets as estimated by FIFO but also to avoid volatility of costs associated to increased demand by the FO supplement industry (Tocher et al., 2019).

Feeding costs can represent 40-70% of the total cost of production (Henry *et al.* 2015) and so, feed practices should be optimized to ensure profitability and lower economic losses. Despite their potential for aquafeeds, current insect meal prices are generally still not competitive with other protein sources (Mulazzani et al., 2020) and are dependent on insect species, live stages, quality and quantity, manufacturing processes, etc. (Llagostera et al. 2019). In the present trial, the diet price and ECR (i.e., feed cost to produce 1 kg of fish) increased with the inclusion of defatted HM. In previous economic analysis, the inclusion of 20 to 60% defatted HM also increased ECR for European perch (Stejskal et al. 2020) while for Siberian sturgeon, diets with 10% and 15% full-fat HM meal inclusion improved profitability by reducing ECR (Rawski et al. 2021). EPI is suggested as a more suitable parameter to evaluate economic profitability, as it considers production, feed costs, and selling price (Moutinho et al. 2017). In the present trial, despite the higher diet price and ECR, similar EPI was observed for diets HM15 and HM30 compared to the FM-based control diet, suggesting a similar economic return when defatted HM was included up to 30%. For European perch, similar EPI was also achieved with 40% defatted HM inclusion (Stejskal et al. 2020) and more than 10% full-fat HM significantly improved profitability of Siberian sturgeon (Rawski et al. 2021). To support the development of a sustainable feed industry through the inclusion of insect meals, the solution must rely on increasing the scale of production, encouraging an increase in price competitiveness and production stability of insect feeds over other protein sources (Arru et al. 2019). Nevertheless, insect production is a fast-growing sector, and it is estimated that the price of insect proteins will become competitive in the coming years (Arru et al. 2019; Mulazzani et al., 2020).

The present study shows that, in diets for gilthead seabream juveniles, it is possible to completely replace FM (at 34% in the control diet) with defatted HM without affecting growth, feed efficiency, and protein digestibility and utilization. This study also showed that high dietary incorporation levels of defatted HM (at least up to 45%) did not affect diet's palatability nor compromised digestibility and reduced FIFO ratio compared to the FM-based diet. These are promising results to the industry as it supports the development and optimization of insect meal production. Providing that price is competitive, feed costs would be reduced by reducing FM in marine carnivorous fish diets while also supporting the sustainable intensification of aquaculture. However, further studies are required to further evaluate the effects of high dietary HM inclusion levels in fish health and welfare, gut microbiota diversity, and fillet composition and acceptability.

Table 2.6: Compilation of studies using *Hermetia illucens* (HI) larvae or prepupae meals as fishmeal (FM) replacement.

Species	IBW (g)[a]	FF DF PDF[b]	HI %CP (%DM)[c]	HI %CL (%DM)[d]	Max. % of HI inclusion	Max. % of FM replacement	Author
African catfish (*Clarias gariepinus*)	4.0	FF	42.6	23	17.2	75	Fawole et al. (2020)
Atlantic salmon (*Salmo salar*)	34.0	FF	42	32	16.1	14	Weththasinghe et al. (2021)
	1398.0	DF	*	*	14.8	100	Belghit et al. (2019)
Eurasian Perch (*Perca fluviatilis)*	21.9	PDF	55.3	17.9	40	42	Stejskal et al. (2020)
European seabass (*Dicentrarchus labrax*)	12.1	FF	46.4	19.7	14.8	50	Abdel-Tawwab et al. (2020)
	50.0	DF	55.8	5.5	19.5	45	Magalhães et al. (2017)
	10.7	FF	*	*	<10.9	<30	Reyes et al. (2020)
Gilthead seabream (*Sparus aurata*)	1.5	FF	31.6	27.2	27.6	30	Karapanagiotidis et al. (2014)
	6.7	FF	40.1	33.9	5.6	15	Fabrikov et al. (2020)
	32.0	DF	61.8	6.8	45	100	Present study
Japanese seabass (*Lateolabrax japonicus*)	14.1	DF	55.4	1.6	19.2	64	Wang et al. (2019)
Jian carp (*Cyprinus carpio*)	34.0	DF	51	*	5.3	50	Li et al. (2017)
	10.9	*	*	*	14	100	Zhou et al. (2018)
Lemon fin bard hybrid (*Hypsibarbus wetmorei* ♂ X *Barbodes gonionotus* ♀)	1.52	DF	60.1	5.6	7.5	75	Kamarudin et al. (2021)
Meagre (*Argyrosomus regius*)	18.0	DF	55.4	10.9	<10	<17	Guerreiro et al. (2020)
Nile tilapia (*Oreochromis niloticus*)	5.7	FF	41.6	23.2	8	7	Devic et al. (2018)
Rainbow trout (*Oncorhynchus mykiss*)	45.0	FF	47.1	20.3	13.2	50	Dumas et al. (2018)
	178.0	DF	55.3	17.8	40	50	Renna et al. (2017)

Table 2.6 (cont.): Compilation of studies using *Hermetia illucens* (HI) larvae or prepupae meals as fishmeal (FM) replacement.

Species	IBW (g)[a]	FF DF PDF[b]	HI %CP (%DM)[c]	HI %CL (%DM)[d]	Max. % of HI inclusion	Max. % of FM replacement	Author
Rainbow trout (*Oncorhynchus mykiss*)	22.6	FF	43.6	33.1	15	25	St-Hilaire et al. (2007)
	120.0	*	*	*	*	50	Stamer et al. (2014)
	55.0	FF	30	33.9	10.9	30	Melenchón et al. (2020)
Rice Field Eel (*Monopterus albus*)	24.0	FF	32.1	32.2	10.5	5	Hu et al. (2020)
Siberian sturgeon (*Acipenser baerii*)	24.2	DF	65.8	4.2	18.5	25	Caimi et al. (2020)
	14.4	FF	35.0	29.8	30	61	Rawski et al. (2020)
Turbot (*Psetta maxima*)	54.9	DF	47.6	11.8	33	39	Kroeckel et al. (2012)
Yellow catfish (*Pelteobagrus fulvidraco*)	1.2	*	*	*	11.3	20	Hu et al. (2017)
	48.5	FF	47	17.7	22.3	40	Xiao et al. (2018)

[a]Initial body weight, [b]Full-fat (FF), defatted (DF) or partially defatted (PDF) HI meal, [c]Crude protein content of HI meal, [d]Crude lipid content of HI meal, *Data not indicated by the authors. The maximum level of HI larvae or prepupae meal was that recommended by the authors. If the authors did not recommend any level, the highest HI meal inclusion with similar performance to the non-HI meal control diet was considered.

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Chapter 3: Use of black soldier fly (*Hermetia illucens*) larvae meal in diets for gilthead seabream juveniles: effects on growth-related gene expression, intermediary metabolism, digestive enzymes, and gut microbiota modulation

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Use of black soldier fly (*Hermetia illucens*) larvae meal in diets for gilthead seabream juveniles: effects on growth-related gene expression, intermediary metabolism, digestive enzymes, and gut microbiota modulation

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Abstract

A study was conducted to evaluate the effects of dietary inclusion of defatted *Hermetia illucens* larvae meal (HM) on the expression of growth-related genes, plasma metabolites, activity of intermediary metabolism enzymes, and gut microbiota modulation in gilthead seabream (*Sparus aurata*) juveniles. Four experimental diets were formulated (43% crude protein, 18% crude lipids) with increasing levels of defatted HM: 0 (HM0), 15 (HM15), 30 (HM30), and 45% (HM45) replacing fishmeal (included at 34% in the control diet) at 0, 22, 60, and 100%, respectively. Fish were fed these diets for 67 days. At the end of the trial, the expression of muscle growth hormone receptors (*ghr1* and *ghr2*), insulin-like growth factors (*igf1* and *igf2*), cathepsins (*cts-a* and *cts-b*), and myogenic determination factor 1 (*myod1*) were not affected, while a downregulation of myogenic determination factor 2 (*myod2*) and upregulation of myostatin (*mstn*) was observed with HM inclusion. Plasma metabolites profile was unaffected by diet composition, except for a linear decrease trend in total lipids. A linear increase of liver alanine aminotransferase (ALT) and glutamate dehydrogenase (GDH) activity and a linear decrease of glucose 6-phosphate dehydrogenase (G6PDH), malic enzyme (ME), and (FAS) activity were observed with HM inclusion. The activity of β-hydroxy acyl-CoA dehydrogenase (HOAD) followed a quadratic trend, being higher in fish fed with diet HM15 than in the control. Likewise, amylase, trypsin, and chymotrypsin activity were higher in the posterior intestine of fish fed with diet HM15. Digesta of fish fed the HM-containing diets showed an increase in the number of operational taxonomic units, microbial richness, and diversity. Conversely, a reduction in these indexes was observed in the mucosa. Among the identified bacteria, Firmicutes represented the predominant phyla. The inclusion of HM increased the abundance of *Oceanobacillus, Lederbergia, Bacillus,* and

Corynebacterium in the digesta while *Lactobacillus* and *Caldalkalibacillus* abundance was reduced. Overall, total FM replacement with defatted HM had no major effects on plasma metabolites and muscle growth-related genes but induced a few differences in intermediary metabolism and digestive enzymes activity. HM inclusion improved gut digesta microbiota richness and diversity, promoting the growth of potential beneficial bacteria.

Keywords: *Sparus aurata*, alternative protein source, muscle growth, gut microbiome, insect meal

1. Introduction

With the increasing human population and demand for fish products, aquaculture has a crucial role in providing global food security, promoting economic development, and preservation of oceans and freshwater resources (FAO 2022; Tacon 2022). However, the growth of aquaculture production must be accompanied by sustainable feed practices. Traditionally, fishmeal (FM) has been recognized as the ideal protein source for carnivorous fish species due to its balanced amino acid profile, high palatability, and digestibility (Oliva-Teles et al. 2015). However, shortage in supply, increased prices, and the environmental impacts associated with FM production have led to substantial efforts in finding alternative protein sources.

Over the past decade, the interest in using insects as an alternative ingredient for animal feeds has been increasing (Tran et al. 2022). Insects are easy to breed, have fast growth and high conversion rates, low land and water requirements, and can convert waste and by-products into valuable and nutritious final products, thus taking part in a circular bioeconomy (Diener et al. 2009). Moreover, insects can be farmed locally, thus reducing the dependency on imports from other countries, which can be affected by social and political issues, disrupting the supply and production chain, besides reducing the carbon footprint related to transportation.

There are currently eight authorized insect species for use in aquaculture feeds in the European Union (Commission Regulation (EU) 2021/1925). Among these, the black soldier fly (*Hermetia illucens*) stands as one of the most promising species (van Huis et al. 2013). Black soldier fly larvae meal (HM) is characterized by a high protein content (30–60% dry matter), a well-balanced amino acid profile, and high amounts of vitamins and minerals (Liland et al. 2021). HM is rich in lipids (up to 30% dry matter) (Henry et al. 2015) but defatting has become standard practice to reduce ingredient composition

variability and increase the protein content (Maulu et al. 2022). HM also contains bioactive compounds, such as chitin, lauric acid, and antimicrobial peptides, which have been shown to improve lipid utilization and immune and antioxidant status in animals (Muller et al. 2017; Gasco et al. 2018; Weththasinghe et al. 2021).

The potential use of HM in aquafeeds has already been established for several fish species of high economic interest including European seabass, *Dicentrarchus labrax* (Magalhães et al. 2017*)*, rainbow trout, *Oncorhynchus mykiss* (St-Hilaire et al. 2007; Melenchón et al. 2020), Atlantic salmon, *Salmo salar* (Belghit et al. 2018; Belghit et al. 2019; Weththasinghe et al. 2021), Japanese seabass, *Lateolabrax japonicus* (Wang et al. 2019) and gilthead seabream, *Sparus aurata* (Moutinho et al. 2022). In these studies, partial or total fish meal substitution by HM was achieved without detrimental effects on growth performance and feed efficiency. Nonetheless, the absence of differences at the somatic level may undercover physiological differences in intermediary metabolism or the molecular processes regulating muscle growth (Chemello et al. 2021). The skeletal muscle comprises over 60% of the total body mass of teleost fish (Alami-Durante et al. 2010; Ramos-Pinto et al. 2019), and its growth is a complex and dynamic process that can be affected by diet composition, including changes in the dietary protein profiles introduced by the use of alternative ingredients (Chemello et al. 2021). Thus, understanding the potential interactions between dietary components and muscle growth mechanisms is crucial for the optimization of fish growth and ultimately enhancing the nutritional quality of farmed fish for consumers.

Further, previous studies have shown that dietary inclusion of insect meal may affect intermediary metabolism in different species, such as European seabass (Basto et al. 2023), rainbow trout (Chemello et al. 2020), gilthead seabream (Mastoraki et al. 2022) and meagre, *Argyrosomus regius* (Guerreiro et al. 2020). Understanding the potential effects of dietary inclusion of insect meals on fish metabolism is also mandatory to improve fish nutrition (Panserat et al. 2019), as it may compromise fish growth performance and nutrient utilization and affect fish health in the long term.

Diet is considered a major driver in shaping gut microbiota in animals (Terova et al. 2019; Weththasinghe et al. 2022), and gut microbiota has been increasingly recognized as playing a crucial role in the nutrition, immune status, and overall health of fish (Weththasinghe et al. 2022). Previous studies have highlighted that several bioactive compounds present in insects can modulate microbiota (Gasco et al. 2018; Huyben et al. 2019). For instance, chitin was shown to have prebiotic properties in fish, promoting a balanced and healthy gut microbiota (Terova et al. 2019; Rangel et al. 2022a) and

increasing chitinase-producing bacteria, that are not commonly found in fish gut (Askarian et al. 2012; Ringø et al. 2012; Rangel et al. 2022b). Thus, it is important to evaluate the impact of dietary alterations on the composition of gut microbiota, as they have the potential to disrupt nutrition, metabolism, and the overall welfare of fish.

Previously, we have shown that it was possible to completely replace fish meal in the diets of gilthead sea bream juveniles by including 45% defatted HM, without detrimental effects on fish growth performance and feed efficiency (Moutinho et al. 2022). The present study aims to build upon this previous research and provide insights into the effects of the dietary inclusion of defatted HM on the expression of genes related to muscle growth, the activity of key enzymes of intermediary metabolism, digestive enzymes, and gut microbiota modulation.

2. Materials and methods

2.1 Ethics statement

All procedures were performed by accredited scientists (following the FELASA category C recommendations). The trial was conducted per the European Union Directive (2010/63/EU) on animal protection for scientific purposes and approved by the Ethics Committee of CIIMAR (reference ORBEA_CIIMAR_27_2019).

2.2 Experimental conditions and sampling

Four experimental diets were formulated (43% crude protein, 18% crude lipids) according to gilthead seabream's nutritional requirements (NRC 2011). A control diet (HM0) contained 34% FM as the main protein source. The other experimental diets were formulated to replace FM with defatted HM at 22, 60, and 100%, corresponding to 15, 30, and 45% HM inclusion levels (diets HM15, HM30, and HM45, respectively). The FM used contained 73.4% crude protein, and 8.9% crude lipids and the defatted HM contained 61.8% crude protein and 6.8% crude lipids. All ingredients were finely ground, well mixed, and dry pelleted in a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA) through a 3 mm matrix. Pellets were dried in an oven (55 °C for 24 h) and stored at -20 °C until use. The proximate composition of ingredients and diets is presented in Table 3.1.

Table 3.1: Formulation and proximate composition of experimental diets and ingredients.

	HM[1]	HM0	HM15	HM30	HM45
Defatted *Hermetia illucens* prepupae larvae meal[1]		-	15	30	45
Fishmeal[2]		34.3	26.7	13.6	0.0
Hemoglobin[3]		4	4	4	4
Corn gluten[4]		11.8	7.7	7.7	7.7
Whole wheat[5]		30.1	26.1	22.0	18.0
Shrimp hydrolysate[6]		1.5	1.5	1.5	1.5
Fish oil[7]		13.7	13.9	14.5	15.1
Vitamins premix[8]		1	1	1	1
Minerals premix[9]		1	1	1	1
Choline		0.5	0.5	0.5	0.5
Binder[10]		1	1	1	1
Betaine		0.2	0.2	0.2	0.2
Soy lecithin		0.5	0.5	0.5	0.5
Calcium biphosphate[11]		-	0.5	1.6	2.7
Taurine		0.3	0.3	0.3	0.3
Arginine		-	-	0.35	0.8
Methionine		-	0.1	0.3	0.5
Lysine		-	-	-	0.3
Dry matter (%)	93.3	95.6	95.4	95.6	96.3
Crude Protein (N x 6.25) (%)	61.8	43.0	43.5	43.4	45.5
Crude Lipid (%)	6.8	17.8	17.8	18.1	18.0
Energy (kJ g^{-1})	11.9	22.8	21.3	22.2	22.6
Ash (%)	21.6	8.77	9.23	8.94	8.74
Chitin (%)[12]	6.20	0	0.93	1.86	2.79

[1]Defatted *Hermetia illucens* prepupae larvae meal; Hermetia Bartuh GmbH, Germany
[2]Fishmeal (87.0% DM, 73.4% CP, 8.9% CL, 15.1% Ash, 19.7 kJ^{-1} Energy), Sorgal, S.A. Ovar, Portugal
[3]Hemoglobin powder AP310P; APC Europe S.A.
[4]Corn gluten meal (90.5% DM, 79.5% CP, 2.9% CL); Sorgal, S.A. Ovar, Portugal
[5]Whole wheat (88% DM, 11.4% CP, 1.8% CL); Sorgal, S.A. Ovar, Portugal
[6]Shrimp hydrolysate (*Litopenaeus vannamei* trimmings; 73% CP, 12.1% CL; 13% Ash); Sorgal, S.A. Ovar, Portugal
[7]Fish oil (tuna, sardine, and mackerel trimmings); Sorgal, S.A. Ovar, Portugal
[8]Vitamin premix (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
[9]Mineral premix (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)
[10]Binder: Carboxymethylcellulose sodium. Sigma-Aldrich Química, Portugal
[11]Calcium biphosphate, Sorgal, S.A. Ovar, Portugal
[12]Estimated from the defatted HM chitin content

The experimental conditions were described previously (Moutinho et al. 2022). Briefly, a feeding trial was conducted in a thermoregulated seawater system (with an average water temperature of 23±0.5ºC), equipped with twelve 300 L fiberglass tanks. Triplicate groups of 13 gilthead seabream juveniles (initial body weight of 32 g) were hand-fed each diet 2 times a day, 6 days a week, to apparent visual satiation, for a total of 67

days. At the end of the trial, 4 hours after the morning meal, 3 fish per tank (n=9) were randomly sampled and blood was collected from the caudal artery-vein complex using heparinized syringes. Blood was immediately centrifuged (10 000 rpm, 10 min) and plasma was collected and stored in aliquots at −20 °C until analysis. Fish were then euthanized with a sharp blow to the head, dissected on chilled trays, and the liver and the digestive tract were excised. The intestine was divided into anterior and posterior sections, in which the posterior intestine was distinguished from the anterior intestine by the increased diameter, darker mucosa and annular rings. The anterior intestine was the portion directly after the stomach and included the pyloric caeca. Intestine and whole liver samples were immediately frozen in liquid nitrogen and stored at -80 °C until analysis. Samples of white skeletal muscle were also collected from the dorsal right side and immediately placed in RNAlater (1:10), left overnight at 4ºC, and stored at -80ºC until RNA extraction.

For microbiota analysis, two fish per tank (n=6 per dietary treatment) were sampled under aseptic conditions (working with an open flame and using sterilized solutions, collecting tubes, and tools). Digesta samples (allochthonous bacteria) were obtained by squeezing the entire intestinal content into a sterile tube. Mucosa samples (autochthonous bacteria) were collected by scraping the internal intestinal mucosal surface after opening the intestine in its longitudinal axis. Samples were immediately frozen in liquid nitrogen and stored at − 80 °C until analysis.

2.3 Plasma biochemistry

The quantification of plasma metabolites was performed using commercial kits from Spinreact, S.A. (Girona, Spain), according to the manufacturer's protocols. For cholesterol (ref. 1001090), triglycerides (ref. 1001312), and total protein (ref. 1001290), plasma samples and standard (provided) were incubated with the corresponding reagent for 5 min at 37ºC. For cholesterol and triglycerides, absorbance was measured at 505 nm, against the blank while total protein was measured at 540 nm. For glucose (ref. 41010), plasma samples and standard (provided) were incubated with the reagent for 10 min at 37ºC, followed by absorbance measurement at 505 nm, against the blank. For total lipids (ref. 1001270), plasma samples and standard (provided) were firstly digested with sulfuric acid at 100ºC for 10 min. After colling, the acid-digested sample and standard were incubated with the reagent at 37ºC, for 15 min and absorbance was measured at 525 nm. All absorbance readings were carried out using a Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, Nanjing, China).

2.4 Intermediary metabolism and digestive enzymes activity

Liver and intestine samples were homogenized (dilution 1:4) in ice-cold buffer (100 mM-Tris-HCl, 0.1 mM-EDTA, 0.1% triton X-100 (v/v), pH 7.8; Sigma). Homogenates were centrifugated (30 min, 30.000 g, 4ºC), and the supernatant was collected and stored in aliquots at -80ºC until analysis.

The activity of intermediary metabolism enzymes was determined as follows: glucose 6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), malic enzyme (ME, EC 1.1.1.40), and fatty acid synthase (FAS; EC 2.3.1.38) activities were assayed as described by Castro et al. (2015). B-hydroxy acyl-CoA dehydrogenase (HOAD, EC 1.1.1.35) and glutamate dehydrogenase (GDH, EC1.4.1.2) activities were assayed as described in Guerreiro et al. (2020). Alanine aminotransferase (ALAT, EC 2.6.1.2), and aspartate aminotransferase (ASAT, EC 2.6.1.1) activities were determined using commercial kits from Spinreact, S.A. (Girona, Spain), according to the manufacturer's protocols (ref. 41280 and 41270, respectively).

Amylase (EC 3.2.1.1) and lipase (EC 3.1.1.3) were performed using commercial kits from Spinreact, S.A. (Girona, Spain), according to the manufacturer's protocols and adjusted for 96-well microplates. For amylase, samples were incubated with the provided reagents for 30 sec and the rate of product formation (2-chloro-4-nitrophenol) was quantified at 405 nm. For lipase, samples were incubated with the provided reagents for 1 min and the formation of methylresorufin was followed at 580 nm. Total protease assay was performed according to Magalhães et al. (2017). Briefly, samples were incubated with a 1% (w/v) casein solution and buffer (0.1 M Tris HCl, pH 8) for 1 hour at 37 °C. Trichloroacetic acid (8% w/v) was used to stop the reaction. After 1 h at 4 °C, samples were centrifuged (1800 *g* for 10 min) and the absorbance read at 280 nm. A tyrosine solution was used to establish a calibration curve. One unit of enzyme activity was defined as the amount of enzyme needed to catalyse the formation of 1.0 µmol of tyrosine min^{-1}. Trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) were determined according to Moutinho et al. (2017), using Nα-benzoyl-DL arginine 4-nitroanilide hydrochloride (0.5 mM BAPNA) or N-succinylAla-Ala-Pro-Phe p-nitroanilide (0.2 mM SAPNA) as substrates, respectively. Briefly, samples were incubated with corresponding substrate and buffer (50 mM Tris-HCl and 20 mM $CaCl_2$, pH 8.5 for trypsin and 25 mM $CaCl_2$, pH 7.8 for chymotrypsin) and the production of p-nitroaniline (molar extinction coefficient, 8.8 $mM^{-1}\,cm^{-1}$) was monitored at 410 nm.

All enzyme assays were carried out at 37 °C and changes in absorbance were monitored using a Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, Nanjing,

China). Enzymatic activity was expressed per mg of tissue soluble protein, determined according to the Bradford method (Bradford 1976), using bovine serum albumin solution as standard.

2.5 Gene expression

Muscle RNA was isolated through homogenization with TRIzol reagent (Direct-zol™ RNA Miniprep, Zymo Research) according to the manufacturer's instructions, in a Precellys evolution apparatus (Bertin Instruments, Montigny-le-Bretonneux, France). RNA concentration was quantified and adjusted to 0.5 µg/8 µL H_2O for complementary DNA synthesis, using the NZY First-Strand cDNA Synthesis Kit (NZYTech. MB12502, Lisbon, Portugal). Gene expression was determined by real-time quantitative PCR analysis (CFX Connect™ Real-Time System, Bio-Rad, Hercules, CA, USA). The mixture contained 0.4 µL diluted cDNA (1:1), 0.2 µL of each primer (10 µM), 5 µL SsoAdvanced Universal SYBR® Green supermix (Bio-Rad), and 4.2 µL DNase/RNase/Protease-free water, in a total volume of 10 µL. The sequences of primers used are presented in Table 3.2. Primer's efficiency was validated with serial two-fold dilutions of cDNA and determined from the slope of the regression line of the quantification cycle (Ct) versus the relative concentration of cDNA (Pfaffl 2001) and only efficiencies between 90 and 110% (slope −3.5 and 3.1, r^2 = 0.99) were accepted. The reaction was initiated with incubation at 95 ºC for 30s for hot-start iTaq™ DNA polymerase activation. A total of forty PCR cycles were performed, each consisting of heating at 95 ºC for 15s for denaturing and 30s for annealing and extension. The annealing temperature of each primer pair is presented in Table 2. Melting curves were systematically monotonized (60 ºC temperature 0.5 ºC $10s^{-1}$ from 60 to 95 ºC). Each PCR run included duplicates of reverse transcription for each sample and negative controls (reverse transcriptase-free samples, RNA-free samples). To normalize the results, previously validated genes, elongation factor 1α (*ef1α*) and *β-actin* were used as reference genes. The expression levels are given as the mean normalized values ± standard error, corresponding to the ratio between copy numbers of the target gene transcripts and the geometric mean of copy numbers of the reference genes (Vandesompele et al. 2002).

Table 3.2: Sequence of primers used for real-time quantitative PCR.

Gene	Sequence 5'-3'	Annealing Temp (ºC)	Efficiency	Accession number
igf1	**F:** TGTCTAGCGCTCTTTCCTTTCA **R:** AGAGGGTGTGGCTACAGGAGATAC	54.4	1.99	AY996779
igf2	**F:** TGGGATCGTAGAGGAGTGTTGT **R:** CTGTAGAGAGGTGGCCGACA	64.0	2.02	AY996778
ghr1	**F:** ACCTGTCAGCCACCACATGA **R:** TCGTGCAGATCTGGGTCGTA	62.0	2.00	AF438176
ghr2	**F:** GAGTGAACCCGGCCTGACAG **R:** GCGGTGGTATCTGATTCATGGT	64.0	1.96	AY573601
myod1	**F:** GTTTTGTTCCAGGCGGTCT **R:** GCTGGTGTCGGTGGAGAT	62.0	2.01	AF478569
myod2	**F:** CACTACAGCGGGGATTCAGAC **R:** CGTTTGCTTCTCCTGGACTC	62.0	1.97	AF478568
mstn	**F:** GTACGACGTGCTGGGAGACG **R:** CGTACGATTCGATTCGCTTG	62.0	2.01	AF258448.1
ctsd-a	**F:** CCTCCATTCACTGCTCCTTC **R:** ACCGGATGGAAAACTCTGTG	59.6	2.10	AF036319
ctsd-b	**F:** AAATTCCGTTCCATCAGACG **R:** CTTCAGGGTTTCTGGAGTGG	64.0	1.92	KJ524456
ef1α	**F:** CTGTCAAGGAAATCCGTCGT **R:** TGACCTGAGCGTTGAAGTTG	59.6	1.84	AF184170
β-actin	**F:** TCCTGCGGAATCCATGAGA **R:** GACGTCGCACTTCATGATGCT	54.4	1.88	X89920

igf1: insulin-like growth factor 1; *igf2*: insulin-like growth factor 2; *ghr1*: growth hormone receptor 1; *ghr2*: growth hormone receptor 2; *myod1*: myogenic determination factor 1; *myod2*: myogenic determination factor 2; *mstn*: myostatin; *ctsd-a:* cathepsins-a; *ctsd-b:* cathepsins-b; *ef1α:* elongation factor 1 alpha; *β-actin*: beta-actin

2.6 Microbial diversity

Bacterial DNA of digesta and mucosa was extracted from 300mg of sample, using the DNeasy PowerSoil Pro Kit (Qiagen). Bacterial 16S rRNA gene fragments were amplified by a touchdown PCR on a T100™ Thermal Cycler (BioRad Laboratories Lda., Amadora, Portugal), using primers 16S-358F (containing a GC clamp at the 5′ end) and 16S-517R (Muyzer et al., 1993). PCR-DGGE conditions were described by Moutinho et al. (2017). Briefly, PCR products were loaded in an 8% acrylamide gel, composed of a denaturing gradient of 30-70% 7 M urea/40% formamide. Electrophoresis (16 hours, 60 ºC, 65V) was performed on a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories Lda.), in 1xTAE buffer, following 1-hour staining of 1xTAE buffer containing SYBR Gold Nucleic Acid Gel Stain (Sigma-Aldrich, Química, S.L., Sintra, Portugal). DGGE gels were imaged on a Gel Doc EZ System (Bio-Rad Laboratories Lda.). Bands

of interest were excised and eluted in 20 ml ultrapure water before DNA re-amplification using the same oligonucleotide primers as previously but without a GC clamp. Amplicons were sequenced (STAB VIDA, Lisboa, Portugal) and phylogenetically analyzed by comparing with the GenBank non-redundant nucleotide database using BLAST (http://www.ncbi.nlm.nih.gov), to identify the closest known species. Only sequences higher than 100 bp reads and 80-100% query coverage were considered valid identification.

2.7 Statistical analysis

Data are presented as mean (n=9) and standard error and were checked for normal distribution and homogeneity of variances and normalized when appropriate. One-way ANOVA was used to assess differences between treatments. Polynomial contrasts were performed to determine if data followed a linear or quadratic response to dietary defatted HM inclusion. The probability level of 0.05 was used to reject the null hypothesis, and significant differences amongst means were determined by Tukey's multiple range test. All statistical analyses were performed using SPSS 27.0 software package (IBM Corp.) for Windows.

DGGE banding patterns were transformed into presence/absence matrices and the band's intensities were measured using Quantity One 1-D Analysis Software v4.6.9 (Bio-Rad Laboratories, Lda. Amadora, Portugal). Relative similarities between dietary treatments and replicates were calculated using Primer software v7.0.5 (PRIMER-E Ltd., Ivybridge, UK). Species richness was assessed using Margalef's measure of richness, species diversity was assessed by the Shannon–Weaver index, and evenness by the Simpson Evenness index. The clustering of DGGE patterns was achieved by the construction of dendrograms using the Unweighted Pair Groups Method with Arithmetic Averages (UPGMA). Parameters were subjected to a one-way ANOVA.

3. Results

Data on growth performance has been published previously (Moutinho et al. 2022). Briefly, the inclusion of defatted HM had no effects growth performance and feed utilization, with fish reaching an average final body weight of 97 g (Table 1a; Supplementary material). Survival rate was high and mortality (n=2) only occurring in the control group due to a technical failure.

3.1 Muscle gene expression

At the end of the growth trial, no differences were found in the muscle expression of growth hormone receptors (*ghr1* and *ghr2*), insulin-like growth factors (*igf1* and *igf2*) nor in the expression of cathepsins (*ctsd-a* and *ctsd-b*), and myogenic determination factor 1 (*myod1*) (Figure 3.1). On the other hand, *myod2* expression was downregulated in fish fed all HM-containing diets compared to those fed the control diet. A linear upregulation was found in myostatin (*mstn*) relative expression with the increase of dietary HM level.

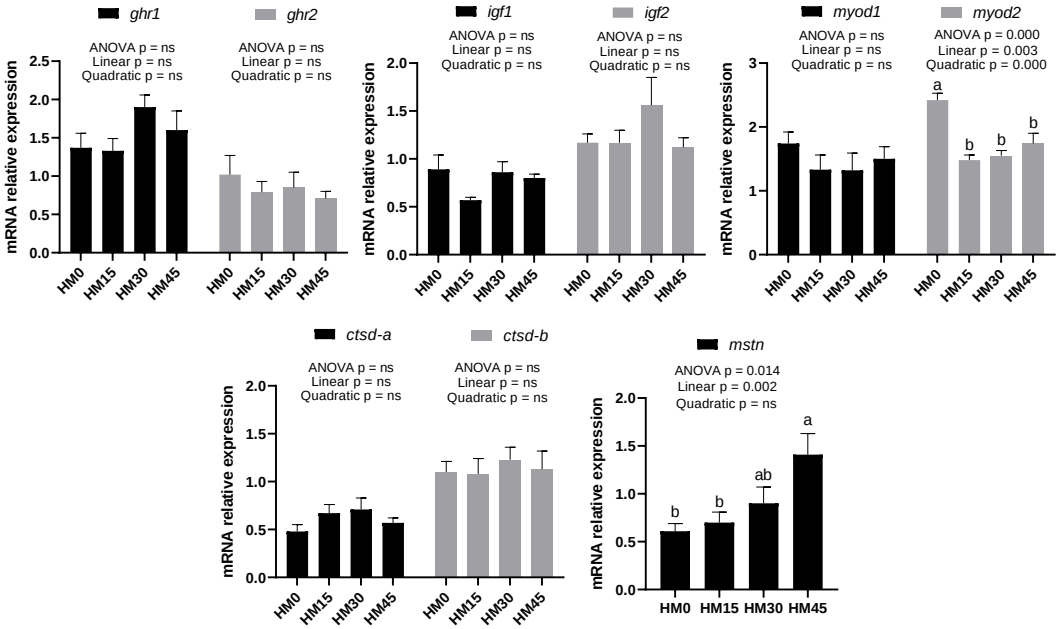

Figure 3.1: Relative expression of genes and markers in the muscle of gilthead seabream fed the experimental diets. Data were normalized with two housekeeping genes, elongation factor 1 alpha (*ef1α*) and *β-actin*. Values are presented as mean (n=9) and standard error represented by error bars. Different superscript letters indicate significant differences between treatments (one-way ANOVA; $p < 0.05$); ns = non-significant p-value.

3.2 Plasma metabolites

No differences were found in plasma cholesterol, glucose, triglycerides, and total protein levels, while total plasma lipid levels followed a decreasing linear trend with increasing dietary HM content (Table 3.3).

3.3 Intermediary metabolism

Regarding intermediary metabolism enzyme activity (Table 3.4), there was a significant linear increase in ALA activity and a trend for linear increase of GDH activity with the increase in dietary HM inclusion level, while no differences were observed in AST

activity. ME and G6PDH activities linearly decreased, and FAS activity followed a linear decreasing trend with dietary HM inclusion level, while HOAD activity followed a quadratic trend, being significantly higher in fish fed the HM15 diet compared to the control.

Table 3.3: Plasma metabolites of gilthead seabream fed the experimental diets.

						Polynomial contrasts		
	HM0	**HM15**	**HM30**	**HM45**	**SEM**	**ANOVA**	**Linear**	**Quadratic**
Cholesterol (nmol L^{-1})	6.52	6.34	5.92	5.83	0.16	ns	ns	ns
Glucose (nmol L^{-1})	3.72	3.87	4.00	3.74	0.09	ns	ns	ns
Total Lipids (g dL^{-1})	4.70	4.23	3.91	3.53	0.18	ns	0.019	ns
Triglycerides (nmol L^{-1})	16.1	16.4	15.1	13.0	0.68	ns	ns	ns
Total Protein (g dL^{-1})	4.53	4.55	4.36	4.32	0.06	ns	ns	ns

Values are presented as mean of n=9. SEM = pooled standard error of the mean. The absence of different superscript letters indicates no significant differences between treatments (one-way ANOVA; $p > 0.05$). ns = non-significant p-value

Table 3.4: Intermediary metabolism enzymes activity (mU mg $protein^{-1}$) of gilthead seabream fed the experimental diets.

						Polynomial contrasts		
	HM0	**HM15**	**HM30**	**HM45**	**SEM**	**ANOVA**	**Linear**	**Quadratic**
ALT[1]	26.9^{b}	35.8^{ab}	31.8^{ab}	38.4^{a}	1.46	0.015	0.010	ns
AST[2]	45.5	42.0	43.5	47.2	1.27	ns	ns	ns
GDH[3]	206.1	240.1	254.1	299.3	13.8	ns	ns	ns
G6PDH[4]	186.3^{a}	168.3^{ab}	160.4^{ab}	138.5^{b}	5.68	0.038	0.006	ns
ME[5]	27.5^{a}	23.6^{ab}	20.2^{ab}	11.9^{b}	1.38	0.004	0.000	ns
FAS[6]	5.90	4.06	3.60	3.53	0.39	ns	0.029	ns
HOAD[7]	8.04^{b}	10.3^{a}	8.87^{ab}	9.28^{ab}	0.24	0.003	ns	0.028

Values are presented as mean of n=9. SEM = pooled standard error of the mean. Different superscript letters indicate significant differences between treatments (one-way ANOVA; $p < 0.05$). ns = non-significant p-value. [1]Alanine aminotransferase; [2]Aspartate aminotransferase; [3]Glutamate dehydrogenase; [4]Glucose 6-phosphate dehydrogenase; [5]Malic enzyme; [6]Fatty acid synthase; [7]β-hydroxyacyl-CoA dehydrogenase

3.4 Digestive enzymes activity

Digestive enzyme activities in the anterior portion of the intestine were not affected by the diets (Table 3.5). On the posterior intestine, lipase and total protease activities were also not affected by the diets, while amylase, trypsin, and chymotrypsin activities were higher in fish fed the HM15 diet than in the control (Table 3.5).

Table 3.5: Digestive enzymes activity (mU mg protein^{-1}) of gilthead seabream fed the experimental diets.

						Polynomial contrasts		
	HM0	**HM15**	**HM30**	**HM45**	**SEM**	**ANOVA**	**Linear**	**Quadratic**
Anterior								
Amylase	211.0	220.6	279.6	204.7	23.1	ns	ns	ns
Lipase	2.04	2.44	2.01	1.74	0.21	ns	ns	ns
Total Proteases	1.45	1.19	1.18	1.16	0.08	ns	ns	ns
Trypsin	131.2	138.3	120.6	116.9	13.0	ns	ns	ns
Chymotrypsin	18.9	18.3	18.2	14.9	1.69	ns	ns	ns
Posterior								
Amylase	391.8^{b}	1084.5^{a}	478.9^{b}	650.8^{b}	70.0	0.000	ns	0.001
Lipase	4.15	6.30	4.80	5.24	0.31	ns	ns	ns
Total Proteases	6.58	7.43	6.35	8.52	0.38	ns	ns	ns
Trypsin	357.1^{b}	525.5^{a}	380.5^{ab}	441.5^{ab}	23.0	0.038	ns	ns
Chymotrypsin	32.4^{b}	57.5^{a}	37.5^{b}	42.8^{ab}	2.44	0.001	ns	0.019

Values are presented as mean of n=9. SEM = pooled standard error of the mean. Different superscript letters indicate significant differences between treatments (one-way ANOVA; $p < 0.05$). ns = non-significant p-value

3.5 Microbiota

The digesta of fish fed the diets including HM showed significantly higher OTUs, richness and diversity indexes than fish fed the control diet, while the opposite was observed in these ecological parameters of the mucosa (Table 3.6).

Table 3.6: Ecological parameters obtained from PCR-DGGE fingerprints of the microbiota found in the intestine recovered from gilthead seabream fed the experimental diets.

						Polynomial contrasts		
	HM0	**HM15**	**HM30**	**HM45**	**SEM**	**ANOVA**	**Linear**	**Quadratic**
Digesta								
OTUs[1]	15.5^{b}	21.0^{a}	21.0^{a}	21.3^{a}	0.63	0.000	0.000	0.001
Richness[2]	0.91^{b}	1.23^{a}	1.22^{a}	1.23^{a}	0.04	0.000	0.000	0.001
Diversity[3]	2.61^{b}	2.99^{a}	2.93^{a}	2.94^{a}	0.04	0.000	0.000	0.000
Evenness[4]	0.92^{b}	0.94^{a}	0.94^{a}	0.94^{a}	0.00	0.000	0.000	0.001
Mucosa								
OTUs[1]	9.67^{a}	7.00^{b}	7.33^{b}	6.83^{b}	0.33	0.001	0.001	0.035
Richness[2]	0.58^{a}	0.40^{b}	0.42^{b}	0.39^{b}	0.02	0.001	0.001	0.027
Diversity[3]	1.96^{a}	1.65^{b}	1.65^{b}	1.64^{b}	0.04	0.013	0.008	ns
Evenness[4]	0.84^{a}	0.79^{ab}	0.78^{ab}	0.77^{b}	0.01	0.027	0.008	ns

Values are presented as mean of n=6. SEM = pooled standard error of the mean. Different superscript letters indicate significant differences between treatments (one-way ANOVA; $P<0.05$). ns = non-significant p-value. [1]Average number of operational taxonomic units; [2]Margalef species richness: $d = (S - 1) / \log(N)$; [3]Shannons diversity index: $H' = -\sum(pi(\ln(pi))$; [4]Simpson evenness index $(1 - \lambda') = 1 - (\sum(ni / N)^2)$

The Bay-Curtis dendrograms (Figure 3.2) based on the DGGE polymorphic analysis of the digesta microbial communities showed that the replicates of HM0/HM15 and

HM45/HM30 groups clustered closely together (except for 3 replicates that failed to cluster). Sequence analysis of selected DDGE bands (Figure 3.2, Table 3.7) showed a strong dominance of autochthonous and allochthonous bacteria from phylum Firmicutes with one species from phylum Actinobacteria. Bands whose closest known species belonged to *Oceanobacillus, Lederbergia, Bacillus,* and *Corynebacterium* genera, were only detected in the digesta of fish fed the HM-containing diets. On the contrary, the inclusion of HM in the diets reduced the abundance of *Lactobacillus sp. And Caldalkalibacillus thermarum*. Regarding the mucosa, only one species was identified, *Oceanobacillus senegalensis*, and analysis of band intensity suggests a reduced abundance with the inclusion of HM.

Table 3.7: Closest relatives (BLAST) to the sequenced PCR-DGGE gel bands (from Figure 2) of the intestinal communities of gilthead sea bream fed the experimental diets.

Band	Closest known species (BLAST)	Phylum	Similarly (%)	Acc. number
Digesta				
1	*Oceanobacillus senegalensis*	Firmicutes	99	MH894249.1
2	*Oceanobacillus senegalensis*	Firmicutes	93	MT537733.1
3	*Lactobacillus* sp.	Firmicutes	97	LC588550.1
4	*Lederbergia ruris*	Firmicutes	98	MT197308.1
5	*Lederbergia galactosidilytica*	Firmicutes	98	NR_144713.1
6	*Bacillus andreraoultii*	Firmicutes	96	MT044137.1
7	*Corynebacterium lizhenjunii*	Actinobacteria	85	CP082237.1
8	*Caldalkalibacillus thermarum*	Firmicutes	95	KU361082.1
9	uncultured bacterium	n/a	100	MH894249.1
Mucosa				
10	*Oceanobacillus senegalensis*	Firmicutes	100	MH894249.1
11	*Oceanobacillus senegalensis*	Firmicutes	97	MH894249.1

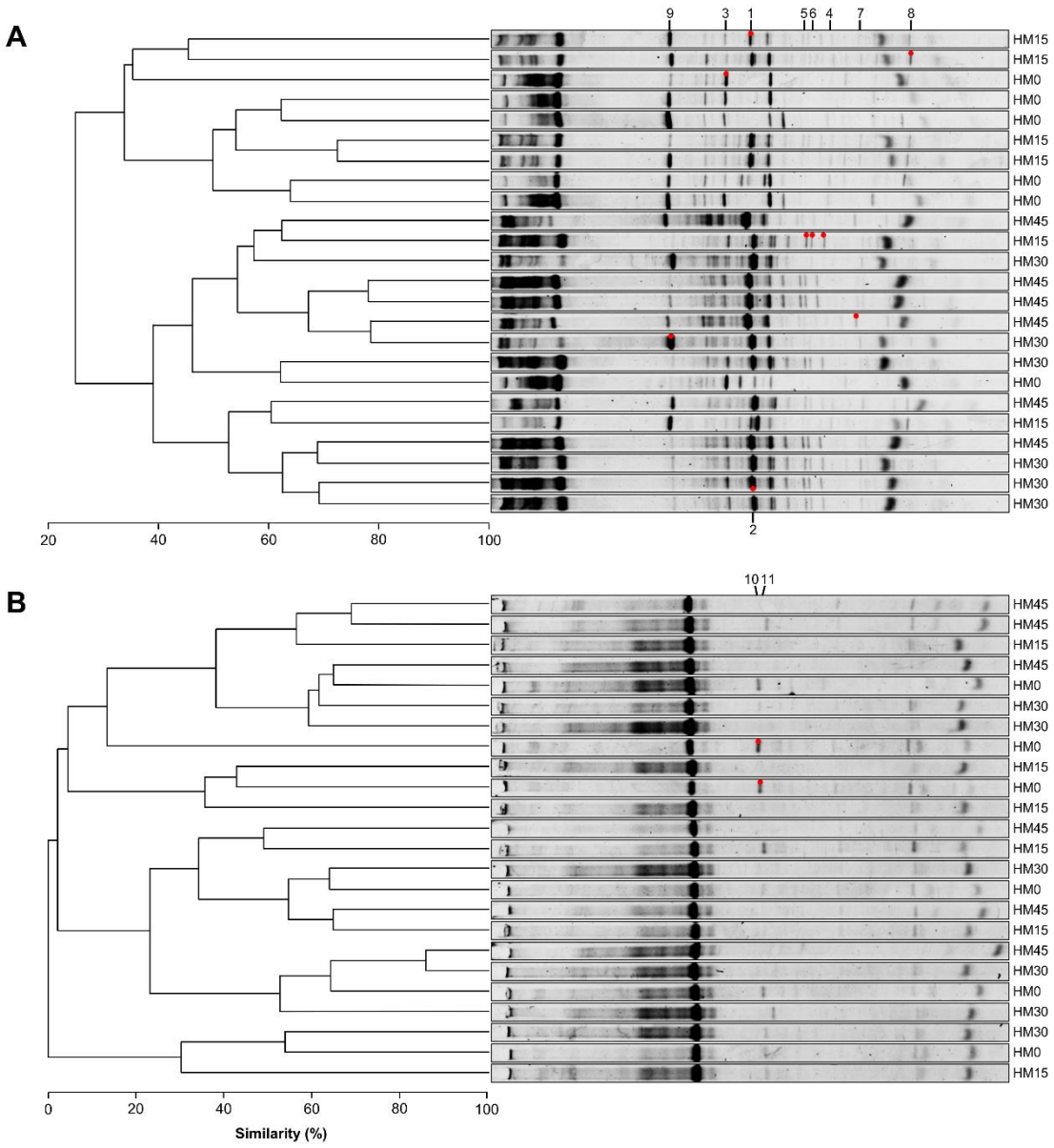

Figure 3.2: Dendrograms and PCR-DGGE fingerprints of the intestinal microbiota of gilthead sea bream fed the experimental diets. A - allochthonous (digesta) bacteria; B - autochthonous (mucosa) bacteria. Numbers (1 to 9 on digesta; 10 and 11 on mucosa) indicate bands excised for sequence analysis, identified in Table 3.7.

4. Discussion

In our companion study (Moutinho et al. 2022), we showed that it is possible a complete replacement of FM with defatted HM (included in the diets up to 45%) without compromising the growth performance and feed efficiency of gilthead seabream juveniles. However, while the lack of negative effects at the somatic level is a promising result, it does not accurately reflect the dietary-related potential changes in metabolism, digestive activity, or microbiota diversity.

Muscle growth is regulated by systemic and local signalling pathways involving the growth hormone (GR)/insulin-like growth factor (IGF) axis (Mommsen 2001; Jiménez-Amilburu et al. 2013). GR, secreted by the pituitary gland, interacts with specific membrane receptors (ghr1 and ghr2) modulating muscle hyperplasia and hypertrophy (Fuentes et al. 2013; Ramos-Pinto et al. 2019). GR also stimulates the production of IGFs, which regulate many aspects of myogenesis and directly stimulate muscle cell proliferation, differentiation, and hypertrophy, and inhibit muscle atrophy (Garcia de la serrana et al. 2012; Fuentes et al. 2013). In the present study, the dietary inclusion of HM had no effects on the muscle gene expression of growth hormone receptors (*ghr1* and *ghr2*) and insulin-like growth factors (*igf1* and *igf2*). These findings indicate that the dietary inclusion of HM replacing FM, besides not affecting growth performance (Moutinho et al. (2022) also seem not to affect the molecular dynamics regulating muscle growth. Similar outcomes were also reported for European seabass, where diets including *Tenebrio molitor* meal (TM) had no effects on growth or gene expression of muscle *ghrs* and *igfs* (Basto et al. 2021).

Muscle growth also represents a balance between protein synthesis and degradation (Bell et al. 2016). Proteolytic systems, such as cathepsins (ctsd), play significant roles in directing muscle fiber turnover (Perelló-Amorós et al. 2021; Li et al. 2023) as they are involved in muscle proteolysis through an autophagy-lysosome system. Cathepsins were shown to be upregulated in gilthead seabream during nutritional stressful conditions, such as fasting (Salmerón et al. 2015). In the present study, the expression of muscle *ctsd-a* and *ctsd-b* remained unchanged, indicating that the breakdown of muscle proteins was not significantly affected by the dietary HM inclusion.

Myogenic determination factors (MYOD) belong to the myogenic regulatory factors family and play a critical role in controlling cell proliferation and muscle lineage determination (Chemello et al. 2021). In the present study, a significant down-regulation of *myod2* expression was observed with the inclusion of HM. Although the expression of *myod1* was not significantly affected, in absolute values, lower expression was noticed

in fish fed the HM-containing diets. In European seabass, TM inclusion also reduced *myod* expression (Basto et al. 2021) while for rainbow trout, *myod* expression was increased (Chemello et al. 2021).

Myostatins (MSTN) also play a crucial role in myogenesis as they act as primary inhibitors of muscle development, negatively regulating the proliferation and differentiation of myocytes (Perelló-Amorós et al. 2021; Yuan et al. 2022). The *mstn* expression in response to dietary inclusion of insect meals seems contradictory among studies. Presently, a linear upregulation of *mstn* expression was observed with increasing dietary HM levels, similar to what was reported in European seabass fed with diets with TM (Basto et al. 2021). For juvenile yellow croaker (*Larimichthys crocea*), an increase in *mstn* expression was also observed, but coincided with a significant reduction in growth performance (Yuan et al. 2022). Conversely, dietary TM inclusion reduced *mstn* expression in the muscle of grass carp (*Ctenopharyngodon idellus*) without affecting growth performance (Li et al. 2023). Thus, further studies are required to elucidate the nutrigenomic effect on *mstn* expression of dietary inclusion of insect meals.

Taken together, the upregulation of *mstn* along with the downregulation of *myod2* in present study may suggest some inhibition or disruption of muscle growth in gilthead seabream juveniles. Indeed, although not significant, a decrease in nitrogen retention was observed in fish fed the HM-containing diets (Moutinho et al 2022), which could partially explain the observed results. Nevertheless, further research is required to elucidate the transcriptional effects of HM and determine whether its long-term use could pose limitations on muscle development.

Besides growth, the impact of novel feed ingredients on fish health and welfare must be considered. Plasma biochemical parameters are often used as bioindicators, reflecting the physiological and health status of fish in aquaculture under various nutritional conditions (Gu et al. 2022). Previous studies replacing FM with insect meals have shown a reduction in plasma cholesterol levels in several fish species (Li et al. 2017; Magalhães et al. 2017; Sankian et al. 2018; Gu et al. 2022). This has been attributed to the presence of chitin and/or chitosan in insect meals, which have been reported to interfere with cholesterol absorption by binding with lipid (cholesterol) micelles and proteins, inhibiting their absorption and increasing bile acid excretion (Magalhães et al. 2017; Gu et al. 2022). In the present study, however, no significant effects were observed on the profile of plasma metabolites, except for a linear decrease in plasma total lipid content with increasing defatted HM in the diets, similar to what was observed in meagre (Guerreiro et al. 2020). Mastoraki et al. (2020), however, showed that different insect meals had

different effects on the plasma metabolites profile in European seabass. The authors concluded that the observed responses could not be solely attributed to chitin, suggesting the involvement of other components present in insect meals or differences in the FA mobilization between species/insect meals used.

Intermediary metabolism enzymes play a crucial role in maintaining cellular homeostasis in various metabolic pathways (Chemello et al. 2020). Amino acids catabolizing enzymes, such as ALT, AST, and GDH, are considered good indicators of amino acid utilization (Fabrikov et al. 2020), as they are involved in transamination and deamination processes (Engelking 2011). In the present trial, there was a trend for increased activity of these enzymes with increasing dietary HM inclusion. Similar findings were reported by Fabrikov et al. (2020) in gilthead seabream fed different insect meals, while other studies report no effect on amino acids catabolism enzymes (Chemello et al. 2020; Guerreiro et al. 2020; Hoffmann et al. 2020; Mastoraki et al. 2020; Melenchón et al. 2020; Mastoraki et al. 2022; Melenchon et al. 2022). Present results suggest that dietary inclusion of HM may decrease the efficiency of protein utilization by fish, eventually by an imbalance of the amino acid profile. These results corroborate those observed in the growth trial (Moutinho et al, 2022) where nitrogen retention, although not significantly different among groups, in absolute values showed a decreasing trend with dietary HM inclusion.

A reduction in the activity of lipogenic-related enzymes (FAS, G6PDH, and ME) was also observed with increasing dietary HM content. Previous studies did not find any differences in the activity of lipogenic enzymes with insect meal inclusion in fish (Chemello et al. 2020; Mastoraki et al. 2022). A possible explanation for the decreased lipogenesis activity observed in the present study could be the decrease of dietary carbohydrates from whole wheat with the inclusion of HM, which may have decreased the substrate available for lipogenesis (Hemre et al. 2002; Dias et al. 2005; Viegas et al. 2016). Another explanation may be the increase in dietary chitin and, eventually, chitosan as chitosan has been shown to decrease the activities of hepatic lipogenic enzymes in rats and chickens (Li et al. 2016; Chiu et al. 2017). Although information on fish is scarce, it was proposed that the reduced lipogenesis in juvenile Japanese seabass fed HM was due to the chitosan present in the insect meal (Wang et al. 2019).

HOAD is an enzyme involved in β-oxidation within the mitochondria and involves fatty acid catabolism (Melenchón et al. 2020). Contrary to lipogenic enzymes, HOAD activity showed a quadratic response to the dietary inclusion of HM, being higher in fish fed with diet HM15 than in the control. Previously, Guerreiro et al. (2020) observed a linear

increase in its activity in meagre fed with diets including HM, while Melenchón et al. (2020) found no differences in rainbow trout fed diets including HM or TM.

In the present study, digestive enzyme activity was not affected by diet composition in the anterior intestine (AI), and overall enzyme activity was lower than in the posterior intestine (PI). This was unexpected, as at the sampling time (4 h after feeding) most of the meal is in AI. Higher digestive enzyme activity in the PI was also observed in gilthead seabream fed poultry by-product meal (Fontinha et al. 2021) and European seabass fed diets with HM (Magalhães et al. 2017). This could be attributed to a possible drag of secreted mucous rather than an actual increased activity, as suggested in previous studies (Pérez-Jiménez et al. 2009; Magalhães et al. 2015; 2017; Fontinha et al. 2021)

Lipase activity was not affected by diet composition, which is in accordance with the results observed in gilthead seabream fed with partially defatted HM (Gai et al. 2023). Amylase activity was higher in the PI of fish fed with diet HM15 than in the other diets. Increased amylase activity was also reported for gilthead seabream when 50% FM was replaced by full-fat HM (Fabrikov et al. 2021) but not when fed partially defatted HM (Gai et al. 2023). Trypsin and chymotrypsin activity in the PI was also higher in fish fed the HM15 diet than in the control. Increased proteolytic activity was also reported for gilthead seabream and grouper fed with HM (Fabrikov et al. 2021; Huang et al. 2022) while no effects were detected in other fish species (Magalhães et al. 2017; Belghit et al. 2019; Wang et al. 2019; Gai et al. 2023). Overall, results on digestive enzyme activity corroborate those of apparent diet digestibility determined in the companion study (Moutinho et al. 2022).

The present study shows a clear influence of the dietary inclusion of defatted HM on fish gut microbiota. Increased microbial richness (OTUs and Margalef index) and diversity (Shannon and Simpson indexes) were observed in the digesta of fish fed the HM-containing diets. On the contrary, these indexes were reduced in the mucosa, regardless of the dietary HM inclusion level. Previous studies have also reported higher gut microbiota diversity and richness in fish fed diets with insect meals (Bruni et al. 2018; Huyben et al. 2019; Terova et al. 2019; Gaudioso et al. 2021; Li et al. 2021). Higher gut microbiota diversity and richness are associated with higher health status of the host while a reduced diversity is associated with dysbiosis and increased risk of diseases (Rimoldi et al. 2019; Terova et al. 2019). This positive effect of insect meals has been attributed to the presence of chitin, as it may act as a prebiotic and is considered a major driver for increased gut microbiota diversity (Gaudioso et al. 2021; Rimoldi et al. 2019). Several studies have shown that dietary chitin inclusion increased intestinal microbial

diversity in Atlantic cod and salmon (Askarian et al. 2012; Ringø et al. 2012; Zhou et al. 2013). Furthermore, the fermentation of chitin and its derivative chitosan results in the production of several beneficial compounds, such as short-chain fatty acids, with beneficial effects on the gut and overall fish health (Gaudioso et al. 2021).

Most of the bacteria identified in this study from the PCR-DGGE fingerprints belonged to the phylum Firmicutes, with one bacterial group belonging to the phylum Actinobacteria. These bacterial groups have been observed to be dominant in previous studies with gilthead seabream (Kormas et al. 2014; Estruch et al. 2015; Moutinho et al. 2017; Panteli et al. 2021).

The dietary inclusion of defatted HM resulted in the appearance of bacteria from the genera *Oceanobacillus*, *Bacillus, Lederbergia* (previously identified as *Bacillus* sp., (Gupta et al. 2020), and *Corynebacterium,* as corresponding bands were only present in the digesta of these fish. In previous studies, *Oceanobacillus* was also detected in the gut of fish fed with insect meals (Tran et al. 2021; Biasato et al. 2022; Weththasinghe et al. 2022). These findings could be partially attributed to the microbiota present in HM, as *Oceanobacillus*, *Bacillus,* and *Corynebacterium* species have been identified in HI larvae (Zheng et al. 2013; Bruno et al. 2019; Jiang et al. 2019; Wynants et al. 2019). Feed-borne gut microbiota modifications have also been suggested by Li et al. (2022) in Atlantic salmon fed with diets including HM.

Present results could also be attributed to chitin present in HM, which may have supported the growth of beneficial chitin degraders, such as *Bacillus spp*. that are well-known chitin degraders (Li et al. 2022; Rangel et al. 2022b) with probiotic potential (Kuebutornye et al. 2019; Soltani et al. 2019; Kuebutornye et al. 2020). *Bacillus* were also the predominant taxa in the mucosa of rainbow trout fed HM (Gaudioso et al. 2021) and Atlantic salmon fed a chitin-supplemented diet, displaying the highest *in vitro* chitinase activity (Askarian et al. 2012). Actinobacteria are also known to display chitinase activity (Lacombe-Harvey et al. 2018). An increased abundance of Actinobacteria following the inclusion of HM was also reported in rainbow trout (Huyben et al. 2019; Terova et al. 2019). In this study, *Corynebacterium* (Actinobacteria) was only detected in fish fed with the HM diets, similar to what was observed in gilthead seabream (Antonopoulou et al. 2019) and rainbow trout (Huyben et al. 2019) fed diets including insect meals.

Dietary inclusion of HM led to a reduction in the abundance of *Lactobacillus* and *Caldalkalibacillus* in the digesta. Unlike observed in previous studies, *Lactobacillus* and other lactic acid bacteria were reported to proliferate in fish fed with HM diets (Huyben

et al. 2019; Rimoldi et al. 2019; Terova et al. 2019). This discrepancy could be due to differences in HI meal used and rearing subtracts (Biasato et al. 2022). For instance, in red drum distinct digesta bacterial communities were found in fish fed HM larvae reared in different substrates (Yamamoto et al. 2022).

5. Conclusion

Overall, this study shows that total FM replacement with defatted HM had no major effects on plasma metabolites but induced a few differences in intermediary metabolism and digestive enzymes activity. Muscle expression of *mstn* was upregulated and *myod2* was downregulated with HM inclusion, which could indicate some limitations on muscle development. Dietary HM improved gut digesta microbiota richness and diversity, promoting the growth of potential beneficial bacteria such as *Oceanobacillus*, *Bacillus*, and *Corynebacterium*.

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8. Supplementary material

Table 1a: Growth performance and nitrogen (N) retention of gilthead seabream fed the experimental diets. A more detailed description of the results and discussion has been published previously (Moutinho et al. 2022).

						Polynomial contrasts		
	HM0	**HM15**	**HM30**	**HM45**	**SEM**	**ANOVA *P*-value**	**Linear *P*-value**	**Quadratic *P*-value**
IBW (g)[1]	32.0	32.0	32.1	32.0	0.02	ns	ns	ns
FBW (g)[2]	97.3	97.8	97.0	94.0	1.39	ns	ns	ns
WG (g kg^{-1} day^{-1})[3]	15.0	15.1	15.0	14.7	0.17	ns	ns	ns
FE[4]	0.80	0.77	0.78	0.79	0.01	ns	ns	ns
DGI[5]	2.12	2.14	2.12	2.04	0.03	ns	ns	ns
PER[6]	1.87	1.82	1.80	1.79	0.03	ns	ns	ns
N retention (% intake)	30.8	29.3	28.2	28.9	0.57	ns	ns	ns

Values are presented as mean (n=3). SEM: Pooled standard error of the mean. Absence of different superscript letter indicate no significant differences between treatments (One-way ANOVA, linear and quadratic polynomial contrasts p-value > 0.05). ns = non-significant. [1]Initial body weight; [2]Final body weight; [3]Weight gain; [4]Feed efficiency = wet weight gain / dry feed intake; [5]Daily growth index = ((final weight$^{1/3}$ − initial weight$^{1/3}$) / days) × 100; [6]Protein efficiency ratio = wet weight gain / crude protein intake.

Chapter 4: Black soldier fly (*Hermetia illucens*) larvae meal as potential modulator of immune, inflammatory, and antioxidant status in gilthead seabream juveniles

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Black soldier fly larvae meal as a potential modulator of immune, inflammatory, and antioxidant status in gilthead seabream juveniles

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Abstract

This study aimed to evaluate the effects of fish meal (FM) replacement with defatted *Hermetia illucens* larvae meal (HM) on the hematological profile, immune parameters, intestinal inflammatory status, and antioxidant response in gilthead seabream (*Sparus aurata*) juveniles. Four experimental diets (43% crude protein; 18% crude lipids) were formulated to include increasing levels of HM. The control diet contained FM at 34% as the main protein source (diet HM0), and for the other diets, FM was replaced by HM at 22, 60, and 100%, corresponding to inclusion levels of 15 (diet HM15), 30 (diet HM30), and 45% (diet HM45), respectively. Fish were fed these diets until apparent visual satiation for 67 days. No differences between groups were observed in the plasma immune humoral parameters or the hematological profile, except for a linear decrease in hemoglobin and hematocrit. In the liver, there was a linear decrease in the activity of glucose-6-phosphate dehydrogenase and glutathione peroxidase, which was lower in fish fed the diet HM45 than in the control. Glutathione reductase activity also linearly decreased with HM inclusion and was lower in fish fed the HM30 diet than with the control. Fish fed the HM15 diet had lower hepatic superoxide dismutase activity than the control, whereas catalase activity and lipid peroxidation (LPO) were unaffected. In the intestine, the inclusion of HM did not affect the activity of antioxidant enzymes. Intestine LPO also linearly decreased with the inclusion of HM, being lower in fish fed the diet HM30 than in the control. Regarding the expression of intestinal pro-inflammatory genes, HM did not affect tumor necrosis factor-alpha (*tnfα*) expression but led to a linear reduction in interleukin-1-beta (*il-1β*) expression, which was lower in fish fed the diet HM45 diet than diet HM15. Cyclooxygenase-2 (*cox2*) expression was also linearly downregulated and was lower in fish fed the diet HM45 compared to the control. As for anti-inflammatory genes, interleukin-*10* (*il-10*) expression followed a negative quadratic

trend, while transforming growth factor (*tgfβ*) expression followed a positive quadratic trend, being higher in the diet HM30 than in the control and HM45 diets. Overall, this study showed that dietary inclusion of up to 45% HM (replacing 100% FM) had no adverse effects on the health of gilthead seabream juveniles. Dietary inclusion of HM improved hepatic antioxidant status and decreased intestinal LPO while upregulating the expression of anti-inflammatory genes and downregulating the expression of pro-inflammatory genes. These results support the use of defatted HM as an ingredient in aquafeeds.

Keywords: insect meal, oxidative stress, inflammation, immunity, *Hermetia illucens*

1. Introduction

Aquaculture has an increasingly important role in ensuring global food security. However, the future of aquaculture production must rely on more sustainable feed practices to reduce the dependency on marine-derived ingredients, such as fish meal (FM) and fish oil (Tacon 2022). The use of insects in aquafeeds as sustainable alternatives to traditional feed commodities has received increasing attention from the scientific community (Tran et al. 2022). Currently, eight species are authorized for use in aquafeeds within the European Union (EU Commission Regulation 2021/1925). Among these, *Hermetia illucens* is the most promising owing to its nutritional quality and environmental benefits (van Huis 2013). *H. illucens* larvae are rich in proteins (30 to 60% dry matter, DM), have a balanced essential amino acid profile, lipids (up to 30% DM), vitamins, and minerals (Henry et al. 2015; Liland et al. 2021). *H. illucens* larvae can be reared in waste and other by-products, taking part in the concept of circular economy (Diener et al. 2009). In addition, *H. illucens* larvae have fast growth and high feed conversion rates, require low water and land resources, and can be farmed locally, improving the economic efficiency and safety of local communities.

Gilthead seabream (*Sparus aurata*) is one of the most economically valuable species in Mediterranean aquaculture. We previously demonstrated that it was possible to replace all FM protein with 45% defatted *H. illucens* larvae meal (HM) without compromising growth performance, feed efficiency, and nutrient digestibility (Moutinho et al. 2022). However, the effects of HM on fish health and well-being still need to be addressed.

Chitin is a biopolymer present in the exoskeletons of arthropods, including insects (Finke 2007). Chitin and its derivatives, such as chitosan, were shown to exert beneficial effects on the antioxidant, immune, and anti-inflammatory responses in fish (Ringø et al. 2012;

Ngo and Kim 2014; Tran et al. 2015; Abdel-Ghany and Salem 2019). Indeed, the immunostimulatory properties of chitin have been shown in several fish species, including gilthead seabream (Esteban et al. 2001), mrigal (Cirrhina mrigala; Mari et al. 2014), hybrid sturgeon (Acipenser baerii ♀ × A. schrenckii ♂; Xu et al. 2023), Kelp grouper (Epinephelus bruneus; Harikrishnan et al. 2012), rainbow trout (Oncorhynchus mykiss; Sakai 1992), and olive flounder (Paralichthys olivaceus; Cha et al. 2008). Similarly, improved antioxidant status was reported for, hybrid sturgeon (Xu et al. 2023), common carp (Cyprinus carpio; Banaee et al. 2015), golden pompano (Trachinotus ovatus; Yu et al. 2023), and Atlantic salmon (Salmo salar; Kim and Thomas 2007) fed diets supplemented with chitin and/or chitosan.

Similarly, in previous studies with insect meals, improved antioxidant, immune and inflammatory responses were also reported in European sea bass (Dicentrarchus labrax; Abdel-Latif et al. 2021), barramundi (Lates calcarifer; Hender et al. 2021), channel catfish (Ictalurus punctatus; Fan et al. 2023), Jian carp (Cyprinus carpio; Li et al. 2017), trench (Tinca tinca; Hidalgo et al. 2022) and rainbow trout (Melenchón et al. 2020), Thus, besides being used as an alternative protein source, insect meals may also have potential as functional ingredients for fish. Therefore, the aim of this was to evaluate the effect of FM replacement with defatted HM on gilthead seabream juveniles' antioxidant and immune status, hematological profile, and intestinal inflammatory response.

2. Materials and methods

This trial was performed according to the EU Directive (2010/63/EU) on animal protection for scientific purposes and approved by CIIMAR's Ethics Committee (ORBEA_CIIMAR_27_2019). The trial was conducted by accredited scientists, following the Federation of European Laboratory Animal Science Associations (FELASA; category C) recommendations.

2.1 Experimental diets and fish growth trial

Four isoproteic (43% crude protein) and isolipid (18% crude lipids) diets were formulated according to the species requirements. The control diet (diet HM0) contained FM as the main protein source at 34%. For the other experimental diets, FM was replaced by defatted HM at increasing levels of 22, 60, and 100%, corresponding to a level of 15, 30, and 45% of inclusion (diets HM15, HM30, and HM45, respectively). Prior to diet manufacturing, all ingredients were ground and mixed, and were dry pelleted in a laboratory pellet mill (California Pellet Mill, Crawfordsville, USA), passing through a 3

mm matrix. After drying in an oven (55 °C for 24 h), feed pellets were stored at -20 °C until use. Table 4.1 shows the proximate composition of diets and ingredients.

Table 4.1: Formulation and proximate composition of experimental diets.

Diet	HM0	HM15	HM30	HM45
Defatted *Hermetia illucens* prepupae larvae meal[1]	-	15	30	45
Fishmeal[2]	34.3	26.7	13.6	0.0
Hemoglobin[3]	4	4	4	4
Corn gluten[4]	11.8	7.7	7.7	7.7
Whole wheat[5]	30.1	26.1	22.0	18.0
Shrimp hydrolysate[6]	1.5	1.5	1.5	1.5
Fish oil[7]	13.7	13.9	14.5	15.1
Vitamins premix[8]	1	1	1	1
Minerals premix[9]	1	1	1	1
Choline	0.5	0.5	0.5	0.5
Binder[10]	1	1	1	1
Betaine	0.2	0.2	0.2	0.2
Soy lecithin	0.5	0.5	0.5	0.5
Calcium biphosphate[11]	-	0.5	1.6	2.7
Taurine	0.3	0.3	0.3	0.3
Arginine	-	-	0.35	0.8
Methionine	-	0.1	0.3	0.5
Lysine	-	-	-	0.3
Proximate composition				
Dry matter (%)	95.6	95.4	95.6	96.3
Crude Protein (N x 6.25) (%)	43.0	43.5	43.4	45.5
Crude Lipid (%)	17.8	17.8	18.1	18.0
Energy (kJ g^{-1})	22.8	21.3	22.2	22.6
Ash (%)	8.77	9.23	8.94	8.74
Chitin (%)[12]	0	0.93	1.86	2.79

[1]Defatted *Hermetia illucens* prepupae larvae meal (93.3% DM, 61.8% CP, 6.8% CL, 21.6% Ash, 11.9 kJ^{-1} Energy, 6.20% chitin); Hermetia Bartuh GmbH, Germany
[2]Fishmeal (87.0% DM, 73.4% CP, 8.9% CL, 15.1% Ash, 19.7 kJ g^{-1} Energy), Sorgal, S.A. Ovar, Portugal
[3]Hemoglobin powder AP310P; APC Europe S.A.
[4]Corn gluten meal (90.5% DM, 79.5% CP, 2.9% CL); Sorgal, S.A. Ovar, Portugal
[5]Whole wheat (88% DM, 11.4% CP, 1.8% CL); Sorgal, S.A. Ovar, Portugal
[6]Shrimp hydrolysate (*Litopenaeus vannamei* trimmings; 73% CP, 12.1% CL; 13% Ash); Sorgal, S.A. Ovar, Portugal
[7]Fish oil (tuna, sardine, and mackerel trimmings); Sorgal, S.A. Ovar, Portugal
[8]Vitamin premix (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.
[9]Mineral premix (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)
[10]Binder: Carboxymethylcellulose sodium. Sigma-Aldrich Química, Portugal
[11]Calcium biphosphate, Sorgal, S.A. Ovar, Portugal
[12]Estimated from the defatted HM chitin content

These experimental diets were previously assessed in a growth trial, and the results were published elsewhere (Moutinho et al. 2022). Briefly, each diet was attributed to

triplicate groups of 13 fish, with an initial body weight of 32 g. For 67 days, fish were hand-fed each diet to apparent visual satiation 2 times a day, 6 days a week. The trial was conducted in a thermoregulated recirculation seawater system (average water temperature of 23±0.5ºC), equipped with twelve 300 L fiberglass tanks.

2.2 Sampling

At the end of the trial, 3 fish were collected randomly from each tank (n = 9) 4 hours after the morning meal. Blood was withdrawn from the caudal vein of each fish with heparinized syringes and was used for total erythrocyte (RBC) and leucocyte (WBC) counts. The remaining blood was centrifuged at room temperature (10,000 × g for 10 min), and plasma was collected and kept at -80◦C until analysis. Fish were euthanized by decapitation, and the liver and digestive tract were excised on chilled trays. A small portion of the distal intestine was removed, kept in RNAlater (1:10) for 24h at 4ºC, and stored at -80ºC until RNA extraction for gene expression analysis. The remaining samples of the intestine and whole liver were frozen in liquid nitrogen and stored at -80 °C until analysis.

2.3 Blood parameters

Total leucocytes (white blood cells; WBC) and erythrocytes (red blood cells; RBC) cell count was performed by a hemocytometer from blood dilutions. Hematocrit (HT) was determined from fresh heparinized blood by microcentrifugation (10,000 x g, 10 min, room temperature) of hematocrit tubes. Hemoglobin (HB) was quantified using a commercial kit (Sprinreact, ref. 1,001,230, Spain) with Drabkin's solution. The following formulas were used to calculate the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin (MCHC) content:

$MCH\ (pg\ cell^{-1}) = (HB / RBC) \times 10$

$MCV\ (\mu m^3) = (HT / RBC) \times 10$

$MCHC\ (g\ 100\ mL^{-1}) = (HB / HT) \times 100$

2.4 Humoral innate immune parameters

Plasma protease activity was measured by azocasein hydrolysis, performed as described by Magalhães et al. (2021). Plasma total antiprotease activity was measured according to Machado et al. (2015) as the ability to inhibit trypsin activity. Plasma peroxidase activity was quantified as described by Quade and Roth (1997) and nitric oxide (NO) using Roche Diagnostic’s Nitrate/Nitrite colorimetric kit (Roche Diagnostics

GmbH, Mannheim, Germany, ref: 11,746,081,001), according to the manufacturer's instructions, adjusted for 96-well microplates.

2.5 Oxidative stress enzyme activity and lipid peroxidation determination

Intestine and liver samples were homogenized on ice (with dilution of 1:4 and a buffer containing Tris-HCl at 100 mM, EDTA at 0.1 mM, and 0.1% of Triton™ X-100 (v/v) with a pH of 7.8). Homogenates were centrifugated at 4ºC (30,000 g, 30 min), supernatant collected, and aliquots were stored at -80ºC until analysis. The assays for each oxidative stress enzyme (G6PDH, glucose-6-phosphate dehydrogenase; GR, glutathione reductase; GPX, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase) were performed as described by Castro et al. (2016). The activity of enzymes GPX, G6PDH, GR, and SOD are expressed as milliunits (mU) per mg of soluble protein, and for CAT, in units (U) per mg of soluble protein. The Bradford method (Bradford 1976) was used to determine protein concentration. The lipid peroxidation levels of the intestine and liver samples were determined based on the concentration of malondialdehyde (MDA), expressed as nmol of MDA per g of wet tissue, calculated from an MDA calibration curve [31]. Changes in absorbance for enzymatic activity and lipid peroxidation were monitored by a microplate spectrophotometer (Multiskan GO; Model 5111 9200; Thermo Scientific, Nanjing, China).

2.6 Gene expression

RNA was extracted from the distal intestine using Direct-zol™ RNA Miniprep kit (Zymo Research) containing TRIzol reagent, and a Precellys Evolution equipment (Bertin Instruments, Montigny-le-Bretonneux, France), according to the manufacturer's protocol. The concentration of RNA was adjusted to 0.5 µg 8 μL^{-1} with H_2O. cDNA was then synthesized using the NZY First-Strand cDNA Synthesis Kit (NZYTech, ref. MB12502, Lisbon, Portugal). Gene expression was determined by real-time quantitative PCR analysis, as described by Moutinho et al. (2024). The annealing temperature and sequences of each pair of primers are presented in Table 4.2. Elongation factor 1 alpha (*ef1α*) and ribosomal protein 18s (*18s*) were used as reference genes for data normalization. Expression levels are presented following the method by Vandesompele et al. (2002) as the mean of the normalized values with standard error. These are calculated as the ratio between copy numbers of the target gene transcripts and geometric mean of copy numbers of the reference genes.

Table 4.2: Sequence of primers used for real-time quantitative PCR.

Gene	Sequence 5'-3'	Annealing Temp. (ºC)	Efficiency	Accession number
tgfβ	**F:** AGAGACGGGCAGTAAAGAA **R:** GCCTGAGGAGACTCTGTTGG	60	1.99	AF424703
il-1β	**F:** GGGCTGAACAACAGCACTCTC **R:** TTAACACTCTCCACCCTCCA	60	1.99	AJ277166
il-10	**F:** TGGAGGGCTTTCCTGTCAGA **R:** TGCTTCGTAGAAGTCTCGGATGT	60	2.13	FG261948
tnfα	**F:** TCGTTCAGAGTCTCCTGCAG **R:** CATGGACTCTGAGTAGCGCGA	60	2.03	AJ413189
cox2	**F:** GAGTACTGGAAGCCGAGCAC **R:** GATATCACTGCCGCCTGAGT	60	2.11	AM296029
ef1α	**F:** CTGTCAAGGAAATCCGTCGT **R:** TGACCTGAGCGTTGAAGTTG	60	1.93	AF184170
18s	**F:** GGGTGTTGGCAGACGTTAC **R:** CTTCTGCCTGTTGAGGAACCA	60	1.96	AM490061.1

tgfβ: transforming growth factor beta; *il-1β*: interleukin-1-beta; *il-10:* interleukin-10; *tnfα:* tumor necrosis factor alpha; *cox2:* cyclooxygenase-2*; ef1α:* elongation factor 1-alpha*; 18s:* ribosomal protein 18s

2.7 Statistical analysis

All statistical analyses were performed using SPSS v27.0 (IBM Corp.) for Windows. All data are expressed as the mean of n = 9 and standard error of the mean (SEM) and were checked for homogeneity of variances and normal distribution. One-way analysis of variance (ANOVA) was used to assess differences between dietary treatments and polynomial contrasts to determine whether data followed a linear or quadratic response to defatted HM inclusion with a significance level of 0.05. Tukey's multiple range test was used to determine significant differences amongst means.

3. Results

Results from the growth performance of the fish fed the experimental diets have been published previously (Moutinho et al. 2022). Briefly, at the end of the trial, fish tripled their weight (going from an average of 32 g to 96.5 g) and was not significantly different between groups. Feed intake and feed efficiency (average of 0.78) were also unaffected by dietary composition.

At the end of the trial, blood MCV, MCH, RBC, WBC, and MCGC were not affected by the dietary inclusion of defatted HM (Table 4.3). Hemoglobin content significantly decreased, and hematocrit showed a trend to decrease with HM inclusion, while a quadratic negative trend was observed for MCGC which presented the lowest value in fish fed the HM30 diet.

Table 4.3: Haematological profile of gilthead seabream fed the experimental diets.

						Polynomial contrasts		
	HM0	**HM15**	**HM30**	**HM45**	**SEM**	**ANOVA *p*-value**	**Linear *p*-value**	**Quadratic *p*-value**
Hemoglobin (g dL^{-1})	2.6^{b}	2.2^{ab}	2.3^{ab}	2.2^{a}	0.08	0.032	0.011	ns
Hematocrit (%)	39.5^{b}	33.8^{ab}	38.6^{ab}	33.3^{a}	0.85	0.014	ns	ns
MCV (µm^{3})^{1}	164.4	170.4	185.5	159.5	6.76	ns	ns	ns
MCH (pg cell^{-1})^{2}	10.9	11.0	10.8	10.1	0.37	ns	ns	ns
MCGC (g 100 mL^{-1})^{3}	6.6	6.1	5.3	6.8	0.26	ns	ns	0.016
RBC (10^{6} µL^{-1})^{4}	2.4	1.9	2.3	2.2	0.07	ns	ns	ns
WBC (10^{4} µL^{-1})^{5}	5.7	6.2	5.4	5.7	0.17	ns	ns	ns

Values are presented as means (n = 9) and standard error of the mean (SEM). Different superscript letters indicate significant differences between treatments (one-way ANOVA; $p < 0.05$). ns: non-significant p value [1]MCV: mean corpuscular volume; [2]MCH: mean corpuscular haemoglobin; [3]MCGC: mean corpuscular haemoglobin concentration; [4]RBC: red blood cells; [5]WHC: white blood cells.

The different dietary treatments had no effects on the immune humoral parameters measured (peroxidase, nitric oxide, antiprotease, and protease activities) (Table 4.4).

Table 4.4. Immune humoral parameters in the plasma of gilthead seabream fed the experimental diets.

						Polynomial contrasts		
	HM0	**HM15**	**HM30**	**HM45**	**SEM**	**ANOVA p-value**	**Linear p-value**	**Quadratic p-value**
Peroxidase (U mL^{-1})	68.7	90.0	75.3	66.0	5.75	ns	ns	ns
Nitric oxide (mM)	0.54	0.52	0.54	0.53	0.02	ns	ns	ns
Antiprotease activity (%)	95.3	93.5	94.8	94.8	0.33	ns	ns	ns
Protease activity (%)	5.4	5.9	6.2	5.3	0.24	ns	ns	ns

Values are presented as means (n = 9) and standard error of the mean (SEM). The absence of different superscript letters indicates no significant differences between treatments (one-way ANOVA; p > 0.05). ns: non-significant p value.

In the liver, G6PDH, GPX, and GR activities linearly decreased with the HM inclusion in the diets (Table 4.5). SOD activity was lower in fish fed the HM15 diet compared to the control and HM30 diets while CAT activity was not affected by the dietary treatments. The hepatic LPO was also unaffected by the dietary treatments.

Table 4.5: Antioxidant enzyme activity and lipid peroxidation in the liver of gilthead seabream fed the experimental diets.

						Polynomial contrasts		
	HM0	**HM15**	**HM30**	**HM45**	**SEM**	**ANOVA p-value**	**Linear p-value**	**Quadratic p-value**
G6PDH[1]	191.4[b]	168.3[ab]	160.4[ab]	138.5[a]	6.58	0.038	0.006	ns
GPX[2]	93.9[b]	76.6[ab]	74.8[ab]	61.1[a]	3.86	0.026	0.005	ns
GR[3]	9.10[b]	9.07[ab]	6.61[a]	7.77[ab]	0.36	0.013	0.006	ns
SOD[4]	218.2[b]	132.8[a]	202.1[b]	172.9[ab]	9.34	0.003	ns	ns
CAT[5]	166.3	190.5	168.6	199	7.34	ns	ns	ns
LPO[6]	10.3	9.72	9.47	9.85	0.66	ns	ns	ns

Values are presented as means (n = 9) and standard error of the mean (SEM). Different superscript letters indicate significant differences between treatments (one-way ANOVA; p > 0.05). ns: non-significant p value. [1]Glucose-6-phosphate dehydrogenase (mU mg protein^{-1}); [2]Glutathione peroxidase (mU mg protein^{-1}); [3]Glutathione reductase (mU mg protein^{-1}); [4]Superoxide dismutase (mU mg protein^{-1}); [5]Catalase (U mg protein^{-1}); [6]Lipid peroxidation (malondialdehyde g tissue^{-1}).

In the intestine, GPX, GR, and SOD enzymatic activities were not affected by the dietary treatments, while G6PDH activity showed a linear trend to decrease with the dietary HM inclusion (Table 4.6). The intestine LPO linearly decreased with the dietary HM inclusion level.

Table 4.6: Antioxidant enzyme activity and lipid peroxidation in the intestine of gilthead seabream fed the experimental diets.

						Polynomial contrasts		
	HM0	**HM15**	**HM30**	**HM45**	**SEM**	**ANOVA p-value**	**Linear p-value**	**Quadratic p-value**
G6PDH[1]	5.24	4.22	3.34	3.94	0.26	ns	0.036	ns
GPX[2]	57.7	72.1	51.6	57.5	3.04	ns	ns	ns
GR[3]	17.5	19.7	15.8	19.1	0.80	ns	ns	ns
SOD[4]	1463.5	1267.9	892.2	1309.0	80.0	ns	ns	ns
LPO[5]	49.2[b]	40.1[ab]	37.2[a]	38.4[ab]	1.5	0.041	0.018	ns

Values are presented as means (n = 9) and standard error of the mean (SEM). Different superscript letters indicate significant differences between treatments (one-way ANOVA; p > 0.05). ns: non-significant p value. [1]Glucose-6-phosphate dehydrogenase (mU mg protein^{-1}); [2]Glutathione peroxidase (mU mg protein^{-1}); [3]Glutathione reductase (mU mg protein^{-1}); [4]Superoxide dismutase (mU mg protein^{-1}); [5]Lipid peroxidation (malondialdehyde g tissue^{-1}).

Regarding intestine gene expression, *il-1β* and *cox2* expression linearly decreased with the dietary inclusion of HM, while *tnfα* expression was unaffected by the dietary treatments (Figure 4.1). The expression of *il-10* showed a negative quadratic trend, while *tgfβ* showed a positive quadratic response.

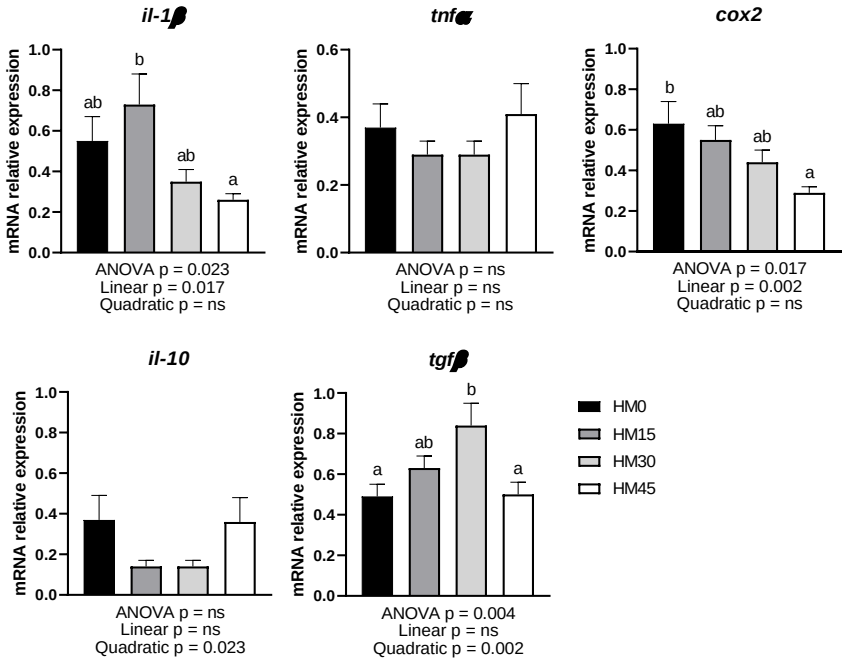

Figure 4.1: Relative expression of selected genes in the intestine of gilthead seabream fed the experimental diets. Data were normalized with two housekeeping genes, elongation factor 1 alpha (*ef1α*) and β-actin. Values are presented as mean (n=9) and standard error represented by error bars. Different superscript letters indicate significant differences between treatments (one-way ANOVA; $p < 0.05$). ns: non-significant p-value.

4. Discussion

Previously, we showed that the total replacement of dietary FM with defatted HM in gilthead seabream juveniles did not compromise growth, feed efficiency, and diet digestibility (Moutinho et al. 2022). This study intends to be a step forward, by evaluating the impact of dietary defatted HM on fish immune and oxidative status.

Hematological parameters are recognized as a valuable tool for evaluating the physiological and health status of fish under different environmental and nutritional conditions (Taufek et al. 2016; Fawole et al. 2020; Witeska et al. 2022). Presently, no differences were observed among the different treatments in the hematological parameters analyzed, except for a reduction in hemoglobin and hematocrit with increasing HM inclusion in the diets. Nevertheless, values are within those previously described for this species (Matias et al. 2018; Teixeira et al. 2022).

It is known that stressful conditions can increase red blood cell indices as an adaptative mechanism to increase oxygen delivery (Witeska et al. 2022). Thus, the lower hemoglobin and hematocrit levels in fish fed the HM diets may indicate reduced stress status of these fish and consequently, a lower metabolic rate leading to lower oxygen

demand in these fish. Contrary to our findings, no differences in those parameters were observed in African catfish (Clarias gariepinus; Fawole et al. 2020), European sea bass (Abdel-Tawwab et al. 2020), Atlantic salmon (Belghit et al. 2019) and trench (Hidalgo et al. 2022) fed diets where FM was replaced by HM, while increased hemoglobin and hematocrit were reported in African catfish, and channel catfish fed diets including cricket meal (Taufek et al. 2016; Fan et al. 2023).

The innate immune system is a primary defense mechanism in fish, playing a crucial role in blocking invading pathogens and triggering adaptive immune reactions (Xu et al. 2023). Previously, it was shown that insect meals had the potential to boost the innate immune response in several fish species, including rainbow trout (Henry et al. 2018a), yellow catfish (Pelteobagrus fulvidraco; Su et al. 2017; Xiao et al. 2018), red seabream (Pagrus major; Ido et al. 2015), olive flounder (Jeong et al. 2021), and European sea bass (Henry et al. 2018b; Abdel-Latif et al. 2021), These beneficial effects on the immune status of fish fed with diets including insect meals have been attributed to chitin, which is present in insects at levels up to 6% (Finke 2007). Indeed, chitin and chitosan (a chitin derivative) have been signaled for their immune-stimulating properties in fish (Esteban et al. 2000; Esteban et al. 2001; Gopalakannan and Arul 2006; Harikrishnan et al. 2012; Mari et al. 2014; Xu et al. 2023). In the present study, however, a lack of immunostimulatory effects were observed in fish fed the diets including HM. Similar results were also reported in trench (Hidalgo et al. 2022).

These apparent discrepancies in results between studies could be due to a multitude of factors, including fish species, experimental conditions, and dietary chitin levels. Further, the immune response is often only boosted after an infection challenge (Su et al. 2017), and in the present study, both the immune and hematological parameters measured, plus the good performance results, indicate that the fish were in good health conditions. Further studies involving bacterial challenges would need to be conducted to assess the beneficial effects of HM inclusion in promoting fish immune status.

Dietary chitin and chitosan supplementation can also improve the inflammatory response in fish (Henry et al. 2015; Yu et al. 2023). In the present trial, a negative linear trend between dietary defatted HM inclusion level and pro-inflammatory *il-1β* and *cox2* gene expression was observed. Interleukin-*1β* is a proinflammatory cytokine and, as in the present trial, its expression was also downregulated in the intestine of Nile tilapia (*Oreochromis niloticus*) fed with diets containing defatted HM (Kishawy et al. 2022). However, an upregulation in the expression of *il-1β* was observed in rainbow trout (Cardinaletti et al. 2019) and barramundi (Hender et al. 2021) intestine and in the liver of

European sea bass (Abdel-Latif et al. 2021) fed diets including HM and in the intestine of channel catfish and liver of snakehead (*Channa argus*) fed diets including cricket meal (Fan et al. 2023; Liu et al. 2023). COX2 is also involved in the production of pro-inflammatory molecules, like prostaglandins, as a response to inflammation (Secombes et al. 2001; Simmons et al. 2004; Gomez-Abellan and Sepulcre 2016). Differently from this study, in a previous study also in gilthead seabream, it was observed that dietary FM replacement with HM did not affect *cox2* expression in the intestine (Carvalho et al. 2023).

Transforming growth factor-beta (*tgfβ*) and *il-10* are anti-inflammatory cytokines and their expression was also modulated by dietary inclusion of HM, although a clear trend was not evident. The anti-inflammatory potential of chitin appears to be dose and particle-size dependent. Excessive dietary inclusion levels may exert opposite effects, as observed in golden pompano (Yu et al. 2023). Also, in contrast to large particles, small chitin fragments may stimulate anti-inflammatory cytokines production (Lee et al. 2008). In other studies, modulation of *il-10* expression was also observed for Nile tilapia (Kishawy et al. 2022), pearl gentian grouper (Epinephelus fuscoguttatus ♀ × E. lanceolatus ♂; Huang et al. 2022), barramundi (Hender et al. 2021), European sea bass (Abdel-Latif et al. 2021), and rainbow trout (Cardinaletti et al. 2019) fed diets containing HM and in channel catfish fed diets containing cricket meal (Fan et al. 2023).

Altogether, results of gene expression further support the good health conditions and reduced inflammatory status of fish fed with the HM-containing diets.

Oxidative stress occurs due to an imbalanced production of reactive oxygen species and the activity of antioxidant mechanisms, causing DNA and cell membrane damage and proteins and lipids peroxidation (Caimi et al. 2020; Abdel-Latif et al. 2021). To mitigate oxidative damage, antioxidant enzymes will act as part of fish antioxidant defense. SOD is an enzyme that catalyzes the dismutation of superoxide anion (O_2^-) to O_2 and hydrogen peroxide (H_2O_2) and is the first to respond to the presence of free oxygen radicals while enzymes CAT and GPX reduce the H_2O_2 produced to O_2 and H_2O (Ighodaro and Akinloye 2018). Glutathione acts as a scavenger of ROS and is oxidized by GPX activity, being molecularly reduced by GR using NADPH supplied by G6PDH (Wu et al. 2004; Ursini and Maiorino 2013).

Fish's antioxidant system has a different response depending on the tissue. The intestine, the first body barrier in contact with food, is usually more susceptible to oxidative stress (Hidalgo et al. 2022; Guerreiro et al. 2023). In this study, similar results were observed as LPO levels were greater in the intestine compared to the liver. Different

antioxidant responses in the liver and intestine were also reported for meagre (Argyrosomus regius; Guerreiro et al. 2023) and pikeperch (Sander lucioperca; Tran et al. 2021) when fed diets including insect meals. Further, a negative linear trend was observed between intestine LPO and dietary HM inclusion levels. However, unlike what was observed in the liver, the activity of intestine antioxidant enzymes was not affected by the experimental diets.

In the liver, GPX, GR, and G6PDH activity linearly decreased with the increase of HM in the diets, while LPO levels were not affected by diet composition. The hepatic activity of CAT was not affected by diet composition while SOD activity was reduced in fish fed with diet HM15 but no differences with the control diet were observed with the other experimental diets. Response of antioxidant enzymes in diets including insect meal has been highly variable, depending on species and tissues analyzed. For instance, decreased SOD activity with dietary HM inclusion was observed in the liver of European sea bass (Moutinho et al. 2021), the serum of African catfish (Fawole et al. 2020), and the gut of trench (Hidalgo et al. 2022). On the contrary, SOD activity increased in rainbow trout's liver fed with diets including HM or TM (Melenchón et al. 2020) and in snakehead fed diets with HM (Siddaiah et al. 2023). In African catfish, diets with HM diets decreased SOD activity which was accompanied by an increase in CAT (Fawole et al. 2020) while in Jian carp, serum CAT activity was increased and SOD activity was unaffected (Li et al. 2017). On the other hand, for rainbow trout, no differences were observed in SOD and CAT activity in the liver and renal tissue when fed diets with HM (Elia et al. 2018).

As in the present study, liver GPX activity was reduced in meagre fed with diets with *Tenebrio molitor* meal replacing FM (Guerreiro et al. 2023), and in Siberian sturgeon (Acipenser baerii; Caimi et al. 2020), rainbow trout (Elia et al. 2018), and pikeperch (Tran et al. 2021) fed diets with HM replacing FM. On the contrary, in channel catfish, hepatic GPX activity increased in fish fed with diets with cricket meal (Fan et al. 2023), and no differences in GPX activity were observed in the liver of European sea bass fed HM (Moutinho et al. 2021). Also contrary to the present results, the inclusion of HM increased liver GR activity in Siberian sturgeon (Caimi et al. 2020) and rainbow trout (Melenchón et al. 2020) but not in European sea bass (Moutinho et al. 2021).

Lipid peroxidation values are used to measure the degree to which lipids are attacked by free radicals (Gu et al. 2022). In the present study, hepatic lipid peroxidation was not compromised by the inclusion of HM. Similarly, no differences in liver lipid peroxidation were also observed in Siberian sturgeon (Caimi et al. 2020), European sea bass (Moutinho et al. 2021) and snakehead (Siddaiah et al. 2023) fed diets with HM.

Similar to our results, a reduction in intestine LPO was also observed in the trench (Hidalgo et al. 2022) and rainbow trout (Henry et al. 2018a) fed diets with insect meals. Overall, results from this study and previous studies suggest a beneficial effect on the oxidative status of fish by including insect meal in the diets. This enhanced antioxidant status may be attributed to the beneficial scavenging activity of chitin and chitosan present in insect meals (Ngo and Kim 2014). Indeed, other studies have shown that dietary chitin or chitosan supplementation improved the oxidative stress response in hybrid sturgeon (Xu et al. 2023), Atlantic salmon (Kim and Thomas 2007), European carp (Banaee et al. 2015), and golden pompano (Yu et al. 2023).

In conclusion, present results indicate that the dietary inclusion of up to 45% HM (replacing 100% FM protein) did not compromise the immune and oxidative status of gilthead seabream juveniles. On the contrary, HM-containing diets contributed to improving the intestinal antioxidant status.

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Chapter 5: Black soldier fly larvae oil as an alternative lipid source in diets for gilthead seabream (*Sparus aurata*) juveniles

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Black soldier fly larvae oil as an alternative lipid source in diets for gilthead seabream (*Sparus aurata*) juveniles

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Abstract

The black soldier fly (*Hermetia illucens*, HI) is a promising insect species to be included in aquafeeds. HI larvae are rich in protein but also in fat, with a fatty acid profile dominated by saturated and medium-chain fatty acids. This study aimed to evaluate the replacement of vegetable oils (VO) with HI larvae oil (HIO) and its effects on growth, whole-body composition, nutrient retention, diet digestibility, and digestive enzyme activity. A control diet (HIO0, 45% crude protein, 18% lipids) was formulated to include 9.5% of a VO blend, and three other diets were formulated similar to the control but with the VO blend replaced at 42% (HIO42), 84% (HIO84), and 100% (HIO100). A growth trial was performed with triplicate groups of fish (initial body weight of 63 g) hand-fed to apparent visual satiation for 10 weeks. A digestibility trial was also performed simultaneously with triplicate groups of fish with similar initial body weights. Results indicate that the total replacement of VO mix with HIO did not affect growth performance, but feed efficiency and protein efficiency ratio linearly improved with the increase of HIO in the diets. No differences between groups were found in whole-body composition and protein and energy retention. There was a trend for protein and energy digestibility to linearly increase with dietary HIO inclusion. Lipid digestibility was not affected by diet composition, however, the apparent digestibility of saturated fatty acids increased and of unsaturated decreased with the increase of dietary HIO inclusion. Total proteases activity was higher in fish fed diet HIO84 and lipase activity followed a positive linear trend with the increase in dietary HIO content. Overall, present results indicate that gilthead seabream can effectively utilize HIO without compromising growth performance and digestibility while improving feed utilization efficiency, thus establishing its potential as an alternative lipid source for aquafeeds.

Keywords: *Hermetia illucens,* alternative ingredients, growth, digestibility, dietary lipids

1. Introduction

Dietary lipids play an important role in fish nutrition, as they act as a major energy source and provide the essential fatty acids required for the growth and well-being of farmed fish (Turchini et al., 2009). Until recently, fish oil (FO) was used as the main lipid source in diets for marine fish due to its high content of n-3 long-chain (≥C20) polyunsaturated fatty acids (LC-PUFA), which are essential for marine fish (Izquierdo et al., 2003). However, due to limited supply, interannual availability fluctuations, and negative environmental impacts associated with its production (Rombenso et al., 2021; Tocher et al., 2019), the aquafeed industry has evolved to use fisheries-derived oils more strategically. The use of vegetable oils (VO) as alternative lipid sources in fish diets has been increasing in the last decades, and it has been demonstrated that it is possible to partially or completely replace dietary FO without compromising fish growth and welfare, as long as the essential fatty acids (EFA) requirements of the animals are satisfied (Turchini et al., 2009). The use of VO in fish diets has however some constraints, as they lack n-3 long-chain PUFAs, which are required for some species, and are often rich in n-6 PUFA. Moreover, the use of VO in diets can lead to land-use conflicts, degradation of water and soil, greenhouse gas emissions, and biodiversity losses (Benzertiha et al., 2020; Xu et al., 2021).

Due to the increasing demand for alternative ingredients, in 2017 the European Union authorized the use of seven insect species as ingredients for aquafeeds (Commission Regulation (EU) 2017/893), and more recently this list was increased to eight insects (Commission Regulation (EU) 2021/1925). Insects have been receiving increasing attention for use as food and feed, as they have fast growth and high feed conversion ratio, have low water and land requirements, and can use wastes and other by-products as food, thus converting poorly valorized sub-products into highly valuable nutritious feedstuffs (Franco et al., 2021; Maldonado-Othón et al., 2022).

The black soldier fly (*Hermetia illucens,* HI) is one of the most promising insect species for use in aquafeeds (Fawole et al., 2021). HI larvae are characterized by having a high protein content (30-60% dry matter), with a balanced amino acid profile, and are also a good source of vitamins and minerals (Liland et al., 2017; Nogales-Mérida et al., 2018). Several studies have already demonstrated the potential of HI larvae meals as an alternative protein ingredient in different fish species, including gilthead seabream

(*Sparus aurata*; Moutinho et al., 2022), European seabass (*Dicentrarchus labrax;* Abdel-Tawwab et al., 2020; Magalhães et al., 2017), Atlantic salmon (Salmo salar; Belghit et al., 2019), Japanese seabass (*Lateolabrax japonicus*; Wang et al., 2019) and rainbow trout (*Oncorhynchus mykiss*; Dumas et al., 2018; Renna et al., 2017).

HI larvae are also rich in fat (up to 40% dry matter), with a fatty acid (FA) profile rich in saturated fatty acids (SFA), with lauric acid (C12:0) being the most abundant, representing 21-50% of total FAs (Belghit et al., 2018a; Liland et al., 2017). Lauric acid is a medium-chain fatty acid (MCFA) and is considered an efficient energy source for animals, since they are preferentially used for β-oxidation and are rapidly catabolized, thus not increasing tissue lipid deposition (Li et al., 2016). Incorporating MCFA in feeds can also increase protein sparing in fish (Davis et al., 1999) and enhance the selective retention of long-chain PUFA, which can be spared for physiologically important processes (Belghit et al., 2018a; Li et al., 2016; Marques et al., 2021). Moreover, dietary inclusion of MCFA, or MCFA-rich ingredients such as HIO, has been suggested to improve the immune, inflammatory, and antioxidant response in fish (Simó-Mirabet et al., 2017; Ullah et al., 2022; Xu et al., 2020), weanling pigs (Li et al., 2015) and broiler chickens (Chen et al., 2022; Kim et al., 2020).

Although often regarded as a by-product of insect meal production (Fawole et al., 2021; Li et al., 2016), the interest in using HI larvae oil (HIO) as an alternative lipid source for animal feeds has been increasing in recent years (Sudha et al., 2022; Xu et al., 2020). Few studies have however investigated the potential of its inclusion in fish diets. Available information indicates that the incorporation of HIO in the diets has no adverse effects on the growth performance of Atlantic salmon (*Salmo salar*; Belghit et al., 2018a; Belghit et al., 2018b), rainbow trout (Oncorhynchus mykiss; Dumas et al., 2018; Fawole et al., 2021), Jian carp (*Cyprinus carpio* var. Jian; Li et al. 2016), and striped catfish (*Pangasianodon hypophthalmus*; Sudha et al. 2022). It was also shown that HIO was used more efficiently than silkworm or yellow mealworm oils by mirror carp (*Cyprinus carpio* var. *specularis*) (Xu et al. 2020).

Therefore, the objective of this study was to evaluate the potential of HIO as a replacement for VO in diets for gilthead seabream juveniles and its effects on growth performance, feed utilization efficiency, whole-body composition, and fatty acid digestibility.

2. Materials and methods

2.1 Ethics statement

All procedures were performed by accredited scientists (following FELASA category C recommendations). Trials were conducted according to the European Union Directive (2010/63/EU) on animal protection for scientific purposes and approved by the Ethics Committee of CIIMAR (reference ORBEA_CIIMAR_27_2019).

2.2 Ingredients and experimental diets

Diet formulation and proximate analysis is presented in Table 5.1. Ingredients were supplied by Sorgal, S.A. (Ovar, Portugal), and the HIO was provided by Hermetia Baruth GmbH (Baruth/Mark, Germany). The HM larvae were reared in a substrate consisting of a mixture of vegetable ingredients for 8 days, after which they were separated from the frass by sieving. After drying, the fat was extracted by a mechanical process using a screw mill. Four experimental diets were formulated to be isoproteic (45% crude protein) and isolipidic (18% crude lipids) and meet the nutritional requirements of gilthead sea bream. The control diet (HIO0) included sardine oil (3.1% of the diet) and a VO blend (9.5% of the diet) as the main lipid sources. The VO blend consisted of 20% rapeseed, 30% palm, and 50% linseed oils. HIO was added to the control diet at 4% (diet HIO42), 7.9% (diet HIO84), and 9.5% (diet HIO100), replacing the VO blend at 42, 82, and 100%, respectively. All ingredients were mixed and cold-pressured pelleted in a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA) through a 3 mm matrix. Pellets were dried in an oven at 55 °C for 24 h and stored at -20 °C until use.

2.3 Growth trial and sampling

Gilthead seabream juveniles were supplied by Sonríonansa S.L. (Pesués, Spain), and were quarantined for two weeks after arriving at the experimental facilities at CIIMAR (Porto, Portugal). Fish were then transferred to the experimental system and allowed to acclimatize to the experimental conditions for additional two weeks. The experimental setup consisted of a thermo-regulated recirculation seawater system equipped with twelve 500 L fiberglass tanks, each supplied with continuous aeration and water flow. During the trial, the temperature averaged 23 ºC, salinity 35‰, dissolved oxygen was kept above 8 mg L^{-1}, nitrogenous compounds were kept below 0.02 mg L^{-1}, and photoperiod was set to 12 h light: 12 h dark. The trial began with 192 fish (initial body weight of 63 g) that were homogeneously distributed into the tanks (16 fish/tank). Five fish from the initial stock were collected and pooled for initial whole-body composition analysis. The experimental diets were randomly assigned to triplicate groups, and fish

were hand-fed twice a day, 6 days a week, until apparent visual satiation for a total of 70 days. Feed consumption was recorded for each tank, and uttermost care was taken to avoid feed waste and to ensure all feed supplied was consumed. At the end of the growth trial, fish fasted for one day and were bulk weighed for each tank under mild anaesthesia (0.3 ml L^{-1} ethylene glycol monophenyl ether). Three fish per tank were euthanized (n=9 per dietary treatment) with a sharp blow to the head and pooled for final whole-body composition analysis and the liver and viscera were excised and weighed to calculate hepatosomatic and visceral indexes. The remaining fish continued to be fed for 3 more days after which the intestine was sampled from 3 fish per tank (n=9 per dietary treatment), 4 hours after the morning meal. Fish were euthanized with a sharp blow to the head and immediately eviscerated in an ice-cold tray. The intestine (with pyloric ceca) was separated from the adherent adipose and connective tissues, immediately frozen and stored at -80ºC until analyses.

2.4 Digestibility trial

A digestibility trial was conducted to evaluate the apparent digestibility coefficients (ADCs) of the experimental diets. For that purpose, chromium oxide (Cr_2O_3) was included as a digestibility marker at 0.5% in the previously used diets. The experimental setup consisted of 12 fiberglass tanks (60 L capacity) supplied with continuous aeration and water flow. Each tank was connected to a feces-settling column, designed according to Cho et al. (1982). Water parameters were maintained as described above for the growth trial. Homogenous groups of 10 fish (initial body weight of 63 g) were allocated in each tank, and diets were randomly assigned and tested in triplicate (n=3 per dietary treatment). Fish were hand-fed twice a day to apparent visual satiation. After five days of adaption to the experimental diets, feces collection was carried out as follows: approximately 30 min after the afternoon meal, tanks, pipes, and settling columns were thoroughly cleaned to ensure no uneaten feed or feces remained in the system. The next day, before the morning meal, feces were collected from the settling column, centrifugated at 4000 *g* for 10 min, and pooled for each tank. The fecal collection was performed daily until an adequate amount of feces was obtained for each tank.

2.5 Chemical analyses

Ingredients, diets, feces, and whole-body were finely ground and homogenized, and analyzed according to the AOAC (2000) procedures: dry matter by drying in an oven at 105 ºC until constant weight, ash by combustion in a muffle furnace at 450 ºC for 16 h, crude protein (N × 6.25) by the Kjeldahl method following acid digestion, using the Kjeltec digestion and distillation units (Tecator Systems, Höganäs, Sweden; models 1015 and

1026, respectively), crude lipids by extraction with petroleum ether using a Soxtec system (Tecator Systems, Höganäs, Sweden; extraction unit model 1043 and service unit model 1046), and gross energy by direct combustion of samples in an adiabatic bomb calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261). Chromium oxide in the diets and feces was determined according to Bolin et al. (1952). To determine the ADC of lipids and fatty acids (FA), the total lipid content of HIO, diets and feces was extracted according to (Folch et al., 1957) and gravimetrically quantified. The FAs in the lipid extract were determined after transesterification to methyl esters (FAME) using a base-catalyzed trans-esterification (Christie, 1982). The FA composition was determined by gas-chromatography (GC) using a Varian GC 430 gas chromatograph (Varian Inc., Palo Alto, CA, USA), equipped with a flame ionization detector (FID) and a Supelco Omegawax™ 320 m capillary column (Supelco, Bellefonte, PA, USA). Chromatograms were recorded with the Galaxie Chromatography Data System 1.9.302.952 (Varian Inc., Palo Alto, CA, USA). FAs were identified by comparing the FAME retention time with those of the Supelco 37 component FAME mix standard (Supelco, Bellefonte, PA, USA) and quantified through calibration curves using tricosanoic acid (C23:0) (Supelco, Bellefonte, PA, USA) as internal standard.

2.6 Digestive enzymes activity

For determination of enzymatic activity, each intestine was homogenized in ice-cold buffer (100 mM Tris–HCl, 0.1 mM EDTA, pH 7.8), centrifuged (30,000 *g*; 30 min; 4 °C) and the supernatant collected and stored at −80 °C until analysis. Total proteases activity was measured by the casein hydrolysis method according to (Walter, 1984) and adapted by Hidalgo et al. (1999). Trypsin (EC 3.4.21.4) activity was determined according to (Faulk et al., 2007), using 1 mM NaBenzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA) as substrate combined with 50 mM Tris–HCl, 20 mM CaCl2, pH 8.2 buffer. Production of p-nitroaniline was monitored at 410 nm. α-Amylase (EC 3.2.1.1) activity was determined with a commercial kit (ref. 41201, Spinreact, Girona, Spain) with modifications; the rate of product formation (2-chloro-4-nitrophenol) was quantified at 405 nm. Lipase (EC 3.1.1.3) activity was determined using a commercial kit (ref. 1001275, Spinreact, Girona, Spain) with modifications; 1-2-O-dilaurylrac-glycero-3-glutaric acid-60-methylresorufin-ester was used as substrate, and the formation rate of methylresorufin was followed at 580 nm. Total soluble protein was determined by the Bradford method (Bradford, 1976), using bovine serum albumin solution as standard. Enzyme activity was expressed as specific activity; one unit (U) of activity was defined as μmol of product generated per minute.

2.7 Calculations

Growth performance and feed efficiency parameters were calculated as follows:

Feed efficiency (FE) = wet weight gain / dry feed intake;

Daily growth index (DGI) = ((final weight$^{1/3}$ – initial weight$^{1/3}$) / days) × 100;

Protein efficiency ratio (PER) = weight gain / crude protein intake;

Hepatosomatic index (HSI) = (liver weight / fish weight) × 100;

Visceral index (VSI) = (visceral weight / fish weight) × 100;

Nitrogen or energy retention (% nitrogen or energy intake) = Whole body nitrogen or energy gain/nutrient or energy intake.

The ADCs (%) of dry matter, protein, lipids, energy, and FA of the experimental diets were calculated as follows:

ADC diet (%) = [1- ((diet Cr_2O_3 × Y in feces))/ ((feces Cr_2O_3 × Y in diets))] × 100

where Y is the nutrient or energy content.

2.8 Statistical analysis

Data are presented as mean (n=3 or n=9) and pooled standard error of the mean (SEM). All data were checked for normal distribution and homogeneity of variances and normalized when appropriate. One-way ANOVA was used to assess differences between treatments, and the probability level of 0.05 was used to reject the null hypothesis. Polynomial contrasts analysis was performed to determine if data followed a linear or quadratic response to dietary HIO inclusion. Tukey's multiple range test was also performed to show the magnitude of differences among means. All statistical analyses were performed using SPSS 25.0 software package (IBM Corp.) for Windows.

Table 5.1: Ingredients (% DM) and proximate composition (% DM) of the experimental diets used in the growth trial and digestibility trial.

	HIO0	HIO42	HIO84	HIO100
Fishmeal[1]	20	20	20	20
Soluble fish protein concentrate[2]	5	5	5	5
Haemoglobin[3]	3	3	3	3
Corn gluten meal[4]	15	15	15	15
Soybean meal[5]	10.9	10.9	10.9	10.9
Rice bran[6]	10	10	10	10
Whole wheat[7]	15	15	15	15
Sardine oil[8]	3.11	3.11	3.11	3.11
VO blend[9]	9.5	5.5	1.5	0
HI larvae oil[10]	0	4.0	7.9	9.5
Taurine[11]	0.3	0.3	0.3	0.3
Phospholipids[12]	1	1	1	1
Shrimp hydrolysate[13]	1.5	1.5	1.5	1.5
Betaine[14]	0.2	0.2	0.2	0.2
Calcium biphosphate[15]	2	2	2	2
Vitamin premix[16]	1	1	1	1
Choline chloride (50%)[17]	0.5	0.5	0.5	0.5
Mineral premix[18]	1	1	1	1
Binder[19]	1	1	1	1
Proximate composition:				
Dry matter, DM (%)	91.6	90.9	92.8	92.1
Crude protein, CP (%)	46.8	46.4	47.0	46.3
Crude lipids, CL (%)	17.6	16.5	17.2	16.4
Ash (%)	9.14	9.21	9.20	9.10
Energy (kJ g^{-1})	22.0	22.1	24.1	23.4

[1]Fishmeal (89.1% DM, 74.9% CP, 10.7% CL, 29.6% Ash); Sorgal, S.A. Ovar, Portugal
[2]Sorgal, S.A. Ovar, Portugal
[3]Hemoglobin powder AP310P; APC Europe S.A.
[4]Sorgal, S.A. Ovar, Portugal
[5]Sorgal, S.A. Ovar, Portugal
[6]Sorgal, S.A. Ovar, Portugal
[7]Whole wheat (88.6% DM, 14.5% CP, 1.2% CL); Sorgal, S.A. Ovar, Portugal
[8]Sardine oil, Sorgal, S.A. Ovar, Portugal
[9]Vegetable oil blend: 50% linseed, 30% palm, and 20% rapeseed oils
[10]*Hermetia illucens* larvae oil; Hermetia Baruth GmbH, Germany.
[11]Sorgal, S.A. Ovar, Portugal
[12]Premix, Viana do Castelo, Portugal.
[13]Shrimp hydrolysate (*Litopenaeus vannamei* trimmings: 73% CP, 12.1% CL, 13% Ash); Sorgal, S.A. Ovar, Portugal
[14]Sorgal, S.A. Ovar, Portugal
[15]Calcium biphosphate, Sorgal, S.A. Ovar, Portugal
[16]Vitamins premix (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
[17]Premix, Viana do Castelo, Portugal.
[18]Minerals premix (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)
[19]Binder: Carboxymethylcellulose sodium; Sigma-Aldrich Química, Portugal

Table 5.2: Fatty acid composition (% of total FAME) of the Hermetia illucens larvae oil (HIO) and the experimental diets.

		Experimental diets			
	HIO	**HIO0**	**HIO42**	**HIO84**	**HIO100**
C12:0	57.1	0.30	12.1	23.7	29.1
C14:0	11.5	1.64	3.99	6.39	7.33
C16:0	12.1	17.9	16.8	15.7	15.2
C18:0	1.59	3.48	3.02	2.55	2.35
C16:1n-7	2.50	1.62	2.18	2.75	2.94
C18:1n-6	6.91	29.7	23.7	18.2	15.9
C18:1n-7	0.31	1.85	1.60	1.40	1.31
C20:1n-9	0.08	1.31	1.22	1.18	1.09
C18:2n-6 (LA)	5.16	16.7	14.8	12.8	12.0
C18:3n-3 (ALA)	0.52	14.8	9.30	3.58	1.38
C20:4n-6 (ARA)	-	0.26	0.28	0.26	0.28
C20:5n-3 (EPA)	-	2.00	2.06	2.07	2.08
C22:6n-3 (DHA)	-	3.88	4.13	4.10	4.12
∑SFA	83.9	24.4	37.2	49.9	55.6
∑MUFA	10.2	36.1	30.4	25.3	22.8
∑n-6 PUFA	5.19	17.5	15.5	13.5	12.7
∑n-3 PUFA	0.58	21.7	16.5	10.9	8.57

The following FAs, found in percentages below 1% of total FAME, were utilised for calculating the classes FAs, but they are not listed in the table: C10:0, C14:1n-5, isoC15:0, C15:0, isoC16:0, C16:1n-9, C16:2n-4, C17:0, C16:3n-4, C17:1, C16:4n-1, C18:2n-4, C18:3n-6, C18:3n-4, C18:4n-3, C18:4n-1, C20:0, C20:1n-11, C20:1n-7, C20:2n-6, C20:3n-6, C20:3n-3, C20:4n-3, C22:0, C22:1n-9, C22:1n-7, C22:2n-6, C21:5n-3, C22:4n-6, C22:5n-6, C22:5n-3, and C24:0. LA: Linoleic acid; ALA: α-Linolenic acid; ARA: Arachidonic acid; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids

3. Results

Diets were comparable in terms of dry matter, protein, lipid, and ash content (Table 5.1) but the dietary FA profile was heavily influenced by the HIO inclusion level (Table 5.2). HIO was predominantly composed of SFAs (83.9%), mainly C12:0 lauric acid (57.1%), and the content of these FAs increased, while total monosaturated fatty acids (MUFA), n-3, and n-6 PUFA decreased with the dietary HIO content (Table 5.2).

All diets were well accepted by the fish and no mortality occurred during the trial. No differences were observed between groups in feed intake, final body weight, weight gain, or DGI (Table 5.3). Feed efficiency and PER linearly increased with the increase of HIO in the diets, being significantly higher in fish fed with HIO84 and HIO100 diets than with the other diets. A trend for a linear increase in nitrogen retention (% nitrogen intake) was also observed, while energy retention (% energy intake) was unaffected by diet composition (Table 5.3).

Table 5.3: Growth performance and nutrient retention (% intake) of gilthead seabream fed the experimental diets.

						Polynomial contrasts		
	HIO0	**HIO42**	**HIO84**	**HIO100**	**SEM**	**ANOVA *p*-value**	**Linear *p*-value**	**Quadratic *p*-value**
Initial body weight, g	63.0	63.0	63.0	63.0	0.03	ns	ns	ns
Final body weight, g	145.9	141.2	156.3	158.6	4.37	ns	ns	ns
Weight gain, g kg^{-1} day^{-1}	11.2	10.9	12.1	12.3	0.36	ns	ns	ns
Daily growth index	1.83	1.75	2.00	2.04	0.08	ns	ns	ns
Feed intake, g kg^{-1} day^{-1}	13.1	12.8	12.9	13.6	0.34	ns	ns	ns
Feed efficiency	0.85^{b}	0.85^{b}	0.93^{a}	0.91^{a}	0.01	0.001	0.001	ns
Protein efficiency ratio	1.94^{c}	2.08^{b}	2.20^{a}	2.22^{a}	0.03	0.000	0.000	ns
Survival (%)	100	100	100	100	0.00	-	-	-
Nitrogen retention (% nitrogen intake)	33.2	34.6	36.2	36.5	0.60	ns	0.039	ns
Energy retention (% Energy intake)	37.6	40.8	38.1	33.4	1.19	ns	ns	ns

Values are presented as a mean of n=3. SEM: Standard error of the mean. Different superscript letters indicate significant differences between treatments (one-way ANOVA, p < 0.05). ns: non-significant p-value (p < 0.05)

The whole-body composition was not significantly affected by diet composition, although a linear trend for a decrease in ash content was observed with the increase in dietary HIO inclusion (Table 5.4). Also, no differences were observed in the hepatosomatic and visceral indexes.

Table 5.4: Whole body composition (% fresh weight) of gilthead seabream fed the experimental diets.

						Polynomial contrasts		
	HIO0	**HIO42**	**HIO84**	**HIO100**	**SEM**	**ANOVA *p*-value**	**Linear *p*-value**	**Quadratic *p*-value**
Moisture	66.5	66.1	66.9	67.3	0.27	0.473	0.209	0.493
Protein	17.5	17.3	17.1	17.1	0.09	0.381	0.113	0.587
Lipid	11.8	12.7	12.4	11.4	0.23	0.232	0.477	0.063
Ash	4.13	3.88	3.88	3.76	0.06	0.128	0.031	0.754
Energy (kJ g^{-1})	8.66	9.11	8.81	8.37	0.14	0.344	0.371	0.139
HSI[1]	1.19	1.12	1.06	1.06	0.03	0.488	0.158	0.662
VSI[2]	5.46	5.12	5.06	5.08	0.10	0.495	0.220	0.401

Values are presented as a mean of n=3. SEM: Standard error of the mean. ns: non-significant p-value (p < 0.05). The absence of different superscript letters indicates no significant differences (one-way ANOVA, p > 0.05). [1]HSI: Hepatossomatic index; [2]VSI: Visceral index.

The ADC of dry matter, protein, and lipids were not significantly affected by diet composition, although a trend for a linear increase in protein digestibility and a quadratic response for lipid digestibility was observed with dietary HIO inclusion (Table 5.5). The ADC of energy linearly increased with dietary HIO inclusion and was significantly higher with diet HIO100 than with diets HIO0 and HIO42.

There was a linear increase of the ADC of total SFA with the incorporation of HIO in the diets (Table 5.5). The ADC of C12:0 (lauric acid) in the control diet was not calculated due to its relative absence in diet and feces. Nevertheless, its digestibility was high (>91%) for the HIO-containing diets. The ADC of unsaturated FA decreased with the dietary inclusion of HIO, and this decrease was statistically significant for n-3 PUFA. The ADC of C20:5n-3 (EPA) and C22:6n-3 (DHA) was not affected by diet composition.

Table 5.5: Apparent digestibility coefficients (%) of nutrients and fatty acids of the experimental diets.

						Polynomial contrasts		
	HIO0	**HIO42**	**HIO84**	**HIO100**	**SEM**	**ANOVA *p*-value**	**Linear *p*-value**	**Quadratic *p*-value**
Dry matter	53.3	50.4	52.9	63.9	2.4	0.238	0.118	0.155
Protein	89.7	88.7	90.1	92.5	0.5	0.068	0.032	0.079
Lipids	85.3	84.7	84.4	88.2	0.6	0.068	0.064	0.038
Energy	70.1^{b}	67.3^{b}	74.6^{ab}	78.4^{a}	1.4	0.004	0.002	0.079
Fatty acids								
C12:0	nd*	91.8^{B}	92.2^{AB}	94.2^{A}	4.6	0.039	0.016	ns
C14:0	85.0^{b}	87.1^{ab}	86.4^{ab}	89.9^{a}	0.6	0.040	0.011	ns
C16:0	74.8^{ab}	77.1^{ab}	73.8^{b}	81.0^{a}	1.0	0.042	0.048	ns
C16:1n-7	89.1	89.9	89.1	92.2	0.5	ns	ns	ns
C18:0	74.3	76.1	71.3	78.4	1.0	ns	ns	ns
C18:1n-9	91.4^{a}	90.5^{ab}	88.4^{b}	91.1^{ab}	0.4	0.048	ns	0.024
C18:1n-7	88.6^{a}	87.3^{ab}	83.6^{b}	87.7^{ab}	0.7	0.030	ns	0.023
C18:2n-6 (LA)	96.9	96.1	94.5	95.7	0.3	ns	0.066	ns
C18:3n-3 (ALA)	99.3^{a}	99.0^{ab}	98.5^{ab}	98.3^{b}	0.1	0.018	0.003	ns
C20:1n-9	89.7	89.0	86.6	89.7	0.5	ns	ns	0.037
C20:5n-3 (EPA)	99.6	99.6	99.5	99.6	0.0	ns	ns	ns
C22:1n-11	90.4	89.6	87.2	89.9	0.5	ns	ns	ns
C22:1n-9	85.0	83.1	77.7	82.6	1.0	ns	ns	ns
C22:6n-3 (DHA)	97.9	98.1	97.3	97.9	0.2	ns	ns	ns
∑SFA	74.8^{c}	82.5^{b}	84.1^{b}	88.9^{a}	1.5	0.000	0.000	ns
∑MUFA	91.0	90.1	88.0	90.9	0.4	ns	ns	0.023
∑n-3 PUFA	99.0^{a}	98.9^{ab}	98.2^{c}	98.5^{bc}	0.1	0.003	0.002	ns
∑n-6 PUFA	97.0	96.2	94.6	95.8	0.3	ns	ns	ns

Values are presented as a mean of n=3. SEM: Standard error of the mean. Different superscript letters indicate significant differences between treatments (one-way ANOVA, $p < 0.05$). ns: non-significant p-value ($p < 0.05$). *nd: not determined due to the very low concentrations in either feed or faeces. LA: Linoleic acid; ALA: α-Linolenic acid; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids.

Amylase and trypsin activities were not statistically affected by diet composition, while lipase activity followed a positive linear trend (Table 5.6). Total protease activity increased with dietary HIO inclusion up to 7.9%, decreasing thereafter.

Table 5.6: Digestive enzymes activity (mU mg protein^{-1}) of gilthead seabream fed the experimental diets.

						Polynomial contrasts		
	HIO0	**HIO42**	**HIO84**	**HIO100**	**SEM**	**ANOVA *p*-value**	**Linear *p*-value**	**Quadratic *p*-value**
Trypsin	36.8	44.6	45.0	41.8	2.10	ns	ns	ns
Total proteases	0.50^{c}	0.68^{ab}	0.84^{a}	0.62^{bc}	0.03	0.000	0.017	0.000
Amylase	95.7	87.4	88.3	80.1	4.7	ns	ns	ns
Lipase	1.61	1.79	1.83	1.93	0.05	ns	ns	ns

Values are presented as a mean of n=9. SEM: Standard error of the mean. ns: non-significant p-value ($p < 0.05$). The absence of different superscript letters indicates no significant differences (one-way ANOVA, $p > 0.05$).

4. Discussion

Insect meals have been proven to be a valuable protein source for aquafeeds, contributing to the formulation of feeds with reduced environmental impact (Belghit et al., 2018b; Henry et al., 2015). Although insect oils have been generally regarded as a by-product of insect meal production, their use in animal feeds has been gaining interest in recent years (Sudha et al., 2022).

The present trial showed that HIO could completely replace VO in diets of gilthead seabream juveniles with no adverse effects on growth performance, feed intake, and whole-body composition. Further, FE linearly increased with HIO incorporation into the diets and N retention also showed a trend to increase with dietary HIO inclusion, and this may be related to the increased ADC of protein and energy. Similar results were observed in other fish species with comparable levels of HIO in the diets. For instance, a lack of negative effects on growth performance has also been reported in rainbow trout with the inclusion of 10% HIO in the diets, totally replacing FO content (Dumas et al. 2018) or with the inclusion of 16% HIO in the diets, totally replacing FO or soybean oil contents (Fawole et al. 2021). Also in barramundi (*Lates calcarifer*), dietary inclusion of 10% HIO replacing 30% FO content did not affect growth performance (Hender et al., 2021). However, other studies present some contradictory results, even with lower dietary inclusion levels of HIO. For instance, while it was possible to include 2% HIO in the diets (replacing 30% FO) of juvenile *Totoaba macdonaldi* without affecting growth, dietary inclusion of 4% HIO (replacing 60% FO) depressed growth performance (Maldonado-Othón et al. 2022). Also in Jian carp, the inclusion of 2.5% HIO in the diets, completely replacing dietary soybean oil, did not affect growth performance (Li et al. 2016). Differently, the dietary inclusion of 2.5% HIO, replacing black cumin seed oil or flax oil, led to higher growth performance of striped catfish (Sudha et al. 2022). Similarly,

in mirror carp, dietary inclusion of 2.5% HIO promoted better growth and feed utilization than silkworm or yellow mealworm oils (Xu et al. 2020), and the replacement of 50% soybean oil (2.5% in the diet) or above by HIO also improved growth and feed utilization (Xu et al., 2021).

In the present trial, no differences in whole-body composition were noticed between experimental groups. Similar results were also observed in other studies with rainbow trout (Dumas et al. 2018), mirror carp (Xu et al. 2020), and striped catfish (Sudha et al. 2021). Differently, in another study also in rainbow trout fed HIO-containing diets it was observed an increase in whole-body moisture content when compared to fish fed an FO-based diet (Fawole et al. 2021), and in black seabream, dietary lauric acid supplementation decreased whole-body moisture content (Ullah et al. 2022).

The FA profile of HIO is dominated by SFA, particularly lauric acid, a MCFA (Rodrigues et al., 2022; Xu et al., 2021). Accordingly, the FA profile of the HIO used in the present study included 84% SFA, of which 68% was lauric acid. This is similar to values described in other studies with HIO (Belghit et al. 2018, Hender et al. 2021, Fawole et al. 2021). MCFAs have been suggested to increase energy availability and be strategically used as a main energy source by fish, therefore not increasing lipid deposition (Li et al., 2016; Røsjø et al., 2000; Xu et al., 2020). In the present trial, there were, however, no differences in whole-body lipid content, HSI, and VSI, indicating that dietary inclusion of HIO did not contribute to decreasing lipid deposition.

MCFAs were also reported to have a protein-sparing effect in fish, allowing for a more effective protein utilization (Davis et al., 1999). Results of the present trial indicate a linear trend for increased PER and N retention (% N intake) which may also indicate a possible protein-sparing effect of HIO related to its high content of MCFA. However, it can not be disregarded that the ADC of protein, energy and FA, namely that of SFA and MCFA also increased with the dietary inclusion of HIO. Thus, more studies are required to confirm if the increased protein utilization efficiency is related to protein-sparing, increased protein and energy availability, or both.

Digestibility is correlated with the activity of digestive enzymes, which can be significantly modified by dietary composition (Ullah et al., 2022). In the present trial, there was a linear increase in total protease activities with the increase in dietary HIO content, which correlates well with the observed increase in protein digestibility. Lipase activity was not significantly affected by dietary composition, although a significative trend to linearly increase with dietary HIO content was observed. In previous studies, 4% coconut oil also enhanced lipase and protease activities for Nile tilapia (Dawood et al., 2021) and diets

with MCT increased proteolytic activity in Atlantic salmon (Nordrum et al., 2000; Nordrum et al., 2003). Higher lipase activity was also found in European seabass larvae fed diets with coconut oil (Morais et al., 2004) and in black seabream fed diets supplemented with 0.1% lauric (Ullah et al. 2022). Although the ADC of lipids was not affected by diet composition in present trial, there were significant differences in the digestibility of FA with the increase in dietary HIO content.

The digestibility of lipids is usually correlated with the degree of unsaturation and chain length of FA (Williams et al., 2006), with SFA generally being less digestible than MUFA and PUFA (Bowyer et al., 2013). Thus, as expected, in the present study, the digestibility of FA increased from SFA<MUFA<PUFA. However, contrary to expected, the ADC of SFA also significantly increased as the SFA content in the diets increased, and this may be related to most SFAs being of medium-chain length, which are more digestible than long-chain length FA. Indeed, in this study, the ADC of SFA highly decreased as the FA chain length increased. Overall, the increase of protein digestibility and SFA digestibility contribute to explaining the increase of ADC of energy with the increase of dietary HIO inclusion. Previously, Belghit et al. (2018b) also showed high lipid digestibility (with minimal impact on individual FA) in Atlantic salmon fed with diets including HIO. On the other hand, more than 4% HIO (replacing 50% FO) reduced lipid digestibility in red hybrid tilapia (Bakar et al., 2021).

5. Conclusion

The present study showed that HIO can be considered a valuable lipid source for gilthead seabream juveniles, and it can completely replace dietary VO without negatively affecting growth performance. Further studies are required to assess the effects of dietary HIO on fillet quality traits, immune status, and oxidative stress response.

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Chapter 6: Effects of black soldier fly (*Hermetia illucens*) larvae oil on fillet quality and nutritional traits of gilthead seabream

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Effects of black soldier fly (*Hermetia illucens*) larvae oil on fillet quality and nutritional traits of gilthead seabream

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Abstract

A study was conducted to evaluate the effects of dietary inclusion of black soldier fly (*Hermetia illucens*) larvae oil (HIO) on fillet fatty acid (FA) profiles and other nutritional traits of gilthead seabream (*Sparus aurata*). Four experimental diets were formulated with 45% crude protein and 18% lipids (circa 50:50 of fish oil and vegetable oil (VO) blend: 20% rapeseed, 30% palm, and 50% linseed oil) and HIO was used to replace VO blend at 42%, 84%, and 100%, corresponding to a dietary inclusion level of 4%, 7.9%, and 9.5%, respectively. After a feeding trial of 10 weeks, several fillet quality traits were analyzed, including morphometric and somatometric indexes, physical and chemical characteristics, FA profile, and lipid peroxidation products. No differences were found in the morphometric and somatometric parameters, or skin and fillet color. The fillets FA profile was significantly modulated by the experimental diets, showing an increase in saturated fatty acids (SFA), particularly lauric acid, and a decrease in monosaturated FA and n-3 and n-6 polyunsaturated fatty acids (PUFA) with the increase of HIO inclusion, while eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) contents were not affected. Fillets FA quality indexes (PUFA/SFA, n-3/n-6 PUFA ratio, hypocholesterolemic/hypercholesterolemic ratio, thrombogenicity, and atherogenicity indexes) were also modulated by dietary HIO inclusion. Fillets pH was reduced and water holding capacity increased with dietary HIO inclusion. Fillet lipid peroxidation level (measured as TBARS) was significantly decreased with dietary HIO inclusion despite an increase in conjugated dienes. Overall, results showed that HIO can efficiently replace VO in gilthead seabream diets, without major negative effects on fillet characteristics and quality traits while decreasing fillet lipid oxidation.

Keywords: insect oil, alternative ingredients, lipids, flesh quality, fatty acids, *Sparus aurata*

1. Introduction

Due to the increasing demand for fish products, aquaculture has an increasingly important role in providing global food security and alleviating the pressure on depleting wild fish stocks. By 2030, it is expected that aquaculture will be responsible for 2/3 of worldwide fish for human consumption (FAO, 2022). The increase in demand for fish products is not only associated with the growth of the human population but also with changes in dietary habits, as consumers progressively acknowledge fish as a healthy source of protein and other key nutrients (Naylor et al., 2021). Seafood is considered an essential component of a healthy human diet due to its high content of the health-beneficial n-3 (or "omega-3") long-chain (≥C_{20}) polyunsaturated fatty acids (n-3 LC-PUFA), including eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3). The inclusion of the so-called "marine ingredients" such as fishmeal and fish oil (both rich in n-3 LC-PUFA) in aquafeeds has traditionally ensured high levels of n-3 LC-PUFA in marine aquaculture finfish (Tocher et al., 2019). However, the finite availability of marine ingredients, along with the expansion of aquaculture production in the last decades (FAO, 2022), have urged the use of alternative ingredients in aquafeeds. These ingredients are generally devoid of n-3 LC-PUFA (Aas et al., 2022; Tocher et al., 2019), and consequently the contents of n-3 LC-PUFA in farmed fish have been declining (Sprague et al., 2016). Also, unlike freshwater species, marine fish require LC-PUFAs in their diets, namely EPA and DHA, as they lack or have low expression of the necessary enzymes for the elongation and/or desaturation of PUFAs to LC-PUFAs, imposing additional constraints on potential alternative lipid sources (Oliva-Teles et al., 2015).

Fish oil (FO) has been regarded as the ideal lipid source for marine fish due to its high content of n-3 LC-PUFA. However, due to increasing demand, prices, and environmental impacts, the industry has shifted towards replacing FO with alternative oils and fats to contribute to the long-term sustainability of aquaculture. Several studies have demonstrated that the use of vegetable oils (VO) to replace FO is achievable without detrimental effects on growth performance and feed utilization, provided that the essential FA requirements of fish are satisfied (Fountoulaki et al., 2009; Izquierdo et al., 2005; Sáez-Royuela et al., 2022). However, while VOs are devoid of n-3 LC-PUFA, they are often rich in monounsaturated fatty acids (MUFA), such as palmitoleic acid (C16:1n-7) and oleic acid (OA, C18:1n-9), as well as C_{18} polyunsaturated fatty acids (C_{18} PUFA) including α-linolenic acid (ALA, C18:3n-3) and linoleic acid (LA, C18:2n-6). Since feed is one of the major modulators of the fillet FA composition (Fabrikov et al., 2021), dietary

replacement of FO with VO in aquafeed formulation impacts the nutritional quality of the final products (Fountoulaki et al., 2009; Sánchez-Moya et al., 2020).

In recent years, the interest in using insects as food and feed has grown remarkably. Insects are rich in protein, lipids, vitamins, and minerals (Liland et al., 2017), have fast growth and high feed efficiency, and their production has efficient water and land utilization, with low carbon footprint and greenhouse gas emissions (Henry et al., 2015). Current European Union (EU) legislation only allows insects to be reared on substrates of vegetable origin or specifically materials of animal origin such as fishmeal and hydrolysed proteins from non-ruminants (Regulation (EC) No 999/2001, 1069/2009, and No 142/2011). Nevertheless, low-valued substrates, including animal manure, food waste, agri-food side streams, and other organic wastes, will need to be specifically addressed in the future, thus allowing insects to contribute to a circular bioeconomy (Diener et al., 2009; van Huis et al., 2013). Currently, in the EU, there are eight authorized insect species for use in aquafeeds (Commission Regulation (EU) 2021/1925), among which is the black soldier fly (HI; *Hermetia illucens*). Besides having a high protein content (up to 60% dry matter), HI larvae are rich in lipids (up to 30% dry matter), which are often considered a by-product of insect meal production (Fawole et al., 2021). Lipids in HI have high levels of saturated fatty acids (SFA), particularly lauric acid (C12:0), and lack n-3 LC-PUFA (Li et al., 2016). However, the FA profiles of insect oils can be modulated to some extent, and if the insects are reared in n-3 LC-PUFA-rich substrates they can accumulate n-3 LC-PUFA, thus improving the nutritional quality of their oil for use in aquafeeds (Hossain et al., 2023; Xu et al., 2021). Additionally, medium-chain fatty acids (MCFA) such as lauric acid are rapidly catabolized and preferentially used for β-oxidation, allowing to spare FA such as EPA and DHA for physiologically important functions (Belghit et al., 2018; Li et al., 2016). Furthermore, the incorporation of lauric acid and other MCFA in aquafeeds has been used as a strategy to improve energy utilization without lipid deposition in tissues (Li et al., 2016).

To date, few studies have evaluated the effects of HIO inclusion in fish diets (Dumas et al., 2018; Hender et al., 2021; Maldonado-Othón et al., 2022; Moutinho et al., 2023; Sudha et al., 2022) and its impact on fillet FA profile and quality traits (Bakar et al., 2021; Fawole et al., 2021; Li et al., 2016).

Gilthead seabream (*Sparus aurata*) is a marine species highly appreciated by consumers, with a worldwide production of 282,100 tonnes in 2020 (FAO, 2022), being one of the most produced species in Mediterranean European aquaculture (FEAP, 2021). In a previous study (Moutinho et al., 2023), it was shown that HIO could fully

replace a VO blend in diets for gilthead seabream juveniles without compromising growth performance, feed efficiency, and nutrient digestibility. This study aimed to further evaluate the impact of these diets on the FA profile, quality traits, and physical and chemical characteristics of gilthead seabream fillets.

2. Materials and methods

2.1 Ethics statement

All procedures were performed by accredited scientists (following FELASA category C recommendations). The growth trial was conducted according to the European Union Directive (2010/63/EU) on animal protection for scientific purposes and approved by the Ethics Committee of CIIMAR (reference ORBEA_CIIMAR_27_2019).

2.2 Ingredients and experimental diets

Ingredients were supplied by Sorgal, S.A. (Ovar, Portugal), and the HIO was provided by Hermetia Baruth GmbH (Baruth/Mark, Germany). According to the supplier's information, HI larvae were reared in a substrate consisting of a mixture of vegetables for 8 days and then separated from the frass by sieving. HIO was separated from the dried larvae using a mechanical screw press. Four isoproteic (45% crude protein) and isolipidic (18% crude lipids) experimental diets were formulated to meet the nutritional requirements of gilthead seabream. A control diet (HIO0) included sardine oil (3.1% of the diet) and a VO blend (9.5% of the diet) as the main lipid sources. The VO blend consisted of a mixture of 20% rapeseed oil, 30% palm oil, and 50% linseed oil. The other experimental diets were formulated with HIO replacing the VO blend at increasing levels: 42% (diet HIO42), 84% (diet HIO84), and 100% (diet HIO100) corresponding to an inclusion level of 4.0%, 7.9%, and 9.5%, respectively. All ingredients were finely ground before mixing and dry pelleted in a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA) through a 3 mm matrix. Pellets were dried in an oven at 55 °C for 24 h and stored at -20 °C until further use. Diets' proximate compositions and fatty acid (% FAME) profile are presented in Table 6.1 and Table 6.2, respectively.

2.3 Experimental conditions

The experimental conditions for the growth trial were previously detailed in Moutinho et al. (2023). Briefly, 192 gilthead seabream juveniles (initial body weight of 63 g) were reared in a thermoregulated seawater system (average temperature 23 ºC), equipped with twelve fiberglass tanks of 500 L capacity with continuous aeration and water flow.

The experimental diets were hand-fed to triplicate groups of fish, two times a day, six days a week, to apparent visual satiation for 10 weeks.

Table 6.1: Ingredients (% DM) and proximate composition (% DM) of the experimental diets.

	HIO0	**HIO42**	**HIO84**	**HIO100**
Fishmeal[1]	20	20	20	20
Soluble fish protein concentrate[2]	5	5	5	5
Haemoglobin[3]	3	3	3	3
Corn gluten meal[4]	15	15	15	15
Soybean meal[5]	10.9	10.9	10.9	10.9
Rice bran[6]	10	10	10	10
Whole wheat[7]	15	15	15	15
Sardine oil[8]	3.11	3.11	3.11	3.11
VO blend[9]	9.5	5.5	1.5	0
HI larvae oil[10]	0	4.0	7.9	9.5
Taurine[11]	0.3	0.3	0.3	0.3
Phospholipids[12]	1	1	1	1
Shrimp hydrolysate[13]	1.5	1.5	1.5	1.5
Betaine[14]	0.2	0.2	0.2	0.2
Calcium biphosphate[15]	2	2	2	2
Vitamin premix[16]	1	1	1	1
Choline chloride (50%)[17]	0.5	0.5	0.5	0.5
Mineral premix[18]	1	1	1	1
Binder[19]	1	1	1	1
Proximate composition:				
Dry matter, DM (%)	91.6	90.9	92.8	92.1
Crude protein, CP (%)	46.8	46.4	47.0	46.3
Crude lipids, CL (%)	17.6	16.5	17.2	16.4
Ash (%)	9.14	9.21	9.20	9.10
Energy (kJ g^{-1})	22.0	22.1	24.1	23.4

[1]Fishmeal (89.1% DM, 74.9% CP, 10.7% CL, 29.6% Ash); Sorgal, S.A. Ovar, Portugal
[2]Sorgal, S.A. Ovar, Portugal
[3]Hemoglobin powder AP310P; APC Europe S.A.
[4]Sorgal, S.A. Ovar, Portugal
[5]Sorgal, S.A. Ovar, Portugal
[6]Sorgal, S.A. Ovar, Portugal
[7]Whole wheat (88.6% DM, 14.5% CP, 1.2% CL); Sorgal, S.A. Ovar, Portugal
[8]Sardine oil, Sorgal, S.A. Ovar, Portugal
[9]Vegetable oil blend: 50% linseed, 30% palm, and 20% rapeseed oils
[10]*Hermetia illucens* larvae oil; Hermetia Baruth GmbH, Germany.
[11]Sorgal, S.A. Ovar, Portugal
[12]Premix, Viana do Castelo, Portugal.
[13]Shrimp hydrolysate (*Litopenaeus vannamei* trimmings: 73% CP, 12.1% CL, 13% Ash); Sorgal, S.A. Ovar, Portugal
[14]Sorgal, S.A. Ovar, Portugal
[15]Calcium biphosphate, Sorgal, S.A. Ovar, Portugal
[16]Vitamins premix (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
[17]Premix, Viana do Castelo, Portugal.
[18]Minerals premix (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)
[19]Binder: Carboxymethylcellulose sodium; Sigma-Aldrich Química, Portugal

Table 6.2: Fatty acid composition (% of total FAME) of the Hermetia illucens larvae oil (HIO) and the experimental diets.

		Experimental diets			
	HIO	**HIO0**	**HIO42**	**HIO84**	**HIO100**
C12:0	57.1	0.30	12.1	23.7	29.1
C14:0	11.5	1.64	3.99	6.39	7.33
C16:0	12.1	17.9	16.8	15.7	15.2
C18:0	1.59	3.48	3.02	2.55	2.35
C16:1n-7	2.50	1.62	2.18	2.75	2.94
C18:1n-7	0.31	1.85	1.60	1.40	1.31
C18:1n-9	6.91	29.7	23.7	18.2	15.9
C20:1n-9	0.08	1.31	1.22	1.18	1.09
C18:2n-6 (LA)	5.16	16.7	14.8	12.8	12.0
C18:3n-3 (ALA)	0.52	14.8	9.30	3.58	1.38
C20:4n-6 (ARA)	-	0.26	0.28	0.26	0.28
C20:5n-3 (EPA)	-	2.00	2.06	2.07	2.08
C22:6n-3 (DHA)	-	3.88	4.13	4.10	4.12
∑SFA	83.9	24.4	37.2	49.9	55.6
∑MUFA	10.2	36.1	30.4	25.3	22.8
∑n-6 PUFA	5.19	17.5	15.5	13.5	12.7
∑n-3 PUFA	0.58	21.7	16.5	10.9	8.57

The following FA, found in percentages below 1% of total FAME, were utilised for calculating the classes FA, but they are not listed in the table: C10:0, C14:1n-5, isoC15:0, C15:0, isoC16:0, C16:1n-9, C16:2n-4, C17:0, C16:3n-4, C17:1, C16:4n-1, C18:2n-4, C18:3n-6, C18:3n-4, C18:4n-3, C18:4n-1, C20:0, C20:1n-11, C20:1n-7, C20:2n-6, C20:3n-6, C20:3n-3, C20:4n-3, C22:0, C22:1n-9, C22:1n-7, C22:2n-6, C21:5n-3, C22:4n-6, C22:5n-6, C22:5n-3, and C24:0. LA: Linoleic acid; ALA: α-Linolenic acid; ARA: Arachidonic acid; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids.

2.4 Biometrics, marketable characteristics, and physical parameter analyses

At the end of the growth trial, three fish per tank (n=9 per dietary treatment) were collected after 24 h fasting, sacrificed by a sharp blow to the head, and kept immersed in an ice-slurry bath. Fish were individually tagged, weighed, and total length measured (cm). Then, fish were dissected to separate the viscera and the different parts to calculate the following parameters:

Condition Factor, K (%) = [(body weight (g)/total length (cm)^3] × 100

Fillet Yield, FY (%) = [(fillet with skin weight (g)/body weight (g)] × 100

Visceral fat (%) = [viscera fat weight (g)/body weight (g)] × 100

Frame (%) = [(frame weight (g)/body weight (g)] × 100

Skin (%) = [(skin weight (g)/body weight (g)] × 100

Fins (%) = [(fins weight (g)/body weight (g)] × 100

Color measurements were performed in three positions (cranial, medial, and caudal) on both sides of the skin and right and left fillets with a CHROMA METER CR-200 (Konica Minolta, Chiyoda, Japan). Cranial was defined as the position closest to the head following the lateral line, medial as the position in the centre following the lateral line and caudal as the position closest to the caudal fin along the lateral line. The color was expressed as lightness (*L**), redness index (*a**), and yellowness index (*b**), according to the CIELab system (CIE, 1977). Mean values for each fillet were utilized for data analysis (n = 9 per dietary treatment).

Muscle pH was measured on the cranial, medial, and caudal positions of the epaxial region of the right and left fillets of each fish and the mean values were used for data analysis (n = 9 per dietary treatment). A pH-meter SevenGo SG2™ equipped with an Inlab puncture electrode (Mettler-Toledo, Schwerzenbach, Switzerland) was used.

Fillet texture analyses were performed using a Warner-Bratzler shear blade (width of 7 cm) by a Zwick Roell® 109 texturometer (Zwick Roell, Ulm, Germany), equipped with a 1 kN load cell, setting the crosshead speed at 30 mm min^{-1}. A section of 3 x 3 cm was cut from the epaxial cranial region of both fillets and subjected to the force of the blade probe to determine the share force (n = 9 per dietary treatment). Test-Xpert2 3.0 by Zwick Roell® software was used for data collection and analysis.

Afterwards, the fillets were skinned and homogenized to determine the Water Holding Capacity (WHC, %) according to Iaconisi et al. (2018). Briefly, 2 g of the homogenate was inserted in plexiglass cylinders equipped with filter net and were centrifuged at 1500 rpm for 5 min. Then, the WHC was calculated as the difference between the initial gross weight of the cylinders and their gross weight after centrifugation, and the value obtained was divided by the water content of the sample. WHC was performed in duplicate for each sample and the mean value was used for data analysis (n = 9 per dietary treatment).

2.5 Fatty acid profile

The lipid content of fillet samples was determined according to Folch et al. (1957). Briefly, 2 g of fillet were homogenized on ice with 34 ml of chloroform-methanol solution (2:1 v/v + 0.015 g BHT). A saline solution (10 ml; 0.88% KCl) was used to separate the homogenate in two phases: an upper phase containing non-lipid substances, and a lower phase with the lipid fraction bound to the chloroform. The chloroform was evaporated at 45ºC with constant N flow, and the resulting lipid extract was concentrated (n=9 per

dietary treatment). The fatty acids (FA) profile was determined in the lipid extract after transesterification to methyl esters (FAME) using a base-catalyzed trans-esterification (Christie, 1982). The FA composition was determined by gas chromatography (GC) using a Varian GC 430 gas chromatograph (Varian Inc., Palo Alto, CA, USA), equipped with a flame ionization detector (FID) and a Supelco Omegawax™ 320 m capillary column (Supelco, Bellefonte, PA, USA). The conditions were as described by Secci et al. (2018): temperature was kept at 100 ºC for 2 min, increased to 160 ºC for 4 minutes at the rate of 12 ºC min^{-1}, and then increased to 220 ºC for 14 min at the rate of 3 ºC min^{-1} and kept at 220 ºC for 25 min. The injector and the detector temperatures were set at 220 ºC and 300 ºC, respectively. A quantity of 1 µL of sample in hexane was injected into the column with the carrier gas (helium) kept at a constant flow of 1.5 mL min^{-1} with a split ratio was 1:20. Chromatograms were recorded with the Galaxie Chromatography Data System 1.9.302.952 (Varian Inc., Palo Alto, CA, USA). FA were identified by comparing the FAME retention time with those of the Supelco 37 component FAME mix standard (Supelco, Bellefonte, PA, USA) (n = 9 per dietary treatment).

2.6 Lipid oxidation products

Primary lipid oxidation products were quantified in homogenized fillets samples (n = 9 per dietary treatment) as conjugated dienes (CD). The CDs were quantified in 0.5 µL of lipid extract dissolved in 3 mL pure hexane. The absorbance at 232 nm was determined using a Scan spectrophotometer Varian, equipped with Cary Win UV Software (Varian Inc., Palo Alto, CA, USA), and the mmol hydroperoxides kg^{-1} sample was calculated using a molar extinction coefficient of 29,000 mL $mmol^{-1}$ × cm (Srinivasan et al., 1996). Results are expressed as mmol hydroperoxides kg^{-1} sample.

Secondary lipid oxidation products (thiobarbituric acid reactive substances, TBARS) were quantified in homogenized fillets (n = 9 per dietary treatment) by the colorimetric method described by Secci et al. (2016). Briefly, TBARS were extracted in 10 ml of trichloroacetic acid (5%) and then adding 5 ml of thiobarbituric acid (TBA) 0.02 mol L^{-1}. After 40 min of incubation at 90 °C, the lipid oxidation products were quantified with reference to calibrations curves of TEP (1,1,3,3-tetra-ethoxypropane) in 5% (w/v) TCA (0.2–3.1 µmol L^{-1}). The absorbance at 532 nm was read with a 50 Scan spectrophotometer equipped with Cary Win UV software (Varian Inc., Palo Alto, CA, USA). The results were expressed as mg of malondialdehyde (MDA) equivalents kg^{-1} sample.

2.7 Fat quality indexes

The thrombogenicity index (TI) and atherogenicity index (AI) of the fillets (n = 9 per dietary treatment) were determined according to Ulbricht and Southgate (1991), and the hypocholesterolemic to hypercholesterolemic FA ratio (h/H) was determined according to Santos-Silva et al. (2002), as follows:

TI = [C14:0 + C16:0 + C18:0] / [0.5 × Σ MUFA) + (0.5 × Σ PUFAn-6) + (3 × Σ PUFAn-3) + (Σ PUFAn-3/ Σ PUFAn-6)];

AI = [C12:0 + (4 × C14:0) + C16:0] / (Σ PUFAn-3 + Σ PUFAn-6 + ΣMUFA);

h/H = (C18:1n-9 + C18:2n-6 + C20:4n-6 + C18:3n-3 + C20:5n-3+C22:5n-3 + C22:6n-3) / (C14:0 + C16:0).

2.8 Statistical analysis

Statistical analyses were performed using SPSS 27.0 software package (IBM Corp.) for Windows. Data are presented as mean (n=9) and pooled standard error of the mean (SEM). All data were checked for normal distribution and homogeneity of variances and normalized when appropriate. One-way ANOVA was utilized to assess differences between treatments, and the probability level of 0.05 was used to reject the null hypothesis. Polynomial contrasts analysis was performed to determine if data followed a linear or quadratic response to dietary HIO inclusion. Tukey's multiple range test was also performed to show the magnitude of differences among means. GraphPad Prism 7 software was used for graphics design and linear regression calculations.

3. Results

The growth trial results are not the aim of this study and were published elsewhere (Moutinho et al., 2023). Briefly, the growth trial lasted 70 days and during this period fish weight increased from 63 g to 141-158 g. The dietary inclusion of up to 9.5% of HIO, replacing 100% of a VO blend in a 17% lipid diet, had no adverse effects on the growth performance, feed efficiency, and whole-body composition of gilthead seabream juveniles.

At the end of the growth trial, the weight of visceral fat, fillet, frame, skin, and fins in percentage of body weight were unaffected by the dietary HIO inclusion levels (Table 6.3). The total lipid content of the fillets was also not affected by diet composition, but the fillet FA profile (% total FAME) was significantly modulated, mirroring the FA profile of the experimental diets (Table 6.4). Total SFA (% total FAME) linearly increased with

dietary inclusion of HIO while MUFA, n-3 PUFA, n-6 PUFA, n-3/n-6 and PUFA/SFA ratios (% total FAME) linearly decreased with dietary inclusion of HIO inclusion. Total EPA and DHA content (% total FAME) was also not affected by the dietary treatments. Regarding the FA profile in mg per 100g of fillet (Table 6.5), results indicate a linear decrease in ALA content, being lower in fish fed diet HIO84 and HIO100, compared to the control and HIO42. While n-6 PUFA content remained unaffected, a linear reduction in n-3 PUFA and n-3/n-6 PUFA ratio were observed. PUFA/SFA ratio followed a negative quadratic trend, decreasing as HIO content increased. Conversely, a linear increase in EPA and DHA was observed, being higher in fish fed diet HIO100 compared to the control.

Table 6.3: Morphometric and somatometric indexes of gilthead seabream juveniles fed the experimental diets.

						Polynomial contrasts		
	HIO0	**HIO42**	**HIO84**	**HIO100**	**SEM**	**ANOVA *p*-value**	**Linear *p*-value**	**Quadratic *p*-value**
Visceral fat (%)	1.12	1.15	1.51	0.94	0.11	0.362	0.852	0.192
Condition factor, K (%)	1.62	1.49	1.62	1.61	0.03	0.227	0.719	0.264
Fillet yield (%)	52.5	51.8	52.1	52.5	0.25	0.735	0.933	0.289
Frame (%)	10.6	9.6	10.0	10.8	0.16	0.055	0.560	0.011
Skin (%)	1.54	1.58	1.67	1.53	0.04	0.692	0.844	0.335
Fins (%)	2.26	2.24	2.33	2.35	0.07	0.928	0.559	0.880

Values are presented as means (n = 9). SEM: Standard error of the mean. Absence of different superscript letters indicate no significant differences between treatments (one-way ANOVA; p-value > 0.05).

Table 6.4: Total lipids (g 100 g^{-1} fillet) and fatty acid profile (% lipids) of the fillets from gilthead seabream fed the experimental diets.

						Polynomial contrasts		
	HIO0	**HIO42**	**HIO84**	**HIO100**	**SEM**	**ANOVA *p*-value**	**Linear *p*-value**	**Quadratic *p*-value**
Total lipids	7.77	8.55	8.21	8.92	0.22	0.294	0.117	0.927
∑SFA	22.7^{d}	31.6^{c}	37.4^{b}	42.7^{a}	1.33	0.000	0.000	0.047
∑MUFA	36.8^{a}	33.8^{b}	32.1^{c}	30.6^{c}	0.43	0.000	0.000	0.060
∑n-3 PUFA	21.1^{a}	16.7^{b}	13.9^{c}	11.1^{d}	0.67	0.000	0.000	0.128
∑n-6 PUFA	18.8^{a}	17.7^{a}	16.0^{b}	15.2^{b}	0.28	0.000	0.000	0.657
EPA+DHA	7.15	6.50	7.67	6.74	0.18	0.109	0.966	0.678
n-3/n-6	1.12^{a}	0.95^{b}	0.86^{b}	0.73^{c}	0.03	0.000	0.000	0.384
PUFA/SFA	1.78^{a}	1.09^{b}	0.83^{c}	0.63^{d}	0.08	0.000	0.000	0.000

Values are presented as means (n = 9). SEM: Standard error of the mean. Different superscript letters indicate significant differences between treatments (one-way ANOVA; p-value < 0.05). ∑SFA: Summation of saturated fatty acids, including: C10:0, C12:0, C14:0, isoC15:0, C15:0; C16:0, C18:0, C20:0, C22:0, and C24:0; ∑MUFA: Summation of monounsaturated fatty acids, including: C14:1n-5, C16:1n-7, C16:1n-9, C17:1, C18:1n-7, C18:1n-9, C20:1n-7, C20:1n-11, C22:1n-7, C22:1n-9; ∑PUFA: Summation of polyunsaturated fatty acids, including C16:2n-4, C16:3n-4, C16:4n-1, C18:2n-4, C18:2n-6, C18:3n-3, C18:3n-6, C18:3n-4, C18:4n-3, C18:4n-1, C20:2n-6, C20:3n-6, C20:3n-3, C20:4n-3, C20:5n-3, C22:2n-6, C21:5n-3, C22:4n-6, C22:5n-6, C22:5n-3, 226n-3. EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid.

Table 6.5: Fatty acid profile (mg 100 g^{-1}) of the fillets of fish fed the experimental diets.

						Polynomial contrasts		
	HIO0	**HIO42**	**HIO84**	**HIO100**	**SEM**	**ANOVA *p*-value**	**Linear *p*-value**	**Quadratic *p*-value**
C18:3n-3 (ALA)	332.9^{a}	291.9^{a}	133.5^{b}	103.3^{b}	17.8	0.000	0.000	0.679
C20:5n-3 (EPA)	61.0^{b}	73.7^{ab}	66.7^{ab}	77.4^{a}	2.0	0.014	0.014	0.790
C22:6n-3 (DHA)	144.6^{b}	154.5^{ab}	158.9^{ab}	171.5^{a}	3.4	0.043	0.006	0.831
EPA+DHA	205.9^{b}	228.2^{ab}	225.6^{ab}	249.0^{a}	5.3	0.040	0.008	0.957
PUFA n-3	595.8^{a}	589.2^{a}	414.1^{b}	412.8^{b}	19.1	0.000	0.000	0.914
PUFA n-6	532.9	621.2	492.7	567.6	17.7	0.061	0.870	0.840
n-3/n-6 PUFA	1.12^{a}	0.95^{b}	0.86^{b}	0.73^{c}	0.03	0.000	0.000	0.383
PUFA/SFA	1.78^{a}	1.12^{b}	0.83^{c}	0.63^{d}	0.08	0.000	0.000	0.000

Values are presented as means (n = 9). SEM: Standard error of the mean. Different superscript letters indicate significant differences between treatments (one-way ANOVA; p-value < 0.05). ALA: α-Linolenic acid; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid. ∑PUFA: Summation of polyunsaturated fatty acids, including C16:2n-4, C16:3n-4, C16:4n-1, C18:2n-4, C18:2n-6, C18:3n-3, C18:3n-6, C18:3n-4, C18:4n-3, C18:4n-1, C20:2n-6, C20:3n-6, C20:3n-3, C20:4n-3, C20:5n-3, C22:2n-6, C21:5n-3, C22:4n-6, C22:5n-6, C22:5n-3, C22:6n-3.

The relationship between each FA in the fillets and the diets is presented in Figure 6.1. Except for C16:0, C20:5n-3, and C22:6n-3, we observed a linear increase of FA deposition in the fillet with the increase of FA in the diet. Fillet C16:0 content decreased with the increase in dietary content while fillet EPA and DHA content was identical in all experimental groups.

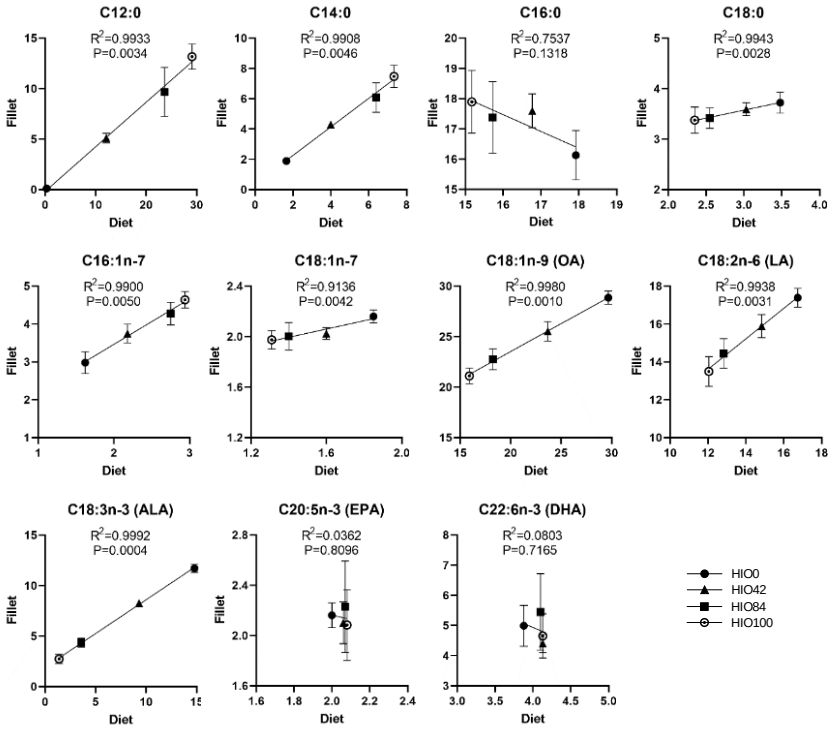

Figure 6.1: Linear regressions of fatty acid profile (% total FAME) of the experimental diets and corresponding fillets. OA: Oleic acid; LA: Linoleic acid; ALA: α-Linolenic acid; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid.

Quality indexes of the fillet were significantly affected by the dietary HIO inclusion, with TI and AI linearly increasing and h/H linearly decreasing with the increase of dietary HIO inclusion (Table 6.6).

Table 6.6: Fatty acid quality indexes of the fillets of gilthead seabream fed the experimental diets.

						Polynomial contrasts		
	HIO0	**HIO42**	**HIO84**	**HIO100**	**SEM**	**ANOVA *p*-value**	**Linear *p*-value**	**Quadratic *p*-value**
TI[1]	0.16^{d}	0.21^{c}	0.26^{b}	0.31^{a}	0.01	0.000	0.000	0.669
AI[2]	0.23^{d}	0.43^{c}	0.61^{b}	0.78^{a}	0.04	0.000	0.000	0.543
h/H[3]	3.69^{a}	2.63^{b}	2.17^{c}	1.79^{d}	0.13	0.000	0.000	0.000

[1]thrombogenicity index; [2]atherogenicity index; [3]hypocholesterolemic/hypercholesterolemic fatty acids ratio. Values are presented as means (n = 9). SEM: Standard error of the mean. Different superscript letters indicate significant differences between treatments (one-way ANOVA; p-value < 0.05).

Fillet texture was not affected by dietary composition while pH linearly decreased and water holding capacity (WHC) linearly increased with dietary HIO inclusion (Table 6.7). Fillet color (assessed by the lightness, redness, and yellowness indexes) was not affected by the dietary inclusion of HIO (Table 6.7). Skin redness index was higher in fish fed the control than the HIO42 diet, but no further differences between groups were observed in skin color indexes. Primary oxidation products (measured as conjugated dienes) were lower in fish fed the control diet than in fish fed the diets including HIO, while the opposite was observed for the secondary oxidation products (measured as TBARS) (Table 6.7).

Table 6.7: Fillet physical and chemical characteristics and primary (CD; mol kg^{-1} muscle) and secondary (TBARS; mg kg^{-1} muscle) oxidation products of gilthead seabream fed the experimental diets.

						Polynomial contrasts		
	HIO0	**HIO42**	**HIO84**	**HIO100**	**SEM**	**ANOVA *p*-value**	**Linear *p*-value**	**Quadratic *p*-value**
pH	6.29^{a}	6.22^{ab}	6.15^{b}	6.16^{ab}	0.02	0.033	0.007	0.248
WHC[1]	79.2^{b}	79.6^{b}	82.2^{ab}	84.6^{a}	0.64	0.004	0.001	0.373
Texture	32.6	32.1	37.7	33.5	1.03	0.212	0.359	0.379
Skin colour[2]								
L*	65.1	69.0	67.5	69.2	0.66	0.092	0.061	0.383
a*	-2.41^{a}	-1.72^{b}	-2.23^{ab}	-1.88^{ab}	0.09	0.014	0.130	0.293
b*	0.91	2.00	1.99	1.66	0.26	0.420	0.342	0.181
Fillet colour								
L*	48.8	48.5	48.7	49.1	0.25	0.844	0.586	0.499
a*	0.34	0.34	0.11	0.35	0.12	0.872	0.856	0.617
b*	0.12	-0.17	-0.33	0.02	0.11	0.506	0.637	0.164

CD[3]	0.18[b]	0.24[a]	0.25[a]	0.25[a]	0.01	0.000	0.000	0.009
TBARS[4]	0.94[a]	0.38[b]	0.31[b]	0.38[b]	0.08	0.015	0.011	0.038

[1]WHC: Water holding capacity; [2]Colour: *L**, lightness; *a**, redness index; *b**, yellowness index. [3]Conjugated dienes; [4]Thiobarbituric acid reactive substances. Values are presented as means (n = 9). SEM: Standard error of the mean. Different superscript letters indicate significant differences between treatments (one-way ANOVA; p-value < 0.05).

4. Discussion

Insect oils have great potential as sustainable lipid sources for aquafeeds. However, it is known that feed lipidic composition plays a major role in modulating the quality of the end product as the muscle FA profile tends to mirror that of the experimental diets (Renna et al., 2017; St-Hilaire et al., 2007). Previously, we showed that dietary inclusion of 9.5% HIO, corresponding to 50% of the dietary lipids and a total replacement of dietary VO, did not impact the health and growth performance of gilthead seabream juveniles while increasing feed efficiency (Moutinho et al., 2023). Here we evaluated the impact of the same diets on the final product quality (Hossain et al., 2023).

At the end of the growth trial, which lasted for 70 days and allowed fish to more than double their initial weight, it was observed a close relationship between the FA profile of diets and fillets. The HIO is very rich in SFA, which corresponds to circa 84% of the total FA of the HIO, with lauric acid comprising 68% of the total SFA. Thus, as expected, SFA levels in the fillets linearly increased with the incorporation of HIO in the diets. Similar results were also observed in other fish species fed with diets including HIO (Fawole et al., 2021; Hender et al., 2021; Sudha et al., 2022), or full-fat or partially defatted black soldier fly meal (Fabrikov et al., 2021; Renna et al., 2017; Secci et al., 2018; St-Hilaire et al., 2007). Nonetheless, lauric acid levels of the fillet (as % total FAME) were considerably lower than those of the corresponding diets, which suggests that this FA was preferably used as an energy source rather than accumulated in the tissues. Similar results were also previously described for other fish species (Bakar et al., 2021; Li et al., 2016; Renna et al., 2017). As for the other SFA, C14:0 levels in the fillets were similar to the dietary levels, while C16:0 levels were decreased and C18:0 levels were increased. Overall, this suggests that C16:0 was elongated to C18:0 and that all these SFA were not utilized as β-oxidation substrates. This was also expected, as the utilization of SFA for oxidation tends to decrease with increasing chain length (Leyton et al., 1987).

The HIO has a low MUFA and PUFA content, and therefore dietary inclusion of HIO led to a decrease in the fillet deposition of MUFA, also reflecting the pattern of the experimental diets. Similar results for MUFA content were also observed in juvenile Jian carp (Li et al., 2016), but not in gilthead seabream or rainbow trout (Fabrikov et al., 2021;

Fawole et al., 2021). Contradictory results regarding PUFA content in the fillets were also observed in previous studies. For instance, dietary HIO inclusion led to a reduced muscle content of n-3 PUFA in striped catfish (Sudha et al., 2022) but not in Jian carp (Li et al., 2016) and red hybrid tilapia (Bakar et al., 2021). On the other hand, in rainbow trout, the dietary inclusion of HIO reduced fillet n-6 PUFA but not n-3 PUFA content (Fawole et al., 2021).

Dietary EPA and DHA were provided by fish oil, which amount was kept constant in all diets, and therefore the level of these FA (as % total FAME) was similar among diets. The EPA and DHA levels in the fillets were also similar to the dietary levels, indicating that these FA were spared and maintained in the fillets. In other studies, EPA and DHA levels were also preserved in rainbow trout muscle when fed diets with HIO compared to soybean oil (Fawole et al., 2021). However, fillet EPA and DHA levels were reduced in gilthead seabream and rainbow trout fed with diets including full-fat HI meal (Fabrikov et al., 2021; St-Hilaire et al., 2007). In these studies, dietary fish oil (FO) levels were also reduced which contributes to explaining the observed results. When evaluating fillet's EPA and DHA content in mg per 100g of fillet, we observed an increased deposition of these FA in fish fed the highest HIO inclusion level, compared to those fed the control diet. According to the World Health Organization, a healthy human diet should consist of regular fish consumption, with a minimum of one to two servings per week, each providing the equivalent of 200–500 mg of EPA+DHA (WHO, 2003). Thus, in this study, a 100 g fillet of gilthead seabream fed HIO would be able to provide an adequate amount of EPA and DHA, aligning with the requirements of a healthy human diet.

The PUFA/SFA and n-3/n-6 PUFA ratios and the AI, TI, and h/H ratios are important parameters to assess the nutritional quality of the fillets for human consumption (Kilar and Kasprzyk, 2021). High PUFA/SFA ratios are recommended as PUFA contributes to decreasing serum low-density lipoprotein and total cholesterol, while SFA contributes to their increase (Kilar and Kasprzyk, 2021). In a healthy human diet, PUFA/SFA ratios above 0.45 are desirable to prevent cardiovascular disease development (Hayes, 2002; Kilar and Kasprzyk, 2021). As expected, the fillet PUFA/SFA ratio in this trial decreased with increasing HIO content, due to the high SFA content of HIO. Nevertheless, PUFA/SFA ratios were higher than 0.45 and therefore the final product was still considered adequate for a healthy human diet.

A high n-3/n-6 ratio is also important for human health since n-3 PUFAs are known for their anti-inflammatory effects, while n-6 PUFAs contribute to inflammation (Fabrikov et al., 2021). In the present trial, HIO inclusion reduced the fillet n-3/n-6 ratio, mainly due to

the low content of C18:3n-3 in HIO. Similar results were also previously observed in this species as well as in other fish species fed diets including HIO (Fabrikov et al., 2021; Fawole et al., 2021; Renna et al., 2017). The AI ratio is an indicator of the balance between pro-atherogenic and anti-atherogenic FA, and indicates the tendency to reduce blood lipid levels, while the TI ratio indicates the balance between pro-thrombogenic and anti-thrombogenic FA, and therefore indicates the tendency to form blood clots (Grigorakis, 2007; Ulbricht and Southgate, 1991). In this study, both AI and TI were negatively affected by HIO inclusion, a result also previously observed in other fish species (Bruni et al., 2020; Fabrikov et al., 2021; Renna et al., 2017). However, the fillet values observed were still well below the recommended threshold (AI < 1.0 and TI < 0.5) for a healthy human diet that contributes to cardiovascular disease prevention (Hender et al., 2021; Kilar and Kasprzyk, 2021).

The hypocholesterolemic and hypercholesterolaemic ratios (h/H) consider the effects of specific FA on cholesterol metabolism, with high h/H ratios being desirable in healthy diets (Testi et al., 2006). In the present study, the fillet's h/H ratio also decreased with increasing dietary HIO inclusion, similar to what was previously observed in rainbow trout fed a diet including full-fat HI meal (Bruni et al. 2020). Nonetheless, the h/H ratios observed in the present study were higher than those observed in wild marine fish species such as mackerel or sardine (Fernandes et al., 2014).

Morphometric and somatometric indexes are important tools in aquaculture, providing insights on growth and overall health of cultured fish while also providing information regarding production and processing efficiency. In the present trial, the inclusion of HIO showed no differences in the measured morphometric and somatometric indexes, agreeing with the growth performance results (Moutinho et al., 2023). These values were also comparable to those obtained by Piccolo et al. (2017) and Pulido-Rodriguez et al. (2021) in gilthead seabream fed diets with insect meals. To the best of our knowledge, there have been no studies evaluating HIO and its effects on morphometric and somatometric indexes in fish. Nevertheless, the inclusion of alternative lipid sources in other studies also did not affect morphometric and somatometric indexes in gilthead seabream (Fountoulaki et al., 2009) and other species like rainbow trout (Caballero et al., 2002) and European seabass (Verge-Mèrida et al., 2022).

Sensory attributes important parameters to assess fish quality and freshness, and can be directly perceived by the consumer, influencing their purchase decision (Álvarez et al., 2020; Renna et al., 2017). In the present trial, the dietary inclusion of HIO had little effect on the color and texture of the fillet and skin, except for a slight decrease in skin

redness (*a**) in group HIO42. In rainbow trout, diets including partially defatted HI meal also had no effects on fillet color (Renna et al., 2017), and diets including full-fat HI meal increased fillet yellowness (*b**) (Bruni et al., 2020).

A high pH is related to a lower shelf-life since an alkaline environment does not inhibit microbial degradation processes (Renna et al., 2017). In this study, there was a slight linear decrease in pH with increasing HIO, indicating that fillet shelf-life was not negatively affected by the dietary inclusion of HIO. The WHC measures the ability of the muscle to retain water and is related to the juiciness of the fillet, and in the present study, there was a linear increase of the fillet WHC with the dietary inclusion of HIO. Differently, in rainbow trout fed a full-fat HI meal-based diet, no effects on the fillet pH or WHC were observed (Bruni et al., 2020).

The replacement of VO with HIO led to decreased lipid peroxidation in the fillet, as indicated by the lower TBARS in fish fed the diets including HIO. This was expected, as HIO has a high amount of SFAs which are less prone to oxidation than unsaturated FA. A reduction in TBARS was also reported in rainbow trout fed with full-fat HI meal diets (Bruni et al., 2020).

The present study showed that dietary replacement of VO with HIO impacted the fillet's FA profile and lipid quality indexes of gilthead seabream juveniles. Nevertheless, the levels of FA important for human nutrition, namely EPA and DHA, were maintained in all experimental groups, and the lipid quality indexes were within the recommended levels for a healthy human diet. Further, dietary HIO inclusion increased WHC and decreased fillet pH and fillet lipid peroxidation, thus contributing to extending the shelf-life of the fillets.

Overall, it can be concluded that HIO can be included in the diets for gilthead seabream, completely replacing VO in practical diets with 10% VO, and having no negative effects on the quality attributes of fillets.

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Chapter 7: Effects of black soldier fly larvae oil on the immune, inflammatory, and oxidative status of gilthead seabream juveniles

Submitted to Fish and Shellfish Immunology

Effects of black soldier fly larvae oil on the immune, inflammatory, and oxidative status of gilthead seabream juveniles

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Abstract

This study aimed to evaluate the effects of dietary inclusion of *Hermetia illucens* larvae oil (HIO) on the immune, inflammatory, and antioxidant status of gilthead seabream (*Sparus aurata*) juveniles. A diet including 18% lipids (50:50 of fish oil and vegetable oil (VO) blend) diet was formulated and three other diets were formulated similar to the control but containing increasing levels of HIO replacing the VO at 42%, 84%, and 100%, corresponding to a dietary inclusion of 4%, 7.9%, and 9.5% of HIO, respectively. Fish with an initial body weight of 63 g were fed the experimental diets to apparent visual satiation for 10 weeks. At the end of the trial, no effects were observed in the plasma immune humoral parameters except for a linear increase in plasma peroxidase activity. Hepatic lipid peroxidation (LPO) was not affected while glutathione peroxidase, catalase, glutathione reductase, and glucose-6-phosphate dehydrogenase activities linearly increased with the dietary HIO inclusion level. In the intestine, LPO was lower in fish fed the HIO diets than in the control, and no differences between groups were observed in the activity of the oxidative stress-related enzymes. Regarding the immune-related genes, interleukin-1β expression and transforming growth factor β expression were not affected by the diets, while it was observed an upregulation of tumor necrosis factor α and interleukin-10, which were higher in fish fed diet with 9.5% HIO than in the other diets. Overall, these results indicate that HIO can be included in diets for gilthead seabream juveniles up to 9.5% without compromising the immune, antioxidant, and inflammatory response while improving intestine LPO.

Keywords: *Hermetia illucens*, lauric acid, *Sparus aurata*, alternative lipid sources, insect oil

1. Introduction

Lipids play an important role in fish nutrition as an energy source but also as a source of essential fatty acids (EFA), necessary not only for growth but also for immunity, disease resistance, and overall health of fish (NRC 2011; Monroig et al. 2018; Turchini et al. 2022). In aquaculture, fish oil (FO) has been regarded as the ideal dietary lipid source, mostly due to its high content in n-3 long-chain polyunsaturated fatty acids (LC-PUFA), such as eicosapentaenoic (EPA, C20:5n-3) and docosahexaenoic (DHA, C22:6n-3) acids. This is especially important for marine fish as they are not capable or have limited capability to convert the C18 precursors to EPA and DHA in sufficient amounts to meet their requirements (Tocher 2010). Thus, these fatty acids (FA) are considered essential for marine fish and must be supplied in their diet. However, due to the increase in demand for FO for aquaculture and human consumption, annual production fluctuations, rise in prices, and biodiversity and environmentally sustainable issues (Tocher et al. 2019), the aquaculture industry has shifted towards using FO more sparingly and strategically.

Terrestrial vegetable oils (VO), such as soybean, linseed, palm, and rapeseed oils, are currently being used as substitutes for FO in commercial aquafeed formulations. Research has shown that, as long as the EFA requirements are met, the partial or total substitution of FO by VO is achievable without adverse effects on the growth and health of marine fish (Fountoulaki et al. 2009; Turchini et al. 2009). However, the FA profile of these alternative oils is suboptimal for marine fish species as they lack LC-PUFA and are rich in C18 polyunsaturated fatty acids, like linoleic (C18:2n-6) and α-linolenic (C18:3n-3) acids, which, in excess, can affect metabolism, compromising health and disease resistance (Montero et al. 2003). Indeed, high dietary levels of VO, especially when used as a single lipid source and not a blend of VO, were shown to have negative effects on the health of several marine fish species, affecting immunity and disease resistance and promoting inflammatory processes (Montero et al. 2003; Montero et al. 2010; Montero et al. 2015; Tan et al. 2016; Tan et al. 2017). Likewise, high levels of VO inclusion were shown to decrease antioxidant capacity in species like Japanese sea bass (Tan et al. 2017), large yellow croaker (Mu et al. 2018), and gilthead seabream (García-Meilán et al. 2023). Besides the effects on fish health, environmental concerns associated with the use of VO, such as land-use conflicts, degradation of water and soil, greenhouse gas emissions, and biodiversity loss, urges the search for more sustainable alternative lipid sources (Benzertiha et al. 2020; Xu et al. 2021).

The interest in insects for food and feed has increased exponentially in the last decade. Among the eight authorized insect species to be included in aquafeeds in the European Union (EU Commission Regulation 2021/1925), the black soldier fly (*Hermetia illucens*, HI) appears as the most promising. HI larvae have fast growth rates and high feed conversion ratios, and their production has lower land and water requirements than plant ingredients (Mohan et al. 2022). HI larvae are also efficient bio-converters, being able to transform low-valued organic waste into highly valuable biomass, thus sustaining a circular bioeconomy (Barragan-Fonseca et al. 2017; Alfiko et al. 2021; Mohan et al. 2022). Also, they can be farmed locally, reducing the dependency on exports and improving economic security and efficiency. Nutritionally, HI larvae are rich in protein (up to 60% dry matter, DM), with a balanced amino acid profile, and have a high lipid content, making up to 30-40% DM (Henry et al. 2015; Nogales-Mérida et al. 2018; Liland et al. 2021). The FA profile of HI larvae oil (HIO) is rich in SFA (up to 52% of total FA content) and is mostly composed of the medium-chain fatty acid (MCFA) lauric acid (Benzertiha et al. 2020; Franco et al. 2021; Liland et al. 2021). To reduce nutritional variation and improve stability and protein content, defatting has become a standard practice in insect meal production (Dumas et al. 2018). This results in high amounts of HI lipids as a byproduct which is currently an underexploited resource.

Studies assessing the potential of HIO for incorporation in aquafeeds are scarce. Up to now, HIO has been evaluated as a replacement for FO or VO in freshwater or diadromous species like Atlantic salmon (Belghit et al. 2018; Hender et al. 2021), Jian carp (Li et al. 2016), mirror carp (Xu et al. 2020; Xu et al. 2021), and rainbow trout (Dumas et al. 2018; Fawole et al. 2021; Kumar et al. 2021), without negative effects on growth and feed utilization. Besides growth, these studies also suggest that HIO can be used as a functional ingredient since the MCFA present in HIO, such as lauric acid, have been suggested to possess anti-inflammatory, antioxidant, and immune-boosting proprieties (Hinton and Ingram 2006; Dayrit 2015; Zhang et al. 2019). Indeed, Xu et al. (2020) showed that HIO was a better lipid source than yellow mealworm and silkworm oils, as it contributed to improving antioxidant and inflammatory responses in mirror carp. Further, MCFAs were shown to have antibacterial, anti-viral, and anti-fungal properties, and could also be used as a strategy to reduce antibiotics use in aquaculture (Stenberg et al. 2019; Franco et al. 2021; Ullah et al. 2022).

In a previous study, we showed that in gilthead seabream juveniles fed diets including 18% lipids (50:50 of fish oil: VO), it was possible to completely replace the VO with HIO without compromising the growth and feed efficiency of the fish (Moutinho et al. 2023). This is a follow-up of that study and evaluates the innate immune, inflammatory, and

antioxidant status of gilthead seabream juveniles fed diets including increasing amounts of HIO.

2. Materials and methods

2.1 Ethics statement

All procedures were performed by accredited scientists, following FELASA category C recommendations. The growth trial was conducted according to the European Union Directive (2010/63/EU) on animal protection for scientific purposes and approved by the Ethics Committee of CIIMAR (reference ORBEA_CIIMAR_27_2019).

2.2 Experimental diets and conditions

Four experimental diets were formulated to be isoproteic (45% crude protein) and isolipidic (18% crude lipids), and to meet gilthead seabream nutritional requirements (NRC 2011). A control diet (HIO0) contained 50:50 of fish oil and a VO blend as lipid sources. HIO (provided by Hermetia Baruth GmbH, Baruth/Mark, Germany) was added to the control diet at 4% (diet HIO42), 7.9% (diet HIO84), and 9.5% (diet HIO100), replacing the VO blend at 42, 82, and 100%, respectively. All 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. Pellets were dried in an oven (55 ºC, 24 h) and stored at − 20 ◦C until use. The ingredient composition and proximate analysis of the experimental diets are presented in Table 7.1.

The feeding trial was conducted at CIIMAR (Porto, Portugal) and the experimental conditions are described in detail by Moutinho et al. (2023). Briefly, 192 gilthead seabream juveniles (initial body weight of 63 g) were reared in a thermoregulated seawater recirculation system (average temperature 23 ºC), equipped with 12 fiberglass tanks, each with 500 L capacity and continuous aeration and water flow. The experimental diets were hand-fed to triplicate groups of fish, two times a day, six days a week, to apparent visual satiation for 10 weeks.

2.3 Sampling

At the end of the feeding trial, blood from 3 fish per tank was collected 4 hours after the morning meal with heparinized syringes. Blood was drawn from the caudal vein and immediately centrifuged (10,000 *g*, 10 min, room temperature) and plasma was collected and stored at -80◦C until analyses. Then, fish were euthanized by decapitation and the digestive tract was separated from adjacent adipose and connective tissues. The whole intestine and liver were collected, immediately frozen in liquid nitrogen, and stored at -

80ºC until analysis. A portion of the distal intestine was also collected and immediately placed in RNAlater, left overnight at 4 ºC, and stored at -80 ºC until RNA extraction.

Table 7.1. Ingredients (% dry matter, DM) and proximate composition (% DM) of the experimental diets.

	HIO0	HIO42	HIO84	HIO100
Fishmeal[1]	20	20	20	20
Soluble fish protein concentrate[2]	5	5	5	5
Haemoglobin[3]	3	3	3	3
Corn gluten meal[4]	15	15	15	15
Soybean meal[5]	10.9	10.9	10.9	10.9
Rice bran[6]	10	10	10	10
Whole wheat[7]	15	15	15	15
Sardine oil[8]	3.11	3.11	3.11	3.11
VO blend[9]	9.5	5.5	1.5	0
HI larvae oil[10]	0	4.0	7.9	9.5
Taurine[11]	0.3	0.3	0.3	0.3
Phospholipids[12]	1	1	1	1
Shrimp hydrolysate[13]	1.5	1.5	1.5	1.5
Betaine[14]	0.2	0.2	0.2	0.2
Calcium biphosphate[15]	2	2	2	2
Vitamin premix[16]	1	1	1	1
Choline chloride (50%)[17]	0.5	0.5	0.5	0.5
Mineral premix[18]	1	1	1	1
Binder[19]	1	1	1	1
Proximate composition:				
Dry matter, DM (%)	91.6	90.9	92.8	92.1
Crude protein, CP (%)	46.8	46.4	47.0	46.3
Crude lipids, CL (%)	17.6	16.5	17.2	16.4
Ash (%)	9.14	9.21	9.20	9.10
Energy (kJ g^{-1})	22.0	22.1	24.1	23.4

[1]Fishmeal (89.1% DM, 74.9% CP, 10.7% CL, 29.6% Ash); Sorgal, S.A. Ovar, Portugal
[2]Sorgal, S.A. Ovar, Portugal
[3]Hemoglobin powder AP310P; APC Europe S.A.
[4]Sorgal, S.A. Ovar, Portugal
[5]Sorgal, S.A. Ovar, Portugal
[6]Sorgal, S.A. Ovar, Portugal
[7]Whole wheat (88.6% DM, 14.5% CP, 1.2% CL); Sorgal, S.A. Ovar, Portugal
[8]Sardine oil, Sorgal, S.A. Ovar, Portugal
[9]Vegetable oil blend: 50% linseed, 30% palm, and 20% rapeseed oils
[10]*Hermetia illucens* larvae oil; Hermetia Baruth GmbH, Germany.
[11]Sorgal, S.A. Ovar, Portugal
[12]Premix, Viana do Castelo, Portugal.
[13]Shrimp hydrolysate (*Litopenaeus vannamei* trimmings: 73% CP, 12.1% CL, 13% Ash); Sorgal, S.A. Ovar, Portugal
[14]Sorgal, S.A. Ovar, Portugal
[15]Calcium biphosphate, Sorgal, S.A. Ovar, Portugal
[16]Vitamins premix (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
[17]Premix, Viana do Castelo, Portugal.
[18]Minerals premix (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)
[19]Binder: Carboxymethylcellulose sodium; Sigma-Aldrich Química, Portugal

2.4 Chemical analysis

Ingredients and diets were analyzed according to the AOAC (2000) procedures: dry matter by drying at 105 °C until constant weight; ash by incineration in a muffle furnace at 450 °C for 16 h; protein (N × 6.25) by the Kjeldahl method after acid digestion (Kjeltec digestion and distillation units; models 1015 and 1026, Tecator Systems; Höganäs); lipid by petroleum ether extraction (Soxtec HT System; Höganäs); gross energy by direct combustion in an adiabatic bomb calorimeter (PARR model 1261, PARR Instruments, Moline, IL, USA).

For FA quantification of HIO and diets, total lipid extraction was performed according to Folch et al. (1957), and FA profile and quantification was done by gas-chromatography (GC) as described in Moutinho et al. (2023). The FA profile of HIO and diets is presented in Table 2.

Table 7.2: Fatty acid composition (% of total FAME) of the *Hermetia illucens* larvae oil (HIO) and the experimental diets.

		Experimental diets			
	HIO	**HIO0**	**HIO42**	**HIO84**	**HIO100**
C12:0	57.1	0.30	12.1	23.7	29.1
C14:0	11.5	1.64	3.99	6.39	7.33
C16:0	12.1	17.9	16.8	15.7	15.2
C18:0	1.59	3.48	3.02	2.55	2.35
C16:1n-7	2.50	1.62	2.18	2.75	2.94
C18:1n-6	6.91	29.7	23.7	18.2	15.9
C18:1n-7	0.31	1.85	1.60	1.40	1.31
C20:1n-9	0.08	1.31	1.22	1.18	1.09
C18:2n-6 (LA)	5.16	16.7	14.8	12.8	12.0
C18:3n-3 (ALA)	0.52	14.8	9.30	3.58	1.38
C20:4n-6 (ARA)	-	0.26	0.28	0.26	0.28
C20:5n-3 (EPA)	-	2.00	2.06	2.07	2.08
C22:6n-3 (DHA)	-	3.88	4.13	4.10	4.12
∑SFA	83.9	24.4	37.2	49.9	55.6
∑MUFA	10.2	36.1	30.4	25.3	22.8
∑n-6 PUFA	5.19	17.5	15.5	13.5	12.7
∑n-3 PUFA	0.58	21.7	16.5	10.9	8.57

The following FA, found in percentages below 1% of total FAME, were utilised for calculating the classes FA, but they are not listed in the table: C10:0, C14:1n-5, isoC15:0, C15:0, isoC16:0, C16:1n-9, C16:2n-4, C17:0, C16:3n-4, C17:1, C16:4n-1, C18:2n-4, C18:3n-6, C18:3n-4, C18:4n-3, C18:4n-1, C20:0, C20:1n-11, C20:1n-7, C20:2n-6, C20:3n-6, C20:3n-3, C20:4n-3, C22:0, C22:1n-9, C22:1n-7, C22:2n-6, C21:5n-3, C22:4n-6, C22:5n-6, C22:5n-3, and C24:0. LA: Linoleic acid; ALA: α-Linolenic acid; ARA: Arachidonic acid; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids.

2.5 Enzymatic assays and lipid peroxidation determination

Liver and whole intestine samples were homogenized (dilution 1:4) in ice-cold buffer (100 mM-Tris-HCl, 0.1 mM-EDTA, 0.1% triton X-100 (v/v), pH 7.8; Sigma) and homogenates were centrifugated (30 min, 30,000 *g*, 4ºC). The supernatant was collected, and aliquots were stored at -80ºC until analysis. All enzyme assays were carried out at 37 °C and changes in absorbance were monitored using a Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, Nanjing, China). The molar extinction coefficients used for H_2O_2 and NADPH were 39 and 6220 $M^{-1} \times cm^{-1}$, respectively.

The specific assay conditions for each enzyme are detailed in Castro et al. (2016). Briefly, catalase (CAT; EC 1.11.1.6) activity was determined by measuring the decrease in H_2O_2 concentration at 240 nm. Glutathione reductase (GR; EC 1.6.4.2) activity was determined at 340 nm by measuring the oxidation of NADPH in the presence of oxidized glutathione (GSSG). Glutathione peroxidase (GPX; EC 1.11.1.9) activity was measured at 340 nm as the NADPH consumption rate by GR to reduce GSSG produced by GPX. Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activity was assayed by measuring the reduction of NADP+ at 340 nm from glucose-6-phosphate.

Enzyme activities were expressed as units (CAT) or milliunits (GPX, GR, and G6PDH) per mg of soluble protein. One unit of enzyme activity was defined as the amount of enzyme required to transform 1 µmol of substrate/min. Protein concentration was determined according to Bradford (1976), using bovine serum albumin as standard.

Lipid peroxidation levels were determined based on the concentration of malondialdehyde (MDA), according to Castro et al. (2016). MDA concentration was expressed as nmol MDA per g of wet tissue, calculated from a calibration curve.

2.6 Immune parameters

Innate immune humoral parameters (nitric oxide, antiproteases, proteases, and peroxidase) were performed in the plasma as described by Magalhães et al. (2021). Briefly, nitric oxide (NO) was determined using a Nitrate/Nitrite colorimetric kit (Roche Diagnostics GmbH, Mannheim, Germany, ref: 11,746,081,001) and adjusted for 96-well microplates. Nitrite and nitrate were determined by comparison with sodium nitrite and potassium nitrate standard curves and were used to quantify NO. Antiprotease activity was determined as the capacity to inhibit trypsin activity. The percentage of inhibition was calculated by comparison with a reference sample. Protease activity was quantified using azocasein hydrolysis and calculated using trypsin as the reference sample (100% protease activity). Peroxidase activity was determined by incubation with 10 mM

3,3',5,5'- tetramethylbenzidine hydrochloride and H_2O_2, and the reaction stopped with H_2SO_4. Peroxidase activity was calculated by defining 1 OD absorbance change as corresponding to 1 unit of peroxidase activity (units mL^{-1} of plasma).

2.7 Gene expression

Total RNA from the distal intestine was isolated by homogenization with TRIzol reagent (Direct-zolTM RNA Miniprep, Zymo Research) according to manufacturer's instructions, in a Precellys evolution apparatus (Bertin Instruments, Montigny-le-Bretonneux, France). RNA concentration was quantified and adjusted to 0.5 µg/8 µL H2O for complementary DNA synthesis, using the NZY First-Strand cDNA Synthesis Kit (NZYTech. MB12502, Lisbon, Portugal).

Gene expression was determined by real-time quantitative PCR analysis (CFX Connect™ Real-Time System, Bio-Rad, Hercules, CA, USA). The mixture contained 0.4 µL diluted cDNA (1:1), 0.2 µL of each primer (10 µM), 5 µL SsoAdvanced Universal SYBR® Green supermix (Bio-Rad), and 4.2 µL DNase/RNase/Protease-free water, in a total volume of 10 µL. The sequences of primers used are presented in Table 7.3. Primers efficiency was validated with serial two-fold dilutions of cDNA and determined from the slope of the regression line of the quantification cycle (Ct) versus the relative concentration of cDNA (Pfaffl 2001) and only efficiencies between 90 and 110% (slope −3.5 and 3.1, r2 = 0.99) were accepted.

The reaction was initiated with incubation at 95 ºC for 30s for hot-start iTaqTM DNA polymerase activation. A total of 40 PCR cycles were performed, each consisting of heating at 95 ºC for 15s for denaturing and 30s for annealing and extension. The annealing temperature of each primer pair is presented in Table 3. Melting curves were systematically monotonized (60 ºC temperature 0.5 ºC 10s−1 from 60 to 95 ºC). Each PCR run included duplicates of reverse transcription for each sample and negative controls (reverse transcriptase-free samples, RNA-free samples).

To normalize the results, previously validated genes, elongation factor 1α (*ef1α*) and ribosomal protein 18s (*18s*) were used as reference genes. The expression levels are given as the mean normalized values ± standard error, corresponding to the ratio between copy numbers of the target gene transcripts and the geometric mean of copy numbers of the reference genes (Vandesompele et al. 2002).

Table 7.3: Sequence of primers used for real-time quantitative PCR.

Gene	Sequence 5'-3'	Annealing Temp (ºC)	Efficiency	Accession number
tgfβ	**F:** AGAGACGGGCAGTAAAGAA **R:** GCCTGAGGAGACTCTGTTGG	58	2.04	AF424703
il-1β	**F:** GGGCTGAACAACAGCACTCTC **R:** TTAACACTCTCCACCCTCCA	60	2.00	AJ277166
il-10	**F:** TGGAGGGCTTTCCTGTCAGA **R:** TGCTTCGTAGAAGTCTCGGATGT	63	2.01	FG261948
tnfα	**F:** TCGTTCAGAGTCTCCTGCAG **R:** CATGGACTCTGAGTAGCGCGA	60	1.92	AJ413189
ef1α	**F:** CTGTCAAGGAAATCCGTCGT **R:** TGACCTGAGCGTTGAAGTTG	60	2.01	AF184170
18s	**F:** GGGTGTTGGCAGACGTTAC **R:** CTTCTGCCTGTTGAGGAACCA	60	2.06	AM490061.1

tgfβ: transforming growth factor beta; *il-1β*: interleukin-1-beta; *il-10:* interleukin-10; *tnfα:* tumor necrosis factor alpha; *ef1α:* elongation factor 1-alpha*; 18s:* ribosomal protein 18s

2.8 Statistical analysis

Data are presented as mean (n = 9) and pooled standard error of the mean (SEM). Data were checked for normal distribution and homogeneity of variances and normalized when appropriate. One-way ANOVA was used to assess differences between treatments, with a probability level of 0.05 being used to reject the null hypothesis. When p-values were significant ($p < 0.05$), differences between means were evaluated with the Tukeys test. Polynomial contrasts analysis was also performed to determine if data followed a linear or quadratic response. All statistical analyses were performed using the SPSS 27.0 software package (IBM Corp.) for Windows.

3. Results

Growth performance results have been published previously (Moutinho et al., 2023). Briefly, fish final body weight was similar and unaffected by the experimental diets. Notably, fish fed diets HIO84 and HIO100 had a significantly higher feed efficiency compared to fish fed the other two diets, HIO0 and HIO42 (Moutinho et al., 2023).

The antioxidant response in the liver and intestine of fish fed the experimental diets is presented in Table 7.4. In the liver, lipid peroxidation (LPO) was not affected by HIO inclusion while a linear increase in the activity of all enzymes analyzed was observed. Differently, in the intestine, LPO linearly decreased with the dietary HIO inclusion, but no differences in the activity of the antioxidant enzymes were observed.

Table 7.4: Specific activities of antioxidant enzymes in the liver and intestine of gilthead seabream juveniles fed the experimental diets.

						Polynomial contrasts		
	HIO0	**HIO42**	**HIO84**	**HIO100**	**SEM**	**ANOVA p-value**	**Linear p-value**	**Quadratic p-value**
Liver								
G6PDH[1]	70.4[b]	84.6[ab]	83.0[ab]	103.5[a]	4.26	0.039	0.008	ns
GR[2]	5.0[b]	5.87[ab]	5.34[ab]	6.38[a]	0.17	0.019	0.014	ns
GPX[3]	58.7	63.7	75.1	78.2	3.61	ns	0.031	ns
CAT[4]	248.7	252.9	241.8	316.5	10.7	0.040	0.036	ns
LPO[5]	6.41	4.16	6.26	5.23	0.39	ns	ns	ns
Intestine								
G6PDH[1]	2.17	2.48	2.58	2.86	0.14	ns	ns	ns
GR[2]	3.90	4.00	4.20	3.67	0.11	ns	ns	ns
GPX[3]	150.8	149.0	146.8	153.9	2.48	ns	ns	ns
LPO[5]	9.36[a]	5.10[b]	4.72[b]	5.44[b]	0.48	0.001	0.002	0.003

Values are presented as mean of n=9. SEM = pooled standard error of the mean.
[1]G6PDH: glucose-6-phosphate dehydrogenase (mU mg protein^{-1}), [2]GR: glutathione reductase (mU mg protein^{-1}), [3]GPX: glutathione peroxidase (mU mg protein^{-1}), [4]CAT: catalase (U mg protein^{-1}), [5]LPO: lipid peroxidation (LPO) (nmol malondialdehyde g tissue^{-1}). Different superscript letters indicate no significant differences between treatments (one-way ANOVA; $P < 0.05$). ns = non-significant p value (> 0.05).

No differences in the plasma immune humoral parameters were observed, except for a linear trend of increase in peroxidase activity with the inclusion of HIO in the diets (Table 7.5).

Table 7.5: Immune humoral parameters measured in the plasma of gilthead seabream fed the experimental diets.

						Polynomial contrasts		
	HIO0	**HIO42**	**HIO84**	**HIO100**	**SEM**	**ANOVA p-value**	**Linear p-value**	**Quadratic p-value**
Nitric oxide (mM)	1.097	1.049	1.005	1.006	0.03	ns	ns	ns
Antiproteases (%)	84.0	86.4	86.3	88.4	0.76	ns	ns	ns
Proteases (%)	8.76	9.04	9.12	8.60	0.29	ns	ns	ns
Peroxidase (U mL^{-1})	87.9	82.7	121.7	136.3	8.44	ns	0.015	ns

Values are presented as mean of n=9. SEM = pooled standard error of the mean.
Absence of different superscript letters indicate no significant differences between treatments (one-way ANOVA; $P > 0.05$). ns = non-significant p value (> 0.05).

Regarding the inflammatory response biomarkers, *il-10* and *tnfα* gene expression was higher in fish fed with the HIO100 diet than with the others (Figure 7.1). In absolute

values, the expression of *il-1β* and *tgfβ* was also higher in fish fed the HIO100 diet, but differences among treatments were not statistically significant.

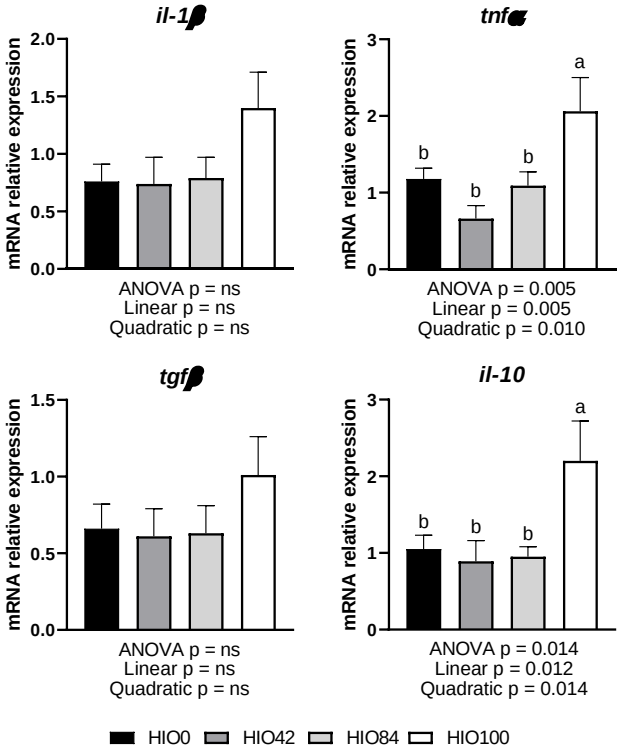

Figure 7.1: Relative expression of genes and markers in muscle of gilthead seabream fed the experimental diets. Data was normalized with two housekeeping genes, elongation factor 1α (*ef1α*) and ribosomal protein S18 (*s18)*. Values are presented as mean (n=9) and standard error represented by error bars. Different superscript letters indicate significant differences between treatments (one-way ANOVA; P < 0.05); ns = non-significant p value (P > 0.05).

4. Discussion

Previously, we showed that it was possible to include up to 9.5% of HIO by replacing a VO blend in diets for gilthead seabream juveniles without compromising the growth, feed efficiency, and digestibility of the fish (Moutinho et al. 2023). However, being a novel feed ingredient, the potential impacts of dietary HIO on fish health still need to be addressed.

Oxidative stress is a process that occurs in all living organisms and is the result of an imbalance between the production of reactive oxygen species and the response of antioxidant mechanisms (Birnie-Gauvin et al. 2017). To protect cells and tissues from oxidative damage, endogenous antioxidant enzymes are activated to counteract the adverse effects of excessive free radicals (Jin et al. 2017; Xu et al. 2020). In the present trial, we observed a linear increase in the activity of liver antioxidant enzyme machinery with dietary HIO inclusion, while liver lipid peroxidation levels were maintained similarly among experimental groups. This suggests an increased oxidative stress in fish fed the

HIO that was counterbalanced by the increased antioxidant enzyme activity. This was unexpected, as the amount of SFA increased and PUFA decreased with the dietary HIO inclusion. As SFA content are less susceptible to oxidative damage than PUFA, they exert an antioxidative effect (Kim et al. 2020; Xu et al. 2020; Gou et al. 2023), and therefore a decrease in lipid peroxidation was expected with the dietary HIO inclusion. Indeed, previous studies have demonstrated that the inclusion of MCFA decreased lipid peroxidation and improved antioxidant response in black sea bream (Ullah et al. 2022) and weanling pigs (Li et al. 2015).

On the other hand, as expected, dietary HIO inclusion reduced lipid peroxidation in the intestine without affecting antioxidant enzyme activities. Previous studies also showed that the inclusion of HIO improved the antioxidant capacity and decreased lipid peroxidation in the liver of *Onychostoma macrolepis* (Gou et al. 2023) and mirror carp (Xu et al. 2020). Likewise, for broiler chickens, the dietary inclusion of HIO increased the activity of plasma antioxidant enzymes and reduced lipid peroxidation (Kim et al. 2020; Chen et al. 2022). It can also not be disregarded the possibility of insect-origin antioxidant components in the oil, such as polyphenols and other bioactive compounds, also contributing to the enhanced antioxidant capacity of HIO (Di Mattia et al. 2019).

The innate immune system acts as the primary defense mechanism in fish, working to block invasive pathogens and trigger adaptive immune reactions (Xu et al. 2023). The dietary incorporation of lauric acid or ingredients rich in lauric acid, such as coconut oil, has been linked to enhanced immune function in fish and other animals (Jackman et al. 2020; Magouz et al. 2020; Dawood et al. 2021; Kumar et al. 2021; Wu et al. 2021). Previously, Cho et al. (2022) attributed the immune-boosting effects and improved disease resistance of rainbow trout fed partially defatted HI meal to its high lauric acid content. In the current study, however, no effects on the measured immune parameters were observed, except for a linear increase in peroxidase activity with the inclusion of HIO. Increased peroxidase was also observed in rainbow trout fed with diets where FO and soybean oil were replaced by HIO (Kumar et al. 2021). Yet, in Jian carp and barramundi, no effects on the immune parameters were observed in fish fed with diets containing HIO (Li et al. 2016; Hender et al. 2021). Nonetheless, studies involving disease challenges are necessary to confirm the potential effects of HIO in fish immune response, as these effects may only be manifested upon an infection challenge (Su et al. 2017).

The immune response is closely linked to inflammation. In line with their immune boosting proprieties, the dietary inclusion of lauric acid-rich ingredients has also been

associated with improved inflammatory status in several animals, including fish, turkeys, and pigs (Intahphuak et al. 2010; Liu 2015; Simó-Mirabet et al. 2017; Sypniewski et al. 2020; Ullah et al. 2022). In the present study, the effects on the expression of two pro-inflammatory genes, *il-1β* and *tnfα*, and two anti-inflammatory genes, *il-10* and *tgfβ*, were evaluated and responses are apparently contradictory. While the dietary inclusion of HIO increased the expression of *il-10*, which is an anti-inflammatory cytokine, an upregulation of the pro-inflammatory gene *tnfα* was also observed. For mirror carp, dietary HIO inclusion also increased hepatic *il-10* expression but decreased *il-1β* and *tnfα* expression when compared to yellow mealworm and silkworm oils (Xu et al. 2020). In a follow-up study, similar findings were observed in the expression of *il-1β* and *tnfα* in the liver of mirror carp fed with diets with HIO enriched in n-3 HUFA, but in the kidney the expression of *il-10* was upregulated (Xu et al. 2021). In the barramundi distal intestine, dietary inclusion of HIO upregulated the expression of *il-1β* but not *il-10* and *tnfα* (Hender et al. 2021) while in rainbow trout, diets with HIO increased the expression of *tnfα* in the kidney (Kumar et al. 2021).

Taken together, these results indicate that HIO can be included in diets for gilthead seabream juveniles up to 9.5% without negatively affecting oxidative status and inflammatory and immune responses. Furthermore, the dietary inclusion of HIO contributed to reducing the lipid peroxidation in the intestine.

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Chapter 8: Effects of black soldier fly larvae oil on lipid metabolism and plasma metabolites profile in gilthead seabream juveniles

Submitted to Aquaculture

Black soldier fly larvae oil as an alternative lipid source for gilthead seabream juveniles: effects on lipid metabolism, liver fatty acid composition, and plasma metabolites profile

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Abstract

The potential of insects as alternative ingredients in animal feeds has already been established. However, limited information is available concerning the use of insect oils as alternative lipid sources for aquafeeds. Therefore, a trial was conducted to assess the effects of the inclusion of black soldier fly (*Hermetia illucens*) larvae oil (HIO) in diets for gilthead seabream (*Sparus aurata*) juveniles. Four diets (45% crude protein, 18% crude lipids) were formulated to replace a vegetable oil (VO) blend (50% linseed, 30% palm, and 20% rapeseed oil) with 0, 42, 84, 100% IO, corresponding to 4, 7.9, and 9.5% inclusion levels, respectively. Three replicate groups of gilthead seabream juveniles (63 g) were fed the experimental diets for 70 days. At the end of the trial, effects on liver fatty acid (FA) profile, plasma metabolites, and expression of lipid metabolism-related genes were assessed. Our results denote that the inclusion of HIO linearly decreased plasma lipids and triglyceride content while high-density lipoprotein levels were increased. Also, the experimental diets influenced the liver's FA composition but without changes in total lipid content. There was an increase in the liver's saturated FA content, like lauric acid, and in monosaturated FAs, like oleic acid. Conversely, n-3 and n-6 polyunsaturated FA content was reduced while EPA and DHA remained unaffected. Also, C16:0 and C18:0 content (% total FA) were higher in the liver than in the corresponding diets. Dietary inclusion of HIO had little effect on the relative expression of genes related to FA synthesis, transport, and β-oxidation. However, downregulation of elongation of very long-chain fatty acids proteins 6 and 1b (*elovl6* and *elovl1b*) was observed with increasing dietary HIO levels. Overall, these results indicate that up to 9.5 % HIO inclusion in the diets is well tolerated by gilthead seabream juveniles, with little effect on plasma metabolites, and expression of key genes involved in fatty acid metabolism. In

summary, these results support the viability of HIO as a lipid alternative for juvenile gilthead seabream, contributing to the sustainable development of aquafeeds.

Keywords: *Hermetia illucens*, alternative ingredient, gene expression, insect oil, *Sparus aurata*

1. Introduction

Dietary lipids are important in fish nutrition serving not only as energy sources (NRC, 2011) but also as providers of essential fatty acids (EFA) essential for normal growth, health, and reproduction (Monroig et al., 2018; Sargent et al., 2003; Turchini et al., 2022). In aquaculture, fish oils (FO) have been regarded as optimal lipid sources for carnivorous and marine fish primarily due to their rich content of n-3 (omega-3) long-chain (≥C_{20}) polyunsaturated fatty acids (LC-PUFA), such as eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3). EPA and DHA are physiologically important nutrients, playing key roles in the development and function of the neural system, in the cell membrane and cellular synthesis, lipid signalling, as well as reproduction and endocrine regulation (Glencross, 2009; Tocher, 2003). Unlike freshwater species, marine fish in general and carnivorous species in particular are largely considered as having limited capacity to biosynthesize EPA and DHA from their precursors despite often having the necessary elongase and desaturase genes (Monroig et al., 2022; Tocher, 2003). Consequently, these n-3 LC-PUFA are EFA for these species and must be provided in diets to prevent deficiency symptoms.

Due to environmental and economic constraints, FO and other marine-derived raw materials in aquafeed formulations have been progressively replaced with terrestrial-origin raw materials (Aas et al., 2022; Hua et al., 2019). Vegetable oils (VO), such as soybean, palm, and rapeseed oils, are nowadays common ingredients in commercial aquafeed formulations (Turchini et al., 2022). Studies have shown that, as long as the essential fatty acid (EFA) needs are met, replacing FO with VO in marine fish diets, either partially or entirely, does not compromise fish growth or health (Turchini et al., 2009). However, VOs lack n-3 LC-PUFA and rather, they are rich in C_{18} polyunsaturated fatty acids (PUFA) like linoleic acid (C18:2n-6, LNA) and α-linolenic acid (C18:3n-3, ALA), impacting tissue composition and lipid metabolism in farmed fish (Riera-Heredia et al., 2020). Furthermore, the use of VO causes environmental concerns related to land use, water and soil degradation, greenhouse gas emissions, and biodiversity decline (Xu et al., 2021).

The interest in insects as food and feed has notably increased. Insects have high conversion ratios, fast growth and require almost no land and water (Sánchez-Muros et al., 2014). Also, insects can transform organic waste and other by-products into nutritionally valuable products (van Huis et al., 2013), and can be reared locally, thus improving economic security and reducing carbon footprint as well as the dependency on imports of raw materials from other countries. Currently, eight insect species have been authorized for use in aquafeeds in the EU (EU Regulation 2017/893; 2021/1925). Among them, the black soldier fly (*Hermetia illucens*; HI) appears as one of the most promising and extensively used in animal feeding. HI larvae are rich in proteins (up to 60% dry matter, DM) with a balanced amino acid profile, and are also rich in lipids (up to 30-35 % DM) (Liland et al., 2017; Nogales-Mérida et al., 2018). Since defatting has become a standard practice in the HI meal industry to enhance protein content, reduce composition variability, and minimize lipid oxidation (Dumas et al., 2018), HI oil (HIO) is currently regarded as a by-product and an underexploited resource with great potential in aquaculture.

HIO has a FA profile characterized by high levels of saturated fatty acids (SFA), the majority of which is composed of the medium-chain (C_{12}-C_{18}) fatty acid (MCFA) lauric acid (C12:0, LA) (Benzertiha et al., 2020). Dietary MCFAs have been proven as adequate and readily available energy sources for several farmed animals including fish, promoting increased growth without increasing tissue lipid deposition (Marten et al., 2006; Sudha et al., 2022). Indeed, MCFAs are characterized by their efficient absorption and rapid utilization as energy sources, facilitated by their direct transport to the mitochondria for β-oxidation, a process independent of the carnitine palmitoyltransferase-1 (Cpt1) system (Baltić et al., 2017; Tseng and Lin, 2019). Furthermore, MCFAs play a pivotal role in promoting lipid metabolism by accelerating the lipolysis of long-chain triglycerides and facilitating the β-oxidation of long-chain ($\geq C_{20}$) fatty acids (LCFA) (Xia et al., 2023). MCFAs have also been linked to improvement in fish health and well-being, reducing plasma triglycerides and increasing high-density lipoprotein (HDL) cholesterol in comparison to longer chain ($\geq C_{20}$) SFA (Panth et al., 2018).

Gilthead seabream (*Sparus aurata*) is one of the most economically important farmed finfish in Mediterranean aquaculture (Manchado et al., 2016). In previous studies, we demonstrated it was possible to include up to 9.5% HIO in the diets (replacing 100% of a VO blend) without negatively affecting the growth performance of gilthead seabream juveniles (Moutinho et al., 2023). The present study is a follow-up of the previous study and aims to gain insight into the impact that dietary HIO inclusion has on lipid

metabolism, by evaluating plasma metabolites profile, as well as liver FA composition and metabolism-related genes in gilthead seabream juveniles fed varying levels of HIO in replacement of dietary VO. More specifically, the genes evaluated included those related to FA elongation and desaturation (elongation of very long-chain fatty acids protein 1b, *elovl1b*; elongation of very long-chain fatty acids protein 5, *elovl5*; elongation of very long-chain fatty acids protein 6, *elovl6*; fatty acid desaturase 2, *fads2*), FA biosynthesis (fatty acid synthase, *fas*), FA transport (liver type fatty acid-binding protein 1, *fabp1*), lipid catabolism (hydroxy acyl-CoA dehydrogenase, *hoad*; carnitine palmitoyltransferase 1a, *cpt1a*; lipoprotein lipase, *lpl*; phospholipase A2, *pla2*), and regulatory transcription factors (peroxisome proliferator-activated receptor alpha, *pparα*; sterol regulatory element-binding protein 1, *srebp1*).

2. Materials and methods

2.1 Experimental diets and rearing conditions

Details on the experimental diets, as well as details on the feeding trial, were provided in Moutinho et al. (2023). Briefly, four isoproteic (45% crude protein) and isolipidic (18% crude lipids) diets were formulated following the nutritional requirements for the species (NRC, 2011). A control diet (HIO0) included sardine oil (3.1% of the diet) and a VO blend (9.5% of the diet) as lipid sources. The VO blend consisted of a mixture of 50% linseed, 30% palm, and 20% rapeseed oil. The remaining three diets included increasing levels of HIO in replacement of VO, namely 4.0%, 7.9%, and 9.5%, representing a VO replacement of 42% (diet HIO42), 84% (diet HIO84), and 100% (diet HIO100), respectively. According to the supplier's information, the HIO was separated from the dried larvae using a mechanical screw press. All dry ingredients were finely ground, mixed, and dry pelleted in a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA) through a 3 mm die. Pellets were dried (55 °C for 24 h) and stored at -20 °C until use. Proximate composition and FA profiles of the experimental diets are presented in Table 8.1 and Table 8.2, respectively.

In the feeding trial, 192 gilthead seabream juveniles, with an initial weight of 63 g, were reared in a thermoregulated seawater system (23.0 ± 0.5 ºC). The system was equipped with 12 fiberglass tanks with 500 L capacity each, equipped with continuous aeration and water flow. The experimental diets were tested in triplicate tanks holding 16 fish each, which were hand-fed to apparent visual satiation twice daily, 6 days per week, for a total of 10 weeks.

Table 8.1: Ingredients (% dry matter, DM) and proximate composition (% DM) of the experimental diets.

	HIO0	HIO42	HIO84	HIO100
Fishmeal[1]	20	20	20	20
Soluble fish protein concentrate[2]	5	5	5	5
Haemoglobin[3]	3	3	3	3
Corn gluten meal[4]	15	15	15	15
Soybean meal[5]	10.9	10.9	10.9	10.9
Rice bran[6]	10	10	10	10
Whole wheat[7]	15	15	15	15
Sardine oil[8]	3.11	3.11	3.11	3.11
VO blend[9]	9.5	5.5	1.5	0
HI larvae oil[10]	0	4.0	7.9	9.5
Taurine[11]	0.3	0.3	0.3	0.3
Phospholipids[12]	1	1	1	1
Shrimp hydrolysate[13]	1.5	1.5	1.5	1.5
Betaine[14]	0.2	0.2	0.2	0.2
Calcium biphosphate[15]	2	2	2	2
Vitamin premix[16]	1	1	1	1
Choline chloride (50%)[17]	0.5	0.5	0.5	0.5
Mineral premix[18]	1	1	1	1
Binder[19]	1	1	1	1
Proximate composition:				
Dry matter, DM (%)	91.6	90.9	92.8	92.1
Crude protein, CP (%)	46.8	46.4	47.0	46.3
Crude lipids, CL (%)	17.6	16.5	17.2	16.4
Ash (%)	9.14	9.21	9.20	9.10
Energy (kJ g^{-1})	22.0	22.1	24.1	23.4

[1]Fishmeal (89.1% DM, 74.9% CP, 10.7% CL, 29.6% Ash); Sorgal, S.A. Ovar, Portugal
[2]Sorgal, S.A. Ovar, Portugal
[3]Hemoglobin powder AP310P; APC Europe S.A.
[4]Sorgal, S.A. Ovar, Portugal
[5]Sorgal, S.A. Ovar, Portugal
[6]Sorgal, S.A. Ovar, Portugal
[7]Whole wheat (88.6% DM, 14.5% CP, 1.2% CL); Sorgal, S.A. Ovar, Portugal
[8]Sardine oil, Sorgal, S.A. Ovar, Portugal
[9]Vegetable oil blend: 50% linseed, 30% palm, and 20% rapeseed oils.
[10]*Hermetia illucens* larvae oil; Hermetia Baruth GmbH, Germany.
[11]Sorgal, S.A. Ovar, Portugal
[12]Premix, Viana do Castelo, Portugal.
[13]Shrimp hydrolysate (*Litopenaeus vannamei* trimmings: 73% CP, 12.1% CL, 13% Ash); Sorgal, S.A. Ovar, Portugal
[14]Sorgal, S.A. Ovar, Portugal
[15]Calcium biphosphate, Sorgal, S.A. Ovar, Portugal
[16]Vitamins premix (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
[17]Premix, Viana do Castelo, Portugal.
[18]Minerals premix (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)
[19]Binder: Carboxymethylcellulose sodium; Sigma-Aldrich Química, Portugal

Table 8.2: Fatty acid profile of *Hermetia illucens* larvae oil (HIO) and the experimental diets.

		Experimental diets			
	HIO	**HIO0**	**HIO42**	**HIO84**	**HIO100**
C12:0 (LA)	57.1	0.3	12.1	23.7	29.1
C14:0	11.5	1.6	4.0	6.4	7.3
C16:0	12.1	17.9	16.8	15.7	15.2
C18:0	1.6	3.5	3.1	2.5	2.3
C16:1n-7	2.5	1.6	2.2	2.7	2.9
C18:1n-7	0.3	1.8	1.6	1.4	1.3
C18:1n-9 (OA)	6.9	29.7	23.7	18.2	15.9
C20:1n-9	0.1	1.3	1.2	1.2	1.1
C18:2n-6 (LNA)	5.2	16.7	14.8	12.8	12.0
C18:3n-3 (ALA)	0.5	14.8	9.3	3.6	1.4
C20:4n-6 (ARA)	-	0.3	0.3	0.3	0.3
C20:5n-3 (EPA)	-	2.0	2.1	2.1	2.1
C22:6n-3 (DHA)	-	3.9	4.1	4.1	4.1
∑SFA	83.9	24.4	37.2	49.9	55.6
∑MUFA	10.2	36.1	30.4	25.3	22.8
∑n-6 PUFA	5.2	17.5	15.5	13.5	12.7
∑n-3 PUFA	0.6	21.7	16.5	10.9	8.6

The following FA, found in percentages below 1% of total FAME, were utilized for calculating the classes FA, but they are not listed in the table: C10:0, C14:1n-5, isoC15:0, C15:0, isoC16:0, C16:1n-9, C16:2n-4, C17:0, C16:3n-4, C17:1, C16:4n-1, C18:2n-4, C18:3n-6, C18:3n-4, C18:4n-3, C18:4n-1, C20:0, C20:1n-11, C20:1n-7, C20:2n-6, C20:3n-6, C20:3n-3, C20:4n-3, C22:0, C22:1n-9, C22:1n-7, C22:2n-6, C21:5n-3, C22:4n-6, C22:5n-6, C22:5n-3, and C24:0. LA: Lauric acid; OA: Oleic acid; LNA: Linoleic acid; ALA: α-Linolenic acid; ARA: Arachidonic acid; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids.

2.2 Sampling

At the end of the trial, three fish per tank (n = 9) were randomly selected 4 hours after the morning meal. Blood was drawn from the caudal vein using a heparinized syringe and promptly centrifuged at room temperature (10,000 × g for 10 minutes). The resulting plasma was collected and stored at -80 ºC until subsequent analyses. Subsequently, the fish were euthanized through decapitation, and their livers were excised on chilled trays. A portion of each liver was preserved in RNAlater (1:10) overnight at 4 ºC and then stored at -80 ºC before RNA extraction for gene expression analysis. The remaining liver samples were rapidly frozen in liquid nitrogen and stored at -80 °C for subsequent fatty acid profile analysis.

2.3 Plasma metabolites

Plasma glucose, lipids, triglycerides, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very-low-density lipoprotein (VLDL) were assessed

using standard clinical procedures. An auto-analyzer (Architect ci8200; Abbot Diagnostics, Saint-Laurent, Canada) was employed for the analyses, conducted by a clinical laboratory certified by Bureau Veritas (NP EN ISO 9001-2000).

2.4 Lipid and fatty acid analysis

Total lipids of diets and livers were extracted according to Folch et al. (1957) and gravimetrically quantified. An aliquot (4 mg) of total lipids was used to prepare fatty acid methyl esters (FAME) using a base-catalyzed trans-esterification method (Christie, 1982). FAMEs were determined by gas-chromatography (GC) as described in more detail by Moutinho et al. (2024).

2.5 Gene expression

Liver sample RNA extraction was conducted using the Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare, UK) following the manufacturer's guidelines. The procedure involved on-column DNase-I treatment, as supplied within the kit and the DNase-treated RNA was then eluted with RNase-free water. The total RNA concentration and the quality ratios 260/280 and 260/230 were measured using a NanoDrop (ThermoFisher Scientific, Waltham, MA, USA). An aliquot of total RNA (1 µg) was employed for cDNA synthesis (Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) from Promega), with random primers from Invitrogen™ (Thermo Fisher Scientific, USA). Gene expression was assessed by real-time quantitative PCR (qPCR) on a CFX Connect™ Real-Time System (Bio-Rad, Hercules, CA, USA), using PyroTaq EvaGreen qPCR Mix Plus NO ROX (Cultek, Madrid, Spain). Each qPCR contained 4 µl of PyroTaq EvaGreen qPCR Mix, 0.4 µl of each primer (forward and reverse), 2 or 5 µl of cDNA sample (1/20 dilution), and PCR-grade water up to a final volume of 20 µl. The volume of cDNA was chosen based on the best standard curve for each gene. The qPCR program included an initial polymerase activation of 95 °C for 15 min, and 40 cycles with denaturation at 95 °C for 15 s, annealing for 30 s, and extension at 72 °C for 20 s. A melting curve (60 ºC to 95 ºC with 0.5 ºC 0.05 s^{-1} increments) was generated after each run to confirm the specificity of the assays. To assess the reaction efficiency, a standard curve was generated in each run by conducting five-fold serial dilutions of cDNA from a mix of all samples (90% to 110% efficiency for all reactions). NormFinder algorithm was used to identify the optimal normalization of reference genes among a set of candidates (***ef1α***, *18s,* and ***β-actin***). Both ***ef1α*** and *18s* were chosen as reference genes to normalize the expression of the target genes. Primer sequence of reference and target genes (*elovl1b*, *elovl5*, *elovl6*, *fads2*, *fas*, *fabp1*, *cpt1a*, *lpl*, *pla2*, ***pparα***, and *srebp1*) is presented in Table 8.3. Relative gene expression was calculated as the ratio between the geometric mean

of the reference genes and the SQ mean of target genes, as described by Vandesompele et al. (2002).

Table 8.3: Sequence of primers used for real-time quantitative PCR.

	Gene	Primer sequence	Accession number
Reference genes	*ef1a*	F: CTTCAACGCTCAGGTCATCAT	AF184170
		R: GCACAGCGAAACGACCAAGGGGA	
	s18	F: GGGTGTTGGCAGACGTTAC	AM490061.1
		R: CTTCTGCCTGTTGAGGAACCA	
Elongases and desaturases	*elovl1b*	F: CTTCCTACACATCTTCCACCACTC	JX975700.1
		R: CCATTCCACCAGGAGCAAAGG	
	elovl5	F: TGCCAGGACACTCACAGTGC	AY660879
		R: GGACGAAGCTGTTTAGGGAGG	
	elovl6	F: GTGCTGCTCTACTCCTGGTA	JX975702
		R: ACGGCATGGACCAAGTAGT	
	fads2	F: GATGTGGAGCAGCTGGGTAT	AY055749
		R: TGTCTACGTTGCTGGTCTGG	
Fatty acid biosynthesis	*fas*	F: TGGCAGCATACACACAGACC	AM952430
		R: CACACAGGGCTTCAGTTTCA	
Fatty acid transport	*fabp1*	F: CATGAAGGCGATTGGTCTCC	KF857311
		R: GTCTCCAAGTCTGCCTCCTT	
Lipid catabolism	*hoad*	F: GAACCTCAGCAACAAGCCAAGAG	JQ308829
		R: CTAAGAGGCGGTTGACAATGAATCC	
	cpt1a	F: GTGCCTTCGTTCGTTCCATGATC	JQ308822.1
		R: TGATGCTTATCTGCTGCCTGTTTG	
	lpl	F: CGTTGCCAAGTTTGTGACCTG	AY495672
		R: AGGGTGTTCTGGTTGTCTGC	
	pla2	F: CCAGACCATCTTCACCATCC	AM972037
		R: CACCCAATCCACAGGAGTTC	
Regulatory transcription factors	*pparα*	F: TCTCTTCAGCCCACCATCCC	AY590299
		R: ATCCCAGCGTGTCGTCTCC	
	srebp1	F: AGGGCTGACCACAACGTCTCCTCTCC	JQ277709
		R: GCTGTACGTGGGATGTGATGGTTTGGG	

ef1a: elongation factor 1-alpha; *s18*: ribosomal protein S18; *elovl1b* elongation of very long-chain fatty acids protein 1b; *elovl5*: elongation of very long-chain fatty acids protein 5; *elovl6*: elongation of very long-chain fatty acids protein 6; *fads2*: fatty acid desaturase 2; *fas*: fatty acid synthase; *lpl*: lipoprotein lipase; *pla2*: phospholipase A2; *fabp1*: liver type fatty acid-binding protein 1; *hoad*: hydroxyacyl-CoA dehydrogenase; *cpt1a*: carnitine palmitoyltransferase 1a; *pparα*: peroxisome proliferator-activated receptor alpha; *srebp1:* sterol regulatory element-binding protein 1;

2.6 Statistical analysis

Statistical analyses were carried out using the SPSS 25.0 software package (IBM Corp.) for Windows. The data are expressed as the mean (n=9) accompanied by the pooled standard error of the mean (SEM). Before analysis, the data underwent checks for normal distribution and homogeneity of variances, and normalization was applied where necessary. Differences between dietary treatments were assessed using one-way

ANOVA, with a significance level of 0.05 used to reject the null hypothesis. Polynomial contrasts analysis was conducted to determine whether the data exhibited a linear or quadratic response. Tukey's multiple range test was employed to elucidate the extent of differences among means.

2.7 Ethics statement

All experimental protocols were executed by certified scientists in adherence to the FELASA category C guidelines. The growth trial adhered to the European Union Directive (2010/63/EU) on animal protection for scientific purposes and received approval from the Ethics Committee of CIIMAR (ORBEA_CIIMAR_27_2019).

3. Results

Growth performance results were published previously (Moutinho et al., 2023). Briefly, the fish's final body weight was similar regardless of the experimental diets. Notably, fish fed with diets HIO84 and HIO100 had a significantly higher feed efficiency compared to fish fed the other two diets (Moutinho et al., 2023).

3.1 Plasma metabolites

Plasma metabolite composition is presented in Table 8.4. At the end of the trial, there was a linear decrease in plasma lipids and triglyceride content and an increasing linear trend in plasma HDL levels with increasing dietary HIO levels. No differences were found for glucose, cholesterol, LDL and VLDL in the plasma of fish fed the experimental diets.

Table 8.4: Plasma metabolites profile of gilthead seabream fed the experimental diets.

							Polynomial contrasts	
	HIO0	**HIO42**	**HIO84**	**HIO100**	**SEM**	**ANOVA P-value**	**Linear P-value**	**Quadratic P-value**
Lipids (g L^{-1})	2.68	2.00	1.77	1.79	0.13	0.041*	0.012	ns
Glucose (mg dL^{-1})	62.3	69.1	68.3	71.2	1.66	ns	ns	ns
Triglycerides (g L^{-1})	14.9	13.3	13.6	9.8	7.59	ns	0.028	ns
Cholesterol (mg dL^{-1})	261.6	287.7	276.3	288.9	5.12	ns	ns	ns
HDL[1] (mg dL^{-1})	34.1	40.2	40.3	45.0	1.51	ns	0.015	ns
LDL[2] (mg dL^{-1})	69.2	80.9	84.6	89.8	3.95	ns	ns	ns
VLDL[3] (mg dL^{-1})	158.2	166.6	160.2	154.1	5.36	ns	ns	ns

Values are presented as mean of n=9. SEM = pooled standard error of the mean. Absence of different superscript letters indicate no significant differences between treatments (one-way ANOVA; P > 0.05). ns = non-significant p value (> 0.05). [1]HDL: high density lipoprotein; [2]LDL: Low density lipoprotein; [3]VLDL: very low-density lipoprotein; *Tukey's post hoc test did not highlight differences between means.

3.2 Liver lipid and fatty acid composition

The dietary inclusion of HIO did not affect the hepatic total lipid content, which ranged between 11.1 and 12.9 g 100 g^{-1} tissue (Table 8.5). However, the FA profiles of the liver were heavily influenced by those of the experimental diets (Table 8.5). Overall, total SFA content linearly increased with dietary HIO inclusion. LA content in the liver was lower than the corresponding diets, ranging from 0.3 to 29.1% of total FAME in the diets and 0.1 to 6.5% of total FAME in the liver. A linear increase was also observed for the MCFA C12:0, C14:0, and C16:0. However, the relative contents (% total FAME) of both C16:0 and C18:0 were higher in the liver than in the corresponding HIO-containing diets. For C16:0, liver content ranged from 16.9 to 20.8 % of total FAME, whereas its dietary content ranged from 15.2 to 17.9 % of total FAME. Also, for C18:0, liver content ranged from 5.7-6.7 % of total FAME whereas its dietary content ranged from 2.3-3.5 % of total FAME. Total MUFA content linearly decreased with dietary HIO content. Oleic acid (C18:1n-9) showed a negative quadratic trend, being lower in fish fed with diet HIO84 and HIO100 compared to the control (Table 8.5). Similarly, a linear decrease in total n-3 and n-6 PUFA contents with the increase of dietary HIO was also observed. However, the EPA, ARA, and DHA contents were not affected by the dietary treatments.

Table 8.5: Liver fatty acid profile (% FAME) and total lipid content (g 100 g^{-1} tissue) of gilthead seabream fed the experimental diets.

						Polynomial contrasts		
	HIO0	**HIO42**	**HIO84**	**HIO100**	**SEM**	**ANOVA P-value**	**Linear P-value**	**Quadratic P-value**
Total lipids	12.9	11.1	11.1	11.3	0.6	ns	ns	ns
C12:0 (LA)	0.1^{a}	2.6^{b}	6.5^{c}	5.8^{c}	0.5	0.000	0.000	0.003
C14:0	1.5^{a}	3.4^{b}	6.5^{c}	6.9^{c}	0.4	0.000	0.000	0.003
C16:0	16.9^{a}	18.5^{a}	20.8^{b}	21.1^{b}	0.4	0.000	0.000	ns
C18:0	6.0^{ab}	6.0^{ab}	5.7^{b}	6.7^{a}	0.1	0.027	ns	0.028
C18:1n-9 (OA)	30.7^{b}	27.8^{ab}	24.9^{a}	25.7^{a}	0.6	0.000	0.000	0.041
C18:2n-6 (LNA)	13.4^{c}	12.6^{bc}	11.4^{ab}	10.2^{a}	0.3	0.000	0.000	ns
C18:3n-3 (ALA)	9.0^{d}	6.0^{c}	2.7^{b}	1.1^{a}	0.5	0.000	0.000	0.011
C20:5n-3 (EPA)	1.7	2.0	1.8	1.7	0.1	ns	ns	ns
C20:4n-6 (ARA)	0.6	0.7	0.6	0.7	0.0	ns	ns	ns
C22:6n-3 (DHA)	6.8	7.6	5.8	6.1	0.3	ns	ns	ns
∑SFA	25.3^{a}	31.1^{b}	40.1^{c}	41.1^{c}	1.7	0.000	0.000	0.003
∑MUFA	38.5^{b}	35.9^{ab}	34.0^{a}	35.4^{ab}	0.6	0.047	0.029	ns
∑n-6 PUFA	15.7^{b}	15.4^{b}	13.6^{a}	12.7^{a}	0.3	0.000	0.000	ns
∑n-3 PUFA	20.3^{b}	18.0^{b}	12.0^{a}	10.5^{a}	0.8	0.000	0.000	ns

The following FA, found in percentages below 1% of total FAME, were utilized for calculating the classes FA, but they are not listed in the table: C10:0, C14:1n-5, isoC15:0, C15:0, isoC16:0, C16:1n-9, C16:2n-4, C17:0, C16:3n-4, C17:1, C16:4n-1, C18:2n-4, C18:3n-6, C18:3n-4, C18:4n-3, C18:4n-1, C20:0, C20:1n-11, C20:1n-7, C20:2n-6, C20:3n-6, C20:3n-3, C20:4n-3, C22:0, C22:1n-9, C22:1n-7, C22:2n-6, C21:5n-3, C22:4n-6, C22:5n-6, C22:5n-3, and C24:0. LA: Lauric acid; OA: Oleic acid; LNA: Linoleic acid; ALA: α-Linolenic acid; ARA: Arachidonic acid; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids. ns = not significant

3.3 Gene expression

The expression of genes related to lipid metabolism is shown in Figure 8.1. Overall, the dietary inclusion of HIO had no significant effects on the relative expression of *elovl5*, *fads2*, *lpl*, *pla2*, *fabp1*, *hoad*, *cpt1a*, *pparα* and *srebp1*. The expression of e*lovl1b* linearly decreased with the dietary HIO content while the expression of *elovl6* followed a negative quadratic response, the minimum expression being observed in fish fed with the diet HIO84 (Figure 8.1). A linear trend for a decrease in *fas* expression with the dietary inclusion of HIO was also observed.

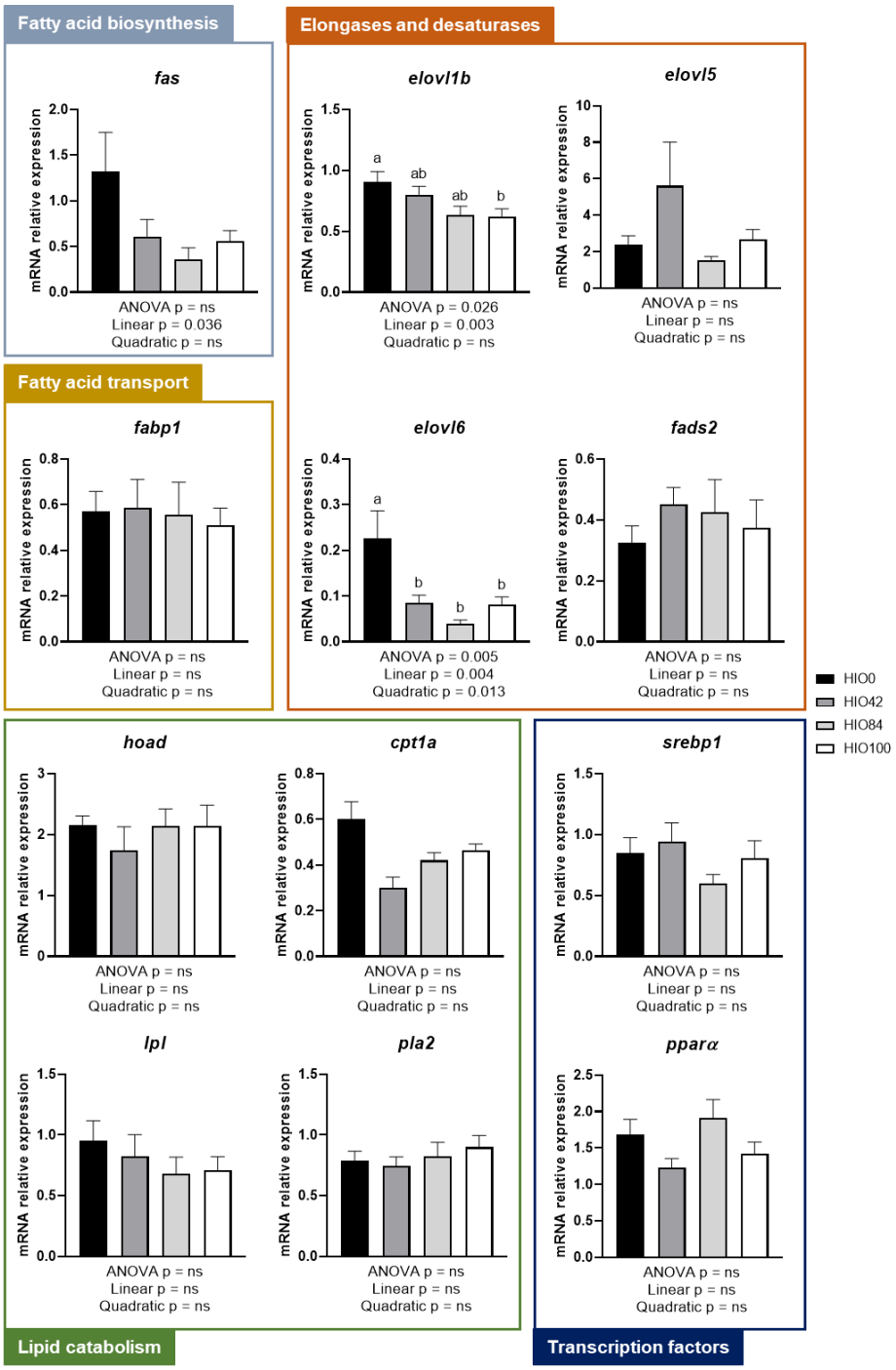

Figure 8.1: Relative mRNA expression of genes in the liver of gilthead seabream fed the experimental diets. Data was normalized with two reference genes, elongation factor 1 alpha (*ef1a*) and ribosomal protein S18 (*s18*). Values are presented as mean (n=9) and standard error represented by error bars. Different superscript letters above bars indicate significant differences between treatments (one-way ANOVA; $p < 0.05$); ns = non-significant p value. *elovl1b:* elongation of very long-chain fatty acids protein 1b; *elovl5*: elongation of very long-chain fatty acids protein 5; *elovl6*: elongation of very long-chain fatty acids protein 6*; fads2*: fatty acid desaturase 2; *fas*: fatty acid synthase; *lpl*: lipoprotein lipase; *pla2*: phospholipase A2; *fabp1*: liver type fatty acid-binding protein 1; *hoad*: hydroxyacyl-CoA dehydrogenase; *cpt1a*: carnitine palmitoyltransferase 1a; *pparα*: peroxisome proliferator-activated receptor alpha; *srebp1*: sterol regulatory element-binding protein 1.

4. Discussion

The use of insect meals as alternative ingredients to partially or completely replace fishmeal has already been demonstrated for many fish species (Kishawy et al., 2022; Magalhães et al., 2017; Rawski et al., 2020; Renna et al., 2017; Wang et al., 2019), including gilthead seabream (Moutinho et al., 2022). However, there is limited information regarding the potential of insect oils as an alternative lipid source in aquafeeds. In a previous study, we showed that it was possible to include up to 10% HIO (replacing 100% of a VO blend) in diets for juvenile gilthead seabream without affecting growth while improving feed efficiency (Moutinho et al., 2023). In the present study, we aimed to provide additional insights into the effects of dietary incorporation of HIO on the lipid metabolism of gilthead seabream juveniles.

Plasma biochemical profile is often used as a bioindicator to assess the health and lipid metabolism status of fish reared under various nutritional conditions (Fawole et al., 2020; Gou et al., 2023; Gu et al., 2022). Previous research has shown that the dietary inclusion of HIO had no effects on the plasma biochemical profile in Jian carp (*Cyprinus carpio* var. Jian; Li et al., 2016), barramundi (*Lates calcarifer*; Hender et al., 2021), and striped catfish (*Pangasianodon hypophthalmus*; Sudha et al., 2022). In the present study, plasma total lipids and triglycerides were reduced, and HDL was increased with dietary HIO inclusion. Similarly, a reduction in plasma triglyceride was also observed in *Onychostoma macrolepis* fed with diets containing HIO (Gou et al., 2023). Reduction of plasma lipids and higher HDL levels is usually associated with better health status (von Eckardstein et al., 2023), thus indicating a positive health benefit of the incorporation of HIO in the diets. This can be attributed to the elevated LA and MCFA content in HIO. Since MCFA can be transported directly to the liver to undergo β-oxidation, without being re-esterified to triglycerides (Xia et al., 2021), a reduction of triglycerides and an increase of HDL were expected. A reduction of plasma triglycerides was also observed in tambaqui (*Colossoma macropomum*) fed with diets including coconut oil, which are characterized by also having high LA and other MCFA content, similar to HIO (do Couto et al., 2022).

A cholesterol-lowering effect of HIO has been reported for red hybrid tilapia (*Oreochromis* sp.; Bakar et al., 2021), and dietary LA supplementation reduced plasma cholesterol in juvenile starry flounder (*Paralichthys olivaceus*) and Japanese flounder (*Platichthys stellatus*) (Kim et al., 2002; Lee et al., 2003). In the present study, despite the lack of an effect on plasma cholesterol, there was a linear increase in HDL with increasing HIO content. Plasma HDL is characterized for having lower triglyceride

content than LDL and VLDL (Weber and Zwingelstein, 1995) and is involved in the transport of cholesterol from plasma to the liver, acting as a cleaner of body tissue cholesterol in mammals (Luo et al., 2014). In humans, high HDL levels are also associated with a reduction in the risk of cardiovascular diseases (Miller and Miller, 1975).

In the present study, dietary inclusion of HIO did not result in increased hepatic lipid deposition, as is typically observed in fish fed with high VO diets (Li et al., 2019; Sun et al., 2011; Torstensen et al., 2011). Similarly, HIO inclusion had no effects on hepatic lipid deposition in *O. macrolepsis* (Gou et al., 2023) or totoaba (*Totoaba macdonaldi*; Maldonado-Othón et al., 2022), while liver lipid content was reduced in mirror carp (*Cyprinus carpio* var. specularis; Xu et al., 2021).

As reported for rainbow trout (*Oncorhynchus mykiss*; Fawole et al., 2021) and totoaba (Maldonado-Othón et al., 2022) fed diets with HIO, in the present study the liver's FA profile also tended to reflect that of the experimental diets. Nevertheless, LA content was much lower in the liver than in the corresponding diets, providing additional evidence that LA was preferentially catabolized as an energy source rather than deposited. Similar results were also noted in the muscle of gilthead seabream, as previously published by Moutinho et al. (2024).

Unlike LCFA, MCFA like LA can pass directly through the mitochondrial membrane without the need for the carnitine shuttle (Schönfeld and Wojtczak, 2016). CPT1A plays a central role in controlling the entry of LCFA for mitochondrial FA β-oxidation (Tseng and Lin, 2019). In the present study, although not statistically significant, fish fed the HIO-containing diets had a lower numerical expression of *cpt1a*, which suggests some decreased LCFA oxidation. Nevertheless, the lack of differences in *hoad* expression, suggests that β-oxidation was not compromised and that LA might be the major substrate. Contrary to present results and unexpectedly, studies in other fish species indicated an upregulation of *cpt1a* with increasing dietary HIO levels (Gou et al., 2023; Xu et al., 2020) or with diets including coconut oil (Tseng and Lin, 2019).

Fish, like other vertebrates, can biosynthesize *de novo* C16:0 and C18:0 by the action of *fas*, which is a multifunctional enzymatic complex that catalyzes cytosolic lipogenesis in animals (Sargent et al., 2003; Tocher, 2003). Thus, given the linear increase of hepatic content of C16:0 and C18:0 with the dietary HIO inclusion, an increased *fas* expression was also to be expected. However, although not different between diets, a linear decrease in *fas* expression was observed. This suggests some reduced *de novo* lipogenesis, possibly indicating that some of the previously formed LA was further

elongated to C16:0 and C18:0. Decreased *fas* expression was also observed in intraperitoneal fat of mirror carp fed diets with HIO (Xu et al., 2020) but not for Jian carp (Li et al., 2016), *O. macrolepis* (Gou et al., 2023) or rainbow trout (Fawole et al., 2021), denoting a species-specific response that deserves further investigation.

The decrease in LNA and ALA liver content with dietary HIO inclusion mirrors that of diet composition and reflects the low LC-PUFA biosynthetic capacity of gilthead seabream (Monroig et al., 2022) since the liver content of ARA, EPA, and DHA remained unchanged. In zebrafish, it was demonstrated that *elovl1a* and *elovl1b* were involved in the elongation of PUFA (Bhandari et al., 2016) while *elovl6* is involved in the elongation of C_{12}, $C_{14,}$ and C_{16} SFA and MUFA (Matsuzaka and Shimano, 2009). Thus, the downregulation of both *elovl1b* and *elovl6* with the inclusion of HIO seems to reflect the decrease of PUFA in the diets (*elovl1b)* and the preferential use of LA for energy purposes instead of being elongated to PUFA (*elovl6)* and stored as an energy reserve.

In marine fish, *fads2* expression is low as endogenous production of LC-PUFA is limited (Geay et al., 2010). Thus, as expected, *fads2* expression was not affected in the present study. However, studies in other species report increased *fads2* expression, in fish fed with diets including HIO, namely in rainbow trout (Fawole et al., 2021), and in zebrafish (*Danio rerio*; Zarantoniello et al., 2021; Zarantoniello et al., 2019). This seems to be related to the different capabilities of elongation and desaturation of PUFA in the different species (Tocher and Glencross, 2015) and the complexity of LC-PUFA biosynthesis regulation mechanisms in fish (Xie et al., 2021).

In the present study, the gene expression associated with FA catabolism (i.e. *hoad*, *cpt1a*, *lpl,* and *pla2*) was not affected by the dietary inclusion of HIO. Further, the expression of *pparα*, a central regulator of mitochondrial FA oxidation and lipid hydrolysis (Huss and Kelly, 2005; Li et al., 2016) was also unaffected by the dietary inclusion of HIO. This seems to indicate that dietary inclusion of HIO did not affect lipid catabolism, although it affected the preferred substrate (LA) used for β-oxidation.

In conclusion, the dietary inclusion of HIO resulted in a positive modulation of plasma metabolites profile, decreasing triglycerides and lipid content and increasing HDL cholesterol levels. The hepatic genes related to lipid metabolism were not markedly affected by HIO, although a downregulation in *elovl1b* and *elovl6* in response to the different dietary FA profiles was observed. Overall, it can be concluded that HIO can be used as an alternative lipid source in diets for gilthead seabream juveniles, without negative effects on fish health and lipid metabolism, thus contributing to the production of more sustainable aquafeeds.

5. Acknowledgements

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Chapter 9: General discussion, conclusion, and future perspectives

1. General discussion

Since the EU approval for use in aquafeeds in 2017, research on the potential of insect meals in fish feeds has increased exponentially. Overall, insect meals are considered a good alternative to traditional feed commodities, with a good nutritional value for fish. Among the authorized insect species to be used in aquafeeds, *Hermetia illucens* (HI) appears to be the most promising for carnivorous fish species. HI meal contains a high protein content with a balanced essential amino acid (EAA) profile, and is rich in lipids, vitamins and minerals. In recent years, several studies have evaluated the inclusion of HI meals in diets for several farmed fish species. However, the results regarding the ideal dietary inclusion level showed significant discrepancies. While in some studies high inclusion levels (> 30%) have been achieved without obvious detrimental effects on growth performance (Sealey et al. 2011; Kroeckel et al. 2012; Renna et al. 2017; Rawski et al. 2020; Stejskal et al. 2020; Biasato et al. 2022), as well as in the present study (Moutinho et al. 2022; Chapter 2), in other studies low to moderate dietary inclusion levels (< 15%) led to adverse effects on growth performance (Hu et al. 2017; Dumas et al. 2018; Adeoye et al. 2020; Fawole et al. 2020; Guerreiro et al. 2020; Kamarudin et al. 2021; Huang et al. 2022; Carvalho et al. 2023; Karapanagiotidis et al. 2023). Such an apparent discrepancy among studies could be attributed to the inconsistency of insect meal quality influenced by several factors, such as life stage, processing techniques, and rearing media, but also to the fish species, life stage, and feeding habits.

Despite the absence of differences in growth in gilthead seabream juveniles fed diets with varying HI meal (HM) inclusion levels (Moutinho et al. 2022; Chapter 2), it is known that dietary inclusion of HM can cause changes at the molecular level, affecting muscle growth regulatory mechanisms (Chemello et al. 2021). In the long term, these changes can compromise fish growth and nutritional quality for human consumption. In the present study, we evaluated the expression of several key genes related to muscle development and growth (Moutinho et al. 2024b; Chapter 3). We observed that the dietary inclusion of defatted HM had no effects on muscle growth hormone receptors, *ghr1* and *ghr2*, nor insulin-like growth factors, *igf1,* and *ifg2*, which are associated with muscle cell proliferation, differentiation and hypertrophy (García de la Serrana et al. 2012; Fuentes et al. 2013). Likewise, no differences were observed in myogenic differentiation factor 1 (*myod1)*, while *myod2* was downregulated with HM inclusion. Furthermore, the expression of myostatin (*mstn*), which inhibits the proliferation and differentiation of muscle cells, was upregulated by the dietary inclusion of defatted HM. These results suggest some inhibition or disruption of muscle growth in gilthead seabream juveniles fed diets including HM, but further research is required to elucidate

the transcriptional effects and determine whether its long-term use of HM could pose limitations on muscle development.

Chitin is considered to be one of the major drawbacks in use of insect meals in aquafeed formulations, as previous reports suggested chitin to be responsible for decreased protein and lipid digestibility in several fish species, affecting nutrient uptake and, consequently, growth (Hu et al. 2017; Dumas et al. 2018; Caimi et al. 2020; Weththasinghe et al. 2021; Stejskal et al. 2022). However, this was not the case in the present study, as both protein and lipid digestibility of the experimental diets were not compromised by the inclusion of defatted HM (Moutinho et al. 2022; Chapter 2). We also observed an increase in dry matter and energy digestibility in the HM-containing diets, which could be partly attributed to some chitinolytic activity, which increased energy availability. Chitinase activity, either through endogenous or exogenous production by gut bacteria, has been detected in some marine fish (Danulat and Kausch 1984; Gutowska et al. 2004) but not others (Kroeckel et al. 2012). Although chitinolytic activity was not measured in the present study, and no reports on chitinase activity of gilthead seabream have been published to the best of our knowledge, some activity is expected since chitin-containing prey, such as crustaceans, is part of this species' natural diet.

Besides growth, the potential of an alternative ingredient must also consider its effects on fish health and well-being. Oxidative stress occurs when there is an unbalance between reactive oxygen species production and the activity of antioxidant mechanisms. Such unbalance can be caused by internal or external factors, like dietary changes, leading to DNA and cell membrane damage and tissue lipid and protein peroxidation (Abdel-Latif et al. 2021). In the present study, we observed an increased activity of antioxidant enzymes in the liver and reduced lipid peroxidation in the intestine with the inclusion of defatted HM, suggesting an improved antioxidant capacity at the intestinal level (Chapter 4). Several reports have also observed improvements in antioxidant response and reduced lipid peroxidation in fish fed with diets including HM (Li et al. 2017; Zhou et al. 2018; Melenchón et al. 2020, 2022; Moutinho et al. 2021; Hidalgo et al. 2022; Siddaiah et al. 2023). This positive effect on antioxidant response has been attributed, at least in part, to the free radical scavenging properties of chitin present in insect meals, protecting tissues against free radical damage (Ngo and Kim 2014). Likewise, chitin has also been linked to improved immune status in fish. Although no effects related to HM inclusion were observed on the measured immune parameters in the present study (Chapter 4), previous studies reported improved immune response in fish fed with diets including HM, with an increase in non-specific immune parameters (Xiao et al. 2018; Abdel-Latif et al. 2021; Kishawy et al. 2022). Thus, more studies are required, including

bacterial challenges, to understand the fish immune modulation of dietary inclusion of insect meals.

Chitin has also been associated with improved inflammatory response. In the present study, we observed an effect associated with the increased dietary content of HM on the intestinal inflammatory condition in gilthead seabream with a downregulation of the expression of intestinal pro-inflammatory genes interleukin-1β (*il-1β*) and cyclooxygenase 2 (*cox2*), and upregulation of anti-inflammatory gene transforming growth factor β (*tgfβ*), suggesting an improved inflammatory status (Chapter 4). Similar findings were also reported for Nile tilapia (Kishawy et al. 2022) and pearl gentian grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂) (Huang et al. 2022), while contradicting results were obtained in zebrafish, with an upregulation of both pro and anti-inflammatory genes with dietary HM inclusion (Zarantoniello et al. 2020).

Gut microbiota has been increasingly recognized as playing a crucial role in fish nutrition, immune status, and overall health (Weththasinghe et al. 2022). Previous studies in fish have shown that chitin and other bioactive compounds present in insect meals have prebiotic properties, promoting a balanced and healthy gut microbiota (Gasco et al. 2018; Huyben et al. 2019; Terova et al. 2019; Rangel et al. 2022a), and increasing content in chitinase-producing bacteria (Askarian et al. 2012; Ringø et al. 2012; Rangel et al. 2022b). In the present study, we observed a modulation of the gut microbiota with the dietary inclusion of defatted HM (Moutinho et al. 2024b; Chapter 3). Fish fed the HM-containing diets had higher microbial richness and diversity in the digesta, which is associated with a higher fish health status (Terova et al. 2019). The inclusion of defatted HI meal also promoted the appearance of bacteria from the genera *Oceanobacillus*, *Bacillus*, *Lederbergia,* and *Corynebacterium*. These results could be partially explained by the microbiota present in HI larvae that colonized the gut (Li et al. 2022) or by the presence of chitin in the HI meal that supported the growth of beneficial chitin degraders, such as *Bacillus* spp. and *Corynebacterium* (Antonopoulou et al. 2019; Huyben et al. 2019). Indeed, higher microbial diversity was also reported by Askarian et al. (2012), Ringø et al. (2012), and Zhou et al. (2013) in fish fed with diets supplemented with chitin.

Insect oil has been regarded as a by-product of insect meal production and is currently an underexploited resource with great potential as an alternative lipid source in aquafeeds (Fawole et al. 2021). Unlike insect meals, the interest in insect oils has only recently begun to be perceived (Sudha et al. 2022). HI larvae oil (HIO) has a very interesting fatty acid (FA) profile since it is rich in saturated fatty acid (SFA), especially in lauric acid. Medium-chain (C_{12}-C_{18}) fatty acids (MCFA), such as lauric acid (C12:0),

have been used as strategic energy sources in animal feeds. MCFAs have been shown to improve animal growth without increasing lipid deposition as they are easily catabolized and preferably used as an energy source rather than storage. In the present study, we evaluated the replacement of a vegetable oil (VO) blend with increasing levels of HIO in practical diets for gilthead seabream containing approximately 6% fish oil (FO). Our results showed that it is possible to replace all of VO (up to 9.5% inclusion in the diets) with HIO without adverse effects on growth performance while having a linear improvement in feed efficiency (Moutinho et al. 2023; Chapter 5). Lack of adverse effects on growth performance with the dietary HIO inclusion has also been reported for Atlantic salmon (Belghit et al. 2018), barramundi (*Lates calcarifer*) (Hender et al. 2021), Jian carp (Li et al. 2016), rainbow trout (Dumas et al. 2018; Fawole et al. 2021) and striped catfish (Sudha et al. 2022), while for mirror carp dietary HIO inclusion even improved growth and feed efficiency (Xu et al. 2020; Xu et al. 2021).

The digestibility of lipids is correlated with the degree of unsaturation and chain length of FAs. Thus, SFAs are expected to be generally less digestible than monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) (Bowyer et al. 2013). This was also observed in the present study, as the digestibility of FAs increased from SFA < MUFA < PUFA (Moutinho et al. 2023; Chapter 5). However, contrary to what was expected, the digestibility of SFAs increased as their dietary content increased. This may be related to most SFAs in the diets being of medium chain length, which are more digestible than longer-chain length FA. Improvement in SFA and protein digestibility may explain the increase in the digestibility of energy of the HIO-containing diets.

Besides being an energy source, HIO may also have functional properties. Dietary inclusion of MCFA and MCFA-rich ingredients such as HIO, have been suggested to improve the immune, inflammatory and antioxidant response in fish (Simó-Mirabet et al. 2017; Xu et al. 2020; Ullah et al. 2022), weanling pigs (Li et al. 2015) and broiler chickens (Kim et al. 2020; Chen et al. 2022). In the present study, we observed an increase in the activity of liver antioxidant enzymes and a reduction in lipid peroxidation in the intestine (Chapter 7), further supporting this hypothesis. Increased activity of antioxidant enzymes was also reported for rainbow trout (Fawole et al. 2021) and mirror carp (Xu et al. 2020; 2021) fed diets with HIO. For the cyprinid *Onychostoma macrolepis*, increased superoxide dismutase activity was associated with decreased liver lipid peroxidation (Gou et al. 2023). Regarding inflammatory status, we observed apparently contradicting results. While the inclusion of 9.5% HIO upregulated the expression of the anti-inflammatory gene *il-10* in the intestine, the expression of the pro-inflammatory gene *tnfα* was also upregulated (Chapter 7). Contradicting results have also been previously

reported in other studies. While for mirror carp, HIO inclusion upregulated the expression of liver anti-inflammatory genes (Xu et al. 2020), for barramundi and rainbow trout, HIO inclusion induced an inflammatory status by upregulating the expression of pro-inflammatory genes (Hender et al. 2021; Kumar et al. 2021). For mirror carp, pro-inflammatory genes were upregulated by HIO inclusion but downregulated the expression of anti-inflammatory genes (Xu et al. 2021). Thus, long-term evaluation of fish inflammatory status when fed HIO is essential since a chronic inflammatory status will compromise fish well-being, and can affect health and disease resistance. Despite this, in the present study, the immune response was not compromised by the inclusion of HIO (Chapter 7). In this study, a linear increase in peroxidase activity was observed, as also reported in rainbow trout fed with diets containing HIO (Kumar et al. 2021).

Understanding how HIO modulates lipid metabolism is essential for its successful incorporation in aquafeeds. This is particularly relevant for marine carnivorous species, such as gilthead seabream, since HIO is rich in SFAs and lacks physiologically essential nutrients like long-chain (≥ C_{20}) polyunsaturated fatty acids (LC-PUFA), including the n-3 (omega-3) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). In the present study, we evaluated several key genes related to lipid metabolism, FA biosynthesis and catabolism, lipoprotein metabolism, and lipid digestion (Chapter 8). Overall, we observed that the inclusion of HIO is well tolerated by gilthead seabream, with minimal effects on the expression of the selected gene markers. However, a decrease in the expression of elongation of very long-chain fatty acids protein 6 (*elovl6*) and 1b (*elovl1b*)*,* and fatty acid synthase (*fas*) was detected with the increased inclusion of HIO. Elovl6 is responsible for the elongation of SFA and MUFA to form C_{18} FA (Weiss-Hersh et al. 2020), while Elovl1b is involved in the synthesis of SFA and MUFA of fatty acyl chains ≥ 22 carbons (Ofman et al. 2010). These results, together with the downregulation of *fas* (synthesis of C16:0 and C18:0) expression noted above, suggest a decreased capacity for *de novo* lipogenesis in fish fed with diets including HIO. This reduction of Fas synthetic activity could be partially attributed to the sparing effect of HIO, allowing the utilization of longer chain FA or other biological processes, thus eliminating the need for their synthesis by the fish.

The use of alternative lipid sources must also consider the potential effects on the nutritional quality of the fillet for human consumption. Up to this point, very few studies have evaluated the impacts of HIO on fillet quality traits. In the present study, we observed a positive correlation between fillet FA profile and FA profile of the experimental diets, with increased content of SFAs, particularly lauric acid (Moutinho et al. 2024a - Chapter 6). Increased SFA/lauric acid muscle deposition in fish fed with diets including

HIO was also observed in barramundi (Hender et al. 2021), rainbow trout (Fawole et al. 2021), and totoaba (Maldonado-Othón et al. 2022), and the whole body of striped catfish (Sudha et al. 2022). Nevertheless, in the present study, also possibly due to the sparing effect of HIO, levels of important FA for human composition, such as the health-promoting EPA and DHA, were maintained in the fillet, regardless of the level of HIO in the diet. Also, the FA quality indicators, such as thrombogenicity and atherogenicity indexes, and hypocholesterolemic/hypercholesterolemic FA, n-3/n-6, and PUFA/SFA ratios, were maintained within the recommended levels for a healthy human diet.

2. General conclusions

Overall, the present results showed that HI meal (HM) and HI oil (HIO) are not only suitable alternative protein and lipid sources in diets for gilthead seabream juveniles but may also have functional properties. This study showed that the dietary inclusion of defatted HM up to 45% and of HIO up to 10% in diets for gilthead seabream juveniles was possible without detriments on growth performance, feed efficiency, and digestibility. Similarly, there were no detriments of antioxidant, immune, and inflammatory status, while the nutritional quality of the fillet for human consumption was maintained.

From the results presented in this thesis, the following conclusions can be drawn:

- Replacing 100% of FM protein (up to 45% in the diets) with defatted HM had no negative effects on the growth performance and feed efficiency of gilthead seabream juveniles.
- Protein and lipid digestibility were not compromised by dietary HM inclusion, while dry matter and energy digestibility were increased.
- Expression of muscle growth-related genes (*ghr1*, *ghr2*, *igf1*, *igf2*, *myod1*) and proteomic enzymes (*ctsd-a*, *ctsd-b*) were not affected by dietary HM inclusion, but the downregulation of *myod2* and upregulation of *mstn* suggests potential constraint on muscle growth with the use of HM.
- The activity of intermediary metabolism enzymes was modulated with increasing dietary HM content, increasing amino acid catabolizing enzymes (ALT and GDH), and decreasing lipogenic-related enzymes (G6PDH, ME, FAS).
- Dietary inclusion of HM modulated gilthead seabream gut microbiota, with an increase in digesta microbial richness and diversity, and an increased abundance of bacteria with probiotic and chitinolytic potential.

- Dietary HM inclusion was shown to modulate the liver antioxidant enzymes without affecting lipid peroxidation status and reduced intestinal lipid peroxidation.
- HM seems to have an anti-inflammatory effect, as supported by the reduction in the expression of pro-inflammatory genes *il-1β* and *cox2* and the increase in anti-inflammatory gene *tgfβ* in the intestine.
- Total VO replacement with HIO (up to 9.5% dietary inclusion) was achieved without negative effects on growth performance while improving feed efficiency.
- Protein and lipid digestibility was not compromised by dietary HIO inclusion, while energy digestibility increased. Likewise, the digestibility of FA was high and the digestibility of SFA and lauric acid increased as HIO increased.
- Fillet FA profiles of gilthead seabream juveniles fed HIO diets reflected those of the experimental diets, showing an increase in SFA and lauric acid, and a decrease in MUFA and n-3 and n-6 PUFA, with increasing the dietary HIO inclusion. Dietary inclusion of up to 9.5% HIO had no effects on the EPA and DHA contents in the fillet, and FA quality indices were kept within the recommended levels for a healthy human diet.
- Gilthead seabream antioxidant status was enhanced by the dietary inclusion of HIO as shown by the decrease in intestine lipid peroxidation.
- Dietary HIO inclusion did not compromise the immune response of gilthead seabream, and up to 9.5% HIO increased the expression of both anti- and pro-inflammatory genes (*il-10* and *tnfα*).
- The immune response of gilthead seabream juveniles was not affected by dietary HIO inclusion, except for the increased peroxidase activity.
- In the intestine, the inclusion of 10% HIO in the diet increased the expression of both anti- and pro-inflammatory genes (*il-10* and *tnfα*).
- Dietary inclusion of HIO reduced plasma lipid and triglyceride content and increased HDL.
- Present results suggest that the dietary inclusion of HIO reduced lipogenesis in gilthead seabream, as demonstrated by a decrease in the expression of genes *elovl6*, *elovl1b,* and *fas*.

3. Future perspectives

Based on present research, the utilization of *Hermetia illucens* (HI) meal and oil as alternative ingredients for aquafeeds shows great potential. However, several key areas deserve further investigation. Despite positive outcomes in the present study, more extensive research is necessary to optimize inclusion levels for different fish species, considering growth performance, nutrient utilization, and long-term impacts on fish health. This optimization process could involve refining insect-rearing methods and adjusting insect diets to tailor their nutritional composition according to fish requirements. Additionally, reducing chitin content through the use of exogenous chitinases or probiotics with chitinolytic potential would be one strategy to improve the bioavailability of nutrients.

Findings from the present study suggest the dietary inclusion of HI meal or HI oil has no adverse effects on fish immune response. Nonetheless, a controlled *in vivo* bacterial challenge would be advantageous for a more detailed assessment of the potential immunomodulatory effects of these ingredients. Additionally, further exploration of the modulatory effects of dietary inclusion of HI oil on the gut microbiota is needed, as alterations in the gut microbiota provide valuable insight into overall gut health and functionality.

Addressing the economic feasibility of dietary incorporating HI products is also pivotal for their sustainable use as feed ingredients in aquafeeds. This will enhance the economic competitiveness of insect-derived ingredients compared to conventional feed sources that are commonly used in current formulations. Considering the positive results of HI meal and HI oil demonstrated in this study, investigating the feasibility of using full-fat insect meals instead of defatted ones could be physiologically and economically advantageous. Utilizing full-fat insect meals might reduce processing costs and potentially render insect-based ingredients more economically viable for large-scale aquafeed production.

Finally, while this study indicates positive impacts on fish health, exploring the safety and nutritional quality of fish reared on insect-based feeds for human consumption is important. Thus, future research should also investigate the nutritional composition and assess potential health benefits or risks associated with consuming fish reared on diets containing insect-derived ingredients.

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