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FEEDING STRATEGIES AND REARING TECHNIQUES FOR A SUSTAINABLE AQUACULTURE

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LIST OF ABBREVIATIONS

AA	Amino acids
ADC	Apparent digestibility coefficients
ADF	Acid detergente fibre
ALA	Alfa-linolenic acid
ALCA	Attributional life cycle assessment
AP	Acidification potential
ARA	Arachidonic acid
CED	Cumulative energy demand
CF	Crude fibre
CLCA	Consequential life cycle assessment
CP	Crude protein
DHA	Docosahexaenoic acid
DM	Dry matter
DO	Dissolved oxygen
DSF	Depleted stock fraction
EAA	Essential amino acids
ECO	ECO: Freshwater ecotoxicity
EE	Ether extract
EFA	Essential fatty acids
EP	Eutrophication potential
EPA	Eicosapentaenoic acid
EUR	European euro
FA	Fatty acid
FCR	Feed conversion ratio
FM	Fish meal
FO	Fish oil
FOFM	Fish meal and fish oil
GE	Gross energy
GHG	Greenhouse gasses
GWP	Global warming potential
HI	*Hermetia illucens*
HUFA	Highly unsaturated fatty acids
LA	Linoleic acid
LC-PUFA	Long chain polyunsaturated fatty acids
LCA	Life cycle assessment
Mt	Million tonnes
MUFA	Monounsaturated fatty acids
NDF	Neutral detergent fibre
NS	Not specified
OA	Oleic acid
OM	Organic matter
PUFA	Polyunsaturated fatty acids
RAS	Recirculating aquaculture system
SD	Standard deviation
SFA	Saturated fatty acids
SGR	Specific growth rate
SMC	Soybean meal concentrate
TAN	Total ammonia nitrogen
TM	*Tenebrio molitor*
TAN	Total ammonia nitrogen
TM	*Tenebrio molitor*
USD	United States Dollars
VO	Vegetable oil
VSI	Viscerosomatic index
WD	Water depletion
WG	Weight gain

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RIASSUNTO

La presente tesi di dottorato ha inteso valuare strategie di alimentazione e tecniche di allevamento dei pesci al fine di migliorare la sostenibilità dell'acquacoltura. A questo scopo, è stato adottato un approccio multidisciplinare, valutando le prestazioni produttive, la salute e la qualità dei pesci, e la produzione di ortaggi. Inoltre, nella parte di discussione generale, è stato valutato l'impatto ambientale di alcune soluzioni studiate, utilizzando un'analisi di Life Cycle Assessment (LCA).

Il **primo contributo** ha studiato l'effetto della sostituzione di olio di pesce con oli vegetali in diete per ricciola mediterranea, *Seriola dumerili* (Risso, 1810), analizzando il profilo acidico di muscoli bianchi e rossi, fegato, cervello e grasso viscerale. A questo scopo, 225 giovanili di ricciola mediterranea (peso vivo iniziale: 176 ± 3,62 g) sono stati alimentati per 109 giorni con una delle seguenti diete sperimentali: una dieta di controllo (FO 100) con olio di pesce come unica fonte lipidica, oppure con diete in cui il 75% (FO 25) o il 100% (FO 0) di olio di pesce era sostituito con una miscela di oli vegetali. Al termine della prova di alimentazione, sono stati campionati i tessuti; il loro grasso è stato estratto ed utilizzato per l'analisi del loro profilo acidico. La composizione in acidi grassi dei muscoli rossi e bianchi, del fegato e del grasso viscerale ha rispecchiato il profilo acidico delle diete sperimentali, mentre quello del cervello è risultato piuttosto robusto rispetto ai cambiamenti nella dieta. I risultati del presente studio rappresentano un contributo utile alla comprensione delle funzioni fisiologiche chiave del pesce e di come queste vengono preservate anche quando il pesce è alimentato con diete a bassi livelli di olio di pesce. Inoltre, lo studio apporta nuove conoscenze sui fabbisogni di acidi grassi nelle diete per la ricciola mediterranea, migliorando la sostenibilità della produzione e il benessere del pesce.

Il **secondo contributo** ha valutato gli effetti di un periodo di *refeeding* con diete a base di olio di pesce sulla composizione acidica di muscoli bianchi e rossi di ricciola mediterranea. Dopo un periodo di accrescimento (109 giorni) durante il quale i pesci sono stati alimentati con una dieta a base di olio di pesce (FO 100) oppure con diete in cui l'olio di pesce è stato parzialmente (75% di sostituzione; FO 25) o completamente sostituito da oli vegetali (FO 0), tutti i pesci sono stati sottoposti ad un periodo di alimentazione con dieta FO 100 (*refeeding* o *wash-out*) per 90 giorni. Il profilo acidico dei muscoli di pesci alimentati prima del *refeeding* con dieta FO 0 rifletteva quello degli oli vegetali inclusi nella dieta, ricchi di acido linoleico (LA) e acido α-linolenico (ALA), mentre risultava carente di acido arachidonico (AA), acido eicosapentaenoico (EPA) e acido docosaesaenoico (DHA). Nei pesci alimentati durante l'accrescimento con una dieta FO 0, gli acidi grassi non essenziali sono stati completamente ripristinati dopo 45 e 90 giorni di *refeeding* con dieta FO 100. D'altra parte, alla fine del periodo di *refeeding*, si è osservato un significativo aumento ($P<0,001$) di AA, EPA e DHA pari al 33%, 16% e 43%, rispettivamente, nei muscoli bianchi della ricciola. Allo stesso modo, l'AA e il DHA nei muscoli rossi sono aumentati rispettivamente del 33% e del 41%, mentre l'EPA è rimasto simile a quello osservato nei

pesci alimentati con diete FO 0. Pertanto, un *refeeding* di 90 giorni può parzialmente migliorare il profilo lipidico nei muscoli di ricciola mediterranea precedentemente alimentate con diete a base di oli vegetali.

Il **terzo contributo** ha studiato gli effetti di due densità di allevamento (bassa - ALD, 3,81 kg m^{-3} vs alta - AHD, 7,26 kg m^{-3}) sulla crescita, la salute e la qualità della carne di trote iridee (*Oncorhynchus mykiss,* Walbaum 1792) e sulla resa e qualità microbiologica di lattuga (*Lactuca sativa,* Linnaeus 1758.) in un sistema acquaponico a basso input tecnologico e confrontate con un sistema di coltivazione idroponica (HYP). A questo scopo sono state utilizzate nove unità sperimentali (tre repliche per trattamento) ed un totale di 123 trote iridee (peso iniziale: 142 ± 35 g), distribuite casualmente in sei vasche da 500 litri (3 per densità di allevamento) e monitorate durante un periodo di prova di 117 giorni. Il peso finale, le performance di crescita, la conversione alimentare e la mortalità del pesce non hanno presentato differenze significative tra le due densità di allevamento. Inoltre, gli indici morfometrici, i risultati di macellazione e la qualità della carne non sono stati influenzati. Allo stesso modo, la quantità di lattuga prodotta durante due cicli consecutivi è risultata simile tra i trattamenti (2,4 kg m^{-2} in media). Infine, al momento della raccolta, la contaminazione microbica (carica batterica totale di *E. coli*, *Enterobacteriaceae*, *Pseudomonas*, muffe e lieviti) sulla pelle del pesce e sulle foglie di lattuga è risultata simile tra i sistemi acquaponici a diverse densità di allevamento, nonché tra lattuga prodotta in acquaponica e in idroponica. In conclusione, il sistema di acquaponica testato è risultato idoneo alla produzione di trota iridea e lattuga, mentre la densità di allevamento del pesce non ha influenzato le performance di crescita e la qualità del prodotto.

Il quarto contributo ha valutato gli effetti della sostituzione alimentare di farina di pesce con farina (HI) di *Hermetia illucens,* Linnaeus 1758*),* sulla crescita, digeribilità del mangime, morfologia intestinale e sulla qualità del filetto di trote iridee allevate in un sistema acquaponico a basso input tecnologico. A tale scopo, 173 trote iridee (peso iniziale: 156 g ± 39,8 g) sono state distribuite in nove unità acquaponiche sperimentali (tre vasche per trattamento) e alimentate per 76 giorni con una delle tre diete contenenti 0 g/kg (H0, dieta di controllo), 62 g /kg (H6), o 124 g/kg (H12) di HI e 200 g/kg, 150 g/kg o 100 g/kg di farina di pesce, rispettivamente. La digeribilità apparente delle diete non è stata influenzata dal livello di inclusione di HI nella dieta. Al termine della prova, la mortalità delle trote è stata stata bassa (2,9%) e non influenzata dal trattamento alimentare. Il tasso di crescita è risultato inferiore nei pesci alimentati con dieta H12 rispetto a quelli alimentati con diete H0 e H6, sia dopo 26 giorni di alimentazione che al termine della prova. Al contrario, l'inclusione alimentare di HI non ha modificato significativamente indice di conversione, peso finale ed indici morfometrici. L'inclusione di HI ha aumentato la densità delle cellule caliciformi nell'intestino dei pesci alimentati con la dieta H12 rispetto a quelli alimentati con dieta H0 ($P < 0,05$). Per quanto riguarda la qualità del filetto, gli indici del rosso e del giallo sono risultati inferiori nei pesci alimentati con dieta H12 rispetto a quelli alimentati con dieta H0. L'inclusione di HI ha avuto uno scarso effetto sulla composizione chimica e sul profilo acidico dei filetti, mentre i livelli di C12:0 e C14:0 sono aumentati in pesci alimentati con

H6 e H12. In conclusione, una sostituzione del 25% di farina di pesce con farina di HI non ha sostanzialmente influenzato le performance di crescita e la qualità del filetto delle trote, mentre una sostituzione del 50% ha influenzato la morfologia intestinale, il colore del filetto e le sue caratteristiche nutrizionali.

La discussione sull'impatto ambientale di alcune delle strategie studiate basata sull'**analisi LCA** ha mostrato che la sostituzione dell'olio di pesce con oli vegetali ha avuto implicazioni positive in termini di potenziale di riscaldamento globale e esaurimento delle risorse marine, mentre ha aumentato il potenziale di eutrofizzazione. Per quanto riguarda la tecnica di allevamento, l'allevamento di trote ad alta densità ha avuto un impatto ambientale inferiore per kg di pesce prodotto rispetto a quello a bassa densità, soprattutto in termini di consumo idrico, eco tossicità delle acque dolci, riscaldamento globale ed impiego di energia.

In conclusione, sulla base dei risultati della presente tesi, oli vegetali e farina di insetti sono ingredienti alternativi promettenti da includere nelle diete per specie marine e d'acqua dolce di alto valore economico. Tuttavia, le future formulazioni dovranno considerare non solo le caratteristiche nutrizionali e i costi degli ingredienti, ma anche il loro impatto ambientale. L'acquaponica è una tecnica promettente per l'allevamento di specie d'acqua dolce di alto valore economico come la trota iridea. Inoltre, l'ottimizzazione della densità di allevamento dei pesci in acquaponica è un aspetto chiave per ottenere elevate rese e una buona qualità del prodotto, riducendo al contempo l'impatto ambientale e assicurando la salute e il benessere dei pesci.

ABSTRACT

The present PhD thesis aimed at improving the knowledge on feeding strategies and rearing techniques that might improve the sustainability of the aquaculture sector through a multidisciplinary approach, evaluating fish growth performance, health and quality, and vegetable production. The environmental impact of the different solutions was also assessed using a Life Cycle Assessment (LCA) analysis as a part of thesis general discussion.

The **first contribution** evaluated how replacing different levels of fish oil by vegetable oils in the diet of Mediterranean yellowtail, *Seriola dumerili* (Risso, 1810), affects the fatty acids (FA) signature, i.e. overall FA profile, in different tissues. A total of 225 Mediterranean yellowtail juveniles (initial live weight: 176 ± 3.62 g) were fed for 109 days with one of three diets: a control diet (FO 100), with FO as the only lipid source, or diets with 75% and 100% of fish oil replaced with a vegetable oil mixture. At the end of the feeding trial, the fish brains, muscles, livers, and visceral fat were sampled and their fat were extracted and used for FA analysis. The FA signatures of red and white muscle, liver, and visceral fat tissues changed when the dietary FA source changed, whereas FA signatures in the brain were rather robust to such dietary changes. These new insights might help to evaluate whether key physiological functions are preserved when fish are fed diets with low FO levels, as well as to define the dietary FA requirements of Mediterranean yellowtail to improve the sustainability of the production and welfare of the fish.

The **second contribution** assessed the effects of a wash-out on the fatty acid composition in the muscles of Mediterranean yellowtail. After 109 days during which fish were fed either a fish oil -based diet (FO 100) or a diet (FO 0) in which FO was completely substituted by vegetable oils, all fish were subjected to a wash-out with FO 100 diet for 90 days. The FA profile of muscles in fish fed FO 0 diet at the beginning of the experiment reflected that of dietary vegetable oils, rich in linoleic acid (LA), and α-linolenic acid (ALA), and was deficient in AA (arachidonic acid), EPA (eicosapentaenoic acid), and DHA (docosahexaenoic acid). Non-essential FA were fully restored in fish previously fed FO 0 diet on the 45^{th} or 90^{th} day of wash-out. At the end of wash-out, the FA composition showed that AA, EPA and DHA in the white muscles increased by +33%, +16%, and +43% ($P < 0.001$), respectively. Similarly, AA and DHA in the red muscles increased by +33% and +41% respectively, while EPA remained similar to fish fed FO 0 diet exclusively. Therefore, a 90-d wash-out can partially improve the FA profile in muscles of Mediterranean yellowtail previously fed vegetable oil-based diets.

The **third contribution** investigated the effects of two stocking densities (low - ALD, 3.81 kg m^{-3} vs. high - AHD, 7.26 kg m^{-3}) on the growth, health, and flesh quality of rainbow trout (*Oncorhynchus mykiss,* Walbaum 1792) and the yield and microbiological quality of lettuce (*Lactuca sativa,* Linnaeus 1758) produced in a low-tech aquaponic system compared to hydroponic cultivation (HYP). Nine experimental units (three replications per treatment) were utilised. A total of 123 rainbow trout (initial body weight: 142 ± 35 g) were randomly distributed in six 500-L tanks (3 per stocking density) and monitored during a 117-day trial

period. The final weight, specific growth rate, feed conversion ratio and mortality of the fish did not differ between stocking densities. The morphometric indices, slaughter results, and flesh quality were not affected. Similarly, the quantity of lettuce produced during two consecutive cycles was similar among treatments (2.4 kg m^{-2} on average). At harvest, microbial contamination (total viable count, *E. coli*, *Enterobacteriaceae, Pseudomonas*, mould, and yeasts) was similar in the fish skin and lettuce produced in aquaponic systems with different stocking densities, as well as in the lettuce produced in aquaponic and hydroponic systems. In conclusion, rainbow trout and lettuce productions were successful in the tested aquaponic system, whereas stocking density did not affect fish growth or flesh quality.

The **fourth contribution** evaluated the effects of dietary substitution of fishmeal with partially defatted (*Hermetia illucens*, Linnaeus 1758) meal (HI) on the growth, feed digestibility, gut morphology, and fillet quality of rainbow trout reared in a low-tech aquaponic system. A total of 173 rainbow trout (initial body weight: 156 g ± 39.8 g) were distributed among nine experimental aquaponic units (three tanks per treatment) and fed one of three diets containing 0 g/kg (H0, control diet), 62 g/kg (H6), or 124 g/kg (H12) of HI and 200 g/kg, 150 g/kg, or 100 g/kg of FM over a period of 76 days, respectively. The apparent digestibility coefficients of diets were unaffected by the inclusion level of dietary HI. At the end of the trial, trout mortality was low and unaffected by dietary treatment. The specific growth rate was, however, lower in fish fed the H12 diet than in those fed H0 and H6 diets after 26 days and at the end of the trial. In contrast, dietary inclusion of HI appeared to have no appreciable effect on the feed conversion ratio, final weight, fish condition factor, viscerosomatic index or hepatosomatic index. The inclusion of HI was, nevertheless, found to promote a 10% increase in the density of goblet cells in the gut of fish fed the H12 diet compared with those receiving the H0 diet ($P < 0.05$). With regards to fillet quality, redness and yellowness indices were lower in fish fed the H12 diet than in those fed the H0 diet. Although dietary HI had little effect on the proximate composition and fatty acid profile of fish, the proportions of C12:0 and C14:0 increased with HI dietary inclusion. In conclusion, fish growth and fillet quality were essentially unaffected by a 25% fish meal replacement with HI, whereas at a replacement rate of 50%, certain effects on gut histology and fillet colour and nutritional characteristics were detected, which warrant further investigation.

The discussion about the environmental impact based on **LCA analyses** showed that fish oil substitution with vegetable oils had positive implications in terms of global warming potential and depletion of marine resources, whereas increased the eutrophication potential. As for farming technique, rainbow trout successfully adapted to a low-tech aquaponic system. The rearing of trout at high density had a lower environmental impact per kg of fish produced compared to that at low density, especially in terms of water use, freshwater ecotoxicity, global warming and cumulative energy demand.

In conclusion, based on the results of the present thesis, vegetable oils and insect meal are promising alternative ingredients to be included in diets for high-value marine and freshwater carnivorous species. Nevertheless, future aquafeed formulations will be required

to focus not only on the nutritional characteristics and costs of the ingredients but also on their environmental impact. Aquaponics is a promising technique to be used for rearing high-value freshwater species such as rainbow trout. Furthermore, the optimization of fish stocking density in aquaponics is a key aspect to obtain high product yields and quality, reducing the environmental impact as well as assuring fish health and welfare.

RESUMEN EN ESPAÑOL

La presente tesis doctoral presentaba como principal objetivo contribuir al conocimiento sobre estrategias de alimentación y técnicas de producción que puedan mejorar la sostenibilidad del sector acuícola a través de un enfoque multidisciplinar, evaluando el crecimiento, la salud y la calidad sensorial de los peces, así como la producción de especies vegetales. También se evaluó El impacto ambiental de las diferentes propuestas mediante el análisis del Ciclo de Vida (LCA) como parte de la discusión general de la tesis.

El primer estudio evaluó el efecto de la sustitución de diferentes niveles de aceite de pescado (FO) por aceites vegetales en piensos para la seriola mediterránea (*Seriola dumerili*, Risso 1810) en el perfil de ácidos grasos (FA) de diferentes tejidos. Un total de 225 *S. dumerili* (peso vivo inicial: 176 ± 3,62 g) fueron alimentados durante 109 días con tres piensos diferentes: un pienso control (FO 100), con FO como única fuente lipídica, y dos piensos donde se sustituyó el 75% y el 100% de FO por VO, respectivamente. Al final de la prueba de alimentación, se tomaron muestras del tejido cerebral, músculo, hígado y grasa visceral de los peces, extrayendo los lípidos para el análisis de FA. El perfíl de FA de la grasa visceral, hígado, músculo rojo y blanco cambiaron cuando se modificó la fuente lipídica en el pienso, mientras que el perfíl de FA del tejido cerebro fue bastante resistente a su alteración por los cambios en la dieta. Estos nuevos datos podrían ayudar a evaluar si las funciones fisiológicas claves se conservan cuando los peces son alimentan con dietas con niveles bajos de FO, así como para definir las necesidades de FA en la dieta de la *S. dumerili* para mejorar la sostenibilidad de la producción y el bienestar de los peces.

El segundo estudio evaluó los efectos de un periodo de realimentación en la composición de ácidos grasos en el músculo de la seriola mediterránea. Después de 109 días durante los cuales los peces fueron alimentados con un pienso a base de aceite de pescado (FO 100) o un pienso (FO 0) en la que el FO fue sustituido completamente por aceites vegetales, todos los peces alimentados con la dieta FO 0 fueron sometidos a una realimentamentación con un pienso FO 100, durante 90 días. El perfil de FA de los músculos de los peces alimentados con la dieta FO 0 al comienzo del experimento reflejaba el de los aceites vegetales del pienso, ricos en ácido linoleico (LA) y ácido α-linolénico (ALA), y con bajo contenidos en AA (ácido araquidónico), EPA (ácido eicosapentaenoico) y DHA (ácido docosahexaenoico). Los FA no esenciales se restauraron por completo en peces previamente alimentados con la dieta FO 0 en el día 45 o 90 de realimentación. Al final de la realimentación, la composición de FA mostró que AA, EPA y DHA en en el músculo blanco aumentaron en + 33%, + 16% y + 43% ($P < 0,001$), respectivamente. De manera similar, el AA y el DHA en el músculo rojo aumentaron un 33% y 41%, respectivamente, mientras que el EPA se mantuvo similar a los peces alimentados exclusivamente con la dieta FO 0. Por lo tanto, un recebo de 90 días puede mejorar parcialmente el perfil de FA en los músculos de la seriola mediterránea previamente alimentada con dietas a base de aceite vegetal.

El tercero estudio analizó los efectos de dos densidades de producción (baja - ALD, 3,81 kg m^{-3} frente a alta - AHD, 7,26 kg m^{-3}) sobre el crecimiento, la salud y la calidad de

la carne de la trucha arco iris (*Oncorhynchus mykiss*, Walbaum 1792) así como el rendimiento y calidad microbiológica de la lechuga (*Lactuca sativa*, Linnaeus 1758) producida en un sistema acuapónico de baja tecnología en comparación con el cultivo hidropónico (HYP). Se utilizaron nueve unidades experimentales (tres repeticiones por tratamiento). Un total de 123 truchas arco iris (peso corporal inicial: 142 ± 35 g) se distribuyeron al azar en seis tanques de 500 L (3 por cada densidad de producción) y se monitorearon durante un período de prueba de 117 días. El peso final (331 g en promedio), la tasa de crecimiento instantáneo (0,73% d^{-1}), la tasa de conversión del alimento (1,58) y la mortalidad (3%) de los peces no difirieron entre densidades de producción. Los índices morfométricos, rendimiento de la canal, de la carne y la calidad de la carne no se vieron afectados. Asimismo, la biomasa de lechuga producida durante dos ciclos consecutivos fue similar entre tratamientos (2,4 kg m^{-2} en promedio). En la cosecha, la contaminación microbiana (recuento total viable, *E. coli*, Enterobacteriaceae, Pseudomonas, moho y levaduras) tanto de la piel de los peces, como de la lechuga producida en el sistema acuapónico en ambas densidades de producción fue similar a de la lechuga producida en acuaponia y sistemas hidropónicos. En conclusión, las producciones de trucha arco iris y lechuga fueron exitosas en el sistema acuapónico probado, mientras que la densidad de población no afectó el crecimiento de los peces ni a la calidad de la carne.

En el cuarto estudio se evaluó el efecto de la sustitución dietaria de la harina de pescado (FM) por harina de *Hermetia illucens (*Linnaeus, 1758*),* parcialmente desgrasada (HI) sobre el crecimiento, la digestibilidad del alimento, la morfología intestinal y la calidad del filete de la trucha arco iris producida en un sistema acuapónico de baja tecnología. Un total de 173 truchas arco iris (peso vivo inicial: 156 g ± 39,8 g) se distribuyeron entre nueve unidades acuapónicas experimentales (tres tanques por tratamiento) y se alimentaron durante un período de 76 días con tres piensos isoproteicos e isoenergéticos que contenían 0 g / kg (H0, dieta de control), 62 g / kg (H6) o 124 g / kg (H12) de HI y 200 g / kg, 150 g / kg o 100 g / kg de FM, respectivamente. Los coeficientes de digestibilidad aparente de los piensos no se vieron afectados por el nivel de inclusión de HI dietético. Al final del ensayo, la mortalidad de la trucha fue baja y no se vio afectada por el tratamiento dietario. Sin embargo, la tasa de crecimiento instantáneo fue menor en los peces alimentados con la dieta H12 que en los alimentados con las dietas H0 y H6 después de 26 días (1.07% d^{-1} *vs* 1.22% d^{-1}; P <0.001) y al final del período de ensayo (0,81% d^{-1} *vs* a 0,88% d^{-1}; P <0,05). Por el contrario, la inclusión dietaria de HI no pareció tener un efecto apreciable sobre la tasa de conversión del alimento, el peso final, el factor de condición, el índice viscerosomático o el índice hepatosomático. No obstante, se observó que la inclusión de HI promueve un aumento de la densidad de las células caliciformes intestinales en un 10% de los peces alimentados con el pienso H12 en comparación con los que se alimentaron con el H0 (P <0,05). Con respecto a la calidad del filete, los índices de enrojecimiento y amarilleamiento fueron más bajos en los peces alimentados con el pienso H12 que en los alimentados con el H0. Aunque el HI en la dieta tuvo poco efecto sobre la composición química y el perfil de ácidos grasos del pescado, los porcentajes de C12:0 y C14:0 aumentaron con la inclusión del HI en la dieta. En

conclusión, el crecimiento de los peces y la calidad del filete no se vieron esencialmente afectados por la sustitución de harina de pescado al 25% con HI. Sin embargo, con una sustitución del 50%, se detectó ciertos efectos sobre la histología intestinal y el color del filete y las características nutricionales, que justifican una mayor investigación.

La discusión sobre el impacto ambiental de algunas de las estrategias estudiadas basadas en el **análisis de LCA** mostró que la sustitución del aceite de pescado por aceites vegetales tenía implicaciones positivas en términos del potencial de calentamiento global y el agotamiento de los recursos marinos, pero aumentaba el potencial de eutrofización. En cuanto a la técnica de producción, la trucha arco iris se ha adaptado con éxito a un sistema acuapónico de baja tecnología. La producción de truchas a densidades altas tuvo un impacto ambiental menor por kg de pescado producido que la producción de truchas a baja densidad, especialmente en términos de consumo de agua, ecotoxicidad del agua dulce, calentamiento global y uso de energía.

En conclusión, en base a los resultados de esta tesis, los aceites vegetales y la harina de insecto son ingredientes alternativos prometedores para ser incluidos en las dietas de especies marinas y de agua dulce de alto valor económico. Sin embargo, las formulaciones futuras deberán centrarse no solo en las características nutricionales y el coste de los ingredientes, sino también en su impacto ambiental. La acuaponía es una técnica prometedora para la cría de especies de agua dulce de alto valor como la trucha arco iris. Además, la optimización de la densidad de producción de peces en acuaponía es un aspecto clave para obtener altos rendimientos y una buena calidad del producto, al tiempo que se reduce el impacto ambiental, asegurando la salud y el bienestar de los peces.

RESUM EN VALENCIÀ

La present tesi doctoral va tenir com a objectiu contribuir al coneixement sobre estratègies d'alimentació i tècniques de producció que puguin millorar la sostenibilitat del sector aqüícola a través d'un enfocament multidisciplinari, avaluant el creixement, la salut i la qualitat sensorial dels peixos, així com la producció de espècies vegetals. El impacte ambiental de les diferents propostes també es va avaluar mitjançant l'anàlisi de l'Cicle de Vida (LCA) com a part de la discussió general de la tesi.

En el primer estudi es va avaluar la substitució de diferents nivells d'oli de peix per olis vegetals en pinsos per a la seriola Mediterrània (*Seriola dumerili*, Risso 1810) en el perfil d'àcids grassos (FA) de diferents teixits. Un total de 225 *S. dumerili* (pes viu inicial: 176 ± 3,62 g) van ser alimentats durant 109 dies amb tres pinsos diferents: un pinso control (FO 100), amb FO com a única font lipídica, i dos pinsos amb el 75 % i el 100% de substitució de FO per VO, respectivament. A la fi de la prova d'alimentació, es van prendre mostres de teixit cerebral, múscul, fetge i greix visceral dels peixos, extraient els lípids per a l'anàlisi de FA. El perfil de FA del greix visceral, fetge i múscul roig i blanc va canviar quan va canviar la font lipídica en el pinso, mentre que el perfil de FA del teixit del cervell va ser bastant resistent a la seva alteració pels canvis en la dieta. Aquestes noves dades podrien ajudar a avaluar si les funcions fisiològiques claus es conserven quan els peixos s'alimenten amb dietes amb nivells baixos de FO, així com per definir les necessitats de FA en la dieta de la *S. dumerili* per millorar la sostenibilitat de la producció i el benestar dels peixos.

El segon estudi es van avaluar els efectes d'un període de realimentació en la composició d'àcids grassos en el múscul de la seriola mediterrània. Després de 109 dies durant els quals els peixos van ser alimentats amb un pinso a base d'oli de peix (FO 100) o un pinso (FO 0) en la qual el FO va ser substituït completament per olis vegetals, tots els peixos (FO) van ser sotmesos a una realimentamentación amb un pinso FO 100, durant 90 dies. El perfil de FA dels músculs dels peixos alimentats amb la dieta FO 0 a l'inici de l'experiment reflectia el dels olis vegetals de el pinso, rics en àcid linoleic (LA) i àcid α-linolènic (ALA), i amb baix continguts en AA (àcid araquidònic), EPA (àcid eicosapentaenoic) i DHA (àcid docosahexaenoic). Els AG no essencials es van restaurar completament en peixos prèviament alimentats amb la dieta FO 0 en el dia 45 o 90 de realimentació. A la fi de la realimentació, la composició de FA va mostrar que AA, EPA i DHA en en el múscul blanc van augmentar en + 33%, + 16% i + 43% ($P < 0,001$), respectivament. De manera similar, l'AA i el DHA en el múscul roig van augmentar en + 33% i + 41%, respectivament, mentre que el EPA es va mantenir similar als peixos alimentats exclusivament amb la dieta FO 0. Per tant, un rentat de 90 dies pot millorar parcialment el perfil de FA en els músculs de la seriola mediterrània prèviament alimentada amb dietes a base d'oli vegetal.

El tercer estudi va estudiar els efectes de dues densitats de producció (baixa - ALD, 3,81 kg m^{-3} enfront de alta - AHD, 7,26 kg m^{-3}) sobre el creixement, la salut i la qualitat de la carn de la truita (*Oncorhynchus mykiss*, Walbaum 1792) i el rendiment i qualitat

microbiològica de l'enciam (*Lactuca sativa*, Linnaeus 1758) produïda en un sistema aquapònic de baixa tecnologia en comparació amb el cultiu hidropònic (HYP). Es van utilitzar nou unitats experimentals (tres repeticions per tractament). Un total de 123 truites (pes corporal inicial: 142 ± 35 g) es van distribuir a l'atzar en sis tancs de 500 L (3 per cada densitat de producció) i es van monitoritzar durant un període de prova de 117 dies. El pes final (331 g de mitjana), la taxa de creixement instantani (0,73% d-1), la taxa de conversió de l'aliment (1,58) i la mortalitat (3%) dels peixos no van diferir entre densitats de producció. Els índexs morfomètrics, rendiment de la canal i de la carn i la qualitat de la carn no es van veure afectats. Així mateix, la biomassa d'enciam produïda durant dos cicles consecutius va ser similar entre tractaments (2,4 kg m^{-2} de mitjana). A la collita, la contaminació microbiana (recompte total viable, I. coli, Enterobacteriaceae, Pseudomonas, floridura i llevats) tant de la pell dels peixos, com de l'enciam produit en el sistema aquapònic en les dues densitats de producció va ser similar a la de l'enciam produit en acuaponia i sistemes hidropònics. En conclusió, les produccions de truita i enciam van tindre èxit en el sistema aquapònic provat, mentre que la densitat de població no va afectar al creixement dels peixos ni a la qualitat de la carn.

En el quart estudi es va avaluar l'efecte de la substitució dietària de la farina de peix amb farina de *Hermetia illucens* (Linnaeus, 1758), parcialment desgreixada (HI) sobre el creixement, la digestibilitat de l'aliment, la morfologia intestinal i la qualitat del filet de la truita produïda en un sistema aquapònic de baixa tecnologia. Un total de 173 truites (pes inicial: 156 g ± 39,8 g) es van distribuir entre nou unitats aquapòniques experimentals (tres tancs per tractament) i es van alimentar durant un període de 76 dies amb tres pinsos isoproteics i isoenergètics que contenien 0 g / kg (H0, dieta de control), 62 g / kg (H6) o 124 g / kg (H12) de HI i 200 g / kg, 150 g / kg o 100 g / kg de FM, respectivament. Els coeficients de digestibilitat aparent dels pinsos no es van veure afectats pel nivell d'inclusió de HI dietètic. A la fi de l'assaig, la mortalitat de la truita va ser baixa i no es va veure afectada pel tractament dietari. No obstant això, la taxa de creixement instantani va ser menor en els peixos alimentats amb la dieta H12 que en els alimentats amb les dietes H0 i H6 després de 26 dies (1.07% d^{-1} vs 1.22% d^{-1}; P <0.001) i a la fi de el període d'assaig (0,81% d^{-1} vs a 0,88% d^{-1}; P <0,05). Per contra, la inclusió dietària de HI no va semblar tenir un efecte apreciable sobre la taxa de conversió de l'aliment, el pes final, el factor de condició, l'índex viscerosomàtic o l'índex hepatosomàtic. Això no obstant, es va observar que la inclusió de HI promou un augment de la densitat de les cèl·lules caliciformes intestinals en un 10% dels peixos alimentats amb el pinso H12 en comparació amb els que es van alimentar amb el H0 (P <0,05) . Pel que fa a la qualitat de l'filet, els índexs d'envermelliment i engroguiment van ser més baixos en els peixos alimentats amb el pinso H12 que en els alimentats amb el H0. Tot i que el HI en la dieta va tenir poc efecte sobre la composició química i el perfil d'àcids grassos del peix, els percentatges de C12: 0 i C14: 0 van augmentar amb la inclusió de l'HI en la dieta. En conclusió, el creixement dels peixos i la qualitat de l'filet no es van veure essencialment afectats per la substitució de farina de peix al 25% amb HI, mentre que amb

una substitució del 50%, es va detectar certs efectes sobre la histologia intestinal i el color del filet i les característiques nutricionals, que justifiquen una major investigació.

La discussió sobre l'impacte ambiental d'algunes de les estratègies estudiades basades en **l'anàlisi de LCA** va mostrar que la substitució de l'oli de peix per olis vegetals tenia implicacions positives en termes de l'potencial d'escalfament global i l'esgotament dels recursos marins, al temps que augmentava el potencial d'eutrofització. Pel que fa a la tècnica de producció, la truita s'ha adaptat amb èxit a un sistema aquapònic de baixa tecnologia. La producció de truites a densitats altes va tenir un impacte ambiental menor per kg de peix produït que la producció de truites a baixa densitat, especialment en termes de consum d'aigua, ecotoxicitat de l'aigua dolça, escalfament global i ús d'energia.

En conclusió, en base als resultats d'aquesta tesi, els olis vegetals i la farina d'insecte són ingredients alternatius prometedors per a ser inclosos en les dietes d'espècies marines i d'aigua dolça d'alt valor econòmic. No obstant això, les formulacions futures hauran de centrar no només en les característiques nutricionals i el cost dels ingredients, sinó també en el seu impacte ambiental. La aquaponia és una tècnica prometedora per a la cria d'espècies d'aigua dolça d'alt valor com la truita. A més, l'optimització de la densitat de producció de peixos en aquaponia és un aspecte clau per obtenir alts rendiments i una bona qualitat del producte, al temps que es redueix l'impacte ambiental, assegurant la salut i el benestar dels peixos.

CHAPTER 1: LITERATURE REVIEW

INTRODUCTION

Aquaculture production and trends

The state of world fisheries and aquaculture

Global aquatic production reached 179 million tonnes (Mt) in 2018, for a total value of USD 401 billion, of which 82 Mt (USD 250 billion) provided by aquaculture production (FAO, 2020a) (Table 1.1). About 87% (156 Mt) of total seafood production is destined for human consumption, equal to an estimated annual amount of 20.5 kg per capita (FAO, 2020a). The remaining 13% (22 Mt) is directed to non-food products, especially fish oil (FO) and fishmeal (FM) (Table 1.1). In 2018, aquaculture products represented the 46% of the total fish production and the 52% for human consumption.

China is the leading seafood producer, accounting for 35% of the global fish supply, followed by Asia (34%), Americas (14%), Europe (10%), Africa and Oceania. During the last years, an increase in total seafood production was recorded in all continents, except for Europe and Americas, whereas in the last two decades it doubled in Asia and Africa.

Table 1.1. World fisheries and aquaculture production (million tonnes, live weight) and utilization (adapted from FAO, 2020a).

	1986-1995	1996-2005	2006-2015	2016	2017	2018
	Average per year					
Production						
Capture						
Inland	6.4	8.3	10.6	11.4	11.9	12.0
Marine	80.5	83.0	79.3	78.3	81.2	84.4
Total capture	86.9	91.4	89.8	89.6	93.1	96.4
Aquaculture						
Inland	8.6	19.8	36.8	48.0	49.6	51.3
Marine	6.3	14.4	22.8	28.5	30.0	30.8
Total aquaculture	14.9	34.2	59.7	76.5	79.5	82.1
Total world fisheries and aquaculture	101.8	125.6	149.5	166.1	172.7	178.5
Utilization						
Human consumption	71.8	98.5	129.2	148.2	152.9	156.4
Non-food uses	29.9	27.1	20.3	17.9	19.7	22.2
Population (billions)	5.4	6.2	7.0	7.5	7.5	7.6
Per capita apparent consumption (kg)	13.4	15.9	18.4	19.9	20.3	20.5

Global aquaculture production

With capture fishery stocks relatively static since the 1980s, aquaculture has been responsible for a continuous impressive growth in the supply of aquatic animals for human consumption. In 2018, global aquaculture production reached an all-time record, with 114.5 Mt, of which 72% aquatic animals, mainly represented by finfish (54.3 Mt) and about 28% aquatic algae (FAO, 2020a). During the period 2001-2018, global aquaculture supply showed a growth of 5.3% per year, with a slight reduction in the last years (4% in 2017, 3.2% in 2018). In 2018, about 63% of the farmed fish were produced in inland aquaculture facilities, with an increasing trend if compared with the same production in 2000 (58%). On the other hand, coastal and marine aquaculture provided about 31 Mt of aquatic animals in 2018.

Projections report that the global seafood supply is expected to rise from the 179 Mt in 2018 to 204 Mt in 2030, with aquaculture production estimated to increase by 32% and reach 109 Mt. Moreover, at a world level, the country's policies in the next years will endure the transition from extensive to intensive aquaculture. Regarding capture fisheries, fish stocks are expected to stay at high levels, showing an increase in catches in stock-recovering areas and in underfished waters, with a desirable, but not confirmed, slight reduction in fish oil and fish meal production.

European aquaculture production

In 2017, the European aquaculture production peaked a 10-year high of about 1.4 Mt, for a total value of EUR 5 billion, increasing the production by 5% (+ 67,172 tonnes) and the share value of 15% if compared with the previous year. During the last decade, the overall value of aquaculture almost doubled, mainly due to an increase in the production of high-value species, such as European seabass (*Dicentrarchus labrax*), bluefin tuna (*Thunnus thynnus*) and Atlantic salmon (*Salmo salar*), and a sharp increase in the price of the main produced species, related to a higher demand and quality of the end product. In 2018, after the peak of the previous year, European aquaculture production showed a decrease both in the total volume (-4%) and value (-5%) (Figure 1.1). However, if compared with the previous 10 years, the current production showed a growth of 3% (+40,000 tonnes), while economic value grew by 36% (+1.30 billion of euros) (EUMOFA, 2020a).

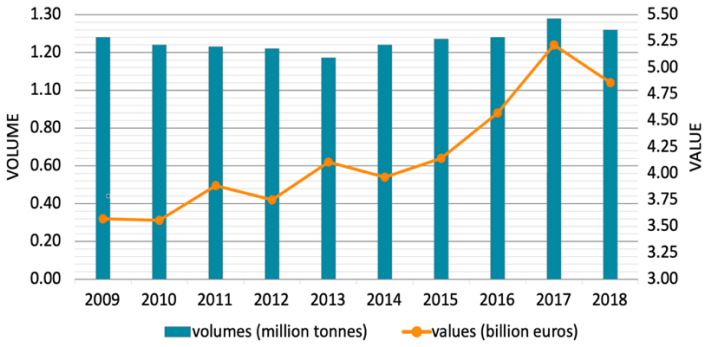

Figure 1.1. European aquaculture production during the last decade (EUMOFA, 2020a)

In 2017, bivalve and other molluscs production reached an 8-year high, due to a rise in the supply of oysters (*C. gigas*) in France and mussels (*Mytilus galloprovincialis*) in Spain from 2016 to 2017 (EUMOFA, 2019a). Mollusc production still covered about half of the total European aquaculture supply, mainly due to oyster farming in France and mussel cultivation in Spain (EUMOFA, 2020a).

Regarding the economic value, all European major species reached a 10-year peak in 2017. Salmonids showed the most important growth, increasing by 18% (EUR 321 million) from 2016 to 2017. Nonetheless, bivalve value raised by 20% and that of freshwater fish by 19%. In 2018, both mollusc and salmonid value decreased significantly if compared with 2017 (−10% and −12%, respectively), due to the reduction in the French production of mussels and British production of salmon (EUMOFA, 2020a). On the other hand, the production of other marine species, mainly European seabass in Spain, reached a five-year peak in 2018 (EUMOFA, 2020a).

The Scientific Advise Mechanism (SAM) of the European Commission indicates aquaculture sustainable development as an explicit priority of the EU and of global policies. However, despite the increasing trends in production, aquaculture development in the European Union has been practically stagnant for the last fifteen years, not exploiting its potential to create wealth and employment, as the FAO has been insistently recommending (APROMAR, 2020; FAO, 2020a).

Spanish and Italian aquaculture production

Spain and Italy are among the top five EU aquaculture-producing countries, along with UK, France and Greece (EUMOFA, 2020a). In 2018, Spanish aquaculture showed an increasing trend, whereas a decrease was recorded in Italy, which showed a drop in volume but an increase in total value over the long term (EUMOFA, 2020a). In 2018, Spanish production of aquatic organisms (aquaculture and fisheries) reached 1.28 Mt (FAO, 2020a). Aquaculture supply reached 348,891 tonnes, for a total value of EUR 452.9 million (Figure 1.2a). The main species cultured were mussel (*Mytilus* spp.) (273,600 tonnes), European seabass (22,460 tonnes), rainbow trout (*Oncorhynchus mykiss*) (18.955 tonnes) and gilthead seabream (*Sparus aurata*) (14, 930 tonnes) (APROMAR, 2020).

According to the most recent statistics collected by APROMAR (2020), Spanish aquaculture production in 2019 is estimated at 342,867 tonnes, for a total value of EUR 501 million. Among species, a slight decrease in the production is estimated for mussel (−4.4%) and gilthead seabream (−9.4%), whereas a significant increase is expected for European seabass (+21.7%) (APROMAR, 2020).

In 2018, Italian aquaculture production of aquatic organisms reached 142,727 tonnes, for a total value of EUR 439 million (Figure 1.2b) (Eurostat, 2020). Among finfish, rainbow trout is the most produced species (37,000 tonnes in 2019), covering the 60% of the total finfish aquaculture production for a total value of EUR 120 million, followed by gilthead seabream (9,700 tonnes), European seabass (7,300 tonnes) and grey mullet (*Mugil cephalus*) (2,500 tonnes) (API, 2020).

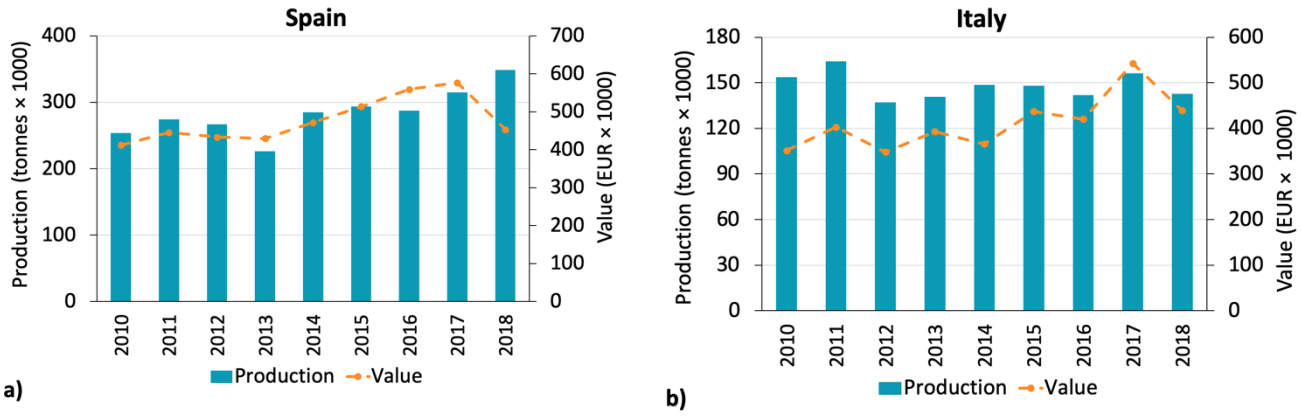

Figure 1.2. Total aquaculture production and value trends from 2010 to 2018 in Spain (a) and Italy (b) (data from Eurostat, 2020)

Major issues of sustainability in aquaculture

Aquaculture is a well-recognized food sector for its high efficiency in producing nutritious protein-rich foods (Osmundsen et al., 2020; Sprague et al., 2017). However, it is often condemned for unsustainable production practices, especially regarding the use of aquafeeds (Cottrell et al., 2020; Osmundsen et al., 2020; Ytrestøyl et al. 2015) and its negative impacts on the environment (Alexander et al., 2016; Naylor et al., 2000, 2021; Perdikaris et al., 2016; Valenti et al., 2018), which may produce costs for fish producers and for the whole society (Ahmed et al., 2019; Neiland et al., 2001).

Aquafeeds typically contain fish oil and fishmeal (hereafter named FOFM) obtained from small pelagic species such as anchovies, herrings, and sardines, and to a smaller extent from fish by-products such as discards and trimmings (FAO, 2020a). FOFM were historically used for their high palatability and for their efficient supply of essential amino acids and fatty acids for most of farmed fish species (Turchini et al., 2019). However, global supply of FOFM is reaching a plateau (Cottrell et al., 2020; Naylor et al., 2021) and to ensure a sustainable growth of the sector, aquaculture must progressively reduce its dependence on these ingredients (Cottrell et al., 2020).

Nowadays, a wide range of raw materials have been examined and adopted to decrease FOFM inclusion in aquafeeds. Among vegetable sources, soy, canola, wheat, corn, palm and sunflower have been included as oil and protein sources in aquafeeds as the price gap with marine sources progressively widened (Cottrell et al., 2020, Troell et al., 2014; Turchini et al., 2009,2019). However, these alternative sources can contain high proportions of anti-nutritional factors and fibre as well as provide low amounts of essential omega 3 polyunsaturated fatty acids, potentially impairing fish health and growth performance and increasing nutrient and feed waste (Francis et al., 2001; Kokou and Fountoulaki, 2018). Thus, considerable uncertainties remain regarding the efficacy of novel feed formulations across

different fish taxa, life stages and about their effects on social, economic and especially environmental challenges (Cottrell et al., 2020).

A wide range of environmental concerns such as resource decline, habitat destruction, water pollution and eutrophication, and impacts on climate change have been associated with the aquaculture sector (Ahmed et al., 2019; Naylor et al., 2021). Therefore, if global aquaculture production will continue to grow at a fast pace (Naylor et al., 2021), new solutions have to be implemented in order to reduce the detrimental pressure on the environment.

In the next years, the intensification of aquaculture production will generate significant resource constraints because of limited freshwater resources and land. The competition between agriculture and aquaculture farms for freshwater is rapidly increasing. Indeed, at a global level, about 201 km^3 of freshwater were utilized by fish farming, which is expected to increase its water demand to 469 km^3 by 2050 (Ahmed et al., 2019). Regarding land use, in 2010 aquaculture production covered about 19 million ha of land and it is estimated to occupy 44 million ha in 2050 (Ahmed et al., 2019) entering in competitions with other agricultural practices.

Other environmental issues associated with aquaculture production are water pollution and eutrophication (Díaz et al., 2019; Wells et al., 2015). The increase in fish stocking density generated by the intensification of farming practices produce high amounts of organic matter and wastes (i.e. uneaten feed and fish feaces) that impair water quality and contribute to eutrophication. In fact, aquaculture is expected to double the eutrophication of freshwater bodies from 0.38 million tonnes P eq. in 2010 to 0.89 million tonnes P eq. in 2050 (Ahmed et al., 2019). Noteworthy, similar trends are also expected for aquaculture-triggered marine eutrophication, which will rise from 1.4 million tonnes in 2010 to 3.2 million tonnes in 2050 (Ahmed et al., 2019). Therefore, new solutions to reduce water pollution and eutrophication are required to ensure aquaculture growth as well as reducing its impact on the environment.

The growth of fish farming has also been associated with the increase of greenhouse gasses (GHG) emissions, which will increase from 332 million tonnes CO_2 eq. in 2010 to 776 million tonnes CO_2 eq. in 2050 (Ahmed et al., 2019). Among various inputs, aquafeed are the largest contributors of GHG emissions in aquaculture, deriving mainly from the production and transport of raw materials and energy required by industry mills to produce the feed (Robb et al., 2017).

To face these challenges, the attention needs to be directed towards new feeding strategies, farming system designs and ecosystem-based management practices that will ensure a sustainable aquaculture production.

ADDRESSING SUSTAINABILITY: KEY SPECIES IN MEDITERRANEAN AQUACULTURE

Diversification of fish species and farming systems represents a promising approach to increase the resilience of the aquaculture sector to face climate changes, market fluctuations and environmental concerns, adding economic, social and ecological insurance to aquaculture systems (Harvey et al., 2017). Fish species diversification is a viable strategy to increase the profitability of the Mediterranean aquaculture, addressing the decrease of market prices of the two main marine species cultured in the Mediterranean area such as the gilthead seabream (*Sparus aurata*) and the European sea bass (*Dicentrarchus labrax*) (Chavez-Pozo et al., 2019). The farming of new fast-growing species with larger final size, high adaptability to farming and good flesh quality will guarantee the expansion of the Mediterranean aquaculture potential providing market diversification and new value-added products (Diversify, 2018).

At a farm and local community scale, diversification of farming systems can add economic, social and ecological insurance to aquaculture productions, particularly for small-scale and family-based enterprises (Harvey et al., 2017). For this purpose, common, well-known and high-value freshwater species such as rainbow trout are more suitable to be farmed using a new or repurposed system or technology (Harvey et al., 2017). In the freshwater European context, rainbow trout is one of the most profitable and farmed species, mainly reared at high stocking densities in flow-through raceways systems supplied with high quality water. However, due to their high adaptability and good market value, rainbow trout can be a promising candidate for aquaponics farming, a new technology that produces fish and plants in a recirculating system, reducing nutrient losses, water consumption and land use.

Mediterranean yellowtail

The Mediterranean yellowtail (*Seriola dumerili*, Risso 1810) (Figure 1.3), also called greater amberjack, has recently received great interest form the aquaculture sector, thanks to its rapid growth rate (Lazzari et al., 2000; Mazzola et al., 2000), higher than Atlantic salmon (Fakriadis et al., 2020a), excellent flesh quality, high consumer acceptance (Sicuro and Luzzana, 2016) and worldwide market availability (Mylonas et al., 2016). Moreover, yellowtail adults can reach a very large size (up to 180 cm in total length and 80.6 kg in body weight) (FAO, 2020b), making this species suitable for a wide variety of high-value seafood products (e.g. sushi preparation) (Asche et al., 2009; Fakriadis et al., 2020a) and fitting profitable market niches (Fakriadis et al., 2020b). Besides, this cosmopolitan species, distributed throughout the temperate zone, successfully adapt to different environmental and captive conditions (Mazzola et al., 2000; Nakada, 2002), representing one of the most promising candidates for species diversification in the aquaculture industry (Mylonas et al., 2016; Sicuro and Luzzana, 2016).

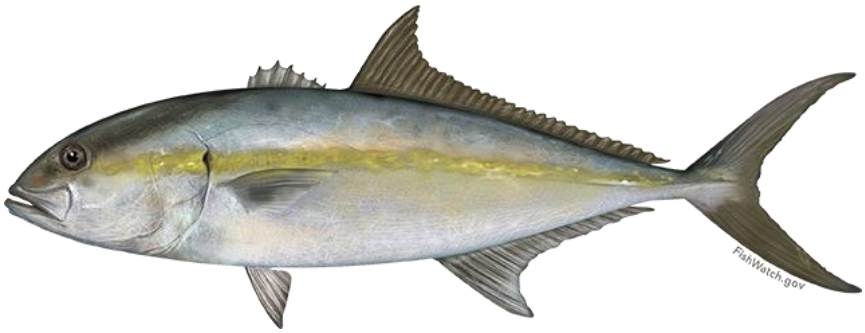

Figure 1.3. Mediterranean yellowtail (www.fishwatch.gov)

Figure 1.4 reports the total aquaculture production and value of *S. dumerili* in the last decades. The great variability (peaks and downs) of the amounts produced are likely related to the wild juveniles' availability of that specific year. In fact, one of the major bottlenecks for the development of Mediterranean yellowtail aquaculture in Europe is a scarce reliable reproduction (Mylonas et al., 2004) and a low number of juveniles produced. Thus, in most cases, juveniles are caught directly in the sea by networks, between April and May, when hatchlings fish gather on water surface. However, this practice caused a depletion of natural reserve with a high environmental impact as for other species.

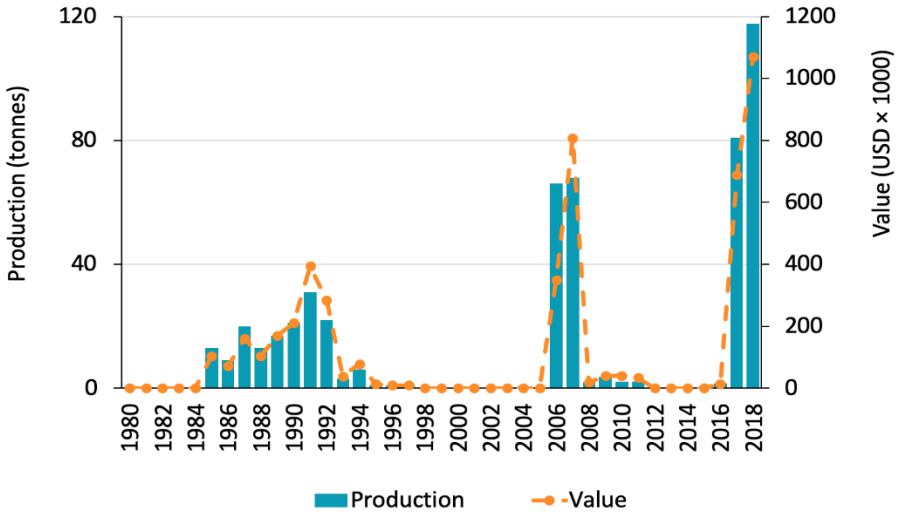

Figure 1.4. Global aquaculture production (tonnes) and total value (USD × 1000) of *S. dumerili* (Data source: FishStatJ, 2020).

Nowadays, Japan, China and South Korea are the most important productive areas for all *Seriola* spp. The Mediterranean production is still quite low but showing an increasing trend during the last years. Indeed, a production of 48 tons was recorded in Spain in 2018 (APROMAR, 2020). However, there is a quite limited commercial activity with hatchery-produced individuals in other Mediterranean countries such as Greece, Italy, and Cyprus. Other producing countries are US and Mexico and Near East (De la Gándara, 2006).

Along with the unreliable reproduction and inconstant supply of juveniles (Fakriadis et al., 2020a), another important bottleneck for *S. dumerili* aquaculture development is the lack of knowledge regarding its nutrient requirements, especially those related to essential fatty acids, which will be analysed and discussed in the following chapters of the present thesis.

Biological features

The Mediterranean yellowtail belongs to the family of Carangidae. It is an euryhaline (lower limit 16‰), carnivorous species that feeds mainly on small pelagic fish (i.e. *Sardinella aurita*, *Sardina pilchardus*, *Boops boops*) and cephalopods (*Loligo* spp. and *Sepia officinalis*) varying its diet according to the life stage and size (Mazzola et al., 2000; Sley et al., 2016).

Regarding water temperature, *S. dumerili* presents a broad range, varying from 15 to 27°C (Fernández-Montero et al., 2018), with an optimum for growth at 20-26°C (Fernández-Montero et al., 2018; Lazzari and Barbera, 1989). Under 15°C the ingestion is null (CULMAREX company, unpublished data), whereas under 9°C the fish dies (FAO, 2020b).

As regards reproduction, *S. dumerili* species presents both sexes separated (Lazzari and Barbera, 1989; Micale et al., 1999), that can be distinguished at early ages (between 4-5 months of age and 24-25.5 cm of length) (FAO, 2020b). Generally, in the Mediterranean area, sexual maturation can be detected at a 61 cm of length (2 years) in males and 80 cm (3 years) in females (Jerez et al., 2006). During the spawning season, which occurs in spring when water temperature increases (Grau, 1992; Lazzari and Barbera, 1988, 1989), sperm emission and oocyte maturation are synchronized and pelagic eggs are produced, with a diameter of about 1.2 mm (De La Gándara and García-Gómez, 2004).

Mediterranean yellowtail farming

Studies on *S. dumerili* have shown high growth rates in the wild (Thompson et al., 1999) as well as in farming conditions (Fernández-Montero et al., 2018; Hossain et al., 2017; Jerez et al., 2006; Jover et al., 1999; Mazzola et al., 2000; Navarro-Guillén et al., 2019; Pastor et al., 2000).

In the Mediterranean area, farming *Seriola dumerili* started in the 80s' with capture-based activities using wild juveniles and evaluating their adaptability in tanks (Cavaliere et al., 1989; García-Gómez, 1993; Gredo et al., 1993; Jover et al., 1999; Lazzari et al., 2000; Papandroulakis et al., 2005; Sawada et al., 2020; Skaramuca et al., 2001), floating cages (Boix et al., 1993; Giovanardi et al., 1984; Marino et al., 1995; Mazzola et al., 1996; Navarro et al., 1987; Lazzari and Barbera, 1989; Porrello et al., 1993) and submerged cages (Mazzola et al., 2000). Juveniles can reach 1 kg of weight after one year of rearing (Cavaliere et al., 1989; de la Gándara and García-Gómez, 2004; García-Gómez, 1993; Jover et al., 1999; Lazzari et al., 2000; Lazzari and Barbera, 1989; Mazzola et al., 1996) and 6 kg in a period of 2.5 years (Jover et al., 1999; Mazzola et al., 2000), showing a growth rate 10 times faster than other commonly farmed species such as European seabass (*Dicentrarchus labrax*) and

gilthead seabream (*Sparus aurata*) over the same rearing period (García-Gómez, 2000) (Figure 1.5).

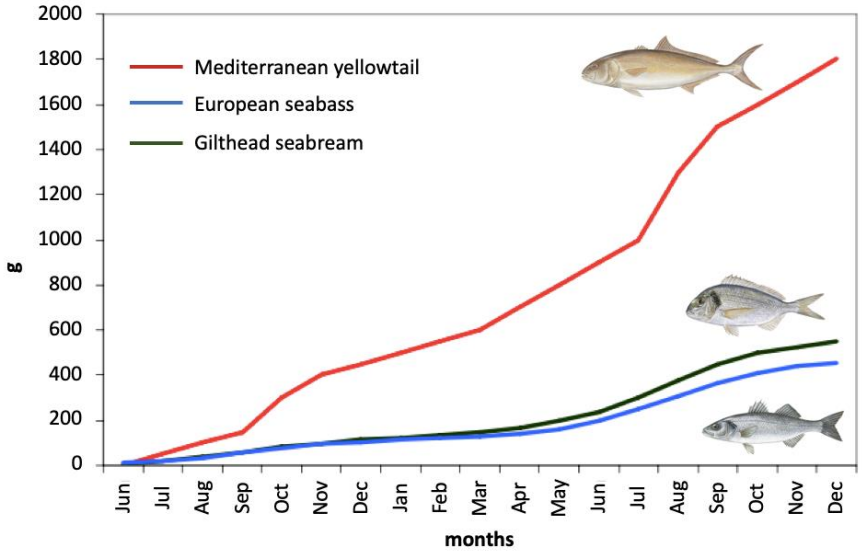

Figure 1.5. Growth curves of Mediterranean yellowtail (red line), European seabass (blue line) and gilthead seabream (green line) (adapted from De la Gándara, 2006).

More recently, thanks to an improved efficiency of the water use in ground systems and the use of marine cages, the stocking density can reach 40-60 fish m^{-3} and 80-100 fish m^{-3} when they weigh 20-50 g and 100 g, respectively. After a growth period of 24-36 months, yellowtails reach the commercial size with a final weight of 3-5 kg (De la Gándara, 2006).

Feeding and nutrition

First successful results on Mediterranean yellowtails farming were obtained with either a pellet-based or a raw-fish diet, showing high survival rates and adaptation to captivity (García-Gómez and Díaz, 1995; Lazzari and Barbera, 1989; Porrello et al., 1993). Then, since the 90's, moist, semi-moist and dry diets have been developed and designed. However, very few information is available about *S. dumerili* essential fatty acid (EFA) requirements (Monge-Ortiz et al., 2018). Being a carnivorous fish, the Mediterranean yellowtail has high protein and energy requirements (Navarro-Guillén et al., 2019). Besides, *S. dumerili* juveniles seem to require a higher protein content (47-55% crude protein) than other marine species (Jover et al., 1999; Takakuwa et al., 2006), and about 18% of crude lipid (see Sicuro and Luzzana, 2016 for a review).

Regarding EFA, carnivorous marine fish such as Mediterranean yellowtail are unable to bio-convert the precursors of polyunsaturated fatty acids (PUFA), like linoleic acid (C18:2 n-6) and α-linolenic acid (C18:3 n-3), into essential n-3 long-chain polyunsaturated fatty acid (n-3 LC-PUFA) such as arachidonic acid (C20:4 n-6), eicosapentaenoic acid (C20:5 n-3) and docosahexaenoic acid (C22:6 n-3). Thus, these essential fatty acids have to be provided

through the diet in order to assure fish growth, health and reproduction (Sargent et al., 1997, 1999; Tocher and Harvie, 1988).

Currently, the information available on FA requirements of Mediterranean yellowtail is scarce. Only one study on juveniles indicated a n-3 LC-PUFA requirement of 12 g/kg dry weight (Monge-Ortiz et al., 2018), whereas more knowledge is available on n-3 LC-PUFA requirement for *S. dumerili* larvae (12-17% of total fatty acids) (Roo et al., 2019). The research works presented in the following chapters aimed at increasing the knowledge regarding the effect of different dietary fatty acid (FA) profiles on *S. dumerili* growth and different tissue FA composition.

Rainbow trout

Rainbow trout (*Oncorhynchus mykiss*) (Figure 1.6) is one of the most widely distributed and studied fish species, thanks to its fast growth (Molony, 2001), high environmental adaptability and tolerance to handling, ease to spawn, and a good flesh quality and value (Ortega and Valladares, 2015; Parisi et al., 2014). This species is native from the Pacific coast of North America. However, it has been gradually introduced all over the world, living also in oceans and seas (EUMOFA, 2020b). The freshwater strain can reach 4.5 kg in 3 years, while the anadromous one, called steelhead, can achieve 7-10 kg during the same period (Parisi et al., 2014). Growth rate of wild *O. mykiss* is highly variable and depends mainly on food abundance and water temperature (Molony, 2001). In farming conditions, the maximum weights reached by *O. mykiss* and reported by previous studies varied between 3.1 kg and 4.8 kg (Aussanasuwannakul et al., 2012; Bugeon et al., 2010; Davidson et al., 2014). In recirculating aquaculture systems (RAS), rainbow trout can achieve 1.3-1.4 kg of weight in one year (Davidson et al., 2009, 2011).

Figure 1.6. Rainbow trout (www.delawarepublic.org)

Rainbow trout aquaculture production showed an exponential growth since 50's, especially in European countries and recently in Chile and Iran (EUMOFA, 2020b). From 2008 to 2017, the world aquaculture production of *O. mykiss* increased by 19%, amounting to a global production of 848,051 tonnes and a total value of 3.9 million USD (FishStatJ, 2020) in 2018 (Figure 1.7). The world-leading producers in 2018 were Iran (173,384 tonnes),

Turkey (103,192 tonnes), Chile (78,252 tonnes) and Norway (68,216 tonnes) (FishStatJ, 2020).

In 2018, Europe provided about the 24% (187,858 tonnes) of the global supply of farmed trout, with a decrease of 9% during the last decade mainly due to the reduction of the French (−24%) and Spanish (−21%) production (EUMOFA, 2020b).

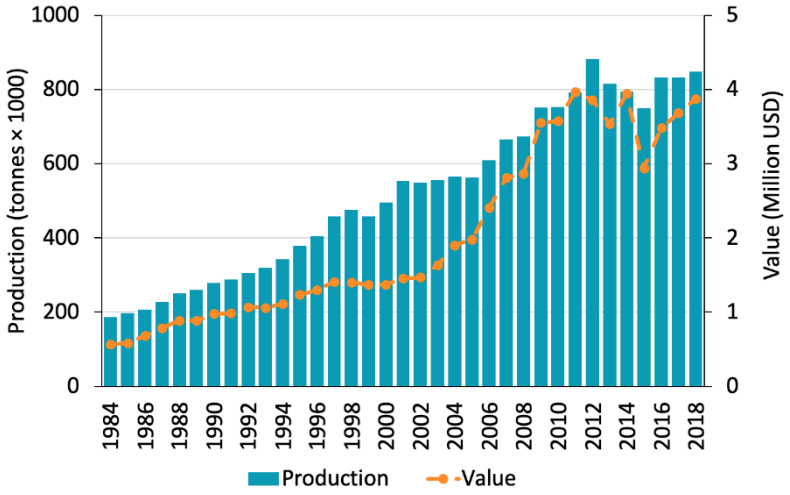

Figure 1.7. Global aquaculture production (tonnes × 1000) and total value (Million USD) of rainbow trout (FishStatJ, 2020).

Biological features

Rainbow trout is a teleost fish belonging to the family of Salmonidae. It is an euryhaline (0-35‰) (Molony, 2001), carnivorous fish, usually feeding on a wide range of zooplankton species, aquatic insects, small fish and crustaceans (Woynarovich et al., 2011). *O. mykiss* shows a wide temperature range (1-27°C), varying according to the life stage. Generally, the optimum temperature for feeding activity and growth is between 7°C and 18°C (Molony, 2001; Woynarovich et al., 2011), while the spawning range is 9-14°C (Molony, 2001).

Regarding reproduction, wild rainbow trout reaches the maturity between three and four years, spawning in spring (January-May) (Liley et al., 1986). However, farmed strains mature earlier and spawn all-year round (Bromage et al., 1992; Gjedrem, 1992). Female egg production can reach 2000 eggs of 3-7 mm per kg of body weight (Estay et al., 1994). In aquaculture systems, trout juveniles are obtained by artificial reproduction, as adults do not spawn naturally.

Rainbow trout farming

Rainbow trout are generally farmed in intensive monoculture systems. Fish are typically reared at stocking densities varying from 15 to 40 kg m^{-3} with a maximum reached of 60 kg m^{-3} (Ellis et al., 2002). However, high densities (> 40 kg m^{-3}) are detrimental for

trout growth and welfare (Ellis et al., 2002). The main farming systems are cages (Azevedo et al., 2011; Ozório et al., 2016; Kaya and Pulatsü, 2017), tanks (Anras et al., 2004; Kientz et al., 2018; Papoutsoglou et al., 2005) and flow-through raceways (Buzby et al., 2016; Meyer and Cassinelli, 2020; Welker et al., 2019), supplied by high-quality water (dissolved oxygen > 5 mg L^{-1}, temperature 12-21°C, pH 6.5-8.5) (Molony, 2001; Woynarovich et al., 2011).

The growing phase starts when fry reach a length of 8-10 cm (250 specimens kg^{-1}) and they are stocked at 25-50 fry m^{-2}. Usually, fish are reared for 9-12 months until the marketable size (30-40 cm, 400-600 g), although some products (e.g. smoked fillets) require fish of a larger size reared over 20 months. During the production cycle, the stock is generally graded four times (at 2-5 g, 10-20 g, 50-60 g and < 100 g). Once reached the table size, trout are supplied to the markets either frozen or fresh, having a shelf-life of about 8-11 days when stored on ice (Giménez et al., 2002; Ninan et al., 2011), and sold as gutted whole fish, fillets, and smoked products.

One of the main issues of rainbow trout farming, especially in the Mediterranean context, is the environmental impact of flow-through systems. In fact, uneaten feed, fish faeces, chemicals and antibiotics can affect water quality and sediment of rivers downstream of the farm, increasing environmental pollution (Bergero et al., 2001). Recently, regulations obliged trout farms to install settling areas in order to remove solid wastes from water before being poured into rivers and streams (Parisi et al., 2014). However, highly nutrient-rich waters deriving from rainbow trout tanks might be utilized to fertilize plants in a closed recirculating cycle, reducing nutrient losses and environmental pollution and, on the other hand, allowing the production of both fish and vegetables. Such farming technique is called aquaponics. To date, from the best to our knowledge no studies are available on the adaptability and production of rainbow trout in aquaponics systems and further research is needed.

Feeding and nutrition

As for other animal and fish species, feed cost is the main voice cost in trout farming, varying between 40-70% of the total (Lasner et al., 2017). Thus, feed preparation, nutritional value and feeding strategy are key factors affecting the profitability of a rainbow trout farm (Kamalam et al., 2019). In 2012, about 1.1 million tonnes of feed were utilized in trout farming (Kamalam et al., 2019), with a feed efficiency ranging from 0.8 to 1.1 depending on farming system and country (Lasner et al., 2017; Tacon and Metian, 2015).

In the 90's, farmed rainbow trout were fed with moist feeds mainly composed by protein-rich animal by-products. Then, semi-moist and later dry feeds were developed, supplying all the essential macronutrients, fatty acids, amino acids and vitamins (Hardy, 2002). Nowadays, nutrient-dense extruded feeds are formulated in order to satisfy rainbow trout requirements (digestible energy: 17.6 kJ/g; protein; 40-50%; and lipids: 16-24%) (Hardy, 2002; NRC, 2011) varying according to fish size, farming environment and systems, and market preferences (Kamalam et al., 2019).

One of the major issues in aquafeed formulation is the reduced reliance on marine fish by-products such as fishmeal and fish oil (Turchini et al., 2009), which availability is gradually reducing and their price increasing (Ido et al., 2020). Thus, during the last two decades, alternative ingredients have been studied and implemented in aquafeeds to replace fishmeal and fish oil, without impairing fish growth, health and welfare. In trout farming, the modern feeds are composed by a blend of plant protein concentrates, fishmeal, cereal by-products, oilseed meals, fish oil, vegetable oils, animal by-products and vitamin-mineral mixes (Hardy, 2010). Recently, novel protein compounds such as insect meals are being studied as alternatives to fishmeal in rainbow trout feeds (Henry et al., 2015; Mancini et al., 2018). Among insects, the black soldier fly (*Hermetia illucens*) seems to be one of the most promising candidates as fishmeal alternative in trout feeds (Renna et al., 2017). Nevertheless, further investigations on the correct fishmeal substitution rates and their effects on trout performance, quality and health are necessary.

ADDRESSING SUSTAINABILITY: FEEDING STRATEGIES

Fish oil and fishmeal are essential ingredients in aquafeeds (Ido et al., 2020), representing essential sources of lipids, especially n-3 PUFA, and proteins (Olsen and Hasan, 2012; Turchini et al., 2009). The production of FO and FM mainly relies on wild-caught pelagic fish like mackerel, herring, sardine and anchovy. According to the most recent scientific reports available (Tacon and Metian, 2015), about 30 million tonnes of wild fish was captured in 2012 to produce FM and FO, of which 68% and 74%, respectively, were utilized to produce fish feeds. Increasing concerns on marine ecosystem preservation were raised regarding the impact of pelagic fish fishing, with consequent limitations in the supply wild-caught fish to produce FO and FM, which declined to 16 million tonnes in 2017 (Naylor et al., 2021).

The increasing demand for aquaculture products triggered a rise in the production of aquafeeds. Thus, the growing need for FO and FM from aquafeed manufactures, coupled with their limited availability, led to a steady increase of FO and FM prices, prompting research and industry to investigate new alternative sources. To date, several alternative lipid and protein sources have been studied. Among them, plants (Gatlin et al., 2007; Shepherd and Bachis, 2014; Tacon et al., 2006; Turchini et al., 2009), insects (Gasco et al., 2019; Henry et al., 2015), microalgae (Sprague et al., 2017), and terrestrial animal by-products (Kureshy et al., 2000; Tacon et al., 2006; Zhou et al., 2011) represent the most promising ones.

The present chapter provides a brief overview of fish oil and fishmeal production and trends and discusses the main effects of the dietary replacement of fish oil with vegetable oils and fishmeal with insect meals, focusing on feed digestibility, fish growth performance, and product quality.

Aquafeed sustainability: fish oil and fishmeal production and trends

A significant amount of world fish catches is processed into fish oil and fishmeal (FOFM). The FOFM can be obtained from whole fish, fish trimmings, or fish by-products. Fishmeal is a protein-rich, flour-type product obtained by milling and drying whole fish or fish parts. On the other hand, fish oil production requires the pressing of pre-cooked fish, followed by the centrifugation of the obtained liquid (FAO, 2020a). To produce FOFM, different small pelagic species such as mackerel (*Scomber* spp.), herring (*Clupea* spp.), sardine (*Sardina pilchardus*) and anchovy (*Engraulis* spp.) are used, mainly as whole fish. Among them, Peruvian anchoveta (*Engraulis ringens)* is utilized in larger volumes.

FOFM supply fluctuates depending on the catch volumes of those species, anchoveta in particular. Over time, the adoption of good management practices and certification schemes has decreased the volumes of unsustainable catches of species suitable for FOFM processing. The total catches used for FOFM production showed a peak in 1994 of over than 30 million tonnes, then steadily decreased to less than 14 million tonnes in 2014.

During the last decade, on average, the amount of fish oil and fishmeal annually produced was 1 million tonnes and 5 million tonnes, respectively (EUMOFA, 2019b). In

2018, the global production of FM was estimated at 5.6 Mt, reaching the highest supply since 2011, mainly due to increased catches of Peruvian anchoveta. However, due to an industrial reduction in the use of FM from whole pelagic species in favour of fish by-products, FM supply is expected to decrease in 2019 (EUMOFA, 2019b) (Figure 1.8).

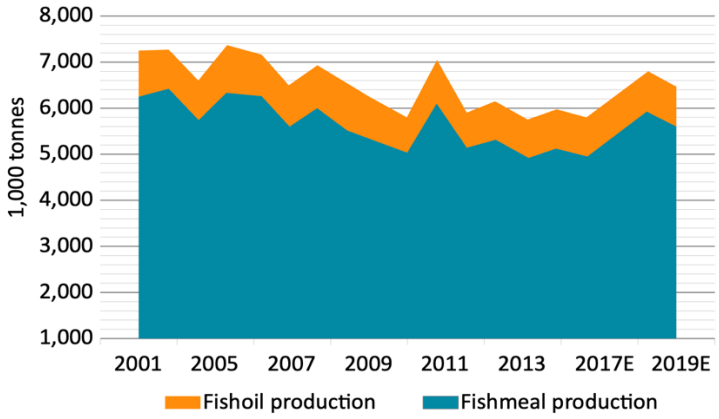

Figure 1.8. Global fishmeal and fish oil production from 2001 to 2019 (Adapted from: EUMOFA, 2019b). Note: the productions from 2017 to 2019 are estimated (E).

The main consumer of FOFM is the aquaculture sector, using about 70% of the global supply in 2017 (EUMOFA, 2019b). Fishmeal is also used to formulate pig feed (22% of the global supply) and poultry feed (5%) (Figure 1.9).

Regarding aquafeeds, in 2016, about 31% of FM was destined to produce feed for crustaceans, 23% for salmonids, and 15% for marine fish (EUMOFA, 2019b). On the other hand, out of the total amount of FO used in fish feeds, 60% was destined to salmonids, 18% to marine species and 6% to crustaceans (EUMOFA, 2019b). Fish oil contains high amount of healthy n-3 fatty acids, especially eicosapentaenoic (EPA) and docosahexaenoic (DHA) acid. Thus, these healthy compounds are highly valued also as food supplements in human nutrition, which transformed about 23% of the global fish oil supply in 2017 (EUMOFA, 2019b).

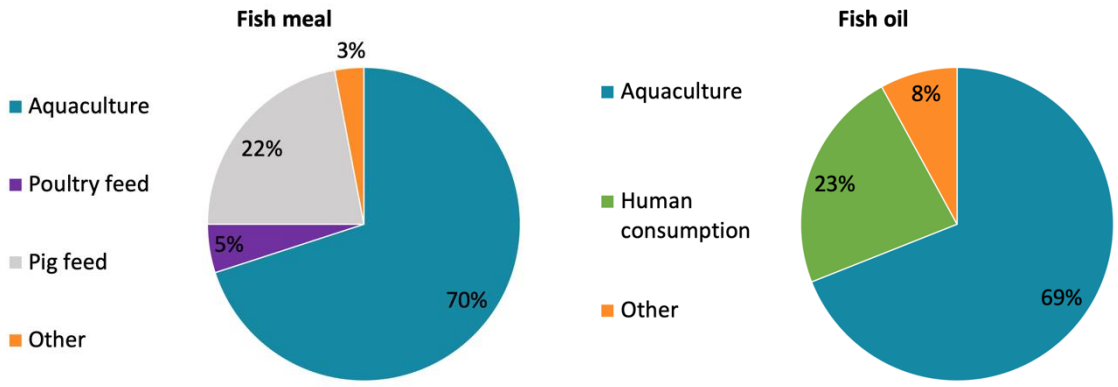

Figure 1.9. Global fishmeal and fish oil usage (volume in tonnes) per destination in 2017 (Adapted from: EUMOFA, 2019b)

Despite FOFM are still considered the most digestible and nutritious ingredients for farmed fish (Olsen and Hasan, 2012; Tacon and Metian, 2015; Turchini et al., 2009), with FO being an essential source of EPA and DHA, their inclusion rates in aquafeeds have shown a downward trend, mainly as a result of price and supply variations, coupled with a steady increasing demand from fish feed industries (FAO, 2020a).

FM prices have generally been declining since mid-2018 (Figure 1.10). However, a premature closure of the second Peruvian anchoveta fishing season in late 2019, coupled with a drop in raw material supply, likely prompted a reversal of trend. Similarly, prices of FO have been increasing since mid-2018 and are expected to increase further (Figure 1.10) (EUMOFA, 2019b).

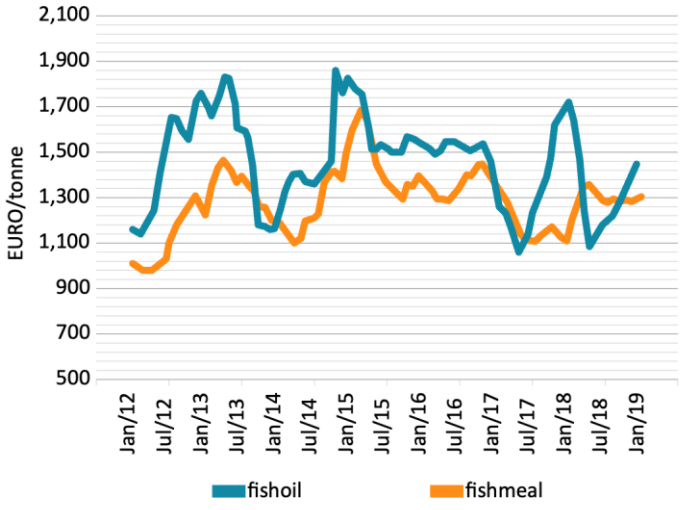

Figure 1.10. Fishmeal and fish oil prices (EUR/tonne) in the European Union from 2012 to 2019 (EUMOFA, 2019b).

Because of the wide variability of FOFM production and related price variations, researchers and industries are investigating alternative sources to be included in fish diets.

Moreover, FOFM are being more selectively included at specific stages of production, such as broodstock, hatchery and finishing or re-feeding diets, whereas FOFM incorporation in grow-out diets is decreasing.

Fish oil replacement: how to satisfy fatty acid requirements and guarantee fish growth and flesh quality?

In recent years, lipids have gained a key role in finfish nutrition due to the implementation and production of energy-dense and high-lipid diets, which improve fish growth, nutrient retention and feed conversion, consequently reducing the length of the grow-out period (Sargent et al., 2002; Turchini et al., 2009). In fish farming, dietary lipids are a key source of essential fatty acids (EFA) necessary for growth, bodily functions, health and reproduction.

Traditionally, marine fish oils have been utilized as unique dietary lipid source in aquafeeds, thanks to their abundance of EFA, ready availability and competitive price. However, the rise of FO prices coupled with the reduction of its supply prompted research and industry to find alternative lipid sources to be included as total or partial FO replacer in aquafeeds. Vegetable oils have been identified as the most promising source, given their high availability and cheap price. However, EFA requirements and metabolism differ among fish species and the effects of different replacement levels of FO with VO on fish growth and product quality need to be carefully evaluated. In the present chapter, the main research results on the effects of dietary replacement of FO with VO are discussed, focusing on fish growth performance and product quality.

Fish fatty acid requirements

Almost all fish have the ability to bioconvert two basic C18 n-3 and C18 n-6 PUFA into the related C20 and C22 n-3 and n-6 PUFA, through alternated steps of elongation and desaturation (Nakamura and Nara, 2004; Turchini et al., 2009). However, several fish species have lost this ability, mainly because of an adaptation to an environment rich in highly unsaturated fatty acids (HUFA), such as the marine one. Therefore, these species present HUFA requirement for C20:4 n-6 (arachidonic acid, ARA), EPA and DHA, respectively. Thus, considering the metabolic transformation from ALA to EPA and DHA, fish species can be divided into two main groups. The first one includes species showing a typical ‘freshwater’ pattern (i.e. omnivorous, carnivorous and herbivorous freshwater species able to bioconvert C18 PUFA into more unsaturated and longer homologues), while the second one comprises fish with a typical ‘marine’ pattern, including most of marine carnivorous species that have lost the ability to bioconvert C18 PUFA and thus require HUFA as essential fatty acids (Mourente et al., 2005; Sargent et al., 2002; Tocher et al., 2006; Turchini et al., 2009).

Fish EFA requirements highly vary among species (i.e. according to their environment and trophic level) and within species (i.e depending on their physiological and developmental state). Generally, cold freshwater and marine species have an essential requirement for n-3

FA, whereas freshwater species living in warm waters require both n-6 and n-3 FA. The requirements for n-6 FA can be easily satisfied by including in the diet LA, which is abundant in about all ingredients of terrestrial and/or marine origins (Turchini et al., 2009). On the other hand, n-3 FA requirements can be met only in a few cases with the unique provision of ALA, whereas in most cases n-3 HUFA have to be included in the diet. Hence, n-3 FA represent the most limiting essential fatty acids (Turchini et al., 2009). The range of fish EFA requirements is relatively wide (Table 1.2), varying from 5.5% dry diet of n-3 HUFA for larvae of some marine carnivorous species to less than 0.5% dry diet of C18 PUFA in juvenile and sub-adult freshwater omnivorous and carnivorous species (Sargent et al., 2002; Turchini et al., 2009).

Table 1.2. Essential fatty acid (EFA) requirements of common fish species (adapted from Turchini et al., 2009).

Common name	Scientific name	EFA requirements (% dry diet)			
		18:2n-6	18:3n-3	n-3 HUFA	n-6HUFA
Arctic charr	*Salvelinus alpinus*		1.0-2.0		
Atlantic salmon	*Salmo salar*		0.5-1.0	0.5-1.0	
Barramundi	*Lates calcifer*			1.0	
Channel catfish	*Ictalurus punctatus*	0.5	1.0-2.0	0.5-0.75	
Chum salmon	*Oncorhynchus keta*	1.0	1.0		
Coho salmon	*Oncorhynchus kisutch*	1.0	1.0		
Common carp	*Cyprinus carpio*	1.0	0.5-1.0		
European sea bass	*Dicentrarchus labrax*			1.0	
Gilthead seabream	*Sparus aurata*			0.5-1.9	
Grass carp	*Ctenopharyngodon idella*	1.0	0.5		
Japanese eel	*Anguilla japonica*	0.5	0.5		
Korean rockfish	*Sebastes schlegeli*			1.0	
Milkfish	*Chanos chanos*	1.0	0.5	1.0	
Nile Tilapia	*Oreochromis niloticus*	0.5			
Rainbow trout	*Oncorhynchus mykiss*	0.8-1.6	0.7-1.0	0.2-1.0	
Red drum	*Sciaenops ocellatus*			0.5-1.0	
Red sea bream	*Pagrus major*			0.5-1.0	
Striped jack	*Pseudocaranx dentex*			1.7	
Tilapia	*Tilapia zilli*	1.0			
Turbot	*Psetta maxima*			0.6-1.3	0.3
Whitefish	*Coregonus lavaretus*			0.5-1.0	
Yellowtail flounder	*Pleuronectes ferrugineus*			2.5	

n-3 HUFA: highly unsaturated fatty acids characterized by 20 or more atoms of carbon and three or more double bonds and the first double bond at the third carbon atom; n-6 HUFA, highly unsaturated fatty acids with 20 or more atoms of carbon and three or more double bonds and the first double bond at the 6th carbon atom.

Vegetable oils

Differently from FO production, which basically remained stagnant over the last three decades, vegetable oil production has remarkably increased, reaching 203 million tons in

2020 (USDA, 2021) (Figure 1.11a). Crude palm oil and soybean oil are the VO mainly produced in the world, amounting to 72 Mt and 57 Mt in 2020, respectively (Figure 1.11b).

Historically, the prices of the major VO (i.e palm oil, soybean oil and rapeseed oil) have been lower (−46%, on average) than FO (Turchini et al., 2009). Moreover, the price differential between VO and FO is expected to widen in the next years, with FO facing a sustained demand from aquafeed producers coupled with a decrease FO supply. Thus, VO became a highly attractive ingredient for aquafeed industries seeking for reliable and cheaper alternatives to FO.

Studies showed that VO can be properly catabolised by fish and used as energy source (Bell et al., 2001; Ng et al., 2007; Regost et al., 2003; Stubhaug et al., 2007; Turchini et al., 2009).

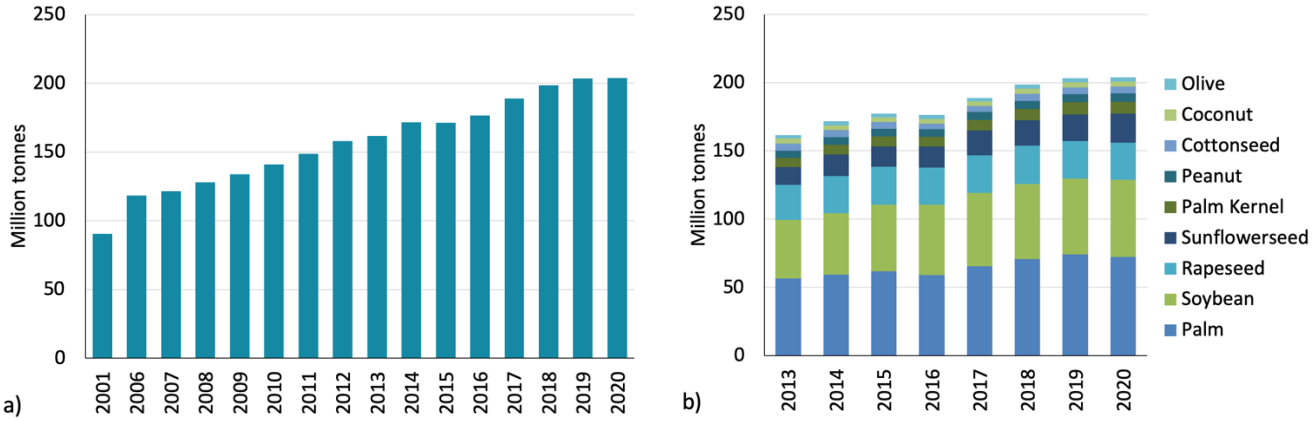

Figure 1.11. Global vegetable oils production by total amount (a) and by plant type (b) (Source USDA, 2021)

Therefore, a significant part of FO included in aquafeeds might be replaced by the more available, sustainable and cost-effective vegetable oil. However, the chemical profile of VO, especially considering its FA composition, limits the unique use of VO as lipid source in aquafeeds (Turchini et al., 2009). Most of vegetable oils lack of n-3 fatty acid if compared to marine fish oil. VO are rich in n-9 and n-6 FA, mainly oleic acid (OA; C18:1 n-9) and linoleic acid (LA; C18:2 n-6), besides linseed oil shows high amounts of alfa-linolenic acid (ALA; C18:3 n-3).

Several studies evaluated the substitution of FO with VO with either a single oil or a blend of VO formulated to reproduce the FA composition of FO concerning total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acid (PUFA) ratios (Francis et al., 2007; Turchini et al., 2009). However, both single and blended VO sources still lack the essential HUFA required by fish, especially marine ones, that are abundant in FO.

The effect of dietary replacement of FO with VO on marine fish growth performance and product quality will be described in the present chapter. Generally, studies showed that VO can substitute considerable amount of FO in the diets of several fish species, as long as appropriate amounts of specific EFA are provided in the diet.

Effect of fish oil replacement with vegetable oils on growth of carnivorous marine fish

Several studies have been performed in order to evaluate the effect of partial and total replacement of fish oil with vegetable oil on growth performance of marine fish. Table 1.3 reports the main results obtained in common temperate and warm water marine carnivorous species.

The European seabass shows a typical "marine" pattern regarding the conversion of ALA into EPA and DHA. Thus, it requires high level of n-3 HUFA in the diet (Turchini et al., 2009). The replacement of 60% FO with two different blends of VO in the diets of European seabass showed no effects on growth performance over a 15-month trial (Richard et al., 2006). Similar results on growth performance were also obtained in European seabass fed either a VO-based diet with soybean oil, linseed oil, rapeseed oil or a mixture of these for 89 days (Izquierdo et al., 2003). Likewise, the partial and complete substitution of FO with soybean oil did not impair seabass growth performance during 84 days of trial (Figueiredo-Silva et al., 2005; Martins et al., 2006). On the other hand, reduced growth performance was observed in European seabass fed a VO blend-based diet (60% FO substitution) compared with a FO-based one for 224 days (Mourente and Bell, 2006) and 168 days (Montero et al., 2008). Finally, the use of VO as unique lipid source led to a significantly reduction in growth performance of both European seabass and gilthead seabream (Alexis, 1997).

In gilthead seabream, no significant effects on growth performance were observed in fish fed diets with 60% substitution of FO with soybean and linseed oil mixtures for 7 months (Menoyo et al., 2004), with soybean oil, rapeseed oil, linseed oil or a mixture of these for 89 days (Izquierdo et al., 2003) and 7 months (Izquierdo et al., 2005), or fed a blend of sunflower oil, cottonseed oil, and either linseed or rapeseed oil for 140 days (Wassef et al., 2007). Similar results were also observer at 72% fish oil substitution with soybean oil in seabream fingerlings fed for 10 months (Martínez-Llorens et al., 2007). Contrarily, a significant reduction in gilthead seabream growth performance was observed with a total dietary replacement of fish oil with either soybean, linseed oil or a blended VO (50% soybean, 50% linseed) during a 168-days trial (Montero et al., 2008).

In sharpsnout seabream (*Diplodus puntazzo*), the replacement of FO with either soybean oil or linseed oil showed no detrimental effects on growth performance (Piedacausa et al., 2007; Nogales-Mérida, 2017). However, fish fed linseed-based diets showed a lower survival than other groups (Piedecausa et al., 2007). On the other hand, no effects on fish survival were observed in sharpsnout seabream fed soybean oil-based and linseed oil-based diet for 8 months if compared with a FO-based diets (Almaida-Pagán et al., 2007).

In red sea bream (*Pagrus major*, Temminck and Schlegel, 1843), the substitution of FO with rapeseed oil at 25%, 48% and 70% did not impair fish growth, feed efficiency and survival (Huang et al., 2007). However, in Australian snapper (*Pagrus auratus*, Forster 1801), the total substitution of FO with rapeseed oil significantly decreased growth performance and increased feed conversion ratio (Glencross et al., 2003).

Japanese yellowtail juvenile (*Seriola quinqueradiata*) fed diets with FO replaced with canola oil at 25%, 50% and 100% for 10 weeks showed reduced growth performance when fed 100%-canola oil diet if compared with other groups (Fukada et al., 2017). However, the total replacement of FO (pollack oil) with a mixture of palm and canola oil did not affect yellowtail growth performance over an 8-week feeding trial (Fukada et al., 2020). Similarly, no effects on yellowtail growth performance were observed when FO was substituted by olive oil at 25%, 50% and 100% during a feeding trial of 40 days (Seno-O et al., 2008). In yellowtail kingfish (*Seriola lalandi*) the total substitution of FO with canola oil significantly reduced fish growth performance during a 5-week feeding trial, whereas a partial substitution (50%) did not impair fish growth if compared with a control diet (100% FO) (Bowyer et al., 2012)

Regarding Mediterranean yellowtail (*Seriola dumerili*, Risso 1810), the complete substitution of FO with a blend of palm oil and linseed oil did not affect growth performance of fish fed experimental diets for 154 days (Monge-Ortiz et al., 2018).

Table 1.3. Effect of FO substitution with VO on growth performance of marine carnivorous fish.

Fish species	VO type	VO Composition	% FO substitution	Days of feeding	Effect on growth performance	Reference
European seabass	Blend	2 Mixtures[1]	60%	448	No significant effects on growth	Richard et al., 2006
	Blend	10% soybean, 60% linseed, 30% rapeseed	60%	89	No significant effects on growth and feed efficiency	Izquierdo et al., 2003
	Single	Soybean	25% and 50%	84	No significant effects on growth	Figueiredo-Silva et al., 2005
	Single	Soybean	25 and 50%	84	No significant effects on growth	Martins et al., 2006
	Blend	2 Mixtures[2]	60%	224	Significant reduction in growth of fish fed 60%-VO diets	Mourente and Bell, 2006
Gilthead seabream	Blend	10% soybean, 60% linseed, 30% rapeseed	60%	89	No significant effects on growth and feed efficiency	Izquierdo et al., 2003
	Blend	50% soybean, 50% linseed	100%	168	Significant reduction in growth of VO-fed fish	Montero et al., 2008
	Single	Either soybean or linseed	100%	168	VO-fed fish showed reduced growth. Worst performance with soybean oil	Montero et al., 2008
Sharpsnout seabream	Single	Either soybean or linseed	100%	92	No significant effect of FO replacement. Higher mortality in LO-fed fish	Piedecausa et al., 2007
	Single	Either soybean or linseed	100%	252	No significant effect of FO replacement on growth and survival	Almaida-Pagán et al., 2007
Yellowtail	Blend	Canola and palm at different percentages	100%	56	No significant effect of FO replacement	Fukada et al., 2020
	Single	Olive oil	25%, 50% and 100%	40	No significant effect of FO replacement	Seno-O et al., 2008
Mediterranean Yellowtail	Blend	80% palm, 20% linseed	50% and 100%	154	No significant effect on growth	Monge-Ortiz et al., 2018

[1]Mix A: 58% linseed, 25% palm, 17% rapeseed; Mix B: 40% linseed, 20% palm, 40% rapeseed. [2]Mix A: 40% rapeseed, 40% linseed, 20% palm; Mix B: 17% rapeseed, 58% linseed, 25% palm.

Effect of fish oil replacement with vegetable oils on tissue fatty acid composition of carnivorous marine fish

The main drawback behind FO substitution with other lipid sources is the inevitable modification of the fatty acid composition of fish flesh, resulting in a loss of the specific healthy characteristics of seafood and fish (Turchini et al., 2009). Health-promoting features related to fish consumption are associated with the intake of long-chain n-3 HUFA, especially EPA and DHA, coupled with a balanced n-6/n-3 ratio. Hence, as seafood and fish are considered to be the main suppliers of EPA and DHA in human diet, these healthy characteristics need to be preserved (Turchini et al., 2009).

As previously described, the substitution of fish oil (rich in EPA and DHA) with vegetable oils is globally increasing. However, contrarily to FO, vegetable oil lacks in n-3 HUFA and shows a high n-6/n-3 ratio. Several studies reported that the partial or total replacement of FO with VO (either a single source or a blend) affected the fatty acid composition of fish edible tissue, from herbivorous to carnivorous and from marine to freshwater species (see Turchini et al., 2009 for a review).

The main results obtained in marine fish are summarized in Table 1.4. As a general trend, the replacement of FO with VO, with the consequent increase of dietary OA, LA and ALA and a reduction in "marine" n-3 EPA and DHA, led to a reflection of the dietary vegetable lipid source in fish whole profile, flesh and organs. Moreover, the differences between a fishmeal-based diet and a vegetable oil-based diet in the proportion of either single FA or FA classes (e.i. saturated, monounsaturated or polyunsaturated FA) are wider than that between the fillets or whole body of fish fed the same diets. For instance, in Atlantic cod (*Gadus morhua*) fed a FO-based diet or a peanut oil-based one, the differences between the two diets for total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), n-6 polyunsaturated fatty acids (PUFA) and n-3 PUFA were −11%, 15%, +791%, and −84%, whereas the differences between the flesh of FO-fed and VO-fed fish were −5%, +9%, +367%, and −24%, respectively (Table 4.3). Similar results can be observed in all the examples provided in Table 4.3 and suggest that, despite dietary changes affected fish FA profile, fish are able to control the magnitude of FA modifications, thriving to maintain a rather "standard" fish fatty acid signature. (Turchini et al., 2009).

Generally, when FO is substituted by VO, the major modifications in FA profile of fish tissue concern an increase of C_{18} PUFA (mainly linoleic acid) coupled with a reduction in the n-3 HUFA proportion and a shift in MUFA composition from C_{20} and C_{22} MUFA to C_{18} MUFA. Thus, the content of linoleic acid in a given alternative lipid source has to be carefully considered, as this FA provides the main negative modifications in the FA composition of fillets from farmed fish (Turchini et al., 2009). On the other hand, studies showed that diets rich in SFA and MUFA might increase the HUFA utilization and/or the efficiency of HUFA transfer from diet to fish tissue (Bowzer et al., 2016; Monge-Ortiz et al., 2018; Pérez et al., 2014; Turchini et al., 2009).

Table 1.4. Fatty acid composition (main FA) of diets and fish (whole body or fillet) of different marine fish species fed a control FO-based diet (FO) or a VO-based diet (VO) (adapted from Turchini et al., 2009).

	OA	LA	ALA	ARA	EPA	DHA	SFA	MUFA	n-6PUFA	n-3PUFA
Atlantic cod (Lie et al., 1986)										
Diet FO	16.0	1.7	1.0	0.6	10.2	16.3	18	48	2.3	31.1
Diet VO (peanut oil, 100%)	52.5	20.5	0.0	0.0	1.0	2.8	20.0	55.0	20.5	5.1
Fillet FO	12.3	1.8	0.5	1.2	14.8	35.8	21.0	22.0	3.0	53.5
Fillet VO (100%)	21.4	13.2	0.6	0.8	10.3	28.4	20.0	24.0	14.0	40.6
Diet difference[1], %	228	1106	−100	−100	−90	−83	11	15	791	−84
Fish difference[2], %	74	663	20	−33	−30	−21	−5	9	367	−24
European seabass (Montero et al., 2005)										
Diet FO	11.3	3.9	0.9	0.9	13.5	11.7	31.7	28.1	6.3	31.9
Diet VO (soybean oil, 60%)	16.7	30.5	4.7	0.4	5.9	5.6	20.1	28.8	15.6	34.6
Fillet FO	18.5	4.6	1.2	0.8	9.2	14.1	31.3	31.9	6.8	28.4
Fillet VO (60%)	20.1	18.9	2.9	0.5	4.9	9.4	27.8	31.2	20.7	19.4
Diet difference, %	47	679	430	−53	−57	−52	−37	2	147	8
Fish difference, %	9	311	142	−38	−47	−33	−11	−2	204	−32
Red sea bream (Huang et al., 2007)										
Diet FO	17.7	4.7	0.8	0.6	8.3	10.0	22.1	44.8	5.8	22.4
Diet VO (rapeseed oil, 70%)	52.8	19.0	5.7	0.2	1.2	4.3	11.5	55.6	19.4	11.7
Whole body FO	22.3	4.2	0.6	0.5	5.6	11.4	24.5	45.8	5.4	20.8
Whole body VO (70%)	52.2	16.2	4.0	0.2	0.9	5.3	14.1	56.0	17.0	11.0
Diet difference, %	198	309	577	−66	−86	−57	−48	24	236	−48
Fish difference, %	134	282	540	−60	−83	−54	−42	22	217	−47
Turbot (Regost et al., 2003)										
Diet FO	nr	5.2	1.3	0.5	7.9	10.6	22.5	47.1	6.3	24.1
Diet VO (soybean oil, 100%)	nr	35.6	4.6	0.3	3.7	5.6	18.5	29.8	36.2	15.5
Fillet FO	nr	5.5	1.3	0.5	6.9	12.3	21.7	45.3	6.9	26.0
Fillet VO (100%)	nr	24.2	3.0	0.5	4.8	9.2	19.1	34.1	26.3	20.5
Diet difference, %	−	585	254	−40	−53	−47	−18	−37	475	−36
Fish difference, %	−	340	131	0	−30	−25	−12	−25	281	−21
Japanese Yellowtail (Seno-O et al., 2008)										
Diet FO	12.7	4.4	0.7	0.6	9.9	9.4	24.1	41.5	1.9	31.0
Diet VO (olive oil, 100%)	53.8	6.4	5.1	0.4	3.1	4.8	19.8	66.3	5.5	9.2
Fillet FO	19.0	3.3	0.9	0.6	7.0	10.4	26.9	43.5	4.1	20.6
Fillet VO (100%)	37.5	5.0	0.7	0.5	3.9	7.5	24.9	52.2	5.6	12.5
Diet difference, %	324	45	629	−33	−69	-49	−18	60	189	−70
Fish difference, %	97	52	−22	−17	−44	-28	−7	20	37	−39
Mediterranean Yellowtail (Monge-Ortiz et al., 2018)										
Diet FO	13.2	7.0	1.1	0.8	15.1	11.1	30.2	22.4	1.1	29.2
Diet VO (soybean oil, 100%)	24.9	11.8	6.0	0.3	5.9	5.1	36.1	29.3	0.5	12.2
Fillet FO	15.9	9.7	1.2	1.3	13.5	10.0	30.3	24.9	2.0	27.8
Fillet VO (100%)	29.7	15.3	5.3	0.6	4.6	5.4	31.3	33.9	1.1	12.0
Diet difference, %	89	69	445	−63	−61	−54	20	31	−55	−58
Fish difference, %	87	58	342	−54	−66	−46	3	36	−45	−57

[1]Diet difference % = (%FA diet VO − % FA diet FO) × (%FA diet FO)$^{-1}$ × 100. [2]Fish difference % = (%FA fish VO − % FA fish FO) × (%FA fish FO)$^{-1}$ × 100. The percentage of FO substitution with VO is between brackets. The percentage difference of the FA percentage between diets and fish tissues is also reported. OA, oleic acid 18:1 n-9; LA, linoleic acid, 18:2 n-6; ALA, a-linolenic acid, 18:3 n-3; ARA, arachidonic acid, 20:4 n-6; EPA, eicosapentaenoic acid, 20:5 n-3; DHA, docosahexaenoic acid, 22:6 n-3; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; n-3 PUFA, polyunsaturated fatty acids with the first double bond at the 3rd carbon atom; n-6 PUFA, polyunsaturated fatty acids with the first double bond at the 6th carbon atom.

Few information is available regarding the effect of FO replacement with VO on Mediterranean yellowtail tissue fatty acid composition. Only one study (Monge-Ortiz et al., 2018) showed that a complete replacement of FO with a mixture of VO (palm oil and linseed oil 4:1) seems to provide a balanced proportion of SFA, MUFA, PUFA and a positive n-6/n-3 ratio, able to maintain a good amount of EPA and DHA in Mediterranean yellowtail (39.2 g initial weight, 387 g final weight) muscle and liver, after a feeding period of 154 days. However, further studies are necessary to assess the effect of FO replacement with other VO mixture in tissues of Mediterranean yellowtail of different life stages and weight. Moreover, as Mediterranean yellowtail FA requirements are still poorly known, the study of the effects of different dietary FA profiles on other tissues rather than muscle such as brain, liver and visceral fat might provide useful insights on FA metabolism and tissue utilization in this species.

Effect of a finishing fish oil-based diet on the fatty acid composition of fish fillets

In order to restore the typical "marine" fatty acid profile in fillets of fish fed diets containing high levels of VO, finishing strategies (also called wash-out) using a FO-based diet before slaughtering have been suggested (Jobling et al., 2004; Robin et al., 2003; Turchini et al., 2006, 2009). The effectiveness of a wash-out with a FO-based diet after a grow-out on VO diets has been already examined in marine species such as European seabass (Montero et al., 2005; Mourente and Bell, 2006), gilthead seabream (Benedito-Palos et al., 2009; Fountoulaki et al., 2009; Izquierdo et al., 2005), Senegalese sole (Reis et al., 2014) and red sea bream (Glencross et al., 2003), and in freshwater fish like rainbow trout (Thanuthong et al., 2012; Yildiz et al., 2018), sunshine bass (Lane et al., 2006) and Murray cod (Turchini et al., 2006, 2007).

Generally, the FA profile of fish grown-out on VO-based diets return similar to those continuously fed FO after several weeks, varying from 4 to 40 weeks (see Table 1.5 for the main results). Nonetheless, studies have commonly observed that the high levels of FA typically found in VO, mainly linoleic acid, can remain present in the fillets until the end of the rearing period, regardless of the length of the wash-out period (Turchini et al., 2009). Alternative lipid sources rich in MUFA and/or SFA, such as palm oils, coconut oils and animal fats seem to show fewer negative effects on the final fillet FA profile compared to vegetable oils high in C_{18} PUFA such as rapeseed oil, soybean oil and linseed oil (Turchini et al., 2009). However, standard criteria for an effective wash-out period and subsequent recovery of the typical FA in fish fillet are not available for all common farmed species, including the Mediterranean yellowtail.

To date, no information is available on the effects of a finishing strategy in Mediterranean yellowtail and on the time required to restore the FA profile. In this thesis, new results on the effects of a FO-based finishing diet in Mediterranean yellowtail grew out on VO-based diets will be discussed, focusing on the recovery on healthy n-3 HUFA such as EPA and DHA.

Table 1.5. Effect of a finishing FO-based diet (refeeding or wash-out period) after a grow-out on VO-based diets on the fatty acid composition of fish fillets

Fish species	VO diet	Days of grow-out	Days of refeeding	Effects on fillet FA profile at the end of refeeding	Reference
Gilthead seabream	Either SO, PO, or RO (70%)	180	120	OA and LA highly retained in fillets. EPA and DHA not restored	Fountoulaki et al., 2009
	Either LO, RO or SO (60-80%)	210	60 and 90	DHA and ARA recovered after 60 days. EPA not recovered even after 90 days. LA strongly retained	Izquierdo et al., 2005
European sea bass	Either LO, RO, or SO (60%)	240	150	LA three folds higher in VO-fed fish than FO-fed ones. DHA levels recovered but not EPA ones	Montero et al., 2005
	Blend RO, LO and PO (60%)	448	140	EPA and DHA not restored	Mourente and Bell, 2006
Senegalese sole	Blend of RO, SO and LO (100%)	140	26	Total restoration of all EFA	Reis et al., 2014
Red sea bream	Either CO or SO	90	32	High retention but reduced LA and ALA levels. EPA and DHA improved but not fully recovered	Glencross et al., 2003
Rainbow trout	Blend 50% LO and 50% SO (100%)	126	56	High retention of LA and ALA, EPA and DHA improved but not fully recovered	Thanuthong et al., 2012
	Blend 50% CSO and 50% CO (100%)	84	84	ARA fully recovered. EPA and DHA improved but not fully recovered	Yildiz et al., 2018
Atlantic salmon	LO	280	20	EPA and DHA partially restored (80%)	Bell et al., 2003a
	LO	280	24	EPA and DHA partially restored (83%)	Bell et al., 2004
	RO	112	84	EPA and DHA recovered after 28 d and 84 d, respectively	Bell et al., 2003b
Sunshine bass	CO (100%)	196	84	All EFA improved but not fully recovered	Lane et al., 2006
Murray cod	Either CO or LO (100%)	175	112	EFA levels recovered	Turchini et al., 2006

FO: fish oil; CO: corn oil; CSO: cotton seed oil; LO: linseed oil; RO; rapeseed oil; SO: sunflower oil; PO: palm oil; EFA: essential fatty acids; LA, linoleic acid, 18:2 n-6; ARA, arachidonic acid, 20:4 n-6; ALA, a-linolenic acid, 18:3 n-3; DHA, docosahexaenoic acid, 22:6 n-3; EPA, eicosapentaenoic acid, 20:5 n-3; The percentage of FO substitution is between brackets

Fishmeal replacement: can insect meals represent an alternative?

In fish farming, the increase in the price of fishmeal prompted the research of alternative protein sources. Insects seem to be the most promising candidates, thanks to their high nutritional value (i.e. rich in proteins, amino acids, fats, minerals and vitamins) and lower environmental impact than other protein-rich ingredients such as FM, whey and egg proteins (Gasco et al., 2019; Lock et al., 2018; Smetana et al., 2019). Moreover, insects are commonly part of fish natural diet (Henry et al., 2015), especially in freshwater fish species such as rainbow trout. In the present chapter, the main research results on the effects of the dietary replacement of fishmeal with insect meals are discussed, focusing on aquafeed digestibility, fish growth performance and product quality.

Insect meals characteristics and utilization in fish diets

Insect chemical composition varies according to rearing conditions, diet (rearing substrate) and life stage (Henry et al., 2015). Several studies evaluated the proximal composition of insects used as animal feed (see Makkar et al., 2014 for a review) and fish feed (see Henry et al., 2015 for a review) (Table 1.6).

The protein content of insect meals ranges between 50% and 82% DM, varying according to the species and processing method (Henry et al., 2015). As a comparison, the protein level of a good fishmeal can show up to 73%, whereas soybean meal can reach a protein content up to 50% (Barroso et al., 2014; Henry et al., 2015). Regarding essential amino acids (EAA), generally, the AA profile of insects is taxon-dependent, with Diptera showing the AA profile closest to that of FM, whereas Orthoptera and Coleoptera show an AA profile closer to soybean meal, with some lacks in methionine and lysine (Henry et al., 2015; Makkar et al., 2014). However, the amino acid profile of most of the insect species tested in aquafeeds seem to satisfy fish requirements (Alegbeleye et al., 2012; Henry et al., 2015; NRC, 2011).

The lipid content of insects generally ranges between 10% and 30% (Barroso et al., 2014). However, the lipid content of insects is extremely variable and mainly depends on insect diets (Barroso et al., 2014; Henry et al., 2015). The FA profile of insect meals greatly varies according to insect species and to the rearing substrate (Gasco et al., 2019). Thus, as the dietary FA profile is generally reflected in that of fish (Turchini et al., 2009), the FA composition of the final product might be influenced. Insects are generally rich in SFA and scarce in PUFA and HUFA (Gasco et al., 2019; Henry et al., 2015). Therefore, the fillets of fish fed diets containing insects might lack in EFA for human health such as EPA and DHA.

As for lipids, the mineral and vitamin composition of insects highly depend on their diet (Henry et al., 2015). Generally, phosphorous and calcium levels of insects are lower than that found in FM, with the exception of high calcium levels in black soldier fly (Makkar et al., 2014).

Table 1.6. Chemical composition of the main insect species tested in aquafeeds (data sources: Makkar et al., 2014; Henry et al. 2015; Ewald et al., 2020; Mancini et al., 2018; Tomotake et al., 2010).

Common name	Black soldier fly	Mealworm	Housefly	House cricket	Silkworm
Scientific name	*Hermetia illucens*	*Tenebrio molitor*	*Musca domestica*	*Acheta domesticus*	*Bombyx mori*
Life stage	Larvae	Larvae	Maggot	Adult	Pupae
CP (%DM)	42.1 ± 1.0	52.8 ± 4.2	50.4 ± 5.3	63.3 ± 5.7	60.7 ± 7.0
AA (g/16g N)					
Alanine	7.7 ± 0.8	7.3 ± 1.0	5.8 ± 1.0	8.8 (8.8, 8.9)	5.8 (5.5, 6.1)
Arginine	5.6 ± 0.3	4.8 ± 1.0	4.6 ± 0.7	6.1 (6.1, 6.1)	5.6 (4.4, 6.8)
Aspartic acid	11.0 ± 1.8	7.5 ± 1.7	7.5 ± 1.5	7.7 (7.1, 8.4)	10.4 (9.9, 10.9)
Cystine	0.1	0.8 ± 0.0	0.7 ± 0.2	0.8 (0.8, 0.8)	1.0 (0.5, 1.4)
Methionine	2.1 ± 0.3	1.5 ± 0.4	2.2 ± 0.8	1.4 (1.3, 1.5)	3.5 (2.3, 4.6)
Lysine	6.6 ± 0.9	5.4 ± 0.8	6.1 ± 0.9	5.4 (5.4, 5.4)	7.0 (6.5, 7.5)
Isoleucine	5.1 ± 0.5	4.6 ± 0.5	3.2 ± 0.5	4.4 (4.3, 4.6)	5.1 (4.4, 5.7)
Leucine	7.9 ± 0.6	8.6 ± 1.8	5.4 ± 0.6	9.8 (9.5, 10.0)	7.5 (6.6, 8.3)
Phenylalanine	5.2 ± 0.4	4.0 ± 0.4	4.6 ± 0.8	3.0 (2.8, 3.2)	5.2 (5.1, 5.2)
Threonine	3.7 ± 1.7	4.0 ± 0.5	3.5 ± 0.7	3.6 (3.6, 3.6)	5.1 (4.8, 5.4)
Tryptophan	0.5	0.6 ± 0.5	1.5	0.6 (0.5, 0.6)	0.9
Glutamic acid	10.9 ± 2.4	11.3 ± 1.1	11.7 ± 1.8	10.4 (10.4, 10.5)	13.9 (12.9, 14.9)
Histidine	3.0 ± 1.0	3.4 ± 0.2	2.4 ± 0.8	2.3 (2.2, 2.3)	2.6 (2.5, 2.7)
Proline	6.6	6.8 ± 0.2	3.3 ± 0.7	5.6 (5.5, 5.6)	5.2 (4.0, 6.5)
Serine	3.1 ± 1.9	7.0 ± 3.5	3.6 ± 0.5	4.6 (4.2, 5.0)	5.0 (4.7, 5.3)
Tyrosine	6.9 ± 0.7	4.9 ± 0.9	4.2 ± 0.4	5.2 (5.1, 5.3)	4.8 (4.6, 4.9)
Valine	8.2 ± 1.4	7.4 ± 0.3	4.7 ± 1.4	5.2 (4.9, 5.5)	5.9 (5.4, 6.4)
EE (%DM)	26.0 ± 8.3	36.1 ± 4.1	18.9 ± 5.6	17.3 ± 6.3	25.7 ± 9.0
FA (% total FA)					
C12:0	45.2± 17.6	0.5 ± 0.5	5.5	Not detected	
C14:0	8.1 ± 1.7	4.0 ± 2.1	31.1 ± 6.0	0.7	0.1
C16:0	8.4 ± 2.3	21.1 ± 6.7	13.4 ± 10.9	23.4	24.2
C16:1 n-7	5.7 ± 3.3	4.0 ± 1.8	3.4	1.3	1.7
C18:0	2.6 ± 0.7	2.7 ± 0.4	24.8	9.8	4.5
C18:1 n-9	13.9 ± 4.3	37.7 ± 8.7	19.8	23.8	26.0
C18:2 n-6	6.0 ± 2.8	27.4 ± 4.0	2.0	38.0	7.3
C18:3 n-3	2.4 ± 0.9	1.3	5.5	1.2	36.3
Ash (%DM)	20.6 ± 6.0	3.1 ± 0.9	10.1 ± 3.3	5.6 ± 2.4	5.8 ± 2.4
CF (%DM)	7.0		5.7 ± 2.4		3.9 ± 1.1
NDF (%DM)			10.0	18.3 ± 2.9	
ADF (%DM)			17.3 ± 6.3	10.0	
GE (MJ/kg DM	22.1	26.8 ± 0.4	22.9 ± 1.4		

AA: amino acids; ADF: acid detergent fibe; CF: crude fibre; CP: crude protein; DM: dry matter; EE: ether extract; FA: fatty acids; GE: gross energy; N: nitrogen; NDF: neutral detergent fiber. Values are expressed as mean ± SD. When data available are two items (n=2), the minimum and maximum values are reported between brackets.

Several different species such as common housefly, black soldier fly, mealworms and crickets were tested as whole or chopped, previously frozen or alive in Channel catfish (*Ictalurus punctatus*), African catfish (*Clarias gariepinus*), Nile tilapia (*Oreochromis niloticus*), blue tilapia (*Oreochromis aureus*) and rainbow trout. Generally, the partial substitution of FM with insects was successful, whereas the complete substitution led to reduced growth and health (see Henry et al., 2015 for a review). Recently, the dietary inclusion of insects has been investigated in the form of a protein-rich, flour-type ingredient called insect meal. Several studies on both freshwater and marine fish investigated the effects of different FM substitution-insect meal inclusion levels on feed digestibility, growth performance and product quality. Results, which will be summarized in the following sections, show a high heterogenicity among studies, varying with and within fish species, fish life stage, insect meal inclusion levels and length of the feeding period.

Effects of the dietary inclusion of insect meal on fish feed digestibility

The digestibility of insect-derived products varies according to the insect species, meal processing and dietary inclusion levels. Moreover, insects generally show a high content of chitin, which can affect nutrient digestibility (Gasco et al., 2019). The digestibility of insect meals has been tested in various fish species and the main results are summarized in Table 1.7. The major insect species included in aquafeeds have been mealworm (*Tenebrio molitor*, TM) and black soldier fly (*Hermetia illucens*, HI) in the form of full-fat, partially defatted and defatted meals. Several trials have been performed on seabream (*Sparus aurata*), rainbow trout (*Oncorhynchus mykiss*), European sea bass (*Dicentrarchus labrax*) and Atlantic salmon (*Salmo salar*), testing full-fat TM meal (Belforti et al., 2015; Gasco et al., 2016; Piccolo et al., 2017), partially defatted HI meal (Belghit et al., 2018; 2019; Dumas et al., 2018; Renna et al., 2017), and defatted HI meal (Magalhães et al., 2017) (Table 1.7).

Regarding the dietary inclusion of TM meals, a lower in vivo digestibility of ether extract (EE) and crude protein (CP) was recorded in seabream fed diet with a full-fat TM inclusion level of 50%, compared to 25% TM inclusion and FM-diets (Piccolo et al., 2017). Similar results were also observed in rainbow trout (Belforti et al., 2015), where a lower apparent digestibility (ADC) of crude protein was recorded in fish fed diets with 50% inclusion of TM compared to 25% TM-diets and FM-diets, whereas no differences were recorded for ADC of ether extract, organic matter and dry matter. In European sea bass, a higher ADC of crude protein was recorded in fish fed a diet containing 25% of TM compared to a standard FM-based diet (Gasco et al., 2016).

Regarding the inclusion of HI meals, a reduction in digestibility of amino acids, crude protein and ether extract was observed in Atlantic salmon fed diets containing 60% of partially defatted HI meal (Belghit et al., 2018), whereas the inclusion of HI meal (4.9%, 9.8% and 14.8% inclusion levels) derived from larvae reared on seaweed did not affect salmon digestibility of amino acids, fatty acids, crude protein and ether extract (Belghit et al., 2019). In rainbow trout, a high dietary inclusion of defatted HI meal (40%) led to a decrease in ADC of dry matter and crude protein, whereas no differences with the control

diet were recorded for ADC of gross energy and ether extract (Renna et al., 2017). Similar results were also observed in rainbow trout fed diets containing 20% of HI (Dumas et al., 2018). The high heterogenicity of results reported in literature indicates that further studies are necessary to assess the effects of different inclusion levels of insect meal on the digestibility of major nutrients in diets of the most common farmed fish.

Table 1.7. Effects of insect meal dietary inclusion on apparent digestibility coefficients in farmed fish in comparison with a control diet (adapted from Gasco et al., 2019).

Fish species	Insect species	Processing	Insect stage	% Insect inclusion	Days of feeding	Effect on nutrient digestibility	Reference
Atlantic salmon	HI	Partially defatted	Larva	4.9%, 9.8% and 14.8%	114	No effect on ADC of CL, CP, AA and FA.	Belghit et al., 2019
	HI	Partially defatted	Larva	60%	56	Significant reduction in ADC of CL, CP and AA	Belghit et al., 2018
European sea bass	HI	Defatted	Pre-pupa	6.5%, 13% and 19.5%	25	No effect on ADC of OM, DM, CP, AA, EE and energy	Magalhães et al., 2017
	TM	Full-fat	Larva	25%	21	ADC of CP significantly higher than the control diet	Gasco et al., 2016
Gilthead seabream	TM	Full-fat	Larva	25% and 50%	21	ADC of CP and EE were lower in fish fed diets with 50% TM inclusion	Piccolo et al., 2016
Rainbow trout	HI	Partially defatted	Larva	25% and 50%	21	No effect on ADC of GE and EE. ADC of DM and CP higher in 25%-HI than 50%-HI diets	Renna et al., 2017
	HI	Partially defatted	Larva	20%	NS	No effect on ADC of major nutrients. Reduction in ADC of CL in HI-diet	Dumas et al., 2018
	TM	Full-fat	Larva	25% and 50%	21	No effect on ASC of DM, EE and OM. ADC of CP lower in TM50 than other groups	Belforti et al., 2015

TM: *Tenebrio molitor*; HI: *Hemetia illucens*; AA: Amino Acid; ADC: apparent digestibility coefficient; CL: crude lipid; CP: crude protein; DM: dry matter; OM: organic matter; EE: ether extract; FA: Fatty acids; GE: Gross energy; NS: not specified.

Effects of dietary inclusion of insect meal on fish growth performance

As for insect meal digestibility, trials on fish growth performance mainly investigated the effects of the dietary inclusion of TM and HI species, which results are summarized in Table 1.8.

Regarding TM, trials were performed on mandarin fish (*Siniperca scherzeri*), rockfish (*Sebastes schlegeli*), European seabass, gilthead seabream, and pearl gentian grouper (*Epinephelus lanceolatus* ♂× *Epinephelus fuscoguttatus* ♀), testing full-fat (Gasco et al., 2016; Khosravi et al., 2018; Piccolo et al., 2017; Sankian et al., 2018) and defatted (Song et al., 2018) TM meals. A dietary inclusion of TM meal up to 16% significantly increased fish specific growth rate (SGR) and weight gain (WG) of juvenile rockfish, whereas higher inclusion levels (>16%) tended to reduce growth performance (Khosravi et al., 2018). On the other hand, juvenile mandarin fish improved their growth rates and nutrient utilization efficiency when fed diets containing up to 20% of TM, then showed a reduction with higher TM inclusion levels (>20%) if compared to a standard FM-based diet (Sankian et al., 2018). Similar results were also observed in European sea bass fed diets containing 25% of TM meal. Indeed, feeding rate, final body weight, SGR, WG of 25%-TM group were similar to FM-based, whereas a worsening in the overall growth performance was observed with the highest TM inclusion level (50%-TM group) (Gasco et al., 2016). Finally, an inclusion of 12.5% and 18.8% of TM in diets for pearl gentian grouper did not affect growth performance compared with the control FM-based diet (Song et al., 2018) (Table 1.8).

Regarding HI, most of the trials reported that low-moderate dietary inclusion levels of HI meal did not impair fish FCR and growth performance. In rainbow trout, FCR, growth performance and survival were not affected with an HI inclusion of 13% (Dumas et al., 2018), 28% (Stadtlander et al., 2017) and 40% (Renna et al., 2017). Similar results were also observed in growth performance of rainbow trout fed diets containing up to 32.8% of HI reared on a fish offal-enriched substrate (Sealey et al., 2011). In Atlantic salmon during the sea-water phase, the inclusion of 10% of defatted HI did not affect feed ingestion, FCR and growth rate (Belghit et al., 2019). Finally, defatted HI meal can be included in diets for Jian carp (*Cyprinus carpio* var. Jian) (Zhou et al., 2018) and European sea bass (Magalhães et al., 2017) at 14% and 19.5%, respectively, without impairing fish growth performance (Table 1.8).

Table 1.8. Effects of insect meal dietary inclusion on growth performance of farmed fish in comparison with a control diet (adapted from Gasco et al., 2019).

Fish species	Insect species	Processing	Insect stage	% Insect inclusion	Days of feeding	Effect on growth performance	Reference
Atlantic salmon	HI	Defatted	Larva	4.91%, 9.84% and 14.8%	114	Growth rate and FI increased and FCR unaffected by HI meal dietary inclusion	Belghit et al., 2019
	HI	Defatted	Larva	60%	56	No effects on FCR and FI.	Belghit et al., 2018
Carp var. Jian	HI	Defatted	Larva	3.5%, 7, 10.5%, 14%	56	No effects on growth performance	Zhou et al., 2018
European seabass	HI	Defatted	Larva	6.5%, 13% and 19.5%	62	No effects on growth performance and feed efficiency	Magalhães et al., 2017
	TM	Full-fat	Larva	25% and 50%	70	50% TM inclusion showed the lowest WG, FW, feeding rate and FCR	Gasco et al., 2016
Gilthead seabream	TM	Full-fat	Larva	25% and 50%	163	25% TM group had higher SGR, WG%, PER and lower FCR than other groups	Piccolo et al., 2016
Mandarin fish	TM	Full-fat	Larva	10%, 20% and 30%	56	Growth and feed efficiency higher in TM-20% group than control.	Sankian et al., 2018
Pearl gentian grouper	TM	Defatted	Larva	2.5%, 5%, 7.5%, 10%, 12.5%	50	Lower WG and FW in TM-2.5% group than other groups	Song et al., 2018
Rainbow trout	HI	Partially defatted	Larva	6.6%, 13.2%, 26.4%	84	Good growth performance with all inclusion levels	Dumas et al., 2018
	HI	Partially defatted	Larva	20% and 40%	78	No effects on survival, FCR and growth performance	Renna et al., 2017
	HI	Fish offal-enriched	Pre-pupa	16.4% and 32.8% (N) 18.12% and 36.24% (E)	56	No effects on growth performance	Sealey et al., 2011
	HI	Partially defatted	Larva	28.1%	49	No effects on growth performance	Stadtlander et al., 2017
Rockfish	TM	Full-fat	Larva	8%, 16%, 24% and 32%	56	Increased growth until 16% TM inclusion. Then growth decreased at 24% and 32% TM inclusion	Khosravi et al., 2018

HI: *Hermetia illucens*; TM: *Tenebrio molitor*; N: normal HI; E: fish offal-enriched HI; FM: fishmeal; FW: final weight; FI: feed intake; FCR: feed conversion ratio; PER: protein efficiency ratio; SGR: specific growth rate; WG: weight gain.

Effects of the dietary inclusion of insect meal on the quality of fish

The effects of the dietary inclusion of TM and HI meal on morphometric indexes, slaughter results, fillet proximate composition and quality have been largely investigated during the last years and main results are summarized in Table 1.9.

Slaughter and morphometric results show a high heterogenicity among studies. In fact, some authors reported no effects of insect meal inclusion in Atlantic salmon (Belghit et al., 2019) and rainbow trout (Renna et al., 2017) fed diets containing HI meal, or in rainbow trout (Iaconisi et al, 2018) and blackspot seabream (Iaconisi et al., 2017) fed TM diets. On the other hand, several studies showed that low (about 11%) and high (from 18.8% to 60%) dietary inclusion levels of HI and TM meals in Atlantic salmon (Belghit et al., 2018; Lock et al., 2016), gilthead seabream (Piccolo et al., 2017) and Jian carp (Li et al., 2016; Zhou et al., 2018) significantly affected morphometric indexes and slaughter results. In detail, higher viscerosomatic index (VSI) (Belghit et al., 2018; Lock et al., 2016) and hepatosomatic index (Belghit et al., 2018; Lock et al., 2016; Zhou et al., 2018) were found in carp (Zhou et al., 2018) and Atlantic salmon (Belghit et al., 2018; Lock et al., 2016) fed HI-diets compared with control ones. Additionally, lower dressing yield (Piccolo et al., 2017), intraperitoneal fat index (Li et al., 2016) and VSI (Piccolo et al., 2017; Li et al., 2016) were observed in carp (Li et al., 2016) and gilthead seabream (Piccolo et al., 2017) fed HI and TM-based diets, respectively.

The dietary inclusion of TM meal did not affect fillet texture and water holding capacity in rainbow trout (Iaconisi et al., 2018), gilthead seabream (Piccolo et al., 2017) and blackspot seabream (Iaconisi et al., 2017). Regarding fish colour, an increased yellowness (b*) and redness (a*) were found both in fillets and skin of blackspot seabream fed diets containing 40% of TM meal (Iaconisi et al., 2017). On the other hand, a decrease in fillet yellowness was observed in rainbow trout fed diets fed the highest inclusion level of HI meal (40%) (Mancini et al., 2018)

The fatty acid (FA) profile of insect meals greatly varies according to the insect species and to the rearing substrate (Gasco et al., 2019). Thus, as the dietary FA profile is generally reflected in that of fish (Turchini et al., 2009), the FA composition of the final product might be influenced. Given the high content of SFA (especially C12:0, lauric acid) in HI larvae, fish fed diets containing increasing levels of HI meal showed an increased content of SFA and C12:0 and decreased levels of healthy PUFA (Mancini et al., 2018; Renna et al., 2017; Secci et al., 2019; Zhou et al., 2018). Nevertheless, Atlantic salmon fed increasing levels of HI meal showed either a decrease (Lock et al., 2016) or an increase (Belghit et al., 2019) in the content of EPA and DHA and in the ∑n-3/∑n-6 ratio. Regarding *Tenebrio molitor*, larvae show a high content of palmitic, linoleic and oleic acid (Gasco et al., 2019). Thus, high dietary inclusion levels of TM meal led to an increase in fillet n-6 PUFA and a decrease in n-3 PUFA, ∑n-3/∑n-6 ratio and a worsening of thrombogenicity index and atherogenicity index (Belforti et al., 2015; Iaconisi et al., 2017, 2018).

Table 1.9. Effects of insect meal dietary inclusion on quality traits of farmed fish in comparison with a control diet (adapted from Gasco et al., 2019).

Fish species	Insect species	Processing	Insect stage	% Insect inclusion	Days of feeding	Effect on flesh quality[1]	Reference
Atlantic salmon	HI	PD	Larva	5% and 25%	105	No differences for odour, flavour/taste or texture among groups (cooked)	Lock et al., 2016
Rainbow trout	HI	PD	Larva	20% and 40%	78	50% HI inclusion increased fillet EE, PUFA and worsened lipid health indexes, decreased fillet yellowness (raw)	Renna et al., 2017
	HI	PD	Larva	20% and 40%	78	Decrease in PUFA, increased SFA (C12:0) and MUFA (raw)	Mancini et al., 2018
	HI	PD	Larva	20% and 40%	78	No effect of HI on fillet pH, shear stress, WHC (frozen/cooked)	Secci et al., 2019
	HI	PD	Larva	28.1%	49	No differences in chemical composition. Decrease in PUFA (EPA and DHA) and increase in SFA (C12:0)	Stadtlander et al. 2017
	HI	Full-fat N and E	Pre-pupa	16.4% and 32.8% (N); 18.12% and 36.24% (E)	56	Enriched HI improved fillet EPA and DHA content. No differences in sensory analysis	Sealey et al., 2011
Rainbow trout	TM	Full-fat	Larva	25% and 50%	90	Increase in CP and decrease in fillet EPA and DHA content. No effects on chemical-physical properties (raw and cooked)	Belforti et al., 2015
	TM	Full-fat	Larva	25% and 50%	90	No effects on prox. composition. Decrease in PUFA content (EPA and DHA)	Iaconisi et al., 2018
Gilthead seabream	TM	Full-fat	Larva	25% and 50%	163	No effects on marketable indexes with 25% TM. No effects on prox. composition. 50% TM vs. FM group: higher yellowness and chroma, lower hue.	Piccolo et al., 2017

PD: partially defatted; N: normal HI; E: fish offal-enriched HI; WHC: water holding capacity; HI: *Hermetia illucens*; TM: *Tenebrio molitor*; FM: fishmeal; EE: ether extract; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; CP: crude protein; CL: crude lipid.

ADDRESSING SUSTAINABILITY: REARING TECHNIQUES

Aquaponics: a sustainable way to produce fish and plants

Sustainable food production is considered a high priority by the 2030 Agenda for Sustainable Development (UN, 2017). Food production depends on the availability of finite resources, such as freshwater, land, nutrients and fossil energy. However, the depletion of these resources in common agricultural practices is higher than their global rate of renovation. Thus, to assure the balance between immediate human needs and the maintenance of the biosphere capacity (Ehrlich and Harte 2015), new sustainable, reliable and alternative methods of food production have to be investigated and installed.

Aquaponics, an innovative technology that combines recirculating aquaculture and soilless plant cultivation (Rakocy et al., 2004) might provide part of the solution (Van Woensel et al., 2015). This alternative farming approach could play a role in addressing both the increase of sustainable food production and planetary limits, especially in nonarable and arid regions and in urban marginal areas (Appelbaum and Kotzen, 2016; Goddek and Körner, 2019).

Briefly, in aquaponic systems, the dissolved nutrients provided by fish faeces and uneaten feed are utilized by bacteria which transform the organic matter, phosphorus and nitrogen into bioavailable forms that can be absorbed by plants cultivated in hydroponics units (Joyce et al., 2019) (Figure 1.12). Therefore, the integration between aquaculture and plant production provides several advantages in terms of its nutrient-use and water-use efficiency, its capacity to produce both fish and plants from only one input source (i.e. fish feed), and its low environmental impact (Buzby and Lin, 2014; Lennard and Goddek, 2019; Roosta and Hamidpour, 2011; Timmons et al., 2002).

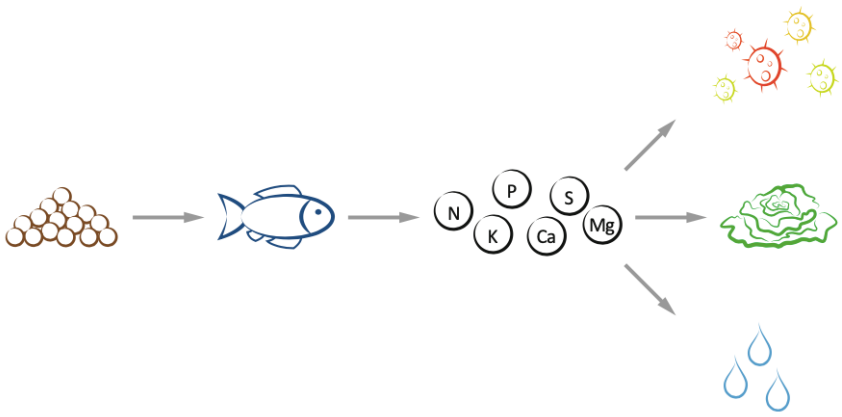

Figure 1.12. Basic scheme of nutrient flows through an aquaponic system. Feed is the main nutrient input. Once fish have eaten the feed and absorbed the nutrients they need, the remaining part is excreted through fish sludge which is then utilized by bacteria, plants and system water (adapted from Lennard and Goddek, 2019).

During the last decades, different methods and approaches have been applied in the aquaponics technology. Thus, the definition of aquaponics varied from a strict recirculating-based context (Cerozi and Fitzsimmons, 2017), to systems in which water from hydroponics units do not return to fish tanks (also called decoupled systems) (Delaide et al., 2016), to scenarios in which both decoupled and recirculating methods are integrated (Knaus and Palm, 2017a). Nevertheless, as stated in the European Aquaponics Hub (COST FA1305, 2017), the emphasis in the definition of aquaponics should be rather placed on the sharing of nutrients, which majority (more than 50%) has to derive from wastes obtained from fish feeding. Thus, in order to provide a comprehensive definition of aquaponics, Lennard and Goodek (2019) stated a new definition and described aquaponics as "*an integrated multi-trophic, aquatic food production approach comprising at least a recirculating aquaculture system (RAS) and a connected hydroponic unit, whereby the water for culture is shared in some configuration between the two units. Not less than 50% of the nutrients provided to the plants should be fish waste derived*"

Regardless the strict definition of aquaponics, there are basic design principles that can be broadly applied to any aquaponic method and approach (Lennard and Goddek, 2019):

1) The use of wastes produced by fish as the major nutrient source for plants.
2) The design of the aquaponic system should focus on aquaculture and hydroponic technologies that preserve the water and the nutrient inputs added.
3) The system should not produce external waste streams of nutrients or water, potentially impacting the environment. If so, wastes should be used in exterior plant production systems.
4) The system should be designed to generate low, or ideally zero, direct environmental impact from wasted nutrients or water.

5) Aquaponic systems should be designed to be easily installed inside environmentally controlled facilities such as greenhouses and fish rooms. This should produce the best productive results for both fish and plants, enhancing the profitability of the farm and financially supporting the high investment and production costs.

In aquaponics, the aquaculture part is mainly designed as a tank-based farming method, where fish are kept inside the tanks and water is filtered through mechanical and biological mechanisms to remove solids and transform ammonia into nitrate, respectively. The dissolved oxygen is guaranteed by direct injection or by aeration (Lennard and Goddek, 2019). Farming fish in concrete tanks allows accumulating a water nutrient concentration suitable for an efficient hydroponic production by satisfying plant nutrient requirements (Lennard and Goddek, 2019). In this context, the recirculating aquaculture system method is generally utilized in aquaponics, because it allows to rear fish in a constant and fix volume of water, guaranteeing low daily replacement of water and allowing fish sludge (i.e plant nutrients) accumulation.

Regarding hydroponics, this cultivation technique when compared with common horticultural practices shows several advantages proper of the soilless culture such as 1) a pathogen-free environment at the beginning of the production cycle and the maintenance of a controlled environment through the use of substrates other than soil, which prevent the spread of soil-borne pathogens; 2) plant growth and yield do not rely on soil type and quality; 3) targeted fertilization with nutrient solutions based on plant requirements and growth rates; 4) the nutrient solutions added into the system water can be reutilized thus maximizing the resources; 5) high control of pests and environmental parameters such as relative humidity and temperature (Maucieri et al., 2019a).

Several studies demonstrated that aquaponics systems of different configurations (i.e decoupled or coupled) can potentially provide plant production rates equal or even better than standard hydroponics (Pantanella et al., 2010). The same results were also observed for fish production, which rates in aquaponics are similar to those of standard RAS (Rakocy, 2012).

Another key advantage of an aquaponic system is the efficient use of water. In fact, water savings in aquaponics can reach 90% or more of the water total volume, with an average water daily consumption rate of 1-1.5% (Love et al., 2015; Maucieri et al., 2018; McMurty 1990), generally due to plant evapotranspiration.

Along with an effective water use, the aquaponics approach provides an efficient utilisation of nutrients (Goddek et al., 2015; Lennard and Goddek, 2019). In standard RAS, up to 75% of the nutrients added through fish feed are wasted (Lennard and Goddek, 2019), whereas aquaponics aims at using these nutrients for plant production, producing two crops from one entry source of nutrients (Timmons et al., 2002).

Balancing the aquaponic ecosystem

Successful aquaponic production strictly relies on the maintenance of a balanced ecosystem in which fish, plants and bacteria are involved in a dynamic equilibrium (Somerville, 2014). Each organism of an aquaponic system presents an ideal range for water quality parameters such as pH, dissolved oxygen, temperature and nitrogen compounds like ammonia, nitrites and nitrates (Somerville, 2014) (Table 1.10). The optimization and best efficiency of the system are achieved when the requirements of the three life forms are matched (Lennard and Goddek, 2019). However, although there are similar and broadly accepted tolerance ranges among fish, bacteria and plants (Delaide et al., 2016; Goddek et al., 2015; Lennard and Goddek, 2019), some compromises are required.

Table 1.10. Main water parameters tolerance of fish, hydroponic plants and bacteria and ideal values for an aquaponics system (adapted from Somerville, 2014)

Organism type	pH	DO (mg/L)	Temp. (°C)	Ammonia (mg/L)	Nitrite (mg/L)	Nitrate (mg/L)
Warm water fish	6-8.5	4-6	22-32	< 3	< 1	< 400
Cold water fish	6-8.5	6-8	10-18	< 1	< 0.1	< 400
Plants	5.5-7.5	> 3	16-30	< 30	< 1	-
Bacteria	6-8.5	4-8	14-34	< 3	< 1	-
Aquaponics	6-8.5	4-8	18-30	< 3	< 1	-

DO: Dissolved oxygen.

The major compromise regarding water quality in aquaponic science is represented by water pH levels (Lennard and Goddek, 2019; Suhl et al., 2016). In fact, the best water pH for hydroponics cultivation is 4.5-6.0, whereas RAS farming is typically performed at water pH settings of 7.0-8.0 (Lennard and Goddek, 2019; Timmons et al., 2002). Thus, an operational pH range of water for aquaponics production is generally accepted between 6.0 and 8.0, which might lead in some cases to suboptimal plant growth (Suhl et al., 2016), but will guarantee paramount transformations by bacteria of toxic ammonia into the less noxious nitrate (Suhl et al., 2016) that are optimal at pH levels around 7.5 (Goddek et al., 2015; Lennard and Goddek, 2019).

Water dissolved oxygen (DO) is usually set considering the requirements of the cultured fish species (Lennard and Goddek, 2019). In fact, microbes and plant roots generally thrive at lower DO levels than most common farmed fish, surviving at concentrations lower than 3 mg/L, whereas fish generally require a DO > 5mg/L (Lennard and Goddek, 2019; Timmons et al., 2002). Thus, if the aquaponic system is maintained with a DO concentration based on fish requirements, also bacteria and plant ones are met.

Regarding water temperature, plant and fish species requirements should be closely matched (Lennard and Goddek, 2019). For example, *Tilapia* spp. require temperature higher

than 25°C, which match with basil plants that can thrive at high temperatures; on the other hand, lettuce need cooler temperatures, matching better with freshwater fish species like rainbow trout (*Oncorhynchus mykiss*). Nevertheless, as for other parameters, water temperature should primarily satisfy fish requirements, as plants show good growth performance at a broader water temperature range than fish. Moreover, a high temperature control (i.e ideal fish temperature range ± 2°C) permits to reach an efficient and optimised fish metabolism and good feed conversion ratios which lead to best growth rates and predictable and constant waste (i.e. nutrients) releases that maintain plant cultivation (Timmons et al., 2002).

In an aquaponic system, the major nitrogen source is represented by the proteins contained in fish feed (Ru et al., 2017; Yildiz et al., 2017). Generally, fish assimilate only the 30% of the nitrogen contained in the feed while the remaining 70% is excreted through faeces, urine and gills (Rafiee and Saad, 2005; Ru et al., 2017). The most relevant waste that fish produce through feed protein catabolism is ammonia (Altinok and Grizzle 2004; Yildiz et al., 2017). This nitrogen compound is accumulated in the water of fish tanks and can reach toxic levels if not treated (Lennard and Goddek, 2019). In the water, ammonia exists in two forms: the ionized one (NH_4^+) that shows a low toxicity to fish and the non-ionized and toxic one (NH_3). The sum of NH_4^+ and NH_3 is represented by the total ammonia nitrogen (TAN). The ratio between NH_3 and NH_4^+ depends on water pH, salinity and temperature (Lennard and Goddek, 2019).

A key process that regulates the balance of an aquaponic systems and directly influences water quality is nitrification, during which NH_3 and/or NH_4^+ produced by fish are transformed by aerobic bacteria into nitrite (NO_2^-) first and then into nitrate (NO_3^-) (Eck et al., 2019). The first step of ammonia transformation is regulated by ammonia-oxidising bacteria like *Nitrosomonas* and *Nitrosococcus* while the second step is carried out by nitrite-oxidising ones such as *Nitrococcus* and *Nitrobacter* (Eck et al., 2019; Rurangwa and Verdegem 2013; Wongkiew et al., 2017). The nitrification process, that requires a high availability of dissolved oxygen (Eck et al., 2019; Shoda et al., 2014), is paramount in aquaponic productions as high concentrations of ammonia (> 0.07 mg/L) and nitrite (> 1 mg/L) are toxic to fish (Eck et al., 2019), affecting their nervous system and reducing their fixation of oxygen. In most of freshwater fish species, an acute-lethal toxicity is reached at a value of 2.79 mg NH_3/L (Randall and Tsui, 2002). Nitrate is less toxic and can be tolerated by fish until levels of 150-300 mg/L (Eck et al., 2019).

In order to optimize the integration between aquaculture and hydroponics productions, a key factor is represented by the ratio between fish wastes (directly depending on fish feed input) and plant nutrient absorption (Goddek et al., 2015). Since the beginning of aquaponics productions, numerous models and general rules have been proposed and developed in order to reach this balance, considering different plant and fish combinations tested at various conditions. The first approach called “The Aquaponic Feeding Rate Ratio” was based on the matching between the area required for plant growing and the daily input of fish feed. This ratio was set at 60-100 grams of fish feed daily added per square meter of

plant growing area (Lennard and Goddek, 2019) and was developed considering *Tilapia* spp. fish feeding on a standard commercial diet (32% of protein) (Rakocy and Hargreaves, 1993). Another study proposed a specific ratio based on plant and fish species with 15-42 g/m^2/day for water spinach (*Ipomoea aquatica*) cultivated with African catfish (*Clarias gariepinus*) (Endut et al., 2010). More recent models aim to determine the feeding rate ratio specifically considering the match between a given plant and fish species both in coupled and decoupled systems (Goddek et al., 2016). Nevertheless, further research is necessary to investigate the effect of different combination of fish and plants on feed input requirements, fish and plant growth performance, health and quality.

Fish species in aquaponics

The choice of fish species is paramount for the economic success of an aquaponic system. In fact, a suitable combination of fish and plant species, coupled with a balanced fish to plant ratio, assure a correct utilization and removal of toxic nutrients by plants, assuring plant and fish growth and welfare. Nevertheless, the effects and benefits of a given fish species in a coupled aquaponic system are not yet fully understood, especially in terms of acceptable ranges of nutrient loads and water quality parameters (Palm et al., 2019).

The first research trials in coupled aquaponics tested 'easy-to-produce' species such as common carp (*Cyprinus carpio*) Naegel (1977), Channel catfish (*Ictalurus punctatus*) (Lewis et al., 1978; Sutton and Lewis, 1982), tilapia (*Tilapia mossambica*) (Naegel, 1977), blue tilapia (*Oreochromis aureus*) (Watten and Busch, 1984), and Nile tilapia (*Oreochromis niloticus*), which has been consequently used in several studies as a model fish species to assess the performance of different plant species in aquaponics (Diem et al., 2017; Palm et al., 2014a; Rakocy 1989, Rakocy et al., 2003, 2004; Rakocy 2012; Simeonidou et al., 2012; Villarroel et al., 2011) while tilapia hybrids-red strain (*Oreochromis niloticus* x blue tilapia *O. aureus* hybrids) were tested in desert and arid environments (Appelbaum and Kotzen 2016; Kotzen and Appelbaum 2010).

During the last years, the choice of fish species used in aquaponic systems is expanding towards indigenous species as well as fish with high consumer acceptance, like African catfish (*Clarias gariepinus*) and European pikeperch (*Sander lucioperca*). Studies showed that African catfish was reared successfully in coupled aquaponics, showing good growth rates and high adaptability to adverse water conditions such as high levels of ammonia and nitrates. Moreover, African catfish farming did not require additional oxygen supply as they are characterized by an air-breathing physiology (Baßmann et al., 2017; Endut et al., 2009; Knaus and Palm, 2017b; Palm et al., 2014b;).

Other important fish species tested in coupled aquaponics belongs to the order of Cypriniformes, such as common carp, koi carp (*Cyprinus carpio* var. koi), tench (*Tinca tinca*) and goldfish (*Carassius auratus*). Studies reported that these species show better growth performance when farmed at low stocking densities and at minimal water flow rates (0.8-1.5 L/min) (Hussein et al., 2015; Nuwansi et al., 2016). The optimal initial stocking density for cyprinids production in aquaponics was found at 1.4 kg m^{-3} for koi carp (Hussein et al., 2015),

2.5 kg m^{-3} for common carp (Maucieri et al., 2019b) and 0.68 kg m^{-3} for tench (Lobillo et al., 2014).

In the European context, fish species of high economic value and market potential have been recently considered one of the key topics in aquaponics research, focusing especially on carnivorous species such as the European pikeperch (Palm et al., 2019). Studies reported that pikeperch showed good growth rates in coupled aquaponics with lettuce (*Lactuca sativa*), without negative effects of high water levels of phosphorous pentoxide and nitrates on fish growth (Blidariu et al., 2013a, b).

To date, most of the research has been performed on warm-water species with broad environmental requirements, whereas no studies investigated the adaptability and growth performance of cold-water species such as rainbow trout, which requires high-quality water with high levels of dissolved oxygen (6-8 mg/L). Indeed, the production of high-value species, such as rainbow trout in aquaponics, might increase the profitability and the competitiveness of aquaponics production, expanding the range of suitable species to be reared in such system.

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CHAPTER 2: THE AIMS

Aquaculture, the world's fastest-growing food producing sector, is widely recognized for its ability to provide nutritious food in a highly efficient way, playing a key role in human nutrition and food security. On the other hand, aquaculture is often criticised for the adoption of unsustainable practices, especially regarding the utilization of feed ingredients and the negative impact on the environment of its farming techniques. Nonetheless, the further increase expected from aquaculture production in the next years will inevitably face environmental, social, and economic challenges. To ensure a sustainable growth of the sector, aquaculture must reduce its dependence on costly and environmental impacting fish oil and fishmeal, maintaining feed efficiency and product quality. However, the effect of alternative lipid and protein sources on fish production and quality need to be carefully investigated, especially when new fish species like *Seriola dumerili* are tested or when novel sources such as insects are used.

Fish oil have been traditionally used as major lipid and essential n-3 HUFA source in marine fish. However, the decrease of its availability coupled with the rise of its price, prompted research and industry to find alternative lipid sources as partial or total FO replacers. **Vegetable oils** (VO), a cheaper, easier to produce and more available sources than FO, have been tested as FO replacers in the diets of farmed fish. Compared to FO, VO are rich in C:18 polyunsaturated fatty acids, but lack in n-3 HUFA, which are essential for marine fish. Thus, several studies evaluated the effect of FO replacement with VO in commonly farmed marine fish such as Atlantic salmon, European seabass and gilthead seabream. The major drawback of FO substitution with VO is the negative effect on fish fillet fatty acid composition, with a reduction of n-3 HUFA such as EPA and DHA, which are beneficial for human health. Therefore, to restore the healthy FA profile of fish fillets during fish farming, a finishing period on a FO-based diets before slaughtering (i.e., wash-out period) have been proposed. The effect of FO substitution with VO during grow-out and the efficacy of a wash-out period with a FO-based diet varies according to vegetable source, refeeding period length, fish species, life stage, and fish fatty acid requirements.

The **Mediterranean yellowtail** (*Seriola dumerili*) is one of the most promising species in aquaculture, thanks to its fast growth, excellent flesh quality and consumer acceptance. As a new species, its fatty acid requirements, especially during grow-out, are not fully known. Moreover, only one study evaluated the impact of FO substitution with VO on growth and fillet quality of *S. dumerili* juveniles. Thus, further research is needed to evaluate the effect of different replacement rates of FO with VO on growth performance, health, and flesh and other tissue fatty acid profile. Moreover, no information is available about the effects of a finishing/wash-out strategy in M. yellowtail with special emphasis on the time required to restore the FA profile and the sensitivity of selected FA to a wash-out diet

During the last years, several novel protein sources have been identified and tested as potential fishmeal replacers. Among them, **insect meals** seem to be the most promising candidates, thanks to their high nutritional value and lower environmental impact than other

protein-rich ingredients. Moreover, insects are commonly part of fish natural diet especially in freshwater fish species such as **rainbow trout**. However, the effects of dietary replacement of fishmeal with insect meals on aquafeed digestibility, fish growth performance and product quality are not yet fully understood and differ between and within fish species, life stages and FM substitution levels.

Along with sustainable feeding strategies, a proper use of nutrients, water and land is essential to ensure a long-term sustainability of the aquaculture sector. Therefore, new **rearing techniques** that preserve water, improve nutrient utilization, reduce nutrient losses, and land utilization have to be investigated and implemented. **Aquaponics**, a farming technique that combines recirculating aquaculture with soil-less plant cultivation in closed recirculating system, represent a viable and more sustainable alternative than common agricultural practices. The main fish species used in aquaponics are low-value and easy-to-produce cyprinids and tilapias, due to their low environmental requirements and high adaptability to handling and farming conditions. However, the use of high-value species such as **rainbow trout** (*Oncorhynchus mykiss*) could improve the profitability and competitiveness of the aquaponics system. To date, to the best of our knowledge, no studies assessed the suitability of rainbow trout in aquaponics, evaluating its growth performance, health, and quality.

Thus, through its four main contributions, the present thesis aimed at improving the knowledge regarding feeding strategies and rearing techniques that can increase the sustainability of aquaculture sector. Specifically, this PhD thesis evaluated the effects of:

- Fish oil replacement with vegetable oil in diets of Mediterranean yellowtail (first contribution; chapter 3).
- Wash-out/finishing period with a fish oil-based diet in Mediterranean yellowtail grew-out on a vegetable-oil based diet (second contribution; chapter 4).
- Stocking density in aquaponic farming of rainbow trout (third contribution, chapter 5).
- Fishmeal replacement with insect meal in diets of rainbow trout reared in aquaponics (fourth contribution, chapter 6).

The general discussion of this thesis (chapter 7) also evaluated the environmental impact through a Life Cycle Assessment (LCA) analysis of:

- Fish oil replacement with vegetable oils during Mediterranean yellowtail grow-out in a recirculating aquaculture system (based on data from the first contribution, chapter 3).
- Rainbow trout farming at two stocking density in a low-tech aquaponic system (based on data from the third contribution, chapter 5).

A multidisciplinary approach was used which included evaluation of growth performance and health (all contributions), feed digestibility (fourth contribution), gut morphology and histological analysis (fourth contribution), tissue fatty acid signatures (first contribution), slaughter results and carcass traits (third and fourth contribution), fillet quality considering fatty acid profile (first, second and fourth contribution) and microbial contamination (third contribution). Moreover, environmental traits such as water losses,

water quality and nutrient availability and dynamics were considered, and vegetable productions were also reported (third and fourth contribution).

CHAPTER 3

FATTY ACID SIGNATURES IN DIFFERENT TISSUES OF MEDITERRANEAN YELLOWTAIL, *SERIOLA DUMERILI* (RISSO, 1810), FED DIETS CONTAINING DIFFERENT LEVELS OF VEGETABLE AND FISH OILS (First contribution)

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ABSTRACT

The study aimed to evaluate how replacing different proportions of fish oil (FO) with vegetable oils (VO) in the diet of Mediterranean yellowtail, *Seriola dumerili* (Risso, 1810), affects the fatty acids (FA) signature, i.e., overall FA profile, in different tissues. A total of 225 Mediterranean yellowtail juveniles (initial live weight: 176 ± 3.62 g) were fed for 109 days with one of three diets: A control diet (FO 100), with FO as the only lipid source, or diets with 75% and 100% of FO replaced with a VO mixture. At the end of the feeding trial, the brains, muscles, livers, and visceral fat were sampled in four fish per tank (12 per treatment), and their fat were extracted and used for FA analysis. The FA signatures of red and white muscle, liver, and visceral fat tissues changed when the dietary FA source changed, whereas FA signatures in the brain were rather robust to such dietary changes. These new insights might help evaluate whether key physiological functions are preserved when fish are fed diets with low FO levels, as well as define the dietary FA requirements of Mediterranean yellowtail to improve the sustainability of the production and welfare of the fish.

Keywords: brain; muscle; liver; greater amberjack; EPA; DHA

INTRODUCTION

The Mediterranean yellowtail, *Seriola dumerili* (Risso, 1810), also called the greater amberjack, is a new, high-value candidate for production in marine aquaculture. This cosmopolitan fish is mainly produced in Japan (Matsunari et al., 2013), Spain (Grau et al., 1999), Italy (Andaloro and Pipitone, 1997), and recently in Vietnam, Korea, and China (Sicuro and Luzzana, 2016). It has high growth rates (reaching 6 kg within 2.5 years of culture), and exceptionally high consumer acceptance (Mazzola et al., 2000; Sicuro and Luzzana, 2016).

Since the Mediterranean yellowtail is an emerging species in aquaculture, its nutritional requirements have been defined in terms of major nutrients (47%–50% crude protein (CP) and 12%–14% crude lipid (CL)) (Haouas et al. 2010; Jover et al., 1999; Papadakis et al., 2008; Takakuwa et al., 2006; Tomás-Vidal, 2008); however, little information is available on its fatty acid requirements (Monge-Ortiz et al., 2018), which thus require further investigation. Only one previous study assessed the effects of dietary fatty acids on Mediterranean yellowtail body composition (Monge-Ortiz et al., 2018).

Fatty acids (FA) play key roles in fish health and nutrition. They maintain the structural and functional integrity of cell membranes, provide metabolic energy through their oxidative metabolism, contribute to visual and brain development, and are precursors of a group of paracrine hormones with relevant biological roles known as eicosanoids (Mourente, 2003; Mourente et al., 1991; Sargent et al., 1999). Fish health, growth, and reproduction are strongly dependent on n-3 and n-6 polyunsaturated fatty acids (PUFA), especially arachidonic acid (AA, C20:4 n-6), eicosapentaenoic acid (EPA, C20:5 n-3), and docosahexaenoic acid (DHA, C22:6 n-3) (Bell et al., 1995; Sargent et al., 1997; Sargent et al., 1999; Tocher and Harvie, 1988). However, marine carnivorous fish such as *S. dumerili* have limited ability to bio-convert the essential precursors of PUFA, such as linoleic acid (LA, C18:2 n-6) and alpha-linolenic acid (ALA, C18:3 n-3), into these essential FA (Tocher and Ghioni, 1999; Turchini et al., 2009).

The overall fatty acid profile within a given tissue, also known as its FA signature (Iverson et al., 2004), is strongly dependent on the physiological function(s) of the tissue itself (Sargent et al., 1993). The liver and muscles are the main sites of β-oxidation (Henderson and Tocher, 1987), whereas the brain stores n-3 LC-PUFA, mainly DHA, which perform neurological functions (Anderson et al., 1990; Bianconi et al., 2018; Furuita et al., 1998). Nevertheless, in nature, the FA signatures of prey tissues can be transferred to their predators with little modification and in a predictable manner (Bergé et al., 2005; Iverson et al., 2004; Thiemann, 2008). Thus, in recent decades, FA have been extensively used as biomarkers in riverine and marine ecosystems (Iverson et al., 2004; Kaushik et al., 2006; Stowasser et al., 2009). Additionally, under farming conditions (Budge et al., 2012; Happel et al., 2016; Magnone et al., 2015), the available literature collectively supports the conclusion that there is a close association between the diet and fillet FA composition in farmed fish, as reviewed by Turchini et al. (2009).

As in other species (Benedito-Palos et al., 2010), understanding the FA distributions and signatures within Mediterranean yellowtail tissues might help us understand whether the physiological needs and essential fatty acid (EFA) requirements of fish are satisfied under farming conditions. This is particularly crucial when diets that contain low levels of fish oil (FO) are used in fish farming. In fact, to ensure the long-term sustainability of the aquaculture sector and to improve its competitiveness, FO has been increasingly replaced by vegetable oils (VO) in fish diets because FO has a high cost and is available in finite and limited amounts (Turchini et al., 2009). Compared to FO, plant seed oils are rich in C:18 PUFA (Turchini et al., 2009), but lacking in n-3 highly unsaturated fatty acids (HUFA), which are essential for marine fish (Sargent et al., 1999).

Most studies performed in farmed fish to date have focused mainly on the effects of dietary lipids on the FA composition of edible muscle tissues (Turchini et al., 2009). Only one study (Benedito-Palos et al., 2010), which investigated FA signatures in gilthead seabream (*Sparus aurata* L.; 1758), assessed how dietary lipids were retained in different tissues (brain, liver, and mesenteric adipose tissue) to obtain further insights into the FA requirements of farmed marine fish.

Therefore, this study was performed to evaluate how the replacement of FO with different proportions of VO in the diet affects the tissue-specific FA signatures and their robustness in the brain, muscle, liver, and visceral fat tissues of Mediterranean yellowtail (*S. dumerili*).

MATERIALS AND METHODS

A feeding trial was performed at the Fish Nutrition Laboratory (LAC) of the Institute of Animal Science and Technology (ICTA) of the Universitat Politècnica de València (Polytechnic University of Valencia, Spain). The experimental protocol was approved by the Committee of Ethics and Animal Welfare of the Polytechnic University of Valencia, following the Spanish Royal Decree 53/2013 on the protection of animals used for scientific purposes. The facility used a thermoregulated recirculating seawater system (65 m^3 capacity), with a rotary drum-type filter and a mechanical gravity biofilter with a volume of 2 m^3, equipped with 9 cylindrical fiberglass tanks with a capacity of 1750 L each with aeration.

Experimental diets

Three isoproteic (59% CP, 50% digestible protein (DP)), and isolipidic (15% CL) extruded diets were formulated, with increasing levels of vegetable oils used to replace the fish oil in the diets as follows: 0% (control diet, FO 100), 75% (FO 25), and 100% (FO 0) replacement of FO with VO (Table 3.1). The mixture of vegetable oils used consisted of linseed oil, sunflower oil, and palm oil (4:3:3). Diets were prepared using a cooking-extrusion processor with a semi-industrial twin-screw extruder (CLEXTRAL BC-45; Firmity, St Etienne, France), at a screw speed of 100 rpm, temperature of 110 °C, and pressure of 40–50 atm, to obtain pellets 2–3 mm in diameter.

Table 3.1. Ingredients (g kg^{-1} as fed) and proximate composition (% dry matter; DM) of the experimental diets.

	Diet		
	FO 100	**FO 25**	**FO 0**
Fishmeal	350	350	350
Wheat	100	100	100
Wheat gluten	140	140	140
Defatted soybean meal	185	185	185
Iberian meat meal	110	110	110
Fish oil	95	24	0
Linseed oil	-	28	38
Sunflower oil	-	21	28
Palm oil	-	22	29
Multivitamin and minerals mix	20	20	20
Proximate composition			
Dry matter, %	87.4	88.8	89.1
Crude protein, % DM	58.8	60.6	58.8
Crude lipid, % DM	15.9	15.1	16.6
Ash, % DM	8.4	10.3	8.3
Gross energy, MJ kg^{-1} DM	24.3	23.8	24.4

Fish oil (FO) 100 diet: Diet formulated with fish oil as lipid source; FO 25 diet: Diet in which fish oil was included at a content of 25%; FO 0 diet: Diet in which fish oil was totally substituted with vegetable oil. [1] Vitamins and mineral mixture (values are g kg^{-1}): Premix, 25; Hill, 10; DL-α-tocopherol, 5; ascorbic acid, 5; (PO4)2Ca3, 5. Premix composition (values are IU kg^{-1}): Retinol acetate, 1,000,000; calcipherol, 500; DL-α-tocopherol, 10; menadione sodium bisulphite, 0.8; hidroclorhidrate thiamine, 2.3; riboflavin, 2.3; pyridoxine hydrochloride, 15; cyanocobalamin, 25; nicotinamide, 15; pantothenic acid, 6; folic acid, 0.65; biotin, 0.07; ascorbic acid, 75; inositol, 15; betaine, 100; polypeptides, 12.

The FA profile of the experimental diets changed depending on the relative proportions of the two lipid sources included in them. Diets containing more FO contained higher proportions of highly unsaturated fatty acids from the n-3 series, whereas diets containing more vegetable oils had higher proportions of LA and ALA (Table 3.2). In all diets, C16:0 accounted for the bulk of the saturated fatty acids (SFA) present, C18:1n-9 for most of the monounsaturated fatty acids (MUFA), and EPA and docosahexaenoic acid (DHA) for most of the n-3 long-chain polyunsaturated fatty acids (PUFA). The inclusion of VO as a lipid source increased the dietary proportion of C18:1 n-9 (27.1%, 29.6%, and 32.7%), C18:2 n-6 (12.7%, 13.4%, and 14.9%), and n-6 PUFA (14.1%, 14.3%, and 15.4%), whereas it decreased the proportions of total SFA, EPA, and DHA in the diets (SFA: 27.8%, 27.1%, and 25.5% in the FO 100, FO 25, and FO 0 diets, respectively; EPA: 5.8%, 4.3%, and 2.8%; DHA: 13.0%, 7.6%, and 4.3%) (Table 3.2).

Table 3.2. Fatty acid composition (% of total fatty acid content) of the experimental diets.

	Diet		
	FO 100	**FO 25**	**FO 0**
14:0	3.28	2.38	1.65
16:0	18.88	19.58	18.93
17:0	0.53	0.24	0.16
18:0	5.07	4.88	4.71
∑ SFA	27.79	27.12	25.46
16:1 n-9	4.22	2.77	1.82
18:1 n-9	27.14	29.64	32.67
18:1 n-7	3.94	2.94	2.44
22:1 n-9	0.32	0.04	0.06
∑ MUFA	35.62	35.39	36.99
18:2 n-6	12.66	13.36	14.86
18:3 n-6	0.10	0.09	0.09
20:3 n-6	0.10	0.04	0.04
20:4 n-6	1.02	0.58	0.35
22:4 n-6	0.24	0.19	0.09
∑ n-6 PUFA	14.12	14.26	15.43
18:3 n-3	2.24	10.48	14.60
20:3 n-3	0.15	0.08	0.06
20:5 n-3	5.81	4.34	2.77
22:5 n-3	1.29	0.73	0.42
22:6 n-3	12.98	7.61	4.28
∑ n-3 PUFA	22.47	23.24	22.13
∑ PUFA	36.59	37.50	37.56
∑ n-6/ ∑ n-3	0.63	0.61	0.70
DHA/EPA	2.23	1.76	1.54

FO 100 diet: diet formulated with fish oil as lipid source; FO 25 diet: diet in which fish oil was included at a content of 25%; FO 0 diet: diet in which fish oil was totally substituted with vegetable oil. Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; DHA/EPA, 22:6 n-3/20:5 n-3.

Fish and experimental design

A total of 225 Mediterranean yellowtail juveniles obtained from a private hatchery (Futuna Blue S.A., Cádiz, Spain) were transported to the Fish Nutrition Laboratory of the Universitat Politècnica de València for use in the feeding trial. After 4 weeks of acclimation, fish were individually weighed (initial weight: 176 ± 3.62 g), randomly distributed among 9 tanks (25 fish per tank), and fed the experimental diets (FO 100, FO 25, or FO 0, with 3 tanks per diet) for 109 days.

During the trial, the water temperature averaged 21.5 ± 2.4°C, the salinity was 31.5 ± 4.1 g L^{-1}, and the dissolved oxygen content was 8.0 ± 0.4 mg L^{-1}. The water pH ranged from

7.5 to 8.0, and the levels of nitrogenous compounds in the water were kept within the limits recommended for marine species.

In vivo recordings

Feed was distributed by hand, twice a day (at 09:00 and 16:00) for six days per week, until apparent satiation. Feed intake was recorded at each administration. Mortality was checked every day. At the beginning and at the end of the trial, fish were individually weighed after one day of feed deprivation and under light anaesthesia (10 mg L^{-1} clove oil containing 87% eugenol; Guinama®, Valencia, Spain). Fish health status during weighing was assessed by direct observation.

After 109 days of feeding, four fish per tank (12 per treatment, 36 in total) were randomly sacrificed by lethal immersion in clove oil (150 mg L^{-1}). Fish were dissected to sample their brain, white and red muscle, liver, and visceral fat tissues, which were then frozen in liquid nitrogen and stored at –80°C until subsequent analyses.

Chemical analyses of diets and fish tissues

Diets were ground up and analysed according to AOAC procedures (AOAC, 1990) to determine their: dry matter (by heating at 105°C until a constant weight was attained), ash (by incinerating them at 550°C for 5 h), crude protein (AOAC official method 990.03, by the DUMAS direct combustion method, using a LECO CN628 apparatus, LECO, ST. Joseph, Michigan, USA), and crude lipid content (by extraction with methyl ether using an ANKOMXT10 Extractor, ANKOM Technology, Macedon, NY, USA). Gross energy content (GE) was calculated according to Brouwer (1965), from the C (g) and N (g) balance in the feed ($GE = 51.8 \times C - 19.4 \times N$). The carbon and nitrogen content were analysed based on the Dumas principle (TruSpec CN; Leco Corporation, St Joseph, MI, USA). All analyses were performed in triplicate.

After thawing, tissues were minced and homogenised. The crude fats in the brain, muscle, liver, and visceral fat tissue sampled were then extracted (Folch et al., 1957). A direct method of fatty acid methyl ester (FAME) synthesis was used for this procedure (O'Fallon et al., 2007). The analysis of brain, muscle, liver, and visceral fat tissues was carried out using 10-30 mg of extracted crude fat from each tissue. First, 1 mL of tridecanoic acid (C13:0) was used as internal standard. Then, 0.7 mL of 10 N KOH and 5.3 mL of HPLC (high-performance liquid chromatography)-quality methanol were added. Tubes were incubated at 55°C in a thermoblock for 1.5 h, and underwent vigorous shaking for 5 s every 20 min. After cooling at ambient temperature in a water bath, 1.5 mL of HPLC-quality hexane was added to the reaction tubes, which were then vortex-mixed and centrifuged at 1006× *g* for 5 min. After this, the hexane layer, containing the FAMEs, was placed into vials for analysis by gas chromatography. The vials were kept at –80°C until gas chromatography was performed. The FAMEs were analysed in a Focus Gas Chromatograph (Thermo, Milan, Italy) equipped with a split/splitless injector and a flame ionisation detector. Separation of the methyl esters was performed in a SPTM 2560 fused silica capillary column (Supelco, PA, USA) (100 m × 0.25

mm × 0.2 μm film thickness). Helium was used as the carrier gas at a flow rate of 20 cm s^{-1}. Samples were injected with a split ratio of 1:100.

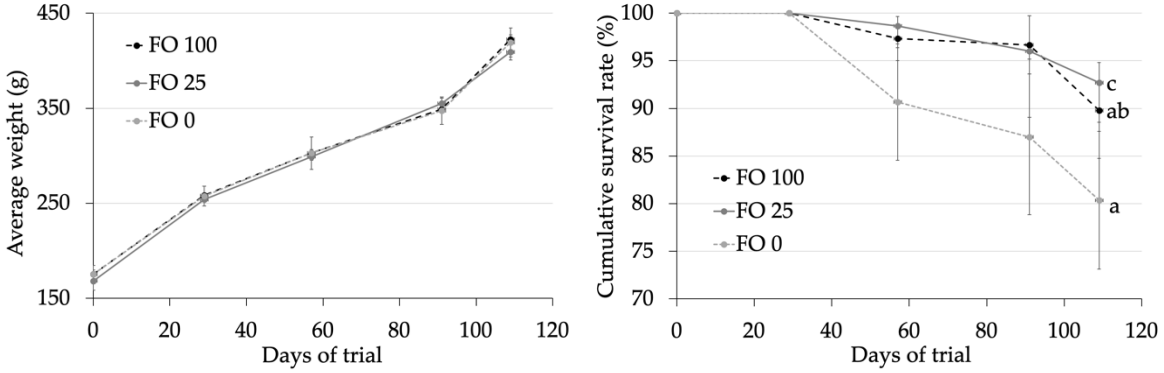
The initial oven temperature, set at 140°C, was held constant for 5 min, and then increased by 4°C min^{-1} to 240°C, at which this temperature was then maintained for 30 min. The FA were identified by comparing their retention times with those of the standards supplied by Supelco. The content of each type of FA was expressed as the percentage of the total FA content.

Statistical analyses

The growth, survival, tissue fat content and FA composition data were compared by analysis of variance (ANOVA), with diet included as the main effect and tank included as a random effect. The PROC MIXED procedure of the Statistical Analysis System (SAS) software (SAS, 2013) was used for all analyses. Adjusted means were compared among treatment levels using Bonferroni's *t*-test. Differences between means with $p \leq 0.05$ were considered statistically significant.

RESULTS

In the present study of *S. dumerili* juveniles (176 g of initial live weight), diets containing only VO as the lipid source had no effect on fish growth (Figure 3.1). In fact, fish fed FO 100, FO 25 and FO 0 diets reached a final weight of 423 g, 409 g and 419 g, respectively ($p > 0.05$), corresponding to a specific growth rate of 0.80% day^{-1} and a feed intake equal to 1.1% of live weight. The survival rate was lower in fish fed diets without fish oil compared with those in fish fed diets containing 100% (FO 100) or 25% (FO 25) fish oil (80.3% *vs.* 89.7% and 92.7%, respectively; $p < 0.05$) (Figure 3.1).



(a) (b)

Figure 3.1. (a) Fish live weight (g) and (b) survival (%) of fish fed experimental diets during the 109 days of trial. Values are expressed as means ± standard error.

Brain

With regard to the brain, the dietary treatment did not affect the proportions of total SFA (32.9% of the total FA on average), MUFA (29.2%), PUFA (37.9%), n 3 PUFA (29.6%), or n 6 PUFA (8.0%) present in the tissue ($p > 0.05$) (Table 3.3). Nevertheless, the replacement of FO with VO decreased the relative content of EPA (3.01% vs. 2.81% and 2.85% in FO 100 vs. FO 25 and FO 0, respectively; $p < 0.001$) and DHA (23.8% vs. 21.4% in FO 100 and FO 25 vs. FO 0; $p < 0.01$), whereas it increased the content of ALA (0.81% vs. 1.63% vs. 2.66% in FO 100 vs. FO 25 vs. FO 0; $p < 0.001$).

Liver

In the liver, the replacement of FO with VO in the diet significantly decreased ($p < 0.001$) the proportion of the total SFA (24.3% and 23.3% *vs.* 20.2% of the total FA in FO 100 and FO 25 *vs.* FO 0), which was due to the lower proportion of C16:0 in VO than FO (13.9% and 13.4% *vs.* 11.6%) (Table 3.4).

Table 3.3. Fat content and fatty acid composition (% of total fatty acid content) of the brain in Mediterranean yellowtail fed the experimental diets. Values are expressed as LS means (n=12).

	Diet			*p*-value	RSD
	FO 100	**FO 25**	**FO 0**		
Fat, % WW	3.22	4.23	3.52	0.999	0.951
Fatty acids, %					
10:0	0.42	0.46	0.57	0.083	0.138
14:0	0.76	0.64	0.68	0.134	0.141
15:0	0.12^{b}	0.10^{a}	0.09^{a}	<0.001	0.000
16:0	16.31	16.54	16.21	0.378	0.563
17:0	0.19^{b}	0.14^{a}	0.14^{a}	0.006	0.000
18:0	11.42	11.76	11.45	0.367	0.625
20:0	0.46	0.44	0.44	0.475	0.045
22:0	0.79	0.76	0.75	0.618	0.105
24:0	2.41	2.35	2.20	0.387	0.318
Σ SFA	32.90	33.17	32.51	0.278	0.932
16:1 n-9	2.67^{b}	2.40^{a}	2.34^{a}	<0.001	0.114
17:1 n-10	0.55	0.49	0.52	0.824	0.235
18:1 n-9	20.33^{ab}	19.93^{a}	21.49^{b}	0.009	1.050
18:1 n-7	2.80^{b}	2.58^{ab}	2.46^{a}	0.005	0.148
20:1 n-9	0.70^{b}	0.59^{a}	0.59^{a}	<0.001	0.063
22:1 n-9	0.32	0.32	0.28	0.271	0.045
24:1 n-9	2.16	2.07	1.98	0.378	0.261
Σ MUFA	29.47	28.44	29.66	0.139	1.336
18:2 n-6c	4.25^{a}	4.39^{a}	5.55^{b}	0.016	1.080
20:2 n-6	0.26	0.23	0.25	0.298	0.055
20:4 n-6	2.65^{b}	2.51^{b}	2.27^{a}	<0.001	0.187
22:2 n-6	0.23^{b}	0.19^{ab}	0.18^{a}	0.028	0.045
22:4 n-6	0.22	0.22	0.18	0.150	0.055
Σ PUFA n-6	7.74	7.69	8.54	0.077	0.967
18:3 n-3	0.81^{a}	1.63^{b}	2.66^{c}	<0.001	0.644
20:3 n-3	0.10^{a}	0.16^{b}	0.19^{b}	<0.001	0.032
20:5 n-3	3.01^{b}	2.81^{a}	2.85^{a}	<0.001	0.118
22:5 n-3	2.13	2.26	2.14	0.483	0.219
22:6 n-3	23.83^{b}	23.83^{b}	21.43^{a}	0.006	1.668
Σ PUFA n-3	29.89	30.71	29.27	0.120	1.127
Σ PUFA	37.63	38.32	37.83	0.176	0.823
Σ n-6/ Σ n-3	0.26	0.25	0.30	0.098	0.041
DHA/EPA	7.95^{ab}	8.50^{b}	7.54^{a}	0.023	0.780

FO 100 diet: diet formulated with fish oil as lipid source; FO 25 diet: diet in which fish oil was included at a content of 25%; FO 0 diet: diet in which fish oil was totally substituted with vegetable oil. Abbreviations: WW, wet weight; RSD, residual standard deviation; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; DHA/EPA, 22:6 n-3/20:5 n-3. The FA <0.1% of the total FA (i.e. C12:0, C14:1, C18:3 n-6 and C30:3 n-6) are not given in table. Different superscript letters indicate significant statistical differences among diets ($p < 0.05$).

Table 3.4. Fat content and fatty acid composition (% of total fatty acid content) of the liver in Mediterranean yellowtail fed the experimental diets. Values are expressed as LS means (n=12).

	Diet			*p*-value	RSD
	FO 100	**FO 25**	**FO 0**		
Fat, % WW	26.6	30.1	25.7	0.721	9.296
Fatty acids, %					
14:0	1.55^{c}	1.08^{b}	0.79^{a}	<0.001	0.129
15:0	0.26c	0.15^{b}	0.10^{a}	<0.001	0.026
16:0	13.88^{b}	13.40^{b}	11.57^{a}	<0.001	0.885
17:0	0.49^{c}	0.32^{b}	0.24^{a}	<0.001	0.037
18:0	7.68^{b}	7.82^{b}	7.01^{a}	0.008	0.621
20:0	0.31^{c}	0.25^{b}	0.21^{a}	<0.001	0.014
24:0	0.06	0.07	0.05	0.369	0.020
Σ SFA	24.32^{b}	23.26^{b}	20.07^{a}	<0.001	1.317
16:1 n-9	3.26^{c}	2.23^{b}	1.64^{a}	<0.001	0.153
17:1 n-10	0.26	0.18	0.19	0.323	0.139
18:1 n-9	32.02^{a}	35.18^{b}	37.55^{c}	<0.001	1.371
18:1 n-7	7.30^{c}	5.77^{b}	4.95^{a}	<0.001	0.289
20:1 n-9	1.88^{b}	1.17^{a}	0.90^{a}	<0.001	0.314
22:1 n-9	0.37^{b}	0.24^{a}	0.16^{a}	<0.001	0.040
24:1 n-9	0.21^{b}	0.12^{a}	0.09^{a}	<0.001	0.063
Σ MUFA	45.29	44.90	45.47	0.613	1.431
18:2 n-6	15.23^{a}	15.21^{a}	16.96^{b}	0.002	1.10
18:3 n-6	0.12	0.11	0.11	0.482	0.026
20:2 n-6	1.23^{b}	0.95^{a}	0.88^{a}	<0.001	0.097
20:3 n-6	0.21^{c}	0.13^{b}	0.08^{a}	<0.001	0.021
20:4 n-6	0.89^{c}	0.53^{b}	0.39^{a}	<0.001	0.097
22:2 n-6	0.79^{c}	0.42^{b}	0.26^{a}	<0.001	0.052
22:4 n-6	0.26^{b}	0.08^{a}	0.05^{a}	<0.001	0.049
Σ PUFA n-6	18.73^{b}	17.43^{a}	18.74^{b}	0.036	1.207
18:3 n-3	2.48^{a}	8.60^{b}	11.27^{c}	<0.001	0.562
20:3 n-3	0.36^{a}	0.81^{b}	0.95^{c}	<0.001	0.117
20:5 n-3	2.21^{c}	1.45^{b}	1.10^{a}	<0.001	0.200
22:5 n-3	2.35^{c}	1.35^{b}	0.83^{a}	<0.001	0.199
22:6 n-3	4.26^{b}	2.29^{a}	1.57^{a}	<0.001	0.607
Σ PUFA n-3	11.66^{a}	14.45^{b}	15.72^{c}	<0.001	0.924
Σ PUFA	30.39^{a}	31.84^{a}	34.46^{b}	<0.001	1.703
Σ n-6/ Σ n-3	1.62^{b}	1.20^{a}	1.20^{a}	<0.001	0.113
DHA/EPA	1.93^{b}	1.59^{ab}	1.36^{a}	<0.001	0.249

FO 100 diet: diet formulated with fish oil as lipid source; FO 25 diet: diet in which fish oil was included at a content of 25%; FO 0 diet: diet in which fish oil was totally substituted with vegetable oil. Abbreviations: WW, wet weight; RSD, residual standard deviation; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; DHA/EPA, 22:6 n-3/20:5 n-3. The FA <0.1% if the total FA (i.e. C12:0, C14:1, C22:0 and C24:0) are not given in table. Different superscript letters indicate significant statistical differences among diets ($p \leq 0.05$).

Moreover, the substitution of FO with VO in the diet decreased the relative content of C16:1 n-9 (3.26% *vs.* 2.23% *vs.* 1.64% in FO 100 *vs*. FO 25 *vs*. FO 0) and C17:1 n-7 (7.30% *vs.* 5.77% *vs.* 4.95%), whereas it increased that of C18:1 n-9 (32.0% *vs.* 35.2% *vs.* 37.6%) ($p < 0.001$). Thus, the proportion of the total MUFA (45.2% on average) was not affected by the dietary treatment ($p > 0.05$). With regard to n-3 PUFA, the inclusion of less FO in the diet decreased the liver tissue's relative content of EPA (2.21% *vs.* 1.45% *vs.* 1.10% in FO 100 *vs*. FO 25 *vs*. FO 0), C22:5 n-3 (2.35% *vs*. 1.35% *vs*. 0.83%), and DHA (4.26% *vs*. 2.29% and 1.57%), whereas it greatly increased ($p < 0.001$) its content of ALA (2.48% *vs.* 8.60% *vs.* 11.3%), and thus the total n-3 PUFA content. As for n-6 PUFA, the substitution of FO with VO decreased the AA proportion of liver tissue (0.89% *vs.* 0.53% *vs.* 0.39%; $p < 0.001$) and increased that of LA (15.2% and 15.2% *vs*. 17.0% in FO 100 and FO 25 *vs*. FO 0; $p < 0.01$).

Visceral fat

In the visceral fat, the total substitution of FO with VO in the diet significantly decreased ($p < 0.001$) the proportion of the total SFA (23.6% and 23.8% *vs*. 22.5% of the total FA in FO 100 and FO 25 *vs*. FO 0) (Table 3.5). Moreover, the replacement of FO with VO decreased the C16:1 n-9 (4.07% *vs.* 2.98% *vs.* 2.48% in FO 100 *vs*. FO 25 *vs*. FO 0) and 18:1 n-7 (4.89% *vs.* 4.11% *vs.* 3.64%) proportions in the visceral fat, whereas it increased that of C18:1 n-9 (27.8% *vs.* 30.0% *vs.* 32.6%) ($p < 0.001$). With regard to n-3 PUFA, the inclusion of less FO in the diet decreased the EPA (3.68% *vs.* 2.76% *vs.* 2.07%), C22:5 n-3 (1.82% *vs.* 1.24% *vs.* 0.9%), and DHA (9.57% *vs.* 5.72% *vs.* 4.01%) proportions in the visceral fat, and increased that of ALA therein (3.76% *vs.* 9.44% *vs.* 12.0%) ($p < 0.001$), whereas the proportion of total n-3 PUFA (19.2% on average) was not affected by diet ($p > 0.05$). As for n-6 PUFA, the substitution of FO with VO increased the LA relative content (14.6% *vs.* 16.2% *vs.* 16.9%), which affected the total proportion of n-6 PUFA in this tissue (17.4% *vs.* 17.9% *vs.* 18.2%) ($p < 0.001$).

Muscle

In the red muscle, the replacement of FO with VO did not affect the proportion of total SFA (24.0% of the total FA on average) (Table 3.6). Among MUFA, the replacement of FO with VO increased the relative content of C18:1 n-9 (27.2% *vs.* 29.0% *vs.* 31.8% in FO 100 *vs.* FO 25 *vs.* FO 0), whereas it decreased that of C18:1 n-7 (4.21% *vs.* 3.65% *vs.* 3.26% in FO 100 *vs.* FO 25 *vs.* FO 0) and C20:1 n-9 (1.91% *vs.* 1.05% *vs.* 0.74%) ($p < 0.001$). For n-3 PUFA, the inclusion of less FO in the diet decreased the proportions of EPA (3.22% *vs.* 2.60% *vs.* 2.14%), C22:5 n-3 (2.12% *vs.* 1.58% *vs.* 1.29%), and DHA (14.0% *vs.* 9.38% *vs.* 7.47%) in red muscle tissue, whereas it increased that of ALA (2.99% *vs.* 7.68% *vs.* 9.84%) ($p < 0.001$). In addition, the partial and total substitution of FO with VO in the diet increased the total n-6 PUFA content of red muscle (14.7% *vs.* 16.0% and 16.7%) due to changes in the proportions of LA ($p < 0.001$).

In the white muscle, the substitution of FO with VO in the diet decreased the proportion of total SFA (24.0% and 23.8% *vs.* 22.6% in FO 100 *vs.* FO 25 *vs.* FO 0) and increased that of C18:1 n-9 (27.6% *vs.* 29.0% *vs.* 31.7%); this also decreased the relative content of C16:1 n-9 (3.78% *vs.* 2.90% *vs.* 2.36%) and C20:1 n-9 (1.76% *vs.* 1.09% *vs.* 0.76%) in white muscle tissue (Table 3.7). For n-3 PUFA, the inclusion of less FO in the diet decreased the EPA (3.41% *vs.* 2.87% *vs.* 2.26%), C22:5 n-3 (1.71% *vs.* 1.38% *vs.* 1.05%), and DHA (11.6% *vs.* 8.59% *vs.* 6.38%) content in white muscle tissue, whereas it increased the ALA content (3.76% *vs.* 9.44% *vs.* 12.0%) ($p < 0.001$). Nevertheless, the proportion of total n-3 PUFA (21.0% on average) was not affected ($p > 0.05$) by dietary treatment. Finally, the LA relative content in the white muscle was increased by this treatment (14.2% *vs.* 15.6% and 16.5%), which affected the total n-6 PUFA content (15.6% *vs.* 16.7% *vs.* 17.3%) ($p < 0.001$).

Table 3.5. Fat content and fatty acid composition (% of total fatty acid content) of the visceral fat in Mediterranean yellowtails fed the experimental diets. Values are expressed as LS means (n=12).

	Diet			*p*-value	RSD
	FO 100	**FO 25**	**FO 0**		
Fat, % WW	37.90	36.90	34.91	0.920	9.529
Fatty acids, %					
14:0	2.55^{c}	1.99^{b}	1.64^{a}	<0.001	0.094
15:0	0.32^{c}	0.22^{b}	0.16^{a}	<0.001	0.018
16:0	14.57^{a}	15.00^{b}	14.36^{a}	<0.001	0.293
17:0	0.44^{c}	0.32^{b}	0.26^{a}	<0.001	0.023
18:0	5.11^{a}	5.64^{b}	5.46^{b}	<0.001	0.207
20:0	0.36^{c}	0.32^{b}	0.29^{a}	<0.001	0.009
22:0	0.14^{a}	0.18^{b}	0.19^{b}	0.001	0.011
24:0	0.10	0.11	0.12	0.307	0.026
Σ SFA	23.63^{b}	23.80^{b}	22.53^{a}	<0.001	0.415
16:1 n-9	4.07^{c}	2.98^{b}	2.48^{a}	<0.001	0.184
17:1 n-10	0.37^{b}	0.20^{a}	0.16^{a}	<0.001	0.049
18:1 n-9	27.8^{a}	30.0^{b}	32.6^{c}	<0.001	0.547
18:1 n-7	4.89^{c}	4.11^{b}	3.64^{a}	<0.001	0.154
20:1 n-9	1.96^{c}	1.11^{b}	0.79^{a}	<0.001	0.116
22:1 n-9	0.40^{c}	0.22^{b}	0.15^{a}	<0.001	0.046
24:1 n-9	0.49^{c}	0.30^{b}	0.23^{a}	<0.001	0.038
Σ MUFA	39.95^{b}	38.87^{a}	40.07^{b}	<0.001	0.495
18:2 n-6c	14.64^{a}	16.17^{b}	16.89^{c}	<0.001	0.534
18:3 n-6	0.14^{b}	0.12^{a}	0.12^{a}	<0.001	0.003
20:2 n-6	0.92^{c}	0.64^{b}	0.48^{a}	<0.001	0.043
20:3 n-6	0.15^{b}	0.07^{ab}	0.06^{a}	0.023	0.075
20:4 n-6	0.67^{c}	0.43^{b}	0.32^{a}	<0.001	0.044
22:2 n-6	0.52^{c}	0.31^{b}	0.26^{a}	<0.001	0.044
22:4 n-6	0.34^{c}	0.18^{b}	0.10^{a}	<0.001	0.046
Σ PUFA n-6	17.37^{a}	17.92^{ab}	18.19^{b}	0.003	0.534
18:3 n-3	3.76^{a}	9.44^{b}	12.0^{c}	<0.001	0.843
20:3 n-3	0.22^{a}	0.26^{b}	0.28^{b}	<0.001	0.022
20:5 n-3	3.68^{c}	2.76^{b}	2.07^{a}	<0.001	0.150
22:5 n-3	1.82^{c}	1.24^{b}	0.90^{a}	<0.001	0.010
22:6 n-3	9.57^{c}	5.72^{b}	4.01^{a}	<0.001	0.634
Σ PUFA n-3	19.05	19.40	19.21	0.266	0.372
Σ PUFA	36.42^{a}	37.33^{b}	37.40^{b}	0.002	0.613
Σ n-6/ Σ n-3	0.91	0.92	0.95	0.104	0.035
DHA/EPA	2.60^{b}	2.07^{a}	1.94^{a}	<0.001	0.153

FO 100 diet: diet formulated with fish oil as lipid source; FO 25 diet: diet in which fish oil was included at a content of 25%; FO 0 diet: diet in which fish oil was totally substituted with vegetable oil. Abbreviations: WW, wet weight; RSD, residual standard deviation; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; DHA/EPA, 22:6 n-3/20:5 n-3. The FA <0.1% of the total FA (i.e. C10:0, C12:0, C14:1) are not given in table. Different superscript letters indicate significant statistical differences among diets (p 0.05).

Table 3.6. Fat content and fatty acid composition (% of total fatty acid content) of the red muscle in Mediterranean yellowtails fed the experimental diets. Values are expressed as LS means (n=12).

	Diet			*p*-value	RSD
	FO 100	**FO 25**	**FO 0**		
Fat, % WW	4.37	4.89	4.49	0.500	0.816
Fatty acids, %					
14:0	1.81	1.81	1.26	0.105	0.602
16:0	14.41	15.01	14.40	0.125	0.455
17:0	0.44^{c}	0.30^{b}	0.24^{a}	<0.001	0.035
18:0	6.53^{a}	6.91^{b}	6.83^{b}	<0.001	0.184
20:0	0.38^{b}	0.33^{a}	0.32^{a}	<0.001	0.017
22:0	0.12^{a}	0.15^{b}	0.16^{b}	<0.001	0.015
Σ SFA	23.88	24.65	23.33	0.061	0.769
14:1 n-9	0.11	0.12	0.10	0.956	0.100
16:1 n-9	3.14^{b}	2.57^{ab}	2.05^{a}	0.005	0.125
17:1 n-10	0.31^{b}	0.21^{a}	0.16^{a}	<0.001	0.011
18:1 n-9	27.21^{a}	29.04^{b}	31.76^{c}	<0.001	0.509
18:1 n-7	4.21^{c}	3.65^{b}	3.26^{a}	<0.001	0.208
20:1 n-9	1.91^{c}	1.05^{b}	0.74^{a}	<0.001	0.134
22:1 n-9	0.36^{b}	0.18^{a}	0.13^{a}	<0.001	0.088
24:1 n-9	0.41^{c}	0.26^{b}	0.20^{a}	<0.001	0.038
Σ MUFA	37.62^{ab}	37.08^{a}	38.37^{b}	0.044	0.651
18:2 n-6c	13.05^{a}	14.83^{b}	15.76^{c}	<0.001	0.552
18:3 n-6	0.12	0.11	0.10	0.053	0.015
20:2 n-6	0.78^{b}	0.58^{a}	0.45^{a}	<0.001	0.027
20:4 n-6	0.91^{c}	0.65^{b}	0.54^{a}	<0.001	0.055
22:2 n-6	0.40^{c}	0.24^{b}	0.16^{a}	<0.001	0.031
22:4 n-6	0.51^{c}	0.29^{b}	0.21^{a}	<0.001	0.040
Σ PUFA n-6	14.70^{a}	15.95^{b}	16.68^{b}	<0.001	0.513
18:3 n-3	2.99^{a}	7.68^{b}	9.84^{c}	<0.001	0.705
20:3 n-3	0.20^{a}	0.25^{b}	0.27^{b}	<0.001	0.021
20:5 n-3	3.22^{c}	2.60^{b}	2.14^{a}	<0.001	0.116
22:5 n-3	2.12^{c}	1.58^{b}	1.29^{a}	<0.001	0.155
22:6 n-3	14.00^{c}	9.38^{b}	7.47^{a}	<0.001	0.946
Σ PUFA n-3	22.56^{b}	21.50^{ab}	21.01^{a}	0.036	0.884
Σ PUFA	38.50	38.27	38.30	0.925	0.881
Σ n-6/ Σ n-3	1.54^{b}	1.35^{a}	1.26^{a}	<0.001	0.083
DHA/EPA	4.34^{b}	3.61^{a}	3.49^{a}	0.001	0.280

FO 100 diet: diet formulated with fish oil as lipid source; FO 25 diet: diet in which fish oil was included at a content of 25%; FO 0 diet: diet in which fish oil was totally substituted with vegetable oil. Abbreviations: WW, wet weight; RSD, residual standard deviation; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; DHA/EPA, 22:6 n-3/20:5 n-3. The FA <0.1% of the total FA (i.e. C12:0, C15:0, C20:3 n-6 and C24:0) are not given in table. Different superscript letters indicate significant statistical differences among diets ($p \leq 0.05$).

Table 3.7. Fat content and fatty acid composition (% of total fatty acid content) of the white muscle in Mediterranean yellowtails fed the experimental diets. Values are expressed as LS means (n=12).

	Diet			*p*-value	RSD
	FO 100	**FO 25**	**FO 0**		
Fat, % WW	5.84	5.94	6.03	0.941	0.770
Fatty acids, %					
14:0	2.28 ^{c}	1.80 ^{b}	1.47 ^{a}	<0.001	0.132
16:0	14.97 ^{b}	14.78 ^{b}	14.22 ^{a}	0.035	0.366
17:0	0.39 ^{c}	0.30 ^{b}	0.23 ^{a}	<0.001	0.037
18:0	5.89	6.21	6.13	0.050	0.171
20:0	0.36 ^{c}	0.33 ^{b}	0.32 ^{a}	<0.001	0.013
22:0	0.12 ^{a}	0.15 ^{b}	0.16 ^{b}	0.020	0.032
Σ SFA	23.94 ^{b}	23.80 ^{b}	22.63 ^{a}	0.009	0.494
14:1 n-9	0.28 ^{c}	0.22 ^{b}	0.15 ^{a}	<0.001	0.038
16:1 n-9	3.78 ^{c}	2.90 ^{b}	2.36 ^{a}	<0.001	0.200
17:1 n-10	0.33 ^{c}	0.22 ^{b}	0.17 ^{a}	<0.001	0.026
18:1 n-9	27.55 ^{a}	29.02 ^{b}	31.72 ^{c}	<0.001	0.759
18:1 n-7	4.15 ^{c}	3.56 ^{b}	3.20 ^{a}	<0.001	0.165
20:1 n-9	1.76 ^{c}	1.09 ^{b}	0.76 ^{a}	<0.001	0.175
22:1 n-9	0.29 ^{c}	0.19 ^{b}	0.12 ^{a}	<0.001	0.045
24:1 n-9	0.35 ^{c}	0.27 ^{b}	0.19 ^{a}	<0.001	0.048
Σ MUFA	38.63	37.50	38.63	0.050	0.615
18:2 n-6c	14.15 ^{a}	15.59 ^{b}	16.46 ^{b}	<0.001	0.552
18:3 n-6	0.14 ^{b}	0.13 ^{a}	0.12 ^{a}	0.001	0.009
20:2 n-6	0.86 ^{c}	0.63 ^{b}	0.49 ^{a}	<0.001	0.059
20:4 n-6	0.79 ^{c}	0.61 ^{b}	0.48 ^{a}	<0.001	0.076
22:2 n-6	0.42 ^{c}	0.28 ^{b}	0.19 ^{a}	<0.001	0.037
22:4 n-6	0.41 ^{c}	0.26 ^{b}	0.18 ^{a}	<0.001	0.052
Σ PUFA n-6	16.45 ^{a}	17.20 ^{ab}	17.80 ^{b}	0.001	0.493
18:3 n-3	3.93 ^{a}	8.25 ^{b}	10.84 ^{c}	<0.001	1.141
20:3 n-3	0.20 ^{a}	0.23 ^{b}	0.25 ^{c}	<0.001	0.019
20:5 n-3	3.41 ^{c}	2.87 ^{b}	2.26 ^{a}	<0.001	0.198
22:5 n-3	1.71 ^{c}	1.38 ^{b}	1.05 ^{a}	<0.001	0.136
22:6 n-3	11.63 ^{c}	8.59 ^{b}	6.38 ^{a}	<0.001	1.196
Σ PUFA n-3	20.60	21.21	20.77	0.830	0.773
Σ PUFA	37.48	38.70	38.74	0.500	0.825
Σ n-6/ Σ n-3	0.80	0.81	0.86	0.086	0.069
DHA/EPA	3.43 ^{b}	2.95 ^{a}	2.82 ^{a}	0.019	0.234

FO 100 diet: diet formulated with fish oil as lipid source; FO 25 diet: diet in which fish oil was included at a content of 25%; FO 0 diet: diet in which fish oil was totally substituted with vegetable oil. Abbreviations: WW, wet weight; RSD, residual standard deviation; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; DHA/EPA, 22:6 n-3/20:5 n-3. The FA <0.1% of the total FA (i.e. C12:0, C15:0, C20:3 n-6 and C24:0) are not given in table. Different superscript letters indicate significant statistical differences among diets (p 0.05).

DISCUSSION

Successful growth performance may be achieved in fish fed with diets containing low levels of FO as long as their minimum EFA requirements are met (Tocher, 2010). In the larval stages, DHA plays a key role in promoting the development of neural membranes and eyes (Ishizaki et al., 2001), improving growth, vitality, and survival (Furuita et al., 1996; Matsunari et al., 2013; Mourente 2003), and preventing swimming disorders like spinning and disorientation (Furuita et al., 1996). The accumulation of DHA in the brain during fish development has been measured in several marine species (Mourente, 2003), which also show very low rates of DHA biosynthesis. Thus, any DHA deficiency during larval development will have serious consequences for the successful performance of fish larvae (Mourente, 2003). Moreover, DHA is essential for the development of schooling behaviour in the Mediterranean yellowtail (Masuda and Tsukamoto, 1999; Masuda et al., 1998; Masuda et al., 1999). Data in the published literature show that larvae (aged 3-7 days after hatching (dah)) of *Seriola dumerili* fed with rotifers (Matsunari et al., 2013) require a supply of DHA equal to 1.5% of the dry matter (DM) in their diet. Larvae (7 mm in length) of the related Japanese amberjack (*S. quinqueradiata* Temminck & Schlegel, 1845) fed with *Artemia* required a diet containing 3.9% of n-3 HUFA (2.5% and 1.3% DM of EPA and DHA, respectively) in a previous study (Furuita et al., 1996). Further, in *S. rivoliana* Valenciennes, 1833 larvae (aged 30 to 50 dah) had higher survival rates and better stress resistance when fed microdiets containing 3.2% DM of DHA (Mesa-Rodriguez et al., 2018).

In this experiment, the dietary treatments tested provided a minimum n-3 HUFA supply of 0.75% DM, which should be sufficient to meet the EFA requirements of most marine species (minimum value: 0.5% DM in juveniles and sub-adults of marine species) (Tocher, 2010). Under different conditions, the total EFA requirements for juveniles (39 to 387 g of live weight in a 154-day feeding trial) of Mediterranean yellowtail have been estimated to be 1.2% DM (Monge-Ortiz et al., 2018). In fact, juvenile fish, although they still need EFA, are likely to require lower levels of EPA and DHA in their diet than those needed in the larval stage. Nevertheless, dietary DHA deprivation in juveniles of pelagic marine species, such as carangids and tunids, can be particularly deleterious because of their fast growth rates (Mourente, 2003), especially when they are fed diets containing low levels of FO.

During the grow-out phase, EFA deficiencies may reduce fish growth. In this regard, a substitution of 60% of the FO with VO in the diet is considered acceptable (Nasopoulou et al., 2012). For carangid species, the growth performance of Japanese yellowtail (*S. quinqueradiata*) (average initial weight: 252 g; final weight 412 g) was not affected by the full replacement of FO with olive oil in a previous short-term feeding trial (40 days) (Bowyer et al., 2012). On the contrary, yellowtail kingfish (*S. lalandi* Valenciennes, 1833) showed impaired growth rates when FO was totally substituted with canola or sunflower oil in their diet during a 5-week-long feeding trial (with a weight change from 96 to 260 g) (Bowyer et al., 2012).

In the present study of *S. dumerili* (grown from 176 to 418 g of live weight, with 109 days of feeding), diets containing only VO as the lipid source had no detrimental impacts on the growth performance of these fish. Nevertheless, the survival rate was lower in fish fed diets without fish oil compared with those in fish fed diets containing 100% (FO 100) or 25% (FO 25) fish oil.

Indeed, Monge-Ortiz et al. (2018) reported that diets including at least 525 g kg^{-1} of fishmeal (FM) are likely to supply enough LC-PUFA to meet the needs of fish, even with the complete substitution of FO (with fish reaching a final weight of 397 g). In fact, FM usually contains up to 8-15% DM of crude lipid, 30-35% of which is composed of n-3 LC-PUFA (Turchini et al., 2009). In the present study, the experimental diets contained 350 g kg^{-1} of FM, which likely met the FA requirements of the studied fish during the grow-out phase.

The present study measured fat storage in different tissues, including the muscles, brain, liver, and visceral fat. Indeed, the locations of fat storage differ within and between fish species (Bell et al., 2006; Stoknes et al., 2004; Tocher and Harvie, 1988). In a previous study (Rodriguez-Barreto et al., 2012), a high lipid content (53% DM) was found in the liver of farmed *S. dumerili*, which was consistent with that recorded in the present study (60% DM) and higher than the fat content of the muscle tissue (12% DM) (Rodriguez-Barreto et al., 2012).

The replacement of FO with VO in fish diets may lead to the accumulation of fat in the fish liver, generating a fatty liver syndrome, which may be the result of inefficient nutrient utilisation and increased rates of lipid peroxidation (Benedito-Palos et al., 2008; Piedecausa et al., 2007). Nevertheless, we did not find differences in the liver fat content of the tested fish, even when they were fed with diets containing only VO as the lipid source, which was consistent with the findings of previous studies done on European seabass (Richard et al., 2006), gilthead sea bream (Bouraoui et al., 2011), and turbot (*Psetta maxima* (L., 1758)) (Regost et al., 2003).

Regardless of whether FO replacement affects fish growth and the lipid content of their tissues, its impact on fatty acid composition will vary depending on the dietary lipid content and source, as well as on the tissues considered (Turchini et al., 2019). The fatty acid signatures of fish tissues are closely related to their dietary FA composition (Turchini et al., 2019). Complete or partial FO replacement with VO blends is known to affect the FA compositions of muscles, fish organs, and fat storage tissues (Turchini et al., 2019). However, the magnitude of the changes in FA signatures varies among different fish species and tissues (Bell et al., 2001; Bell et al., 2003; Torstensen et al., 2004).

The FA signature of several wild and farmed fish was previously measured in muscle and liver tissues, but to our knowledge, few studies have investigated the FA signatures of other fatty tissues, like those of the visceral fat and brain. Specifically, in wild and farmed Mediterranean yellowtail, the FA composition has been analysed in the muscles, liver, ovary (Haouas et al., 2010; Monge-Ortiz et al., 2018; Rodriguez-Barreto et al., 2014; Rodriguez-Barreto et al., 2017; Saito, 2012), and eggs (Rodriguez-Barreto et al., 2014). Other studies have been performed in other *Seriola* species, such as *S. lalandi* (Bowyer et al., 2012; O'Neill

et al., 2015; Rombenso et al., 2018), *S. quinqueradiata* (Seno-O et al., 2008; Fukada et al., 2017), *S. dorsalis* (Gill, 1863) (Bergman et al., 2018; Stuart et al., 2018), and *S. rivoliana* (Mesa-Rodriguez et al., 2018), which mainly focused on the FA composition of muscle tissues.

In the liver of Mediterranean yellowtail, we found a high proportion of MUFA and a low proportion of SFA, likely because their C16:0, C18:0, and C22:0 content was sufficient to maintain or even exceed the requirements of liver metabolic functions. In the brain and visceral fat, PUFA were highly represented (representing on average 37.9 and 37.1% of the total FA content in the brain and visceral fat, respectively). In fact, PUFA (especially C18 FA, such as LA and ALA) are generally stored in non-lipogenic tissues, especially the visceral fat, and are likely used as metabolic substrates for β-oxidation (Torstensen et al., 2004; Bell et al., 2003; Stubhaug et al., 2007). Moreover, the brain contained a high proportion of DHA (on average 23.0% of the total FA), which is known to regulate membrane fluidity, the blood-brain barrier, and the activities of certain enzymes, such as ionic channel proteins and nerve growth factors, both in mammals (Ikemoto et al., 2000; Kitajka et al., 2020) and fish (Matsunari et al., 2013; Mesa-Rodriguez et al., 2018; Mourente, 2003). In salmonids, Atlantic cod, and flatfishes, DHA is also present at high levels in the brain (Mourente et al., 1991; Kreps et al., 1969; Tocher and Harvie, 1988), which agrees with our findings.

Consistent with other studies (Bell et al., 2001; Caballero et al., 2002; Campos et al., 2019), the muscle, liver, and visceral fat FA signatures of Mediterranean yellowtail found in our study reflected the dietary FA profiles of these fish, with the FA signatures of these tissues thus showing a low robustness to changes in the dietary lipid sources from FO to VO. In contrast, the brain FA signature was less strongly affected by the dietary FA composition, whereas only ALA, EPA and DHA changed. Nevertheless, in European seabass, brain lipids appeared to be sensitive to dietary lipid inputs (Pagliarani et al., 1986). On the contrary, a more recent study in juvenile European seabass fed n-3 LC-PUFA-deficient diets showed that polar lipids in their neural tissues had the highest capacity to regulate and preserve their DHA content (Skalli et al., 2006). In the present study, fish fed the diet with 100% VO showed a 10% lower DHA content in the brain than those fed diets with fish oil (FO 100 and FO 25) and a lower survival, which could have been related to some effects of EFA deficiency in them. In contrast, the administration of the FO 25 diet did not decrease the DHA proportion in the brain, which suggests that the substitution of 75% of the FO in the diet with VO could be suitable for use in diets for rearing Mediterranean yellowtail juveniles from 175 to 420 g of live weight. Further studies are necessary to confirm these results over longer feeding periods.

CONCLUSIONS

This study provided new findings on the FA signatures in different tissues of *Seriola dumerili* fed diets with decreasing levels of FO included in them. The FA composition of the diet had a strong effect on the FA compositions of muscle, liver, and visceral fat tissues. The

brain had a FA signature that was more robust to dietary changes, whereas only some EFA (EPA and DHA) decreased when fish oil was totally replaced by vegetable oil.

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CHAPTER 4

RECOVERY OF FATTY ACID COMPOSITION IN MEDITERRANEAN YELLOWTAIL (*SERIOLA DUMERILI*, RISSO 1810) FED A FISH-OIL FINISHING DIET (Second contribution)

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ABSTRACT

The present study evaluated the effects of wash-out on the fatty acid (FA) composition in the muscles of Mediterranean yellowtail. After 109 days during which fish were fed either a fish oil (FO)-based diet (FO 100) or a diet (FO 0) in which FO was completely substituted by vegetable oils, all fish were subjected to a wash-out with FO 100 diet for 90 days. The FA profile of muscles in fish fed FO 0 diet at the beginning of the experiment reflected that of dietary vegetable oils, rich in linoleic acid (LA), and α-linolenic acid (ALA), and was deficient in AA (arachidonic acid), EPA (eicosapentaenoic acid), and DHA (docosahexaenoic acid). No essential FA were fully restored in fish previously fed FO 0 diet on 45th or 90th day of wash-out. At the end of wash-out, the FA composition showed that AA, EPA, and DHA in the white muscles increased by +33%, +16%, and +43% ($p < 0.001$), respectively. Similarly, AA and DHA in the red muscles increased by +33% and +41% respectively, while EPA remained similar to fish fed FO 0 diet exclusively. Therefore, a 90-d wash-out can partially improve the FA profile in muscles of Mediterranean yellowtail previously fed vegetable oil-based diets.

Keywords: wash-out; greater amberjack; thrombogenicity; atherogenicity; EPA; DHA

INTRODUCTION

Species diversification is considered as a tool for sustainable development of aquaculture in near future (Chaves-Pozo et al., 2019). The Mediterranean yellowtail (*S. dumerili*, Risso 1810) is one of the most interesting candidates for European aquaculture diversification (Roo et al., 2019). This carnivorous fish shows high growth rates (weighs 6 kg within 2.5 years of culture) and has excellent flesh quality and worldwide consumer acceptance (Roo et al., 2019; Sicuro and Luzzana, 2016).

Fish oil (FO) has been considered as the major lipid source in aquafeeds for carnivorous fish for a long time (Codabaccus et al., 2013; Turchini et al., 2009). However, FO and fishmeal production are no longer sustainable (Fountoulaki et al., 2009; Shepherd et al., 2013). During the last couple of decades, the global supply of FO has been tightly regulated and has remained low and stable, whereas the aquaculture industry has expanded rapidly. Consequently, FO prices have increased, prompting researchers and industries to develop alternative lipid sources that can be included in aquafeeds (Naylor et al., 2009).

Vegetable oils (VO) (e.g., soybean oil, linseed oil, palm oil, and rapeseed oil) have been widely tested as alternatives to FO in aquafeeds, specifically for their impact on fish growth and flesh quality (Izquierdo et al., 2005; Monge-Ortiz et al., 2018; Montero et al., 2005). However, VO are rich in C18 polyunsaturated fatty acids (PUFA), but are devoid of highly unsaturated fatty acids (HUFA), such as eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3), which are essentials for growth, health, reproduction, and body functions of fish (Turchini et al., 2009). Most freshwater fish are capable of desaturating and elongating C18 fatty acids (i.e. α-linolenic acid C18:3 n-3) into EPA and DHA (Tocher, 2010), whereas marine carnivorous species have lost this bioconversion ability (Mourente and Bell, 2006; Turchini et al., 2009). Therefore, these essential fatty acids (EFA) must be included in marine aquafeeds to meet their EFA requirement (approximately 4%) (Tocher, 2010). According to the literature, the partial substitution (up to 60%) of FO with VO in the diets of marine fish during the grow-out phase does not affect their mortality and growth rates (Fountoulaki et al., 2009; Izquierdo et al., 2005).

Nevertheless, a major limitation of replacing dietary FO with VO is its effect on fillet composition. Since the fatty acid (FA) profile of fish tissues reflects those of the diets consumed by the fish (Turchini et al., 2003; Turchini et al., 2009), fillets of fish fed diets containing high VO levels may contain a low amount of n-3 HUFA (Jobling et al., 2004; Monge-Ortiz et al., 2018), which are considered beneficial for human health (Connor, 2000; Ruxton et al., 2004; Seierstad et al., 2005; Williams, 2000). High intakes of EPA and DHA are recommended to prevent premature birth and low birth weight (Olsen and Secher, 2002), and also to reduce cardiovascular disease risks (Breslow, 2006; Rosenberg, 2002). These FA are also anti-arrhythmic in nature and reduce platelet aggregation and blood triacyl glyceride levels (Hu et al., 2002). For these reasons, health organizations of several countries

recommend a daily intake of 1.2-2.0 g/d of n-3 HUFA (Izquierdo et al., 2005), adjusted according to eating habits, age, and sex.

Therefore, to restore the healthy FA profile of fish fillets during fish farming, finishing strategies before slaughtering (i.e., wash-out period) have been proposed (Jobling, 2004; Robin et al., 2003; Turchini et al., 2006; Turchini et al., 2009). The efficacy of a finishing strategy including FO diets after a grow-out period supplemented with alternative lipid sources has been tested in marine fish species such as gilthead seabream (Fountoulaki et al., 2009; Izquierdo et al., 2005), European seabass (Montero et al., 2005; Mourente et al., 2006), red seabream (Glencross et al., 2003), and Senegalese sole (Reis et al., 2014), as well as in freshwater species such as Murray cod (Turchini et al., 2006; 2007), sunshine bass (Lane et al., 2006), and rainbow trout (Yildiz et al., 2018; Thanuthong et al., 2012), and in Atlantic salmon (Bell et al., 2003ab; 2004).

Finishing strategy can also differently affect the FA composition of the red and the white muscles that make up the fish fillets (Carpene et al., 1998). The former (approx. 10% of the fillet) are located in strips along the midline and assure a steady aerobic swimming by an aerobic metabolism based on lipids (McKenzie et al., 2011; Teulier et al., 2019); the latter represent the bulk of the fillet [38] and use carbohydrates for their energy metabolism (Teulier et al., 2019). Additionally, white and red muscles show different sensorial traits and fatty acid composition (Palmeri et al., 2007).

To date, there is no information about the effects of a finishing/wash-out strategy in Mediterranean yellowtail with special emphasis on the time required to restore the FA profile and the sensitivity of selected FA to a wash-out diet. Thus, the present study aimed to evaluate the effects of a wash-out diet and the time required to restore the FA composition of the white and red muscles in Mediterranean yellowtail that was previously fed a vegetable oil-based diet.

MATERIALS AND METHODS

Experimental diets

Two isoproteic (59% crude protein, 50% digestible protein) and isolipidic (15% crude lipid) extruded diets were formulated: a control diet (FO 100) with fish oil as a unique lipid source and a diet (FO 0) in which FO was completely substituted by a blend of vegetable oils (linseed oil, sunflower oil, and palm oil in the ratio of 4:3:3). Diets were prepared using a cooking-extrusion process with a semi-industrial twin-screw extruder (CLEXTRAL BC-45; Firmity, St Etienne, France), at 100 rpm speed screw, 110 °C temperature, and at 40–50 atm pressure to obtain pellets with 2–3 mm diameter. Ingredients and chemical composition of the experimental diets are presented in Table 4.1 and their fatty acid composition is given in Table 4.2.

Table 4.1. Ingredients (g kg^{-1} as fed) and proximate composition (% dry matter) of the experimental diets.

	Diet	
	FO 100	**FO 0**
Fishmeal	350	350
Wheat	100	100
Wheat gluten	140	140
Defatted soybean meal	185	185
Iberian meat meal	110	110
Fish oil	95	0
Linseed oil	-	38
Sunflower oil	-	28
Palm oil	-	29
Multivitamin and minerals mix	20	20
Proximate composition		
Dry matter, %	87.4	89.1
Crude protein, % DM	58.8	58.8
Crude lipid, % DM	15.9	16.6
Ash, % DM	8.4	8.3
Gross energy, MJ kg^{-1} DM	24.3	24.4

Fish oil (FO) 100 diet: Diet formulated with fish oil as lipid source; FO 0 diet: Diet in which fish oil was totally substituted with vegetable oil. [1] Vitamins and mineral mixture (values are g kg^{-1}): Premix, 25; Hill, 10; DL-α-tocopherol, 5; ascorbic acid, 5; $(PO_4)_2Ca_3$, 5. Premix composition (values are IU kg^{-1}): Retinol acetate, 1,000,000; calcipherol, 500; DL-α-tocopherol, 10; menadione sodium bisulphite, 0.8; hidroclorhidrate thiamine, 2.3; riboflavin, 2.3; pyridoxine hydrochloride, 15; cyanocobalamin, 25; nicotinamide, 15; pantothenic acid, 6; folic acid, 0.65; biotin, 0.07; ascorbic acid, 75; inositol, 15; betaine, 100; polypeptides, 12.

Table 4.2. Fatty acid composition (% of total fatty acid content) of the experimental diets.

	Diet	
	FO 100	**FO 0**
14:0	3.28	1.65
16:0	18.88	18.93
17:0	0.53	0.16
18:0	5.07	4.71
∑ SFA	27.79	25.46
16:1 n-9	4.22	1.82
18:1 n-9	27.14	32.67
18:1 n-7	3.94	2.44
22:1 n-9	0.32	0.06
∑ MUFA	35.62	36.99
18:2 n-6	12.66	14.86
18:3 n-6	0.10	0.09
20:3 n-6	0.10	0.04
20:4 n-6	1.02	0.35
22:4 n-6	0.24	0.09
∑ n-6 PUFA	14.12	15.43
18:3 n-3	2.24	14.60
20:3 n-3	0.15	0.06
20:5 n-3	5.81	2.77
22:5 n-3	1.29	0.42
22:6 n-3	12.98	4.28
∑ n-3 PUFA	22.47	22.13
∑ PUFA	36.59	37.56
∑ n-6/ ∑ n-3	0.63	0.70
DHA/EPA	2.23	1.54

FO 100 diet: diet formulated with fish oil as lipid source; FO 0 diet: diet in which fish oil was totally substituted with vegetable oil. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; DHA/EPA, 22:6 n-3/20:5 n-3.

In vivo trial

The trial was performed at the Laboratory of Aquaculture (LAC) of the Department of Animal Science at the Polytechnic University of Valencia (Valencia, Spain) in accordance with the protocol approved by the Committee of Ethics and Animal Welfare of the Universitat Politècnica de València (UPV) and following the Spanish Royal Decree 53/2013 about the protection of animals used for scientific purposes. The facility presented a thermo-regulated recirculation seawater system (65 m^3 capacity), with a rotary drum type filter and a mechanical gravity biofilter of 2 m^3, equipped with aerated cylindrical fiberglass tanks of 1750 L capacity. Water temperature during the trial was 21.5 ± 2.4°C.

Yellowtails of both sexes, 11-12 months of age, used in this trial underwent a grow-out period in which they were fed FO 100, FO 25 (75% vegetal oil) and FO 0 diets (three tanks/treatment; 25 fish/tank) for 109 d (Bordignon et al., 2020). Then, the fish that were fed FO 0 and FO 100 diet were moved into 6 tanks and were fed FO 0 diet as the grow-out or FO 100 diet for the next 90 d (wash-out period), as per the following feeding plans: FO 0/FO 0, FO 0/FO 100, and FO 100/FO 100 (2 tanks per experimental group; 15 fish per tank) (Figure 4.1). Fish that were previously fed FO 25 diet were not considered for the present study.

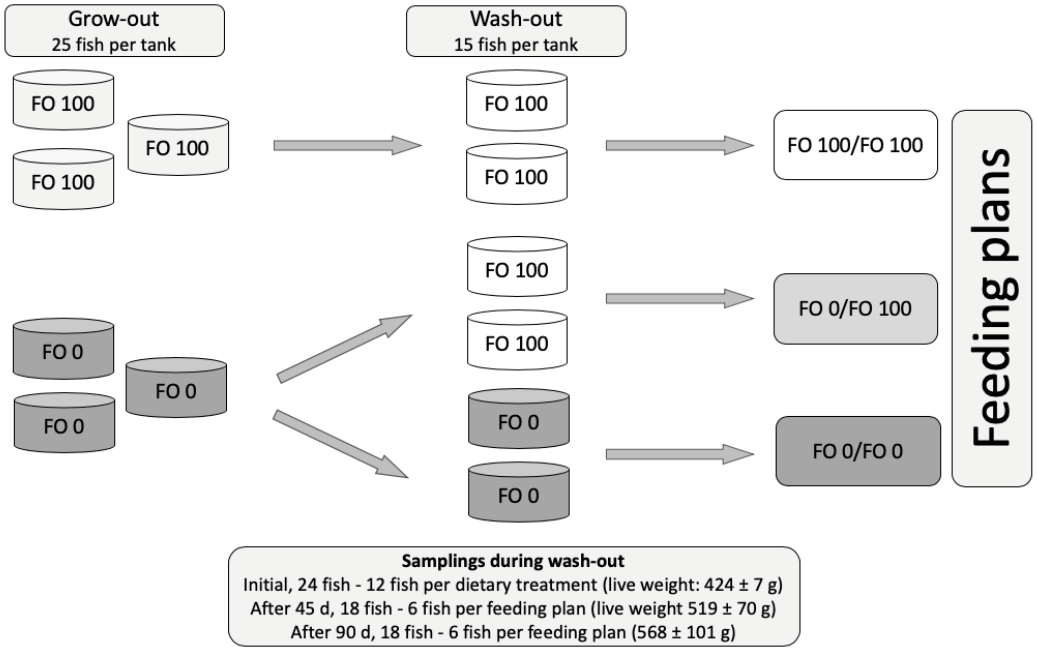

Figure 4.1. Scheme of feeding plans and samplings. During grow-out (109 d), fish were fed FO 100 diet (formulated with fish oil as lipid source) or FO 0 diet (formulated with vegetable oil as lipid source) (three tanks per experimental diet). At the end of grow-out, the same fish were moved into 6 tanks and fed FO 100 diet or FO 0 diet for the wash-out period (90 d) resulting into three feeding plans: FO 0/FO 0, FO 0/FO 100, and FO 100/FO 100 (two tanks per feeding plan). Fish were sampled at the beginning, and after 45 d and 90 d of wash-out.

Feed was offered by hand, twice a day (at 09.00 and 16.00), six days a week, until apparent visual satiation. Fish were weighed at the beginning, at 45 d and at 90 d of wash-out. Fish weight was used to calculate the specific growth rate.

At the beginning of the wash-out phase, 12 fish per treatment were euthanized with a lethal bath of clove oil (150 mg L^{-1}) and were dissected for fillet sampling. During the wash-out period, other 6 fish per treatment were euthanized and dissected with the same procedure after 45 d and after 90 d (Figure 4.1). At each sampling (beginning, 45 d and 90 d of wash-out), the right whole fillets (without skin and bone) were excised, vacuum packed, and stored at –80 °C until analyses.

Chemical analysis

Experimental diets were analysed following AOAC procedures (AOAC, 1990): dry matter (incinerated at 105°C to constant weight), ash (incinerated at 550 °C to constant weight) and crude lipid of diets and fish muscles were extracted with diethyl ether (ANKOM^{XT10}; ANKOM Technology, Macedon, NY, USA). Crude protein was determined following the Dumas combustion method, using a LECO CN628 apparatus (LECO, St Joseph, MI, USA). Gross energy content (GE) was calculated according to Brouwer (1965), from the C (g) and N (g) balance in the feed (GE = 51.8 × C − 19.4 × N). All analyses were performed in triplicate except for fatty acids, which were analysed as single samples.

For each fish, the white and red muscles of each fillet were separately ground, freeze-dried and ground again before their chemical analyses. Fatty acid methyl esters (FAMEs) were obtained directly from freeze-dried samples (O'Fallon et al., 2007). One millilitre of tridecanoic acid (C13:0) was used as an internal standard. Then, 0.7 mL of 10 N KOH and 5.3 mL of HPLC grade methanol were added to the tubes. Tubes were incubated at 55 °C in a thermoblock for 1.5 h, and underwent vigorous shaking for 5 s at an interval of 20 min. The tubes were cooled down to ambient temperature in a water bath, and 1.5 mL of HPLC grade hexane was added to the reaction tubes, which were then vortexed and centrifuged at 1006 × *g* for 5 min. In the next step, the hexane layer containing the FAMEs, was transferred into separate vials for gas chromatography. The vials were kept at –80 °C until they were analysed by gas chromatography. The FAMEs were analysed in a Focus Gas Chromatograph (Thermo, Milan, Italy) equipped with a split/splitless injector and a flame ionisation detector. Separation of the methyl esters was performed in a SPTM 2560 fused silica capillary column (Supelco, PA, USA) (film thickness: 100 m × 0.25 mm × 0.2 μm). Helium was used as the carrier gas, at a flow rate of 20 cm s^{-1}. Samples were injected with a split ratio of 1:100.

The initial oven temperature set at 140 °C, was kept constant for 5 min, and then increased to 240 °C at the rate of 4 °C min^{-1}, which was then maintained for 30 min. The FA were identified by comparing their retention times with those of the standards supplied by Supelco (Sigma-Aldrich, St. Louis, Missouri, USA). The content of each FA was expressed as the percentage of the total FA content.

Nutritional indexes

The index of atherogenicity (IA) and the index of thrombogenicity (IT) were calculated according to Ulbricht and Southgate (1991) as stated below, where MUFA are monounsaturated fatty acids:

$$IA = (C12:0 + 4 \times C14:0 + C16:0) / (MUFA + \sum n\text{-}6 + \sum n\text{-}3)$$

$$IT = (C14:0 + C16:0 + C18:0) / [(0.5 \times MUFA + 0.5 \times \sum n\text{-}6 + 3 \times \sum n\text{-}3) + (\sum n\text{-}3 / \sum n\text{-}6)$$

Statistical analysis

The data of growth performance, muscle lipid content, FA composition and nutritional indexes at the start of the wash-out phase were analysed by analysis of variance with the diet fed during grow-out phase (FO 0, FO 100) as the main effect. The same data of fillets collected on the 45th and 90th day of wash-out were analysed by ANOVA with the feeding plan (FO 0/FO 0, FO 0/FO 100, FO 100/FO 100) as the main effect. The PROC GLM of the Statistical Analysis System (SAS, 2013) was used for all analyses. Adjusted means were compared by Bonferroni t-test. Differences between means with $p \leq 0.05$ were accepted as statistically significant differences.

The data of FA per mg of tissue as affected by the dietary treatments were analysed as described for FA composition and are available as supplementary materials (Tables S4.1, S4.2 and S4.3).

RESULTS

The feeding plan did not affect fish growth (Table 4.3). Fish reached an average weight of 490 g and 624 g after 45 and 90 d of wash-out, respectively, corresponding to SGR (specific growth rate) equal to 0.45% and 0.55 % per d (Table 4.3).

At the beginning of the wash-out period, the FA profile in the white muscles of the fish fed FO 0 diet showed lower levels of saturated FA (SFA) (−6.1%), arachidonic acid (AA; C20:4 n-6) (−38%), EPA (C20:5 n-3) (−34%), DHA (C22:6 n-3) (−44%) but showed higher ALA (C18:3 n-3) (+176%; $p < 0.001$), and total n-6 (+11%) when compared to those seen in fish fed FO 100 diet. The higher total n-6 could be attributed to changes in the levels of LA (C18:2 n-6) (+16%) (Table 4.4). Similarly, the red muscles of the fish fed FO 0 diet showed lower levels of AA (−41%), EPA (−35%), and DHA (−47%), and higher ALA (+238%; $p < 0.001$) and total n-6 (+14%) (+21%), when compared to those seen in fish that were fed FO 100 diet (Table 4.4). The increase in total n-6 (+14%) could be attributed to the changes in LA.

The dietary treatment did not affect the average levels of C16:0 (14.47%), C18:0 (6.29%,), and total n-3 (21.39%) in the white muscles after 45 days of wash-out (Table 4.5). The FA profile of fish that were fed the finishing fish-oil diet (FO 0/FO 100) was similar to that of fish which were fed the VO diet exclusively. Fish that were previously fed FO 0 diet (groups FO 0/FO 0 and FO 0/FO 100) showed lower total SFA (−4%; $p < 0.01$), AA (−36%), DHA (−42%), but higher LA (+19%), total n-6 (+12%), and ALA (+193%) ($p < 0.001$) levels when compared with those seen in fish which were fed FO 100 diet exclusively (Table 4.5). On the other hand, the FA profile of the white muscles was partially restored as EPA showed an increase (+13%) when fish from FO 0/FO 0 dietary treatment were compared with fish that were fed FO 0/100 diet. The EPA levels of fish from FO 0/FO 0 dietary treatment were lower (−33%) than those seen in fish which were fed FO 100 diet exclusively ($p < 0.001$).

Table 4.3. Growth performance of *S. dumerili* during the wash-out period. Values are expressed as least square (LS) means.

	Feeding plan			*p*-Value	RSD
	FO 100/FO 100	**FO 0/FO 0**	**FO 0/FO 100**		
Live weight (g)					
Initial	401	397	397	0.223	5.39
45 d	494	486	491	0.947	49.8
90 d	629	629	612	0.798	62.5
Specific growth rate, %/d					
0-45 d	0.46	0.42	0.46	0.904	0.23
45-90 d	0.53	0.61	0.50	0.706	0.29
0-90 d	0.49	0.51	0.48	0.861	0.11

Diet FO 100: diet formulated with fish oil as lipid source. Diet FO 0: diet in which fish oil was totally substituted by vegetable oil. The feeding plan gives the diet fed during the grow-out trial/the diet fed during the wash-out period. RSD: residual standard deviation.

The dietary treatment did not affect the average levels of C16:0 (14.16%) and C18:0 proportions (6.78%) in the red muscles of the fish after 45 days of wash-out. Fish that were exclusively fed FO 0 diet and fish that were fed FO 0/FO 100 both showed lower total SFA (−4%) and DHA (−36%), but showed elevated proportions of LA (+18%) and total n-6 (+12%) ($p < 0.001$) when compared with those seen in fish fed FO 100 diet exclusively (Table 4.5). As seen in the white muscle, the FA profile of the red muscles was also partially restored as EPA showed an increase (+16%) when fish from FO 0/FO 0 dietary treatment were compared with fish that were fed FO 0/FO 100 diet. The EPA levels of fish from FO 0/FO 0 dietary treatment were lower (−21%) than those seen in fish which were always fed FO 100 diet ($p < 0.001$). Moreover, ALA decreased (−25%) in fish fed FO 0 diet exclusively, when compared with those seen in fish fed FO 0/FO 100 diets ($p < 0.001$) (Table 4.5).

At the end of wash-out phase, the dietary treatment did not affect the average levels of C16:0 (14.58%), C18:0 (6.13%), and total n-3 (20.91%) in the white muscles of the fish (Table 4.6). The differences recorded after 45 d of wash-out between fish that were exclusively fed FO 100 diet and those that were exclusively fed FO 0 diet or fed FO 0/FO 100 diets were confirmed for total SFA (+5% in FO 100; $p < 0.001$) and total *n*-6 (−6% in FO 100; $p < 0.001$). On the other hand, a partial restoration of the FA profile was observed for 74% of fatty acids in fish that were previously fed the FO 0 diet, due to the wash-out phase with FO 100 diet. Fish subjected to wash-out treatment showed higher ratios of AA (+33%), EPA (+16%), and DHA (+43%) ($p < 0.001$) and lower ratios of LA (−6%) and ALA (−29%) ($p < 0.001$) when compared with those seen in the fish that were not subjected to wash-out treatment (Table 4.6).

After 90 d of wash-out, the SFA and EPA ratios seen in the red muscles of fish that were exclusively fed FO 0 diet or in those which were subjected to wash-out treatment with FO 100 diet were lower than that seen in fish that were exclusively fed FO 100 diet [total SFA (−7%; $p < 0.001$) and EPA (−20%; $p < 0.05$)]. On the other hand, a partial restoration for 65% of fatty acids was observed in fish that were previously fed the FO 0 diet due to the

wash-out treatment with FO 100 diet. Fish subjected to wash-out treatment showed higher ratios of AA (+33%) and DHA (+41%) ($p < 0.001$), but lower ratios of LA (−7%), and ALA (−29%) ($p < 0.001$) compared to fish exclusively fed with FO 0 diet (Table 4.6).

As for the nutritional quality of lipids, the index of atherogenicity (IA) was higher in white muscles of fish that were exclusively fed FO 100 diet when compared with those seen in the other groups of fish at the first (+23%), 45th (+17%), and 90th day of wash-out (+16%) ($p < 0.001$). No differences among treatments were recorded for the average value of the index of thrombogenicity (IT) at the first, 45th, and 90th day of wash-out treatment (0.23) (Table 4.7).

Table 4.4. Fat content (%) and fatty acid composition (% of total fatty acids content) of white and red muscles at the beginning of the wash-out period in *S. dumerili* always fed FO 100 and FO 0 diets (12 fish per diet): effect of the grow-out diet. Values are expressed as least square (LS) means.

	White muscle				Red muscle			
	FO 100	**FO 0**	***p*-Value**	**RSD**	**FO 100**	**FO 0**	***p*-Value**	**RSD**
Fat, % WW	5.95	6.04	0.803	0.791	4.33	4.47	0.74	0.928
Fatty acids, %								
14:0	2.27	1.47	<0.001	0.141	1.82	1.26	<0.01	0.358
15:0	Tr	Tr			0.15	0.03	0.02	0.104
16:0	14.96	14.22	<0.01	0.437	14.50	14.40	0.70	0.558
17:0	0.40	0.23	<0.001	0.035	0.44	0.24	<0.001	0.042
18:0	5.89	6.12	0.02	0.197	6.53	6.83	<0.01	0.181
20:0	0.36	0.32	<0.001	0.011	0.38	0.32	<0.001	0.020
22:0	0.12	0.17	0.03	0.039	0.12	0.16	<0.001	0.016
∑ SFA[1]	24.06	22.59	<0.001	0.639	24.01	23.34	0.10	0.873
14:1 n-9	0.28	0.15	<0.001	0.023	0.11	0.10	0.96	0.109
16:1 n-9	3.78	2.36	<0.001	0.210	3.27	2.06	<0.001	0.361
17:1 n-10	0.33	0.17	<0.001	0.025	0.32	0.16	<0.001	0.026
18:1 n-7	4.12	3.20	<0.001	0.179	4.22	3.27	<0.001	0.278
18:1 n-9	27.55	31.72	<0.001	0.821	27.00	31.70	<0.001	0.799
20:1 n-9	1.72	0.76	<0.001	0.168	1.91	0.74	<0.001	0.169
22:1 n-9	0.28	0.12	<0.001	0.046	0.36	0.13	<0.001	0.112
24:1 n-9	0.34	0.19	<0.001	0.047	0.41	0.20	<0.001	0.046
∑ MUFA	38.40	38.67	0.46	0.712	37.60	38.36	0.05	0.771
18:2 n-6	14.20	16.45	<0.001	0.541	13.01	15.77	<0.001	0.522
18:3 n-6	0.14	0.13	<0.01	0.009	0.13	0.10	<0.01	0.017
20:3 n-6	0.10	0.05	<0.001	0.012	0.11	0.07	0.07	0.040
20:4 n-6	0.77	0.48	<0.001	0.071	0.92	0.54	<0.001	0.059
22:4 n-6	0.39	0.18	<0.001	0.050	0.51	0.21	<0.001	0.046
∑ n-6 PUFA	15.60	17.29	<0.001	0.466	14.68	16.69	<0.001	0.460
18:3n-3	4.08	10.84	<0.001	1.177	2.90	9.81	<0.001	0.948
20:3n-3	0.20	0.25	<0.01	0.022	0.20	0.27	<0.001	0.029
20:5n-3	3.44	2.26	<0.001	0.209	3.27	2.14	<0.001	0.156
22:5n-3	1.68	1.05	<0.001	0.143	2.12	1.29	<0.001	0.183
22:6n-3	11.28	6.37	<0.001	1.319	14.02	7.49	<0.001	1.063
∑ n-3 PUFA	20.68	20.77	0.86	1.159	22.51	21.00	<0.01	0.972
20:2	0.85	0.49	<0.001	0.056	0.80	0.45	<0.001	0.067
22:2	0.41	0.19	<0.001	0.037	0.40	0.16	<0.001	0.037
∑ PUFA	37.53	38.74	0.03	1.093	38.39	38.30	0.98	0.892
DHA/EPA	3.27	2.81	<0.01	0.258	4.30	3.50	<0.001	0.344

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; RSD: residual standard deviation; DHA/EPA: C22:6 n-3/ C20:5 n-3. Diet FO 100: diet formulated with fish oil as lipid source. Diet FO 0: diet in which fish oil was totally substituted by vegetable oil. [1] Total SFA include fatty acids not listed (<0.1 % of total FA), C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C21:0, C23:0, C24:0.

Table 4.5. Fat content and fatty acid composition (% of total fatty acid content) of white and red muscle in *S. dumerili* fed diets FO 100/FO 100, FO 0/FO 0 and FO 0/FO 100 after 45 d of wash-out (6 fish per feeding plan): effect of the feeding plan. Values are expressed as least square (LS) means.

	White muscle					Red muscle				
Feeding plan	**FO 100/FO 100**	**FO 0/FO 0**	**FO 0/FO 100**	***p*-Value**	**RSD**	**FO 100/FO 100**	**FO 0/FO 0**	**FO 0/FO 100**	***p*-Value**	**RSD**
Fat, % WW	4.79	4.65	5.42	0.554	1.057	4.37	4.67	4.42	0.842	0.808
Fatty acids, %										
14:0	2.11^{b}	1.41^{a}	1.60^{a}	<0.001	0.103	1.80^{b}	1.21^{a}	1.33^{a}	<0.001	0.087
15:0	0.27^{c}	0.14^{a}	0.17^{b}	<0.001	0.014	0.25^{c}	0.12^{a}	0.16^{b}	<0.001	0.011
16:0	14.68	14.36	14.36	0.42	0.431	14.39	14.22	13.88	0.17	0.385
17:0	0.39^{c}	0.22^{a}	0.26^{b}	<0.001	0.020	0.40^{c}	0.22^{a}	0.27^{b}	<0.001	0.017
18:0	6.20	6.38	6.29	0.50	0.223	6.71	6.82	6.82	0.64	0.224
20:0	0.36^{b}	0.30^{a}	0.32^{a}	<0.001	0.018	0.37^{b}	0.30^{a}	0.32^{a}	<0.001	0.017
22:0	0.12^{a}	0.16^{b}	0.15^{b}	<0.001	0.010	0.12^{a}	0.17^{b}	0.14^{b}	<0.01	0.013
∑ SFA[1]	24.19^{b}	23.03^{a}	23.24^{a}	<0.01	0.427	24.12^{b}	23.15^{a}	23.03^{a}	<0.01	0.496
16:1 n-9	3.60^{c}	2.28^{a}	2.61^{b}	<0.001	0.134	3.19^{c}	2.03^{a}	2.29^{b}	<0.001	0.112
17:1 n-10	0.34^{b}	0.17^{a}	0.21^{a}	<0.001	0.020	0.33^{c}	0.17^{a}	0.21^{b}	<0.001	0.020
18:1 n-7	4.20^{c}	3.09^{a}	3.39^{b}	<0.001	0.145	4.41^{c}	3.37^{a}	3.68^{b}	<0.001	0.119
18:1 n-9	26.97^{a}	32.09^{b}	30.69^{b}	<0.001	0.886	26.83^{a}	32.10^{c}	30.38^{b}	<0.001	0.854
20:1 n-9	1.80^{c}	0.72^{a}	0.98^{b}	<0.001	0.129	1.85^{c}	0.75^{a}	1.10^{b}	<0.001	0.124
22:1 n-9	0.31^{b}	0.11^{a}	0.15^{a}	<0.001	0.041	0.35^{c}	0.14^{a}	0.21^{b}	<0.001	0.029
24:1 n-9	0.41^{b}	0.19^{a}	0.22^{a}	<0.001	0.038	0.41^{b}	0.21^{a}	0.26^{a}	<0.001	0.033
∑ MUFA	37.63	38.65	38.25	0.15	0.769	37.37^{a}	38.77^{b}	38.13^{ab}	0.02	0.644
18:2 n-6	13.19^{a}	15.92^{b}	15.44^{b}	<0.001	0.436	12.50^{a}	15.16^{b}	14.33^{b}	<0.001	0.489
18:3 n-6	0.14^{b}	0.13^{a}	0.13^{a}	<0.001	0.006	0.12	0.11	0.11	0.45	0.010
20:3 n-6	0.10^{b}	0.05^{a}	0.06^{a}	<0.001	0.013	0.11^{b}	0.06^{a}	0.07^{a}	0.001	0.017
20:4 n-6	0.91^{b}	0.54^{a}	0.61^{a}	<0.001	0.084	0.95^{b}	0.59^{a}	0.74^{ab}	<0.001	0.073
22:4 n-6	0.48^{b}	0.18^{a}	0.25^{a}	<0.001	0.045	0.53^{c}	0.22^{a}	0.32^{b}	<0.001	0.045
∑ n-6 PUFA	14.82^{a}	16.82^{b}	16.49^{b}	<0.001	0.402	14.21^{a}	16.14^{b}	15.57^{b}	<0.001	0.406
18:3 n-3	3.27^{a}	10.30^{b}	8.83^{b}	<0.001	0.945	2.91^{a}	9.21^{c}	7.35^{b}	<0.001	0.813
20:3 n-3	0.20^{a}	0.26^{b}	0.25^{b}	<0.01	0.026	0.20^{a}	0.29^{b}	0.30^{b}	<0.001	0.033
20:5 n-3	3.47^{c}	2.18^{a}	2.47^{b}	<0.001	0.126	3.13^{c}	2.13^{a}	2.48^{b}	<0.001	0.126
22:5 n-3	1.84^{b}	1.10^{a}	1.25^{a}	<0.001	0.153	2.21^{b}	1.48^{a}	1.72^{a}	<0.001	0.154
22:6n-3	13.32^{b}	7.00^{a}	8.44^{a}	<0.001	1.296	14.67^{b}	8.19^{a}	10.63^{a}	<0.001	1.293
∑ n-3 PUFA	22.10	20.84	21.24	0.06	0.741	23.12^{b}	21.30^{a}	22.48^{ab}	0.04	0.945
20:2	0.84^{b}	0.48^{a}	0.55^{a}	<0.001	0.051	0.79^{c}	0.47^{a}	0.56^{b}	<0.001	0.045
22:2	0.42^{b}	0.18^{a}	0.23^{a}	<0.001	0.036	0.39^{c}	0.17^{a}	0.23^{b}	<0.001	0.027
∑ PUFA	38.17	38.31	38.52	0.79	0.772	38.51	38.08	38.84	0.39	0.763
DHA/EPA	3.83	3.23	3.42	0.13	0.438	4.69^{b}	3.86^{a}	4.28^{ab}	0.04	0.428

SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, RSD: residual standard deviation, DHA/EPA: C22:6 n-3/ C20:5 n-3. Diet FO 100: diet formulated with fish oil as lipid source. Diet FO 0: diet in which fish oil was totally substituted by vegetable oil. The feeding plan gives the diet fed during the grow-out trial/the diet fed during the wash-out period. [a,b,c] Means with different superscript letter statistically differ. [1] Total SFA include fatty acids not listed (<0.1 % of total FA), C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C21:0, C23:0, C24:0.

Table 4.6. Fat content and fatty acid composition (% of total fatty acid content) of white and red muscle in *S. dumerili* fed diets FO 100/FO 100, FO 0/FO 0 and FO 0/FO 100 after 90 d of wash-out (6 fish per feeding plan): effect of the feeding plan. Values are expressed as least square (LS) means.

	White muscle					Red muscle				
Feeding plan	**FO 100/FO 100**	**FO 0/FO 0**	**FO 0/FO 100**	***p*-Value**	**RSD**	**FO 100/FO 100**	**FO 0/FO 0**	**FO 0/FO 100**	***p*-Value**	**RSD**
Fat, % WW	5.67	4.68	5.21	0.51	1.455	5.22^{b}	3.65^{a}	4.99^{b}	<0.01	0.986
Fatty acids, %										
14:0	2.24^{c}	1.46^{a}	1.74^{b}	<0.001	0.110	1.88^{b}	1.22^{a}	1.51^{a}	<0.001	0.143
15:0	0.29^{c}	0.14^{a}	0.20^{b}	<0.001	0.012	0.27	0.13	0.39	0.07	0.176
16:0	14.79	14.41	14.53	0.27	0.393	14.74^{b}	14.04^{a}	14.17^{a}	0.03	0.446
17:0	0.41^{c}	0.23^{a}	0.30^{b}	<0.001	0.015	0.42^{c}	0.23^{a}	0.31^{b}	<0.001	0.017
18:0	6.15	6.18	6.07	0.80	0.258	6.92^{b}	6.69^{b}	6.42^{a}	0.01	0.226
20:0	0.36^{c}	0.29^{a}	0.33^{b}	<0.001	0.021	0.37^{b}	0.32^{a}	0.32^{a}	<0.001	0.016
22:0	0.11^{a}	0.15^{b}	0.14^{b}	<0.001	0.012	0.12^{a}	0.17^{c}	0.14^{b}	<0.001	0.010
∑ SFA[1]	24.41^{b}	22.94^{a}	23.38^{a}	<0.001	0.519	24.83^{b}	22.90^{a}	23.37^{a}	<0.001	0.612
16:1 n-9	3.76^{c}	2.34^{a}	2.85^{b}	<0.001	0.144	3.21^{c}	2.04^{a}	2.56^{b}	<0.001	0.204
17:1 n-10	0.33^{c}	0.16^{a}	0.23^{b}	<0.001	0.023	0.34^{c}	0.17^{a}	0.24^{b}	<0.001	0.025
18:1 n-7	4.15^{c}	3.00^{a}	3.69^{b}	<0.001	0.363	4.32^{c}	3.30^{a}	3.80^{b}	<0.001	0.133
18:1 n-9	27.13^{a}	33.07^{c}	30.97^{b}	<0.001	0.685	26.41^{a}	32.84^{c}	30.39^{b}	<0.001	0.819
20:1 n-9	1.84^{c}	0.70^{a}	1.16^{b}	<0.001	0.079	1.90^{c}	0.75^{a}	1.25^{b}	<0.001	0.076
22:1 n-9	0.33^{c}	0.09^{a}	0.20^{b}	<0.001	0.029	0.38^{c}	0.14^{a}	0.23^{b}	<0.001	0.011
24:1 n-9	0.42^{c}	0.18^{a}	0.26^{b}	<0.001	0.039	0.51^{b}	0.23^{a}	0.30^{a}	<0.001	0.043
∑ MUFA	37.96^{a}	39.54^{b}	39.36^{b}	0.02	0.883	37.07^{a}	39.47^{b}	38.77^{a}	<0.01	1.114
18:2 n-6	13.35^{a}	15.39^{c}	14.51^{b}	<0.001	0.384	12.41^{a}	14.84^{c}	13.81^{b}	<0.001	0.361
18:3 n-6	0.12	0.12	0.11	0.80	0.024	0.12	0.11	0.14	0.43	0.040
20:3 n-6	0.19	0.07	0.13	0.21	0.110	0.11^{c}	0.07^{a}	0.08^{b}	<0.001	0.004
20:4 n-6	0.92^{c}	0.48^{a}	0.64^{b}	<0.001	0.056	1.02^{c}	0.54^{a}	0.72^{b}	<0.001	0.095
22:4 n-6	0.38^{b}	0.20^{a}	0.27^{ab}	<0.001	0.061	0.50^{b}	0.21^{a}	0.31^{a}	<0.001	0.092
∑ n-6 PUFA	14.96^{a}	16.26^{b}	15.66^{b}	<0.001	0.405	14.16^{a}	15.74^{c}	15.06^{b}	<0.001	0.327
18:3 n-3	2.70^{a}	10.84^{c}	7.73^{b}	<0.001	0.271	2.26^{a}	9.56^{c}	6.82^{b}	<0.001	0.208
20:3 n-3	0.18^{a}	0.28^{b}	0.25^{b}	<0.001	0.026	0.18^{a}	0.33^{c}	0.26^{b}	<0.001	0.030
20:5 n-3	3.44^{c}	2.23^{a}	2.58^{b}	<0.001	0.150	3.00^{b}	2.31^{a}	2.48^{a}	0.02	0.396
22:5 n-3	1.86^{c}	1.04^{a}	1.28^{b}	<0.001	0.052	2.14^{c}	1.41^{a}	1.64^{b}	<0.001	0.064
22:6 n-3	13.24^{c}	6.20^{a}	8.87^{b}	<0.001	0.909	15.23^{c}	7.61^{a}	10.71^{b}	<0.001	1.453
∑ n-3 PUFA	21.42	20.59	20.71	0.28	0.897	22.81	21.22	21.91	0.13	1.362
20:2	0.83^{c}	0.49^{a}	0.62^{b}	<0.001	0.026	0.75^{c}	0.49^{a}	0.60^{b}	<0.001	0.054
22:2	0.42^{c}	0.18^{a}	0.27^{b}	<0.001	0.022	0.38^{c}	0.18^{a}	0.29^{b}	<0.001	0.042
∑ PUFA	37.63	37.52	37.26	0.71	0.697	38.10	37.63	37.86	0.81	1.302
DHA/EPA	3.86^{b}	2.78^{a}	3.44^{b}	<0.001	0.297	5.10^{b}	3.41^{a}	4.33^{ab}	<0.01	0.714

SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, RSD: residual standard deviation, DHA/EPA: C22:6 n-3/ C20:5 n-3. Diet FO 100: diet formulated with fish oil as lipid source. Diet FO 0: diet in which fish oil was totally substituted by vegetable oil. The feeding plan gives the diet fed during the grow-out trial/the diet fed during the wash-out period. [a,b,c] Means with different superscript letter statistically differ. [1] Total SFA include fatty acids not listed (<0.1 % of total FA), C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C21:0, C23:0, C24:0.

Table 4.7. Indices of nutritional quality of lipids and EPA+DHA content (g/100 g of muscle) of white and red muscles in *S. dumerili* at the beginning of the wash-out period (0 d) and after 45 and 90 d: effect of the feeding plan. Values are expressed as least square (LS) means

	Feeding plan			*p*-Value	RSD
	FO 100/FO 100	FO 0/FO 0	FO 0/FO 100		
White muscle					
Initial sampling					
Index of Atherogenicity	0.32	0.26	-	<0.001	0.014
Index of Thrombogenicity	0.24	0.23	-	0.062	0.015
PUFA n-6/PUFA n-3	0.76	0.83	-	0.008	0.053
EPA+DHA	2.57	1.73	-	<0.001	0.322
Sampling at 45 d					
Index of Atherogenicity	0.31^{b}	0.26^{a}	0.27^{a}	<0.001	0.011
Index of Thrombogenicity	0.23	0.23	0.23	0.986	0.010
PUFA n-6/PUFA n-3	0.67^{a}	0.81^{b}	0.78^{b}	<0.001	0.032
EPA+DHA	2.55^{b}	1.57^{a}	1.98^{ab}	<0.001	0.362
Sampling at 90 d					
Index of Atherogenicity	0.32^{c}	0.27^{a}	0.28^{b}	<0.001	0.010
Index of Thrombogenicity	0.23	0.23	0.23	0.858	0.010
PUFA n-6/PUFA n-3	0.70^{a}	0.79^{b}	0.76^{ab}	0.02	0.045
EPA+DHA	2.64^{b}	1.68^{a}	2.27^{ab}	<0.01	0.413
Red muscle					
Initial sampling					
Index of Atherogenicity	0.29	0.26		0.013	0.029
Index of Thrombogenicity	0.22	0.23		0.172	0.014
PUFA n-6/PUFA n-3	0.65	0.80		<0.001	0.041
EPA+DHA	2.54	1.58		<0.001	0.177
Sampling at 45 d					
Index of Atherogenicity	0.29^{b}	0.25^{a}	0.25^{a}	<0.001	0.010
Index of Thrombogenicity	0.22	0.23	0.21	0.328	0.010
PUFA n-6/PUFA n-3	0.62^{a}	0.76^{b}	0.69^{ab}	<0.01	0.040
EPA+DHA	2.72^{b}	1.69^{a}	2.06^{a}	<0.001	0.221
Sampling at 90 d					
Index of Atherogenicity	0.30^{b}	0.25^{a}	0.27^{b}	<0.001	0.014
Index of Thrombogenicity	0.22	0.22	0.22	0.888	0.017
PUFA n-6/PUFA n-3	0.63^{a}	0.74^{b}	0.69^{ab}	<0.01	0.044
EPA+DHA	2.36	1.97	2.40	0.10	0.377

PUFA: polyunsaturated fatty acids, RSD: residual standard deviation, EPA+DHA: C20:5 n- 3+C22:6 n- 3. Diet FO 100: diet formulated with fish oil as lipid source. Diet FO 0: diet in which fish oil was totally substituted by vegetable oil. The feeding plan gives the diet fed during the grow-out trial/the diet fed during the wash-out period. ^{a,b,c} Means with different superscript letter statistically differ.

DISCUSSION

In the last few decades, the widespread use of FO as lipid source in aquafeeds has drastically challenged sustainability of aquaculture. The substitution of FO with alternative sources (such as vegetable oils) has been successful in terms of fish performance, but has faced concerns about their health and flesh nutritional quality. New feeding strategies are being developed to address those concerns. In fact, the replacement of FO with VO in aqua feed increases dietary oleic acid, LA and ALA and reduces the n-3 fatty acid such as EPA and DHA, which increases the vegetable lipid profile of the whole fish, as well as its organs and flesh (Turchini et al., 2009). Since n-3 PUFA play a specific role in inflammatory processes and immune response (Calder, 2012), the change in the dietary FA profile can affect fish response both during growth and wash-out. Moreover, the presence of vegetable FA leads to a decrease in the nutritional value of fish flesh for humans as a result of changes in FA profile as well as an unbalanced ratio of n-3/n-6 (Leaf, 2001). This can affect both the white and red muscles of fish, whereas a greater contribution to the nutritional value of the flesh comes from the former due its higher proportion on the whole fillet compared to the latter (in the present trial, red muscles: 6.8-9.8% of total fillet weight).

In the present trial, two indices based on the functional effects of FA were used to evaluate the nutritional quality of the fish fillet lipid fraction, i.e. the indexes of atherogenicity (IA) and thrombogenicity (IT). Briefly, IA indicates the relationship between main classes of saturated FA (considered pro-atherogenic, i.e. favouring lipid adhesion to cells) and those of unsaturated FA (anti-atherogenic; i.e. inhibiting the aggregation of lipid plaque and reducing the levels of cholesterol, phospholipid and esterified FA) (Ghaeni et al., 2013). The IT is defined by the relationship between the pro-thrombogenic saturated FA and the anti-thrombogenic FA (MUFA, PUFA n-6 and PUFA n-3) (Ghaeni et al., 2013). The IT of flesh in our trial was consistent with values (0.22−0.23) found in most common marine species (Durmus, 2019), whereas the higher IA in fish fed FO 100 diet is related to the higher content of myristic acid (C14:0) in their red and white muscles. In fact, myristic acid in FO 100 diet was almost twice than in FO 0 one. Nevertheless, for all fish, IA values were within recommended values for human health (< 1.0) (Ouraji et al., 2009; Stancheva et al., 2014).

Moreover, in the present trial, after the growth phase (109 d), the FA profile of *S. dumerili* in the white and red muscles reflected the FA composition of the fish-oil (FO 100) or vegetable-oil (FO 0) diets, ingested by the fish, which agreed with our assumptions. Nevertheless, the differences in AA, EPA and DHA rates in both the white and red muscles of the fish that were fed the two dietary treatments were lower than the difference in the diets, which agrees with previously published studies on gilthead seabream (Fountoulaki et al., 2009; Izquierdo et al., 2005; Martínez-Llorens et al., 2007) and rainbow trout (Yildiz et al., 2018).

To restore the fillet nutritional value (in terms of high levels of EPA and DHA) in fish grown on diets containing vegetable oils, specific feeding strategies can be used during the finishing period to wash-out fish that were previously fed VO diets (Codabaccus et al.,

2013; Jobling, 2004; Yildiz et al., 2018). However, fish FA levels are also affected by the fish biosynthesis ability for the different FA, besides the dietary supply. Nevertheless, standards for a successful wash-out and recovery of the desired FA in fish flesh are not yet available for all species including greater amberjack. On the other hand, the available literature about other species is inconsistent.

In gilthead seabream, oleic acid and LA are retained in flesh of the fish even after 120 d of wash-out (Fountoulaki et al., 2009). In European sea bass, Montero et al. (2005) reported that LA was 3-fold higher in fish previously fed vegetable-oil diets compared to those that were fed on fish oil exclusively even after 150 d of wash-out, which agrees with the results stated by Izquierdo et al. (2005) for seabass after a 104-d wash-out. In turbot, even after a wash-out of eight months, high levels of LA in muscle phospholipids have been reported, which could be attributed to the poor LA utilization in a species that can convert LA to 20:2 n-6 (Regost et al., 2003).

According to Mourente and Bell (2006) the wash-out (150 d, 160 g LW) treatment in sea bass is insufficient for restoring EFA. On the contrary, Montero et al. (2005) report that a wash-out period of 150 d, after 8 months of a diet containing 60:40 ratio of vegetable oil and fish oil (75–366 g LW), was able to recover flesh DHA, but could not increase EPA level. Similarly, in gilthead seabream, EFA levels could not be restored after a 120-d wash-out (Fountoulaki et al., 2009). On the other hand, Izquierdo et al. (2005) report that DHA and AA levels were recovered in gilthead seabream following a wash-out of 60 d (after 7 months of feeding with diets containing vegetable oils at 60 % and 80 %; 85–452 g LW), but EPA levels were not recovered even after 90 d of wash-out treatment.

In rainbow trout, 8–12 weeks of wash-out cannot recover EPA and DHA levels (Thanuthong et al., 2012; Yildiz et al., 2018), whereas in Atlantic salmon that has been previously fed linseed oil-based diets for 40 weeks, levels of EPA and DHA in flesh can be restored by 80% after 20 weeks of wash-out treatment [34] and by 83% after 24 weeks of wash-out treatment [32]. On the other hand, Bell et al. (2003) report that in post-smolt salmon (200 g) previously fed a diet containing increasing rates of rapeseed oil for 16 weeks, EPA and DHA levels in flesh can be recovered after 4 and 12 weeks of wash-out, respectively. In Senegalese sole a total restoration of all EFA levels in flesh can be achieved after 26 d of wash-out (Reis et al., 2014).

In the present study, EFA levels in the Mediterranean yellowtail did not recover completely after a 90-d wash-out, but some differences between white and red muscles were recorded. In the white muscles, AA, EPA and DHA levels were partially restored, despite remaining lower when compared with those of fish fed fish-oil diets exclusively. On the other hand, in the red muscle, a partial restoration was observed only for AA and DHA levels, whereas EPA level remained low.

Based on literature, EFA recovery in fish muscles depends upon several factor such as fish species, fish size, duration for which fish were fed vegetable-oil based diets, duration of the wash-out period, and the specific FA. Additionally, FA incorporation in fish muscles can be altered by different metabolic factors such as FA elongation and desaturation, β-

oxidation (Kiessling and Kiessling, 1993), preferential incorporation (Linares and Henderson, 1991), lipogenic activity, environmental factors (Tocher and Sargent, 1990), size and age of animals (Kiessling et al., 2001), and their physiological state (Jeong et al., 2002).

In the present trial, after 90 d of wash-out, DHA was partially recovered and selectively retained by the muscles. This observation agrees with previously published studies on a wide variety of species (Izquierdo et al., 2003). The mechanism of selective deposition has been likely influenced by the high specificity of fatty acid transferases for DHA and the relative resistance of DHA to β-oxidation (Bell et al., 2001). Fish size and fish physiological state can also play a role in DHA recovery. DHA content in flesh of the fish has been found to be negatively affected by the increase in fish size (Tinsley et al., 1973) and the competition between muscles and developing gonads for its incorporation at the time of sexual maturation (Jeong et al., 2002), which was not the case of our trial.

The recovery of EPA in the flesh of the fish following a wash-out strategy was unsuccessful in the present trial. Our observations agree with previously reported studies done with other species [seabass, (Montero et al., 2005); seabream, (Izquierdo et al., 2005); rainbow trout, (Thanuthong et al., 2012; Yildiz et al., 2018)]. According to Madsen et al. (1998), a preferential oxidation of EPA occurs over DHA; and EPA is mainly oxidized by mitochondria, whereas DHA seems to be oxidized by the peroxisomes. Thus, the failure of EPA recovery in the white muscle of Mediterranean yellowtail in our trial could be attributed to the fact that mitochondrial β-oxidation prevails over peroxisomal oxidation in white muscles (Madsen et al., 1998).

The definition of the wash-out duration is also crucial to produce fish with healthy FA profile. An n-3 HUFA deficiency in the fish muscle lowers the nutritional value of the fish for humans. To overcome this, a dilution model has been proposed (Jobling, 2004) to predict the FA restoration at a given time after a dietary change. This model has been used in some fish species such as gilthead seabream and Atlantic salmon (Benedito-Palos et al., 2009; Jobling, 2004). Nevertheless, the model does not fully represent changes in all FA and the variation in different species. For instance, in the Murray cod, the mobilization of oleic acid, LA and ALA during the wash-out has been found to be at a lower rate than the rate predicted by the model, with major changes occurring during the first days of wash-out (Turchini et al., 2006). According to some studies (Jobling, 2004; Lane et al., 2006), the model can provide misleading results when it is used to study the FA changes of ‘lean’ fish fillets, such as the Mediterranean yellowtail.

Thus, under the conditions of our trial, a wash-out period of 90 d partially improved the final FA profile in muscles of Mediterranean yellowtail that was previously fed VO-based diets. In fact, based on our results, the quantity of muscle necessary to cover the daily-recommended ingestion of EPA and DHA (average requirement of 1.6 g/d of n-3 HUFA) decreased from 90 g/d (group FO 0/FO 0) to 70 g/d (FO 0/FO 100). However, further studies are necessary to define the time and the dietary fish oil level that can restore EFA to the same levels seen in the fish that were exclusively fed on fish oil for *S. dumerili*, under different conditions of growth and at different sizes.

Supplementary materials

Table S1: Fatty acid (FA) composition (mg of FA/g of tissue) of white and red muscles at the beginning of the wash-out period in S. dumerili always fed diets FO 100 and FO 0 (12 fish per diet): effect of the grow-out diet. Values are expressed as least square (LS) means. Table S2: Fatty acid (FA) composition (mg of FA/g of tissue) of white and red muscle in *S. dumerili* fed diets FO 100/FO 100, FO 0/FO 0 and FO 0/FO 100 after 45 d of wash-out (6 fish per feeding plan): effect of the feeding plan. Values are expressed as least square (LS) means. Table S3: Fat content and fatty acid composition (mg of FA/g of tissue) of white and red muscle in *S. dumerili* fed FO 100/ FO 100, FO 0/ FO 0 and FO 0/ FO 100 diets after 90 d of wash-out (6 fish per feeding plan): effect of the feeding plan. Values are expressed as least square (LS) means.

Table S1. Fat content and fatty acid composition (mg of FA/g of tissue) of white and red muscles at the beginning of the wash-out period in *S. dumerili* always fed diets FO 100 and FO 0 (12 fish per diet): effect of the grow out diet. Values are expressed as least square (LS) means.

	White muscle				Red muscle			
	FO 100	**FO 0**	***p*-Value**	**RSD**	**FO 100**	**FO 0**	***p*-Value**	**RSD**
Fat, % WW	5.95	6.04	0.803	0.791	4.33	4.47	0.744	0.928
Fatty acids								
14:0	4.04	2.96	<0.001	0.475	2.69	2.07	0.044	0.615
15:0	Tr	Tr			0.22	0.05	0.035	0.166
16:0	26.62	28.66	0.176	3.111	21.42	23.71	0.092	2.795
17:0	0.71	0.47	<0.001	0.087	0.65	0.39	<0.001	0.103
18:0	10.44	12.34	0.004	1.212	9.64	11.25	<0.01	1.202
20:0	0.63	0.64	0.757	0.078	0.56	0.52	0.301	0.075
22:0	0.22	0.33	0.01	0.087	0.18	0.27	<0.001	0.044
24:0	0.14	0.17	0.063	0.027	0.13	0.16	0.063	0.030
∑ SFA[1]	42.79	45.57	0.239	4.891	35.49	38.42	0.182	4.577
14:1 n-9	0.50	0.29	<0.001	0.061	0.15	0.16	0.875	0.160
16:1 n-9	6.74	4.74	<0.001	0.744	4.84	3.38	<0.001	0.750
17:1 n-10	0.59	0.38	<0.001	0.070	0.47	0.27	<0.001	0.063
18:1 n-7	7.32	6.46	0.044	0.854	6.25	5.39	0.042	0.849
18:1 n-9	49.15	64.12	<0.001	7.757	39.96	52.30	<0.001	6.100
20:1 n-9	3.06	1.52	<0.001	0.416	2.83	1.22	<0.001	0.374
22:1 n-9	0.50	0.24	<0.001	0.109	0.53	0.21	<0.001	0.163
24:1 n-9	0.61	0.39	<0.001	0.115	0.61	0.33	<0.001	0.113
∑ MUFA	68.47	78.09	0.044	9.531	55.64	63.27	0.045	7.673
18:2 n-6	25.33	33.18	<0.001	3.670	19.25	25.99	<0.001	2.917
18:3 n-6	0.25	0.25	0.902	0.041	0.19	0.17	0.368	0.037
20:3 n-6	0.18	0.11	<0.001	0.030	0.16	0.12	0.257	0.074
20:4 n-6	1.37	0.97	<0.001	0.136	1.34	0.88	<0.001	0.090
22:4 n-6	0.69	0.35	<0.001	0.088	0.74	0.35	<0.001	0.071
∑ n-6 PUFA	27.80	34.86	0.001	3.812	21.68	27.51	<0.001	3.059
18:3n-3	7.31	21.98	<0.001	3.351	4.31	16.19	<0.001	2.099
20:3n-3	0.36	0.50	0.002	0.084	0.29	0.45	<0.001	0.070
20:5n-3	6.11	4.55	<0.001	0.689	4.82	3.52	<0.001	0.487
22:5n-3	2.99	2.11	<0.001	0.366	3.12	2.12	<0.001	0.382
22:6n-3	19.99	12.77	<0.001	2.586	20.56	12.26	<0.001	1.436
∑ n-3 PUFA	36.76	41.91	0.043	5.065	33.10	34.54	0.343	3.215
20:2	1.52	0.98	<0.001	0.181	1.19	0.75	<0.001	0.164
22:2	0.74	0.37	<0.001	0.098	0.40	0.16	<0.001	0.084
∑ PUFA	66.82	78.12	0.013	8.800	56.57	63.07	0.038	6.281
DHA/EPA	3.27	2.81	0.002	0.258	4.30	3.50	<0.001	0.344

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; RSD: residual standard deviation; DHA/EPA: C22:6 n-3/ C20:5 n-3. Diet FO 100: diet formulated with fish oil as lipid source. Diet FO 0: diet in which fish oil was totally substituted by vegetable oil.
[1] Total SFA include fatty acids not listed (<0.01 mg FA/g of tissue), C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C21:0, C23:0.

Table S2. Fat content and fatty acid composition (mg of FA/g of tissue) of white and red muscle in *S. dumerili* fed diets FO 100/FO 100, FO 0/FO 0 and FO 0/FO 100 after 45 d of wash-out (6 fish per feeding plan): effect of the feeding plan. Values are expressed as least square (LS) means.

	White muscle					Red muscle				
Feeding plan	**FO 100/FO 100**	**FO 0/FO 0**	**FO 0/FO 100**	***p*-Value**	**RSD**	**FO 100/FO 100**	**FO 0/FO 0**	**FO 0/FO 100**	***p*-Value**	**RSD**
Fat, % WW	4.79	4.65	5.42	0.554	1.057	4.37	4.67	4.42	0.842	0.808
Fatty acids, %										
14:0	3.45^{ab}	2.27^{a}	2.92^{a}	0.033	0.596	2.80^{c}	1.97^{a}	2.08^{b}	0.011	0.383
15:0	0.43^{c}	0.22^{ab}	0.31^{b}	0.002	0.069	0.38^{c}	0.20^{a}	0.24^{b}	<0.001	0.038
16:0	24.00	23.04	26.16	0.547	4.038	22.24	23.26	21.79	0.764	2.897
17:0	0.64^{b}	0.36^{a}	0.48^{ab}	0.003	0.097	0.61^{b}	0.36^{a}	0.43^{a}	<0.001	0.067
18:0	10.08	10.20	11.47	0.357	1.514	10.31	11.17	10.74	0.578	1.252
20:0	0.58	0.48	0.58	0.261	0.097	0.57	0.49	0.51	0.286	0.077
24:0	0.13	0.13	0.15	0.385	0.021	0.13	0.16	0.15	0.081	0.017
∑ SFA	39.51	36.94	42.34	0.508	6.362	37.22	37.89	36.16	0.871	4.667
16:1 n-9	5.88^{b}	3.68^{a}	4.76^{ab}	0.012	0.931	4.93^{b}	3.32^{a}	3.57^{a}	0.004	0.620
17:1 n-10	0.58^{b}	0.28^{a}	0.38^{ab}	<0.001	0.078	0.51^{b}	0.28^{a}	0.34^{a}	<0.001	0.046
18:1 n-7	6.84	4.95	6.19	0.059	1.078	6.80	5.52	5.80	0.077	0.838
18:1 n-9	44.24	51.48	56.17	0.140	8.707	41.63	52.49	47.73	0.067	6.472
20:1 n-9	2.93^{b}	1.16^{a}	1.79^{a}	<0.001	0.444	2.84^{b}	1.23^{a}	1.74^{a}	<0.001	0.291
22:1 n-9	0.51^{b}	0.18^{a}	0.28^{ab}	0.002	0.111	0.53^{b}	0.22^{a}	0.33^{a}	<0.001	0.065
24:1 n-9	0.67^{b}	0.30^{a}	0.40^{a}	0.002	0.127	0.62^{b}	0.34^{a}	0.41^{a}	<0.001	0.058
∑ MUFA	61.62	62.02	69.96	0.489	11.21	57.86	63.41	59.93	0.591	8.190
18:2 n-6	21.60	25.53	28.12	0.059	3.801	19.39	24.78	22.52	0.051	3.027
18:3 n-6	0.23	0.20	0.24	0.413	0.042	0.18	0.18	0.17	0.879	0.027
20:3 n-6	0.17^{b}	0.09^{a}	0.12^{ab}	0.011	0.035	0.17^{b}	0.10^{a}	0.11^{a}	0.008	0.033
20:4 n-6	1.46^{b}	0.86^{a}	1.11^{a}	<0.001	0.146	1.45^{c}	0.96^{a}	1.16^{b}	<0.001	0.091
22:4 n-6	0.77^{b}	0.29^{a}	0.46^{a}	<0.001	0.084	0.80^{c}	0.36^{a}	0.51^{b}	<0.001	0.050
∑ n-6 PUFA	24.22	26.97	30.03	0.125	4.018	22.00	26.38	24.47	0.139	3.169
18:3 n-3	5.50^{a}	16.62^{b}	16.20^{b}	<0.001	2.832	4.66^{a}	15.12^{b}	11.54^{b}	<0.001	2.125
20:3 n-3	0.33	0.41	0.46	0.099	0.090	0.31^{a}	0.47^{b}	0.48^{b}	0.025	0.093
20:5 n-3	5.67^{b}	3.50^{a}	4.50^{ab}	0.012	0.921	4.82^{b}	3.50^{a}	3.90^{ab}	0.017	0.617
22:5 n-3	2.95^{b}	1.76^{a}	2.27^{a}	<0.001	0.350	3.37^{b}	2.42^{a}	2.71^{a}	0.004	0.359
22:6 n-3	21.38^{b}	11.11^{a}	15.27^{a}	<0.001	2.202	22.30^{b}	13.39^{a}	16.72^{ab}	<0.001	1.686
∑ n-3 PUFA	35.83	33.40	38.71	0.377	5.140	35.46	34.90	35.35	0.978	4.178
20:2	1.37^{b}	0.77^{a}	1.02^{ab}	0.005	0.228	1.21^{b}	0.77^{a}	0.89^{a}	0.003	0.157
22:2	0.69^{b}	0.28^{a}	0.42^{a}	<0.001	0.116	0.60^{b}	0.27^{a}	0.37^{a}	<0.001	0.072
∑ PUFA	62.11	61.42	70.17	0.358	9.392	59.27	62.37	61.08	0.811	7.402
DHA/EPA	3.83	3.23	3.42	0.13	0.438	4.69^{b}	3.86^{a}	4.28^{ab}	0.04	0.428

SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, RSD: residual standard deviation, DHA/EPA: C22:6 n-3/ C20:5 n-3. Diet FO 100: diet formulated with fish oil as lipid source. Diet FO 0: diet in which fish oil was totally substituted by vegetable oil. The feeding plan gives the diet fed during the grow-out trial/the diet fed during the wash-out period. ^{a,b,c} Means with different superscript letter statistically differ. [1] Total SFA include fatty acids not listed (<0.01 mg FA/g of tissue), C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C21:0, C23:0.

Table S3. Fat content and fatty acid composition (mg of FA/g of tissue) of white and red muscle in *S. dumerili* fed FO 100/ FO 100, FO 0/ FO 0 and FO 0/ FO 100 diets after 90 d of wash-out (6 fish per feeding plan): effect of the feeding plan. Values are expressed as least square (LS) means.

	White muscle					Red muscle				
Feeding plan	**FO 100/FO 100**	**FO 0/FO 0**	**FO 0/FO 100**	***p*-Value**	**RSD**	**FO 100/FO 100**	**FO 0/FO 0**	**FO 0/FO 100**	***p*-Value**	**RSD**
Fat, % WW	4.68	5.67	5.21	0.511	1.455	3.65^{a}	5.55^{b}	4.99^{ab}	0.009	0.986
Fatty acids										
14:0	3.87	2.91	3.49	0.242	0.970	2.60	2.43	2.76	0.735	0.710
15:0	0.50^{b}	0.28^{a}	0.40^{ab}	0.016	0.117	0.37	0.25	0.67	0.107	0.315
16:0	25.14	28.68	28.90	0.513	5.734	19.78^{a}	27.93^{b}	25.79^{ab}	0.017	4.658
17:0	0.70^{b}	0.45^{a}	0.59^{ab}	0.026	0.141	0.57	0.46	0.56	0.272	0.134
18:0	10.34	12.29	12.03	0.268	2.068	9.21^{a}	13.28^{b}	11.68^{ab}	0.006	1.977
20:0	0.60	0.58	0.66	0.654	0.142	0.51	0.64	0.59	0.168	0.122
22:0	0.18^{a}	0.30^{b}	0.28^{ab}	0.010	0.056	0.17^{a}	0.33^{b}	0.26^{a}	<0.001	0.047
24:0	0.12	0.15	0.16	0.205	0.032	0.12^{a}	0.19^{b}	0.19^{b}	0.037	0.044
∑ SFA[1]	41.45	45.65	46.50	0.658	9.193	33.33^{a}	45.50^{b}	42.54^{ab}	0.031	7.686
16:1 n-9	6.48	4.67	5.71	0.158	1.572	4.42	4.06	4.68	0.667	1.161
17:1 n-10	0.56^{b}	0.32^{a}	0.47^{ab}	0.009	0.119	0.47	0. 34	0.44	0.154	0.120
18:1 n-7	7.12	5.97	7.35	0.388	1.823	5.86	6.56	6.92	0.465	1.374
18:1 n-9	46.61	65.99	61.80	0.056	12.90	35.87^{a}	65.35^{b}	55.45^{a}	<0.001	9.702
20:1 n-9	3.18^{b}	1.39^{a}	2.33^{ab}	0.003	0.746	2.58^{b}	1.49^{a}	2.27^{ab}	0.012	0.600
22:1 n-9	0.57^{b}	0.18^{a}	0.40^{b}	<0.001	0.132	0.51^{b}	0.28^{a}	0.42^{ab}	0.006	0.114
24:1 n-9	0.70^{b}	0.35^{a}	0.52^{ab}	<0.001	0.119	0.67^{b}	0.46^{a}	0.54^{ab}	0.048	0.142
∑ MUFA	65.21	78.86	78.57	0.358	17.12	50.38^{a}	78.55^{b}	70.73^{ab}	0.004	13.07
18:2 n-6	22.84	30.68	28.97	0.100	5.967	16.76^{a}	29.47^{c}	25.20^{b}	<0.001	4.247
18:3 n-6	0.21	0.24	0.23	0.824	0.073	0.17	0.22	0.26	0.253	0.077
20:3 n-6	0.39	0.14	0.27	0.365	0.305	0.15	0.13	0.15	0.418	0.032
20:4 n-6	1.54^{b}	0.96^{a}	1.26^{ab}	0.003	0.238	1.31^{b}	1.06^{a}	1.32^{b}	0.037	0.185
22:4 n-6	0.66^{b}	0.39^{a}	0.54^{ab}	0.035	0.161	0.65^{b}	0.41^{a}	0.57^{ab}	0.033	0.155
∑ n-6 PUFA	25.63	32.40	31.27	0.215	6.553	19.04^{a}	31.31^{b}	27.50^{a}	<0.001	4.605
18:3 n-3	4.59^{a}	21.64^{c}	15.40^{b}	<0.001	2.809	3.09^{a}	19.03^{c}	12.44^{b}	<0.001	1.680
20:3 n-3	0.31^{a}	0.57^{b}	0.49^{ab}	0.006	0.117	0.25^{a}	0.65^{b}	0.48^{a}	<0.001	0.114
20:5 n-3	5.87	4.42	5.15	0.175	1.282	4.07	4.54	4.53	0.697	1.096
22:5 n-3	3.16^{b}	2.07^{a}	2.55^{ab}	0.031	0.636	2.86	2.81	2.99	0.894	0.609
22:6 n-3	21.84^{b}	12.25^{a}	17.51^{b}	<0.001	2.894	19.53^{b}	15.11^{a}	19.48^{ab}	0.022	2.933
∑ n-3 PUFA	35.76	40.96	41.09	0.384	6.872	29.79^{a}	42.15^{b}	39.92^{a}	0.003	5.645
20:2	1.42	0.98	1.24	0.098	0.333	1.04	0.97	1.11	0.734	0.278
22:2	0.73^{b}	0.37^{a}	0.54^{ab}	0.010	0.179	0.53	0.36	0.55	0.094	0.164
∑ PUFA	63.54	74.71	74.15	0.358	13.89	50.40^{a}	74.78^{b}	69.06^{a}	0.002	10.58
DHA/EPA	3.86^{b}	2.78^{a}	3.44^{b}	<0.001	0.297	5.10^{b}	3.41^{a}	4.33^{ab}	<0.01	0.714

SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, RSD: residual standard deviation, DHA/EPA: C22:6 n-3/ C20:5 n-3. Diet FO 100: diet formulated with fish oil as lipid source. Diet FO 0: diet in which fish oil was totally substituted by vegetable oil. The feeding plan gives the diet fed during the grow-out trial/the diet fed during the wash-out period. a,b,c Means with different superscript letter statistically differ. 1Total SFA include fatty acids not listed (<0.01 mg FA/g of tissue), C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C21:0, C23:0.

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CHAPTER 5

EFFECTS OF STOCKING DENSITY ON THE GROWTH AND FLESH QUALITY OF RAINBOW TROUT (*ONCHORHYNCHUS MYKISS*) REARED IN A LOW-TECH AQUAPONIC SYSTEM (Third contribution)

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ABSTRACT

In the present study, we evaluated the effects of two stocking densities (low - ALD, 3.81 kg m^{-3} vs. high - AHD, 7.26 kg m^{-3}) on the growth, health, and flesh quality of rainbow trout (*Oncorhynchus mykiss*) and the yield and microbiological quality of lettuce (*Lactuca sativa*) produced in a low-tech aquaponic system compared to hydroponic cultivation (HYP). Nine experimental units (three replications per treatment) were utilised. A total of 123 rainbow trout (initial body weight: 142 ± 35 g) were randomly distributed in six 500-L tanks (3 per stocking density) and monitored during a 117-day trial period. The final weight (331 g on average), specific growth rate (0.73% d^{-1}), feed conversion ratio (1.58), and mortality (3%) of the fish did not differ between stocking densities. The morphometric indices, slaughter results, and flesh quality were not affected. Similarly, the quantity of lettuce produced during two consecutive cycles was similar among treatments (2.4 kg m^{-2} on average). At harvest, microbial contamination (total viable count, E. coli, Enterobacteriaceae, Pseudomonas, mould, and yeasts) was similar in the fish skin and lettuce produced in aquaponic systems with different stocking densities, as well as in the lettuce produced in aquaponic and hydroponic systems. In conclusion, rainbow trout and lettuce productions were successful in the tested aquaponic system, whereas stocking density did not affect fish growth or flesh quality.

Keywords: water quality, hydroponics, slaughter results, microbial contamination

INTRODUCTION

Aquaponics is an emerging sustainable food production system, which combines fish farming (aquaculture) and soilless crop cultivation (hydroponics) in integrated multi-trophic systems where animals, plants, and microorganisms are in symbiosis (Rakocy et al., 2006; Goddek et al., 2015; König et al., 2018). These systems provide short and eco-friendly food supply chains with increased resource-use efficiency, high economic and environmental sustainability, and food resilience (Van Woensel et al., 2015). Low-tech aquaponic systems based on a simple design, easy management, and low capital costs are suitable for implementation in different geographic regions and production areas (Palm et al., 2018; Palm et al., 2019). Moreover, aquaponics could also play a key role in future urban farming developments and socio-economic progress in smart cities (dos Santos, 2016).

A vital requirement for one-loop aquaponic systems is the need for maintaining the optimal levels for fish and plants concerning the water temperature, pH, and chemical composition (Monsees et al., 2017; Gichana et al., 2018). In this regard, the stocking density of fish is a key factor for balancing aquaponic ecosystems, since it directly affects water quality in terms of nutrients, gases, and waste by-products, thus influencing plant growth as well as fish health and growth (Somerville et al., 2014; Yildiz et al., 2017; Palm et al., 2019).

Several 'easy-to-produce' fish species, such as Blue tilapia (*Oreochromis aureus*), Nile tilapia (*Oreochromis niloticus*), Common carp (*Cyprinus carpio*), tench (*Tinca tinca*), and African catfish (*Clarias gariepinus*), have been successfully reared in aquaponic farming (Somerville et al., 2014; Goddek et al., 2015). Most of this research has focused on warm-water fish species, whereas, to our knowledge, no data are available on cold-water species, such as rainbow trout (*Oncorhynchus mykiss*). Indeed, the choice of fish species depends upon its economic values, the market demand, and the geographic localisation of the production system (Forchino et al., 2017).

On the other hand, several crops have been grown in aquaponics, including herbs, fruiting species, and leafy vegetables (Thorarinsdottir, 2015). Lettuce (*Lactuca sativa*) is one of the main plant species used in aquaponic systems (Love et al., 2015), showing growth rates similar to those in hydroponics (Lennard and Ward, 2019; Monsees et al., 2019). Moreover, all studies have focused on fish performance/mortality and vegetal biomass production, whereas, to our knowledge, no information is available on the rheological and microbiological quality of the fish produced in aquaponic systems. Finally, few studies have tested the microbiological quality and safety of the vegetables in aquaponics (Elumalai et al., 2017; Schmautz et al., 2017; Mori and Smith, 2019).

Thus, our study aimed at evaluating the effects of two stocking densities in fish tanks on the growth performance and flesh quality traits of rainbow trout (*Oncorhynchus mykiss*) as well as the microbiological load of the end-products (both fish and vegetables) in a low-tech aquaponic system.

MATERIALS AND METHODS

Experimental conditions for the aquaponic and hydroponic systems

The research was conducted at the experimental farm of the University of Padova, North-East Italy (45°20′N, 11°57′E, 6 m a.s.l.), inside a plastic greenhouse. The experiment consisted of nine identical independent units (Figure 5.1), i.e., three hydroponic units (HYP) without fish, three aquaponics units with a low stocking density (ALD), and three aquaponics units with a high stocking density (AHD). The hydroponic section worked as a biofilter. The system size was designed basing on the recommendations of Somerville et al. (2014) for small-scale aquaponics units, i.e. 10 kg maximum fish biomass in a 500-L fish tank coupled with a biofilter having a minimum volume equal to 10–30% of the total fish tank volume. Before the start of the present trial, the system had been previously used for another cycle with common carp, which guaranteed about the regular functioning of the biofilter (Maucieri et al., 2019). Municipal water was used without any pre-treatment to fill the systems at the start of the European carp cycle. Then, to restore losses due to fish splashing, evaporation and transpiration during the trial, municipal water was used after stocking for at least 48 h. No energy to regulate water temperature, no probe for the continuous evaluation of water quality or remote management, and no device for water sanitation were used. No correction for alkalinity was performed.

Figure 4.1. Rendering of the aquaponic unit. A, main tank for fish (500 L); B, sedimenter; C, tanks for vegetables/biofilters (275 L); D, storage tank for water collection (50 L) and pumping to A tank.

Each independent unit consisted of: 1) a main tank (volume 500 L, height 0.80 m, diameter 0.90 m), in which the fish were kept in the aquaponic units or where the nutritive

solution was present in the hydroponic units; 2) two tanks for vegetables (volume 275 L each, height 0.35 m, diameter 1.00 m) for a total crop area of 1.6 m^2, filled with 225 L of light expanded clay aggregates (specific area 250 m^2 m^{-3}, packing density 300 kg m^{-3}, total porosity 0.55 m^3 m^{-3}; LECA Laterlite, Solignano, Italy), which received water from the main tank and acted both as a biofilter and hydroponic substrate for vegetable growth; and 3) a storage tank (volume 50 L, height 0.45 m) for collecting water from the vegetable tanks, before pumping it back into the main tank (Figure 5.1). The three parts of the system had water at different heights so that the water flow was guaranteed by the overflow (from the main tank to the vegetable tanks, and then to the storage tank). A single pump (Newa Jet 1700, NEWA TecnoIndustria Srl, Loreggia, Italy) returned the water from the storage tank to the main tank. The flow rate was 300 L h^{-1}, which corresponded to a complete recirculation of water every 2 h. The nine main tanks were aerated by a porous stone (4.0 cm × 4.0 cm × 15.0 cm, 14 L min^{-1}; Sweetwater® AS15S, Pentair, Cary, NC, USA) connected to an aerator (Scubla D100, Scubla Srl, Remanzacco, Italy) and covered with a net to prevent fish from jumping.

In vivo trial and recordings

The experiment was run during the winter season (November–February), under a natural photoperiod, and lasted 117 days. In all 9 units before the beginning of the trial, 132 g $unit^{-1}$ of KH2PO4, 197 g $unit^{-1}$ of K2SO4, 273 g $unit^{-1}$ of MgSO4·7H2O, 10 g $unit^{-1}$ of Fe-EDTA, and 5 g $unit^{-1}$ of micronutrients were added. On day one, fish were placed in the 3 ALD and 3 AHD units. Meanwhile, 333 g $unit^{-1}$ of $Ca(NO_3)_2$ and 480 g $unit^{-1}$ of (NH_4NO_3) were added to the 3 HYP units. The nutrient solution was calculated by the free software HydroBuddy based on optimal conditions for lettuce in hydroponics.

Six of the nine main tanks were stocked with a total of 123 rainbow trout, with an initial live weight of 142 ± 35 g, obtained from a commercial farm (Troticoltura Santa Cristina, Treviso, Italy). The three ALD units received 14 fish per tank (average initial stocking density of 3.81 kg m^{-3}), while the three AHD units received 27 fish per tank (average initial stocking density of 7.26 kg m^{-3}). The low and the high stocking density were chosen based on recommendations for small-scale aquaponics systems, i.e. 10–20 kg m^{-3} (Somerville et al., 2014). The high stocking density was chosen also in view of the maximum final biomass of the fish (estimated as 3.0–3.5 times the initial weight), consistent with recommendations for organic aquaculture (i.e., 25 kg m^{-3} for rainbow trout reared in freshwater; Commission Regulation 889/2008). Consistently with practice in not intensive farms, the fish were manually fed once a day, until apparent satiation and in two rounds separated by 20–30 min, with a commercial diet of extruded pellets (Skretting, Verona, Italy; composition: 40% crude protein, 11.5% crude fat, 4% crude fibre, 8% ash, 0.2% sodium, 1.5% calcium, and 0.8% phosphorus, as-fed basis). The quantity of feed administered daily was calculated for each aquaponic unit on the basis of the biomass present at the moment of each weighing, i.e., 1.5% of the biomass from the beginning of the experiment to 20 days, 1.0% from 23 to 53 days, and 1.5% from 56 days to the end of the experiment.

During the trial, two crop cycles of lettuce (*Lactuca sativa* L.) were cultivated in succession during 77 days for the first cycle and 44 days for the second one. At the beginning of each cycle, 20 plants per experimental unit (10 plants per tank, plant density of 13 plants m^{-2}) were transplanted during the third true leaf stage. The first cycle began 3 days before fish addition in the systems, the second cycle was harvested the day after fish harvesting. Plants were obtained from an external supplier. Neither pesticides nor antibiotics were used in the water or feed during the entire experiment.

Water quality

Throughout the trial, water lost from each unit was daily recorded and manually refilled. Two times per week, the outflow water of the fish tanks was monitored for the temperature, dissolved oxygen (DO), pH, redox potential (ORP), and electric conductivity (EC), using a portable multi-parameter apparatus (HQ40d Portable Multi-Parameter Meter, Hach Lange GmbH, Germany). The chlorophyll content was measured with a fluorescence detector (HHLD Fluorescence-Chlorophyll, Turner Designs, USA). The anion (NO_2^-, NO_3^-, and PO_4^{3-}) and cation (NH_4^+) contents in the water were determined by ion chromatography (Maucieri et al., 2019).

In vivo recordings of fish

Fish health was monitored daily. Fish were individually weighed by a scale (precision 1 g; Wunder Sa.Bi. srl, Trezzo sull'Adda, Italy) at the beginning of the trial (day 0), and then on a monthly basis at 22, 55, 84, and 117 days (end of the rearing period). For this, fish were removed from their tank, placed in a separate one, and anaesthetised with 10 mg L^{-1} of clove oil containing 87% eugenol. Fish were not fed for 48 h before and 24 h after weighing. The specific growth rate (SGR) and feed conversion ratio (FCR) were calculated as follows:

$$SGR\ (\%\ d^{-3}) = [(Log_e\ Final\ weight - Log_e\ Initial\ weight) / No.\ of\ days] \times 100$$

$$FCR = weight\ of\ dry\ feed\ distributed / net\ wet\ weight\ gain\ of\ fish$$

Recordings at fish slaughtering and vegetable harvest

At the end of the rearing period, all fish were slaughtered. Before slaughtering, fish were fasted for 24 h. Fish were harvested with a handling net, anaesthetised with 10 mg L^{-1} of clove oil in a separate tank, and then manually restrained using a plastic knob so that a percussion could be applied to the head of fish. All fish were weighed and 36 fish (6 per tank; 18 per experimental treatment), representative of their experimental groups regarding average body weight and variability, were selected, individually tagged, and stored in polystyrene boxes with ice in a cold room (0 to 2 °C). Eighteen fish (3 per tank; 9 per experimental treatment) were analysed one day after slaughter, whereas the other eighteen fish (3 per tank; 9 per experimental treatment) were analysed seven days after slaughter. The rigor mortis index (RI) was determined using the following formula (Bito et al., 1983):

$$RI\ (\%) = [(L_0 - L_t) / L_0] \times 100$$

where, L0 (cm) is the vertical distance between the base of the caudal fin and the table surface, measured immediately after death, and Lt (cm) is the vertical distance between the base of the caudal fin and the table surface at 1 and 7 days after slaughter.
For each storage time, the total length, standard length, head length, and maximum height were measured (Luxinger et al., 2018), and the following morphological indices (Di Marco et al., 2017) were calculated:

$$Condition\ factor = (body\ weight / total\ length^3) \times 100$$

$$Cranial\ index = head\ length / total\ length$$

$$Relative\ profile = maximum\ height / total\ length$$

Thereafter, the L*, a*, and b* colour indices of the skin were mea- sured at three points on the dorsal side with a Minolta CM–508C spectrophotometer (Minolta Corp., Ramsey, NJ, USA). A texture profile analysis (TPA) was performed at a central position on the lateral side under the first dorsal fin, using a TA.XT.plus Texture Analyser (Stable Micro Systems, Godalming, UK) with a 20-mm diameter cylindrical probe, with 5 mm compressions at a constant speed of 2 mm/s for two consecutive cycles, separated by a 5-s interval. The muscle pH was measured at three points on the dorsal side with a pH meter (Basic 20; Crison Instruments Sa, Carpi, Italy) equipped with a specific electrode (cat. # 5232; Crison Instruments Sa). Then, the fish were dissected and the carcasses were weighed. The carcass and fillet yields were calculated using the following formulae:

$$Carcass\ yield\ (\%) = (carcass\ weight / slaughter\ weight) \times 100$$

$$Fillet\ yield\ (\%) = (fillet\ weight / slaughter\ weight) \times 100$$

The skin was separated from the fillets, and then the flesh colour indices were measured at three points on the dorsal side of the fillets, taken from the right side of the fish (the right fillets) according to the procedure describe above. The left fillets were used to measure the total volatile base-nitrogen (TVB-N) content (EEC, 1995).
At harvesting (77 days: first crop cycle; 44 days: second crop cycle), all lettuce plants were divided into aboveground and belowground parts, and the fresh weight of the leaves was immediately recorded to determine the marketable yield following the same procedure reported by Maucieri et al. (2019).

Microbiological analysis of vegetables and fish

At the time of fish slaughter, leaves from all plants (i.e. those of the second cycle) were collected and pooled (one pool per tank, 9 pools). The pooled samples were rinsed with 400 mL of Buffer Peptone Water (BPW) and rinsates were used for the detection and enumeration of different microbial targets.

Then, the same 36 fish selected for rheological analyses were used for sampling at the skin level immediately after slaughter (18 specimens; 3 fish × 3 tanks × 2 groups) and 7 days later, after storage at 2 °C (18 specimens; 3 fish × 3 tanks × 2 groups). For sampling, a standard area of the skin (25 cm^2) was swabbed with sterile cotton swabs, which were placed in 10 mL of Maximum Recovery Diluent (8 g NaCl/L, 1 g of bacteriological peptone/L) and then serially diluted.

Targets bacteria included specific spoilage organisms (SSO) associated with fish spoilage during storage (i.e. psychotrophyc bacteria such as *Pseudomonas* and putative H2S producers), besides bacteria associated with faecal contamination (i.e. *Escherichia coli*). Total mesophilic count (as total viable count), mould, yeast, and *Enterobacteriaceae* were also evaluated being generic food quality indicators.

In both vegetables and all fish sampled at slaughter and after 7 d, the total viable count (TVC) was evaluated on Plate Count Agar (Biokar Diagnostics, Beauvais, France), incubated at 30 °C for 72 h (ISO 4833- 1:2013). The contamination provided by *Enterobacteriaceae* was assessed using Violet Red Bile Glucose Agar (Biokar Diagnostics), incubated at 37 °C for 24 h (ISO 21528-2:2017). *Escherichia coli* counts were performed on a Tryptone Bile X-Glucuronide (TBX, Oxoid Ltd., Basingstoke, Hampshire, UK) medium, incubated at 44 °C for 18–24 h (ISO 7251:2005). The count of H2S-producing bacteria (putative *Shewanella* spp.) was carried out on iron agar (Lyngby, Laboratorios Conda, Torrejón de Ardoz, Spain), incubated at 25 °C for 48 h (Andreani and Fasolato, 2016). The *Pseudomonas* spp. count was evaluated on *Pseudomonas* Agar Base supplemented with cetrimide, fucidine, and cephaloridine (Oxoid), incubated at 25 °C for 48 h (Andreani and Fasolato, 2016). In trout samples, yeast and mould counts were performed on Oxytetracycline Glucose Yeast Extract Agar (OGYE, Oxoid), incubated at 25 °C for 3–5 days (ISO 21527-1:2008). The results were reported as $\log_{10}$ CFU/cm^2 or mL of rinsate.

Ethics statement

The study was approved by the Ethical Committee for Animal Experimentation (Organismo per la Protezione del Benessere Animale, OPBA) of the University of Padova (project no. 6/2017; prot. n. 15,132). All animals were handled according to the principles stated by the EC Directive 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes. Research staff involved in animal handling were animal specialists (PhD or MS in Animal Science) and veterinary practitioners.

Statistical analysis

The data concerning the water quality, vegetable yields and microbiological quality were analysed by a one-way ANOVA with the experimental group (ALD, AHD, and HYP) as the main effect. The growth performance and slaughter data of the fish were analysed by a one-way ANOVA with the stocking density (ALD and AHD) as the main effect. The rigor mortis, physicochemical traits, and microbiological quality data of the fish were analysed by a two-way ANOVA with the stocking density, storage time (1/7 days), and their interaction as the main effects. The PROC GLM of SAS (Statistical Analysis System, 2013) was used for all analyses. Bonferroni's test was used to compare means. Differences among means with $p < .05$ were assumed to be statistically significant.

RESULTS

Water characteristics

Throughout the trial, the daily water losses averaged 2.45 L d^{-1}, i.e., 0.41% of the total water contained in each unit, without significant differences among groups (data not reported in tables). The EC, redox potential, and contents of ammonium, nitrite, and nitrate in the water varied according to the following pattern: ALD < AHD < HYP ($p < .001$). However, the phosphate content was higher in aquaponic units compared to hydroponic ones, and increased significantly with the stocking density of fish ($p < .001$; Table 5.1).

Table 5.1. Physicochemical traits of water over the experimental period (117 days).

	Experimental groups				
	ALD	AHD	HYP	P-value	RMSE
Tanks (n)	3	3	3		
Temperature (°C)	10.8	10.8	10.6	0.722	2.0
Dissolved oxygen (mg L^{-1})	9.58^{A}	8.69^{B}	11.31^{C}	<0.001	0.85
pH	7.43^{B}	7.24^{A}	7.25^{A}	0.005	0.45
Chlorophyll (µg L^{-1})	55.3^{B}	80.2^{C}	14.1^{A}	<0.001	38.2
Electrical conductivity (ds cm^{-1})	1.63^{A}	1.89^{B}	2.81^{C}	<0.001	0.20
Redox potential (mV)	75.2^{A}	83.1^{AB}	90.6^{B}	<0.001	24.9
NH_4^+ (mg L^{-1})	0.55^{Aa}	0.80^{Ab}	21.20^{B}	<0.001	13.41
NO_2^- (mg L^{-1})	0.07^{Aa}	0.18^{Ab}	7.74^{B}	<0.001	7.74
NO_3^- (mg L^{-1})	314^{A}	417^{B}	1405^{C}	<0.001	202
PO_4^{3-} (mg L^{-1})	86.3^{B}	106.5^{C}	42.9^{A}	<0.001	16.8

ALD: aquaponic units at low stocking density of fish; AHD: aquaponic units at high stocking density of fish; HYP: hydroponic units. RMSE: Root mean square error. Means with different superscript letters are statistically different (a, b: $p < .05$; A, B: $p < .01$).

Trends in water characteristics during the trial are shown in Figure 5.2. Water temperature changed with external environmental conditions (Figure 5.2a); dissolved oxygen showed a similar pattern in the three groups, being always higher in HYP tanks (Figure 5.2b); pH decreased in all groups from about 8.00 until about 7.00 (Figure 5.2c). Ammonia added

with the fertilizer in the HYP tanks at the beginning of the trial disappeared by the 60th d of trial (Figure 5.2d), being converted in nitrite (Figure 5.2e) and then in nitrate (Figure 5.2f). Ammonia produced by fish was higher in AHD tanks compared to ALD tanks during the first weeks; it was similar in the two groups in following period, whereas it showed an increase after 100 d when fish biomass increased (Figure 5.2d). Nitrite sharply increased in AHD tanks from 100 d onwards (Figure 5.2e) whereas nitrate showed a constant increase in both groups until about 600 mg L^{-1} at the end of the trial (Figure 5.2f).

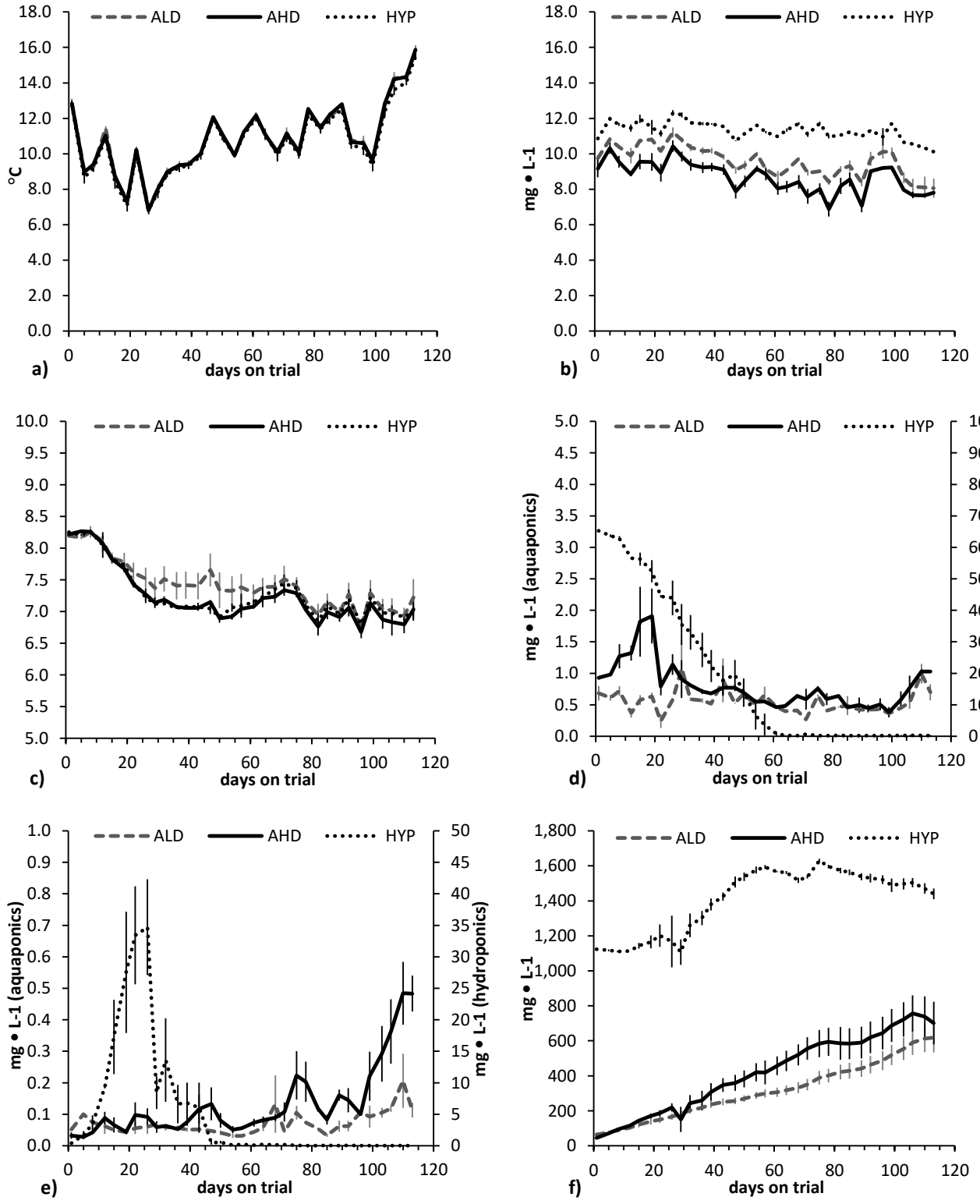

Figure 5.2. Temperature (a), dissolved oxygen (b), pH (c), ammonium (d), nitrite (e) and nitrate (f) values (means ± SE) measured in water of the main tanks with fish during the trial. ALD: aquaponic system with low stocking density of fish; AHD: aquaponic system with high stocking density of fish; HYD: hydroponic system.

Fish growth performance and biomass production

Throughout the trial, only four fish died (2 from ALD and 2 from AHD treatments) without showing any previous symptoms of disease. The stocking density did not affect the live weight, SGR, or FCR of the fish (Table 5.2). At the end of the trial, the live weight and SGR averaged 331 ± 78 g and 0.73 ± 0.06% d^{-1}, respectively, while the FCR reached 1.58 ± 0.16 (average of AHD and ALD fish). The stocking density at the end of the experiment reached 8.86 and 16.94 kg m^{-3} in ALD and AHD groups, respectively (Table 5.2).

Table 5.2. Growth performance of rainbow trout

	Stocking density			
	ALD	AHD	P-value	RMSE
Total fish per treatment (n)	40	78		
Tanks (n)	3	3		
Fish weight (g)				
0 days	143	140	0.657	6
22 days	170	166	0.726	12
55 days	217	215	0.886	19
84 days	264	256	0.660	20
117 days	333	329	0.871	26
Specific growth rate (% d^{-1})				
0-22 days	0.79	0.76	0.850	0.17
22-55 days	0.74	0.77	0.693	0.08
55-84 days	0.69	0.62	0.298	0.06
84-117 days	0.70	0.76	0.449	0.08
0-117 days	0.72	0.73	0.928	0.04
Feed conversion ratio				
0-22 days	1.52	1.58	0.868	0.38
22-55 days	1.28	1.14	0.317	0.15
55-84 days	1.84	1.87	0.842	0.20
84-117 days	1.73	1.44	0.255	0.27
0-117 days	1.65	1.51	0.323	0.15
Biomass (kg m^{-3})				
0 days	3.81	7.26	<0.001	0.49
22 days	4.53	8.57	<0.001	0.36
55 days	5.78	11.04	<0.001	0.29
84 days	7.04	13.20	<0.001	0.49
117 days	8.86	16.94	<0.001	0.51
Biomass growth (kg m^{-3})				
0-22 days	0.72	1.31	0.031	0.22
22-55 days	1.25	2.47	0.002	0.21
55-84 days	1.26	2.16	0.010	0.24
84-117 days	1.82	3.74	<0.001	0.21
0-117 days	5.05	9.68	<0.001	0.08

ALD: aquaponic units at low stocking density of fish; AHD: aquaponic units at high stocking density of fish. RMSE: Root mean square error.

Biometric traits and flesh quality of fish

The stocking density did not affect the morphologic traits/indices, slaughter results (Table 5.3), or flesh quality of the fish (Table 5.4). On average, the carcass yield was 89.0% of the slaughter weight. Meanwhile, the fillet weight and yield were 163 g and 49.1% of the slaughter weight, respectively.

Regarding the storage time, after 7 days the rigor index (−75.5 units), muscle pH (−0.095 units), hardness (−15.8 N), and chewiness (−5.09 units) significantly decreased ($.001 < p < .01$). Meanwhile, the lightness of the skin (+12.7 units) and fillet (+5.7 units) and the yellowness of the skin (+2.8 units) increased ($p \leq .001$). Whereas, the redness of the skin (−3.6 units) and the yellowness of the fillet (−1.8 units) decreased ($p < .001$).

Table 5.3. Morphometric indices and slaughter results of rainbow trout

	Stocking density			
	ALD	AHD	P-value	RMSE
Fish (n)	18	18		
Total length (mm)	289	290	0.858	15
Standard length (mm)	250	248	0.797	13
Head length (mm)	61	61	0.644	3
Maximum height (mm)	71	73	0.402	7
Condition factor (%)	1.37	1.35	0.774	0.17
Relative profile	0.25	0.25	0.299	0.02
Cranial index	0.21	0.21	0.356	0.01
Carcass weight (g)	287	289	0.881	73
Carcass yield (%)	89.0	89.0	0.825	0.5
Fillet weight (g)	165	160	0.632	32
Fillet yield (%)	49.9	48.3	0.095	2.9

ALD: aquaponic units at low stocking density of fish; AHD: aquaponic units at high stocking density of fish. RMSE: Root mean square error.

Table 5.4. Rigor index and flesh quality measured at different times (1 day and 7 days after slaughter) in rainbow trout stored in ice (0 to 2°C).

Stocking density (D)	ALD		AHD		P-value			
Time (T)	1 d	7 d	1 d	7 d	D	T	D×T	RMSE
Fish (n)	9	9	9	9				
Whole fish								
Rigor index (%)	92.4	18.7	89.2	11.6	0.216	<0.001	0.645	0.1
Muscle pH	6.55	6.45	6.54	6.45	0.956	0.014	0.885	0.11
Hardness (N)	25.0	6.9	20.2	6.7	0.279	<0.001	0.329	6.8
Cohesiveness	0.82	0.77	0.83	0.71	0.606	0.101	0.457	0.15
Springiness (mm)	0.39	0.34	0.32	0.45	0.902	0.807	0.598	0.47
Chewiness (N mm)	7.74	1.88	5.59	1.28	0.105	<0.001	0.357	2.47
TVB-N (mg 100 g^{-1})	16.3	16.5	16.9	16.7	0.293	0.931	0.540	1.1
Skin								
L*	41.0	48.6	37.1	54.8	0.544	<0.001	0.136	12.8
a*	4.06	0.02	3.34	0.11	0.324	<0.001	0.201	0.93
b*	6.94	11.00	7.40	9.03	0.321	0.001	0.117	2.27
Fillets								
L*	38.3	43.8	39.1	45.0	0.111	<0.001	0.775	1.8
a*	-1.71	-1.32	-1.65	-1.06	0.558	0.088	0.724	0.84
b*	9.72	7.58	9.78	8.33	0.402	0.001	0.466	1.42

ALD: aquaponic units at low stocking density of fish; AHD: aquaponic units at high stocking density of fish. RMSE: Root mean square error.

Marketable yield of vegetables

The marketable lettuce yield was 2.82 ± 0.64 kg m^{-2} at the end of the first cycle and 1.89 ± 0.18 kg m^{-2} at the end of the second cycle (average of the three groups). On average for the two cycles, the lettuce production in the aquaponic system was similar to that of the hydroponic cultivation, even if a higher production was recorded in aquaponic units compared to hydroponic ones in the second cycle (on average 1.98 vs. 1.75 kg m^{-2}, $p < .01$; Figure 5.3).

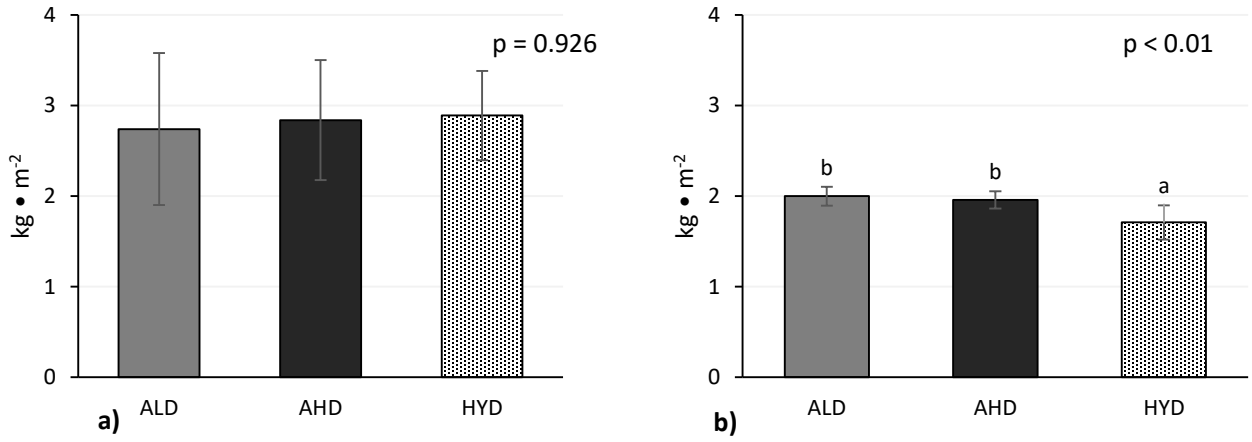

Figure 5.3. Lettuce production in the first cycle (a) and in the second cycle (b). ALD, aquaponic system with low stocking density of fish; AHD: aquaponic system with high stocking density of fish; HYD: hydroponic system.

Microbiological quality of fish and vegetables

At the skin level, the TVC and *Enterobacteriaceae* counts were not affected by the stocking density or sampling time (Table 5.5). *Pseudomonas* increased (0.36 to 0.81 $\log_{10}$ CFU cm^{-2}; p = .024) and H2S-producing bacteria tended (0.41 to 0.86 $\log_{10}$ CFU cm^{-2}; p = .072) to increase from ALD to AHD groups. *Escherichia coli*, moulds and yeasts were not found in the skin or lettuce and thus their counts are not given in tables. In addition, regarding the lettuce, the TVC, *Enterobacteriaceae*, and *Pseudomonas* counts did not differ between the aquaponic and hydroponic systems (Table 5.6).

Table 5.5. Total viable count, *Enterobacteriaceae*, *Pseudomonas* and H_2S-producing bacteria (Log_{10} CFU 20 cm^{-2}) measured at skin level at different times (at slaughter, 0 d, and 7 d after slaughter) in rainbow trout stored in ice (0 to 2°C).

Stocking density (D)	ALD		AHD		P-value			
Time (T)	0 d	7 d	0 d	7 d	D	T	D×T	RMSE
Total viable count	2.06	2.41	2.59	2.44	0.401	0.982	0.119	0.64
Enterobacteriaceae	0.63	1.00	0.92	0.93	0.665	0.469	0.502	0.78
Pseudomonas	0.39	0.33	0.87	0.74	0.024	0.637	0.846	0.57
H_2S-producing bacteria	0.41	0.41	0.89	0.82	0.072	0.889	0.890	0.72

ALD: aquaponic units at low stocking density of fish; AHD: aquaponic units at high stocking density of fish. RMSE: Root mean square error.

Table 5.6. Total viable count, *Enterobacteriaceae* and *Pseudomonas* (Log_{10} CFU ml^{-1}) measured in lettuce at harvesting.

	Experimental group				
Parameters	ALD	AHD	HYD	P-value	RMSE
Lettuce					
Total viable count	5.41	5.89	5.64	0.536	0.50
Enterobacteriaceae	3.43	2.44	1.98	0.274	1.00
Pseudomonas	6.04	5.70	6.03	0.492	0.38

RMSE: Root mean square error.

DISCUSSION

Growth performance of fish

In the present study, the stocking density did not affect the growth performance of rainbow trout reared in low-tech aquaponic systems. The water conditions were maintained within acceptable values for trout growth (Azevedo et al., 1998; Bregnballe, 2015). In fact, the water temperature ranged from 7.0 to 15.9 °C (Figure 4.2a), with an average of 10.7 °C. The DO values were consistently within suitable ranges for the health and welfare of rainbow trout (Ellis et al., 2002), nutrient adsorption capacity of plant roots, and productivity of nitrifying bacteria (Somerville et al., 2014). As expected, the lowest DO values were recorded in the AHD units (Figure 4.2b) due to the higher fish and bacteria respiration (Hussain et al., 2014; Rayhan et al., 2018; Maucieri et al., 2019). The water pH (on average 7.3, range 8.1–6.7; Figure 4.2c) was also consistent with the requirements for rainbow trout ($7.0 < pH < 8.0$; Bregnballe, 2015) and bacteria ($7.0 < pH < 9.0$; Antoniou et al., 1990; Goddek et al., 2015). Considering the water pH and temperature during the trial, the content of unionised ammonia was consistently below the critical threshold for rainbow trout (0.06–1.10 mg L^{-1}; Ellis et al., 2002) in both ALD and AHD treatments. Similarly, the nitrites remained within a safe range (Kroupova et al., 2008). On the other hand, the water nitrate content overpassed values recommended for cold species in aquaponic systems (400 mg L^{-1}; Somerville et al., 2014) for about half of the trial in the AHD tanks and about one third in the ALD tanks (Figure 4.2f).

The activity of nitrifying bacteria in ALD and AHD units was proven by the nitrate content, which was higher than previously observed in other one-loop aquaponic systems (Lennard and Leonard, 2006; Palm et al., 2014; Maucieri et al., 2019). The progressive accumulation of nitrates in the aquaponic systems could be related to the reduction in the nutrient uptake by plants at higher pH values (Wortman, 2015) or an unbalance between available nitrate and vegetable biomass. Nevertheless, changes in water ammonia and nitrite during the trial can be related to changes in water temperature and pH, which can affect the values: 6.9 and 11.5 kg m^{-3}) (Maucieri et al., 2019). To our knowledge, only one study is available concerning trout reared in aquaponic systems focused on microbiological analysis of lettuce (Alcarraz et al., 2019), whereas commercial aquaponics systems are successfully working in Columbia, Chile and Canada. Other authors found that the mortality rate

simultaneously increased when the initial stocking density increased, both in *Cyprinus carpio* var. Koi (from 2.1 to 2.8 kg m^{-3}) (Hussain et al., 2014) and *Oreochromis niloticus* (from 0.5 to 1.6 kg m^{-3}) (Rayhan et al., 2018) cultured in aquaponics. However, the response to different stocking densities could vary dramatically among fish species (Ashley, 2007), and is largely affected by the biological requirements of the fish and water quality.

Nevertheless, in the present trial, the SGR of trout were considerably lower and FCR higher than that observed in open or recirculating aquaculture systems (Naderi et al., 2017; Zahedi et al., 2019). Low water temperatures (10.7 °C on average) and moderate feeding levels likely accounted for these results. However, rainbow trout show feeding activity at water temperatures as low as 6 °C, while growth and feed conversion are optimized at 15–16 °C (Azevedo et al., 1998; Bregnballe, 2015). In intensive aquaculture systems, a high stocking density can impair the feed efficiency and growth performance of trout (see Ellis et al., 2002 for a review). Recent studies have confirmed the negative impacts of a high stocking density on the growth performance of different fish species (Li et al., 2012; de las Heras et al., 2015; Ni et al., 2016), including trout (Suárez et al., 2014; Naderi et al., 2017; Zahedi et al., 2019). However, regarding rainbow trout, these effects are detectable only when the fish biomass surpasses 24 kg m^{-3} (677 g final weight) (Zahedi et al., 2019), which did not occur in the current experiment. On the other hand, North et al. (2006) did not observe significant differences in the final weight of female rainbow trout (180 g initial weight; approximately 400 g final weight) stocked at densities of 10, 40, and 80 kg m^{-3}. Indeed, multiple factors, such as fish age, fish size, and water quality, could modify the effects of stocking density on the growth performance and welfare of fish (Ashley, 2007; Poli, 2009). Furthermore, the competition for food and physiological stress due to crowding conditions may also reduce the growth performance of fish (Qi et al., 2016).

Rheological and microbiological shelf life of fish

As for the growth performance, the stocking density did not modify the slaughter results or morphometric indices of trout. In contrast, Suárez et al. (2014) found a decreased final weight, total length, fillet weight, and viscerosomatic index when the initial stocking density increased from 15 to 40 kg m^{-3} in one-year-old rainbow trout (100 g initial weight) cultured in tanks continuously supplied with 1 L s^{-1} of water at 14 °C.

In our trial, we did not detect significant differences in the flesh quality according to the stocking density. In standard land-based production systems, a high stocking density is a major chronic stressor in farmed fish (Hoyle et al., 2007), which may also affect the end-product quality. In fact, a high rainbow trout stocking density (40 kg m^{-3}) has been known to reduce the muscle pH and water holding capacity, and enhance the rigor strength and firmness (Suárez et al., 2014). Nevertheless, in the low-tech aquaponic system we tested, the highest stocking density used did not produce stressful conditions for the rainbow trout and, as a consequence, there were no negative effects on the flesh quality.

The changes in fish texture during storage could be related to autolytic and microbial processes that take place after death, which make the muscle softer and less elastic (Li et al.,

2011; Cai et al., 2014). This is usually associated with an increase in flesh lightness during cold storage, as observed in our trial and extensively reported in fillets of rainbow trout (Jouki et al., 2014; Dehghani et al., 2018) and European sea bass (Dicentrarchus labrax; Chéret et al., 2005). Moreover, after one week of storage in ice, lighter skin has also been found in the Atlantic salmon (*Salmo salar*; Erikson and Misimi, 2008). However, other authors have found a decreased L* (Álvarez et al., 2008) with an increasing storage time. Therefore, in the present study, the reduction in the rigor index, and hardness and chewiness of the flesh was expected and consistent with previous results concerning trout fillets (Jouki et al., 2014; Concollato et al., 2016), whole gilthead seabream (*Sparus aurata*; Álvarez et al., 2008), and whole red seabream (*Pagrosomus major*; Cai et al., 2014). In fact, the L* index changes based on the muscle structure and amount of free water, which affects light scattering (Chéret et al., 2005). Moreover, significant a* and b* changes can occur in fish during storage because of the oxidation of heme pigments and lipids, respectively (Dehghani et al., 2018).

Under the conditions used in this trial, the flesh pH decreased from 24 h to 7 days of storage, which could be attributed to the anaerobic process of muscle glycogen breakdown with the consequent production and accumulation of lactic acid (Grigorakis et al., 2003; Daskalova, 2019). Nevertheless, several authors have reported that the muscle pH of fish remained stable (Grigorakis et al., 2003; Álvarez et al., 2008) or increased (Dehghani et al., 2018; Secci et al., 2018) after 6–8 days of cold storage, due to the formation of alkaline by-products of microbial origin (Mokrani et al., 2018). In the current study, the TVB-N concentration in the fish muscle did not change after one week of storage in ice and remained below the acceptability threshold (25–27 mg TVB-N 100 g^{-1} of sample) (Giménez et al., 2002; Ninan et al., 2011). The shelf life for rainbow trout stored on ice is approximately 8–11 days (Ninan et al., 2011; Sampels, 2014). In our study, the skin microbial contamination remained stable during the 7-day storage, which is consistent with the absence of change concerning TVB-N. The low levels of spoiler targets, such as *Pseudomonas* and *Shewanella*, ex- plain the dynamics of chemical markers of freshness, such as TVB-N. Nevertheless, the surface washing of fish by melting ice could have also contributed to this result, as reported in other studies on rainbow trout and other species using slurry ice (Aubourg et al., 2009; Rodríguez et al., 2006). Moreover, in our trial, the initial and final mesophilic counts were lower than those previously measured in other species reared in aquaponics (Elumalai et al., 2017), whereas the initial skin contamination was consistent with previous recordings in the same species farmed under conventional aquaculture systems (Aubourg et al., 2009).

Lettuce production and microbiological contamination

The increasing concerns regarding water consumption in food pro- duction should be solved by a comprehensive approach that includes several strategies focused on the improvement of water-use efficiency, especially where the availability of water for agricultural purposes is a critical factor (Goddek et al., 2015). In the present trial, the daily water consumption was within ranges reported in most of the literature (0.05–5.0%; see Maucieri et al., 2018 for a review). As expected, results obtained during winter were 70%

lower than those achieved in the same aquaponic system during summer (Maucieri et al., 2019). Other authors have reported that aquaponic systems can equal or even overcome the hydroponic method in the production of lettuce, herbs, and fruiting vegetables (Suhl et al., 2016; Lennard and Ward, 2019). This is consistent with our results that showed the comparable (first crop cycle) or greater (second crop cycle) marketable yield of lettuce in aquaponics compared with the HYP units. The vegetable production in aquaponics is supported by the continuous supply of N compounds from the fish and the dense community of microbial flora in the system, which may assist plants in nutrient access and uptake (Lennard and Ward, 2019).

Despite Pantanella et al. (2012) reporting that the yield of lettuce decreased in aquaponics with respect to hydroponics when the fish density dropped from 8 to 5 kg m^{-3}, we did not find any effect of the stocking density on lettuce production which means that nutrient supply was sufficient also in the case of the low stocking density. In addition, Maucieri et al. (2019) observed similar yields of lettuce in hydroponics and aquaponics at a low fish stocking density (Cyprinus carp, 2.5 kg m^{-3}). Overall, the marketable yield of lettuce achieved in the present study was lower than that reported by other authors (Lennard and Leonard, 2006; Maucieri et al., 2019). Differences in the variety of lettuce, environmental conditions, nutrient concentrations, water temperature, and solar irradiation justify the different results among studies.

Regarding the microbial quality of the lettuce, leafy vegetables are often largely contaminated. The results of the present trial are consistent with previous ones concerning whole vegetables, such as red chicory, on which *Pseudomonas* (6 Log_{10} CFU g^{-1}) and *Enterobacteriaceae* (4 Log_{10} CFU g^{-1}) were dominant microorganisms (Alfonzo et al., 2018). Under the conditions of the present study, the average mesophilic count (reported as TVC) at harvest in the lettuce rinsate (5.65 Log_{10} CFU mL^{-1}) was comparable to the values measured after 63 days (4.5 Log_{10} CFU $^{g-1}$) but higher than those after 118 days of cultivation (2.8–3.5 Log_{10} CFU g^{-1}) in a previous study cultivating lettuce in aquaponics (Elumalai et al., 2017). Moreover, we did not find differences in the counts of mesophilic bacteria and *Enterobacteriaceae* between lettuce leaves produced in aquaponics or in hydroponics as already found by Alcarraz et al. (2019) who did not find differences neither in psychrophilic bacteria. Finally, in lettuce, as with fish samples, the absence of faecal indicator bacteria, such as *E. coli*, suggests the aquaponic system was suitably hygienic (Elumalai et al., 2017). On the other hand, a recent study (Wang et al., 2020) highlighted the risk for foodborne pathogens (e.g. *E. coli* STEC) in aquaponic and hydroponic systems.

CONCLUSIONS

The economic margin of aquaponic systems deployed in cold environments could be increased by rearing species characterised by a higher market value than the warm-water species usually cultured, such as rainbow trout. Based on our results, rainbow trout can be successfully farmed in a low-tech aquaponic system until a final density of approximately 17 kg m^{-3} without negative effects on the growth and flesh quality. The marketable yield of

lettuce, similar to that obtained in hydroponics, confirmed that aquaponics is a viable integrated production system for both fish and vegetables. The chemical, physical, and microbiological indicators proved that food-safe products can be obtained from a low-tech aquaponic system.

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CHAPTER 6

PERFORMANCE AND FILLET TRAITS OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) FED DIFFERENT LEVELS OF *HERMETIA ILLUCENS* MEAL IN A LOW-TECH AQUAPONIC SYSTEM (Fourth contribution)

Presented at:

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ABSTRACT

In this study, we evaluated the effects of dietary substitution of fishmeal (FM) with partially defatted *Hermetia illucens* meal (HI) on the growth, feed digestibility, gut morphology, and fillet quality of rainbow trout reared in a low-tech aquaponic system. A total of 173 rainbow trout (initial body weight: 156 g ± 39.8 g) were distributed among nine experimental aquaponic units. Three diets were fed to fish (three aquaponic units per dietary treatment) over a period of 76 days, i.e. H0 diet (control), H6 diet, and H12 diet containing 0 g/kg, 62 g/kg, or 124 g/kg of HI and 200 g/kg, 150 g/kg, or 100 g/kg of FM, respectively. We found that the apparent digestibility coefficients of diets were unaffected by the inclusion level of dietary HI. At the end of the trial, trout mortality was low (2.9%) and unaffected by dietary treatment. The specific growth rate was, however, lower in fish fed the H12 diet than in those fed H0 and H6 diets after 26 days (1.07% d^{-1} vs. 1.22% d^{-1}; $P < 0.001$) and at the end of the trial (0.81% d^{-1} vs. 0.88% d^{-1}; $P < 0.05$). In contrast, dietary inclusion of HI appeared to have no appre- ciable effect on the feed conversion ratio (on average 1.53), final weight (303 g), fish condition factor (1.40), viscerosomatic index (10.9%), or hepatosomatic index (1.22%). The inclusion of HI was, nevertheless, found to promote a 10% increase in the density of goblet cells in the gut of fish fed the H12 diet compared with those receiving the H0 diet ($P < 0.05$). With regards to fillet traits, redness and yellowness indices were lower in fish fed the H12 diet than in those fed the H0 diet. Although dietary HI had little effect on the proximate composition and fatty acid profile of fish, the proportions of C12:0 and C14:0 increased with HI dietary inclusion. In conclusion, fish growth and fillet quality were essentially unaffected by a 25% fish meal replacement with HI in isonitrogenous and isoenergetic diets (control diet containing 200 g/kg of fish meal), whereas at a replacement rate of 50%, we detected certain effects on gut histology and fillet colour and nutritional characteristics, which warrant further investigation.

INTRODUCTION

The global demand for food products is expected to grow by between 1.1% and 1.5% per year by 2050 (Alexandratos and Bruinsma, 2012). However, meeting these demands will necessitate an ever-increasing reliance on natural resources such as water, land, and nutrients, which are already unsustainably exploited by modern agricultural practices (Lennard and Goddek, 2019). To ensure sufficient food production and at the same time conserve natural resources and maintain environmental integrity, it will be necessary to develop alternative and more sustainable agricultural methods (Goddek et al., 2015; Maucieri et al., 2019). Aquaponics (AP), that combines recirculating aquaculture systems (RAS) with soilless hydroponic plant production and reduces water consumption (Verdegem et al., 2006; Endut et al., 2011), land use (Barbosa et al., 2015; dos Santos, 2016), and nutrient wastes (Nichols and Savidov, 2012; Graber and Junge, 2009; Wongkiew et al., 2017), can make an important contribution in this regard. In particular, aquaponic systems have relevant advantages with respect to nutrient cycling, by avoiding the discharge of fish effluents enriched with dissolved nitrogen and phosphorus into groundwater (van Rijn, 2013), and facilitating the fertilization of soilless crops with organic inputs derived from fish faeces (Goddek et al., 2015).

Fish feed serves as a primary nutritional source for aquaponic systems, providing nutrients that sustain fish, bacteria, and plants (Lennard and Goddek, 2019). The main protein sources in aquafeeds are fishmeal (FM) and soybean meal (Naylor et al., 2009; Turchini et al., 2009; Gasco et al., 2018). However, the use of both these nutrient sources raises important sustainability concerns on account of the increasing price of FM (Naylor et al., 2000; Tacon and Metian, 2018; FAO, 2018) and the environmental costs associated with protein-rich plant production (Foley et al., 2011). In this context, insect-based meals could represent a sustainable alternative source of protein for fish production (Mancini et al., 2018). Among insects with potential utility in this respect is the black soldier fly (*Hermetia illucens*; HI), which is considered one of the most promising species owing its low environmental impact, rearing requirements, and high adaptability to low-cost substrates such as manure, food by-products, and waste (Mancini et al., 2018; Gasco et al., 2020). Moreover, HI prepupae meal is characterized by an essential amino acid profile similar to that of FM (Henry et al., 2015) and is part of the natural diet of fish (Henry et al., 2015). In addition, the production of these insects leaves a small ecological footprint, results in minimal releases of greenhouse gases and ammonia, and has limited need for arable land (Van Huis 2013; Makkar et al., 2014; Henry et al., 2015). Notably, the inclusion of processed insects in aquafeeds has recently been permitted in Europe (EC, 2017). With regards to the species of fish used for AP, high-value species such as rainbow trout (*Oncorhynchus mykiss* Walbaum) have the potential to enhance the profitability and competitiveness of systems compared with the cyprinids and tilapias that are typically farmed using this technique (Palm et al., 2019). However, rainbow trout are notably more demanding in their requirements, particularly in terms of water temperature (7–18 °C) (Woynarovich et al., 2011) and dissolved oxygen (6–8 mg/L) (Timmons and Ebeling, 2013). To the best of our knowledge, there has to date been only a

single study that has investigated the growth performance and quality of rainbow trout reared under aquaponic conditions (chapter 5, third contribution), although some commercial farms have already been established and are currently operational in Canada, Chile, and Colombia. Moreover, only two studies have investigated the inclusion of insect meal in aquafeed of fish raised in aquaponic systems, namely, the Nile tilapia (Kessen, 2016) and Siberian sturgeon (Zarantoniello et al., 2021). Thus, in this study, we aimed to assess the growth performance, diet digestibility, gut morphometry, and product quality of trout reared in a low-tech aquaponic system and fed different levels of HI meal.

MATERIALS AND METHODS

The growth trial conducted in the present study was performed at the Experimental Farm of the Department of Agronomy, Food, Natural Resources, Animal, and Environment of the University of Padova (Legnaro, Padova, Italy). Fish diets were prepared and a digestibility trial was carried out at the Experimental Facility of the Department of Agricultural, Forest and Food Sciences of the University of Torino (Torino, Italy). The study was approved by the Ethical Committee for Animal Experimentation (Organismo preposto al Benessere degli Animali) of the University of Padova (project no. 6/2018; prot. n. 15132 approved on 25/01/2018). The digestibility trial protocol was approved by the Ethical Committee of the University of Torino (Prot. N. 143,811). Animals were handled in accordance with the principles stated by the EU Directive 2010/63/EU regarding the protection of animals used for experimental and other scientific purposes. The research staff involved in animal handling were animal specialists (PhD or MS in Animal Science) and veterinary practitioners.

Equipment

The trial was performed at the farm of the University of Padova (Northeast Italy, 45◦ 20′ N; 11◦ 57′ E; 6 m a.s.l.) in a plastic greenhouse with 50% shading. The experimental system consisted of nine independent units, each of which comprised a main tank containing fish (volume 500 L; height 0.80 m) and a sedimenter (volume 100 L; height 0.60 m) (Fig. 6.1). These units also included two tanks containing plants (volume 275 L each; height 0.35 m), filled with 225 L of expanded clay (LECA Laterlite, Solignano, Italy), which received water from the main tank containing fish and acted both as biofilter and substrate for plant growth, as well as a water storage tank (volume 50 L; height 0.45 m), in which water derived from the tanks containing plants was collected and subsequently recirculated to the main tank containing fish via the operation of a single pump (Newa Jet 1700; NEWA Tecno Industria Srl, Loreggia, Italy). The tanks used in the present trial were made of high-density polyethylene (HDPE). The aquaponic units were designed to be “low-tech”, as they were characterized by: i) a simple hydroponic section which acted also as a biofilter; ii) no energy utilization to regulate water temperature; iii) very low environmental control, i.e. the absence of probes and systems for the continuous evaluation of water and for remote management, and the absence of devices for water sanitation, such as UV and ozone chamber systems.

Water flow throughout the system was guaranteed by overflow (from the main tank with fish to the tanks with plants, and thereafter to the storage tank). A flow rate of 120 L h^{-1} permitted complete water turnover at 5-h intervals, and the oxygenation of water was facilitated by a porous stone connected to an aerator (Scubla D100; Scubla Srl, Remanzacco, Italy), which was placed within the main tanks containing fish.

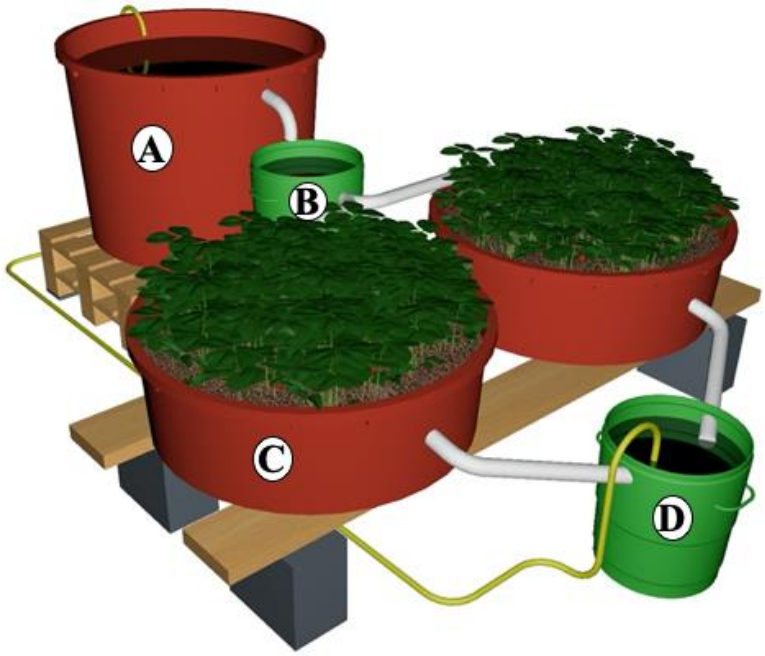

Figure 6.1. A 3D sketch of one of the nine aquaponics experimental unit. Fish tank (A); Sedimenter (B); Tanks for vegetables (C); Water storage tank (D).

Plants, fish, and experimental diets

Each of the two designated plant tanks contained seven strawberries (*Fragaria* × *ananassa* Duch.) plantlets (germinated in peat pots) bearing five to seven fully developed leaves, for a total of 14 plants per experimental unit. An ever-bearing cultivar was used for fruit ripening from April to August, and during the experimental period, ripe strawberries were collected daily.

A total of 173 rainbow trout (initial body weight: 156 g ± 39.8 g) were purchased from a commercial farm. A different number of fish per tank (19–20) was allocated to the main tanks to balance the initial biomass among three experimental groups to be fed different diets (6.074 ± 0.69 kg m^{-3} per tank in H0 group; 6.026 ± 0.55 kg m^{-3} in H6 group; 6.073 ± 0.46 kg m− 3 in H12 group) for 76 days.

The three dietary treatments assessed in the present study were based on partially defatted HI larval meal (Mutatec, Caumont-sur-Durance, France), which was used to replace different proportions of a standard FM (0%, 25%, and 50%) in diets H0, H6, and H12, respectively. These three dietary treatments were formulated as follows: diet H0, the control diet containing 200 g/kg FM and no HI; diet H6, containing 150 g/kg FM and 62 g/kg HI; and diet H12, containing 100 g/kg FM and 124 g/kg HI (Table 1). Owing to the lower crude

protein concentration of HI meal (60.5% DM) compared with that of FM (74.3% DM), the HI meal was included at higher rates than the those of the substituted FM. Furthermore, the level of included gelatinized starch was slightly modified. The HI larvae were commercially reared on plant by-products and partially defatted using a mechanical process. However, no other information was provided by the producer regarding either the rearing substrate or the processing methodologies, as this information is deemed confidential.

The experimental diets were prepared at the experimental facility of DISAFA. The ground ingredients were individually weighed (KERN PLEN v.2.2; KERN & Sohn GmbH, Balingen-Frommern, Germany; d: 0.01) and subsequently mixed with fish oil using a blender (Brevetti S.A.G.A., Milano, Italy). From 250 to 500 mL kg^{-1} of water was added to the mixture to facilitate the pelleting process. The pelletizing was performed using a meat grinder (LABOR 32; Rheninghaus Factory, San Mauro Torinese, Italy). The pellets (3.0 mm) were subsequently dried (50 ◦ C for 48 h) and stored in black bags at – 20 °C until use. A total of 20 kg were prepared per each diet before the start of the trial.

The diets were isonitrogenous [crude protein: 44% dry matter (DM)], isolipidic (crude lipid: 17% DM), and isoenergetic (gross energy: 21 MJ/ kg). The diets were formulated according to rainbow trout requirements (NRC, 2011). The chemical compositions of the HI and experimental diets are listed in Table 6.1; the fatty acid composition of the experimental diets is reported in Table 6.2.

Table 6.1. Ingredients (g/kg as fed) and proximate composition (% DM) of the experimental diets and *Hermetia illucens* meal (HI).

	HI	**Diets**		
		H0	H6	H12
Ingredients				
Fishmeal (CP 73% DM)		200	150	100
Hermetia illucens larva meal		0	62	124
Gelatinized starch, D500		150	138	126
Corn gluten meal		119	119	119
Soybean (SB) meal		215	215	215
SB protein concentrate		70	70	70
Porcine haemoglobin		30	30	30
Wheat flour		55	55	55
Fish oil		70	70	70
Soybean oil		70	70	70
Hydrolysed krill		5	5	5
Mineral premix[1]		2.5	2.5	2.5
Vitamin premix[2]		2.5	2.5	2.5
DL-methionine		8	8	8
L-lysine		3	3	3
Proximate composition				
Dry matter, %	94.0	93.6	93.5	93.5
Crude protein, %DM	60.5	44.7	44.1	44.0
Crude lipid, %DM	7.43	17.0	16.7	17.3
Ash, %DM	10.8	6.40	6.12	6.00
Gross energy, MJ/kg DM	21.5	22.6	23.0	21.8

H0, H6 and H12 diets, fishmeal replaced with *Hermetia illucens* meal (HI) at 0%, 25% and 50%, respectively. [1]Mineral premix (mg/kg diet): bicalcium phosphate 500 g, calcium carbonate 215 g, marine salt 40 g, potassium chloride 90 g, magnesium chloride 124 g, magnesium carbonate 124 g, iron sulfate 20 g, zinc sulfate 4 g, copper sulfate 3 g, potassium iodide 4 mg, cobalt sulfate 20 mg, manganese sulfate 3 g, sodium fluoride 1 g (Granda Zootecnici, Cuneo, Italy). [2]Vitamin-premix (mg/kg diet): DL-αtocopherolacetate, 60 IU; sodium menadione bisulfate, 5 mg; retinylacetate, 15,000 IU; DL-cholecalciferol, 3000 IU; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; Vitamin B12, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg; choline chloride, 2000 mg (Granda Zootecnici, Cuneo, Italy).

Table 6.2. Fatty acid profile (% of total FAME) of the experimental diets.

	Diet		
	H0	H6	H12
C12:0	0.15	2.37	3.81
C14:0	5.90	6.16	6.64
C16:0	19.6	20.1	23.6
C18:0	3.96	3.44	4.22
C16:1n-7	5.88	5.54	5.67
C18:1n-9	15.8	15.9	18.6
C18:1n-7	2.16	1.99	2.09
C18:2n-6	24.2	24.2	27.1
C18:3n-3	3.54	3.37	2.45
C20:4n-6	0.37	0.33	0.23
C20:5n-3	5.91	5.18	3.16
C22:6n-3	3.99	3.76	2.44
SFA[1]	31.62	34.08	40.99
MUFA[1]	24.82	24.34	27.14
PUFA[1]	43.56	41.58	31.88
∑n-3	16.02	14.55	9.56
∑n-6	25.07	24.95	20.97
∑n-6/n-3	1.58	1.74	2.22
PUFA/SFA	1.38	1.22	0.78

H0, H6 and H12 diets, fishmeal replaced with *Hermetia illucens* meal (HI) at 0%, 25% and 50%, respectively. FAME: Fatty acid methyl esters; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids. [1]Including minor FAs.

Water quality

Throughout the trial, water lost via evapotranspiration from each unit was recorded daily and manually replenished. Dissolved oxygen and water temperatures were recorded daily, whereas pH, redox potential, electric conductivity, and chlorophyll content in the fish tanks were monitored at weekly intervals using a portable multi-parameter apparatus (HQ40d Portable Multi-Parameter Meter; Hach Lange GmbH, Germany). Similarly, total ammonia nitrogen (TAN) was measured once a week using an Ammonia Rapid Kit (Megazyme; Astori Tecnica, Poncarale, Italy).

During the trial period, the daily loss of water due to evapotranspiration averaged 7.86 L d^{-1}, which represented 1.31% of the total water contained in each unit, without differences among groups (data not reported in tables). Similar values for the physicochemical properties of water were obtained among all experimental groups: average water temperature 19.4 ± 1.7 °C, dissolved oxygen 7.96 ± 0.61 mg L^{-1}, pH 7.4 ± 0.2, and TAN 0.13 ± 0.10 mg L^{-1}. Additionally, on average, water chlorophyll levels were 76.0 ± 22.5 μg L^{-1}, electrical conductivity 976 ± 171 dS cm^{-1} and redox potential −30.5 ± 18.2 mV (data not reported in tables).

In vivo recordings

The health and mortality of fish were monitored daily. Fish were individually weighed at the beginning of the trial (0 d), and thereafter once a month at 26, 59, and 76 days (end of the rearing period) of the trial,after being anaesthetized in a separate tank with 10 mg $L^{-1}$of clove oil containing 87% eugenol. Fish were fasted for 48 h before and 24 h after weighing.

Fish were fed by hand twice daily (08:00 and 15:00), 6 days a week, until visually assessed apparent satiation. The amount of feed was recorded daily and the specific growth rate (SGR) and feed conversion ratio (FCR) were calculated as follows:

$$SGR\ (\%\ d^{3}) = [(Log_e\ final\ weight - Log_e\ initial\ weight)/No.\ of\ days] \times 100$$

$$FCR = weight\ of\ dry\ feed\ distributed/net\ wet\ weight\ gain\ of\ fish$$

In vivo digestibility

An in vivo digestibility trial was performed to assess the apparent digestibility coefficient (ADC) of nutrients. Two hundred and sixteen rainbow trout (100.6 ± 8.53 g) were allocated to 12 cylinder-conical tanks of 250 L (four tanks per treatment, 18 fish per tank) connected to a flow-through open system (tank water inflow: 8 L min^{-1}) supplied with artesian well water (constant temperature: 13 ± 1 °C; dissolved oxygen level: 8.5 mg L^{-1}). After 14 days of adaptation to the experimental diets, the fish were fed by hand to apparent satiety twice daily (08:00 and 15:00), 6 days per week. ADC values were obtained using the indirect acid-insoluble ash method. To this end, 1% Celite ® (Fluka, St. Gallen, Switzerland) was added to the diets as an inert marker to replace 1% wheat meal. Faeces were collected daily from each tank for four consecutive weeks, using a continuously operated automatic device (Choubert's system; Chemello et al., 2020), and then freeze-dried and frozen (− 20 °C) until used for analyses. The ADC values of dry matter, crude protein, ether extract, and gross energy were calculated as described by Caimi et al. (2020).

Recordings at the time of fish slaughter

At the end of the trial, all fish were slaughtered after fasting for 24 h. Fish were caught as for previous weighing and then stunned by a percussion applied to the head of manually restrained fish using a plastic knob. Dead fish were weighed and total and standard lengths were measured, from which Fulton's condition factor (K) was calculated as follows:

$$K = (fish\ weight / total\ length^{3}) \times 100$$

The fish were then dissected, and the carcasses were weighed. The somatic indices and carcass and fillet yields were calculated as follows:

$$Hepatosomatic\ index\ (VSI, \%) = (liver\ weight / fish\ weight) \times 100$$

$$Viscerosomatic\ index\ (VSI, \%) = (viscera\ weight / fish\ weight) \times 100$$

Carcass yield (%) = (carcass weight / slaughter weight) × 100

Fillet yield (%) = (fillet weight / slaughter weight) × 100

The skin was separated from the fillets and the L* a* b* colour indices (CIE, 1976) were measured at three points on the dorsal side of the right fillets using a Minolta CM–508C spectrophotometer (Minolta Corp., Ramsey, NJ, USA). The colour difference (ΔE) between pairs of fillet samples was calculated according to Mokrzycki and Tatol (2011).

Both the right and left fillets were minced. The right fillets were freeze-dried, placed under vacuum in plastic bags, and stored at 4 °C until used for analysis of the proximate composition of meat. The left fillets were stored under vacuum at −18 °C until used for analysis of meat fatty acid profiles.

Proximate composition and fatty acid analysis

The experimental diets, freeze-dried fillets, and collected faeces were analysed according to AOAC (2000) methods to determine the contents of dry matter (934.01), ash (967.05), and crude protein (2001.11). Ether extract contents were measured after acid hydrolysis treatment (EC, 1998). The gross energy content of diets was assessed using an adiabatic calorimetric bomb (C7000; IKA, Staufen, Germany), and the inert marker content in diets and faeces was analysed using the acid-insoluble ash method, as described by Atkinson et al. (1984).

For the fatty acid composition of diets and fillets, the fat was extracted based on accelerated solvent extraction (Application Note 334; ASE®, Dionex, Sunnyvale, CA, USA) using two extraction cycles. The extracted lipids were initially transmethylated as fatty acid methyl esters (FAMEs). Prior to methylation, an internal standard (13:1 methyl ester) was added to the extract. After centrifugation, the supernatant was subjected to two-dimensional gas chromatography (GC × GC) using an Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a modulator (Agilent G3486A CFT, CA, USA), an automatic sampler (Agilent 7693, CA, USA), and an FID detector connected to chromatography data system software (Agilent ChemStation, CA, USA). A Supelco SP 2560 column (80 m × 0.18 mm internal diameter, 0.14-μm film thickness; Sigma-Aldrich, St. Louis, MO, USA) was used as the first capillary column with hydrogen gas as a carrier. As the second capillary column, we used a J&W HP 5 ms column (3.8 × 0.25 mm internal diameter, 0.25-μm film thickness; Agilent Technologies) with hydrogen again being used as the carrier. Fatty acids were identified by comparing with the retention times of the standard FAME mixture (Supelco 37 component FAME Mix, 47,885 – U). Individual FAMEs were expressed as a percentage of the total volume of the eluted FAMEs.

Gut histological analyses

At slaughter, we selected 36 fish (4 fish per tank, 12 fish per dietary treatment), based on treatment-wise average live weight, and dissected to sample gut tissue. From these, a 2-

cm sample was taken from the proximal intestine (the tract between the pyloric sphincter and the ileum-rectal valve) (Verdile et al., 2020), and the annexed pyloric caeca were removed. Samples were washed in phosphate-buffered saline (PBS) solution and approximately 1 cm was fixed in paraformaldehyde in PBS (0.1 M, pH 7.4), dehydrated, and embedded in paraffin. Four 4-μm sections per sample were obtained using a microtome and stained with haematoxylin/eosin for morphometric evaluation. Intestinal villus lengths were measured using NIH ImageJ software (Rueden et al., 2017), according to the procedure described by Hampson (1986), with 20 measurements being obtained from each gut sample. The goblet cells identified on 10 different villi per trout were counted along 300 μm of the villus surface.

Statistical analysis

The data obtained for growth performance, gut morphology, ADC, slaughter results, and fillet quality of fish were analysed via a one-way ANOVA, with the experimental diet (H0, H6, and H12) serving as the main effect. The PROC GLM procedure of SAS (Statistical Analysis System, 2013) was used for all analyses. Bonferroni's test was used for the comparison of means, with differences among means assumed to be statistically significant at $P \leq 0.05$. The data obtained for fillet fatty acid profiles were preliminarily assessed for normal distribution using the Shapiro-Wilk test and the PROC UNIVARIATE procedure of SAS.

RESULTS

Plant productivity, fish production, and in vivo digestibility

With regards to plant productivity, strawberries yielded on average 38 ± 19 fruits m^{-2} with an average fruit weight of 7.4 ± 1.4 g, which did not differ significantly among dietary treatments (Fig. 6.2).

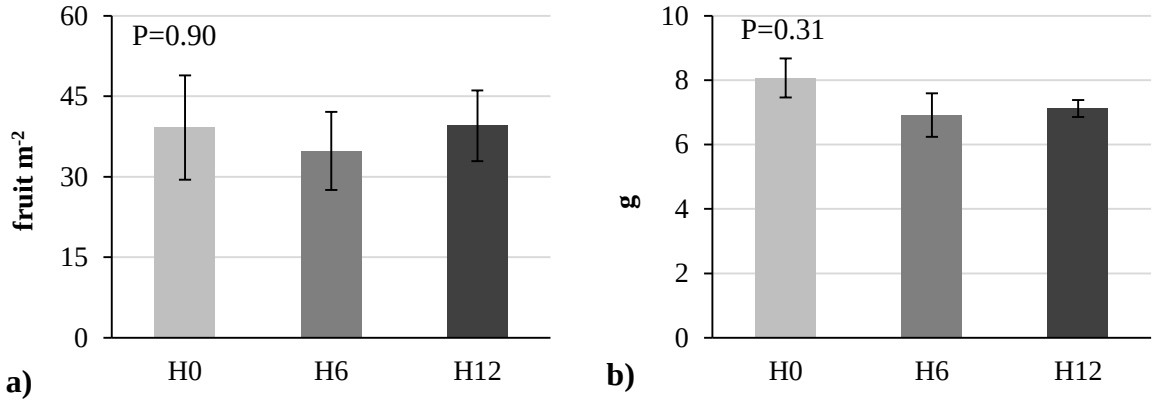

Figure 6.2. Number of fruits (a) and fruit weight (b) (means ± SE) of strawberries cultivated in aquaponics systems with trout fed diets with different replacement rates of fishmeal with *Hermetia illucens* (HI) meal (0%, 25% and 50% of replacement for H0, H6 and H12 diets, respectively).

Among the experimental trout, only five fish died during the course of treatment (four fed the H0 diet and one fed the H6 diet) without any prior visible symptoms of disease, whereas four fish (one and three from the H0 and H6 diets, respectively) were discarded at slaughter, given the lack of difference between their initial and final live weights. During the initial 26 days of the trial, SGR was lower in fish fed the H12 diet than in those receiving the H0 and H6 diets (−12.3% on average; $P < 0.001$) (Table 6.3). Similarly, at the end of the trial, the SGR of H12 fish was lower than that of either H0 or H6 fish (−7.9%; $P = 0.035$). In contrast, we recorded no significant differences among the experimental groups with respect to final live weight (304 g on average), FCR (1.53), final fish biomass (11.0 kg m^{-3}), or biomass growth (5.28 kg m^{-3}). Similarly, no significant differences were detected among dietary treatments in terms of the ADC of dry matter (89.0%, on average) or crude protein (96.4%), ether extract (98.0%), or gross energy (93.2%) contents (Table 6.4).

Table 6.3. Growth performance of rainbow trout.

	Diet				
	H0	H6	H12	P-value	RMSE
Total fish per treatment (n)	53	53	58		
Tanks (n)	3	3	3		
Fish weight (g)					
0 days	158	160	154	0.681	40
26 days	218	221	202	0.150	53
59 days	291	293	270	0.195	75
76 days	310	313	285	0.142	82
Specific growth rate (% d^{-1})					
0-26 days	1.22^{b}	1.22^{b}	1.07^{a}	<0.001	0.26
26-59 days	0.87	0.84	0.86	0.714	0.23
59-76 days	0.35	0.38	0.32	0.135	0.18
0-76 days	0.88^{b}	0.87^{b}	0.81^{a}	0.035	0.16
Feed conversion ratio					
0-26 days	1.21	1.23	1.27	0.799	0.13
26-59 days	1.54	1.60	1.50	0.642	0.12
59-76 days	2.28	2.37	2.77	0.396	0.43
0-76 days	1.50	1.54	1.55	0.785	0.10
Biomass (kg m^{-3})					
0 days	5.60	5.67	5.95	0.644	0.48
26 days	7.69	7.80	7.82	0.951	0.54
59 days	10.3	10.4	10.4	0.973	0.80
76 days	11.0	11.1	11.0	0.980	0.82
Biomass growth (kg m^{-3})					
0-26 days	2.09	2.13	1.87	0.391	0.23
26-59 days	2.61	2.56	2.62	0.981	0.34
59-76 days	0.67	0.73	0.60	0.607	0.15
0-76 days	5.35	5.41	5.08	0.734	0.54

H0, H6 and H12 diets, fishmeal replaced with *Hermetia illucens* meal (HI) at 0%, 25% and 50%, respectively.
RMSE: Root mean square error.

Table 6.4. Apparent digestibility coefficient (ADC) of nutrients of experimental diets.

	Diets				
	H0	H6	H12	P-value	RMSE
Dry matter (%)	88.6	88.7	89.6	0.31	0.84
Crude protein (%)	96.8	96.3	96.2	0.12	0.36
Ether extract (%)	97.8	97.7	98.4	0.51	0.40
Gross energy (%)	93.5	93.0	93.2	0.65	0.59

H0, H6 and H12 diets, fishmeal replaced with *Hermetia illucens* meal (HI) at 0%, 25% and 50%, respectively. RMSE: Root mean square error.

Gut histological analysis

Dietary treatment had no significant effect on proximal gut villus height, the average of which was 505 ± 73.5 μm (Fig. 6.3a). However, the density of goblet cells was significantly higher in fish fed the H12 diet than in those fed the H0 diet (8.36 vs. 7.45 cells × 300 μm^{-1}; $P < 0.05$), with intermediate values being obtained for fish fed the H6 diet (Fig. 6.3b).

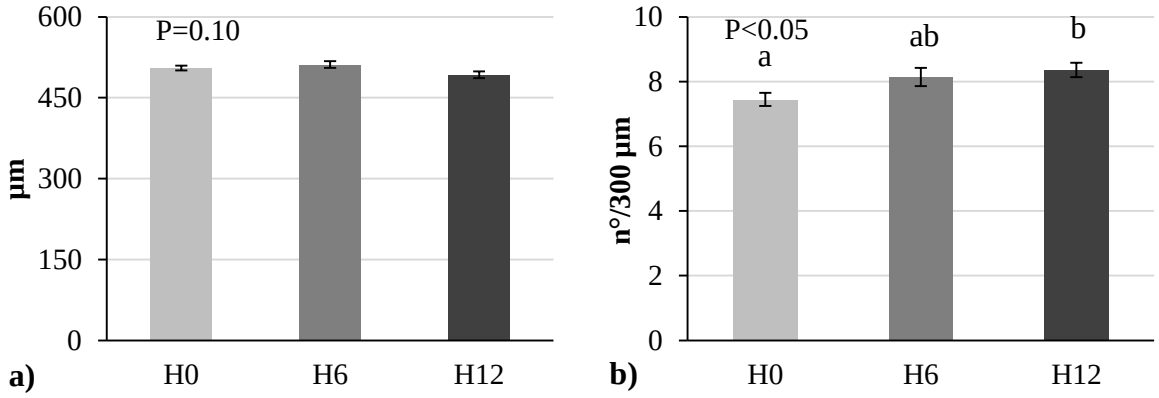

Figure 6.3. Villi height (a) and goblet cells density (b) (means ± SE) in the proximal intestine of rainbow trout cultured in aquaponics and fed diets with different replacement rates of fishmeal with *Hermetia illucens* (HI) meal (0%, 25% and 50% of replacement for H0, H6 and H12 diets, respectively).

Biometric traits and flesh quality of fish

Dietary treatment had no significant effect with respect to Fulton's condition factor (average 1.40), VSI (10.9%), HSI (1.22%), carcass yield (88.2%), fillet weight (156 g), or fillet yield (57.8%) (Table 6.5). In contrast, fillet redness (a*) and yellowness (b*) were significantly lower in fish fed the H12 diet than in those fed the H0 diet (−58% and −19%, respectively; $P < 0.001$; Table 6.5).

Table 6.5. Morphometric and somatic indices, slaughter results and fillet characteristics of trout.

	Diets				
	H0	H6	H12	P-value	RMSE
Fish (n)	53	53	58		
Total length (mm)	276	281	273	0.267	25
Standard length (mm)	252	255	249	0.463	23
K	1.43	1.39	1.37	0.101	0.13
VSI (%)	10.8	11.0	10.8	0.850	1.4
HSI (%)	1.22	1.24	1.19	0.703	0.32
Carcass weight (g)	267	277	254	0.260	73
Carcass yield (%)	87.6	88.2	88.9	0.607	6.6
Fillets (n)	24	24	24		
Weight (g)	158	162	148	0.553	44
Yield (%)	56.7	58.5	58.1	0.611	6.3
Colour					
L*	39.7	40.3	40.9	0.164	2.2
a*	-1.21^{b}	-1.54^{ab}	-1.91^{a}	<0.001	0.57
b*	8.69^{b}	7.50^{ab}	7.01^{a}	<0.001	1.26

H0, H6 and H12 diets, fishmeal replaced with *Hermetia illucens* meal (HI) at 0%, 25% and 50%, respectively. K: Fulton's condition factor; VSI: viscerosomatic index; HIS: hepatosomatic index. RMSE: Root mean square error.

We detected no significant differences among the experimental groups for fillet chemical composition: average moisture (73.0%), crude protein (21.2%), and crude lipids (4.17%) (Table 6.6). In terms of fatty acid profiles, fish fed the H12 diet were found to have higher proportions of C12:0 (1.33% vs. 0.42%; $P < 0.001$) and C14:0 (4.38% vs. 3.96%; $P < 0.01$) compared with those fed the H0 diet, with intermediate values being recorded for fish fed the H6 diet, whereas an opposite trend was observed for C18:1 n-7 (2.31% vs. 2.18%; $P < 0.001$). In contrast, dietary treatment was found to have no significant effect on the proportions of eicosapentaenoic acid (EPA, C20:5 n-3: 2.72% on average), docosahexaenoic acid (DHA, C22:6 n-3: 6.01%), and saturated (32.6%), monounsaturated (30.0%), n-3 (13.3%), or n-6 (23.1%) fatty acids (Table 6.6).

Table 6.6. Fillet proximate composition, fatty acid profile and dietary indices of trout.

	Diet				
	H0	H6	H12	P-value	RMSE
Fillets (n)	12	12	12		
Proximate composition					
Moisture (%)	72.8	73.0	73.1	0.84	1.2
Ash (%)	1.38	1.39	1.41	0.29	0.04
Protein (%)	21.2	21.3	21.2	0.72	0.4
Lipid (%)	4.24	4.14	4.12	0.97	1.35
Fatty acids (% of total FAME)					
C12:0	0.43[a]	0.84[b]	1.33[c]	<0.001	0.26
C14:0	3.96[a]	4.18[ab]	4.38[b]	<0.01	0.30
C16:0	22.3	22.2	22.6	0.77	1.4
C18:0	3.79	3.82	3.74	0.80	0.30
C16:1n-7	6.09	6.17	5.96	0.60	0.50
C18:1n-9	20.8	20.1	20.8	0.21	1.2
C18:1n-7	2.31[b]	2.25[ab]	2.18[a]	<0.001	0.08
C18:2n-6	20.7	21.1	21.4	0.22	0.9
C18:3n-3	2.50	2.49	2.43	0.53	0.15
C20:4n-6	0.57	0.55	0.50	0.21	0.09
C20:5n-3	2.88	2.78	2.51	0.21	0.52
C22:6n-3	6.27	6.41	5.35	0.14	1.36
SFA[1]	31.9	32.5	33.5	0.10	1.8
MUFA[1]	30.4	29.5	30.0	0.35	1.4
PUFA[1]	37.7	38.0	36.5	0.35	2.5
∑n-3	13.8	13.8	12.2	0.14	2.2
∑n-6	22.9	23.2	23.3	0.45	0.9
∑n-6/n-3	1.73	1.72	1.93	0.10	0.26
PUFA/SFA	1.19	1.18	1.10	0.20	0.13

H0, H6 and H12 diets, fishmeal replaced with *Hermetia illucens* meal (HI) at 0%, 25% and 50%, respectively. FAME: Fatty acid methyl esters; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids. RMSE: Root mean square error. [1]Including minor FAs.

DISCUSSION

From the perspective of AP, the rainbow trout is not considered among the most suitable of species for culturing, primarily due to its high water quality requirements (Molony, 2001). Nevertheless, some successful commercial farms are currently operational outside of Europe, and the results obtained in the present study confirm the adaptability of trout to aquaponic conditions, even when a low level of technology (basic system components, no environmental control, no water sanitation) is adopted (Chapter 5, third contribution). Indeed, we recorded a notably low fish mortality (2.9% on average) during the course of trial, which is consistent with the findings of previous studies that have examined the performance of European carp (*Cyprinus carpio*; Maucieri et al., 2019), rainbow trout (Chapter 5, third contribution), and largemouth bass (*Micropterus salmoides*; Bordignon et al., 2020) using the same type of system. This is in spite of the fact that on some warm days during the trial, water temperatures reached peaks higher than the temperature considered optimal for rainbow trout (<20 °C; Ineno et al., 2005; Chen et al., 2015), although it remained lower than the limit of thermal tolerance for this species (24 °C; Bear et al., 2007). Otherwise, the dissolved oxygen concentration (6.08 to 10.2 mg L^{-1}) varied within a suitable range for rainbow trout growth and welfare (Ellis et al., 2002), plant nutrient absorption, and biofilter activity (Lennard and Goddek, 2019). Similarly, water pH, which ranged from 6.4 to 8.5 (average 7.38), was consistent with the optimal ranges of fish (6.5 to 8.5; Molony, 2001) and bacteria (6.5 to 8.0; Lennard and Goddek, 2019; Timmons et al., 2002). In addition, recorded values for the TAN content of water were found to be below the chronic toxicity threshold for rainbow trout (approx. 4 mg NH_3-N L^{-1}; Thurston et al., 1984).

With regards to production, the results obtained in AP can be similar to those achieved in stand-alone RAS (see Lennard and Goddek, 2019 for a review). However, we found that trout performance in the present trial was lower than that typical of open and RAS systems (Naderi et al., 2017; Zahedi et al., 2019). Nevertheless, SGR was found to be higher (+18%), whereas FCR was lower (− 3%) than the values previously obtained for rainbow trout (from 141 g to 331 g) using the same aquaponic system (Chapter 5, third contribution).

In terms of the composition of fish diets with respect to the replacement of FM with HI meals, the findings of a previous study on rainbow trout (179 to 541 g live weight over 78 days) revealed no sig- nificant differences in performance in response to an inclusion of 20%– 40% partially defatted HI meal (Renna et al., 2017). Similar results have been reported for rainbow trout (137 g to 277 g live weight over 98 days) fed a full-fat HI meal (10.5% and 21%; Cardinaletti et al., 2019). Conversely, however, other authors have reported reductions in the FCR of rainbow trout juveniles in response to the provision of diets con- taining increasing proportions of defatted HI meal (from 6.5% to 26%; Dumas et al., 2018), and with the inclusion of HI prepupae at 30% (St- Hilaire et al., 2007) and 33% (Sealey et al., 2011). This is partially consistent with the impairment of growth performance observed during the first period of the present trial with respect to the highest HI rate. Comparatively, reduced fish growth performance during entire trials has previously been reported in Nile tilapia

farmed in AP (Kessens, 2016) and Siberian sturgeon (*A. baerii*) (Zarantoniello et al., 2021) fed diets containing 68% and 50% HI meal, respectively.

The reduction in SGR observed in the present study at the highest HI inclusion was, however, not found to be associated with any obvious differences in diet digestibility.

Although a number of previous studies on rainbow trout fed diets containing different inclusion levels of HI (Renna et al., 2017) and *Tenebrio molitor* meal (Belforti et al., 2015; Chemello et al., 2020; Rema et al., 2019) have not reported the effects on diet digestibility, other authors have noted that the apparent digestibility of major nutrients might be affected by the inclusion of insect meals in aquafeeds (for a review see Gasco et al., 2019). Furthermore, reductions in the digestibility of crude protein have been observed in trout fed diets containing high levels of HI (20%; Renna et al., 2017) and *T. molitor* meal (20%; Chemello et al., 2020; 50%, Belforti et al., 2015). To a large extent, such disparities among studies are assumed to be attributable to substantial differences in insect meal quality associated with the origin of raw materials and their processing, as well as differences in other compound feed ingredients and FM replacement rates (Gasco et al., 2019).

Defence-related responses of fish gut to insect-based diets have also been described in other fish, including the black tetra (*Gymnocorymbus ternetzi*; Leknes, 2014), rice field eel (*Monopterus albus*; Dai et al., 2007), and tiger barb (*Puntius tetrazona*; Leknes, 2014), which are assumed to be associated with an increase in the density of goblet cells, as has been observed in the gut of trout fed the highest percentage HI inclusion in the trials conducted by Elia et al. (2018) and Cardinaletti et al. (2019), as well as in the present trial. Additionally, a shortening of villus height in the gut of fish fed diets containing insects has been reported in the literature, indicating potential intestinal inflammation and a diminished absorptive surface (Li et al., 2017; Zhang et al., 2018; Cardinaletti et al., 2019). In contrast, consistent with the findings of the present study, other authors have observed no appreciable alterations in the proximal (Elia et al., 2018) or distal (Dumas et al., 2018) intestine of rainbow trout fed diets containing different inclusion levels of partially defatted HI meal for 78 and 84 days, respectively.

As to whether the replacement of FM with insect meals can affect product quality requires the evaluation of both the rheological and nutritional properties of fillets. With respect to rheological properties, consistent with the findings of the present study, certain changes in fillet colour have previously been detected in rainbow trout fed a diet containing 10.5% full-fat HI meal (Bruni et al., 2020) or 20% of defatted HI meal (Mancini et al., 2018), although other studies have reported no differences in trout fed full-fat mealworms (*T. molitor*; Iaconisi et al., 2019) or defatted HI meal (Renna et al., 2017). In the present study, we observed differences in colour indices of the fillets of trout fed the H0 and H12 diets ($\Delta E = 3.74$), which were within a range of colour difference ($3.5 < \Delta E < 5$) that can be appreciated even by inexperienced observers (Mokrzycki and Tatol, 2011). Such changes are conceivably attributable to the variations in feed pigments associated with the substitution of FM with HI meal, as has previously been observed in studies where FM is replaced by vegetable sources (Iaconisi et al., 2018; Tibaldi et al., 2015). However, the effects of HI meal pigments on fish

fillets are poorly understood and may be dependent on multiple factors, including species, rearing substrates, and the processing of insects (Gasco et al., 2019; Larouche et al., 2019; Leni et al., 2019).

With regards to the influence of dietary HI inclusion on the nutritional quality of fillets, the findings of previous studies have been somewhat inconsistent. Some studies have reported changes in the lipid and dry matter contents of fish flesh (Renna et al., 2017; Sealey et al., 2011), whereas others have not (Dumas et al., 2018; Mancini et al., 2018). In the present study, we detected increases in C12:0 and C14:0 contents in the fillets of trout fed insect meal, as has previously been reported (Bruni et al., 2020; Mancini et al., 2018; Renna et al., 2017). Noteworthy, despite the lower dietary supply in insect-based diets, the EPA and DHA contents in fillets were apparently unaffected, which can probably be attributed to the fact that experimental diets contained the same amount of fish oil and that the partially defatted HI meal have low amounts of ether extract (approx. 7.5% DM).

Turning to other components of the evaluated system, namely, the plants cultivated in AP, the major nitrogen source for plants is the proteins in fish feed (Lennard and Goddek, 2019). Thus, it is reasonable to assume that the use of different protein sources (i.e., FM or HI meal) might have an influence on plant growth in instances where there is a change in ADC. However, we found no evidence to indicate that this was the case in the present study. Indeed, the yields obtained for strawberries were found to be comparable with those obtained in AP with different carp species (Roosta and Afsharipour, 2012), as well as in a closed hydroponics system (Talukder et al., 2019) and plastic bag cultivation (Saidimoradi et al., 2019).

Finally, based on the costs at the feed mill of the raw materials used for producing the diets, we calculated that diet H6 and H12 had a cost equal to 1.13 and 1.26 times the cost of diet H0. Thus, since fish biomass and strawberry production were not affected by the dietary treatment, we can state that the production cost of trout increased with the inclusion level of HI meal.

CONCLUSIONS

In the present study, we demonstrated the feasibility of rainbow trout production based on a low-tech aquaponic system with limited environmental control. With respect to the use of HI meals, we established that diets with the highest level of insect meal inclusion had a slight effect on fish growth performance and also promoted an increase in the density of gut goblet cells, thereby tending to indicate certain detrimental effects at the gut level, which clearly warrant further investigation. Moreover, the effects on fillet traits require careful evaluation in view of consumers' perception of fish quality. Overall, our findings indicate that rearing high-value fish species in aquaponic systems, combined with the substitution of dietary FM with insect meal, could contribute to enhancing the competitiveness and attractiveness of aquaponic products, provided that costs for insect meals will decrease.

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CHAPTER 7: GENERAL DISCUSSION

To evaluate the impact of some of the proposed strategies from an environmental perspective, a life cycle assessment approach was used in the following discussion of the present PhD thesis to assess the environmental impact of (i) fish oil substitution with vegetable oils in diets for Mediterranean yellowtail (Case study 1) and of (ii) rainbow trout farmed at two stocking densities in a low-tech aquaponic system (Case study 2).

ENVIRONMENTAL IMPACT OF THE NEW STRATEGIES THROUGH A LIFE CYCLE ASSESSMENT (LCA)

The LCA approach in aquaculture

Despite aquaculture is often considered to be sustainable (Tlutsy and Thorsen, 2016), its rapid growth is frequently associated with a negative environmental impact caused by the eutrophication of aquatic ecosystems, the intensive exploitation of water and land, the introduction of allochthonous fish species and the ecotoxicity produced by the usage of chemicals (Bohnes et al., 2019; Ottinger et al., 2016). Thus, the identification of new strategies and production systems that are environmentally sustainable is paramount.

The broad definition of environmental sustainability is the 'maintenance of natural capital' (Bohnes et al., 2019). In practice, it follows the input/output rules according to which the emissions from wastes produced by a system or action should not exceed the capacity of the local ecosystem (output rule), and the rate of harvesting of natural resources should allow their regeneration (input rule) (Goodland and Daly, 1996).

To assess the environmental sustainability of food-producing systems, a valuable tool is represented by the LCA, which is demonstrating a key role on the path towards a more sustainable food production (Ziegler et al., 2016). The LCA is an ISO-standardized method (ISO, 2006) that quantifies the impacts on natural resources, human health and ecosystems generated by production systems and products throughout their entire life cycle, i.e. from raw material extraction, through their utilization, and up to their final discharging and removal (EC, 2010; Ghamkhar et al., 2020).

The LCA method can be applied to evaluate multiple impact categories, such as eutrophication to water bodies, toxicity to human health and ecosystems produced by the release of chemicals, and climate change. Thus, LCA can be utilized as a decision-making tool in aquaculture industry. In fact, the LCA method can detect the hotspots of a given system, which impacts can be subsequently reduced, identifying new production strategies or alternatives characterized by a lower environmental impact. The assessment of the impact of a given system can be applied at macro-level, i.e evaluating the whole impact of a sector or a country), at meso-level, i.e. an entire farm, and at micro-level, i.e focusing on a specific input or process like feed production (Bohnes et al., 2019).

Two different life cycle assessment approaches, attributional LCA (ALCA) and consequential LCA (CLCA), are commonly used to assess the environmental impact related to the production of a given product. The ALCA calculates the environmental impact that can be attributed to the production, disposal or consumption of a given product, assuming a static system (Bamber et al., 2019; Kua and Kamath, 2014). The CLCA considers future scenarios to determine the environmental impact as a consequence of a change (e.g. effects of the market, material substitutions, new methods of production, etc.) in the production or consumption of a product (Bamber et al., 2019; Ekvall et al., 2016).

In attributional assessments, the system boundary is represented by the material flows and processes directly used in the production, disposal or consumption of an item. In the consequential approach all the material flows and processes that are directly or indirectly affected by a small changes (marginal changes) in the output of a given product are included in the system boundary. Moreover, ALCA uses average data, e.g. global warming potential emitted from a business-as-usual production of 1 kg of a given product, whereas CLCA uses marginal data, e.g., data related to the consequences of the marginal production (+1 kg) of a given product (Brander et al., 2008; Weidema et al., 1999).

To comprehensively understand the impacts related to a product, a hotspot analysis is also frequently used in LCA studies. To the purposes of the present discussion, the main objective of a hotspot analysis is to identify the major impacts (i.e. hotspots) generated from the ouput of a given product and then to formulate strategies and recommendations to achieve the reduction of such impacts (Barthel et al., 2015).

A considerable set of LCA studies have been conducted in aquaculture (see Bohnes et al., 2019 for a comprehensive review), evaluating different production systems (Aubin et al., 2006, 2009; Samuel-Fitwi et al., 2013; Forchino et al., 2017; Chen et al., 2020; Maucieri et al., 2018; Medina et al., 2016; Sherry and Koester, 2020; d'Orbcastel et al., 2009), farming locations (Ghamkhar et al., 2020; Chen et al., 2020; Little et al., 2018), and feed ingredients (Papatryphon et al., 2004).

Most of the studies indicated aquafeed production as one of the key contributors to the environmental impact of this sector. As a result, the effect of new solutions such as novel feed formulations have been explored in recent years (Ayer and Tyedmers, 2009; García García et al., 2016; Henriksson et al., 2017; Iribarren et al., 2012). The impacts of commercial fish feed mainly derive from the supply and production of raw materials such as fish oil and fish meal (FOFM), either due to the low efficiency of production plants or intensive use of fuels from fisheries vessels (Fréon et al., 2017; Iribarren et al., 2012). On the other hand, aquafeed industries are progressively reducing their dependence on FOFM because of their increasing price and low availability (Papatryphon et al., 2004). Consequently, recent LCA studies focused on the replacement of FM with crop-based protein sources, and the majority of them found that FM substitution improved environmental results (Avadí et al., 2015; Grönroos et al. 2006; Nhu et al., 2016; Samuel-Fitwi et al., 2013; Smárason et al., 2017; Pelletier and Tyedmers, 2007) whereas three (Boissy et al., 2011; Iribarren et al., 2012;

Papatryphon et al., 2004) reported comparable impacts. As for FO, few information (Papatryphon et al., 2004; Boissy et al., 2011) is available regarding the environmental impact of total FO substitution with vegetable oils in marine fish diets, and no studies addressed the environmental effects of dietary changes in diets for *S. dumerili*.

During the last years, integrated multitrophic aquaculture systems such as aquaponics have received an increasing interest in LCA studies (Maucieri et al., 2018; Forchino et al., 2017; Chen et al., 2020; Ghamkar et al., 2020). In fact, these systems have the potential to decrease the environmental impact of food production, reducing nutrient release, water utilization and land use (Lennard and Goddek, 2019). Aquaponics systems mainly rely on modern farming techniques and highly controlled environments (Bohnes et al., 2019). However, infrastructures are considered one of the major environmental hotspots in aquaponics, and the choice of low-tech systems with less impacting materials and low environmental controls might help to the decrease the environmental effect of plants and fish production (Forchino et al., 2017; Maucieri et al., 2018).

Studies found that the life cycle impacts per unit of farmed fish are sensitive to fish stocking density (Ghamkar et al., 2021). A high stocking density might reduce electricity use and water dependence per unit of fish produced (Ghamkar et al., 2020). On the other hand, a high stocking density can produce more wastes (Siddiqui and Al-Harbi, 1999; Maucieri et al., 2020) and impair fish welfare (Yildiz et al., 2017), with a consequent reduction of feed efficiency (Rowland et al., 2006), increasing the amount of feed consumed and the associated impacts on the environment. Thus, the optimization of fish stocking density is one of the suggested environmental improvement measures (Song et al. 2019) to be considered in both aquaculture and aquaponics systems.

To date, no studies evaluated the environmental impact of (i) a low-tech aquaponic system and (ii) aquaponic farming using different fish stocking density.

Thus, to evaluate the impact of some of the proposed strategies from an environmental perspective, a life cycle assessment approach was used in the following discussion of the present PhD thesis to assess the environmental impact of (i) fish oil substitution with vegetable oils in diets for Mediterranean yellowtail (Case study 1) and of (ii) rainbow trout farmed at two stocking densities in a low-tech aquaponic system (Case study 2).

CASE STUDY 1: LIFE CYCLE ASSESSMENT OF FISH OIL SUBSTITUTION WITH VEGETABLE OILS IN DIETS FOR *SERIOLA DUMERILI*

Presented at:

Bordignon, F., Sturaro, E., Trocino, A., Martínez-Llorens, S., Tomás Vidal, A., Berton, M. Life cycle assessment of fish oil substitution with vegetable oils in diets for *Seriola dumerili*. 29th International Symposium Animal Science Days (ASD2021), 15-17/09/2021, Gödollo, Hungary.

Based on the contribution of the Chapter 3 of the present thesis, the following case study aimed at assessing the environmental consequences of the substitution of fish oil with vegetable oils in diets for Mediterranean yellowtail (*Seriola dumerili*) juveniles. Thus, the evaluation of the environmental footprint associated with the marginal production of 1 kg of fish-oil based diet and of 1 kg of vegetable oils-based diet was performed using a consequential life cycle assessment (CLCA) model.

MATERIALS AND METHODS

The foreground data about the aquaculture system settings, the production inputs utilized, and the growth performance of the Mediterranean yellowtail juveniles derived from Milián-Sorribes et al. (2021) and Chapter 3 (first contribution). The studies explored the effect of three different diets (0%, 25% and 100% of FO substituted by VO mixture including linseed, sunflower, and palm oils – FO 0, FO 25 and FO 100 diet, respectively) on fish performance and meat quality. The two extreme scenarios (FO 0 *vs* FO 100) were retained for consequential analysis. For each diet, the relative CLCA model was contructued to respond to the experiment question about the environmental consequences to produce a marginal 1 kg of each diet. The CLCA models calculated for the two scenarios were based on the same assumptions, system boundaries and co-products management.

As for the general settings of the models, the system boundaries were set to include the direct impacts related to the production of the single feedstuffs included in the diets and the indirect impacts related to the modifications caused by the production and handling of these feedstuffs. The impact categories assessed were global warming potential (GWP, kg CO_2-eq) without and with land use change (GWP and GWP_LUC) and eutrophication potential (EP, g PO_4-eq). Moreover, per each diet, we calculated the depleted stock fraction (DSF) index as follows:

$$DSF = 1 - \frac{B}{K}$$

where B is the current biomass (tonnes) of a given stock and K is the carrying capacity of a population in the Schaefer model (Heliàs et al., 2018).

The following general assumption were applied to our models: 1) the additional production of FO and/or FM not directly used for FO 0 and FO 100 diet production must replace an equivalent amount of FO and/or FM in a second production system; 2) the marginal VO, i.e. the VO that are marginally produced to cover a new/displaced demand of vegetable oils, was soybean oil. 3) co-products, i.e. the additional soybean meal co-production as a consequence of a new production of soybean oil were assumed to be handled in two different ways: I) to substitute existing products used by a second production system (Substitution scenario); II) to be credited to an expanding production system (Credit scenario). In the Substitution scenario, the affected production system was beef cattle intensive system (i.e., the animal system with the lowest production increase rate in Europe), whereas poultry was assumed to be the expanding production system (FAOSTAT, 2021).

Production of marginal fish oil-based diet

The FO 100 diet, with no substitution of FO with VO, was the fish diet in the business-as-usual scenario. The specific FO 100 diet formulation was reported in Table 3.1 of the first contribution (Chapter 3). This diet contained 350 g/kg of FM and 95 g/kg of FO. Data about the FM and FO production were derived from Samuel-Fitwi et al. (2013). To produce 95 g FO, 2100 g of fish was needed, with the coproduction of 450 g of FM. Since the FM inclusion in FO 100 diet was 350 g/kg, 100 g of FM were available and were assumed to substitute an equivalent 100 g FM in a second production system (Figure 7.1). As a consequence, the FO co-produced with the avoided 100 g FM used in the secondary production system was not produced anymore and should be replaced by another system. In this case, we assumed that the FO substitution was operated through a VO mixture equal to that used to produce the FO 100 diet (i.e. mixture of linseed, sunflower and palm oils). The details about the amount of the single vegetable oils and of the consequences due to their production are reported in next heading "Production of marginal vegetable oils-based diet". As for the other diet ingredients, no indirect environmental consequences were considered, as they would be the same observed in the marginal production of FO 100 diet and CLCA modelling should focus firstly on the specific consequences that discriminated the two alternative systems (EC, 2010).

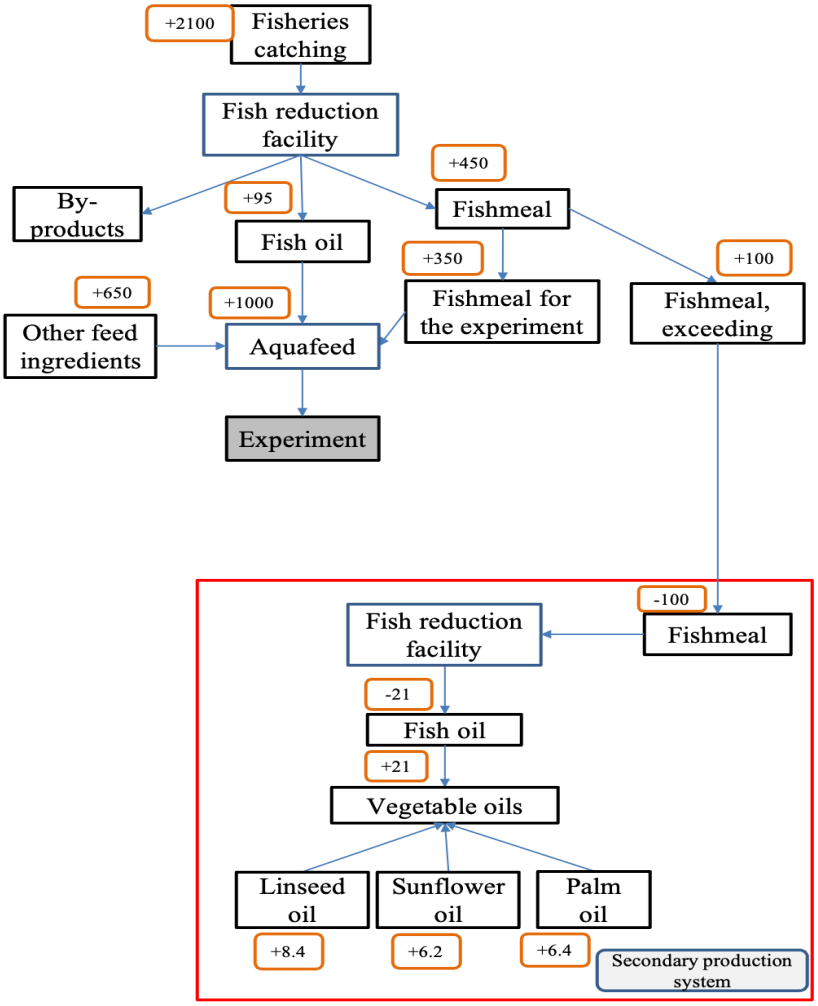

Figure 7.1. Scheme modelling the marginal production of (+1 kg) of FO 100 diet from a CLCA perspective. The exceeding (+100 g) FM generated by the production of 95 g of fish oil and not directly used in the experimental diet substituted an equal amount of FM in a secondary production system

Production of marginal vegetable oils-based diet

As the FO 0 maintained the same level of FM inclusion in diet FO 0 compared to diet FO 100 while substituting FO, the implication was that the FO coproduced with FM was available to be used by a secondary production system. In the production of FO 100 diet, the production of 95 g FO implied the production of FM amount greater than that included in the diet (450 g vs 350 g). Thus, in the FO 0 scenario, as the unique fish-related feed inclusion was 350 g of FM, two consequences had to be modelled: the coproduction of 74 g of FO (coproduced with 350 g of FM) (Fig. 7.2) and the avoided production of 100 g of FM (Fig. 7.3). The marginal FO production, as explained in the general settings of the model, substituted an equal FO amount used by a secondary production system, since FO and FM were assumed as limited in the provision. The avoided production of 74 g of FO implied the displace of 350 g of co-produced FM. Considering the market trend, the marginal protein meal was assumed to be soybean meal concentrate (SMC) with a 1:1 substitution rate with FM (Samuel-Fitwi et., 2013). Soybean meal concentrate is obtained from soybean meal, whose production implied the coproduction of soybean oil. Nearly 100 g of soybean oil were produced as a consequence of 350 g of SMC, and this production was assumed to displace an equal amount of palm oil (Dalgaard et al., 2008). The consequences due to the displacement of palm oil with soybean oil were modelled following Dalgaard et al. (2008), with the use of a marginal meal composed of barley grain and soybean meal to replace the avoided co-production of palm kernel meal associated to palm oil. The consequences due to the avoided production of 100 g of FM, related to the difference between 95 g and 74 g of FO were modelled using the same methodological approach.

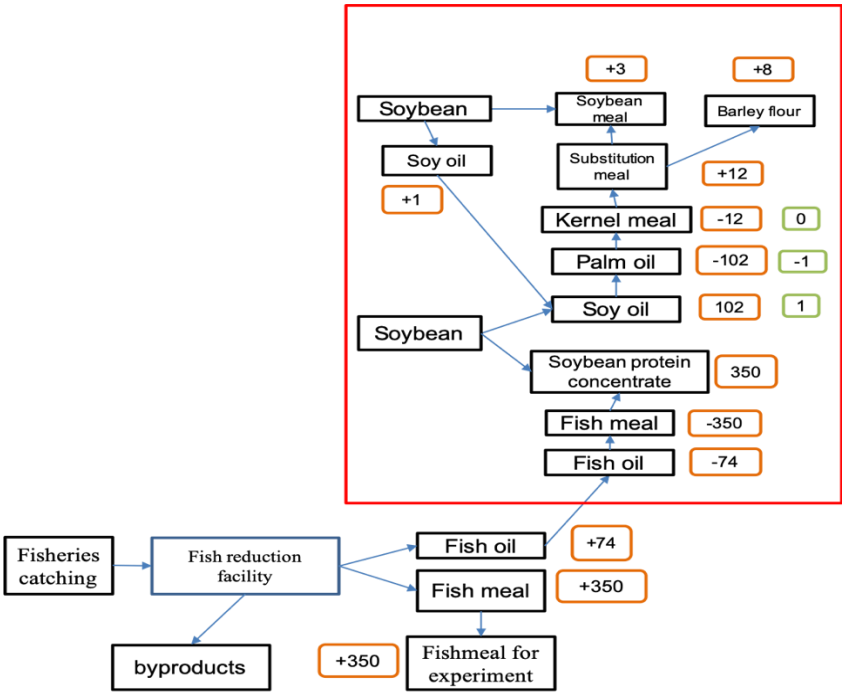

Figure 7.2. Scheme modelling the marginal production (+1 kg) of FO 0 diet from a CLCA perspective. The consequences related to the coproduction of 74 g of FO (generated by the dietary inclusion of 350 g of FM) and its displace in a secondary production system are modelled.

As for the VOs utilized for FO substitution in FO 0 diet, the production of the linseed oil (38 g), sunflower oil (28 g) and palm oil (29 g) implied the co-production of linseed meal (47 g), sunflower meal (42 g) and palm kernel meal (3.5 g), respectively. The new production of linseeds, sunflower seeds and palm fruits were assumed to be reached through agricultural intensification of existing production. The oils and co-produced meals yield data were derived from the Ecoinvent database v3.6 (Wernet et al., 2016). In the Credit scenario, the protein meals were credited to the poultry system (Fig. 7.4), whereas in the Substitution scenario their production (for linseed and sunflower meals) implied the displacement of other feedstuffs consumed by beef cattle in intensive systems (Fig. 7.5). For palm kernel meal, the modelling of the consequences was equal to that explained earlier in the previous section. The displacements were based on the chemical composition of the linseed and sunflower meals (INRAE, 2019), with a meal constructed to maintain constant the provision of the nutrients to beef cattle (e.g. 1 kg of linseed provides 366 g of crude protein and the substituted meal was modelled to provide the same crude protein amount). The basic assumptions in the substituted meals were that the first affected protein meal used in beef cattle diet was rapeseed meal and that the displaced rapeseed oil was replaced by soy oil with 1:1 substitution rate. The final coproduction of soybean meal was credited to poultry systems.

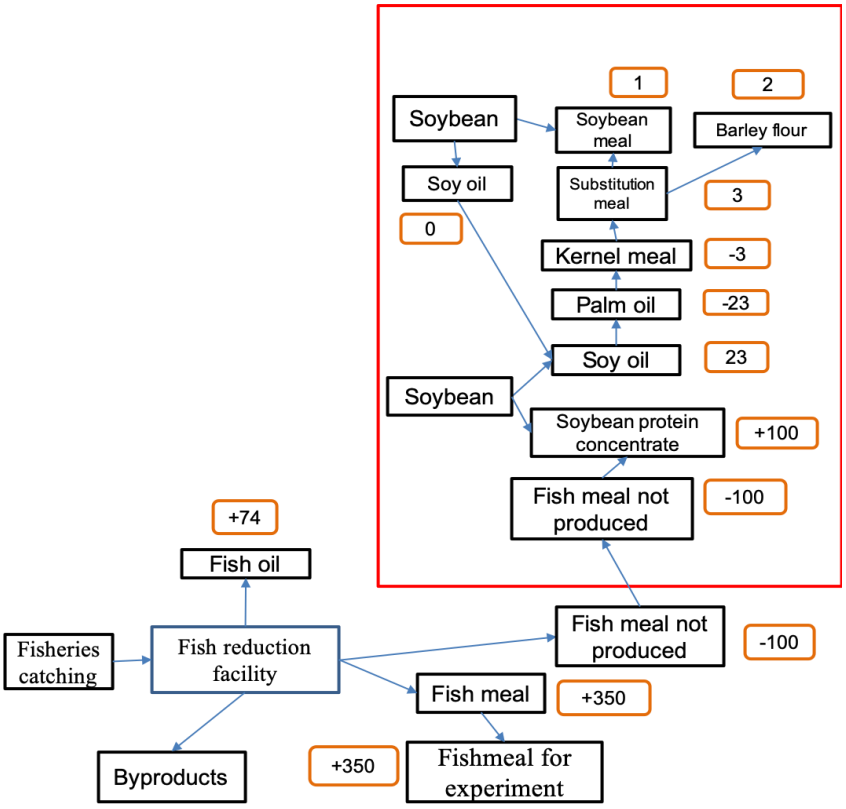

Table 7.3. Scheme modelling the marginal production (+1 kg) of FO 0 diet from a CLCA perspective. The consequences generated in a secondary production system due to the avoided production of 100 g of FM are modelled. The FM not produced was substituted an equal amount (100 g) of soybean meal concentrate in the secondary system.

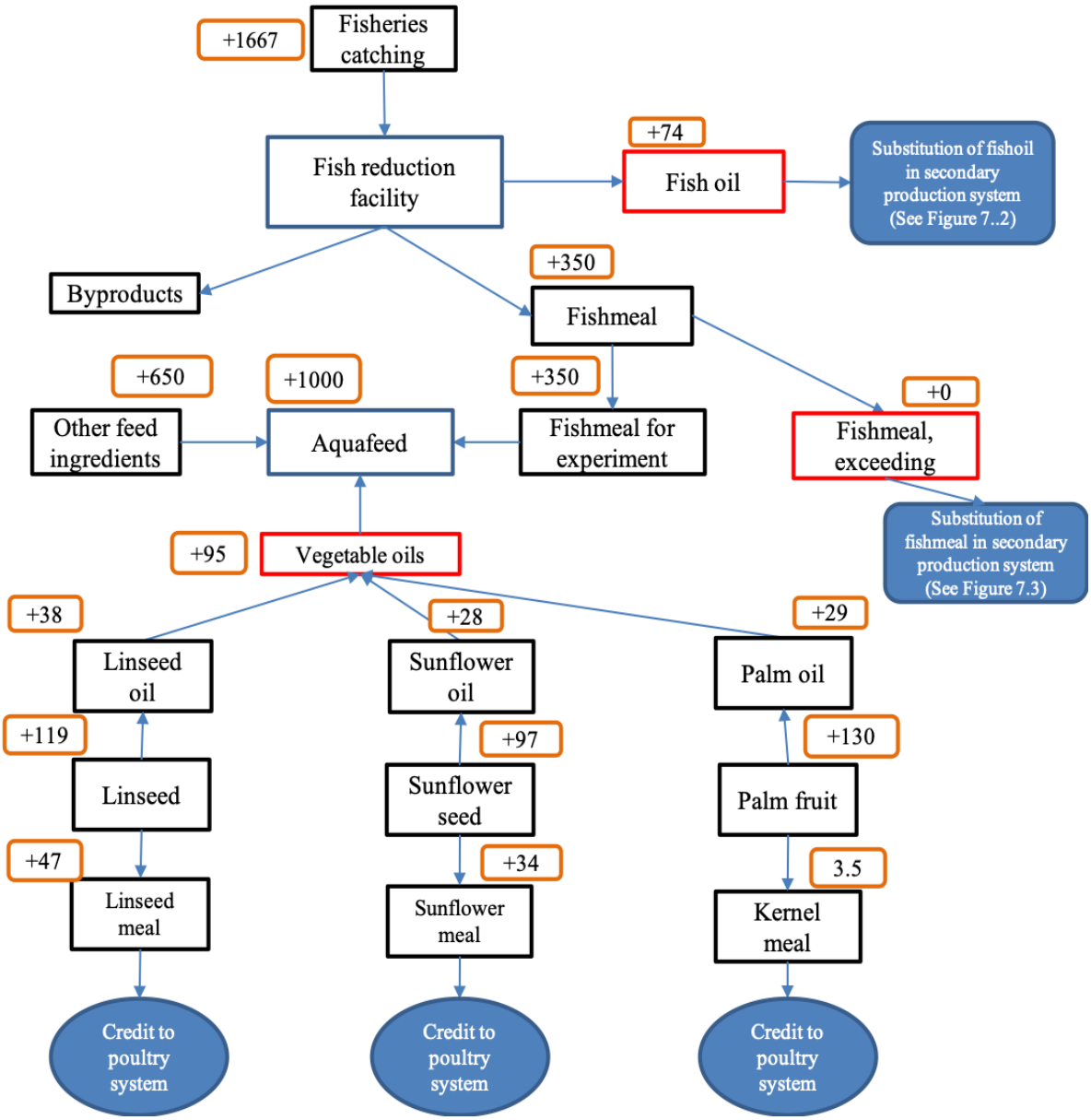

Figure 7.4. Scheme modelling the marginal production (+1 kg) of FO 0 diet from a CLCA perspective and according to the credit scenario: the production of vegetable oils (38 g of linseed oil, 28 g of sunflower oil and 29 g of palm oil) substituting 95 g of fish oil in the FO 0 diet implied the co-production of protein meals (47 g of linseed meal, 42 g of sunflower meal and 3.5 g of palm kernel meal) which were credited to the poultry system as the most expanding sector.

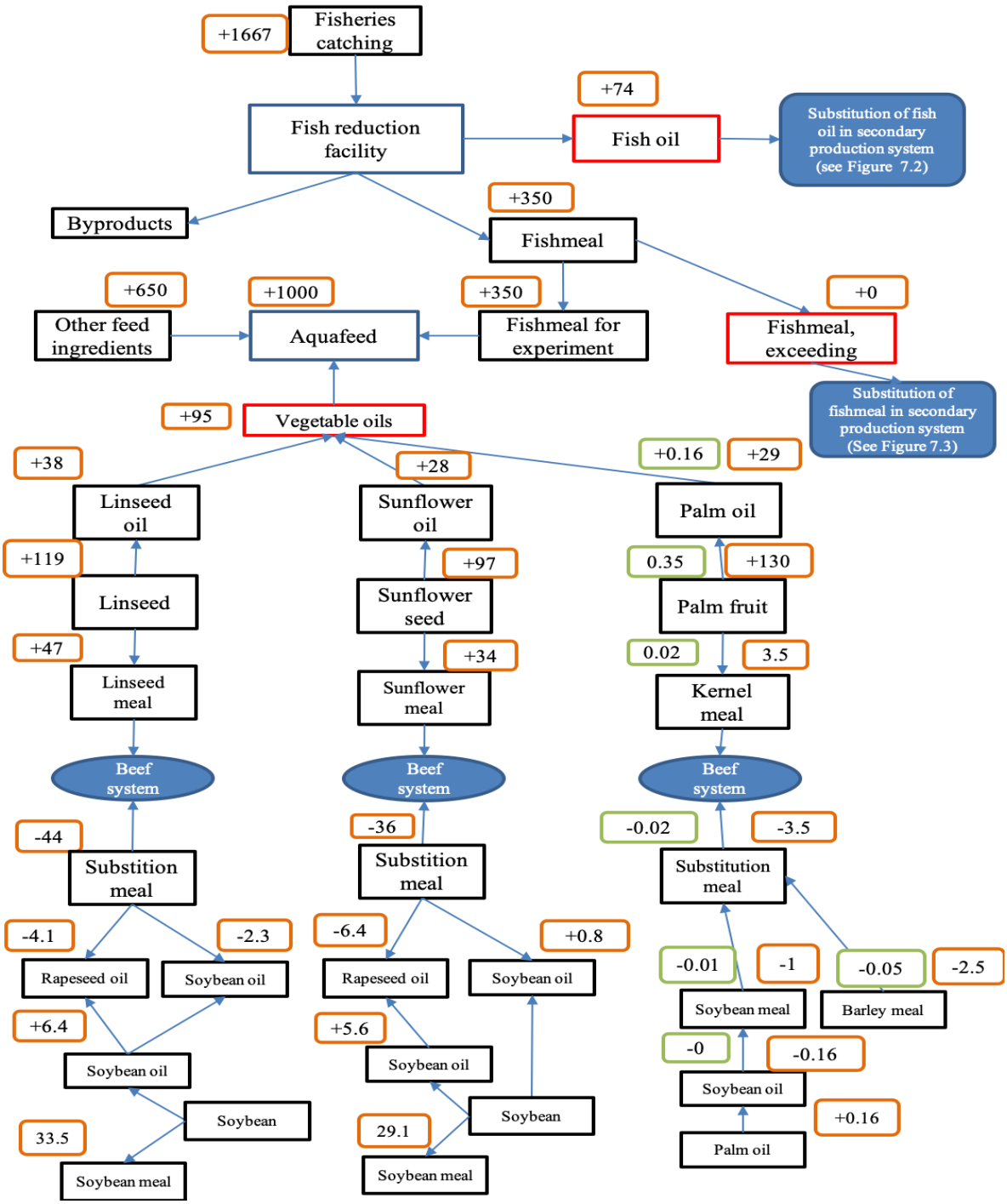

Figure 7.5. Scheme modelling the marginal production (+1 kg) of FO 0 diet from a CLCA perspective and according to the substitution scenario: the production of vegetable oils (38 g of linseed oil, 28 g of sunflower oil and 29 g of palm oil) substituting 95 g of fish oil in the FO 0 diet implied the co-production of protein meals (47 g of linseed meal, 42 g of sunflower meal and 3.5 g of palm kernel meal) which were used to substitute similar feedstuffs consumed by beef cattle in intensive systems.

Impact categories computation and life cycle impact assessment

The impact categories assessed were GWP, EP and DSF. The main references for the emission factors (EFs) used were Ecoinvent database v 3.6 (Wernet et al., 2016), Agrobalyse v3.0 (Colomb et al., 2015; ADEME, 2018) and Agri-footprint v3 databases. The emissions related to methane (CH_4), nitrous oxide (N_2O) and carbon dioxide (CO_2) were included in the computation of GWP category, whereas those related to N- and P-compounds in EP calculation. When available, consequential emission factors were used, whereas in the case that consequential EFs were not available, attributional EFs were adopted, since the modifications were small-scale and can be included in Situation A ("micro-level decision support") described in ILCD protocol (EC, 2010). The characterization factors to compute the single compounds to the common unit of GWP and EP were derived from Myhre et al. (2013) and CML-IA method (Oers et al., 2016), respectively.

Regarding DSF category, the scheme proposed by Heliàs et al. (2018) was followed. This scheme is based on the computation of the FM and FO needed to produce one unit of fish output and the selection of the EFs based on the origin of FM and FO. The amount of FM and FO used was derived from previous sections "Production of marginal vegetable oils-based diet" and the feed conversion ratios published in Milián-Sorribes et al. (2021) (1.8 kg/kg fish for FO 100 and 1.9 kg/kg fish for FO 0 diets), whereas the relative origin of these feed materials was assumed equal to that adopted in the assessment of the European sea bass production system in Agribalyse database (Colomb et al., 2015; ADEME, 2018).

RESULTS AND DISCUSSION

Attributional life cycle assessment

To have an overview of the environmental performance of the aquafeed tested, the impact associated with the production of the given FO 0 and FO 100 diets was assessed by an attributional LCA, which considered the environmental impact attributed to the production of 1 kg of experimental diets (FO 0 and FO 100 diet), assuming a static system and with system boundary including only the material flows and processes directly used in diet production. On average, the production of 1 kg of FO 100 diet released 1.10 kg CO_2-eq, 1.60 kg CO_2-eq when considering changes in land use (GWP_LUC) and 8.6 g PO_4-eq. The DSF was equal to $2.8E^{-07}$. On the other hand, the production of 1 kg of FO 0 diet produced 1.16 kg CO_2-eq (+5% compared to FO 0), 1.67 of CO_2-eq (GWP_LUC) (+4%) and 13.6 g PO_4-eq (+58%). Finally, DSF was equal to $1.95E^{-07}$ (Fig. 7.6).

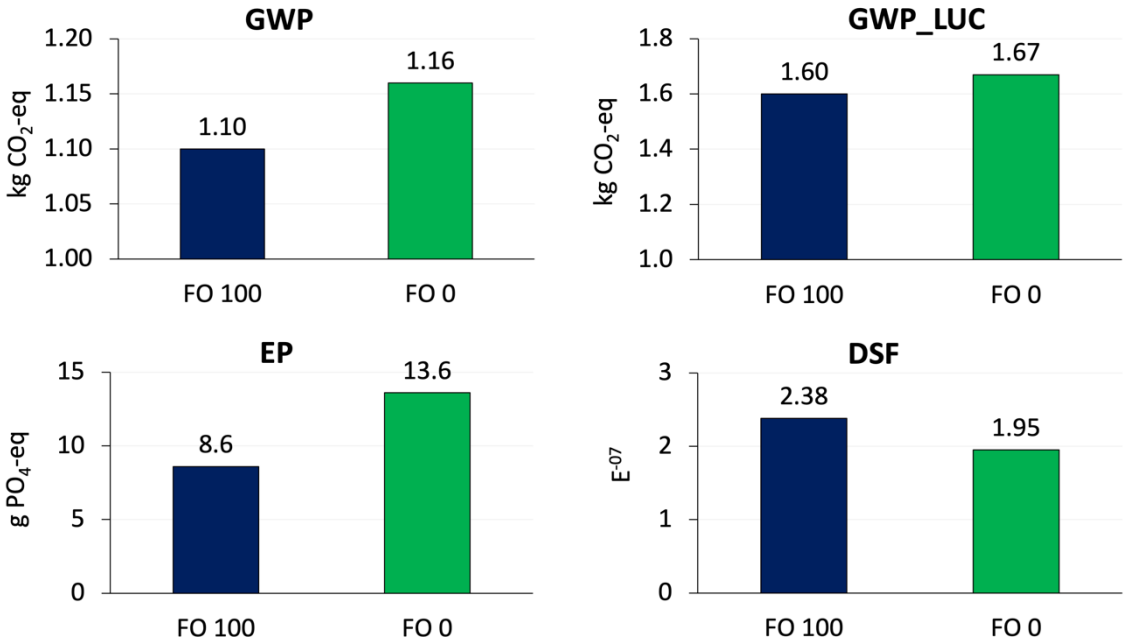

Figure 7.6. Environmental impact related to the production of 1 kg of FO 100 and FO 0 diet and calculated using an attributional life cycle assessment. GWP: Global warming potential; GWP_LUC: Global warming potential with land use change; EP: Eutrophication potential; DSF: Depleted stock fraction.

Consequential life cycle assessment

Data were also analysed using a CLCA approach, which considers future scenarios to determine the environmental impact because of a change (fish oil substitution with vegetable oils) in the production of the diets tested.

According to the CLCA, when the Substitution scenario was applied, the marginal production of 1 kg of FO 0 diet compared to the FO 100 one produced a lower GWP (-68%), similar GWP_LUC (+2%) and higher EP (+35%) (Fig. 7.7). Considering the Credit scenario, the marginal production of 1 kg of FO 0 diet compared to the FO 100 one gave a lower GWP (-37%), GWP_LUC (-14%) and a higher EP (+36%) (Fig. 7.8).

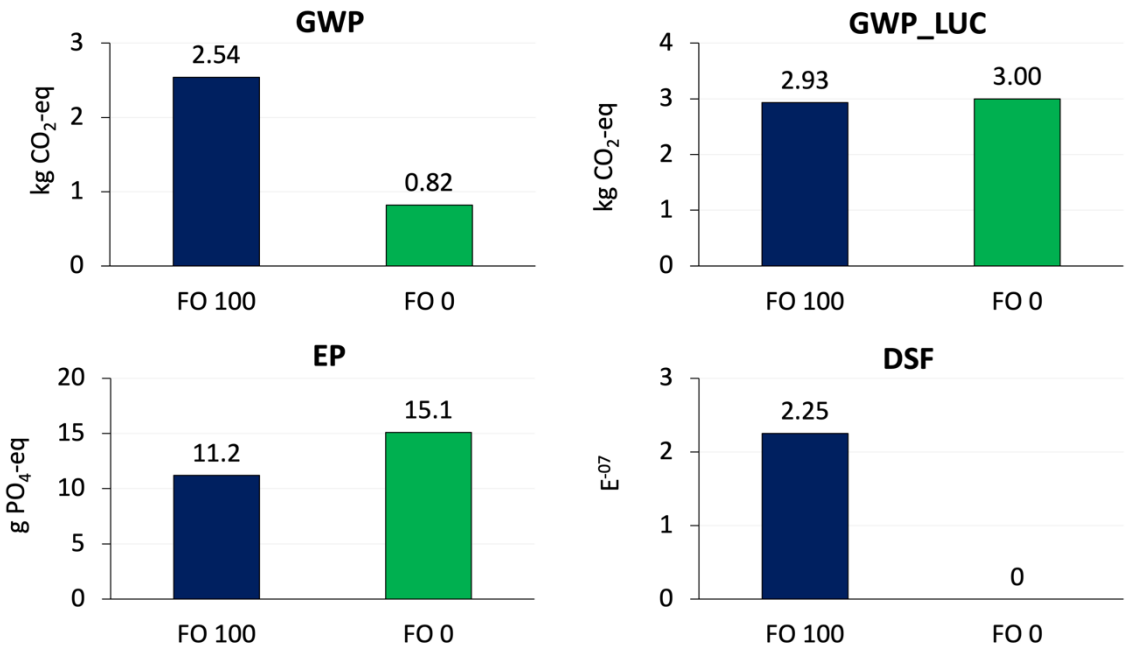

Figure 7.7. Environmental impact related to the marginal production of 1 kg of FO 100 and FO 0 diet and calculated using a consequential life cycle assessment (Substitution scenario). GWP: Global warming potential; GWP_LUC: Global warming potential with land use change; EP: Eutrophication potential; DSF: Depleted stock fraction.

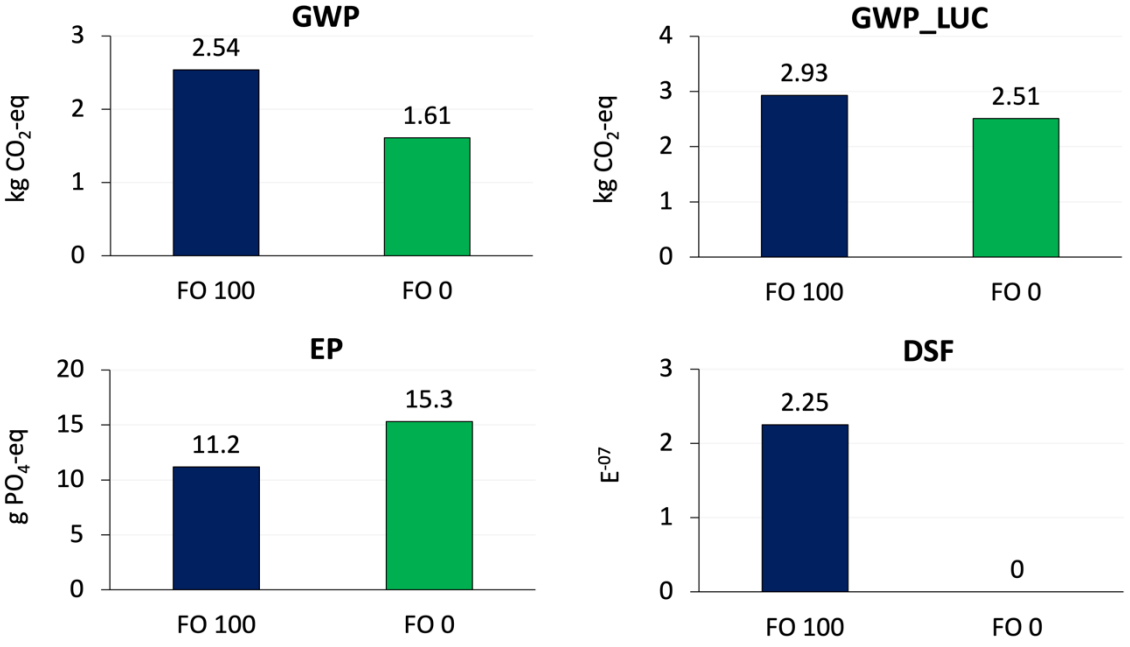

Figure 7.8. Environmental impact related to the marginal production of 1 kg of FO 100 and FO 0 diet and calculated using a consequential life cycle assessment (Credit scenario). GWP: Global warming potential; GWP_LUC: Global warming potential with land use change; EP: Eutrophication potential; DSF: Depleted stock fraction.

Considering an attributional perspective, the production of a fish oil-based diet has positive implications in terms of global warming potential. Similar results were also observed by Papatryphon et al. (2004), who reported that the total substitution of fish-derived ingredients with plant ingredients might not lead to any improvements in terms of global warming. However, when considering land use change, the positive effects of FO 100 production on global warming were neutralized, suggesting that the emissions related to land use change (i.e. cultivation of new virgin soils to produce oilseed crops) have a strong impact on the overall calculation. Therefore, further studies are needed to test the environmental performance of alternative ingredients that do not require a new soil cultivation (Maiolo et al., 2020). Among possible other options, poultry by-products and insects might be promising alternatives to reduce the impact of land changes, whereas the use of fisheries by-products seem to remarkably increase the environmental impacts of feed production, mainly due to a high energy consumption during processing and to the low oil/meal yield obtainable from such by-products (Pelletier and Tyedmers, 2007).

Several studies showed that the increase of plant ingredients in aquafeeds, even until a total substitution of fishmeal and fish oil, can lower the overall environmental impact and decrease the pressure on wild fish stocks (Pelletier and Tyedmers, 2007; Boissy et al., 2011; Smárason et al., 2017). However, plant-based diets produce a higher eutrophication potential compared to conventional ones (Smárason et al., 2017), with the total replacement of fish-derived ingredients, especially fish oil replacement, being the major voice of impact on eutrophication (Papatryphon et al., 2004), as observed in the present case study.

The impact associated with the production of plant oil greatly relies on the crops selected as raw materials (Boissy et al., 2011). Among the three vegetable oils tested, sunflower oil resulted to have the greatest eutrophication potential. Thus, further research should evaluate whether the use of a sunflower oil alternative might reduce the overall impact of a VO-based diet. Among plant oils, palm oil appeared to be the best substitute for fish oil in terms of environmental performance (Boissy et al., 2011; Schmidt, 2010). Thus, the formulation of a new VO-based diet replacing sunflower with palm oil, i.e. a diet formulated with linseed oil and palm oil (4:6) as lipid sources, might be tested in Mediterranean yellowtail, evaluating fish growth, health, muscle fatty acid profile and environmental performance.

The attributional LCA generally gives a lower input compared to consequential ones (Samuel-Fitwi et al., 2013). Indeed, an attributional scenario will not capture the increase in production of a given product, the secondary effects on other ingredients and on the context where ingredients are sourced (Samuel-Fitwi et al., 2013). Therefore, consequential LCA is preferred to describe all the affected processes.

In the present case study, the consequential approach gave similar results to the attributional one in terms of eutrophication potential, with the marginal production of FO 0 having a higher impact compared to that of FO 100. On the other hand, the marginal production of the VO-based diet resulted in positive implications in terms of GWP, with the

substitution scenario (protein co-products substituted feedstuffs in beef cattle intensive systems) showing the lowest global warming potential.

Finally, as expected, the marginal production of a VO-based diet did not impact on the depletion of marine biotic resources. However, potential trade-offs between the reduced impact on the biodiversity and stocks in marine ecosystems and the increased pressure on terrestrial biodiversity should be carefully considered, especially when considering tropical biomes where oilseed crops such as soybean are largely cultivated.

In conclusion, given the increasing demand for a sustainable development of fish production, future feed formulations will be required to focus not only on the nutritional characteristics and costs of the ingredients, but also on their environmental impact. This evolution will demand the use of methodological approaches, namely LCA, to identify the major environmental hotspots during the feed production chain, using standardized methods and open high-quality databases.

CASE STUDY 2: COMPARATIVE LIFE CYCLE ASSESSMENT OF RAINBOW TROUT FARMING AT TWO STOCKING DENSITIES IN A LOW-TECH AQUAPONIC SYSTEM

Based on the data obtained on the frame of the contribution of the Chapter 5 of the present thesis, the following case study aimed at evaluating the effect of two initial stocking densities on the environmental footprint associated with the production of 1 kg of table-size rainbow trout (300 g) farmed in a low-tech aquaponic system.

MATERIALS AND METHODS

The environmental impact was assessed using an attributional life cycle assessment model. The environmental footprint was computed by applying the ILCD Handbook protocol for attributional LCA (European Commission, 2010). The construction and application of the LCA model followed the scheme described by ISO standards 14040 and 14044 (ISO, 2006): goal and scope definition, life cycle inventory, life cycle impact assessment and interpretation of the results.

Goal and scope definition

The LCA model settings were defined to evaluate the environmental footprint associated with the rainbow trout production in the experimental low-tech aquaponic system described in Chaper 5 (third contribution), with the scope of analyzing the effect of two different fish stocking densities (low - ALD, 3.81 kg m^{-3} *vs* high - AHD, 7.26 kg m^{-3}). In this aquaponic system, the production of rainbow trout was associated with the production of lettuce. On these bases, a gate-to-gate model was used, with the system boundaries set to include the impact related to the fish rearing, the production of the fish feedstuffs and the input needed to set (tanks, water, initial nutrients, expanded clay, pumps, aerators) and maintain (electricity, refilling water due to evapotranspiration) the trout-lettuce aquaponic system (Fig. 7.9).

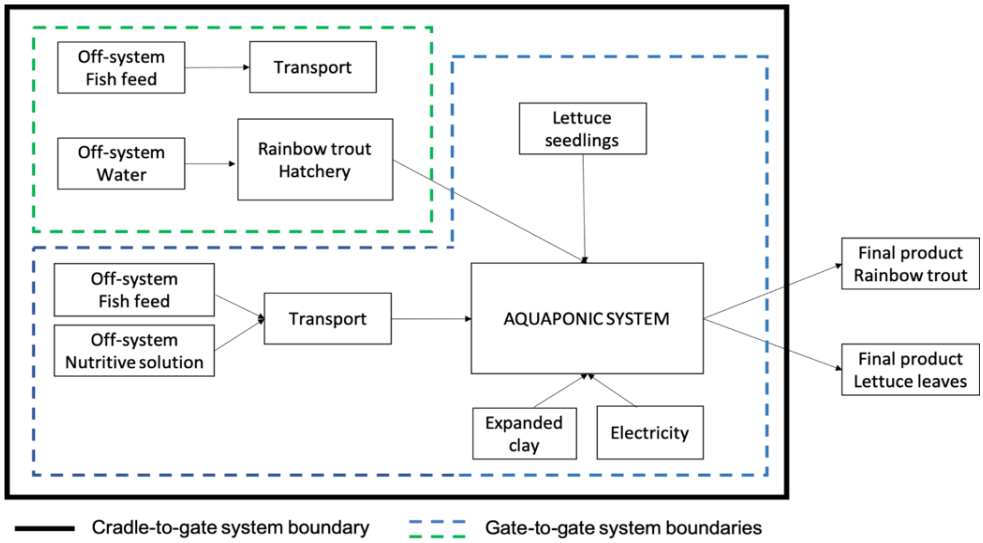

Figure 7.9. System boundaries (dotted lines) for the gate-to-gate Life Cycle Assessment of the production of rainbow trout in a low-tech aquaponic system

The functional unit (FU) was 1 kg of rainbow trout at the end of the experiment (bodyweight – BW: 329-333 g/fish). As the aquaponic system produced more than one product (fish plus lettuce), the need to resolve how to allocate the whole impact to the two different products emerged. Following the ISO guidelines (ISO, 2006), two different methods to resolve the multifunctionality of the analyzed system were applied: 1. mass allocation (whole impact allocated on the basis of the mass – kilograms - of fish and lettuce produced during the experiment); 2. economic allocation (whole impact allocated on the basis of the relative economic value of the fish and lettuce produced). The impact categories assessed were global warming (GWP, kg CO_2-eq), acidification (AP, g SO_2-eq) and eutrophication (EP, g PO_4-eq) potentials, cumulative energy demand (CED, MJ), water depletion (WD, m^3 water equivalent) and freshwater ecotoxicity (ECO, CTUe). Moreover, all the impact contributions lower than 1% were not considered (cut-off).

Life cycle inventory

Data about the aquaponic structures, experimental design and the inventories of inputs and outputs were originated from Chapter 5 (third contribution). The local emission of N and P due to fish input-output balance was estimated on the basis of the procedure suggested by Cho and Kaushik (1990), computing the N and P intake from fish feeds and their retention in fish bodyweight. The impact related to all the inputs used during the experiment were calculated by multiplying the quantity of the single input by the relative impact factor. Impact factors were derived from the Ecoinvent database v3.6 (Wernet et al., 2016) implemented in Simapro software v8.0.5. As the tanks used for the experiment were recovered from previous uses, the relative impact of their production was cut-off. The impact related to the lettuce production from an alternative system was derived from the Ecoinvent database (Wernet et al., 2016).

Data about the background phase related to the fish during the hatchery phase were obtained from the producers through an interview which regarded the inputs and the management to obtain the fish at a bodyweight equal to the initial body weight of the fish in the experiment. Data for the impact computation related to the experimental phase were obtained by the third contribution of the present thesis (Chapter 5). On the other hand, the production of the lettuce plants at the third true leaf stage was cut-off due to its very small contribution to the whole impact.

Life cycle impact assessment

The single substances and contributions were standardized to the common unit of the related impact category. The CML-IA method (Oers, 2016) was applied to GWP, AP and EP; Cumulative Energy Demand method (implemented in Simapro v8.0.5 software) to CED; ILCD Midpoint (EC, 2012) to WD and ECO categories.

The contribution of each production phase to each impact category was assessed by using the hotspot analysis (EC, 2010). As aquaponic systems are multifunctional, producing more than one output, a sensitivity analysis was used to test different allocation methodologies on the variability of the impact results.

RESULTS AND DISCUSSION

To produce 1 kg of table-size rainbow trout (from 140 to 330 g of live weight) at a low fish stocking density (3.81 kg m^{-3}) on average 1.64 kg of fish feed, 563 g of nutrient solution, 33 kWh of electricity and 470L of water (water depleted from the aquaponic system) were required. At a high fish stocking density (7.26 kg m^{-3}), 1.52 kg of feed, 302 g of nutrient solution (−46% compared to low stocking density), 18 kWh of electricity (−47%) and 252 L of water (−46%) were used. Regarding system outputs, the production of 1 kg of rainbow trout at a low stocking density released 34.6 g of nitrogen and 4.3 g of phosphorous and yielded 1.70 kg of lettuce leaves. The same production obtained from a high stocking density released 27.9 g of nitrogen and 3.5 g of phosphorous and obtained 0.91 kg of lettuce (Table 7.2).

In Table 7.2 the gate-to-gate life cycle impacts associated with the production of 1 kg of rainbow trout, farmed in aquaponics at two different stocking densities, are reported. Results were calculated considering mass allocation and economic allocation between fish and lettuce.

As for the mass allocation, on average, the production of 1 kg of rainbow trout in the low-tech aquaponic system emitted 6.2 kg CO_2-eq (GWP), 47 g of SO_2-eq (AP) and 61 g of PO_4-eq, whereas the CED was 106 MJ, the ECO 109 CTUe and the water used was 60 L. The farming of rainbow trout at a low stocking density generated a greater impact compared to a high stocking density in terms of GWP (+25%), AP (+16%), CED (+27%), ECO (+32%), and water use (+40%), whereas no differences were observed for EP (Table 7.2). On the other hand, considering the economic allocation, the production at a low stocking density showed

a greater impact (+56%, on average) compared to a high stocking density in all the categories considered, ranging from +29% in EP to +70% in ECO. The only exception was represented by WD, which was lower (−28%) in low compared to high density systems (Table 7.3).

Table 7.2. Life cycle inventory (gate-to-gate aquaponic system) of rainbow trout farming at two initial stocking densities (Low: 3.81 kg m^{-3} vs. High: 7.26 kg m^{-3}). Data are expressed per 1 kg of table-size trout (from about 140 to 300g of live weight) produced.

Variable	Unit	Stocking density	
		Low	High
INPUTS			
Fish feed	g	1644	1524
Plant nutrient solution			
KH_2PO_4	g	51.8	27.8
K_2SO_4	g	77.5	41.7
$MgSO_4$	g	107.3	57.8
Fe-ETDA	g	4.19	2.09
Micronutrients	g	2.09	1.07
$Ca(NO_3)_2$	g	130.9	70.1
NH_4NO_3	g	188.5	101.1
Expanded clay	kg	0.52	0.53
Electricity	kWh	33	18
Transport	tkm	0.126	0.118
Water	L	469.6	252.4
OUTPUTS			
Nutrient released in the water			
Nitrogen	g	34.6	27.9
Phosphorous	g	4.29	3.48
Rainbow trout	g	1000	1000
Lettuce leaves	g	1702	914
ALLOCATION			
Mass allocation to fish	%	37%	52%
Economic allocation to fish	%	80%	88%

Table 7.3. Impact category values (gate-to-gate) per 1 kg of live rainbow trout from a low-tech aquaponic system characterized by two initial fish stocking densities (Low: 3.81 kg m^{-3} vs. High: 7.26 kg m^{-3}). The coproduction of fish and lettuce was resolved by using mass allocation or economic allocation

		Stocking density			
		Low		High	
Impact category[1]	Unit	mass	economic	mass	economic
GWP	kg CO_2-eq	6.9	15.1	5.5	9.3
AP	g SO_2-eq	50	108	43	73
EP	g PO_4-eq	61	133	61	103
CED	MJ	118	255	93	157
ECO	CTUe	125	270	94.1	159
WD	m^3 eq	0.013	0.028	0.023	0.039
Water use	m^3	0.07	0.15	0.05	0.09

[1]GWP: Global warming potential; AP: Acidification potential; EP: Eutrophication potential; CED: Cumulative energy demand; ECO: Freshwater ecotoxicity; WD: Water depletion.

In the present case study, the gate-to-gate LCA model showed that the production of rainbow trout at a low stocking density was associated to a greater environmental impact than at the production at a high density in terms of global warming potential, acidification potential, cumulative energy demand, freshwater ecotoxicity and water use. On the other hand, eutrophication potential was greater at a high fish stocking density. Although this is the first study evaluating the environmental effects of different fish stocking densities in aquaponics, similar results were also observed in previous LCA studies in Indonesian (Mungkung et al., 2013) and Egyptian (Yacout et al., 2016) tilapia traditional farms with different production practices (intensive vs. semi-intensive) and stocking densities. Overall, the increase of system productivity has led to a decrease in the environmental impact of trout aquaponic farming, as already observed in other aquaculture (Mungkung et al., 2013; Yacout et al., 2016) and animal production systems (Cesari et al., 2017; Penati et al., 2010)

The hotspot analysis performed in the present study confirmed that the main contributors to the environmental impact in an aquaponic system is the electricity used for system functioning and the production of fish feed (Fig. 7.10) as already observed by other authors (Chen et al., 2020; Ghamkar et al., 2020; Forchino et al., 2017; Maucieri et al., 2018).

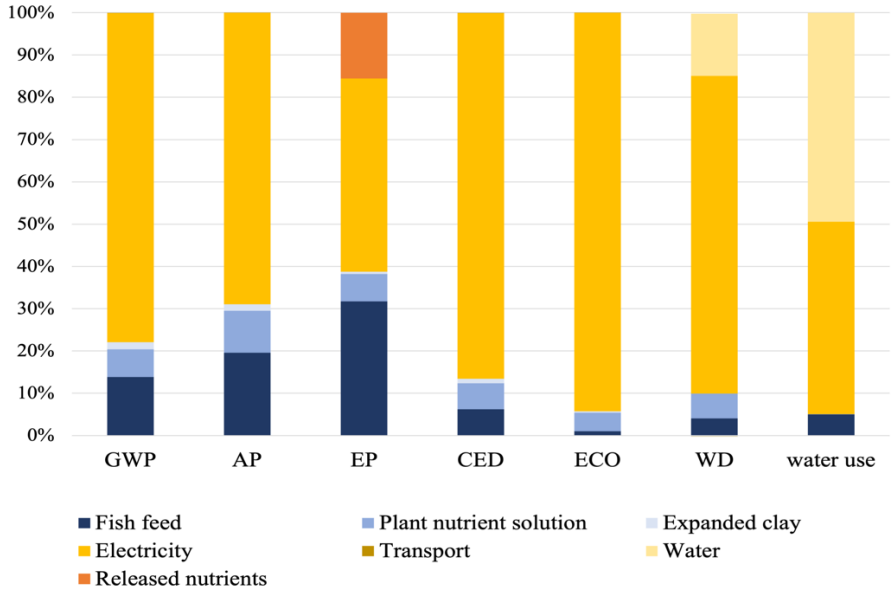

Figure 7.10. Gate-to-gate hotspot analysis for the Life Cycle Assessment of the production of 1 kg of rainbow trout in a low-tech aquaponic system. GWP: Global warming potential; AP: Acidification potential; EP: Eutrophication potential; CED: Cumulative energy demand; ECO: Freshwater ecotoxicity; WD: Water depletion.

The production of 1 kg of rainbow trout in the tested aquaponic system used 25.5 kWh, a slightly higher energy consumption if compared with previous studies on a virtual (16 kWh; Roque d'Orbacastel et al., 2009) and commercial (19.6 kWh; Samuel-Fitwi et al., 2013) rainbow trout RAS farm, and lower than that consumed in a small-scale aquaponic system to produce 1 kg of Nile tilapia (96.2 kWh; Delaide et al., 2017), or in a cold-weather aquaponic system to produce 1 kg of hybrid walleye (423.3 kWh; Ghamkar et al., 2020) or in a commercial RAS to produce 1 kg of turbot (80.6 kWh; Aubin et al., 2009). Nevertheless, results on raw energy consumption are difficult to compare due to differences among farm settings and husbandry practices (Forchino et al., 2017). Generally, in recirculating aquaculture systems, including aquaponics, electricity can contribute to the 50% of GWP and CED and up to 30% of AP (Chen et al., 2020). Therefore, the reduction of energy consumption is one of the major challenges for aquaponic production. In this sense, the use of renewable energy sources such as wind power or solar panels could be a viable solution.

Fish feed has been found to be the major material contributor to the environmental impact in aquaculture (Aubin et al., 2009; Samuel-Fitwi et al., 2013; Avadí and Fréon, 2015; Roque D'Orbacastel et al., 2009; Yacout et al., 2016; Mungkung et al., 2013) and aquaponics (Chen et al., 2020; Ghamkhar et al., 2020), being the main nutrient input to provide protein

and essential nutrients to fish diet in the case of carnivorous fish. Nevertheless, the reduction of the environmental impact generated by fish feed seem to be more difficult to achieve (Forchino et al., 2017) and largely depends on the improvement of FCR. The average FCR obtained in our system was 1.6, higher than those previously reported in trout RAS and aquaponics (Forchino et al., 2017). The higher FCR could be explained by the low environmental control and low farming intensity of our low-tech system (Mungkung et al., 2013) where fish were kept sometimes under sub-optimal temperature and oxygen conditions, which may have reduced the overall feed efficiency. In fact, previous studies showed that as farming intensity increases (i.e. high technology, environmental control and fish stocking density) FCR improves (Ghamkhar et al., 2021).

The hotspot analysis revealed that electricity had a great impact on GWP, while the contribution of feed was mainly observed in AP and EP. For this reason, two different scenarios using a sensitivity analysis were then tested: 1) the shift of energy source from grid mix to a photovoltaic plant; 2) the improvement of FCR from 1.6 (present study; see Chapter 5) to 1.2, as the average of FCR reported in previous studies in rainbow trout RAS and aquaponic farming (Forchino et al., 2017; Roque d'Orbacastel et al., 2009; Aubin et al., 2009).

The sensitivity analysis performed considering the first scenario showed that the replacement of the Italian electricity grid mix with a photovoltaic plant as electricity provider could abate substantially the emissions in terms of GWP associated to 1 kg of rainbow trout, from -69% to -98% in the low stocking density scenario and from -60% to -84% in the high one, depending on the method used to resolve the multifunctionality problem (Fig. 7.11). Besides, this GWP abatement could be reached without notably worsening the impact in terms of the other impact categories (AP, EP, CED, ECO, WD and water use) (Fig. 7.11).

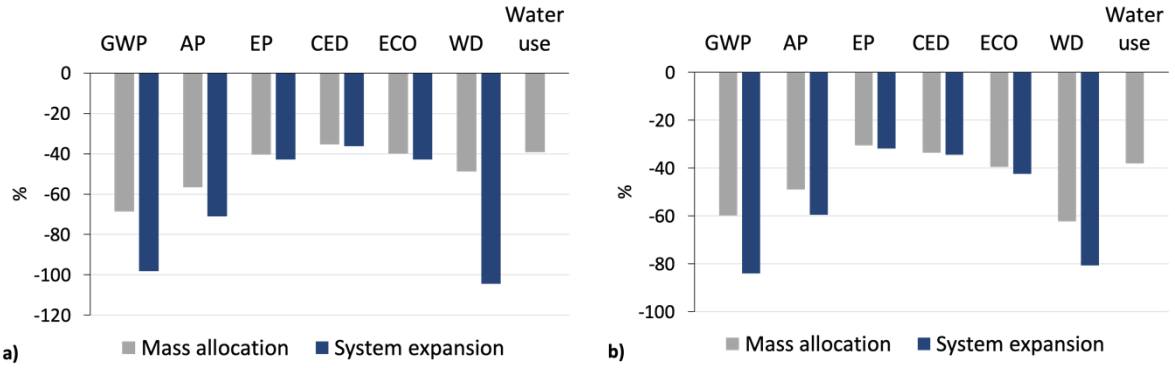

Figure 7.11. Percentage reduction in the environmental impact of the tested low-density (a) and high-density (b) aquaponic systems when changing from using the Italian grid mix (present system) to a photovoltaic plant as electricity source. GWP: Global warming potential; AP: Acidification potential; EP: Eutrophication potential; CED: Cumulative energy demand; ECO: Freshwater ecotoxicity; WD: Water depletion. Data were analyzed considering mass allocation (whole impact allocated on the basis of the mass – kilograms - of fish and lettuce produced during the experiment) and system expansion (expanded the

system to include the production of lettuce through a traditional system and subtracting this impact to the whole impact associated to the aquaponic system)

Considering the second scenario, the reduction of FCR from 1.6 to 1.2 brought also to a decrease in the environmental impact compared to the first scenario, depending on the impact category considered and the allocation method used (Fig. 7.12). The most important reduction was observed in terms of eutrophication potential, with a decrease from -12% in the low-density scenario to -16% in the high-density one (Fig. 7.12).

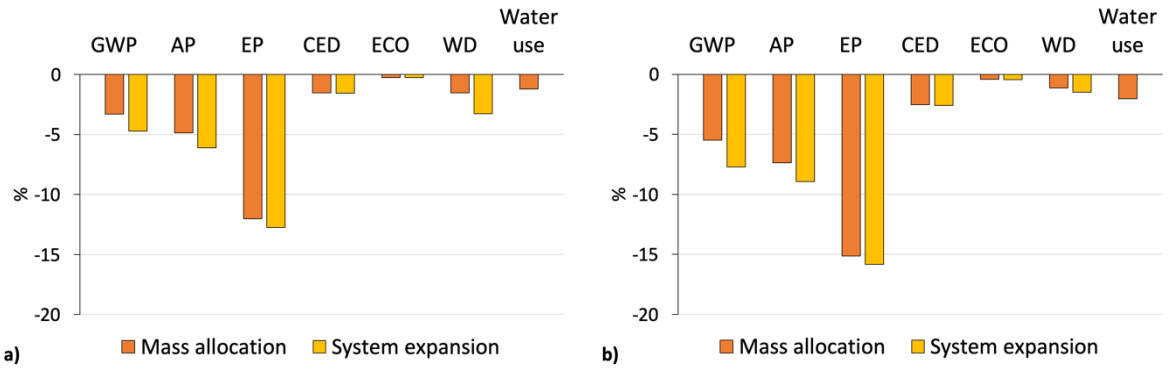

Figure 7.12. Percentage reduction in the environmental impact of the tested low-density (a) and high-density (b) aquaponic system resulted from the reduction of feed conversion ratio from 1.6 to 1.2. GWP: Global warming potential; AP: Acidification potential; EP: Eutrophication potential; CED: Cumulative energy demand; ECO: Freshwater ecotoxicity; WD: Water depletion. Data were analyzed considering mass allocation (whole impact allocated on the basis of the mass – kilograms - of fish and lettuce produced during the experiment) and system expansion (expanded the system to include the production of lettuce through a traditional system and subtracting this impact to the whole impact associated to the aquaponic system).

Another main contributor to the environmental impact of the present aquaponic system was the plant nutrient solution. In fact, despite nutrients such as zinc (Zn), manganese (Mn) and iron (Fe) can derive from fish feed, while copper (Cu) and boron (B) from tap water (Delaide et al., 2017), key micronutrients in aquaponic systems are often present at very low levels to sustain optimal plant performance (Delaide et al., 2017; Bittsanszky et al., 2016; Roosta, 2014; Nozzi et al., 2018). Therefore, the addition of a micronutrient-rich solution is necessary to obtain plant yields comparable to those observed in hydroponic cultivations. This is the first study that addressed the environmental impact of a nutrient solution added to an aquaponic system. Indeed, given the importance of obtaining high plant yields and the relative high contribution of plant nutrient solution observed in most of the impact categories studied, further LCA studies should carefully consider also the impact derived from plant micronutrients when used in aquaponic farms.

As for macronutrients, the release of nitrogen and phosphorous contributed for 16% of the eutrophication potential, while the remaining part derived from electricity and feed production. Fish stocking density had an impact on EP, which was lower in low- compared to high-density systems. In the same aquaponic set-up, higher nitrogen losses and a lower nitrogen use efficiency were observed when European carp were farmed at high compared to low stocking density (Maucieri et al., 2020). On the other hand, other studies performed in RAS and semi-intensive systems showed a reduced EP when farming intensity and stocking density increased (Ghamkhar et al., 2021). The reduction of the eutrophication impact in high-density aquaponic systems might be achieved through the improvement of feeding strategies (i.e. reduction of FCR) and the upgrading the system set-up, enhancing the removal of solids derived from fish faeces and uneaten feed.

In the Mediterranean context, rainbow trout are typically farmed in flow-through systems, known to use higher amount of water (+90-99%) if compared with closed RAS, such as aquaponics (Ghamkar et al., 2021). In our study, the production of 1 kg of rainbow trout used 60 L of water, about 99% less than the average amount of water used to produce 1 kg of trout in flow-through farms (Samuel-Fitwi et al., 2013; Roque d'Orbacastel et al., 2009; Aubin et al., 2009). In addition, studies showed an improvement in water use efficiency with the increase of farming intensity (Aubin et al., 2009), with low-stocking density systems having higher (+28%) water use compared with high ones (Mungkung et al., 2013). These results were observed also in our aquaponics system when applied a gate-to-gate approach (i.e. when considering only the impact related to the rearing of trout in our aquaponic system), where a greater water use was found to produce 1 kg of trout at low compared to high stocking density when considering both mass allocation (+40%) and economic allocation (+67%).

In conclusion, the farming of rainbow trout in a low-tech aquaponic system is suitable at the highest density tested, without any negative effects on fish and plant growth performance and water quality. From the environmental point of view, a high fish stocking density showed a lower impact per kg of fish produced, especially in terms of water use, freshwater ecotoxicity, global warming and cumulative energy demand. The electricity used for the system functioning was the major hotspot observed in the tested system. Nevertheless, the replacement of the energy source from of common grid mix to renewable sources such as photovoltaic systems can substantially reduce the environmental impact derived from electricity, especially in terms of global warming. The farming of rainbow trout in aquaponics is a promising alternative to common flow-through systems, particularly in view of reducing water use. Nevertheless, the impact derived from water use should be carefully evaluated in cradle-to-gate perspective, i.e from raw material extraction to the farm gate (before product being transported to the consumer), as off-system stages (i.e. hatchery phase) could strongly increase the overall water consumption required for fish production.

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CHAPTER 8

MAIN CONCLUSIONS AND PERSPECTIVES

The main objective of the present PhD thesis was to evaluate new feeding strategies and alternative rearing techniques to improve the sustainability of the aquaculture production.

The goal was pursued by focusing on three specific objectives: (i) replacing fish oil with vegetable oil in diets for a new fast-growing marine species such as Mediterranean yellowtail (First contribution and second contribution); ii) developing low-tech aquaponic system for farming high-value freshwater species such as rainbow trout (Third contribution and fourth contribution); iii) replacing fishmeal with insect meal (*Hermetia illucens*) in rainbow trout reared in a low-tech aquaponic system (Fourth contribution).

Moreover, to evaluate the impact of some of the proposed strategies from an environmental perspective, a life cycle assessment approach was used in the general discussion of the present PhD thesis to assess the environmental impact of (i) fish oil substitution with vegetable oils in diets for Mediterranean yellowtail (Case study 1) and of (ii) rainbow trout farmed at two stocking densities in a low-tech aquaponic system (Case study 2).

The findings obtained from the four contributions highlight that:

I. The total **substitution of fish oil with vegetable oils** (linseed oil, sunflower oil, and palm oil; 4:3:3) in diets for Mediterranean yellowtail juveniles did not impact on growth performance, whereas a slight reduction in fish survival was observed, which warrant further investigations.

II. The fatty acid profiles of the experimental diets fed Mediterranean yellowtail juveniles were mirrored in target fish tissues such as muscle, liver, and visceral fat, whereas brain was found to be more robust to dietary changes.

III. After 109 days of feeding with a vegetable-oil based diet, a reduction in health-promoting eicosapentaenoic acid and docosahexaenoic acid was found in muscles of Mediterranean yellowtail juveniles.

IV. The refeeding with a fish-oil based diet for 90 days can only partially improve the FA profile in muscles of Mediterranean yellowtail previously fed on vegetable oil-based diets.

V. The substitution of fish oil with vegetable oils seemed to have positive implications regarding global warming potential, whereas led to a higher impact in terms of eutrophication potential.

VI. Among the three vegetable oils included in the alternative diets, the production of sunflower oil was found to be the most impacting hotspot.

VII. Rainbow trout can be successfully farmed in a low-tech **aquaponic** system.

VIII. Trout can be farmed in aquaponics at a high initial stocking density (7.26 kg m^{-3} vs. 3.81 kg m^{-3}), without negative effects on growth performance, health, and flesh quality.

IX. A high fish stocking density had a lower environmental impact per kg of fish produced, especially in terms of water use, freshwater ecotoxicity, global warming and cumulative energy demand.

X. The electricity used for system functioning was the major hotspot observed in the tested aquaponic system.

XI. Trout growth, survival and fillet traits were essentially unaffected by a 25% **fish meal replacement with *Hermetia illucens* meal**, whereas at a replacement rate of 50%, certain effects were detected on gut histology, fillet colour and nutritional characteristics.

The present thesis confirmed that vegetable oil-based diets are successful and can be used in juveniles of Mediterranean yellowtail, whereas more evidence are required regarding fish health. Further studies are also required to evaluate the feasibility of different combination of oilseed crops to be include in dietary blends to improve the environmental impact associated with their production.

Regarding protein sources, the partial replacement of fish meal with defatted *Hermetia illucens* meal is a viable strategy to reduce aquafeed dependence on marine-based products. In the next years, other insect species should be tested as potential fish meal alternative, evaluating the best replacement rates to assure optimal growth performance, promote immune response, high product quality and reduce environmental burdens.

Finally, aquaponics farming was found to be a valuable technique for rainbow trout farming, reducing the dependence on water, land, and nutrient supply. However, LCA analysis revealed that energy consumption and feed production are the major hotspots of this system. Thus, further studies should focus on the optimization of the aquaponic set-up, on the use of alternative energy sources for system functioning as well as on the improvement of farming technique and feed distribution to achieve optimal conversion rates. Additionally, further research is also required to evaluate the adaptability and the production of different combinations of plant and fish species in aquaponics considering different water qualities and water availability at a global level with severe challenges for water use.